

Design, Synthesis and Applications of Fluorescent Small Organic Molecules and Nucleoside

**A
Dissertation Submitted to the
Indian Institute of Technology Guwahati
As Partial Fulfilment for the Award of Degree of**

**Doctor of Philosophy
In
Chemistry**

By

**Rajen Kundu
Roll No. 08612208**



**Department of Chemistry
Indian Institute of Technology Guwahati
Guwahati - 781039, India
June 2012**

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Dedicated to
My
Parents
&
Grand Mother
And
Those who have helped me

INDIAN INSTITUTE OF TECHNOLOGY, GUWAHATI
Department of Chemistry



DECLARATION

I do hereby declare that the research work embodied in this thesis entitled “*Design, Synthesis and Applications of Fluorescent Small Organic Molecules and Nucleoside*” has been carried out by me under the supervision of **Dr. Subhendu Sekhar Bag** in the Department of Chemistry, Indian Institute of Technology Guwahati, India.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

IIT Guwahati
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CERTIFICATE

This is to certify that the research work presented in this thesis entitled
“Design, Synthesis and Applications of Fluorescent Small Organic Molecules and Nucleoside” is an authentic record of the results obtained from the research work done by **Mr. Rajen Kundu** under my supervision in the Department of Chemistry, Indian Institute of technology Guwahati, India. This work is original and has not been submitted elsewhere for a degree.

IIT Guwahati
June, 2012

Dr. Subhendu Sekhar Bag
(Thesis Supervisor)

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Area of Interest

Design and synthesis of small organic fluorophores and fluorescent biomolecules for chemical and biochemical application

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- ❖ Junior Research Fellowship and Eligibility for Lectureship (**CSIR JRF-NET**), **Dec J 2008**, awarded by Council of Scientific & Industrial Research and University Grants Commission, India.
- ❖ Qualified **GATEJ2008** (Graduate Aptitude Test in Engineering) examination organized by Ministry of Human Resource Development, Government of India.

List of Publications

1. •Installation/Modulation of the Emission Response via Click Reaction• Bag, S. S.; **Kundu, R.**• *J. Org. Chem.* **2011**, 76 (9), 3348–3356.
2. •Click-Reagent Version of Sonogashira Coupling Protocol to Conjugated Fluorescent Alkynes with No or Reduced Homocoupling• Bag, S. S.; **Kundu, R.**; Das, M.• *J. Org. Chem.* **2011**, 76 (7), 2332–2337 (Highlited in *ChemInform* **2011**, 42 (26), 2332–2337).
3. •Singly and doubly labeled base-discriminating fluorescent oligonucleotide probes containing oxo-pyrene chromophore• Bag, S. S.; **Kundu, R.**; Katsuhiko, M.; Saito, Y.; Saito, I.• *Bioorg. Med. Chem. Lett.* **2010**, 20, 3227–3230.
4. •Suppressed β -Effect of Silicon in 3-Silylated Monocyclic β -Lactams: The Role of Antiaromaticity• Bag, S. S.; **Kundu, R.**; Basak, A.; Slania, Z.• *Org. Lett.* **2009**, 11(24), 5722–5725.
5. •Triazolyl-Donor/Acceptor Chromophores Decorated Unnatural Nucleosides and Oligonucleotides with Duplex Stability Comparable to that of Natural A/T Pair• Bag, S. S.; Talukdar, S.; Matsumoto, K.; **Kundu, R.**• *J. Org. Chem.* **2013**, 78, 278.
6. •Fluorometric sensing of Cu^{2+} ion with smart fluorescence light-up probe, triazolylpyrene ($^{\text{TNDMB}}$ Py)• Bag, S. S.; **Kundu, R.**; Talukdar, S.• *Tetrahedron Lett.* **2012**, 53, 5875.
7. •Highly solvatochromic fluorescent naphthalimides: design, synthesis, photophysical properties and fluorescence switch-on sensing of ct-DNA• Bag, S. S.; Pradhan, M. K.; **Kundu, R.**; Jana, S.• *Bioorg. Med. Chem. Lett.* **2013**, 23, 96.
8. •Sensing of biomolecules and label-free discrimination of DNA containing a triple T–C/T–G mismatch pair with a fluorescence light-up probe, triazolylpyrene ($^{\text{TNDMB}}$ Py)• Bag, S. S.; **Kundu, R.**; Jana, S.• *Tetrahedron Lett.* **2013**, 54, 2627.
9. •Sensing of micellar microenvironment with dual fluorescent probe, triazolyl pyrene ($^{\text{TNDMB}}$ Py) • Bag, S. S.; **Kundu, R.**• *Journal of Fluorescence* **2013** DOI: <http://dx.doi.org/10.1007/s10895-013-1218-6>

Communicated

10. ● Stabilizing a Duplex DNA Containing an Abasic site with Unnatural Nucleosides ● Bag, S. S.; **Kundu, R.**; Talukdar, S.●

Articles/Manuscripts 4, 5, 7 and 10 are not included in the thesis.

List of Conferences/Symposiums

1. National Conference on Frontiers in Chemical Sciences (FICS) – 2010, Organized by *Department of Chemistry, IIT Guwahati, India.*
2. International Conference on Chemistry: Frontiers and Challenges – 2011, Organized by *Department of Chemistry, Aligarh Muslim University, India.*
3. Symposium on Chemical Research in the First Decade of 21st Century – 2011, Organized by *Department of Chemistry, Visva-Bharati, India.*
4. Diamond Jubilee Symposium on Recent Trends in Chemistry (DJSRTC – 2011), Organized by *Department of Chemistry, IIT Kharagpur, India.*
5. Junior National Organic Symposium Trust (J-NOST – 2012), Organized by *Department of Chemistry, IIT Guwahati, India.*



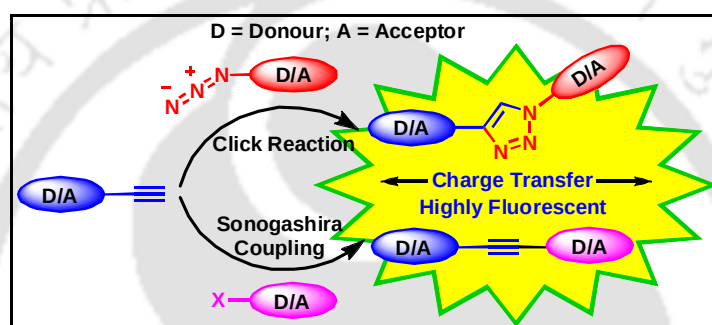
ABSTRACT

The dissertation entitled “**DESIGN, SYNTHESIS AND APPLICATIONS OF FLUORESCENT SMALL ORGANIC MOLECULES AND NUCLEOSIDE**” is an embodiment of research aimed towards: (a) the importance of small organic solvatochromic fluorophores and fluorescent nucleosides, (b) the use of “Click” reaction and Sonogashira coupling to the design and synthesis of solvatochromic fluorophores and fluorescent nucleoside, (c) probing of biomolecular (Protein and DNA) and micellar microenvironment with small fluorescent molecules, (d) the detection of metal ions, and (e) the detection of DNA with fluorescently labeled oligonucleotide probes. Towards this journey several novel solvatochromic fluorophores and a fluorescently labeled nucleoside were synthesized either *via* click reaction, modified Sonogashira coupling or *via* classical Sonogashira coupling reaction and their photophysical and biophysical properties were evaluated.

The thesis contains 6 chapters. **Chapter 1** describes briefly the use of solvatochromic fluorophores towards biological applications and the “click reaction” and “Sonogashira” coupling reaction to the synthesis of fluorescent small organic molecules. **Chapter 2** deals with the installation/modulation of fluorescence response into non-fluorescent/weakly fluorescent molecules *via* “click” chemistry. **Chapter 3** comes with the development of a “click-reagent version” of Sonogashira coupling protocol to the synthesis of fluorescent small molecules. **Chapter 4** deals with the application of one of our synthesized fluorophore, triazolyl pyrene (^{TNDB}Py) in probing of biomolecular (Calf Thymus DNA and Bovine Serum Albumin) microenvironment. **Chapter 5** explores triazolyl pyrene, (^{TNDB}Py) as a fluorescence sensor of micellar microenvironment and of Cu²⁺ ion. Finally, **Chapter 6** deals with synthesis of fluorescently labeled nucleoside *via* classical Sonogashira coupling and its application in genotyping single nucleotide polymorphisms (SNPs).

Chapter 1: Application of “Click” Reaction and “Sonogashira” Coupling to the Synthesis of Fluorescent Small Organic Molecules: A Review

This chapter introduces a brief literature study of fluorescent small molecules, their synthesis *via* click reaction and Sonogashira coupling, and their applications in chemical/biochemical world (**Scheme 1**).

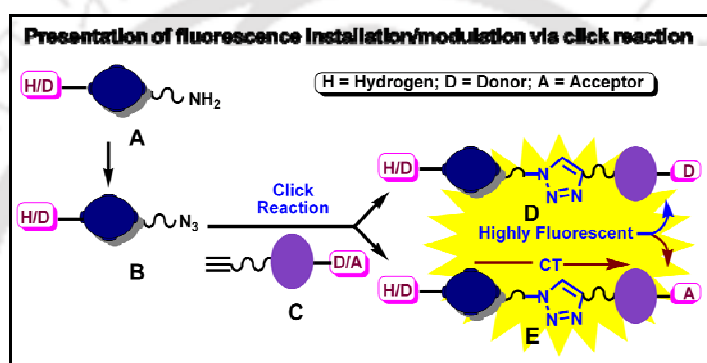


Scheme 1: Schematic presentation of synthesis of fluorophores *via* “click” reaction and Sonogashira coupling.

Since the advent of click chemistry philosophy in 2001, it has become a powerful tool for the synthesis of small organic fluorescent molecules that are widely used in biological study, drug discovery and as ion sensor. On the other hand, Sonogashira coupling, because of its mild reaction condition and tolerance to a variety of substrates drew much attention and became the most popular, widely used practical tool for the generation of several terminal and internal π -conjugated acetylenic compounds of interesting photophysical and photochemical properties of widespread applications ranging from chemistry, biology to molecular organic materials. Also, in the field of chemical genomics, for DNA detection, Sonogashira coupling has widely been used for the generation of fluorescently labelled nucleosides. Thus, this chapter contains a critical survey of these two main reactions to the generation of conjugated fluorescent molecules/ fluorescently labeled nucleosides aiming their application in biophysical/biochemical world.

Chapter 2: A Click Chemistry Strategy for the Installation/Modulation of the Emission Response of Non-Fluorescent/Weakly Fluorescent Molecules

This chapter deals with the novel concept of installation/modulation of the emission response *via* click reaction and describes the design, synthesis, and photophysical properties of some of the click chemistry derived fluorophores.



Scheme 2: Schematic presentation of fluorescence installation/modulation *via* “click” reaction.

Here, we have demonstrated the installation of a fluorescence property into a non-fluorescent precursor and modulation of an emission response of a pyrene fluorophore *via* click reaction (**Scheme 2**). The synthesized fluorophores show different solvatochromicity and/or intramolecular charge transfer (ICT) feature as is revealed from the UV-visible, fluorescence photophysical properties of these fluorophores, and DFT/TDDFT calculation. We observed that some of the synthesized fluorophores showed purely ICT character while emission from some of them arose from LE state. A structureless and solvent polarity sensitive dual emission behavior was observed for one of the fluorophore, triazolylpyrene that contains an electron donating –NMe₂ substituent (**Figure 1b-c**). The effect of ICT on the photophysical properties of these fluorophores were studied by fluorescence emission spectra, and DFT/TDDFT calculations. All of our findings revealed the delicate interplay of structure and emission properties and thus having broader general utility. As the ICT to LE

intensity ratio can be employed as a sensing index, the dual emissive fluorophore can be utilized in designing molecular recognition system too. We envisage that our investigation is of importance for the development of new fluorophores with predetermined photophysical properties that may find a wide range of application in chemistry, biology, and in material science.

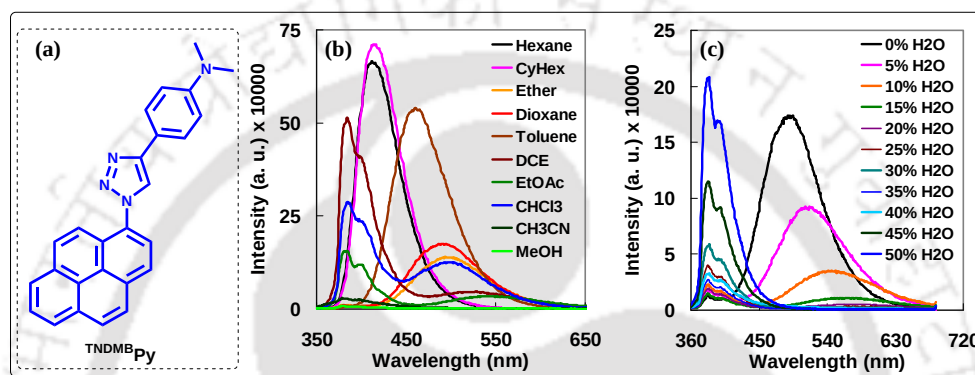
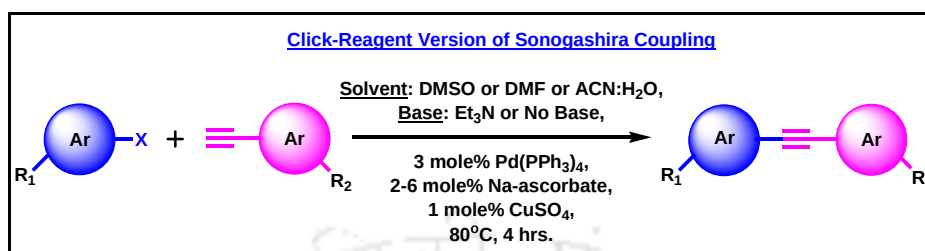


Figure 1: (a) Chemical structure of triazolyl pyrene. Fluorescence spectra of triazolyl pyrene (b) in different solvents and (c) in dioxane titrated with water. (10 μ M, r.t.; $\lambda_{ex} = \lambda_{max}^{abs} \approx 340$ -345 nm of each solvent).

Chapter 3: A Modified Sonogashira Coupling Protocol to the Synthesis of Fluorescent Small Molecules

This chapter describes the development of “click-reagent version” of Sonogashira coupling protocol. Diarylalkynes with donor and/or acceptor substituents have been synthesized *via* this protocol at moderate to excellent yields and with no or drastically reduced quantities of undesired homocoupled side products. This protocol is green-solvent compatible, air-insensitive, and effective under microwave condition.



Scheme 3: General procedure of our developed protocol to the synthesis of fluorescent small molecules.

There has been a tremendous research effort over the course of the last decade to overcome the shortcomings of classical Sonogashira coupling; however, no single protocol has overcome all of the shortcomings, especially the problem of alkyne-alkyne homocoupling. Therefore, overcoming the limitations, especially the problem of the homocoupling side reaction, remains a challenge for researchers.

As a part of our ongoing research effort on reaction, we became curious to know whether the same click reagents can serve as a source of active Cu(I) for Sonogashira coupling. Also we wanted to know the followings:

- (a) can Na-ascorbate maintain a reducing atmosphere in this system, and
- (b) if so, would it be more effective to reduce the incidence of homocoupling side reaction in comparison to CuI or other reported modified Sonogashira methods?

With this aim we attempted to answer our curiosities and we were successful in developing a “click-reagent version of Sonogashira coupling” protocol under mild reaction conditions without any requirement of skillful reaction tailoring (**Scheme 3**).

Chapter 4: Probing Biomolecular Microenvironment with Small Fluorescent Molecule

This chapter describes the studies on the interaction of biomolecular microenvironment with our synthesized fluorophore, triazolyl pyrene (^{TNDBM}Py, **Chapter 2**). In particular, we have exploited the interesting emissive property of

triazolyl pyrene to study its interaction with (a) Calf Thymus DNA (ct-DNA), and (b) Bovin Serum Albumin (BSA) spectroscopically (**Figure 2**).

We envisaged that the introduction of 1,2,3-triazole residues to an aromatic unit may significantly enhance the interaction ability *via* stacking/H-bonding/electrostatic interaction with the amino acid residues in a protein and/or with the aromatic bases in DNA; thereby allow one to gather information on the protein's/DNA's microenvironment. With this idea in mind, we have exploited ^{TNDMB}Py probe to study the interaction with ct-DNA and BSA. This chapter is divided into two subchapters; **Chapter 4A and 4B**; each contains their individual introduction, background, objective, and results and discussion.

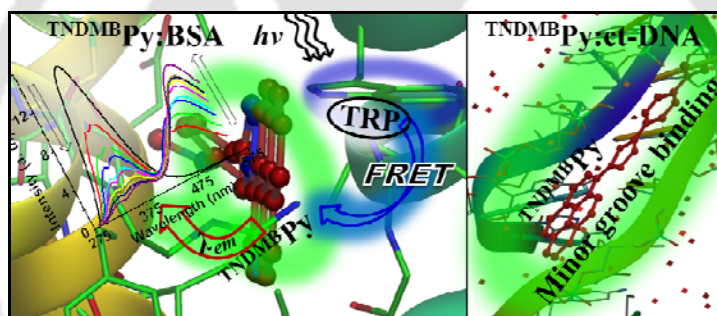


Figure 2: Graphical presentation of sensing of biomolecules BSA and ct-DNA by our probe, triazolyl pyrene.

Chapter 4A: Studies of the Interaction of Smart Fluorescent Probe, Triazolyl Pyrene (^{TNDMB}Py), With Calf Thymus DNA

In this chapter a brief literature review on probes for ct-DNA is presented along with a discussion of our observations. In UV-Visible spectra a clear hyperchromicity with a minimal shift in absorption wavelength maxima of ^{TNDMB}Py was observed as [ct-DNA] was increased gradually. Similarly the fluorescence intensity ($\lambda_{em} = 525$ nm) of ^{TNDMB}Py was increased with increasing [ct-DNA] with a binding constant in the order of $7.2 \times 10^3 \text{ M}^{-1}$, as was revealed from fluorescence spectra (**Figure 3a**). Hoechst 33258 displacement from Hoechst 33258-ct-DNA complex by the probe ^{TNDMB}Py (**Figure 3b**) suggests a minor groove binding of the probe with ct-DNA.

Amber* energy minimized geometry supports the minor groove binding event (Figure 3c).

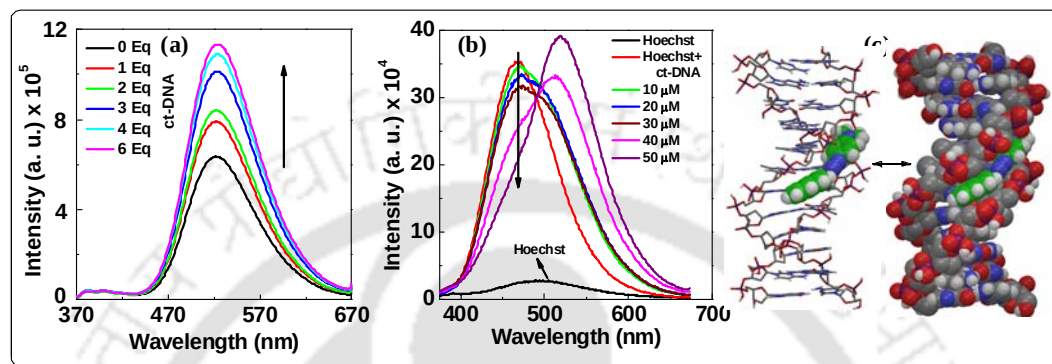


Figure 3: (a) Fluorescence spectra ($\lambda_{\text{ex}} = 345 \text{ nm}$) of ^{TNDMB}Py (50 μM) in presence of increasing ct-DNA concentration. (b) Emission spectra ($\lambda_{\text{ex}} \approx 340 \text{ nm}$) of hoechst 33258 (black line at bottom), hoechst-ct-DNA complex and hoechst-ct-DNA complex mixed with ^{TNDMB}Py. (c) Amber* energy minimized geometry of ^{TNDMB}Py with DNA (PDB Id: 1DNH), showing the minor groove binding of the probe ^{TNDMB}Py.

Chapter 4B: Studies of the Interaction of Smart Fluorescent Probe, Triazolyl Pyrene (^{TNDMB}Py), With Bovin Serum Albumin

A brief literature report of fluorescent probes of BSA as well as detailed findings of our experiment has been presented in this chapter. Thus, A hyperchromicity with very little (7 nm) blue shift of the absorbance wavelength maxima of ^{TNDMB}Py was revealed upon addition of increasing amount of [BSA] from the UV-Vis spectra. From the fluorescence spectra, we observed increased fluorescence intensity with 41 nm blue shift (Figure 4a) of the probe ^{TNDMB}Py with increasing [BSA]. The blue shifting event of the probe ^{TNDMB}Py with increasing [BSA] was further supported by fluorescence image of probe in absence and presence of BSA under UV-light of 254 nm (Figure 4b). The observed binding constant of the probe with BSA was $5.1 \times 10^4 \text{ M}^{-1}$, as was revealed from the fluorescence spectra. The experimental free energy of

binding thus calculated was $\Delta G = -6.41$ kcal/mol, good accorded with the theoretical calculated value $\Delta G = -7.72$ kcal/mol.

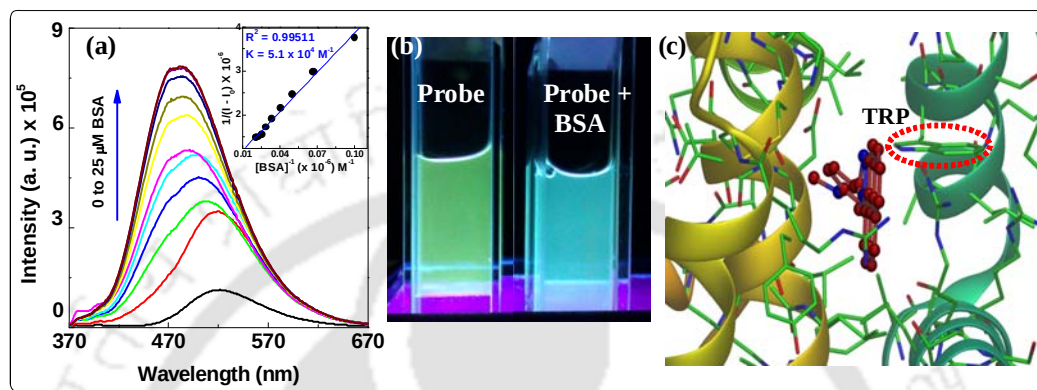


Figure 4: (a) Fluorescence spectra ($\lambda_{\text{ex}} = 345$ nm) of $^{\text{TNDMB}}\text{Py}$ ($20 \mu\text{M}$) in presence of increasing [BSA]. (b) Fluorescence image of probe in absence and presence of BSA under UV-light of 254 nm. (c) Docking pose of $^{\text{TNDMB}}\text{Py}$ in presence of BSA.

Steady state fluorescence anisotropy suggests that the probe is involved in tight binding inside the hydrophobic pocket of BSA and experiences a highly restricted rotational motion. The time resolved fluorescence anisotropy also supported this result. Overlap spectra of BSA's emission and the $^{\text{TNDMB}}\text{Py}$'s absorbance satisfied the possible FRET process. Fluorescence titration experiment of BSA with $^{\text{TNDMB}}\text{Py}$ and the docking study (**Figure 4c**) supported the possible occurrence of FRET process.

Chapter 5: Fluorimetric Sensing of Micellar Microenvironment and Cu^{2+} Ion with Smart Fluorescent Probe, Triazolyl Pyrene ($^{\text{TNDMB}}\text{Py}$)

This chapter deals with fluorimetric sensing of micellar microenvironment and Cu^{2+} ion with smart fluorescent probe, Triazolyl pyrene ($^{\text{TNDMB}}\text{Py}$). This chapter is divided into two subchapters, **Chapter 5A and B** each contains their individual introduction, background, objective, and results and discussions.

Chapter 5A: Probing Micellar Microenvironment with Smart Fluorescent Probe, Triazolyl Pyrene (^{TN}DMBPy)

A brief review of fluorescent probes of micellar microenvironment is presented in this chapter. The absorption spectra of ^{TN}DMBPy in an aqueous solution of varying surfactant concentration, CTAB, SDS and TX-100 showed that as the surfactant concentration was increased the absorbance was increased with no shift in wavelength maxima. The increase of absorbance in each surfactant solution with increase in surfactant concentration was due to the enhanced solubilization of ^{TN}DMBPy in surfactant solutions.

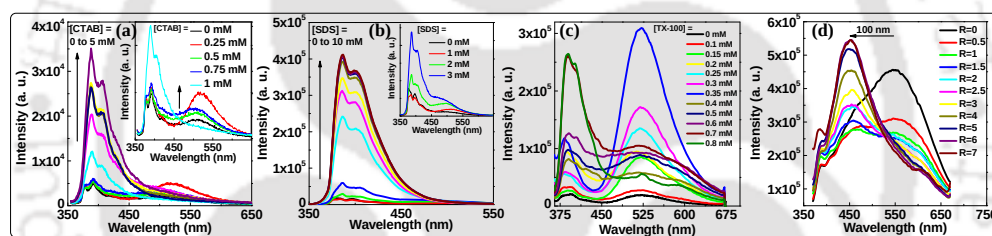


Figure 5: Fluorescence spectra ($\lambda_{\text{ex}} = 344 \text{ nm}$) of ^{TN}DMBPy in aqueous solution of varying surfactant concentration of (a) CTAB, (b) SDS, (c) TX-100 and (d) in reverse micelle of TX-100 in benzene-hexane-water system at 298 K [^{TN}DMBPy] = 10 μM .

From the steady state fluorescence spectra, in ionic surfactant solution (CTAB and SDS), we observed a dual emission (LE and ICT) at low surfactant concentration (insets **Figure 5a-b**). The ICT band showed a blue shifting pattern with enhanced intensity that disappeared as the concentration of surfactant increases ($> 1 \text{ mM}$ for CTAB and $> 3 \text{ mM}$ for SDS, **Figure 5a** and **Figure 5b** respectively). In non-ionic surfactant (Triton X-100) solution, the fluorophore showed dual emission with dominant ICT emission over LE emission at low concentration (up to 0.35 mM, **Figure 5c**). In reverse micelle we observed a blue shifted ICT band with no LE band with increasing molar concentration of water (**Figure 5d**). We found 100 nm blue shifting when we moved from $R = 0$ to $R = 7$, where R is the molar ratio of water to

TX-100 ($R = [H_2O]/[TX-100]$). The blue shifting of ICT band is because of movement of the probe from hydrophilic core to hydrophobic core (surface) of the reverse micelle. Thus from the steady-state fluorescence study it was observed that the ICT band of the probe, ^{TNDBM}Py was more influenced by the micellar environment in comparison to the LE band.

Chapter 5B: Sensing of Cu²⁺ ion with Smart Fluorescent Probe, Triazolyl Pyrene (^{TNDBM}Py)

A brief literature review of Cu²⁺ ion chemosensors is presented in this chapter. Here, we explored the sensing capability of ^{TNDBM}Py towards Cu²⁺ ion in acetonitrile in presence of other metal ions like, Na⁺, K⁺, Mg²⁺, Ca²⁺, Co²⁺, Ni²⁺, Cu⁺, Zn²⁺, Ag⁺ ions spectroscopically (**Figure 6a**). We observed a remarkable change in the emission behavior of triazolyl pyrene in presence of Cu²⁺ ion. With increasing Cu²⁺ ion concentration an excimer band centered at 466 nm appeared along with the monomer band. The intensity of the bands, I_M and I_E and the quantum yields were found to be increased with [Cu²⁺] and it reached maxima when the Cu²⁺ ion concentration was 2.5 equivalents of that of the probe, triazolyl pyrene (**Figure 6b**). The association constant of Cu²⁺ with triazolyl pyrene, was determined by Benesi-Hildebrand plot which comes in the order of $2.23 \times 10^5 \text{ M}^{-1}$. From the Job's plot a 2:1 ligand to metal complexation/association was evidenced which was further supported by presence of mass peak at $m/z [(2L+Cu)^{2+}]$ 841.65 in ESI-MS spectra. DFT calculation supported the experimentally observed stoichiometry as well as the possible occurrence of the excimer emission.

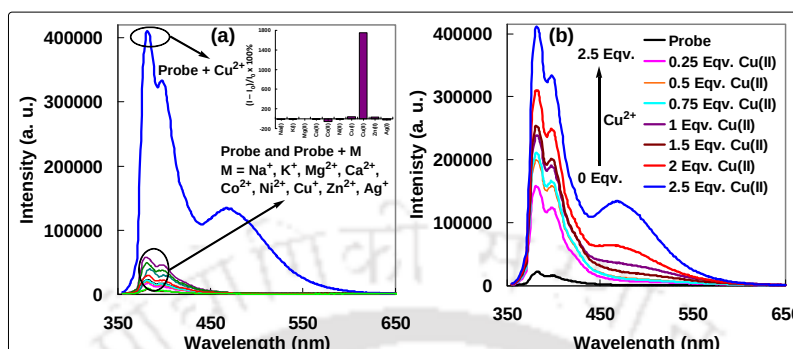


Figure 6: (a) Fluorescence spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of probe (TNDMB-Py) ($10 \mu\text{M}$) in presence of various metal ions (2.5 equiv.). (b) Fluorescence spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of probe (TNDMB-Py) ($10 \mu\text{M}$) in presence of increasing Cu^{2+} concentration.

Chapter 6: Synthesis of Fluorescently Labeled Nucleoside via Sonogashira Coupling and Its Application in Genotyping Single Nucleotide Polymorphisms (SNPs)

A brief literature review of fluorescent probes for detection of SNPs is presented first. This chapter describes the synthesis of fluorescently labeled nucleoside and its application in SNPs detection *via* our conceptual “Just-Mix and Read” strategy (Figure 7).

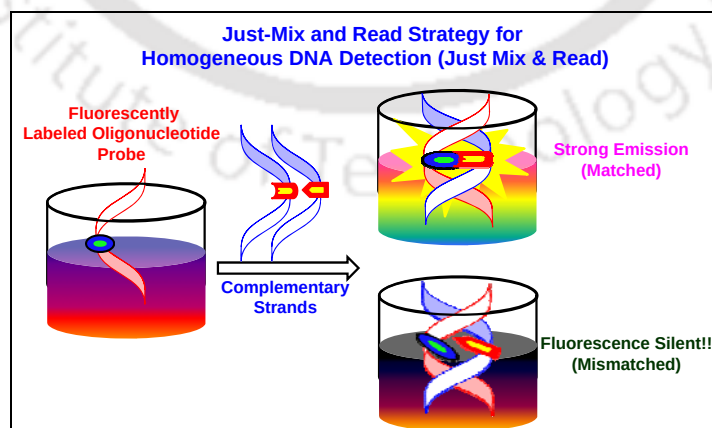


Figure 7: Graphical presentation of our concept of homogeneous DNA detection.

Here, we have developed a new oxo-pyrene labeled fluorescent nucleoside, Oxo-PyU which showed a strong solvent polarity dependent long wavelength fluorescence emission. The designed Oxo-PyU labeled fluorescent oligonucleotide probe was found to be highly efficient for the detection of matched base A of a target DNA opposite to the labeled base, Oxo-PyU of probe DNA *via* an enhancement of fluorescence signal (**Figure 8b**). Furthermore, the doubly labeled probe was also capable of detecting opposite matched consecutive –AA– bases of target DNA with a strong fluorescence signal generation and a large fluorescence brightness factor (**Figure 8c**).

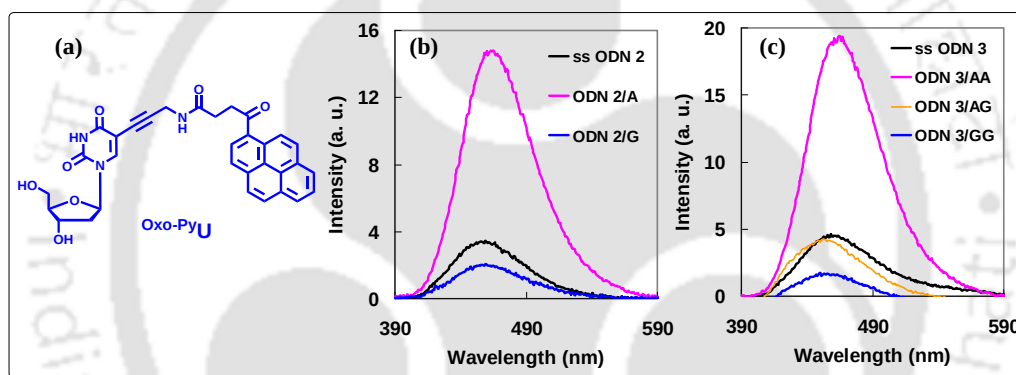


Figure 8: (a) Chemical structure of Oxo-PyU . Fluorescence emission spectra of (b) ss ODN 2 [5'-d(CGCAAT Oxo-PyU TAACGC)-3'] and ODN 2 hybridized with ODN 5'-d(GCGTTA N ATTGCG)-3' [$\text{N} = \text{A}, \text{G}$], and (c) ss ODN 3 [5'-d(CGCAAT Oxo-PyU TAACGC)-3'] and ODN 3 hybridized with ODN 5'-d(GCGTTA NN ATTGCG)-3' [$\text{N} = \text{AA}, \text{AG}, \text{GG}$] in 50mM phosphate buffer of pH 7 containing 100 mM NaCl. [Each single strand concentration was 2.5 μM ; $\lambda_{\text{ex}} = \lambda_{\text{max}}^{\text{abs}}$].

Thus, the doubly labeled ODN which exhibit unique fluorescence properties depending on the number of adenines on the complementary strands, would be effective for the detection of consecutive –AA– sequences and discrimination of –GG– sequences located at a specific site of the target DNA. The probes also could be useful for discrimination of A/G or AA/GG allele as revealed from their fluorescence behavior.

List of Abbreviations

A	Adenine
Ac	Acyl
Ad	Adamantly
AIE	Aggregation-induced emission
A-T-A	Acceptor-triazole- acceptor
Ar	Aryl
A-T-D	Acceptor-triazole-donor
Ag ₂ O	Silver oxide
Abs	Absorbance
Bn	Benzyl
BSA	Bovine serum albumin
B3LYP	Becke, three-parameter, Lee-Yang-Parr
BDF	Base-discriminating fluorescent
BODIPY	4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene.
C	Cytosine
CD	Circular Dichroism
CMC	Critical micelle concentration
CT	Charge Transfer
CTAB	Cetyltrimethylammonium bromide
Cu	Copper
CPMV	Cowpea mosaic virus
CuI	Copper iodide
Cyhex	Cyclohexane
CHCl ₃	Chloroform
CuSO ₄	Copper sulfate
CR	Click reagent

CH ₃ CN	Acetonitrile
D-T-A	Donor-triazole-acceptor
D-T-D	Donor-triazole- donor
Diox/Dx	1,4-Dioxane
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DCE	Dichloroethane
DNA	Deoxyribonucleic acid
DFT	Density functional theory
DCM	Dichloromethane
DIPEA	N,N-Diisopropyl ethyl amine
DMAP	N, N-Dimethylamino pyridine
Et ₃ N	Triethylamine
EtOAc	Ethylacetate
EtOH	Ethanol
eV	Electron volt
ESF	Environment sensitive fluorescent
E. coli	Escherichia coli
Eth	Ethynylphenylalanine
EDC.HCl	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide- hydrochloride
Eqv.	Equivalent
Fl	Fluorescence
FET	Field-effect transistors
FISH	Fluorescence In Situ Hybridization
FRET	Fluorescence Resonance Energy Transfer
G	Guanine

HBr	Hydrogen bromide
HOBT	1-hydroxy-benzotriazole
HRMS	High Resolution Mass Spectroscopy
HSA	Human serum albumin
HTS	Highthroughput Screening
Hpg	Homopargylglycine
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HOMO	Highest Occupied Molecular Orbital
ICT	Intramolecular Charge Transfer
IR	Infrared Spectroscopy
KOH	Potassium hydroxide
K ₂ CO ₃	Potassium carbonate
k	Rate Constant
LE	Local emission
LUMO	Lowest Unoccupied Molecular Orbital
LED	Light-emitting diode
Max	Maxima
MeOH	Methanol
mM	Mili molar
m.p.	Melting point
MALDI-ToF	Matrix Assisted Laser Desorption Ionization-Time of Flight
Na	Sodium
Na ₂ CO ₃	Sodium carbonate
Ni	Nickel
NMR	Nuclear Magnetic Resonance
NRD	Non-radiative decay
nM	Nano molar

nm	Nanometer
ODN	Oligodeoxyribonucleotide
ORTEP	Oak Ridge Thermal Ellipsoid Plot
OLED	Organic light emitting diodes
O ₂	Oxygen
PhCN	Phenyl cyanide
Py	Pyrene
Per	Perylene
PNA	Protein nucleic acid
PCR	Polymerase chain reaction
PRODAN	6-Propionyl-2-dimethylaminonaphthalene
PPh ₃	Triphenyl phosphine
Pd	Palladium
PET	Photoinduced electron transfer
Ph	Phenyl
PLC	Preparative Thin Layer Chromatography
PPTS	Pyridinium <i>para</i> -toluene sulfonate
ppm	Parts per million
r.t.	Room temperature
RNA	Ribonucleic acid
SDS	Sodium Dodecyl sulphate
SNP	Single Nucleotide Polymorphism
SMFM	Single molecule fluorescence microscopy
SATE	S-acetyl-2-thioethyl
ss	Single strand
T	Thymine
TBAF	Tetrabutylammonium fluoride

TBAB	Tetrabutylammonium bromide
TBAOH	Tetrabutylammonium hydroxide
Triton -X-100	4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol
TFE	Trifluoroethanol
TANA	Thioacetamido nucleic acids
TMV	Tobacco mosaic virus
TLC	Thin layer chromatography
THF	Tetrahydrofuran
TDDFT	Time dependent density functional theory
TEA	Triethyl amine
TFA	Trifluoroacetic Acid
THP	Tetrahydropyran
TBS	<i>tert</i> -butyldimethylsilyl
TMS	Trimethylsilyl
TLC	Thin Layer Chromatography
UV	Ultra violet
μM	Micro molar
Wt	Water
Φ	Quantum Yield
ϵ	Molar extinction co-efficient
τ	Decay time
$\text{\AA}$	Angstrom (10^{-8}cm)
$\tilde{\nu}$	Wave Number
λ	Wave Length
λ_{max}^{abs}	Absorption maxima
λ_{max}^{fl}	Fluorescence maxima
A_f	Solvent polarity parameter

μ_e	Excited state dipole moment
μ_g	Ground state dipole moment

NMR Data

δ	Chemical shift in NMR
s	singlet
d	doublet
t	triplet
q	quartet
m	multiplet
bs	broad singlet
dd	double doublet
dt	doublet of triplet
ddd	doublet of doublet of doublet
J	coupling constant in Hz

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Chapter 1

**APPLICATION OF “CLICK” REACTION
AND “SONOGASHIRA” COUPLING TO
THE SYNTHESIS OF FLUORESCENT
SMALL ORGANIC MOLECULES: A
REVIEW**

1.1. Introduction

Unraveling the structure, functions, dynamics, and intermolecular interactions of biological macromolecules, cells, and organisms relied on the development of fluorescence-based techniques.¹ Fluorescence is one of the most informative, sensitive, and operationally simple analytical techniques.² Therefore, it has found widespread applications in chemical and biochemical sensing processes, and in many areas/disciplines ranging from environmental science, medicine, pharmacy, cellular biology, nano materials to genetics.³ Fluorescence based detection techniques are now used routinely in numerous applications, such as DNA sequencing,¹ⁱ in situ genetic analysis,^{1j} single nucleotide polymorphism (SNPs) typing through techniques like fluorescence in situ hybridization (FISH),^{1k} and highthroughput screening (HTS).^{1l} However, many of the biomolecules are complex in nature and do not show any inherent emissive properties in these intricate interactions. Excluding a few amino acids, the common biomolecular building blocks lack significant fluorescence properties. For example, the intrinsic fluorescence of the naturally occurring nucleotide bases in nucleic acids is extremely weak. These bases exhibit very short fluorescent decay times in the range of a few picoseconds, and do not provide much structural information since signals are normally averaged over all bases in the oligonucleotide sequence.

Therefore, the development of ideal probe, the small fluorescent molecules/fluorescent biomolecular building blocks having solvofluorochromic properties for monitoring microenvironmental change around biomolecules is a very important research target for understanding biological events associated with interbiomolecular interactions. In particular, monitoring the change of local microenvironments such as dielectric properties in DNA, proteins, cell membrane is highly important for understanding structures, functions, dynamics of the biomolecules and interbiomolecular interactions. In such a probing scenario, the fluorophore's emission property may be modulated such as fluorescence can be

enhanced/quenched and/or emission can be shifted to red/blue region thereby enabling visual observations of the biomacromolecular structure, dynamics, and functions.

An ideal probe for monitoring various structures and dynamics of such biomolecules and its surroundings should be sensitive to its local biomolecular microenvironment, including pH, viscosity, biological analytes, and solvent polarity, and should interact strongly with the biomolecules via electrostatic/H-bonding and other noncovalent interactions. Also, design of small organic fluorophores having strong absorption and long emission wavelength have attracted attention in recent years because they can extract inner biomolecular informations when conjugated to or complexed with a biomolecule. In this respect the best suited fluorescent probes are mostly solvatochromic fluorophores that are being widely used as reporter probes for investigating chemical, and biochemical phenomena because of their ability in sensing a small variation in dielectric constants within a biomolecular microenvironment. Also, for biomolecular application, long wavelength emission, especially emission at visible region is highly desirable; otherwise the auto fluorescence from biological macromolecules would inhibit the detection sensitivity.

As a result of tremendous research effort, several fluorescent probes have been reported for probing biological microenvironment with a generation of distinct and readable fluorescence signal. As for example, secharide sensor, fructose sensor, fluorescence sensors for metal ions in living cells were developed and reported. Also, a number of fluorescent nucleosides/fluorescently labeled nucleosides, fluorescent amino acids, *etc.*, have appeared in the literature as biomolecular building blocks for probing of nucleic acid's /protein's structures, dynamics, and functions. All these efforts are aimed at understanding the structure, dynamics and functions of such biomolecular entity by studying the detectable signal generated from sensitive fluorescence techniques. All the research efforts are in success with the use of microenvironment sensitive solvatochromic fluorophores. Few of such examples of fluorophores used as biosensors⁴ and chemosensors⁵⁻⁶ are shown in **Figure 1.1**.

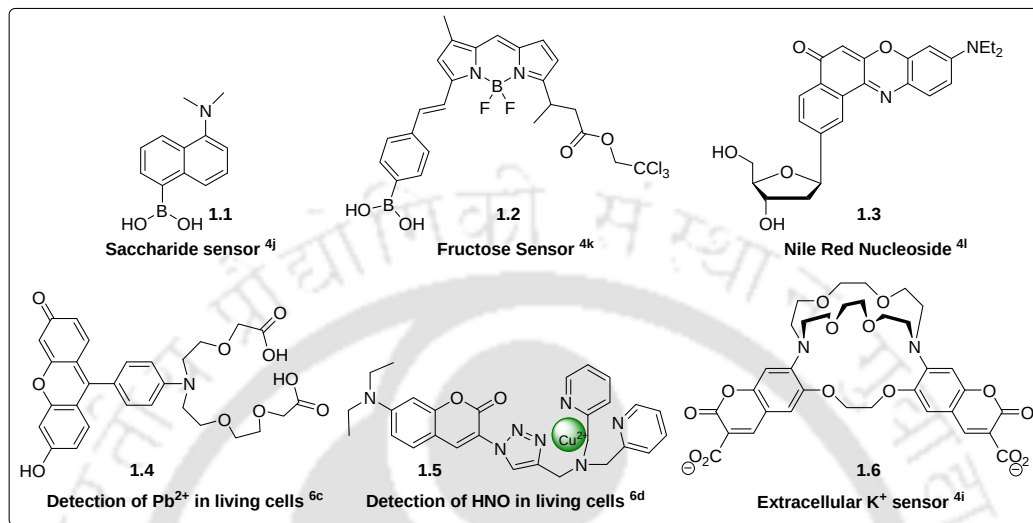


Figure 1.1: Examples of some fluorophores used in chemical/biochemical sensing events.

Fluorescence based detection of cations and anions are also of great interest to many scientists including chemists, biochemists, clinical biochemists and environmentalists. Essential trace elements like sodium, chlorine, potassium, calcium, magnesium, zinc, iron, manganese, copper, iodine *etc.*, are required to support human biochemical processes. Their insufficiency in human body causes various diseases. So, detection of those essential trace elements and study of their level of insufficiency is of high importance. Metal ions like arsenic, cadmium, and heavy metal ions like mercury, lead present in water are toxic for living organism. Their detection in the environment is thus also highly desirable. As a result of tremendous research effort, a large number of fluorophores and detection methodologies have been developed to address the metal ion causing pollution to the environment and the trace metal ions essential for maintaining human health. Few of such examples of fluorophores used as sensors for metal ions⁷⁻⁸ are shown in **Figure 1.2**.

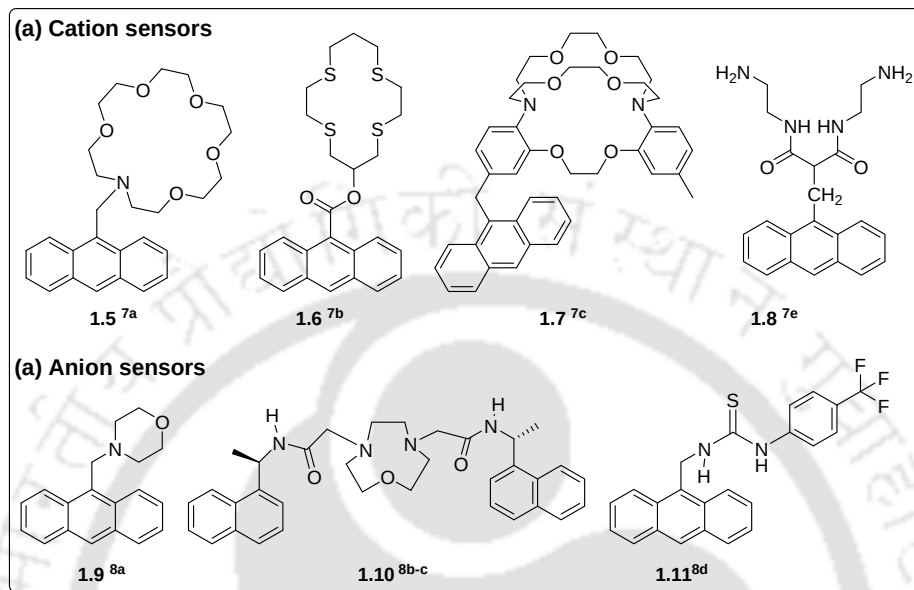


Figure 1.2: Examples of some fluorophores used as cation and anion sensors.

In this chapter, we would like to describe in brief few more fluorescent small molecules generated *via* two important reactions, "click reaction", and Sonogashira coupling and their applications in various field of chemical and biological sciences. A general description of fluorescence spectroscopy, solvatochromism that have been applied to chemical and biomolecular studies is also presented here.

1.2. Fluorescence Spectroscopy and Its Applications

Spectroscopy-based technique is highly sensitive which depends on the nature of the transitions involved. Upon excitation at the absorbance wavelength maxima of a chromophore Franck–Condon¹⁰ state is generated within 10^{-15} sec, the efficiency of which depends on the chromophore's molar absorptivity. The vibrational relaxation, soon after excitation, quickly populates the lowest vibrational level of the chromophore's excited electronic state (**Figure 1.3**, Jablonski diagram).² This relaxation process, generating the emissive state, accounts for the lower emission energy of a chromophore compared to its excitation energy which is popularly known

as Stoke's shift. Typical organic chromophores reside in their excited state for a period of $(0.5\text{--}20) \times 10^{-9}$ s. The excited state lifetime reveals the sum of the various radiative and nonradiative processes that the excited chromophore undergoes while decaying back to the ground state (τ_0). The parameter quantum yield, Φ , of the chromophores is defined as the fraction responsible for emitting a photon, or the fluorescence lifetime (τ) ($\Phi = \tau/\tau_0$). The fluorescence brightness ($\epsilon \times \Phi$) of a fluorophore is currently an important measure which is the product of the molar absorptivity (ϵ) and the fluorescence quantum yield (Φ) and denote a fluorophore's efficiency to visualize an event. This becomes useful parameter when comparing the utility of two fluorophores with similar fluorescence quantum yields but with different molar absorptivities.

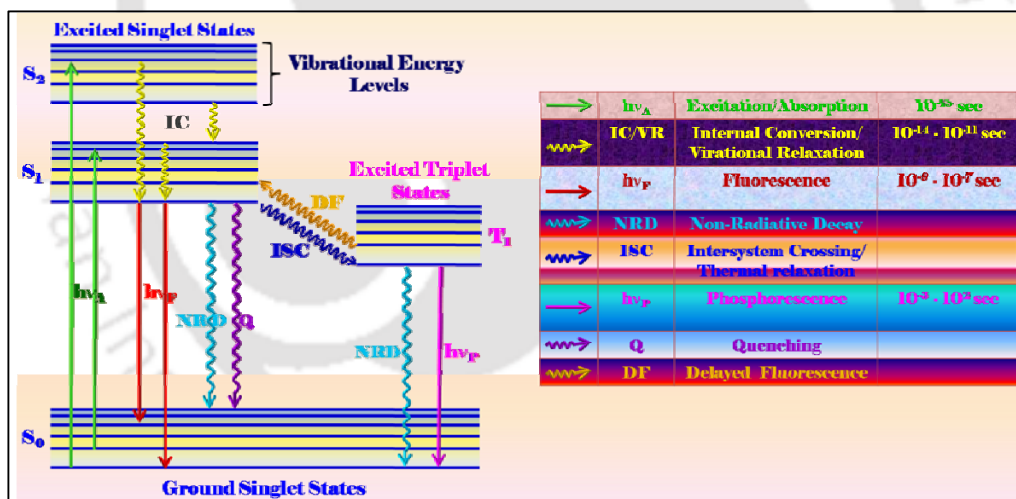


Figure 1.3: A simplified Jablonski diagram.

Several fluorescence based techniques are widely used in elucidation of many chemical and biological events. As for example (a) Steady-State Fluorescence,^{2,11a} (b) Fluorescence Resonance Energy Transfer (FRET),^{2,11b} (c) Time-Resolved Fluorescence,^{2,11c} (d) Fluorescence Anisotropy,^{2,11d} (e) Fluorescence Microscopy and Single Molecule Spectroscopy,^{2,11e} and (f) *In vivo* Fluorescence Imaging,^{2,11f-g} are

commonly used for their versatility and sensitivity. Creative probe design can provide chromophores with appropriate excitation and emission wavelengths, while minimizing interference by any other background auto fluorescence from cellular constituents. Selective excitation coupled with sensitivity of many chromophores to various environmental parameters like pH, polarity, viscosity, presence of quenchers, etc., makes molecular fluorescence an extremely versatile and efficient tool for in vitro biophysical and biochemical analyses, high-throughput screening assays^{12a} for drug discovery, in vivo cellular imaging, as well as in cellular studies to provide spatial and temporal information by super-resolution microscopy.^{12b}

1.3. Solvatochromic Fluorophores

The phenomenon of solvent polarity sensitive emission by a fluorophore is known as solvatochromism and the fluorophore is said to be solvatochromic. For such fluorophores, the dipole moment of the excited state, in general, is greater than that of the ground state. Therefore, rearrangement of solvent molecules can lower the energy of the excited state prior to the emission, resulting in a red shift of the emission wavelength maximum. Solvatochromic fluorophores exhibit emission properties, such as emission wavelengths, quantum yields and fluorescence lifetimes, are highly sensitive to their local environment. Such dynamic behavior makes these parameters particularly well suited for investigating biomolecular interactions because it provides information on the state of a biomolecule within their microenvironment. For example, if a solvatochromic fluorophore is appended to nucleic acid or the surface of a protein or a membrane at a site that is involved in a transient binding interaction or undergo a conformational change, then the probe would report such events provided they are coupled to modifications in the local solvent sphere.

1.4. Origin of Solvatochromism

The effect of solvent polarity on the emission properties of a solvatochromic fluorophore are generalized in the Jablonski diagram² in **Figure 1.4**, which depicts the energies of the different electronic states of the system.

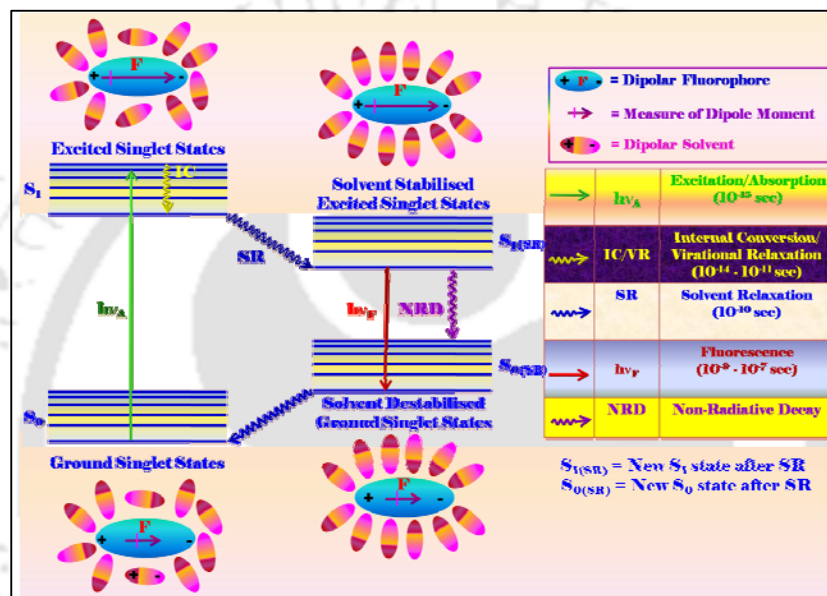


Figure 1.4: Origin of solvatochromic fluorescence emission.

As shown in the diagram, the dipolar fluorophore resides in the ground electronic state, S_0 , surrounded by a sphere of polar solvent molecules. Upon absorption of light energy ($h\nu_A$), the fluorophore-solvent system is rapidly promoted to singlet excited state, S_1 . During this event, the system adopts a new electronic configuration with an increased dipole moment that differs significantly from that of the ground state. This process of electronic excitation of solvent bound fluorophore occurs in a much faster rate than that of the motions of atomic nuclei (Frank–Condon principle¹⁰). Then, the solvent spheres reorient dipoles to accommodate the generated larger dipole of the fluorophore in the picosecond time-scale leading to the development of highly ordered arrangement in S_1 state. This step is known as solvent relaxation which ultimately lowers the energy of the excited singlet state ($S_{1(SR)}$) while simultaneously

destabilizes the ground state ($S_{0(SR)}$). Therefore, the energy gap between the S_1 and S_0 state decreases down significantly. The system finally returns to the ground state ($S_{0(SR)}$) with the emission of photon of a much longer wavelength (lower energy, $h\nu_F$; a fluorescence event) than that was originally absorbed during excitation. Thus, the solvatochromic fluorophores emit at a much longer wavelength with increase in solvent polarity as the degree of solvent relaxation increases with increasing solvent polarity.

As the energy gap between the S_1 and S_0 states is reduced, there is a high possibility that solvatochromic fluorophores exhibit a marked increase in non-radiative decay. Thus, in many instances, the fluorophore returns spontaneously to the ground electronic state through a thermal non-radiative decay process (NRD, k_{nr}) that competes with fluorescence. This effect is much more prominent particularly in polar protic solvents such as water, methanol, etc., and results in a decrease in the fluorescence quantum yield. The mechanisms for such processes include a range of events such as internal charge transfer, H-bonding, tautomerization, isomerization, and intersystem crossing to an excited triplet state (T_1). In such a competing scenario in polar solvents, perturbing the ordered solvent sphere, one can exploit the fluorophore to show sensitive switch-like emission property. **Figure 1.5** is a pictorial presentation of shifting of fluorescence wavelength maxima as the polarity of the solvent increases.

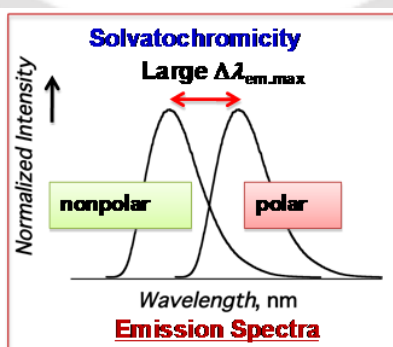


Figure 1.5: Schematic representation of solvatofluorochromicity.

1.5. Importance of Solvatochromic Fluorophores in Biochemical Studies

Monitoring the change of local microenvironments such as dielectric properties in biomolecules is highly important for understanding interbiomolecular interactions. However, because the naturally occurring biomolecular building blocks such as, DNA bases, are faintly fluorescent, studies on biomolecules/nucleic acids are mostly dependent on the use of extrinsic fluorescent species. However, study of protein biomolecules can be done with the use of both intrinsic as well as extrinsic fluorescent species. For example, the fluorescence of the natural amino acid, tryptophan has long been known to be environment-dependent and has been widely used in studying protein folding and ligand-binding events in solution. However, its complex photophysics, short wavelength excitation, and the relative abundance of tryptophan in nature strongly limit its potential for applications in complex protein systems. Therefore, the development of covalent extrinsic fluorophores and their linkage to protein functional groups has been used to solve this problem.

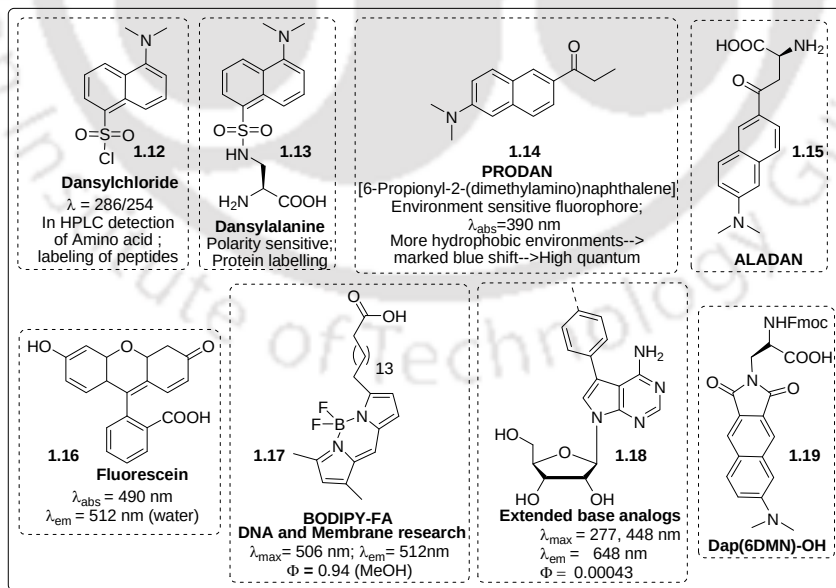


Figure 1.6: Examples of some solvatochromic fluorophores/fluorescent biomolecular building blocks used in biochemical studies.

Similarly, in nucleic acid world, the natural nucleobases are faintly fluorescence, therefore, their use in sensing or detection of DNA comes with no information. Thus, the lack of significant fluorescence of naturally occurring nucleobases or lack of large abundance or numbers of naturally occurring fluorescent amino acids or other biomolecular building blocks, has prompted the design and application of extrinsic synthetic fluorophores with improved photophysical properties. Thus, several probes have been reported based on the fluorophore's properties like intercalation, stacking, and/or groove binding for DNA detection with a generation of enhanced fluorescence signal. On the other hand, protein binding ability of several chromophores also have been studied *via* an improved fluorescence signal generation. These efforts have led to the emergence of a number of solvatochromic probes with diverse properties. Some examples of such solvatochromic fluorescent molecules/biomolecular building blocks are depicted in **Figure 1.6**.

1.6. Use of Solvatochromic Fluorophores in Probing Biochemical Events

Fluorophores with emission maxima that display sensitivity to solvent polarity, popularly known as solvatochromic fluorophores, are widely used in the study of biomolecular microenvironment surrounding the chromophore. Therefore, they are widely being used in various research field of biochemical interest which includes:

- (a) In Carbohydrates Research
- (b) In Phospholipids and Fatty Acids Research
- (c) In Amino Acids and Protein Research
- (d) In Nucleic Acid Research

Thus, fluorescent analogues of biomolecular building blocks or small solvatochromic fluorophores are used to get information about the function of carbohydrates in biological systems; they are being widely used as sensors for saccharides^{13a-b} and in metabolic saccharide engineering^{13c-d}. Fluorescent probes also

have greatly contributed to our understanding of the properties and function of biological membranes. As for example, they are widely used for studying membrane polarity, permeability, and bilayer permeability. For studying the activity of membrane-bound proteins,^{14a-b} and protein–lipid interactions, the fluorescent probes are being exploited. Also, in amino acids and protein research, fluorescence spectroscopy has proven extremely useful instrumental technique in shedding light on their intricacies. Therefore, solvatochromic fluorescent probes have found applications in (a) determination of structure, conformation and function of proteins, (b) studying folding/unfolding state of protein,^{15a} (c) the estimation of local polarity or in the study of binding events *via* the different response from solvatochromic probes to changes in polarity, (d) photocontrol of a biological function by using photoswitchable chromophores, and in (e) building protein models in synthetic polypeptides.^{15b} Few examples of solvatochromic fluorescent probes used in probing biochemical events are shown in **Figure 1.7**.

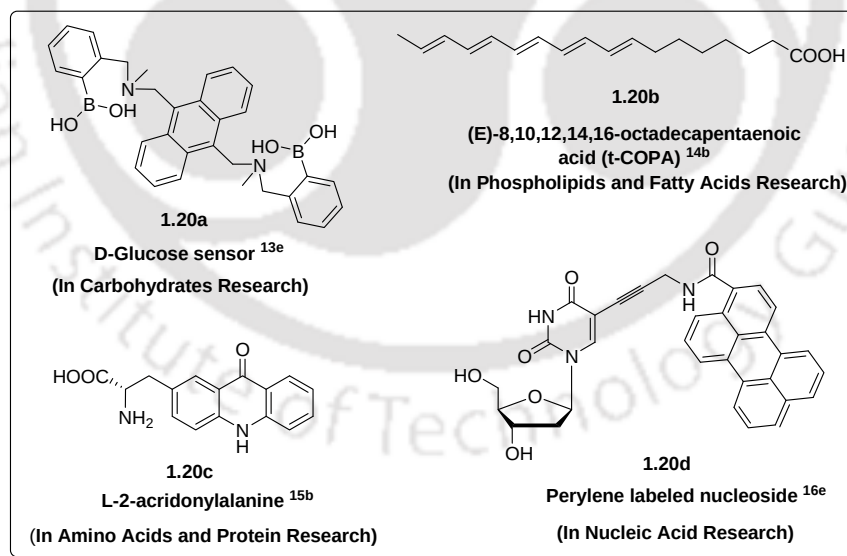


Figure 1.7: Some more examples of fluorescent analogues of biomolecular building blocks/fluorescent molecules used in probing biochemical events.

Fluorescent nucleosides analogues, like, (a) chromophoric base analogs, (b) pteridines, (c) expanded nucleobases, (d) extended nucleobases, (e) isomorphous nucleobases and (f) modified nucleosides-covalently linked with solvatochromic fluorophores, have greatly contributed to our still growing understanding of nucleic acids' structures, recognitions, and functions. Small solvatochromic organic fluorophore labeled oligonucleotide probes are now a days widely used in (a) single nucleotide polymorphisms (SNPs) typing which has attracted attention due to their relevance to human health and ultimately for the realization of the concept of personalized medicine,^{16a-b} (b) determination of nucleic acid structure and function,^{16c-d} (c) investigating change in local microenvironment around nucleic acids, (d) monitoring ligand binding, and in (e) the study of DNA-protein interactions.^{16c-d}

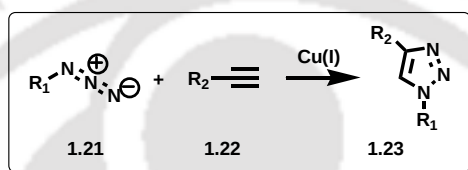
Now a days, a combination of a large number of tools, techniques and methodologies of organic chemistry along with the various design architectures focusing the research interest has led to the creation of various fluorescent small molecules/biomolecular building blocks of interesting photophysical properties applicable for several research purposes, such as sensing and probing of various chemical/biochemical events.

Out of several organic named and unnamed reactions applicable for the synthesis of fluorescent molecules, we are interested mainly on “click reaction”, and palladium (0) mediated Sonogashira coupling as two important organic reaction strategies to generate such fluorescent small molecules. Hence, here, we want to describe few of such fluorescent small molecules generated *via* these two reactions and applications of few of such molecules in various research fields of chemical and biological sciences.

1.7. Click Reaction to the Synthesis of Fluorescent Small Molecules

Since the introduction of “click” reaction by K. B. Sharpless and his co-workers in 2001, it has become a very useful as well as a powerful reaction as a chemoselective ligation strategy for labeling of biomolecules. Click reaction is very simple, mild and

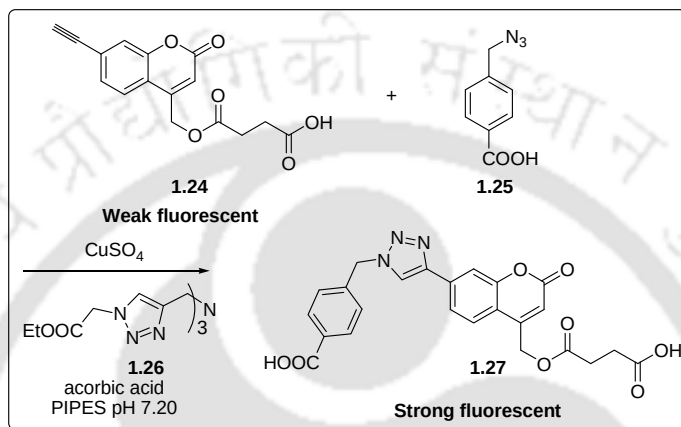
does not need tedious reaction steps (**Scheme 1.1**). In this respect Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction of azides with terminal alkynes^{17a-c} is a powerful and very robust transformation that has been successfully used for the fluorescent labeling of proteins,^{17d} fluorescent labeling and imaging of glycans,^{17e-f} the cell surface labeling of *Escherichia coli*,^{17g} the activity-based protein profiling in live mice^{17h} and in many other cases in many research area.



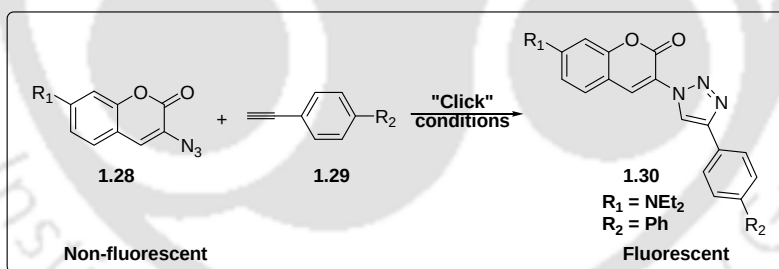
Scheme 1.1: Cu(I) catalysed Huisgen 1,3-dipolar cycloaddition.

Recently, the click reaction has been widely used not only as a mere ligation strategy but also for the generation of several molecules with various designed architectural framework utilized for various applications such as, in host guest chemistry, in metal coordination for sensing and detection of metal ions and in the generation of modulated solvatochromic fluorescent small organic molecules for possible application in sensing in chemical and biochemical research. Several research groups jumped into this sophisticated reaction and utilized it for various purposes. As for example, Fahrni *et al.*,^{18a} installed the fluorescence property into the non-fluorescent or weakly fluorescent azido or alkynyl coumarin derivative by using Cu(I) catalyzed click chemistry (**Scheme 1.2**). Wang *et al.*,^{18b} also did the same job *via* Cu(I) catalyzed click chemistry (**Scheme 1.3**). As the reaction condition is mild, so, it would allow to synthesize a large library of fluorescently labeled dyes. Both the azide and alkyne are quite inert to biological systems, so this reaction has potential in bioconjugation and bioimaging applications. This feature of the click reaction results in useful applications in (a) fluorescence labeling and imaging of glycans inside the cells,^{17e-f} (b) monitoring the conformational change of the protein by Förster resonance energy transfer (FRET) measurements,^{19a} (c) selective dye-labeling of

newly synthesized proteins in bacterial cells,^{19b} (d) fluorescence visualization of newly synthesized proteins in mammalian cells,^{19c} and also in (e) fluorescence visualization of lipids in living cells.^{19d}



Scheme 1.2: Synthesis of highly fluorescent triazolyl coumarin derivative *via* click reaction.



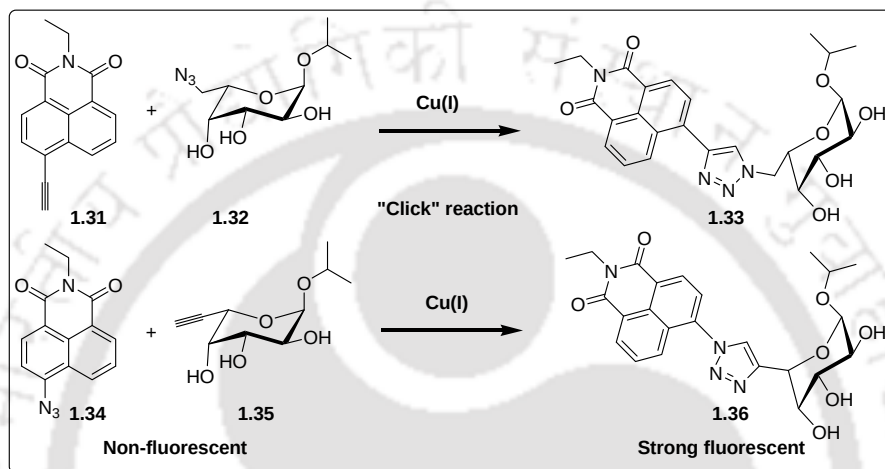
Scheme 1.3: Synthesis of fluorescent triazolyl coumarin *via* click reaction.

1.7.1. Biological Applications of Click Chemistry/Click Fluorophores

1.7.1.1. Fluorescence Labeling of Glycans

Labeling of biomolecules is dependent on the use of solvatochromic fluorophores and has got attention in recent years. Thus, Wong group^{17e} have developed a fluorescent labeling technique based on click chemistry which allowed rapid, versatile, and specific covalent labeling of cellular glycans bearing azide groups (Scheme 1.4). The fluorescent probe has been generated from a nonfluorescent

precursor, 4-ethynyl-*N*-ethyl-1,8-naphthalimide *via* the click reaction with an azido-modified sugar. This fluorogenic click-product permits imaging of glycans at the cell surface and inside the cell (**Figure 1.8**).



Scheme 1.4: Schematic representation of the synthesis of strongly fluorogenic probes **1.33** and **1.36** *via* click reaction.

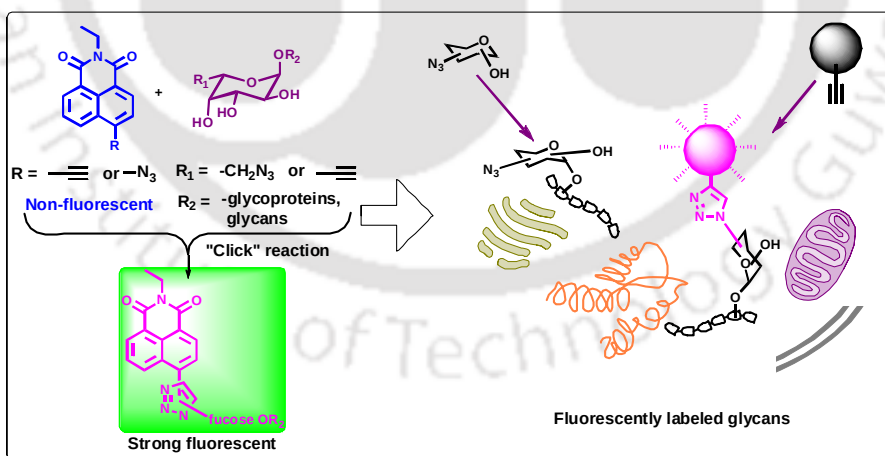
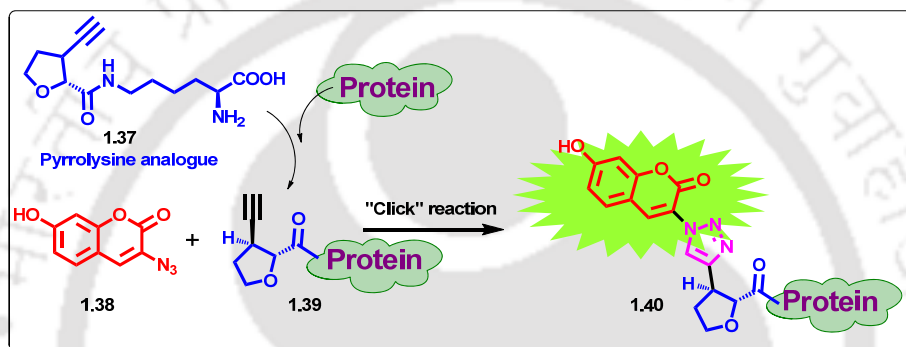


Figure 1.8: Strategy for fluorescent labeling of glycans in cells.

1.7.1.2. Fluorescence Labeling of Proteins

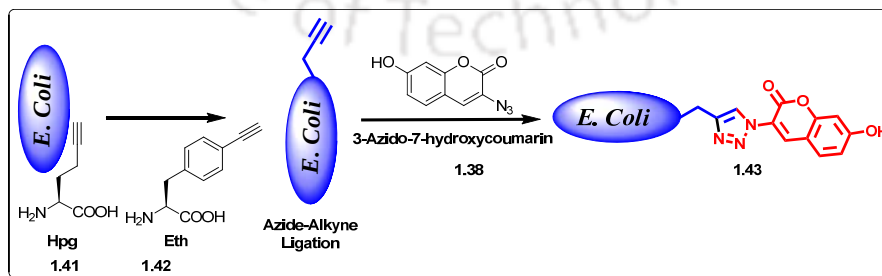
Highly solvatochromic fluorescent molecules find application as fluorescent tag of protein for visualization of their conformation and interaction with other

biomolecules. Thus, Chan *et al.*,^{19a} have introduced a new pyrrolysine congener containing a terminal acetylene and incorporated into recombinant protein through the UAG codon. This protein containing ethynyl pyrrolysine upon reaction with azidocoumarin *via* click reaction afforded highly fluorescent triazolyl coumarin labeled protein (**Scheme 1.5**). With this technique, they monitored the conformational change of the protein by Förster resonance energy transfer (FRET) measurements.

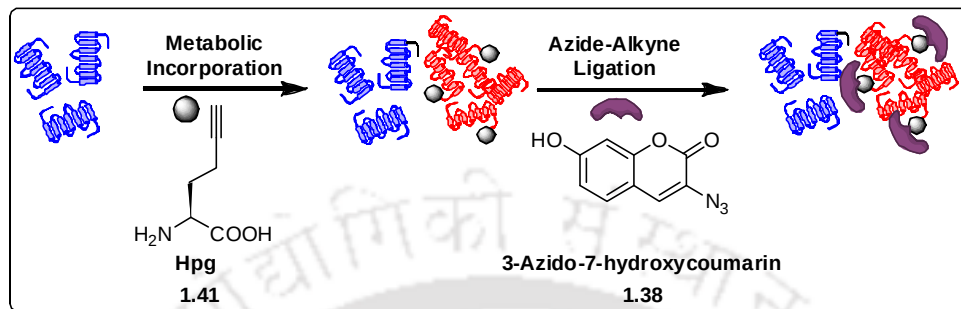


Scheme 1.5: Schematic representation for the synthesis of fluorescently labeled protein.

Fluorogenic labeling of protein in bacterial cell surface and in mammalian cells with the help of click chemistry have been reported by Tirrell *et al.*,^{19b-c} (**Scheme 1.6** and **Scheme 1.7** respectively). They incorporated the alkynylamino acid into recombinant proteins to get alkynyl sites for attachment of fluorogenic dye 3-azido-7-hydroxycoumarin (**1.38**) *via* click reaction. This technique can be utilized to visualize proteins of unknown sequence, structure, and function.



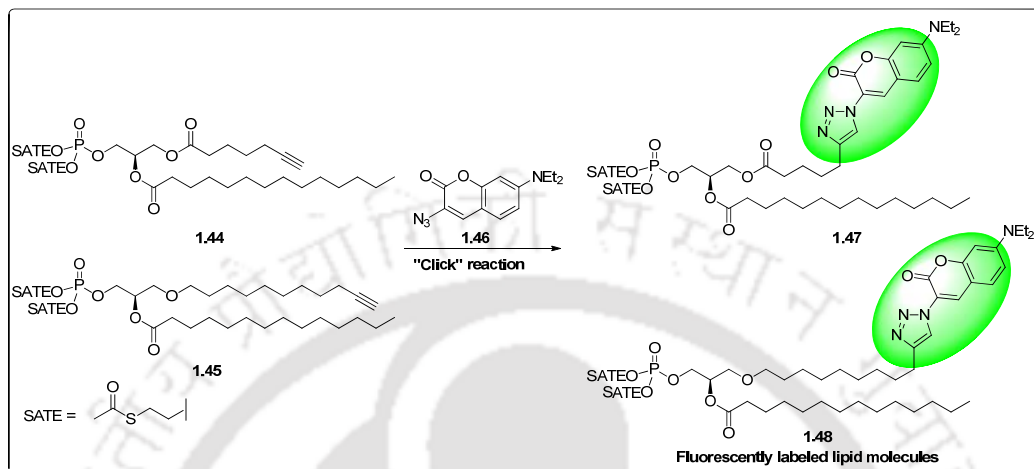
Scheme 1.6: Schematic representation of fluorogenic labeling in *E. coli* cells *via* click reaction.



Scheme 1.7: Fluorescent labeling of proteins *via* click chemistry for fluorescence visualization of mammalian cells.

1.7.1.3. Fluorescence Labeling of Lipids in Living Cells

Monitoring the structure, functions and dynamics of biomolecules, like lipids or cell, need labeling with a fluorescent molecules so as to visualize the biomolecules with the help of highly sensitive fluorescence based technique. As for example, Schultz *et al.*,^{19d} have reported for the first time, the fluorescent labeling of lipids by click reaction in fixed and living cells (**Scheme 1.8**). Thus, Cu(I) catalyzed 1,3-dipolar cycloaddition reaction between fluorogenic dye 3-azido-7-*N,N*-diethylcoumarin (**1.46**) and alkynyl lipid molecules **1.44** and **1.45** afforded fluorescent triazolyl coumarin labeled lipid molecules **1.47** and **1.48** respectively. This labeling technique would enable the monitoring of lipid dynamics in both fixed and living cells and would give a new insight into cellular distribution, lipid interaction partner, and behavior of lipid in response to endogenous or environmental stimuli.



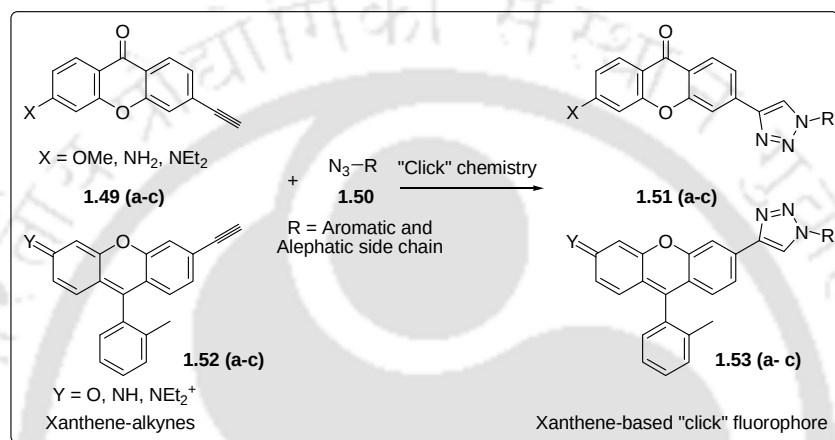
Scheme 1.8: Fluorescence labeling of lipids *via* click chemistry.

Up till now, a very few fluorogenic click substrates are available in which "click" chemistry directly involved to install the fluorescence property. Even many of them absorb light in the UV region which makes them inefficient for a wide range of applications. As the biological system containing fluorophores absorb light in the UV-region, so those UV-excited click fluorophores are not a good choice or suitable for bioimaging applications. Thus, it is very important to develop triazole based long wavelength emissive fluorescent molecules so as to apply them in biological study avoiding the interference of the background auto fluorescence signal generated from fluorescent biomolecules.

1.7.1.4. Click-Fluorophore with Long Wavelength Absorbance for Possible Bioimaging Application

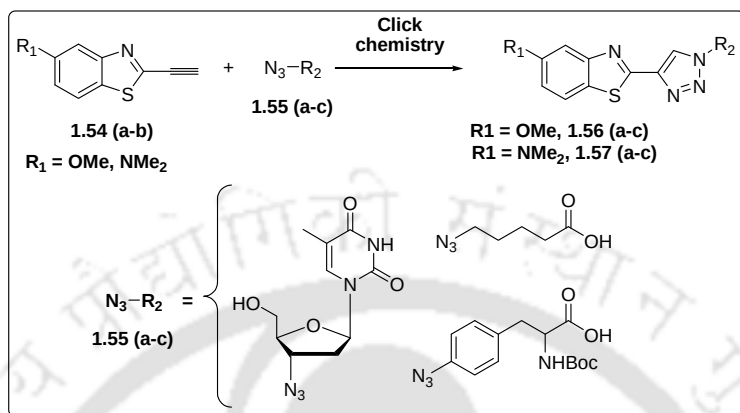
Highly solvatochromic and long wavelength emissive fluorescent molecules are useful in bioconjugation and possible bioimaging to acquire information about biomolecular structures, functions, dynamics and interbiomolecular interaction. Towards this end, Yao *et al.*,^{20a} have introduced some xanthene-based click fluorophores (1.51 and 1.53) using xanthene-alkynes (1.49 and 1.52) and aromatic or

aliphatic azides (**1.50**) (**Scheme 1.9**). The synthesized fluorophore showed long wavelength emission and absorption in near visible region. These fluorophores could potentially be used as green light-emitting substitutes for the existing "click" fluorophores in bioconjugation and bioimaging applications.



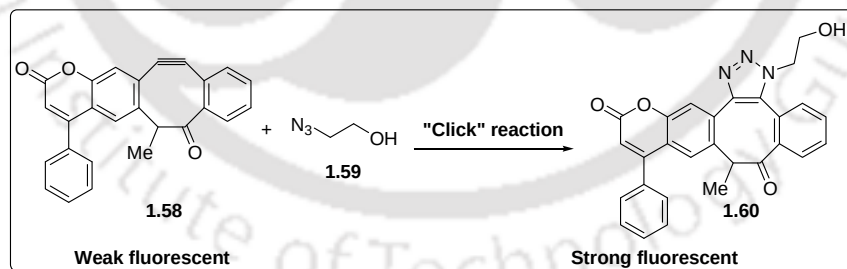
Scheme 1.9: Synthesis of xanthone based fluorophores using click chemistry.

Recently, Tung *et al.*,^{20b} have developed some benzothiazole based visible fluorogenic click fluorophores (**1.56** and **1.57**, **Scheme 1.10**) for cellular imaging. They have shown that two alkynyl benzothiazole derivatives with an electron-donating dimethylamino or methoxy group at the 6-position of 2-ethynyl-benzothiazole showed 30 to 130 nm red-shift in fluorescence wavelength upon click reaction with various azides, compared to the parent benzothiazole fluorogenic dye. Thus, their model reactions with functionalized nucleoside and amino acid (**Scheme 1.10**) indicated that the developed 'click-on' dye could be applied to labeling of various biomolecules, such as nucleic acids, proteins, and other molecules, in cells.



Scheme 1.10: Synthesis of triazolyl benzothiazole as visible fluorogenic 'click-on' dyes.

A triazole based fluorogenic cyclooctene derivatives with the help of click chemistry have been reported by Bertozzi *et al.*,^{20c} (**Scheme 1.11**). Cyclooctyne probe (**1.58**) becomes fluorescent upon reaction with azide (**1.59**) under click condition to form fluorogenic triazolylcyclooctene derivative (**1.60**) with strongly fluorescent emission property. Thus, this type of cyclooctyne probe can be used for fluorescence imaging of azide-labeled biomolecules.



Scheme 1.11: Synthesis of triazole based fluorogenic cyclooctene derivatives.

1.7.2. Chemical Applications of Click Chemistry/Click Fluorophores

Apart from biological applications, click fluorophores are increasingly used as chemosensor. Though the triazole moiety mainly acts as a ligation for biological application, its hetero atoms, nitrogens, have potential to bind both with metal cations

as well as anions. At present very few triazole based fluorescent chemosensor are available, few of them with their application are shown in **Figure 1.9**.

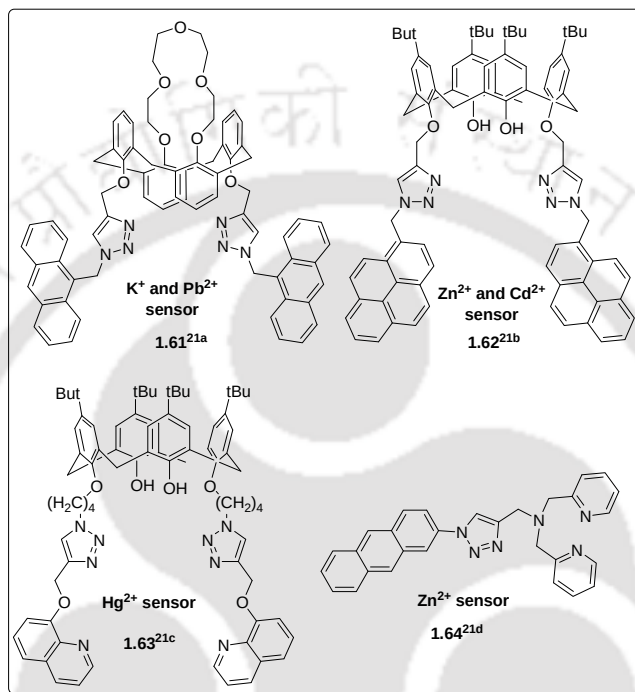


Figure 1.9: Chemical structure and applications of some triazole based chemosensors.

Imperiali *et al.*,²² have developed long wavelength emissive triazole-substituted 8-hydroxyquinoline derivatives *via* click reaction (**Figure 1.10**) and successfully incorporated into peptide, containing protein kinase recognition sequences to act as a reporter of kinase activity. This triazole derivative showed red shift in excitation and emission wavelength when complexed with Mg²⁺ ion in HEPES buffer (pH = 7.4). Comparative study of synthetic phosphorylated and unphosphorylated peptides in the presence of Mg²⁺ showed that the emission of incorporated fluorophore was greater for phosphorylated peptides than the unphosphorylated peptides. Binding of Mg²⁺ with ring nitrogen and hydroxyl oxygen of 8-hydroxyquinoline derivative results in a stronger chelation-enhanced fluorescence of phosphorylated peptide.

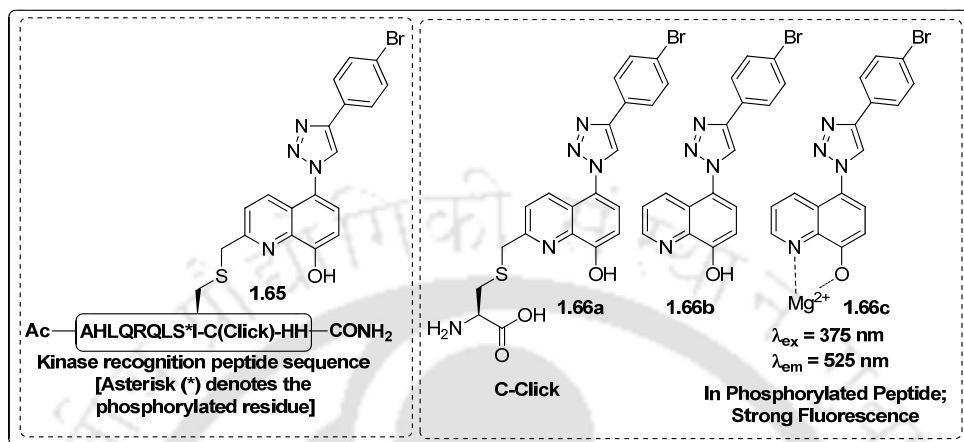


Figure 1.10: Chemical structure of synthesized click fluorophore and its probable binding with Mg^{2+} ion.

From the above discussed click-fluorophores, it is clear that click chemistry have a great impact to the generation/modulation of fluorescence response; thereby enabling efficient fluorescence based strategy for chemical/biochemical application. As was mentioned earlier, we will now focus on the strength of Sonogashira coupling protocol to the synthesis of highly conjugated molecules of various designed architectures applicable for various chemical/biochemical researches.

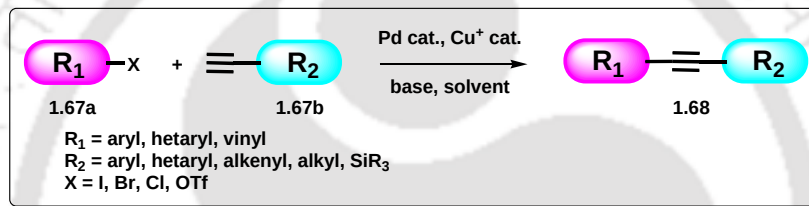
1.8. Sonogashira Coupling to the Synthesis of Fluorescent Small Molecules

Since the discovery of Sonogashira coupling²³ in 1975, it has become a powerful tool and has got widespread applications for the synthesis of various alkynyl conjugated fluorescent molecules for the applications in various research fields such as,

- in synthesis of conjugated molecules for sensory applications,
- synthesis of fluorescent enediynes, pharmacophore,
- synthesis of molecules for electronic and electrooptical applications,

- (d) synthesis of molecules for nanomaterial applications,
- (e) synthesis of conjugated fluorescent nucleosides for DNA detection.

Details of Sonogashira coupling will be discussed in **Chapter 3**. In brief Sonogashira coupling reaction is the Pd(0)/Cu(I) catalyzed coupling reaction between aryl halide and a terminal alkyne in the presence of base resulting in a conjugated alkynyl derivative. **Scheme 1.12** shows the classical Pd(0)/Cu(I) catalyzed Sonogashira coupling reaction.



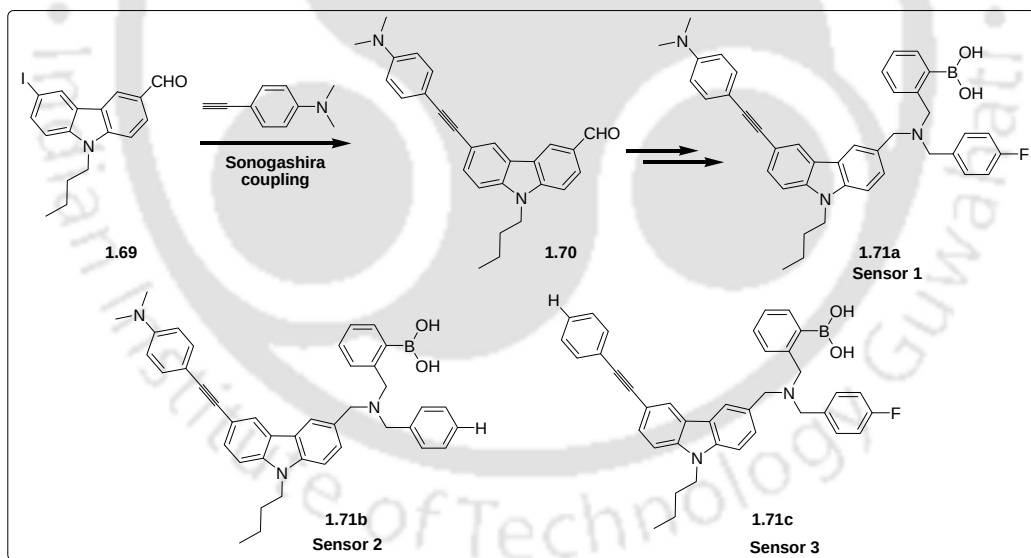
Scheme 1.12: Schematic representation of Sonogashira coupling reaction.

1.8.1. Synthesis of Conjugated Molecules via Sonogashira Coupling for Sensory Applications

The Sonogashira coupling is used for the synthesis of conjugated molecules. The extended conjugation endows new and modulated electronic/photophysical properties to the molecules. These modulated electronic/photophysical properties can be exploited and utilized in various research field of material science, nanotechnology and in sensory application in chemical/biological science.

As for example, Zhao *et al.*,^{24a} have reported three boronic acid sensors **1.71(a-c)** based on phenylethynylated carbazole unit as the fluorophore (**Scheme 1.13**). The synthesis was achieved by using Sonogashira cross coupling reaction. The synthesized fluorophores (**1.71a** and **1.71b**) containing N,N-dimethylphenylethynyl donors were predicted to display a d-PET (photoinduced electron transfer) based fluorescence sensor where fluorophore acts as an electron donor. However, fluorophore **1.71c** containing simple phenylethynyl unit did not show any d-PET

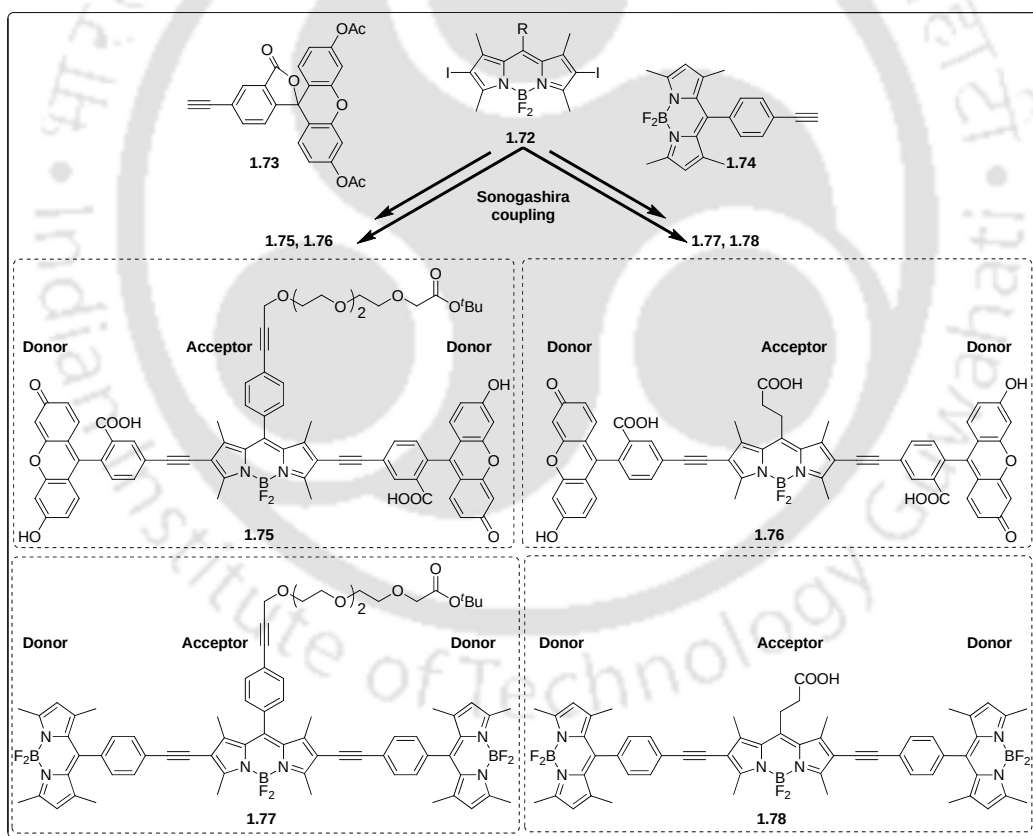
(fluorophore as an electron donor of the PET) effect; rather the normal a-PET (fluorophore as an electron acceptor of the PET) effect was observed. The fluorescence study of the fluorophore **1.71a** showed that the fluorescence intensity of the fluorophore increases in presence of mandelic acid at pH 5.6, while the fluorescence intensity decreases in presence of tartaric acid. Thus, the fluorophore can be used as a sensor for the selective detection of α -hydroxyl carboxylic acids. The effect of pH and the interaction with analytes on the fluorescence property of the fluorophore **1.71a** was also rationalized by DFT/TDDFT calculations. The authors believed that the DFT/TDDFT calculations aided design of rational sensor. Thus, using free energy changes and electron transfer rate constants to study the emission properties of PET sensors would become an essential tool in the design of new fluorophores or fluorescent sensors with predetermined photophysical properties.



Scheme 1.13: Synthesis of phenylethynylated-carbazole monoboronic acid based fluorescent sensors *via* Sonogashira coupling.

Energy transfer based fluorescent proton sensors **1.75-1.78** (Scheme 1.14) have been synthesized *via* Sonogashira coupling and reported by Burgess *et al.*^{24b} Thus, compounds **1.75** and **1.76** have been synthesized starting from compound **1.72**

containing two different “R”-groups upon reaction with compound **1.73** via Sonogashira coupling. By the similar way compounds **1.77** and **1.78** have also been synthesized via Pd(0)-mediated Sonogashira coupling between compound **1.72** containing two different “R”-groups and compound **1.74**. The synthesized fluorescein and BODIPY-based donor and BODIPY-based acceptor containing fluorophores were highly pH sensitive and the energy transfer between donor and acceptor were fully controlled upon pH of the medium. Thus, these energy transfer fluorophores with distinct fluorescent properties provide a new, and potentially useful approach to sensors for biomedical applications.



Scheme 1.14: Synthesis of fluorescent proton sensors via Sonogashira coupling.

1.8.2. Synthesis of Fluorescent Eneidyne

Eneidyne are a class of natural products having antitumor, anticancer activity. Their biological DNA cleaving activity originates from the generation of benzenoid biradical intermediate from the ene-di-yne pharmacophoric unit *via* classical Bergmann cyclisation reaction. The synthesis of this class of natural products or the model eneidyne compounds involves Sonogashira coupling as a key reaction step. Thus, Sonogashira coupling is useful in the synthesis of natural product of biological importance. Fluorophore labeled natural products or fluorescent eneidyne can also be synthesized using the same protocol. Thus, various novel fluorescent eneidyne have been synthesized *via* Sonogashira cross coupling (**1.79-1.82**, **Figure 1.11**). Basak *et al.*,^{25a} have synthesized fluorescent eneidyne (**1.79-11.80**) *via* Sonogashira coupling. They^{25b} also have reported eneidyne based β -turn mimetic peptide (**1.81**, **Figure 1.11**) which can detect Cu^{2+} ion and gold nano partical *via* the generation of an enhanced fluorescence signal.

Recently, Ghosh *et al.*,^{25c} reported eneidyne scaffold - based fluorescence chemosensor (**1.82**, **Figure 1.11**) which can recognize H_2PO_4^- in CH_3CN solution.

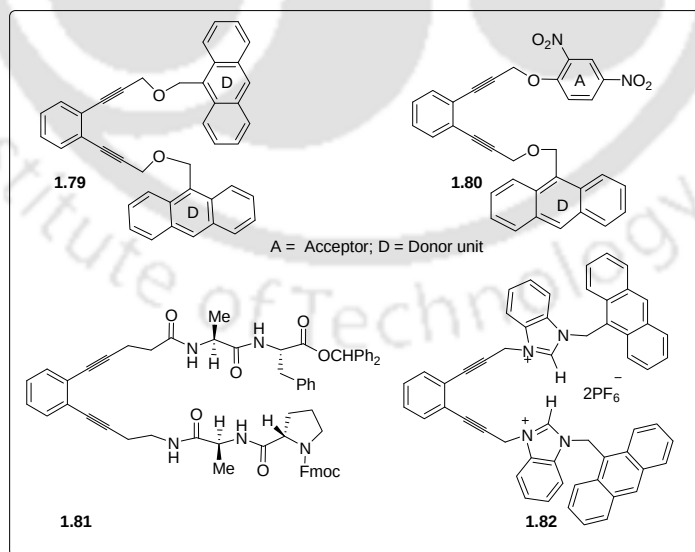
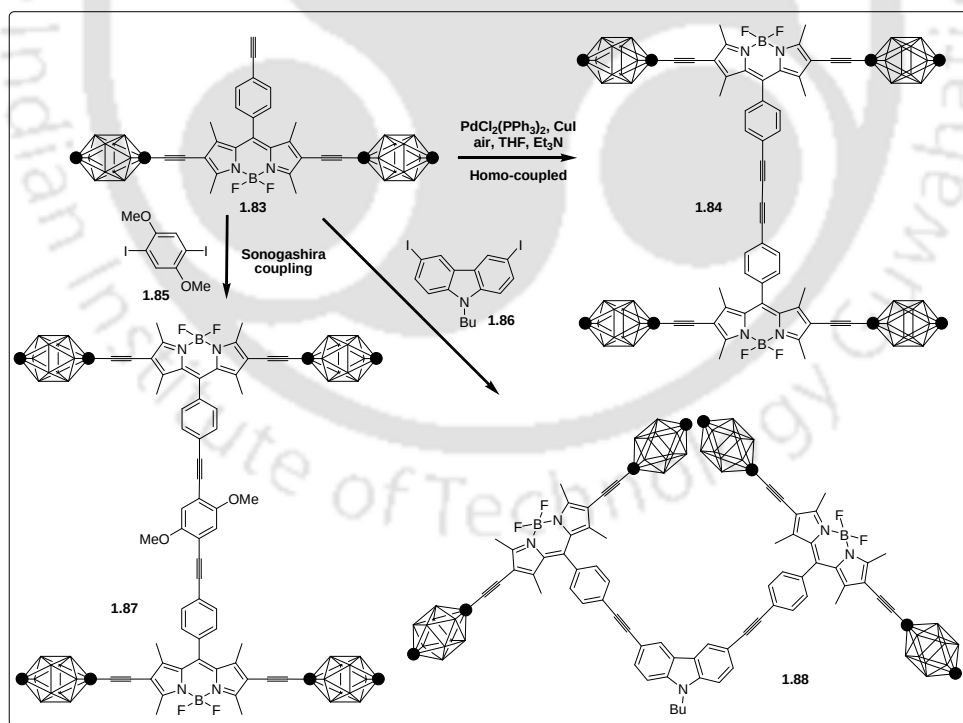


Figure 1.11: Chemical structure of fluorescent eneidyne.

1.8.3. Synthesis of Conjugated Molecules for Application in Nanomaterial

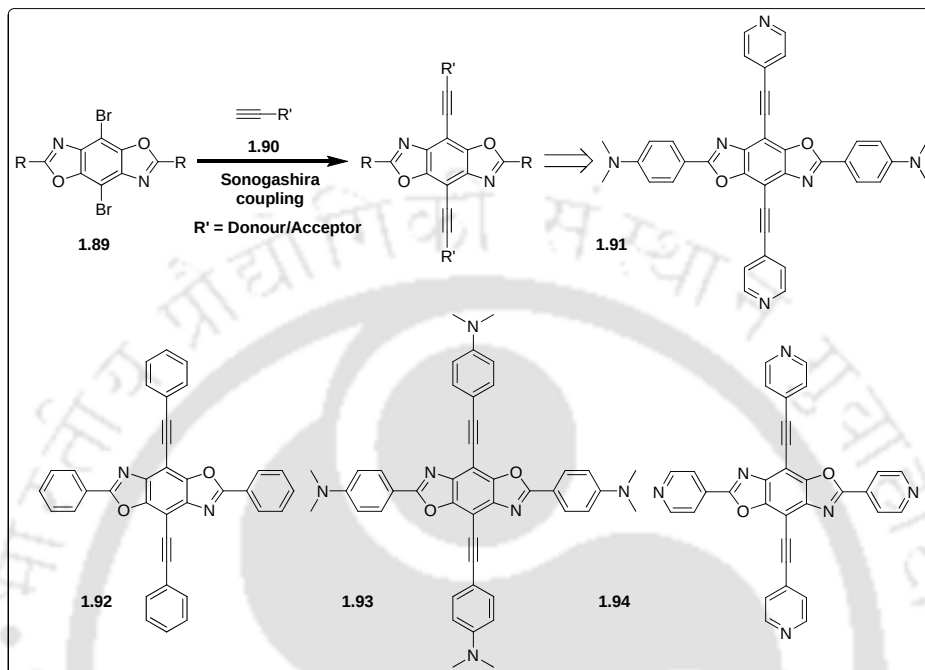
The organic molecules like conjugated polymers, polycyclic aromatic hydrocarbons, dyestuffs, dendrimers have significant advances in the synthesis of nanomaterials and in research of nanoscience. Conjugated polymers have attracted attention in recent years as organic semiconductors for use in devices such as light-emitting diodes (LEDs),^{26a} solar cells,^{26b} and field-effect transistors (FETs).^{26c} There has also been interest in monodisperse conjugated oligomers, both as active materials and as models for polymers.^{26d-e} Since these materials are well-defined molecular species, reliable structure–property relationships can more easily be obtained from them than from the corresponding polydisperse polymers. A novel and interesting feature of the conjugated materials is that they can self-assemble into well-defined nanostructures.



Scheme 1.15: Synthesis of highly fluorescent BODIPY-based nanocars *via* Sonogashira coupling.

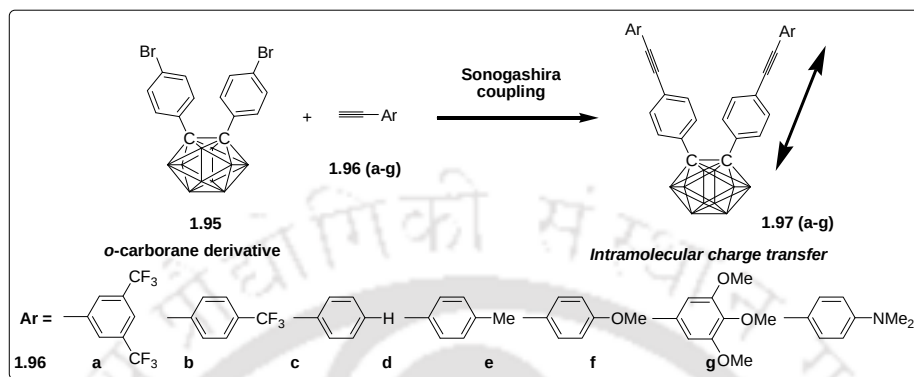
In 2010, Tour *et al.*,^{26f} have reported a convergent synthesis of highly fluorescent nanocars of BODIPY containing dye *via* Sonogashira coupling (**Scheme 1.15**). Thus, compound **1.83** was converted to **1.87** and **1.88** *via* Sonogashira coupling with compounds **1.85** and **1.86** respectively. Compound **1.84** was prepared from **1.83** in presence of air catalyzed by $\text{PdCl}_2(\text{PPh}_3)_2$ and CuI . The synthesized highly fluorescent H-shaped BODIPY-containing nanocars with p-carborane wheels can move on smooth surfaces depending on their chassis. They observed that the absorption maxima of the synthesized BODIPY containing nanocar derivatives centered at around 550 nm and the position of emission maxima lie at around 570 nm to 590 nm. These molecules possess high fluorescence quantum yields ($\Phi > 0.7$). These high quantum yields of the nanocars make them suitable for single molecule fluorescence microscopy (SMFM) experiments.

The synthesis of benzobisoxazole-based cruciform-shaped conjugated molecules have been reported by Miljanić *et al.*,^{26g} towards the design of molecules for optical application. Thus, compound **1.89** upon reaction with various donor and acceptor containing arylacetylenes (**1.90**) *via* Sonogashira coupling afforded several cruciform products **191-1.94** (**Scheme 1.16**). These synthesized cruciforms products can potentially be applied as materials with nonlinear optical properties.^{26h-i} The geometrically regular benzobisoxazole cruciforms with crystalline structures could easily develop the way for their use as solid-state sensors.



Scheme 1.16: Synthesis of heterocyclic fluorophores, benzobisoxazole cruciforms via Sonogashira coupling.

Aggregation-induced emission (AIE) property possessed by a series of diphenyl-*o*-carborane derivatives (**1.97**, **Scheme 1.17**) have been reported by Chujo *et al.*^{26j} Thus, the synthesis of diphenyl-*o*-carborane compounds (**1.97**) was accomplished via a Pd(0)-mediated Sonogashira coupling between bis(4-bromophenyl)-*o*-carborane (**1.95**) and donor/acceptor arylacetylenes (**1.96**). The emission maxima of these derivatives lie in the visible spectrum region ($\lambda_{\text{max}} = 452\text{--}662\text{ nm}$) originated from an intermolecular charge transfer state (ICT) which was supported by a computational study. Thus, these π -conjugated *o*-carborane derivatives with electron-donating and electron-accepting groups possessing aggregation-induced emission (AIE) property can potentially be applied for organic light emitting diodes (OLEDs), semiconductor lasers as well as fluorescence sensors.^{26k-o} In addition, the high solubility of these organic molecules allows them for simple and low-cost processing of large-area optoelectronic devices.



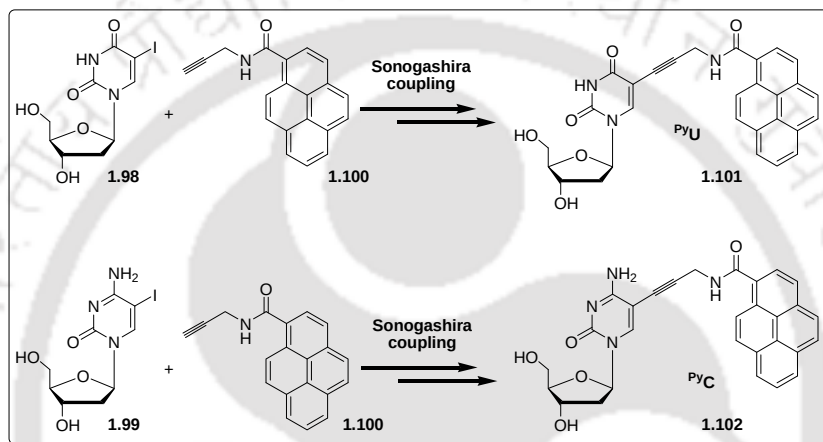
Scheme 1.17: Synthesis of diphenyl-*o*-carborane derivatives *via* Sonogashira coupling.

1.8.4. Synthesis of Fluorescently Labeled Nucleosides *via* Sonogashira Coupling for DNA Detection

Fluorescent nucleosides and oligonucleotide probes are powerful tools for structural studies of nucleic acids, sequencing, molecular diagnostics, and other applications relating to genomics. Therefore, a great deal of research efforts have been put forth for the development of ideal fluorescent probe, small fluorescent molecules, fluorescent biomolecular building blocks, especially fluorescent nucleoside analogues in DNA research having solvofluorochromic properties for monitoring microenvironmental change around nucleic acids for understanding biological events associated with interbiomolecular interactions.

Saito *et al.*,^{27a} have designed and synthesized base-discriminating fluorescent (BDF) nucleosides with the help of Sonogashira coupling (**Scheme 1.18**) and used them for DNA detection. Thus, pyrene labeled 2'-deoxyuridine (^{Py}U, **1.101**) and 2'-deoxycytidine (^{Py}C, **1.102**) were synthesized *via* Sonogashira coupling²³ of N-(prop-2-yn-1-yl)pyrene-1-carboxamide (**1.100**) with 5'-protected of 5-iodouridine (**1.98**) and 4 and 5'-protected of 5-iodocytidine (**1.99**) respectively. These fluorescent labeled nucleosides were incorporated into short oligonucleotide sequence and exploited in single nucleotide polymorphism typing. The ODNs containing ^{Py}U and ^{Py}C selectively emit enhanced fluorescence signal when the base in complementary target

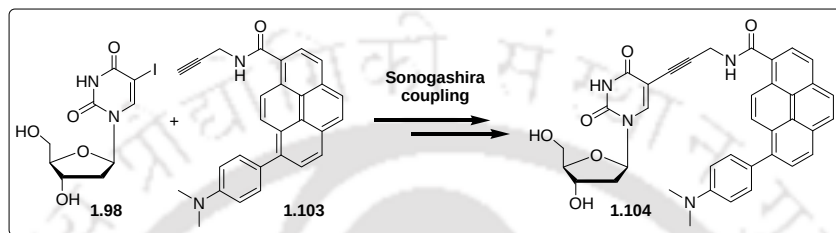
ODNs opposite to BDF nucleoside of probes are **A** and **G** respectively. Thus, Sonogashira coupling reaction finds applications in synthesizing fluorescently labeled nucleoside which are efficiently fluorescent building block of nucleic acid biomolecule usable for sensing local microenvironment of DNA and detection of DNA *via* the generation of distinct fluorescent signal.



Scheme 1.18: Synthesis of BDF nucleosides ^{Py}U and ^{Py}C *via* Sonogashira coupling.

In 2005 Okamoto and Saito *et al.*,^{27b} have reported a pyrene labeled dual fluorescent nucleoside, **1.104** which was synthesized *via* Sonogashira coupling of 5-iodo-2'-deoxyuridine (**1.99**) with *N*-propargyl 8-(4'-(*N,N'*-dimethylamino)phenyl)pyrene-1-carboxamide (**1.103**, **Scheme 1.19**). The nucleoside **1.104** was incorporated into short oligonucleotide sequence for monitoring the change of local microenvironment of DNA. They studied the potential of the dual fluorescence of the probe generated from two excited singlet state, a locally excited (LE) state, and an intramolecular charge transfer (ICT) state for monitoring the DNA structure and hybridization. Thus, efficiently controlled dual fluorescence emission into a duplex allowed them to probe DNA hybridization by a colour change. An orange coloured fluorescence has been observed for the single strand ODN containing dual fluorescent nucleoside **1.104**, while the double strand DNA emitted a pink fluorescence as a mixture of two colours of orange ICT and blue LE fluorescence.

Thus, a change in fluorescence colour allowed them to sense the change in microenvironment around the fluorophore in the single strand state and in the duplex conformation.

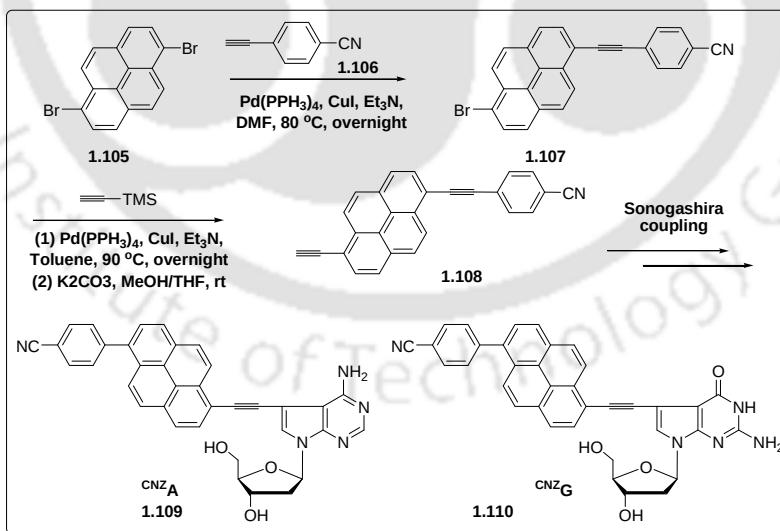


Scheme 1.19: Synthesis of dual fluorescent nucleoside (**1.104**) via Sonogashira coupling.

Most of the efforts to impart useful photophysical properties upon non-emissive natural nucleobases involve the linking of natural nucleobases to fluorescent aromatic or heteroaromatic chromophores *via* an ethynyl linker or the direct attachment of fluorescent chromophores on the natural nucleobases. By extensive studies on the substituent effects on 8-arylethynylated 2'-deoxyguanosine derivatives, Saito *et al.*, have found that aromatics with donor/acceptor functional groups if linked *via* π -conjugation to the 8-position of guanosine/adenosine, 2-position of adenosine and 5-position of deoxyuridines, can modulate the photophysical properties of the nucleobases and might give rise to the design of a large library of solvatochromic fluorescent nucleosides.

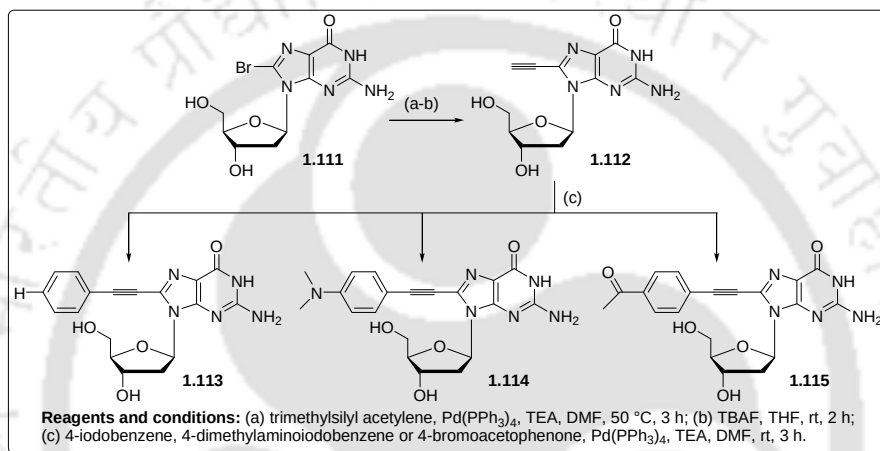
With this design concept several environment sensitive fluorescent (ESF) nucleosides based on the combination of natural nucleobases and aromatic donor/acceptor chromophoric units were synthesized and their solvatochromic emission properties were exploited. Thus, environment sensitive donor/acceptor aromatics containing several fluorescent deoxyuridine, deoxyadenosine and deoxyguanosine derivatives were prepared mainly *via* Pd(0) mediated Sonogashira cross-coupling reaction and their solvatochromicity were examined. As for example, Saito *et al.*,^{27c} have reported an approach for the synthesis of push-pull type of

fluorescent 7-deazapurine nucleosides, ^{CNZ}A and ^{CNZ}G nucleosides (**1.109** and **1.110**, **Scheme 1.20**). The designing concept was based on the electron withdrawing property of –CN group which would pull the electron density toward itself and the electron rich pyrene ring would push the electron density toward the conjugated pyrene ring. Thus, the compound **1.107** has been synthesized from **1.105** by Sonogashira cross coupling with 4-ethynyl benzonitrile. Compound **1.107** was then converted to **1.108** by Sonogashira cross coupling with trimethylsilylethynyl, followed by deprotection to get the compound **1.108**. Finally, the push-pull type of fluorescent nucleosides, ^{CNZ}A and ^{CNZ}G were obtained from the Sonogashira cross coupling of compound **1.108** with corresponding iodo derivatives, 7-iodo-7-deaza-2'-deoxyadenosine and 7-iodo-7-deaza-2'-deoxyguanosine, respectively. They studied the photophysical properties of the synthesised nucleosides and found to be highly solvatochromic. Ultimately they have incorporated ^{CNZ}A into short oligonucleotides. From the thermal denaturation study of the duplexes containing ^{CNZ}A revealed that ^{CNZ}A can form a stable base pair with both thymine and cytosine.



Scheme 1.20: Synthetic scheme of push-pull-type solvatochromic 7-deaza-2'-deoxypurine nucleosides, ^{CNZ}A and ^{CNZ}G via Sonogashira coupling.

Recently, they have also synthesized 2'-deoxyguanosine analogues of ESF nucleoside.^{27d} Thus, 8-aryl ethynylated-2'-deoxyguanosines or adenosines were prepared from the cross-coupling reaction of the corresponding 8-bromo-2'-deoxyguanosine or adenosines. By following **Scheme 1.21**, all the 8-alkynyl aromatics substituted 2'-deoxyguanosines were prepared.



Scheme 1.21: Synthesis of 8-substituted 2'-deoxyguanosine analogues of ESF nucleoside.

The synthesized donor or acceptor containing compounds showed high solvatochromicities when tested in various organic solvents of different dielectric properties. Both the UV-visible and steady state emission spectral study revealed that they possess high degree of solvatochromicity. Examination of the photophysical properties of 8-arylethynylated deoxyguanosine derivatives in various solvents revealed that all the deoxyguanosines *viz.*, 8-ethynylphenyl-2'-deoxyguanosine (**1.113**, ^{8-EP}G), 8-ethynyl-N,N-dimethylanilino-2'-deoxyguanosine (**1.114**, ^{8-EDMA}G), and 8-ethynyl-4-acetylphenyl-2'-deoxyguanosine (**1.115**, ^{8-EAP}G) showed strong fluorescence at around 475 nm in non-polar solvents like ethyl acetate but relatively weak fluorescence in polar solvents like methanol and acetonitrile. More interestingly, fluorescence intensity of acetyl analogue ^{8-EAP}G (**1.115**) showed a strong solvent dependency, presumably because of intermolecular charge transfer between donor

purine moiety and the conjugated acceptor acetylphenyl unit. Therefore, this example showed that introduction of acetyl group on the aromatic moiety could induce a strong solvatochromic fluorescence property to the donor purine bases/nucleosides.

The highly solvatochromic fluorescent deoxyguanosine analogue 4-acetylphenylethynyl-2'-deoxyguanosine, ^{8-EAP}G (1.115), was incorporated into oligodeoxynucleotides (ODNs) *via* automated DNA synthesis for possible detection of SNP. Two ODNs containing ^{8-EAP}G were synthesized-one with flanking AT base pair and other with flanking GC base pair-for testing whether SNP typing could be done universally or the detection ability of the probe would be dependent on the sequence. Fluorescence emission of ODN 1, [5'-d(CGCAAC X CAACGC)-3', X = ^{8-EAP}G] and ODN 2 [3'-d(GCGTTG N GTTGCG)-5', N = A, T, G, C] in the absence and presence of various complementary target strands revealed the following observations. Fluorescence intensities of single stranded ODN 1 and ODN 2 and of the duplexes with complementary strands cODN 3 [5'-d(CGCAATXTAACGC)-3', X = ^{8-EAP}G] and cODN 4 [3'-d(GCGTTA N ATTGCG)-5', N = A, T, G, C], respectively, were very weak. On the contrary, the fluorescence spectra of duplex ODN 1/cODN 3 (N = T) and ODN 2/cODN 4 (N = T) showed an intense fluorescence at around 510 nm.^{27d} Thus, the probe containing ESF nucleoside, ^{8-EAP}G, is highly efficient in sensing of the presence of T base of target DNA opposite to the ESF nucleoside of probe DNA with drastically enhanced fluorescence signal which is again independent of sequence or flanking bases. The fluorescence emission was visible with naked eye only for T opposite ESF base ^{8-EAP}G under illumination with 365 nm transilluminator. Thus, acetyl substituted fluorescent deoxyguanosine ^{8-EAP}G is exceptionally useful as a T specific ESF base regardless of the flanking sequences in a fluorescence hybridization assay. The reason for the T specific fluorescence emission was explained on the basis of location of the chromophore at a hydrophobic site inside the groove.

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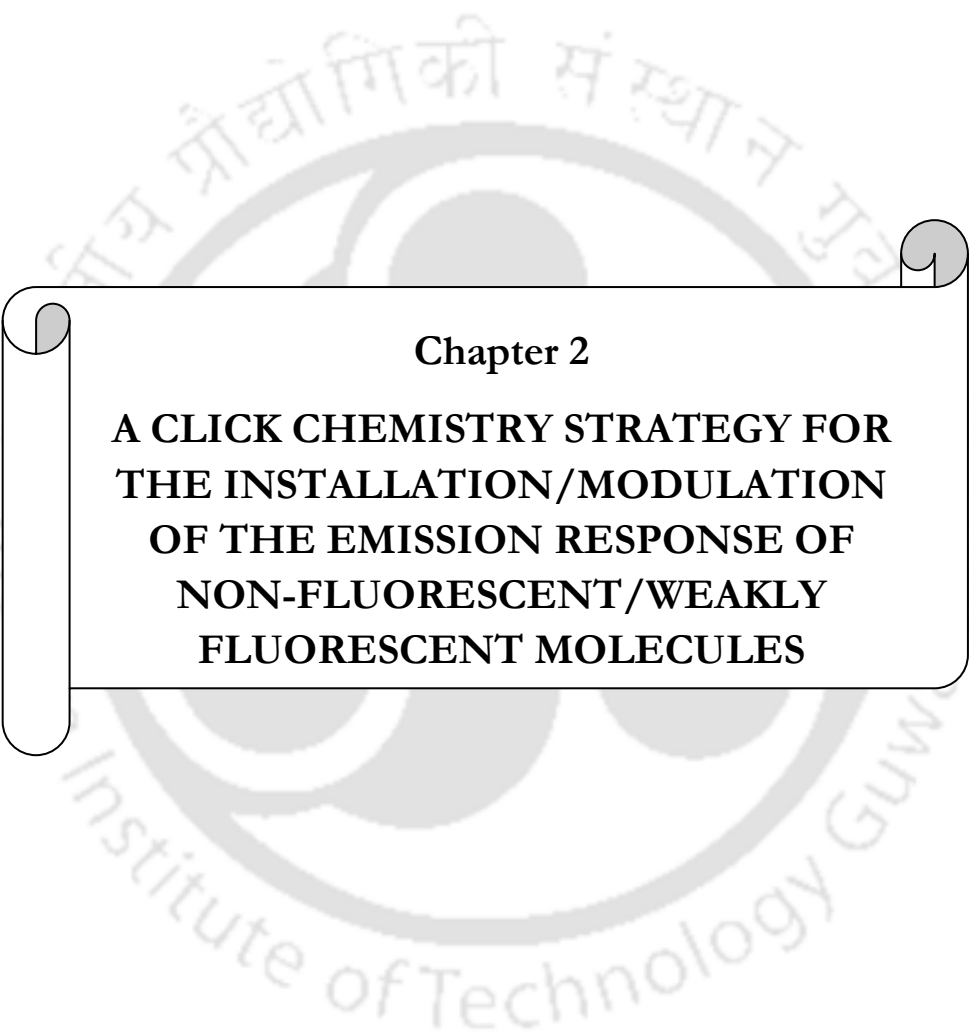
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Chapter 2

A CLICK CHEMISTRY STRATEGY FOR THE INSTALLATION/MODULATION OF THE EMISSION RESPONSE OF NON-FLUORESCENT/WEAKLY FLUORESCENT MOLECULES

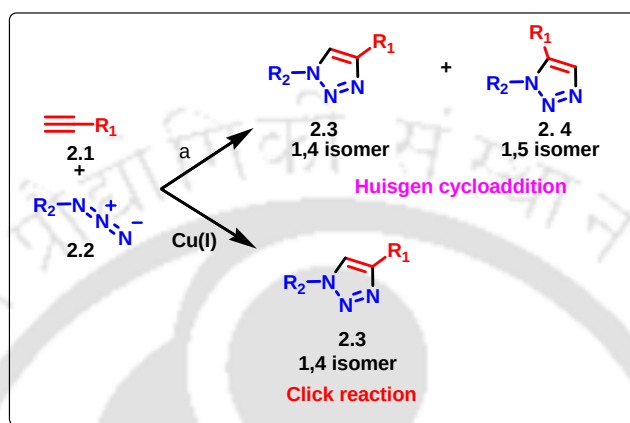
2.1. Introduction

Organic small fluorescent molecules are well known since long back for their widespread applications ranging from material science, chemical science, nanomaterials, organic electronic to biology for probing biomolecular structure, properties, and functions.¹ However, their photophysical properties sometimes could not be correlated with their structural design. Moreover, many of the fluorophores are not long wavelength emissive and not solvatochromic, thus, disabling their use in cell or in sensing the change in microenvironment in biological world. Therefore, design of microenvironment sensitive long wavelength emissive organic fluorophores of predictable photophysical properties in relation to their designed structure *via* a simple and versatile method is highly desirable for many of chemical/biochemical applications.¹ The excellent regioselectivity, simplicity along with the versatility of the reaction conditions, render “click chemistry” advantageous, for synthesizing small organic fluorescent molecule and for achieving the selective coupling of small fluorogenic molecules within a complex biological environment which enhances greatly the efficiency of biological studies aimed at understanding the natural biological processes *via* a distinct and quantifiable signal generation.^{1,2}

2.2. The Click Reaction

The cycloaddition reaction of a 1,3-dipolar molecule to an alkene/alkyne for the synthesis of five-membered triazole ring system is a classic reaction in organic chemistry widely used for the preparation of molecules of fundamental importance for both research and development and in industry. The history of 1,3-dipoles goes parallel with the discovery of diazoacetic ester by Curtius in 1883.³ The general application of 1,3-dipoles in organic chemistry was first established by Huisgen in the 1960s.⁴ At the same time, the new concept of conservation of orbital symmetry, developed by Woodward and Hoffmann, appeared.^{5,6} On the basis of the concept of

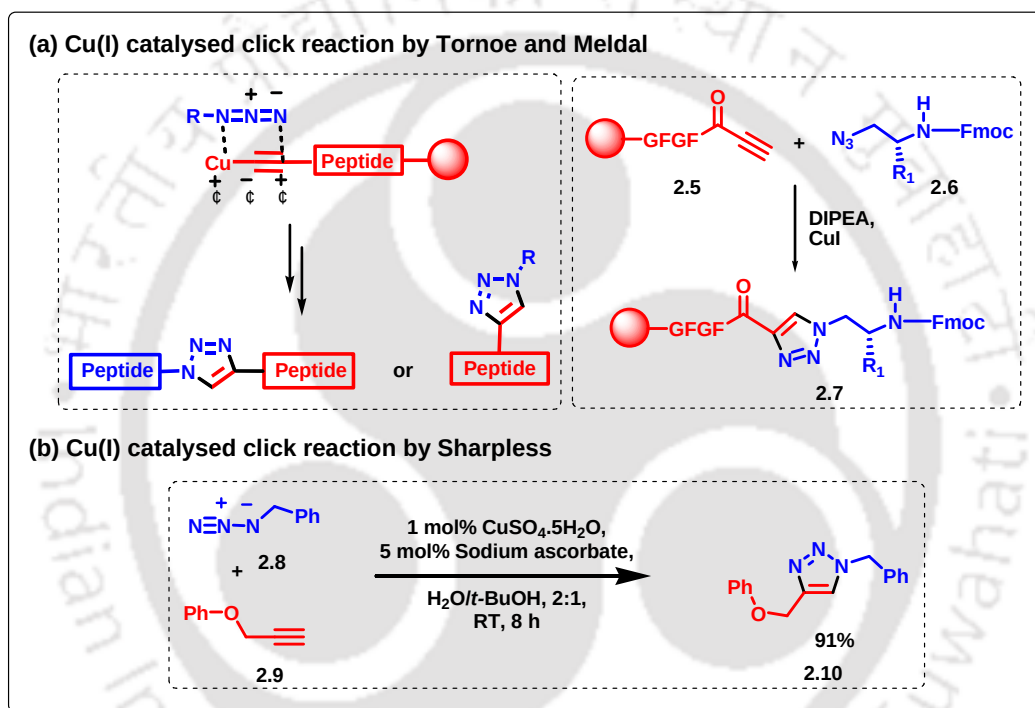
conservation of orbital symmetry, Houk *et al.* have further contributed to shed more light on the reactivity and regioselectivity of 1,3- dipolar cycloaddition reactions.⁷⁻⁹



Scheme 2.1: Schematic representation of 1,3-dipolar cycloaddition between azides and alkynes.

Pioneered by Huisgen, the 1,3-dipolar cycloaddition reaction between terminal acetylenes and azides was brought back into the focus of researchers of various research fields due to the introduction of Cu(I)-catalyzed cycloaddition by Tornøe and Meldal in 2001.¹⁰ Later on Sharpless and his co-workers¹¹ introduced the concept of “click chemistry” for the same reaction. In original Huisgen 1,3-dipolar cycloaddition reaction (a 3+2 cycloaddition), a 1,3-dipole such as azides, nitroxides or diazoalkanes react with a dipolarophile like alkenes, alkynes or carbonyls *via* a concerted pathway to form a five-membered triazole heterocycle. This reaction gives poor regioselectivity which leads to a mixture of 1,4- and 1,5-disubstituted triazoles and may require elevated temperatures because alkynes are poor 1,3-dipolar acceptors (**Scheme 2.1**). The discovery of copper(I) salts as a catalyst for the reaction by Meldal (**Scheme 2.2a**) and then by Sharpless (**Scheme 2.2b**) allowed it to evolve from a reaction requiring harsh conditions leading to a mixture of 1,4- and 1,5- regioisomers to a regioselective (1,4- regioisomers) one. This regioselective and mild Cu(I)-catalyzed Huisgen cycloaddition reaction is now a day popularly known as “click reaction” that can be performed at room temperature in very short reaction

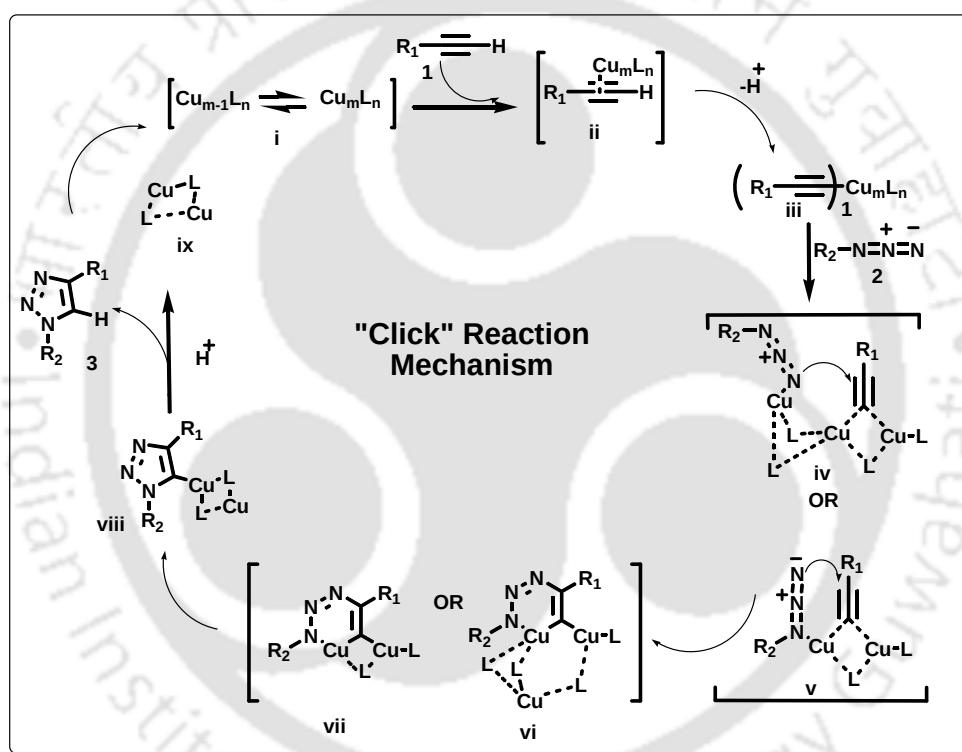
times (**Scheme 2.1**). Compared to other metal-catalyzed reactions, the use of Cu(I) presents the major advantages because it is inexpensive and easy to handle. Most of the protocols involve the reduction of stable sources of Cu(II), such as CuSO₄, with sodium salts or the comproportionation of Cu(II)/Cu(0) species to generate active Cu(I)-catalyst which catalyses the present click reaction.



Scheme 2.2: Schematic presentation of Cu(I) catalyzed click reactions by (a) Tornøe and Meldal and (b) Sharpless *et al.*

The plausible mechanism of Cu(I)-catalyzed 1,3- dipolar cycloaddition, the click reaction, is shown in **Scheme 2.3**. Thus, the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction between the azide and the alkyne proceeds through a stepwise mechanism. Thus, the copper (I) forms a pi complex with the triple bond of a terminal alkyne. In the presence of a base, the terminal hydrogen, being the most acidic is deprotonated first to give a Cu acetylide intermediate (**iii**). Studies have shown that the reaction is second order with respect to Cu. It has been suggested that the transition state involves two copper atoms. One copper atom is bonded to the acetylide while the

other Cu atom serves to activate the azide. In the next step, the azide (**2**) replaces one of the ligands and the copper complex binds to the nitrogen adjacent to the carbon, forming intermediate **iv** or **v**. Attack by the terminal nitrogen of **v** on C-2 of the acetylide forms the unusual six-membered Cu(III) intermediate **vii**. Rearrangement of **vii** generates the five-membered specie **viii**, which is ultimately transformed to the product **3** by protonation.



Scheme 2.3: Postulated catalytic cycle for azide-alkyne click reaction.

2.3. Types of Click Reaction

Various types of click reaction are reported in literature, which are as follows:

- (a) Cycloaddition [1,3-dipolar/ hetero-Diels-Alder],
- (b) Addition to C-C multiple bonds [Oxidation, Michael addition],

(c) "Non-aldol" carbonyl chemistry [oxime ethers, hydrazones, aromatic heterocycles],

(d) Nucleophilic reactions [Ring opening].

Among the various types of "click" reactions as shown in **figure 2.1**, the most powerful example is the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition between an azide and a terminal alkyne to afford the 1,2,3-triazole with the selective generation of 1,4-regioisomer (**Scheme 2.1**).

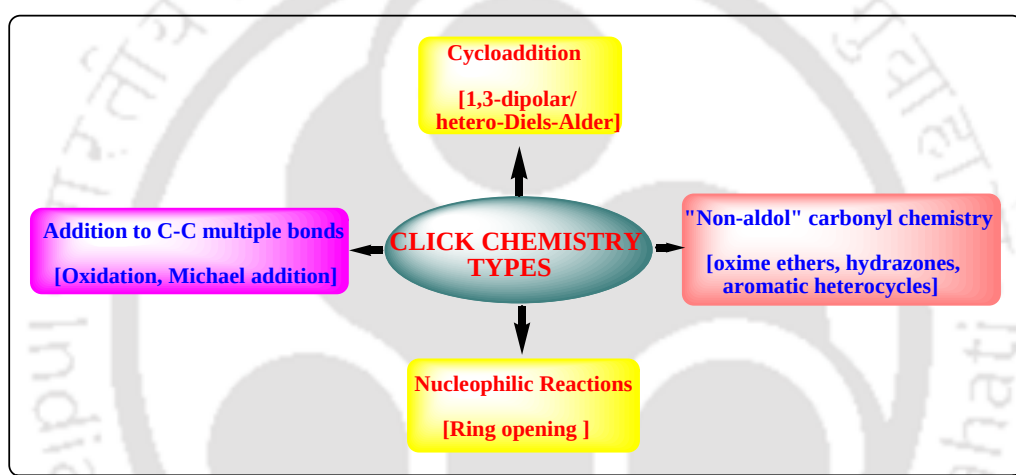


Figure 2.1: Pictorial presentation showing types of click reactions.

2.4. Applications of Click Reaction

The development of the copper(I)-catalyzed cycloaddition reaction between azides and terminal alkynes has led to many interesting applications of click reactions including the synthesis of natural product derivatives.

Some of the widespread applications of click chemistry are listed below:

- synthesis of 1,4-substituted triazoles in organic chemistry
- modification of peptide function with triazoles
- modification of natural products and pharmaceuticals
- drug discovery
- macrocyclizations using Cu(I) catalyzed triazole couplings

- modification of DNA and nucleotides by triazole ligation
- supramolecular chemistry: calixarenes, rotaxanes, and catenanes, and in dendrimer design
- carbohydrate clusters and carbohydrate conjugation by Cu(I) catalyzed triazole ligation reactions
- polymer sciences, material science and in nanotechnology, and
- bioconjugation

Few of the particular applications are presented in following sections.

2.4.1. In Peptide Chemistry: α -Turn Peptidomimetic

Local structural motifs in protein loops are crucial to their interaction with other molecules or protein receptors. As for example, α -turn structural motifs are often incorporated into peptide mimetics to maintain peptide's bioactive conformations. Oh *et al.*,^{12a} have investigated that the triazole moiety can induce a α -turn in a modified peptide (**Figure 2.2**). They found that the 1,4-substituted triazole formed via a Cu(I) catalyzed click reaction of 4-pentynylamine and 4-azidobutanoic acid is an efficient turn mimetic with four atom extensions of both substitutions. Their simple and mild strategy of triazole based stable α -turn mimetic might find applications in areas including bioorganic, supramolecular and material chemistry.

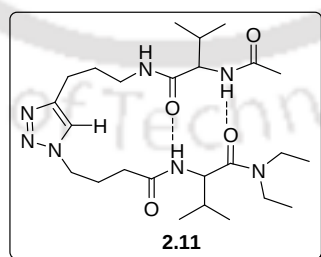
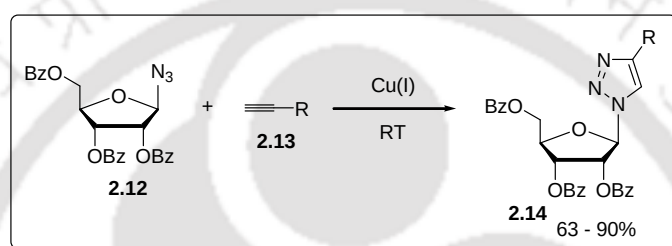


Figure 2.2: Chemical structure of a triazole induced α -turn mimetic.

2.4.2. In Carbohydrates Chemistry

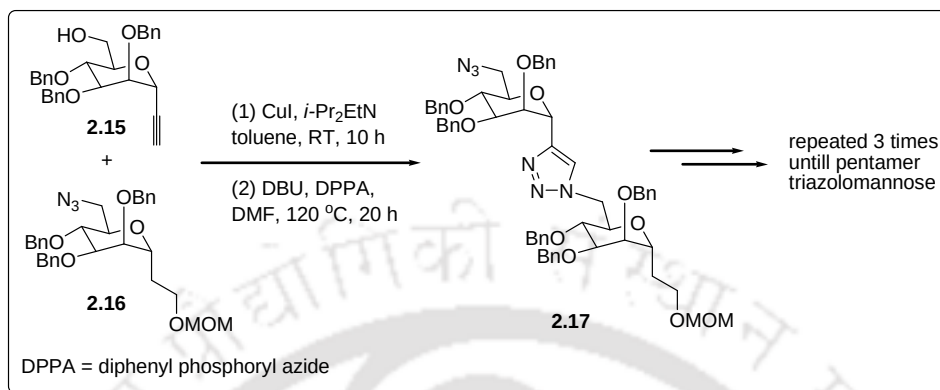
Click reactions can be utilized to construct building blocks for the rapid synthesis of molecules with diverse structure and function. Agrofoglio *et al.*,^{12b} have recently

utilized click reaction for the synthesis of new 1,4-disubstituted-1,2,3-triazolo-nucleosides for the drug discovery program. Analogous compounds have been successfully developed into DNA virus and retrovirus therapeutic agents, against various viruses. Thus, protected 4-azido-ribose (**2.12**) reacted with terminal alkyne (**2.13**) in presence of Cu(I) at room temperature afforded the expected 1,4-disubstituted-1,2,3-triazolo-nucleosides (**2.14**) (**Scheme 2.4**).



Scheme 2.4: Synthesis of new 1,4-disubstituted-1,2,3-triazolo-nucleosides.

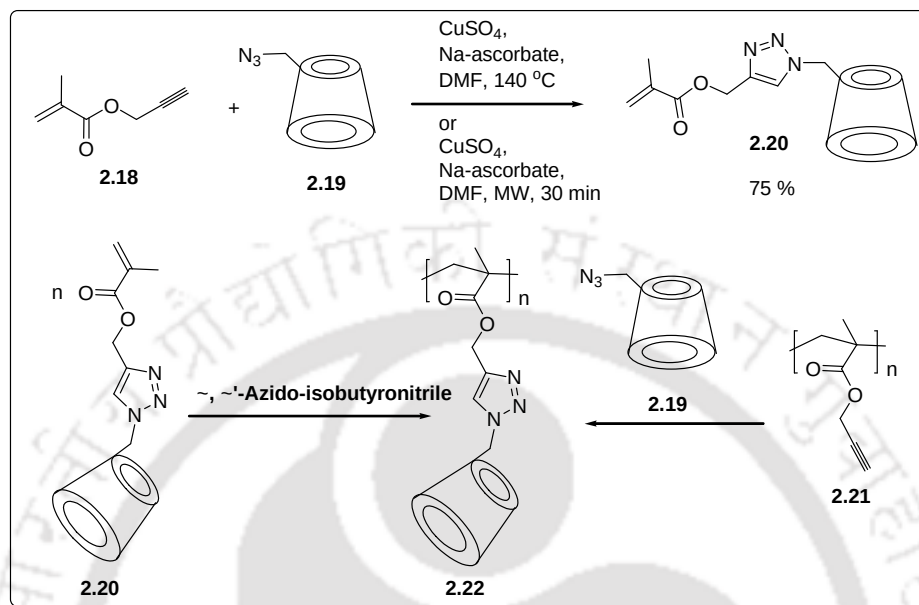
Click chemistry derived carbohydrate-based triazol libraries in the search for biophysical/biochemical probes usable in glycobiology, have been designed and synthesized by Dondoni and co-workers.^{12c} Thus, they have designed triazolyl oligosaccharide where 1,2,3-triazole ring acts as a rigid and stable linker between the two glucoside (**Scheme 2.5**). Thus, the Cu(I) catalyzed click-ligation strategy is an efficient methodology to the generation of the new class of (1,6)-oligomannose analogues containing triazol as a linker. Thus a library of carbohydrate analogues devoid of glycosidic oxygen atom can be prepared to obtain glycosidase resistant substrates as probes and inhibitors of many important biological processes.



Scheme 2.5: Synthesis of triazolyl oligosaccharide.

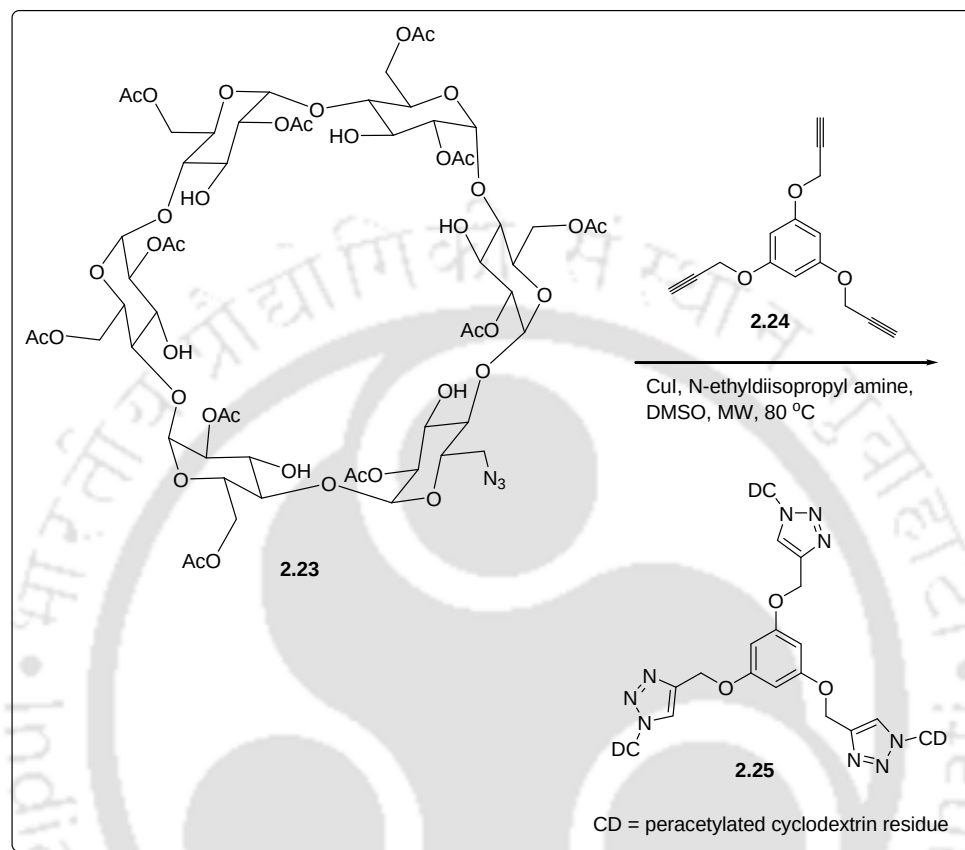
2.4.3. In Macromolecular Chemistry

Macromolecules often display remarkable biological activities. Thus, click chemistry has got recent research interest to engineer well-defined macromolecules with increasing complexity and functionality. Ritter and co-workers^{12d} have reported the synthesis of triazolyl cyclodextrin monomethacrylate to obtain a polymer with potential application in supramolecular chemistry (**Scheme 2.6**). Thus, Cu(I) catalyzed reaction of propargyl methacrylate (**2.18**) with 6I-azido-6I-deoxycyclomaltoheptaose (**2.19**) afforded a new monomer mono- (1H-1,2,3-triazol-4-yl)(methyl)2-methylacryl- -cyclodextrin (**2.20**) useful for the design of polymeric architecture. They expected that triazolyl cyclodextrin decorated polymer with a significant content of covalently attached cyclodextrins might find applications in supramolecular chemistry, drug delivery, and analytical sciences.



Scheme 2.6: Synthesis of triazole containing cyclodextrin derivative *via* click reaction.

Later on, Delattre and co-workers^{12e} reported a synthesis of tripod molecule containing an aromatic core bearing three peracetylated cyclodextrins *via* Cu(I) catalyzed click reaction to study the photoluminescent properties of complexation phenomena with different pesticides (**Scheme 2.7**). Thus, a fluorescence study revealed that the fluorescent tripod is efficient in generating a differential fluorescence response depending on the nature of a guest pesticide inside the cyclodextrin cavity. Thus, the new peracetylated cyclodextrin trimer bearing three triazole linkers can efficiently be utilized as fluorescent host molecules for the differentiation of various pesticides *via* a generation of distinct fluorescence signal.

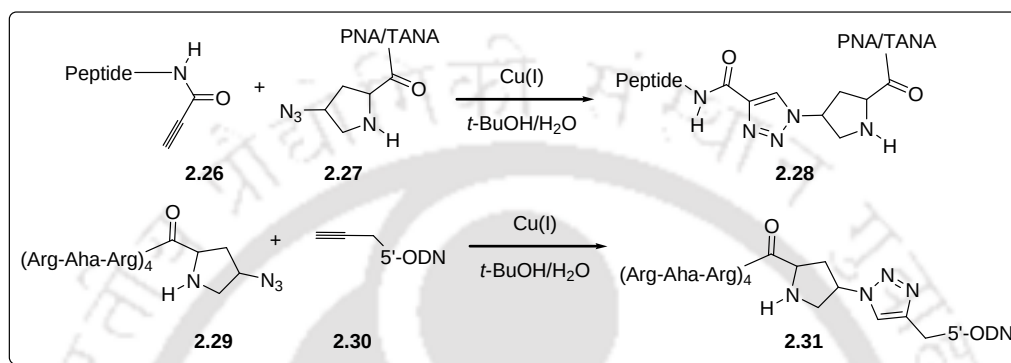


Scheme 2.7: Synthesis of a fluorescent tripodal system using click chemistry usable for fluorimetric differentiation of various pesticides.

2.4.4. Conjugates of Peptides with DNA and PNA via Click Reaction

The click reaction has also been successfully adopted to generate conjugates of biomolecules such as DNA/PNA-protein conjugate. As for example, click chemistry has been exploited by Kumar *et al.*,^{12f} to generate PNA-peptide, DNA-peptide, conjugate molecules usable for therapeutic application. The synthesis were carried out by reacting alkyne derivatised peptides (2.26) with azide labeled PNA or TANA (2.27) to produce peptide-PNA/TANA conjugates 2.28 (Scheme 2.8), or azido peptide (2.29) with alkyne labeled DNA (2.30) to give oligonucleotide-peptide conjugate 2.31 (Scheme 2.8). Thus authors claim that the peptide-DNA/PNA/TANA conjugation strategy might be a unique solution for CPP conjugation to

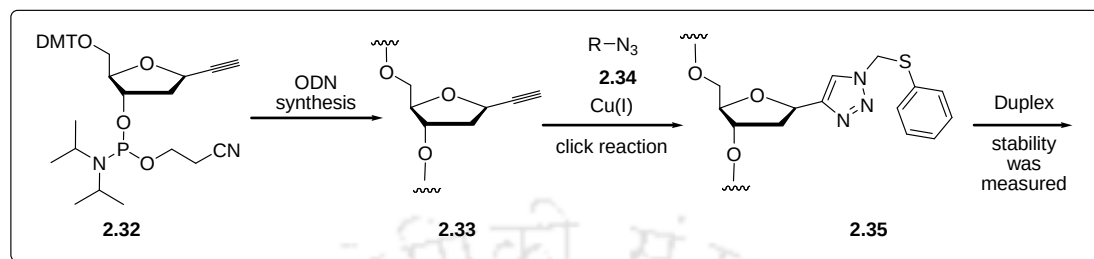
antisense/siRNA therapeutics. These conjugates also might find applications in antisense therapy. Some of these conjugates were also found as a potential alternative to the use of transfection agents to improve the efficiency of siRNA.



Scheme 2.8: Conjugation of peptides to DNA and PNA via click reaction.

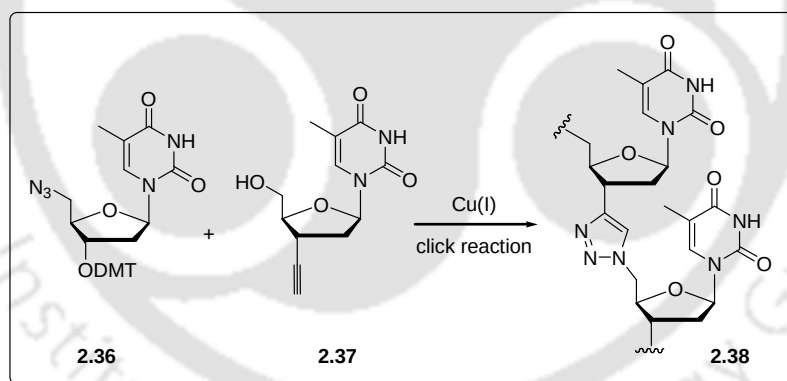
2.4.5. Synthesis of Artificial DNA

There is a great deal of interest in developing new synthetic methods to construct chemically modified DNA oligonucleotides (ODNs) for biological and nanotechnological applications and it quickly became apparent to researchers in the nucleic acids field that the Cu(I)-catalyzed click reaction has great potential. Therefore, it has got much recent interest to decorate nucleic acid bases, therefore the DNA with several ways. As for an example, Nakahara *et al.*,^{12g} reported the synthesis of triazolyl DNA using click chemistry. Thus, the oligonucleotides (2.33) containing 1-ethynyl-2-deoxy- β -D-ribofuranose were reacted with a series of azides (2.34) using the Cu(I)-catalyzed click reaction to produce ODNs containing artificial triazole nucleobases 2.35 (Scheme 2.9). The thermal denaturation study of the post synthetically modified DNAs with their targets showed that the base 2.35 could act as a universal base. Thus the authors expected that the nucleobase analogue may be used as an ambiguous site in primers for PCR and sequencing.



Scheme 2.9: Synthesis of triazolyl DNA by post synthetic modification via click reaction

A modified DNA analogues in which the phosphodiester linkages were replaced by triazole linkages through the reaction of alkyne nucleosides **2.37** with azide nucleosides **2.36** via Cu(I) catalyzed click reaction have been developed by Nuzzi *et al.*,^{12h} (**Scheme 2.10**). They have demonstrated the ability of the method by preparing a triazole-linked trinucleoside, *i.e.*, a non-natural “triazole codon”.



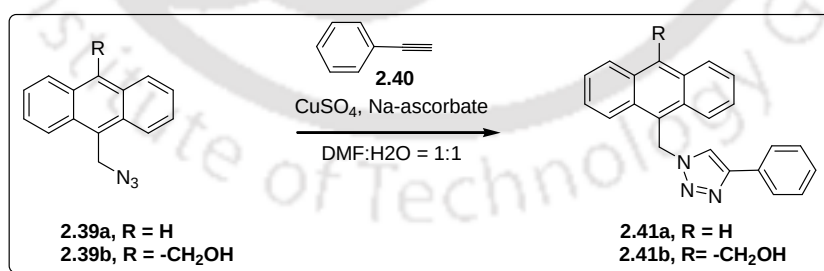
Scheme 2.10: Synthesis of triazole linked DNA analogues.

2.5. Background: Design and Synthesis of Triazole Based Fluorophore

Synthesis of triazole based fluorophore via Cu(I)-catalyzed click reaction of alkyne and azide is a powerful tool for bioconjugation, material science, organic synthesis and drug discovery. The first triazole based fluorophore via Cu(I)-catalyzed click reaction was based on coumarin derivatives and was individually reported by

Fahrni *et al.*,^{13a} and Wang *et al.*^{13b} Thus, Fahrni *et al.*,^{13a} reported the synthesis of highly fluorescent molecule *via* click reaction which was already discussed in **Chapter 1**. A series of coumarin based click fluorophore have also been synthesized *via* the Cu(I) catalyzed click reaction by Wang *et al.*,^{13b} Thus, they were able to show that the click reaction can able to modulate the fluorescence property of the precursor fluorophore.

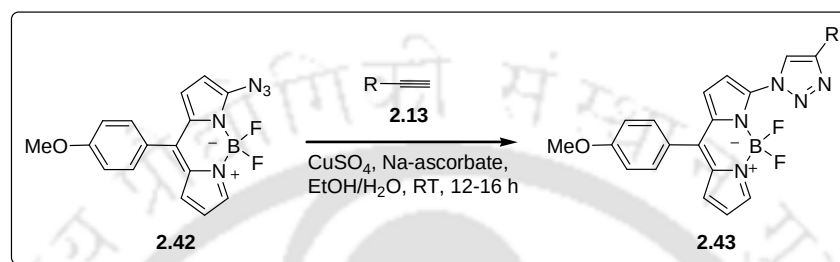
Later on, Wang *et al.*, have reported the synthesis of a series of triazolyl fluorescent dye *via* click reaction. They have synthesized several anthracene derivatives, **2.39** containing an azido group near the anthryl core (**Scheme 2.11**)^{13c}. The azido group permits an electron transfer to the excited anthracene which quenches the fluorescence of the dye. However, the formation of triazole compounds **2.41** *via* the Cu(I)-catalysed click reaction between azides (**2.39**) and alkyne (**2.40**) reduces the electron donating effect of the nitrogen, blocks the PET process and thus restores the fluorescence property of the anthracene chromophore. Thus, the bioorthogonal fluorogenic reaction based on photoinduced electron transfer (PET) might find application in the emerging field of cell biology and functional proteomics due to the high reaction efficiency of click reaction and the distinct fluorescence properties of the products.



Scheme 2.11: Synthesis of triazolyl anthracene derivatives *via* click reaction.

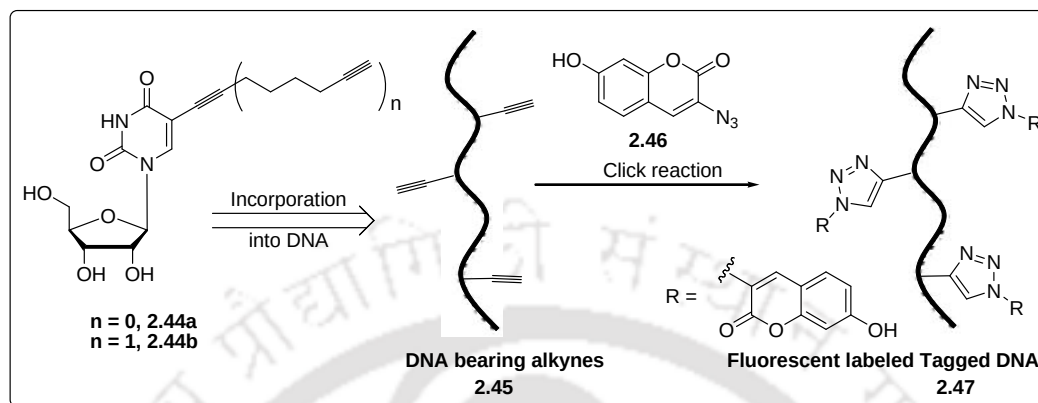
Wang *et al.*,^{13d} also have reported the synthesis of highly fluorescent triazolyl BODIPY derivative (Boron-dipyrromethenes) from the non-fluorescent BODIPY *via* the click reaction. Thus, the non-fluorescent BODIPY **2.42** with an azido-group at the 3-position of the pyrrole ring (which quenches the fluorescence property) reacted with

an alkyne (**2.13**) *via* Cu(I)-catalyzed click reaction to afford a triazolyl fluorophore **2.43** (Scheme 2.12). Thus, they have shown that the formation of triazole ring in the product **2.43** has lead to the enhancement of fluorescence property.



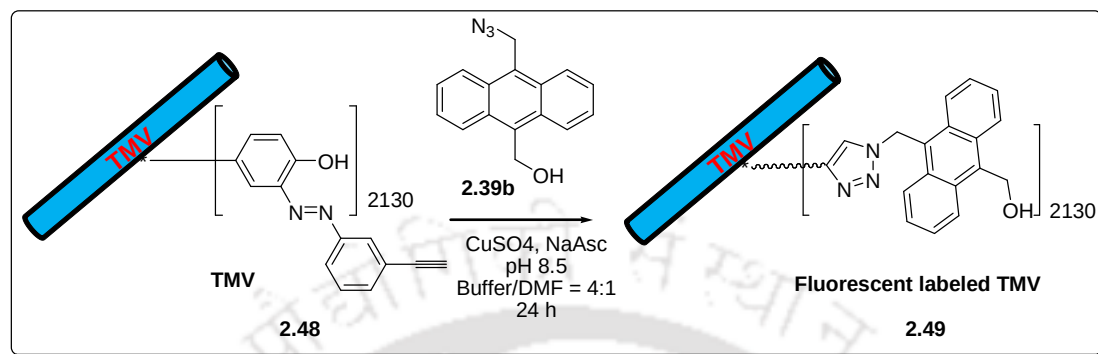
Scheme 2.12: Synthesis of triazolyl BODIPY derivative *via* click reaction.

An efficient post synthetic method using click chemistry to functionalize high-density alkyne-modified DNA have been reported by Carell and coworkers.^{13e} They have incorporated the alkyne substituted uridine nucleosides (**2.44a** and **2.44b**) into a series of oligodeoxyribonucleotides (ODNs) *via* solid-phase synthesis. A Cu(I) catalyzed click reactions were then performed between alkyne-modified ODNs (**2.45**) and 3-azido-7-hydroxycoumarin (**2.46**) to afford triazolyl coumarin tagged fluorescent DNA, **2.47** (Scheme 2.13). Fully labeled DNA was obtained with flexible alkynes **2.44b**, and partially labeled DNA was obtained with rigid alkynes **2.44a**. The synthesized DNA tagged can be detected via fluorescence study. This method is highly efficient for the conversion of alkyne-modified DNA into fluorescently labeled DNA even with multiple labeled DNA for various biotechnological applications. This method can even be utilized to modify long DNA fragments obtained by PCR with various labels without DNA cleavage and in modification or functionalization of several genes.



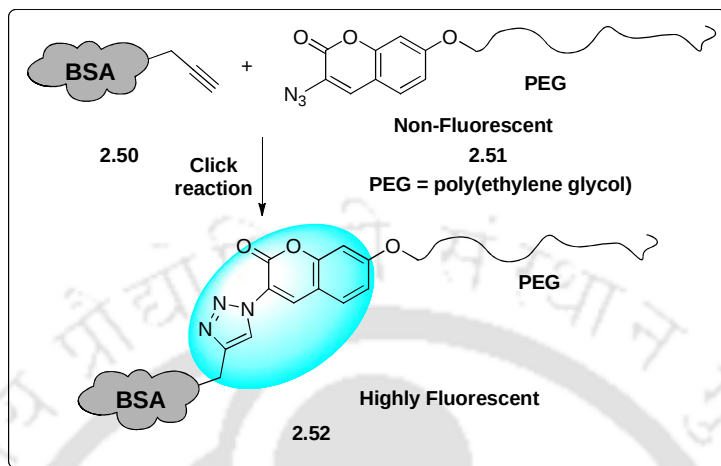
Scheme 2.13: Synthesis of triazolyl coumarin tagged fluorescent DNA *via* click reaction.

The click reaction has also got attention in the labeling of viral particles as was revealed by the report of Finn and coworkers.^{13f} They have derivatized cysteine and lysine residues of cowpea mosaic virus (CPMV) with alkyne and azide groups for further derivatization with suitable fluorophoric units containing azide/alkyne moiety *via* Cu(I)-catalyzed click reaction to ultimately label the viruses. Therefore, the fluorogenic click reaction is an ideal alternative method for the labeling of viral particles for biomedical applications. As for example, Wang and coworkers,^{13c} have modified tobacco mosaic virus (TMV) *via* the Cu(I)-catalyzed click reaction. To accomplish this, first they introduced triple bonds on the tyrosine residues of TMV by electrophilic substitution reaction at the ortho position of the phenol ring to get the alkyne-modified TMV (2.48). Then the click reaction was performed between alkyne-modified TMV (2.48) and non-fluorescent azido derivatives (2.39b) to afford fluorescently labeled TMV virus, 2.49 (Scheme 2.14).



Scheme 2.14: Fluorescent labeling of TMV virus particles *via* click reaction.

As protein-polymer conjugates constitute a versatile and interesting class of materials that are beneficial for applications in medicine and biotechnology,^{13g-i} click chemistry has got recent interest to generate various protein-polymer conjugate.^{13j-l} Recently, Cornelissen and coworkers^{13m} have used click reaction to monitor protein-polymer conjugation. They prepared several 3-azidocoumarin terminated poly(ethylene glycol) (2.51) and performed the click reaction with alkyne-functionalized bovin serum albumin (BSA), 2.50 to afford a highly fluorescent triazole-linked protein-polymer conjugate 2.52 (**Scheme 2.15**). The generation of the fluorescence can precisely be determined spectroscopically which can provide the information about the efficiency of the click reaction. Thus, it is clear that click chemistry can offer a possibility of monitoring the bioconjugation process by a study of fluorescence assay that is based on the progression of the reaction. This concept might find application in the formation of protein-polymer biohybrid amphiphiles.



Scheme 2.15: Synthesis of fluorescently labeled triazole-linked protein-polymer conjugate *via* click reaction.

2.6. Objective

Thus, from the above background it is clear that there are a few reports of click fluorescent molecules; however, the synthesis of microenvironment sensitive fluorophores in which the fluorescence property is installed into a non-fluorescent precursor or modulated for weakly fluorescent molecules *via* “click chemistry” has got very less attention. With this background and aim, therefore, we framed our objectives as below:

- The design and synthesis of click fluorophores from nonfluorescent/weakly fluorescent precursor azides/alkynes. In our design, we have chosen donor and/or acceptor aromatic azides and/or alkynes so as to achieve highly solvatochromic fluorophores with internal charge transfer (ICT) character.
- The evaluation of their photophysical properties of the “click” chemistry-generated new fluorophores.

- (c) Establishment of the concept of installation/modulation of fluorescence properties.
- (d) We also want to investigate the relation between solvatochromic emission property and the ICT feature by theoretical study.

The structures of the azides/alkynes and the targeted fluorophores are shown in **Figure 2.3**.

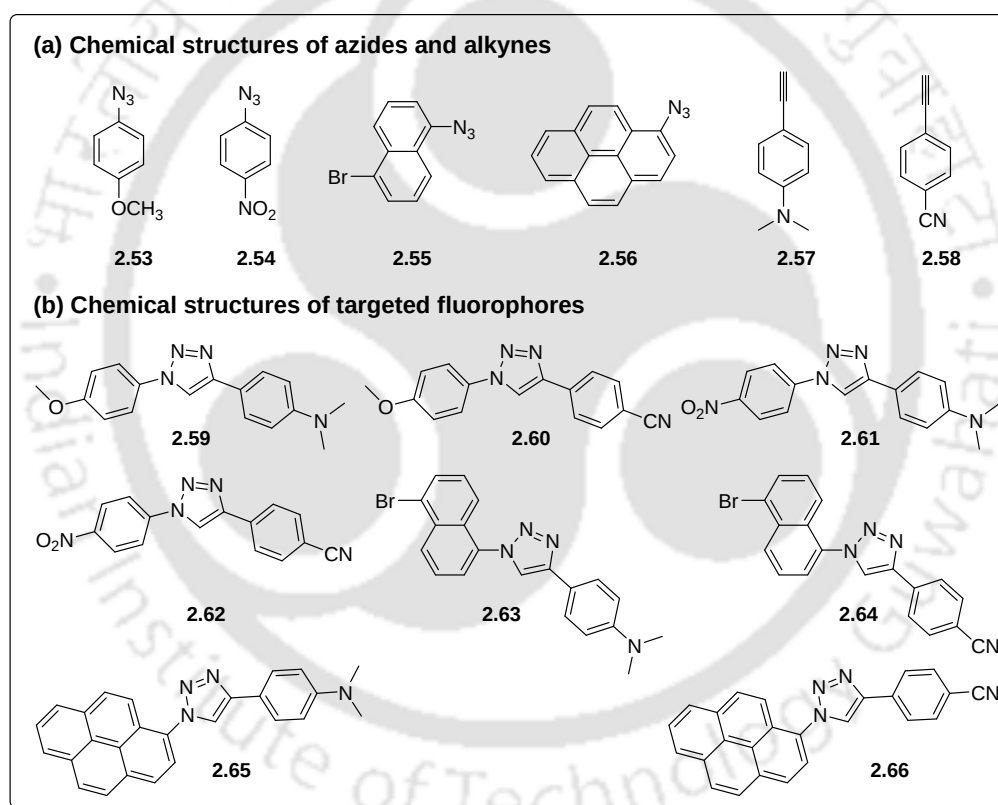


Figure 2.3: Chemical structures of the azides/alkynes and synthesized fluorophores.

2.7. The Concept of Our Design of Solvatochromic Fluorophores

A study of literature reveals that to design an environment sensitive fluorophore one needs to consider the following:

- (a) **Key Physical Parameters:** High molar extinction coefficients, high emission intensity and long excitation and emission wavelengths, high quantum yields, large Stoke's shift.
- (b) **Donor-Acceptor Units:** Environment sensitive fluorophores frequently possess an electron donor (**D**) and an electron acceptor (**A**) substituent attached to the same aromatic ring system, or linked *via* a linker or spacer.
- (c) **Environment Sensitive Aromatics:** The fluorescence of unsubstituted aromatic hydrocarbons is insensitive to the environment due to the high degree of symmetry between the ground and lowest singlet excited states.
- (d) **Possess ICT Character:** The electron donating and accepting groups on the aromatic ring confer a low energy excited state with marked intramolecular charge transfer character (ICT).
- (e) **Potential D-A Pairs:** The most important potential donor groups are amino or alkylamino, while sulfonyl, carbonyl, and nitrile groups are among the best acceptors.
- (f) **Connecting the Donor and Acceptor Units:** There are mainly three ways of connecting the donor and acceptor units as shown below (**Figure 2.4**).

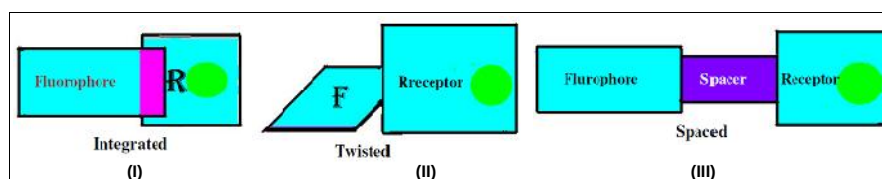


Figure 2.4: Three different ways of connecting fluorophores (F) and receptors (R).

Considering the above design criteria, we, therefore, designed the fluorophores in such a way that the donor and the acceptor units are linked *via* the triazole unit thereby the triazolyl donor part can act as full donor to the acceptor unit allowing a charge transfer process leading to installation of emission on to the non fluorescent

precursor. We also designed pyrene based fluorophore keeping in mind that the electron donating properties of triazolyl ring might effectively modulate the emission properties of an electronically coupled pyrene when it is coupled with a donor or an acceptor unit. **Figure 2.3** showed the list of targeted new fluorophores of two categories-one where fluorescence is installed and other where fluorescence is modulated. While compounds **2.59-2.64** were designed to show the installation of fluorescence response on to the non-fluorescent precursor amines or alkynes, the compounds **2.65-2.66** were targeted to show the modulation of the fluorescence response of pyrene (**Figure 2.3**). The concept of design strategy leading to fluorescence enhancement/modulation is presented in **Figure 2.5**.

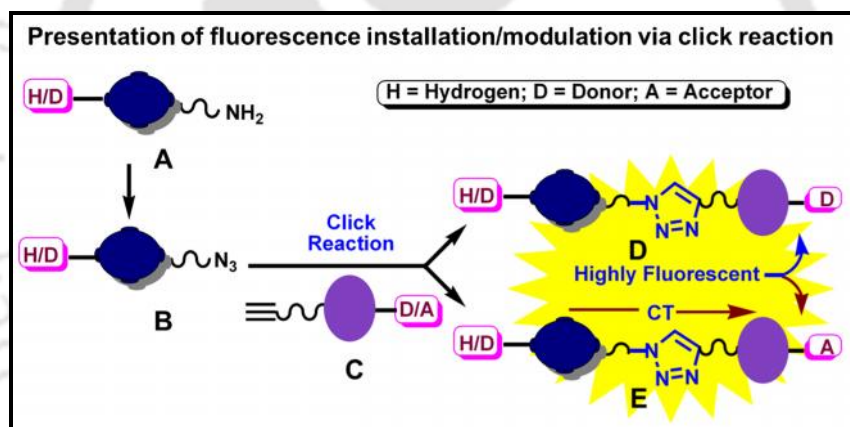


Figure 2.5: Presentation of fluorescence installation/modulation *via* click reaction.

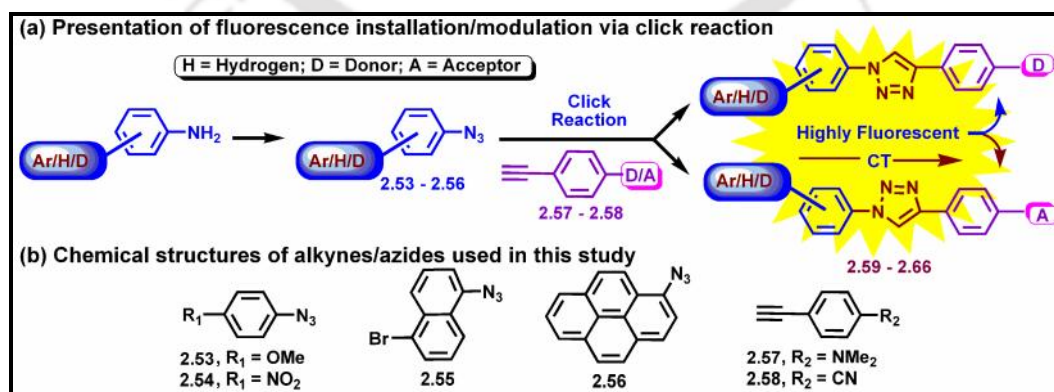
2.8. Results and Discussion

In our design a donor and an acceptor unit are linked *via* a triazole moiety so as to allow a charge transfer process ultimately leading to an installation of a fluorescence emission on to a non-fluorescent precursor (**Figure 2.5**). We have also focused on the design of triazolyl linked intrinsic fluorophore for modulation of its emission response. For this purpose we have chosen a versatile fluorophore, pyrene, which showed featured excimer/monomer emission and long fluorescence lifetime. However, pyrene fluorophore suffers from short emission wavelength which makes it difficult to use for

the applications such as *in vivo* fluorescence imaging. Therefore, preparation of long wavelength emissive and solvatochromic pyrene derivatives is of immense importance.¹⁴⁻¹⁶ Thus, we have designed pyrene based fluorophores keeping in mind that the electron donating property of the triazolyl ring may effectively modulate the emission response of an electronically coupled pyrene.

2.8.1. Synthesis of Click Fluorophores

The synthesis was accomplished (**Scheme 2.16**) *via* a modified click reaction protocol² and the products obtained in moderate to high yields were characterized well by NMR, mass, and in some cases by single crystal X-ray analysis. Thus, to a 1:1 dichloromethane:ethanol solution of aryl azides (**2.53-2.56**, synthesized *via* a diazotization reaction of the corresponding aryl amines and subsequent treatment with sodium azide), 1.5 equivalent of alkyne (**2.57-2.58**, **Scheme 2.16**) was added. After 5 minutes of degassing with N₂ and stirring, a solution of sodium ascorbate (6 mol%) followed by a solution of CuSO₄ in water (1 mol%) was added to the reaction mixture. After completion of the reaction monitored by TLC, the reaction mixture was evaporated to dryness, partitioned between water and ethyl acetate. The organic layer was washed with water, brine, and then dried over Na₂SO₄. The final product was obtained in purity by column chromatography and characterized.



Scheme 2.16: General procedure for the synthesis of aromatic azides and click fluorophores.

2.8.2. Spectral Characterization of Click Fluorophore

The ^1H NMR study of the fluorophore **2.59** showed that the two methyl ($-\text{NMe}_2$) group appeared as singlet at 3.01 ppm, the other methyl ($-\text{OMe}$) group appeared as singlet at 3.87 ppm. The two aromatic protons near NMe_2 group appeared as doublet at 6.81 ppm, another two protons of the same benzene ring appeared as doublet at 7.77 ppm. While two ortho protons with respect to the $-\text{OMe}$ group of methoxybenzene unit resonate at 7.03 ppm and appeared as doublet, other two protons of the same unit also appeared as doublet and resonate at 7.68 ppm. The triazole ring's hydrogen appeared as singlet at 7.98 ppm (**Figure 2.6**). The X-ray single crystal structure (ORTEP diagram is shown in **Figure 2.6**) corroborated the assigned structure.

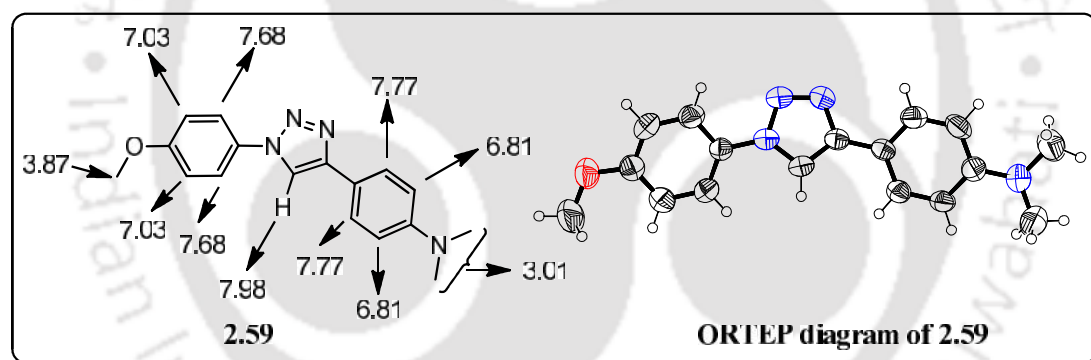


Figure 2.6: ^1H -NMR assignment and the ORTEP diagram with ellipsoid at 50% probability of **2.59**.

For fluorophore **2.64** two aromatic protons near $-\text{CN}$ group appeared as doublet at 7.75 ppm in ^1H NMR spectrum. The other two protons in the same benzene ring appeared as doublet at 8.05 ppm. The triazole ring's hydrogen appeared as singlet at 8.22 ppm. We observed two triplet signals for two separate hydrogen of naphthalene ring at 7.38 and 7.69 ppm which is good accord with the structure (**Figure 2.7**). The X-ray single crystal structure (ORTEP diagram is shown in **Figure 2.7**) corroborated the assigned structure.

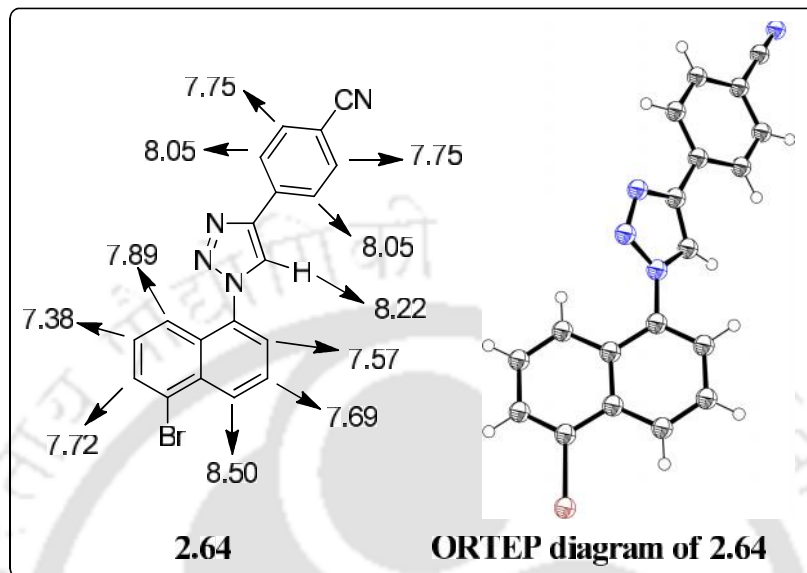


Figure 2.7: ^1H -NMR assignment and the ORTEP diagram with ellipsoid at 50% probability of **2.64**.

The ^1H NMR characterization data of compound **2.65** is also presented here as another representative compound. Thus, the ^1H NMR study of the fluorophore **2.65** revealed that all the six hydrogen of $-\text{NMe}_2$ group appeared as singlet at 3.03 ppm. The two aromatic ortho protons with respect to $-\text{NMe}_2$ group appeared as doublet at 6.84 ppm, and other two protons in the same benzene ring came as doublet at 7.87 ppm. The triazole ring's hydrogen resonates at 8.18 ppm and appeared as a singlet. The pyrene ring's protons lie in the chemical shift range of 7.97-8.29 ppm (**Figure 2.8**). The X-ray single crystal structure (ORTEP diagram is shown in **Figure 2.8**) finally corroborated the assigned structure.

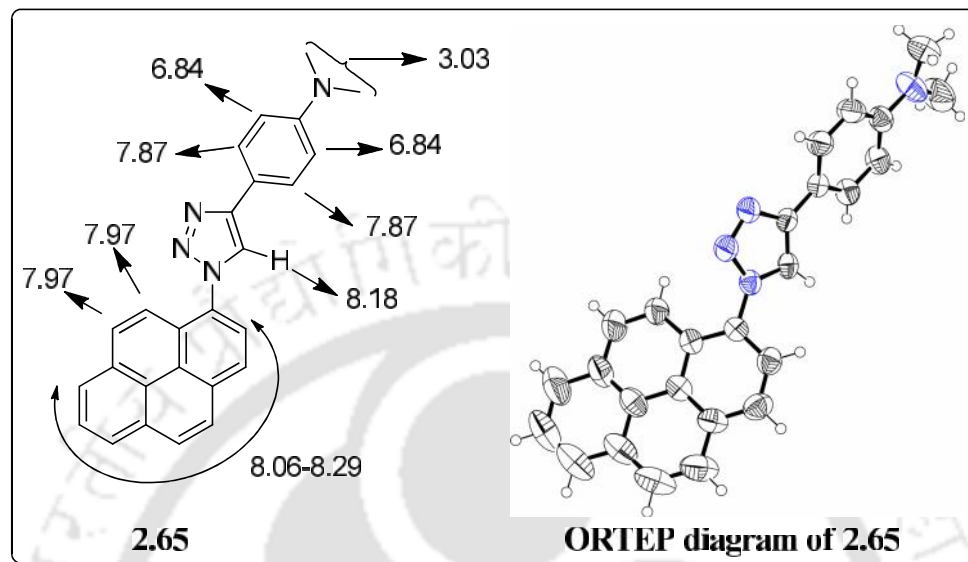


Figure 2.8: ^1H -NMR assignment and the ORTEP diagram with ellipsoid at 50% probability of **2.65**.

2.8.3. X-Ray Crystallographic Data

X-ray intensity data were collected at 296 K with Mo K α radiation ($\lambda = 0.71073$ Å) using a Bruker Nonius SMART CCD diffractometer equipped with graphite monochromator and Apex CD camera. The SMART software was used for data collection and for indexing the reflections and determining the unit cell parameters; the collected data were integrated using SAINT software.¹⁷ The structures were solved by direct methods and refined by full-matrix least-squares calculations using SHELXTL software. All the non-H atoms were refined in the anisotropic approximation against F^2 of all reflections. The single crystals were collected from chloroform-ethylacetate mixture at room temperature for all the cases and the X-ray crystallographic data of representative compounds are given in **Table 2.1**.

Table 2.1: Crystal data of the representative fluorophores.

Fluorophore Data	2.59	2.64	2.65
Z	4	4	4
Molecular Formula	C ₁₇ H ₁₈ N ₄ O	C ₁₉ H ₁₁ N ₄ Br	C ₂₆ H ₂₀ N ₄
Temperature (K)	296	296	296
Space Group	P 21 21 21 Orthorhombic	P -1 Triclinic	P 21/c Monoclinic
a (Å)	5.7361(3)	10.989(2)	5.7090(2)
b (Å)	13.5039(6)	11.956(2)	16.6315(5)
c (Å)	19.7921(8)	14.877(4)	20.8904(7)
α (degree)	90	105.234(5)	90
β degree)	90	94.855(5)	95.408(2)
γ (degree)	90	116.184(4)	90
M _r	294.35	375.22	388.46
V (Å ³)	1533.09(12)	1646.4(6)	1974.70(11)
μ (mm ⁻¹)	0.083	2.503	0.079
F(000)	624.0	752.0	816.0
R(reflections)	0.0380(1540)	0.0438(3115)	0.0440(2091)
wR2(reflections)	0.0531(3850)	0.1167(8196)	0.0767(4921)
Correction method	Multi Scan	Multi Scan	Multi Scan

2.8.4. Studies of Photophysical Properties

Having all the products in very pure recrystallized form in hand, we studied, first, their photophysical properties in different solvents of varying dielectric constants. The

aim of this study was to explore the effect of solvent polarity on the fluorophore's photophysical properties and to correlate these effects to their structure.

We observed that while solvent polarity had only a minor influence on the absorptive properties of almost all the fluorophores, it drastically affected their emission properties. We observed that all the products showed an increase in fluorescence intensity over their parent amine or the alkyne precursors. It has also been found that the responses of some of the products are highly sensitive toward a solvent's dielectric constant leading to a red shifted emission.

We observed structureless, broad, and strong solvent polarity dependent dominant emission from the fluorophore **2.59**, [a Donor-Triazole-Donor (**D-T-D**) system] (**Figure 2.9c**). Thus, **2.59** showed a 122 nm red shifted emission when compared between the solvent hexane (almost zero intensity) and MeOH. Upon titration of a dioxane solution of **2.59** by water a red shift of 129 nm was observed in 50% water (**Figure 2.9d**). These features indicated an emission from an ICT state.¹⁸⁻¹⁹ Fluorescence quantum yields were also found to be increased with the increase in solvent polarity (**Table 2.2**). This clearly indicated a highly polar emissive state. However, quantum yields decreased in MeOH and ethanol which may be due to a protic solvent-solute interaction.^{18a,20}

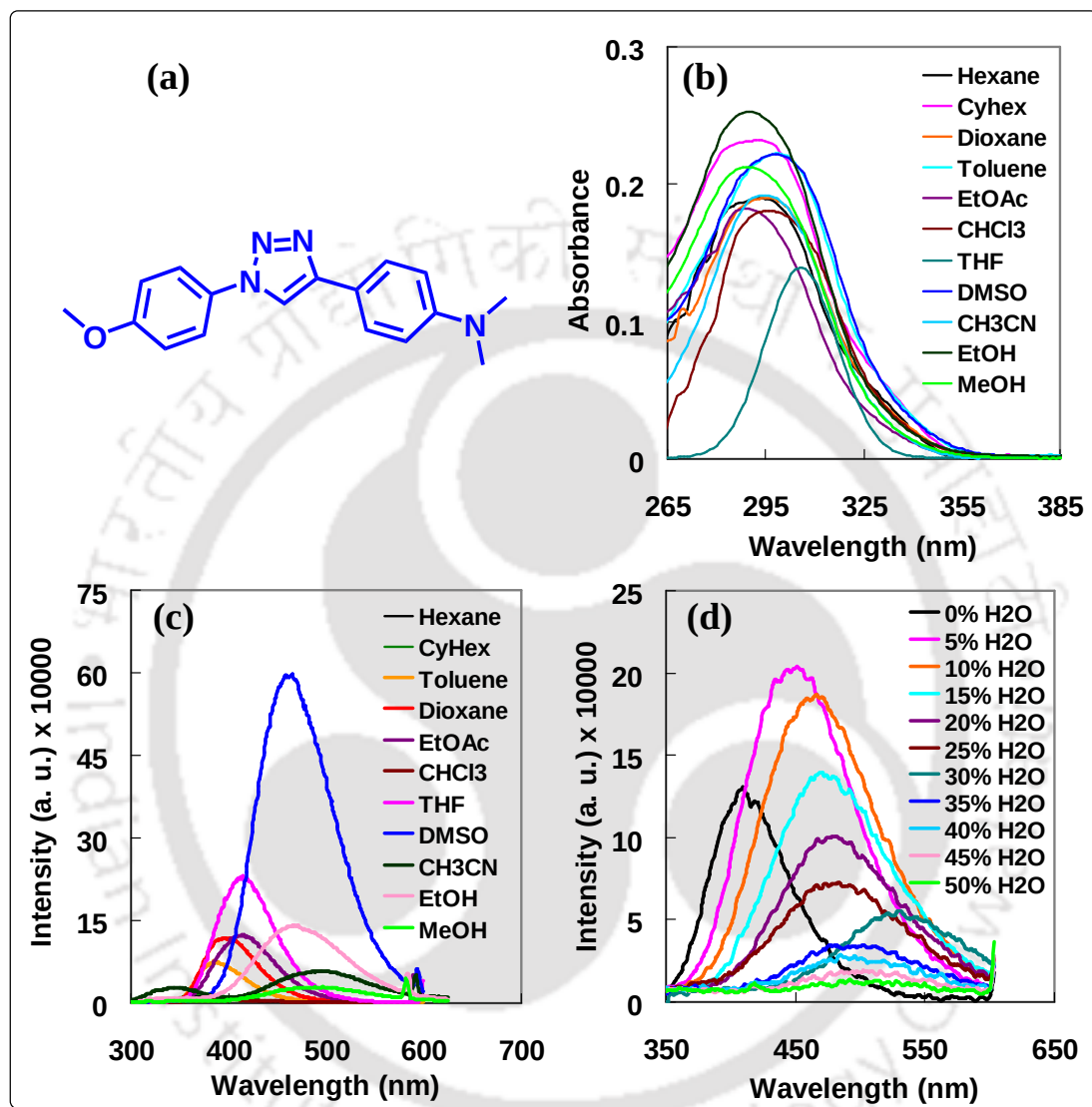


Figure 2.9: (a) The structure of the fluorophore, (b) UV-visible, (c) fluorescence spectra of **2.59** in different solvents and (d) fluorescence in dioxane-water solvent system [10 μ M, r.t.; $\lambda_{\text{ex}} = \lambda_{\text{max}}^{\text{abs}}$ (286 – 304 nm) of each solvent].

Table 2.2: Summary of photophysical property of the fluorophore **2.59**.

Entry	Solvents	af	UV-Vis & Fluorescence			Entry	Solvents	af	UV-Vis & Fluorescence		
			λ_{abs} [nm]	λ_{em} [nm]	Φ				λ_{abs} [nm]	λ_{em} [nm]	Φ
2.59	Cyhex	0.000	292	371	0.008	2.59 Dioxane : Water	Dx ₉₅ Wt ₅	0.186	294	452	0.111
	Hexane	0.001	294	371	0.007		Dx ₉₀ Wt ₁₀	0.229	296	468	0.115
	Toluene	0.013	294	387	0.027		Dx ₈₅ Wt ₁₅	0.249	297	474	0.094
	Diox	0.021	291	405	0.053		Dx ₈₀ Wt ₂₀	0.262	294	482	0.063
	CHCl ₃	0.148	293	405	0.005		Dx ₇₅ Wt ₂₅	0.270	291	485	0.045
	EtOAc	0.201	286	414	0.063		Dx ₇₀ Wt ₃₀	0.277	294	534	0.035
	THF	0.210	304	415	0.177		Dx ₆₅ Wt ₃₅	0.283	288	502	0.022
	DMSO	0.265	296	463	0.309		Dx ₆₀ Wt ₄₀	0.287	294	500	0.020
	EtOH	0.290	287	470	0.094		---	---	---	---	---
	CH ₃ CN	0.307	291	496	0.052		---	---	---	---	---
	MeOH	0.309	287	493	0.020		---	---	---	---	---

The fluorophore of the type **D**(donor)-**T**(triazole)-**A**(acceptor) wherein the precursor amine and alkyne contain a donor –OMe group and an acceptor –CN group respectively, as in the case of **2.60**, showed a structureless emission with a red shift (**Figure 2.10c**). We observed 54 nm red shifting from hexane to CH₃CN, and 92 nm red shifting upon titration of a dioxane solution of **2.60** by water. Also, the quantum yield was increased as the solvent polarity was increased (**Table 2.3**). These results infer an intramolecular charge transfer (ICT) feature of the lowest-lying excited state¹⁸ which was supported by a solvatochromicity test (**Figure 2.10d**).

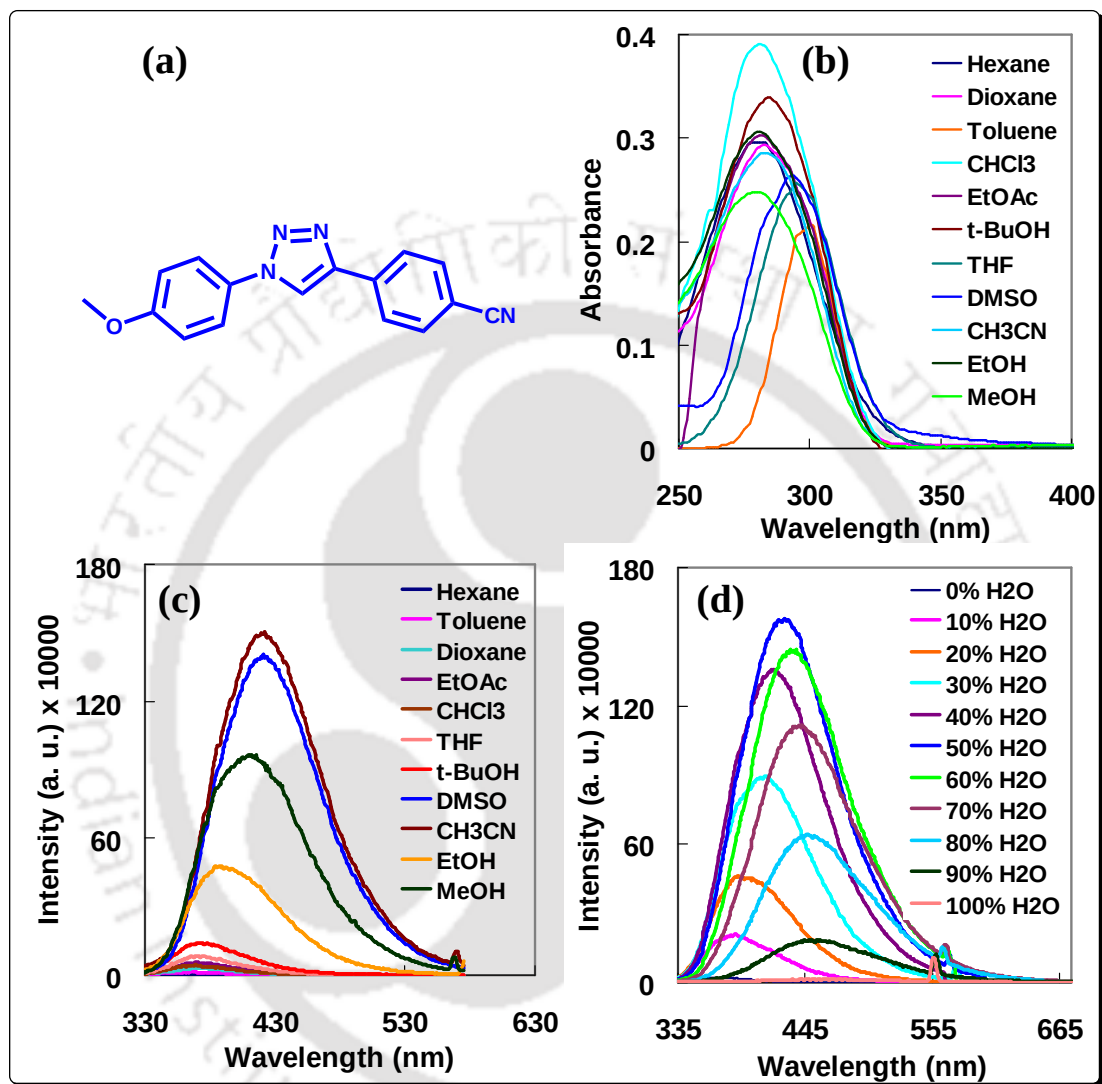


Figure 2.10: (a) The structure of the fluorophore, (b) UV-visible, (c) fluorescence spectra of **2.60** in different solvents and (d) fluorescence in dioxane-water solvent system [10 μ M, r.t.; $\lambda_{\text{ex}} = \lambda_{\text{max}}^{\text{abs}}$ (278 – 294 nm) of each solvent].

Table 2.3: Summary of photophysical property of the fluorophore **2.60**.

Entry	Solvents	af	UV-Vis & Fluorescence			Entry	Solvents	af	UV-Vis & Fluorescence		
			λ_{abs} [nm]	λ_{em} [nm]	Φ				λ_{abs} [nm]	λ_{em} [nm]	Φ
2.60	Hexane	0.001	278	368	0.004	2.60	Dx ₁₀₀ Wt ₀	0.021	283	372	0.010
	Toluene	0.013	293	367	0.006		Dx ₉₀ Wt ₁₀	0.229	282	385	0.077
	Diox	0.021	288	372	0.010		Dx ₈₀ Wt ₂₀	0.262	282	390	0.158
	CHCl ₃	0.148	279	370	0.010		Dx ₇₀ Wt ₃₀	0.277	281	411	0.263
	EtOAc	0.201	280	374	0.016		Dx ₆₀ Wt ₄₀	0.287	281	419	0.552
	THF	0.210	294	373	0.025		Dx ₅₀ Wt ₅₀	0.294	280	427	0.623
	DMSO	0.265	292	422	0.619		Dx ₄₀ Wt ₆₀	0.300	279	435	0.583
	EtOH	0.290	279	386	0.169		Dx ₃₀ Wt ₇₀	0.306	278	441	0.448
	CH ₃ CN	0.307	281	422	0.610		Dx ₂₀ Wt ₈₀	0.311	277	449	0.275
	MeOH	0.309	278	412	0.460		Dx ₁₀ Wt ₉₀	0.315	274	455	0.235
	---	---	---	---	---		Dx ₀ Wt ₁₀₀	0.320	274	464	0.087

Fluorophore **2.61**, **2.62** and **2.63** showed only a faint emission under the condition used for others. However, at large slit width, the following behaviors were observed where quantum yields could not be evaluated. A highly solvent polarity dependent emission was exhibited by fluorophore **2.61**, an **A-T-D** system. Thus, as the solvent polarity increased, a shift of 56 nm from cyclohexane to MeOH with decreased intensity was observed which is characteristic of ICT emission (**Figure 2.11**, **Table 2.4**). However, the fluorophore **2.62** (**A-T-A** system), showed a solvent polarity independent structured emission (**Figure 2.12**, **Table 2.4**).

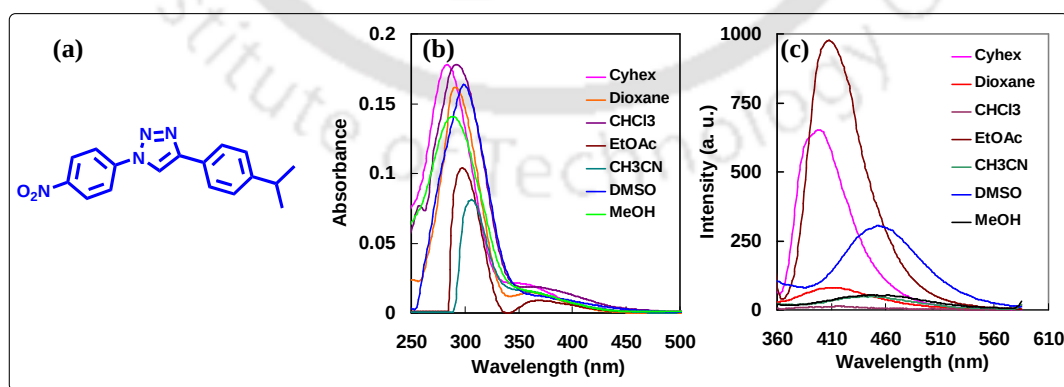
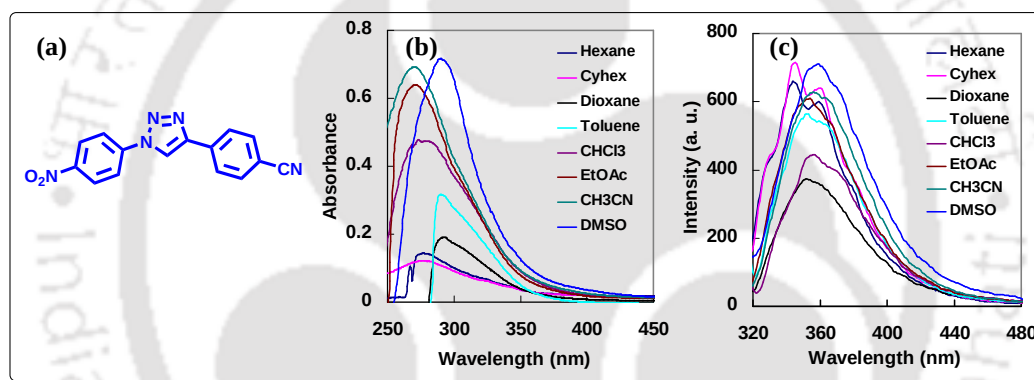


Figure 2.11: (a) The structure of the fluorophore, (b) UV-visible and (c) fluorescence spectra of **2.61** in different solvents [10 μ M, r.t.; $\lambda_{\text{ex}} = \lambda_{\text{max}}^{\text{abs}}$ (283 – 305 nm) of each solvent].

Table 2.4: Summary of photophysical property of the fluorophores **2.61-2.62**.

Entry	Solvents	af	UV-Vis & Fluorescence		Entry	Solvents	af	UV-Vis & Fluorescence	
			λ_{abs} [nm]	λ_{em} [nm]				λ_{abs} [nm]	λ_{em} [nm]
2.61	Cyhex	0.000	283, 358	399	2.62	Cyhex	0.000	277, 343	345
	Toluene	0.013	286, 361	393		Hexane	0.001	276, 343	344
	Diox	0.021	290, 363	410		Toluene	0.013	290, 340	353
	CHCl ₃	0.148	291, 371	418		Dioxane	0.021	292, 338	352
	EtOAc	0.201	296, 369	408		CHCl ₃	0.148	280, 337	356
	DMSO	0.265	298, 370	452		EtOAc	0.201	271, 341	354
	CH ₃ CN	0.307	305, 367	449		DMSO	0.265	289, 343	359
	MeOH	0.309	287, 368	446		CH ₃ CN	0.307	270, 342	356

**Figure 2.12:** (a) The structure of the fluorophore, (b) UV-visible and (c) fluorescence spectra of **2.62** in different solvents [10 μ M, r.t.; $\lambda_{\text{ex}} = \lambda_{\text{max}}^{\text{abs}}$ (270 – 292 nm) of each solvent].

Fluorophore **2.63** exhibited a dual behavior similar to **2.59** in low polar solvents, but a single emission centered at 357 nm was appeared in high polar solvent like in CH₃CN and in MeOH. In low polar solvents like, hexane, cyclohexane, 1,4-dioxane, toluene, chloroform, ethylacetate, the second band at 399 nm in hexane shifted gradually with decrease in intensity as the polarity increases and reaches to 537 nm in ethylacetate which again inferred an emission from ICT state (**Figure 2.13**, **Table 2.5**).

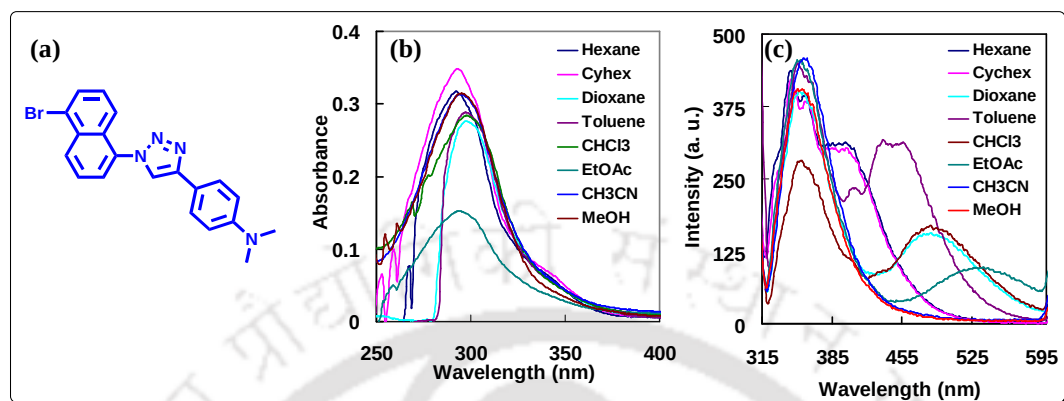


Figure 2.13: (a) The structure of the fluorophore, (b) UV-visible and (c) fluorescence spectra of **2.63** in different solvents [10 μ M, r.t.; $\lambda_{\text{ex}} = \lambda_{\text{max}}^{\text{abs}}$ (292 – 297 nm) of each solvent].

Fluorophore **2.64** showed a comparatively less solvent polarity sensitive emission that was centered at around 406 nm. A spectral shift of only 23 nm was observed with decrease in intensity as we moved from hexane to MeOH. This result is clearly an indication of an ICT emission (**Figure 2.14**, **Table 2.5**).

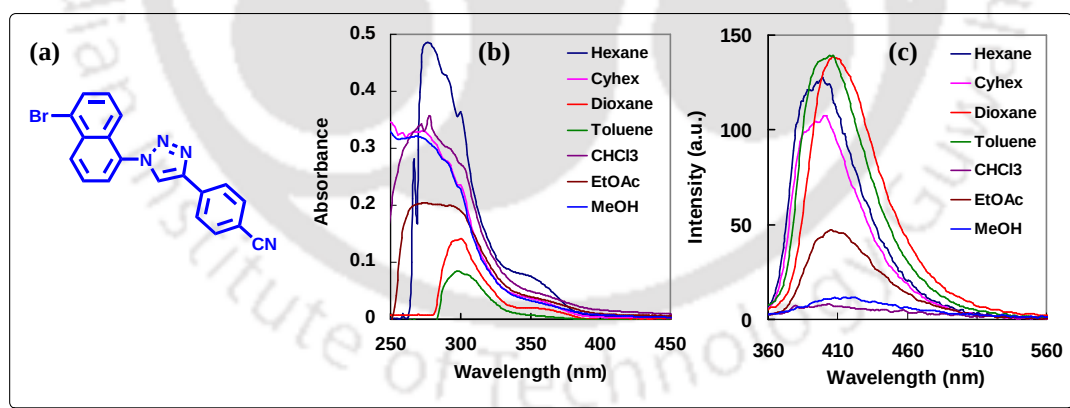


Figure 2.14: (a) The structure of the fluorophore, (b) UV-visible and (c) fluorescence spectra of **2.64** in different solvents [10 μ M, r.t.; $\lambda_{\text{ex}} = \lambda_{\text{max}}^{\text{abs}}$ (297 – 300 nm) of each solvent].

Table 2.5: Summary of photophysical property of the fluorophores **2.63-2.64**.

Entry	Solvents	af	UV-Vis & Fluorescence		Entry	Solvents	af	UV-Vis & Fluorescence	
			λ_{abs} [nm]	λ_{em} [nm]				λ_{abs} [nm]	λ_{em} (nm)
2.63	Cyhex	0.000	293, 345	344, 399	2.64	Cyhex	0.000	300, 357	401
	Hexane	0.001	292, 344	344, 399		Hexane	0.001	300, 350	399
	Toluene	0.013	297, 344	351, 447		Toluene	0.013	297, 350	406
	Dioxane	0.021	297, 343	352, 484		Dioxane	0.021	300, 361	406
	CHCl ₃	0.148	298, 345	354, 484		CHCl ₃	0.148	301, 360	403
	EtOAc	0.201	294, 342	354, 537		EtOAc	0.201	297, 360	405
	CH ₃ CN	0.307	295, 344	357		MeOH	0.309	300, 362	422
	MeOH	0.309	295, 344	355		---	---	---	---

We observed an interesting fluorescence photophysical property exhibited by the fluorophore **2.65** (an **A-T-D** system), a triazolyl modified pyrene. Thus, the fluorophore **2.65** showed a strong structureless emission centered at 415 nm in hexane and cyclohexane. However, in ether, dioxane, toluene, CHCl₃, and EtOAc, a dual emission behavior was observed with a strong red shifted pattern of the ICT band as the solvent polarity was increased from toluene (466 nm) to EtOAc (544 nm) (**Figure 2.15c, Table 2.6**). In highly polar solvents like CH₃CN, and MeOH, a structured band characteristic of a pyrene emission with a very low intensity was observed which was also supported by a solvatochromicity test (**Figure 2.15d**). Thus, in pure dioxane, the ICT emission was highly dominated over the LE emission and as the % of H₂O was increased, the intensity of the LE emission was also going to increase. However, in 50% H₂O in dioxane, we observed a pure LE emission (**Figure 2.15d, Table 2.6**).

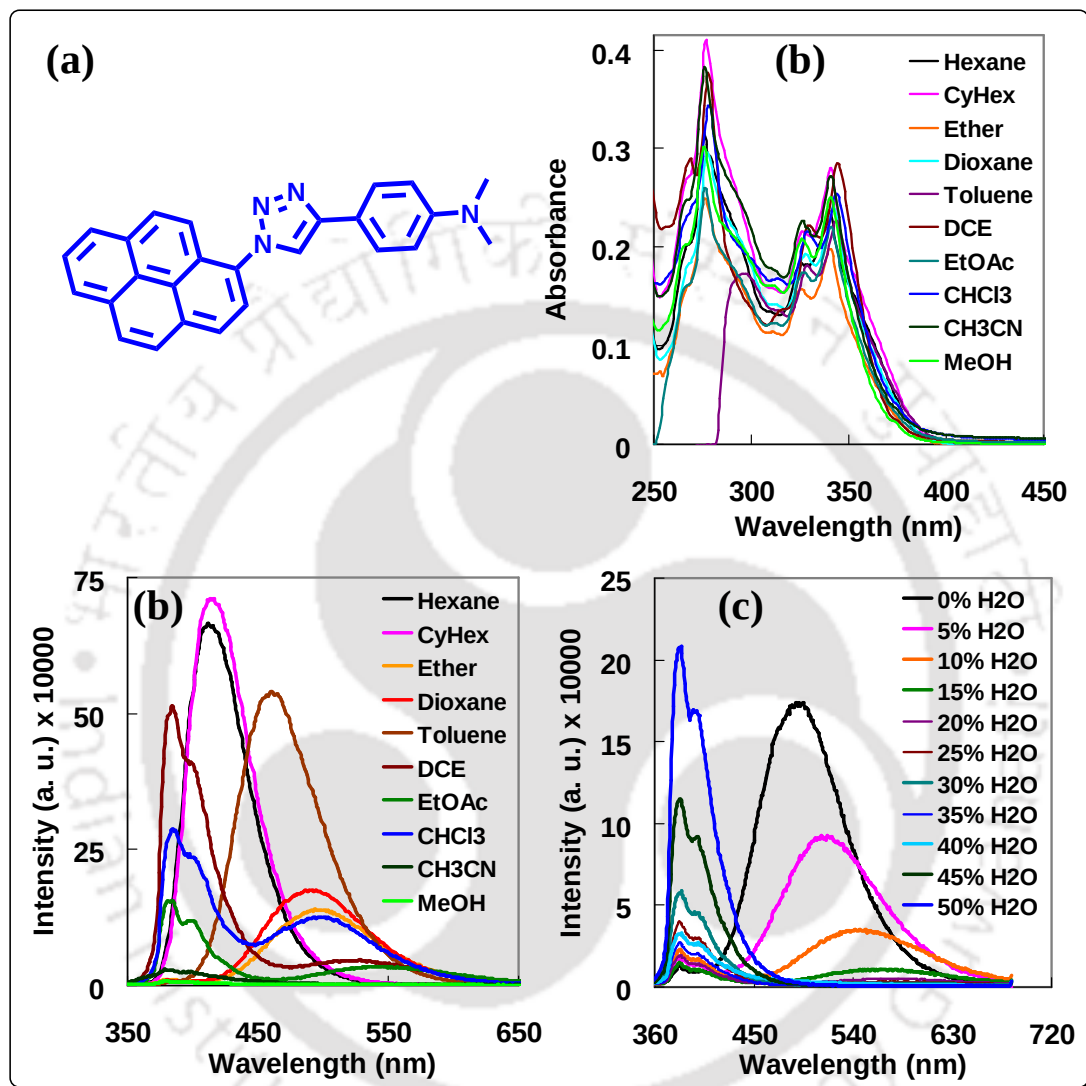


Figure 2.15: (a) The structure of the fluorophore, (b) UV-visible, (c) fluorescence spectra of **2.65** in different solvents and (d) fluorescence in dioxane-water solvent system showing a dual behavior [$10\ \mu\text{M}$, r.t.; $\lambda_{\text{ex}} = \lambda_{\text{max}}^{\text{abs}}$ (340 – 344 nm) of each solvent].

Table 2.6: Summary of photophysical property of the fluorophore **2.65**.

Entry	Solvents	af	UV-Vis & Fluorescence			Entry	Solvents	af	UV-Vis & Fluorescence		
			λ_{abs} [nm]	λ_{em} [nm]	Φ				λ_{abs} [nm]	λ_{em} [nm]	Φ
2.65	Cyhex	0.000	341	415	0.165	2.65 Dioxane : Water	Dx ₁₀₀ Wt ₀	0.021	342	384, 490	0.078
	Hexane	0.001	340	413	0.181		Dx ₉₅ Wt ₅	0.186	342	383, 516	0.047
	Toluene	0.013	343	461	0.202		Dx ₉₀ Wt ₁₀	0.229	342	383, 548	0.022
	Dioxane	0.021	342	490	0.078		Dx ₈₅ Wt ₁₅	0.249	342	384, 567	0.009
	CHCl ₃	0.148	343	384, 499	0.107		Dx ₈₀ Wt ₂₀	0.262	342	384, 580	0.008
	Ether	0.163	340	499	0.078		Dx ₇₅ Wt ₂₅	0.270	342	384, 585	0.009
	EtOAc	0.201	341	384, 544	0.052		Dx ₇₀ Wt ₃₀	0.277	342	384	0.014
	DCE	0.221	344	384, 530	0.104		Dx ₆₅ Wt ₃₅	0.283	343	384	0.006
	CH ₃ CN	0.307	341	379	0.008		Dx ₆₀ Wt ₄₀	0.287	342	383	0.008
	MeOH	0.309	341	380	0.003		Dx ₅₅ Wt ₄₅	0.291	342	383	0.025
							Dx ₅₀ Wt ₅₀	0.294	342	383	0.039

Unlike compound **2.65**, the fluorophore **2.66** (a **D-T-A** system) which contains an electron withdrawing –CN substituent, the emission was centered at 400 nm, and the vibronic structure of a pyrene emission was maintained *i.e.*, the emission originates from a purely LE state (**Figure 2.16c**).^{18c,21} The fluorescence quantum yield of **2.66** was increased as the solvent polarity was increased with no solvatochromicity (**Table 2.7**) while it was lower in a polar solvent than that in a non-polar solvent in case of **2.65** (**Table 2.6**). These results infer an ICT feature of the lowest-lying excited state in **2.65** (with the pyrene as an electron acceptor) but not in **2.66**.^{18,19,21}

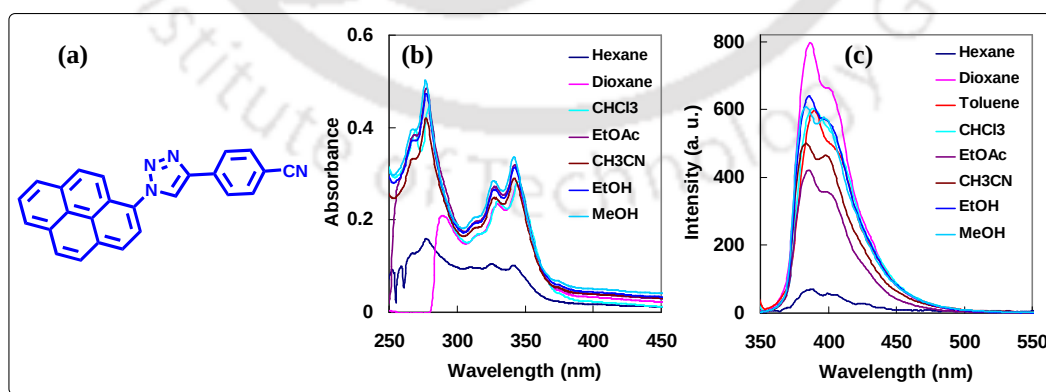


Figure 2.16: (a) The structure of the fluorophore, (b) UV-visible and (c) Fluorescence spectra of **2.66** in different solvents [10 μM , r.t.; $\lambda_{\text{ex}} = \lambda_{\text{max}}^{\text{abs}}$ (340 – 345 nm) of each solvent].

Table 2.7: Summary of photophysical property of the fluorophore **2.66**.

Entry	Solvents	af	UV-Vis & Fluorescence		
			λ_{abs} [nm]	λ_{em} [nm]	Φ
2.66	Hexane	0.001	341	388	0.071
	Toluene	0.013	344	390	0.098
	Dioxane	0.021	344	387	0.188
	CHCl ₃	0.148	345	388	0.152
	EtOAc	0.201	342	385	0.085
	EtOH	0.290	342	386	0.141
	CH ₃ CN	0.307	342	384	0.122
	MeOH	0.309	341	383	0.130

Therefore, our study clearly showed that a “click” reaction between an azide and an alkyne sufficiently installs a fluorescence emission property into a non-fluorescent precursor and modulates the emission response of a pyrene fluorophore. It is important to note that these fluorophores show a direct trend between the fluorescence intensities and solvent dielectric constants and thus have a broader general utility (**Figure 2.9 - 2.16**). Examination of the band-shape and fluorescence quantum yields of the fluorophores revealed the delicate interplay of structure and emissive states. Thus, compound **2.59**, **2.60**, and **2.65** showed a highly emissive, pure ICT state as was indicated by a high quantum yield, broad band-shape, and red shifted pattern as the solvent polarity was increased. The solvent polarity independent LE emission of **2.66** suggests the emitting state as a non-polar $^1\pi - \pi^*$ state. The dual emission exhibited by **2.65** indicated the presence of a mixed LE and CT state. Switching between these two states depends on the structure and the solvent polarity.^{19,21e}

2.8.5. Fluorescence Life Time Measurement

To interpret the photophysical properties in a more intuitive manner, the fluorescence lifetimes of **2.59**, **2.60**, **2.64**, **2.65** and **2.66** were measured in different solvents. Thus, biexponential decay was observed in nonpolar dioxane or hexane and the lifetimes of the major components are less than or closer to 1.0 ns (**Table 2.8**).

However, as the solvent polarity was increased, lifetimes were also increased. As for example, in dioxane, the major component (68%) of fluorophore **2.60** was short lived ($\tau^2 = 0.04$ ns) and its relative contribution was increased to 100% in 1:1 dioxane:water solution ($\tau^2 = 3.45$ ns), which is an indication of a π - π^* state. In protic polar solvent, like methanol, compared to DMSO, the life time of **2.60** was decreased which may be because of the fluorescence quenching through non-radiative pathway *via* hydrogen bonding.^{18a} The relative contribution of the longer lifetime component of **2.65** ($\tau_2 = 2.5$ ns) in hexane was increased from 27% to 100% as the solvent polarity was increased (in CHCl_3 , $\tau_1 = 2.62$ ns). In methanol, the minor component showed a decay time τ_1 , of 1.87 ns, for which a weak emission from LE state was observed. No ICT emission has been observed as the decay time of the major component ($\tau_1 = 14.30$ ns) was very large and the fluorophore followed a non-radiative pathway.

Lifetime data were also collected at two emission maxima for **2.65** in solvents displaying biexponential decays (**Table 2.8**). Thus, it was observed that, the value of τ_1 and τ_2 remain consistent, but the relative contributions of the two lifetimes vary according to the observed wavelength. As for example, for **2.65**, in dioxane, the relative contribution of the longer lifetime τ_1 was increased from 29% to 83% as the observed wavelength was increased from 406 nm to 496 nm. Similar behavior was observed in 10% water in dioxane solvent. As the intensity of the LE band was increased from dioxane to 10% water in dioxane solution, the lifetime is also increased from 3.78 ns to 4.27 ns (monitoring wavelength is the LE band). This observation correlates with the K_{nr} value that was decreased from 3.98 to 3.64 ns. When the monitoring emission wavelength was the ICT band, we observed that with decrease in intensity, the lifetime was also decreased from 2.95 ns to 1.70 ns which again correlate the increased K_{nr} value of 4.28 ns from 2.83 ns.

Table 2.8: Summary of steady state and time resolved fluorescence properties of the fluorophores.^[a]

Entry	Solvent ^[b]	Φ	λ [nm]	τ_1 [ns]	τ_2 [ns]	$\langle\tau\rangle$ [ns]	k_f [10 ⁸ s ⁻¹]	k_{nr} [10 ⁸ s ⁻¹]	χ^2
2.59	Diox	0.052	410	0.78 (88.57%)	2.71 (11.43%)	0.84	0.79	11.01	1.38
	DMSO	0.308	357	2.09 (41.53%)	14.36 (58.47%)	4.18	0.73	1.65	1.32
			471	0.41 (100%)	---	0.41	7.5	17	1.14
	EtOH	0.093	349	1.42 (93.65%)	8.24 (6.35%)	1.50	0.61	6.0	1.06
			482	7.08 (100%)	---	7.08	0.13	1.28	1.15
	15% Wt in Dx	0.094	472	8.26 (100%)	---	8.26	0.11	1.09	1.01
2.60	Diox	0.010	395	0.04 (67.84%)	3.06 (32.16%)	0.06	1.63	165.0	1.02
	DMSO	0.619	427	3.06 (100%)	---	3.06	2.02	1.24	1.04
	MeOH	0.46	420	1.75 (100%)	---	1.75	2.6	3.08	1.11
	50% Wt in Dx	0.623	433	3.45 (100%)	---	3.45	1.8	1.09	1.07
2.64	Hexane	0.183	399	0.20 (53.56%)	3.27 (46.44%)	0.35	5.17	23.08	1.15
	DMSO	0.564	415	2.91 (50.76%)	11.31 (49.24%)	4.58	1.23	0.95	1.21
	MeOH	0.067	422	1.64 (46.69%)	5.63 (53.31)	2.62	0.25	3.55	0.99
2.65	Hexane	0.180	412	0.81 (72.98%)	2.50 (27.02%)	0.98	1.82	8.31	1.04
	Toluene	0.202	463	1.59 (30.62%)	3.26 (69.38%)	2.46	0.82	3.23	1.04
	Diox	0.001	406	3.78 (29.02%)	2.00 (70.98%)	2.31	0.33	3.98	1.09
		0.076	496	2.95 (82.68%)	6.57 (17.32%)	3.25	0.23	2.83	1.01
	EtOAc	0.030	385	3.27 (5.92%)	1.27 (94.98%)	1.30	0.39	7.26	1.05
		0.022	548	3.87 (100%)	---	3.87	0.13	2.44	1.05
	CHCl ₃	0.058	384	7.20 (100%)	---	7.20	0.14	1.24	1.09
		0.059	498	2.62 (100%)	---	2.62	0.40	3.40	0.94
	MeOH	0.003	388	1.87 (8.90%)	14.30 (91.10%)	8.98	0.003	1.1	1.05
	10% Wt in Dx	0.003	401	4.27 (11.73%)	2.56 (88.27%)	2.68	0.08	3.64	1.13
		0.019	577	1.70 (62.97%)	5.47 (37.03%)	2.28	0.09	4.28	0.99
2.66	Hexane	0.071	388	1.40 (5.91%)	8.71 (94.09%)	6.65	0.10	1.39	1.09
	MeOH	0.13	383	14.99 (100%)	---	14.9	0.08	0.58	1.02

^[a]Excitation at λ_{ex} nm and recorded at λ_{max} . $\langle\tau\rangle$, k_f , and k_{nr} are weighted means from the biexponential fits: $\langle\tau\rangle = 1/(\alpha_1/\tau_1 + \alpha_2/\tau_2)$, $k_f = \Phi/\langle\tau\rangle$, and $k_{nr} = (1 - \Phi)/\langle\tau\rangle$.^{18c,18g}

^[b]Diox = Dx = Dioxane, Wt = Water.

2.8.6. Test for Solvatochromicity via Lippert and Mataga Polarity Parameter

To get an insight into the different solvatochromic behaviors of **2.59**, **2.60** and **2.65** the spectral dependency on solvent polarity was studied on the basis of Lippert-Mataga model.²² The Lippert-Mataga polarity parameter (Δf) was considered as the

measure of the polarity of different solvents and solvent mixtures used and was calculated using following equation 1.²²⁻²⁵

$$\Delta f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \quad (1)$$

where, ε and n are the dielectric constant and refractive index of the medium.

Thus, the absorption (a_{max}^{abs}) and fluorescence (a_{max}^{fl}) maxima of the more intense fluorophores **2.59**, **2.60** and **2.65** were measured in different solvents and solvent mixtures along with the polarity parameter Δf of the different solvent systems calculated using equation 1 (**Table 2.9**). As was revealed from the UV-visible, and fluorescence spectra (**Figure 2.9-2.10** for **2.59-2.60** and **Figure 2.15** for **2.65** respectively), the a_{max}^{fl} values gradually undergo a red shift when the solvent polarity increases for all the fluorophores. However, the red shift of a_{max}^{fl} is much larger than the shift in a_{max}^{abs} values, suggesting the fluorescent states of all the fluorophore to be more polar in nature than their ground states.^{18c,21g,22b-c,25b,26-28} In particular, fluorophore **2.59-2.60** showed a slight red shift with Δf in the lower solvent polarity region ($\Delta f < 0.265$) but at the higher solvent polarity region the a_{max}^{abs} values showed a small blue shift (**Table 2.9**). The a_{max}^{fl} values gradually undergo a more red shift when the solvent polarity increases for **2.59-2.60**. In case of fluorophore **2.65**, the a_{max}^{abs} values remain almost unchanged with solvent polarity; however, the a_{max}^{fl} (ICT) values gradually increase with increase in solvent polarity.

Next, we examined the correlations of the absorption and fluorescence maxima ($\tilde{\nu}_{max}^{abs}$ and $\tilde{\nu}_{max}^{fl}$ respectively in cm^{-1}) of the fluorophore **2.59**, **2.60** and **2.65** respectively, with the solvent polarity function Δf (**Figures 2.17a-c**). Thus, from figure **2.17b** and **2.17c** it was seen that for the fluorophore **2.60** and **2.65**, the $\tilde{\nu}_{abs}$ values apparently correlate linearly with Δf , suggesting that the ground state of these fluorophores is moderately polar in nature. For the fluorophore **2.59**, a less correlation

was observed for the $\tilde{\nu}_{abs}$ vs. Δf plot (**Figure 2.17a**). However, following the UV-visible spectra (**Figure 2.9b** and **2.10b** for **2.59-2.60** respectively), which was quite similar to that of the **2.60**, we expect that the ground state of the **2.59** is also of polarity similar to that of the **2.60**.

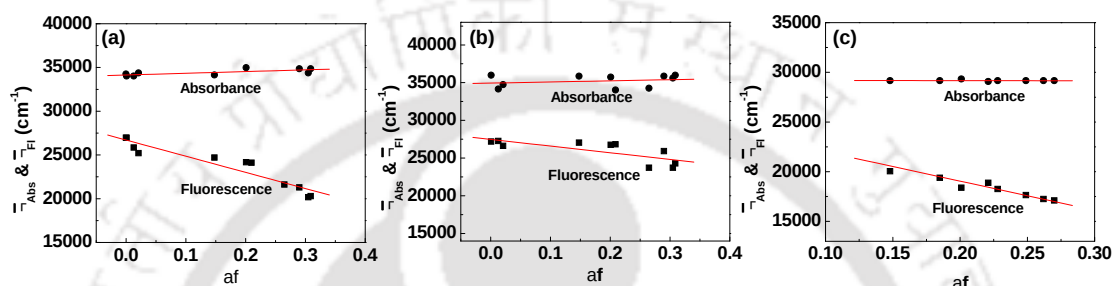


Figure 2.17: Plots of $\tilde{\nu}_f$ & $\tilde{\nu}_{abs}$ values against Δf for fluorophore (a) **2.59**, (b) **2.60** and (c) **2.65** ($\tilde{\nu}_{f(ICT)}$ and $\tilde{\nu}_{abs}$ values) in different solvents.

However, as is shown in **Figure 2.17a-c**, the $\tilde{\nu}_f$ values for all the fluorophores showed a very good linear correlation with Δf for the whole range of the solvent polarity tested. This observation, therefore, suggests that for all the fluorophores the nature of the fluorescent states remains essentially unchanged in all the solvents used. However, the spectral feature changes significantly due to the solvent induced modulation. The reasonably high slopes of the $\tilde{\nu}_f$ vs. Δf plots for all the fluorophores further suggest that the fluorescent states of these fluorophores are highly polar in nature, most possibly of intramolecular charge transfer (ICT) character.^{18,19,21,26-28}

Table 2.9: Summary table of $\tilde{\nu}_{max}^{abs}$ and $\tilde{\nu}_{max}^{fl}$ of the fluorophores and solvent polarity parameter (Δf).

Solvents	Δf	2.59		2.60		2.65			
		$\tilde{\nu}_{max}^{abs}$ [cm ⁻¹]	$\tilde{\nu}_{max}^{fl}$ [cm ⁻¹]	$\tilde{\nu}_{max}^{abs}$ [cm ⁻¹]	$\tilde{\nu}_{max}^{fl}$ [cm ⁻¹]	Solvents ^[a]	Δf	$\tilde{\nu}_{max}^{abs}$ [cm ⁻¹]	$\tilde{\nu}_{max}^{fl(ICT)}$ [cm ⁻¹]
Hexane	0.001	34013	26954	35971	27173	5% Wt in Dx	0.185	29154	19379
Cyhex	0	34246	26954	--	--	10% Wt in Dx	0.228	29154	18248
Dioxane	0.021	34364	25188	34722	26595	15% Wt in Dx	0.249	29154	17636
Toluene	0.013	34013	25839	34129	27247	20% Wt in Dx	0.262	29154	17241
EtOAc	0.201	34965	24154	35714	26737	25% Wt in Dx	0.27	29154	17094
CHCl ₃	0.148	34129	24691	35842	27027	DCE	0.221	29069	18867
THF	0.21	32894	24096	34013	26809	EtOAc	0.201	29325	18382
DMSO	0.265	33783	21598	34246	23696	CHCl ₃	0.148	29154	20040
CH ₃ CN	0.305	34364	20161	35587	23696	---	---	---	---
EtOH	0.29	34843	21276	35842	25906	---	---	---	---
MeOH	0.309	34843	20283	35971	24271	---	---	---	---

^[a] Dx = Dioxane, Wt = Water, DCE = Dichloroethane

We have also plotted $\tilde{\nu}_{fl}$ and $\tilde{\nu}_{abs}$ values against $E_T(30)$ of the fluorophores **2.59**, **2.60** and **2.65** in different solvents (**Figure 2.18**). Like the plots of $\tilde{\nu}_{fl}$ & $\tilde{\nu}_{abs}$ values against Δf , the fluorophores (**2.59**, **2.60** and **2.65**) showed similar types of behavior with $E_T(30)$ value in different solvents. The $E_T(30)$ values of different solvents and the absorption ($\tilde{\nu}_{max}^{abs}$) and fluorescence ($\tilde{\nu}_{max}^{fl}$) maxima of the fluorophores **2.59**, **2.60** and **2.65** are shown in **Table 2.10**.

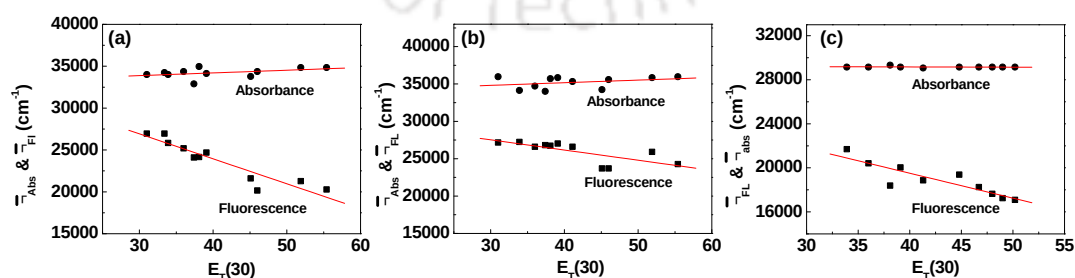
**Figure 2.18:** Plots of $\tilde{\nu}_{fl}$ & $\tilde{\nu}_{abs}$ values against $E_T(30)$ for fluorophore (a) **2.59**, (b) **2.60** and (c) **2.65** ($\tilde{\nu}_{fl(ICT)}$ & $\tilde{\nu}_{abs}$ values) in different solvents.

Table 2.10: Summary table of $\tilde{\nu}_{max}^{abs}$ and $\tilde{\nu}_{max}^{fl}$ of the fluorophores and corresponding $E_T(30)$ values of the solvents.

Solvents	$E_T(30)$	2.59		2.60		2.65			
		$\tilde{\nu}_{max}^{abs}$ [cm ⁻¹]	$\tilde{\nu}_{max}^{fl}$ [cm ⁻¹]	$\tilde{\nu}_{max}^{abs}$ [cm ⁻¹]	$\tilde{\nu}_{max}^{fl}$ [cm ⁻¹]	Solvents ^[a]	$E_T(30)$	$\tilde{\nu}_{max}^{abs}$ [cm ⁻¹]	$\tilde{\nu}_{max}^{fl(ICT)}$ [cm ⁻¹]
Hexane	31	34013	26954	35971	27173	0% Wt in Dx	36	29154	20408
Cyhex	33.4	34246	26954	---	---	5% Wt in Dx	44.8	29154	19379
Dioxane	36	34364	25188	34722	26595	10% Wt in Dx	46.7	29154	18248
Toluene	33.9	34013	25839	34129	27247	15% Wt in Dx	48	29154	17636
EtOAc	38.1	34965	24154	35714	26737	20% Wt in Dx	49	29154	17241
CHCl ₃	39.1	34129	24691	35842	27027	25% Wt in Dx	50.2	29154	17094
THF	37.4	32894	24096	34013	26809	Toluene	33.9	29154	21691
t-BuOH	41.1	---	---	35335	26595	DCE	41.3	29069	18867
DMSO	45.1	33783	21598	34246	23696	EtOAc	38.1	29325	18382
CH ₃ CN	46	34364	20161	35587	23696	CHCl ₃	39.1	29154	20040
EtOH	51.9	34843	21276	35842	25906	---	---	---	---
MeOH	55.4	34843	20283	35971	24271	---	---	---	---

^[a]Dx = Dioxane, Wt = Water, DCE = Dichloroethane

In different solvents, the absorption and fluorescence spectra involve the electronic transitions between the same two ground and excited electronic states, the Stokes' shift ($\Delta\tilde{\nu}$) is expected to follow a linear relation with Δf , as suggested by the Lippert and Mataga equation 2²³⁻²⁵

$$\Delta\tilde{\nu} = \Delta\tilde{\nu}_0 + \frac{2(\epsilon_e - \epsilon_g)^2}{hcr^3} \Delta f \quad (2)$$

where μ_e and μ_g are the excited (fluorescent) state and the ground state dipole moments of the fluorophore, h is Planck's constant, c is the velocity of light, and r is the Onsager radius of the dipole-solvent interaction sphere. The $\Delta\tilde{\nu}$ values for **2.59**, **2.60** and **2.65** (for the long wavelength emissive band) in different solvents were calculated (in cm⁻¹) (**Table 2.11**) and the $\Delta\tilde{\nu}$ was plotted against the solvent polarity Δf following equation 2. Thus, it is seen from the plot that the $\Delta\tilde{\nu}$ (**Figure 2.19a**) values correlate linearly well with the Δf values for all the solvents studied. Linear

correlation with large slopes of the $\Delta\nu$ vs. Δf plots suggests that the fluorescence states are highly polar in nature for all the fluorophores. Considering the Onsager radius r values for the fluorophores (**Table 2.12**, obtained from geometry optimized structures at B3LYP/6-31G* level²⁹), the $(\mu_e - \mu_g)$ values for **2.59**, **2.60** and **2.65** were estimated using the slopes of the respective $\Delta\nu$ vs. Δf plots and were found to be about 24.0, 21.0, and 21.0 Debye, respectively. Substantially high values of $(\mu_e - \mu_g)$ thus obtained from the plot (**Figure 2.19a** and **Table 2.12**) suggest that the fluorescence states of all these fluorophores are of strong ICT character which is inferred earlier. The $(\mu_e - \mu_g)$ value for **2.59** is somewhat higher than that of the **2.60** and **2.65** which indicates a higher ICT character of **2.59** compared to others. Comparing the spectral behaviors and all the experimental results, it is clear that the ICT state is developed from the LE state in case of **2.65** (**Figure 2.19b**).

Table 2.11: Summary table of $\Delta\nu$ of the fluorophores and solvent polarity parameter (Δf).

Fluorophores		2.59	2.60	2.65
Solvents	Δf	$\Delta\nu$ [cm ⁻¹]	$\Delta\nu$ [cm ⁻¹]	$\Delta\nu_{\text{ICT}}$ [cm ⁻¹]
Dioxane	0.021	9176	8127	---
5% H ₂ O in Dioxane	0.185	11889	---	9775
10% H ₂ O in Dioxane	0.228	12416	8628	10906
15% H ₂ O in Dioxane	0.249	12572	---	11518
20% H ₂ O in Dioxane	0.262	13266	9081	11913
25% H ₂ O in Dioxane	0.27	13745	---	12060
30% H ₂ O in Dioxane	0.277	15287	10513	---
Toluene	0.013	8174	6882	7463
EtOAc	0.201	10811	8977	10943
CHCl ₃	0.148	9438	8815	9114
DMSO	0.265	12185	10550	---
CH ₃ CN	0.305	14203	11891	---
EtOH	0.29	13567	9936	---
MeOH	0.309	14560	11700	---
Dichloroethane	0.221	---	---	10202

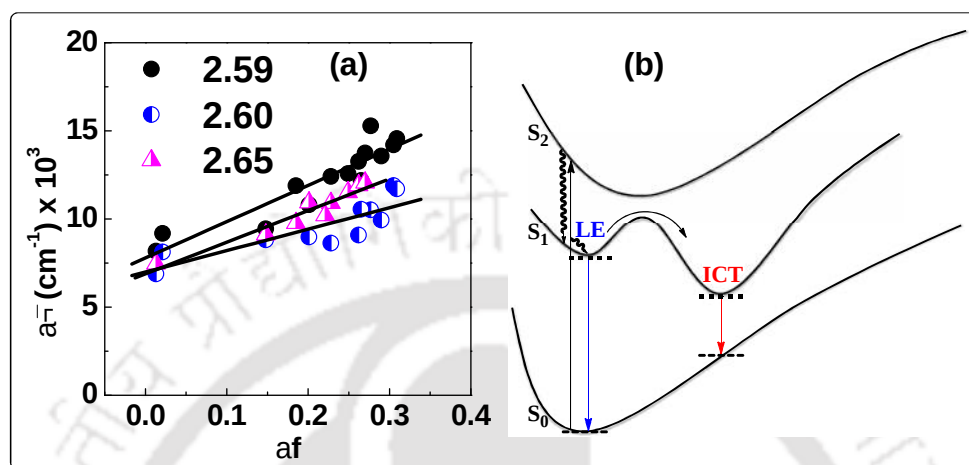


Figure 2.19: (a) A plot of $\Delta\tilde{\nu}$ vs. Δf of 2.59, 2.60 and 2.65 and (b) proposed photophysical process for the dual emission.

Table 2.12: The dipole moment and excitation energy values of 2.59, 2.60 and 2.65.

$\Delta\mu$ in Debye	2.59	2.60	2.65
$\Delta\mu^{[a]} = (\mu_e - \mu_g)$	24	21	21
$\mu_g^{[b]}$	5.23	9.31	3.92
Excitation energy (Calc.) in eV	4.55	4.14	3.54
Excitation energy (Exp.) in eV	4.27	4.35	3.61
Onsager radii $^{[b]}$ (Å)	6.6	7.2	6.3

^[a] Calculated from Eq. 2 and slope from the plots of $\Delta\tilde{\nu}$ values against Δf . ^[b] Obtained from B3LYP/6-31G* optimized geometry.

2.8.7. Theoretical Calculation

To investigate the relation between the polarity-dependent emission and the ICT feature, we have carried out TDDFT calculations using Gaussian 03 program package¹⁹ (Figure 2.20–2.23 and Table 2.13). Thus, as revealed from the overlapping of HOMO-LUMO as well as the transition oscillator strength (f), the electronic transitions of $S_0 \rightarrow S_1$ are fully allowed for all the fluorophores (Figure 2.20–2.23 and

Table 2.13). This means that reverse transition, *i.e.*, $S_0 \leftarrow S_1$, is also fully allowed indicating the potential fluorophoric nature of the molecules. The emissive state of **2.62** and **2.66** is basically a locally excited (LE) state as is suggested by the HOMO–LUMO distributions. For **2.60**, **2.61** and **2.64** however, the emissive state is characterized with more significant electron redistribution, *i.e.*, ICT feature. Calculated results supported our above explanation.³⁰

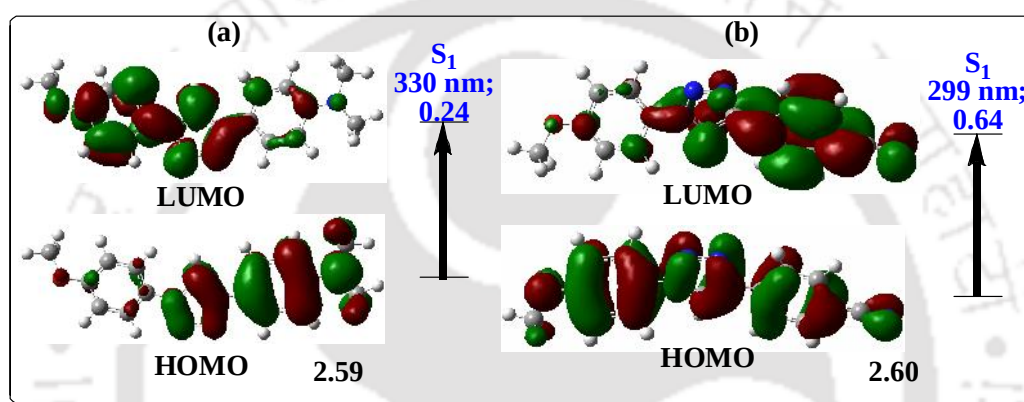


Figure 2.20: MO diagram with electronic transition of fluorophores **2.59** and **2.60**.

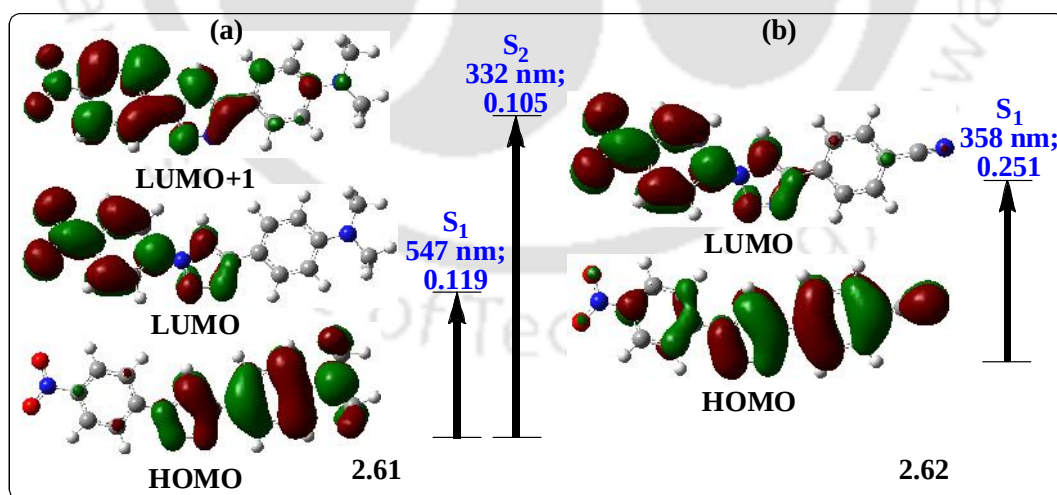


Figure 2.21: MO diagram with electronic transition of fluorophores **2.61** and **2.62**.

Calculated excitation energy for the transition of $S_0 \rightarrow S_1$ of **2.59**, **2.60**, **2.62**, **2.64**, and **2.66** are 330, 299, 358, 318 and 355 nm (in vacuum) respectively. These values

are close to the experimental result of 294 nm (in hexane), 288 nm (in dioxane), 343 nm (in hexane), 300 nm (in dioxane) and 344 nm (in dioxane) respectively. For fluorophore **2.61**, the $S_0 \rightarrow S_1$ transition is excited at much longer wavelength of 547 nm ($f = 0.11$). The transition $S_0 \rightarrow S_2$ (332 nm, in vacuum) is however, closer to the experimental value (in dioxane 290 nm) with almost similar f value ($f = 0.10$).

Fluorophore **2.63** and **2.65** showed dual emission which again depends on solvent polarity, thus showing an emission from both LE and CT states. The calculated excitation energy (in vacuum) for the transition of $S_0 \rightarrow S_1$ of **2.63** and **2.65** are 419 nm and 447 nm respectively which are slightly higher than the experimental results (experimental, 344 nm and 340 nm in hexane respectively). However, the transition $S_0 \rightarrow S_2$ (349 nm, in vacuum) for the fluorophore **2.65** is closer to the experimental value with higher f value ($f = 0.39$ for $S_0 \rightarrow S_2$ and $f = 0.10$ for $S_0 \rightarrow S_1$) indicating that the S_2 state is populated well (**Figure 2.23a** and **Table 2.13**). Similarly for the fluorophore **2.63**, the excitation energy for $S_0 \rightarrow S_2$ transition is 306 nm (in vacuum) which is closer to the experimental value, 344 nm (in hexane) with a higher f value ($f = 0.33$ for $S_0 \rightarrow S_2$ and $f = 0.06$ for $S_0 \rightarrow S_1$) (**Figure 2.22a** and **Table 2.13**).

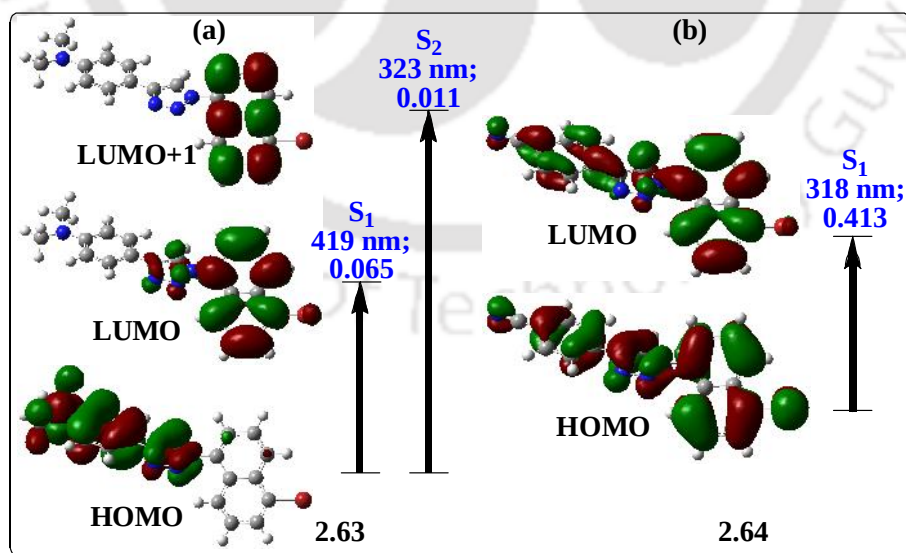


Figure 2.22: MO diagram with electronic transition of fluorophores **2.63** and **2.64**.

As is revealed from the overlapping of HOMO-LUMO as well as the transition oscillator strength for all the fluorophores except for **2.63** all the electronic transitions of $S_0 \rightarrow S_1$ are allowed. This means that reverse transition, *i.e.*, $S_0 \leftarrow S_1$, is also fully allowed indicating the potential fluorophoric nature of the molecules. For molecule **2.63**, however, the $S_0 \rightarrow S_1$ transition is excited at much longer wavelength of 419 nm with f value of 0.0651 (**Figure 2.22a**). This small f value indicated the very weakly fluorescent properties of **2.63** which was true as we did not see any fluorescence for it under the same condition as for the other fluorophores. For **2.61**, however, the $S_0 \rightarrow S_1$ transition is excited at much longer wavelength of 547 nm (experimental value, 290 nm in dioxane) with f value of 0.119 (**Figure 2.21a**).

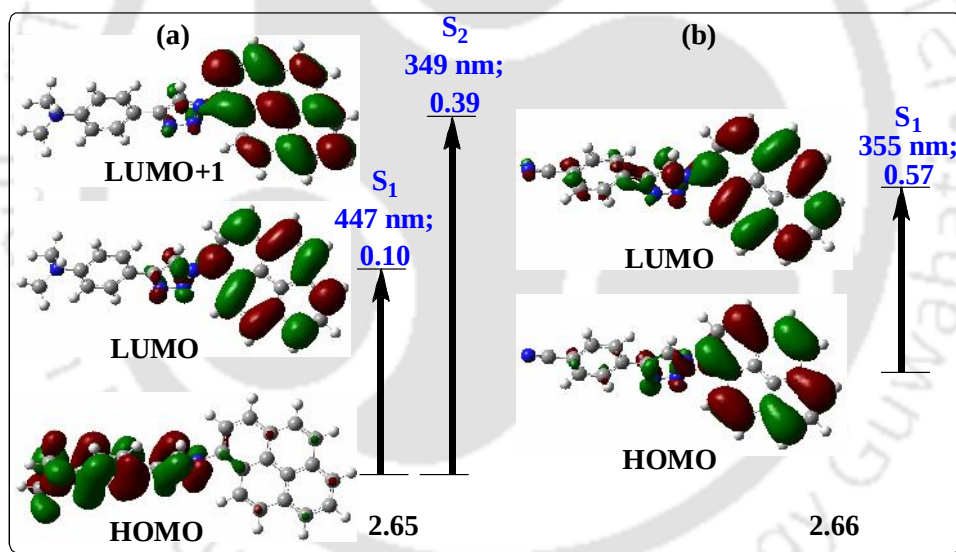


Figure 2.23: MO diagram with electronic transition of fluorophores 2.65 and 2.66.

Table 2.13: Summary table of theoretical calculations.

Entry	Optimized Energy ^[a] (a.u.)	Electronic transition	Energy (eV) (Wavelength)	<i>f</i>	Composition	CI
2.59	-952.82911387	S ₀ -> S ₁	3.7516 eV 330.48 nm	0.2406	HOMO -> LUMO (78 -> 79)	0.68214
2.60	-911.13732175	S ₀ -> S ₁	4.1435 eV 299.22 nm	0.6495	HOMO -> LUMO (72 -> 73)	0.66671
2.61	-1042.8444926	S ₀ -> S ₁	2.2657 eV 547.23 nm	0.1199	HOMO -> LUMO (81 -> 82)	0.69364
		S ₀ -> S ₂	3.7273 eV 332.64 nm	0.1053	HOMO -> LUMO+1 (81 -> 83)	0.67852
2.62	-1001.1180349	S ₀ -> S ₁	3.4542 eV 358.94 nm	0.2518	HOMO -> LUMO (75 -> 76)	0.68867
2.63	-3563.0506440	S ₀ -> S ₁	2.9547 eV 419.62 nm	0.0651	HOMO -> LUMO (100 ->101)	0.69991
		S ₀ -> S ₂	4.0411 eV 306.81 nm	0.3382	HOMO-1 -> LUMO (99 ->101)	0.63774
2.64	-3521.3266593	S ₀ -> S ₁	3.8986 eV 318.02 nm	0.4131	HOMO -> LUMO (94 -> 95)	0.66376
2.65	-1221.8273429	S ₀ -> S ₁	2.7704 eV (447.54 nm)	0.1015	HOMO -> LUMO (102 -> 103)	0.69830
		S ₀ -> S ₂	3.5453 eV (349.71 nm)	0.3975	HOMO -> LUMO+1 (102 ->104)	0.33709
2.66	-1180.1039717	S ₀ -> S ₁	3.4832 eV 355.95 nm	0.5740	HOMO -> LUMO (96 -> 97)	0.61413

^[a] B3LYP/6-31G* optimized energy, E(RB+HF-LYP)

2.9. Conclusion

In conclusion, we have been able to install the fluorescence response into a non-fluorescent precursor and modulate the emission response of a pyrene fluorophore *via* click reaction. Our findings reveal the delicate interplay of structure and emission properties and thus having a broader general utility. The relation between the polarity-dependent emission and the ICT feature of the synthesized fluorophores was studied by fluorescence emission spectra and DFT/TDDFT calculations. As the ICT to LE intensity ratio can be employed as a sensing index, the dual emissive fluorophore can be utilized in designing molecular recognition systems. Our investigation on the photophysical properties of click chemistry derived fluorophores is of immense importance for the development of new fluorophores with predetermined photophysical properties that may find a wide range of applications in chemistry, biology, or material sciences. The exploration of the dual fluorescent probe, triazolyl pyrene, as a switch on fluorescent sensor for biomolecules, micellar microenvironment and metal ion is described in **Chapter 4-5**.

2.10. Experimental Section

2.10.1. General Methods

All reactions were carried out under a nitrogen atmosphere. Organic extracts were dried over anhydrous sodium sulphate. Solvents were removed in a rotary evaporator under reduced pressure. Silica gel (60-120 mesh size) was used for the column chromatography. Reactions were monitored by TLC on silica gel 60 F254 (0.25mm). ^1H NMR spectra were measured with Varian 400 (400 MHz) and ^{13}C NMR spectra were measured with Varian 400 (100 MHz) spectrometer. Coupling constant (J value) was reported in hertz. The chemical shifts were shown in ppm downfield from tetramethylsilane, using residual chloroform ($\delta = 7.24$ in ^1H NMR, $\delta = 77.23$ in ^{13}C NMR), dimethyl sulfoxide ($\delta = 2.48$ in ^1H NMR, $\delta = 39.5$ in ^{13}C NMR), as an internal standard. Mass spectra were recorded using WATERS MS system, Q-tof premier and data analyzed using Mass Lynx 4.1. IR spectra were recorded in KBr or neat on a Perkin Elmer Spectrum one FT-IR spectrometer.

2.10.2. Crystallographic Description

X-ray intensity data were collected at 296 K with Mo $\text{K}\alpha$ radiation ($\lambda = 0.71073$ Å) using a Bruker Nonius SMART CCD diffractometer equipped with graphite monochromator and Apex CD camera. The SMART software was used for data collection and for indexing the reflections and determining the unit cell parameters; the collected data were integrated using SAINT software.¹⁷ The structures were solved by direct methods and refined by full-matrix least-squares calculations using SHELXTL software. All the non-H atoms were refined in the anisotropic approximation against F^2 of all reflections. The single crystals were collected from chloroform-ethylacetate mixture at room temperature.

2.10.3. General Procedure for the Preparation of Aryl Azides

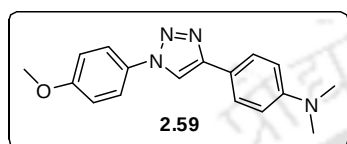
To an ice cold solution of sodium nitrite (3 eqv.) in water was added dropwise to a cold solution of aryl amine 1 (1 eqv.) in water and concentrated hydrochloric acid at 0 °C over 7 – 10 min. The reaction mixture was slowly stirred for 1 - 2 min before an ice cold solution of sodium azide (6 eqv.) in water was added dropwise at 0 °C over 10 min. The mixture was stirred for 15 min at 0 °C. The resulting mixture was extracted with ether. The organic layer was washed with water, followed by a brine solution, dried over anhydrous Na₂SO₄ and then concentrated to yield pure aryl azides **2.53-2.56**. Formation of the product azide was confirmed from IR study (1-azido-4-methoxybenzene (**2.53**): Yield: 60%; IR: 2105 cm⁻¹; 1-azido-4-nitrobenzene (**2.54**): Yield: 62%; IR: 2126 cm⁻¹; 1-azido-5-bromonaphthalene (**2.55**): Yield: 89%; IR: 2134 cm⁻¹; 1-azidopyrene (**2.56**): Yield: 75%; IR: 2133 cm⁻¹) and the produced azides were then immediately used for the next step without further purification.

2.10.4. General Procedure for the [3+2] Cycloaddition of Azides and Terminal Alkynes

In a round bottom flask fitted with a septum, azide (**2.53-2.56**) (1.0 eqv.) was dissolved by dry 1:1 dichloromethane and ethanol and was degassed for 5 min with N₂. Then, alkyne (**2.57-2.58**) (1.5 eqv.) was added and both the stirring and degassing were continued for next 5 min. After that a 6 mol % sodium ascorbate was added. The reaction mixture was stirred for 5 min before 1 mol% powdered CuSO₄ was added. Degassing was continued for next 10 min. The reaction was allowed to proceed to room temperature and monitored by TLC. After total consumption of the starting azide, the reaction mixture was evaporated completely to a solid material and partitioned between water and ethyl acetate. The organic layer was washed with water followed by brine solution, dried over Na₂SO₄ and then concentrated. The title triazolyl-compounds (**2.59-2.66**) were separated by column chromatography and characterized. The average yields were 50 to 85%.

2.10.4.1. Synthesis of 4-(1-(4-methoxyphenyl)-1H-1,2,3-triazol-4-yl)-N,N-dimethylbenzenamine (2.59)

Using the general procedure, starting from 0.116 g of 1-azido-4-methoxybenzene **2.53** and 0.169 g of **2.57**, 0.137 g of the title compound **2.59** was isolated as a white

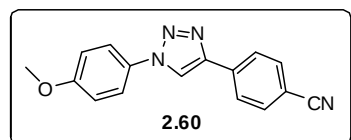


solid (60%). IR (KBr): 1618, 1593, 1518, 1503, 1354, 1250, 1040, 808 cm^{-1} ; ^1H NMR (CDCl_3 ; 400 MHz) δ 3.01 (6H, s), 3.87 (3H, s), 6.81 (2H, d, $J = 7.2$ Hz), 7.03 (2H, d, $J = 7.6$ Hz), 7.68 (2H, d, $J = 8.0$ Hz), 7.77 (2H, d, $J = 8.0$ Hz), 7.98 (1H, s); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 40.7, 55.8, 112.8, 114.9, 116.5, 118.9, 122.2, 126.9, 130.9, 148.9, 150.6, 159.8; HRMS calcd. for $\text{C}_{17}\text{H}_{18}\text{N}_4\text{O}$ ($[\text{M}+\text{H}]^+$) 295.1559, found 295.1562.

Crystallographic description of 2.59: Crystal dimension (mm): 0.45 x 0.25 x 0.15. $\text{C}_{17}\text{H}_{18}\text{N}_4\text{O}$, Mr = 294.35; Orthorhombic, space group P2(1)2(1)2(1); a = 5.7361(3) Å, b = 13.5039(6) Å, c = 19.7921(8) Å; $\alpha = 90.00^\circ$, $\beta = 90.00^\circ$, $\gamma = 90.00^\circ$, V = 1533.09(12) Å³; Z = 4; $\rho_{\text{cal}} = 1.275 \text{ Mg/m}^3$; $\mu (\text{mm}^{-1}) = 0.083$; $F(000) = 624$; Refinement method = Full-matrix least-squares on F^2 ; Final R indices [$I > 2\sigma_I$] R1 = 0.0355, wR2 = 0.0645, R indices (all data) R1 = 0.0743, wR2 = 0.0723; goodness of fit = 1.013.

2.10.4.2. Synthesis of 4-(1-(4-methoxyphenyl)-1H-1,2,3-triazol-4-yl) benzonitrile (2.60)

Using the general procedure, starting from 0.116 g of 1-azido-4-methoxybenzene **2.53** and 0.148 g of **2.58**, 0.144 g of the title compound **2.60** was isolated as a white

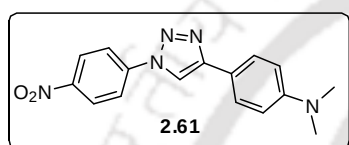


solid (67% yield). IR (KBr): 2231, 1611, 1519, 1489, 1258, 1025, 825 cm^{-1} ; ^1H NMR (CDCl_3 ; 400 MHz) δ 3.87 (3H, s), 7.04 (2H, d, $J = 8.8$ Hz), 7.66 (2H, d, $J = 8.8$ Hz), 7.73 (2H, d, $J = 8.0$ Hz), 8.00 (2H, d, $J = 8.0$ Hz), 8.18 (1H, s); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 55.8, 111.8, 115.1, 118.9, 119.2, 122.4, 126.3, 130.3, 132.9,

134.9, 146.5, 160.3; HRMS calcd. for $C_{16}H_{12}N_4O$ ($[M+H]^+$) 277.1089, found 277.1091.

2.10.4.3. Synthesis of *N,N*-dimethyl-4-(1-(4-nitrophenyl)-1*H*-1,2,3-triazol-4-yl)benzenamine (2.61)

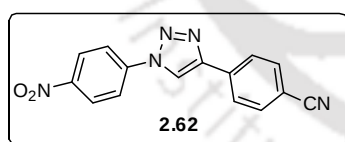
Using the general procedure, starting from 0.067 g of 1-azido-4-nitrobenzene **2.54** and 0.090 g of **2.57**, 0.050 g of the title compound **2.61** was isolated as an orange



solid (50%). IR (KBr): 1621, 1595, 1517, 1497, 1368, 1335, 853 cm^{-1} ; 1H NMR ($CDCl_3$; 400 MHz) δ 3.00 (6H, s), 6.78 (2H, d, $J = 6.8$ Hz), 7.08 (2H, d, $J = 8.8$ Hz), 7.59 (2H, d, $J = 8.8$ Hz), 7.77 (1H, s), 8.28 (2H, d, $J = 8.8$ Hz); ^{13}C NMR ($CDCl_3$; 100 MHz) δ 41.1, 113.5, 125.0, 125.5, 126.2, 129.9, 131.1, 133.6, 138.5, 141.9, 147.6; HRMS calcd. for $C_{16}H_{15}N_5O_2$ ($[M+H]^+$) 310.1304 found 310.1309.

2.10.4.4. Synthesis of 4-(1-(4-nitrophenyl)-1*H*-1,2,3-triazol-4-yl)benzonitrile (2.62)

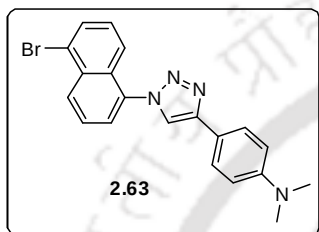
Using the general procedure, starting from 0.064 g of 1-azido-4-nitrobenzene **2.54**



and 0.075 g of **2.58**, 0.017 g of the title compound **2.62** was isolated as a yellow solid (51%). IR (KBr): 2228, 1612, 1594, 1521, 1490, 1366, 1338, 854 cm^{-1} ; 1H NMR ($CDCl_3$; 400 MHz) δ 7.35 (2H, d, $J = 8.4$ Hz), 7.54 (2H, d, $J = 8.8$ Hz), 7.70 (2H, d, $J = 8.0$ Hz), 7.95 (1H, s), 8.33 (2H, d, $J = 8.8$ Hz); ^{13}C NMR ($CDCl_3 + d_6$ -DMSO; 100 MHz) δ 113.2, 117.4, 124.8, 125.3, 129.0, 130.1, 132.6, 134.4, 136.0, 140.3, 147.5; HRMS calcd. for $C_{15}H_9N_5O_2$ ($[M+H]^+$) 292.0834, found 292.0840.

2.10.4.5. Synthesis of 4-(1-(5-bromonaphthalen-1-yl)-1H-1,2,3-triazol-4-yl)-N,N-dimethylbenzenamine (2.63)

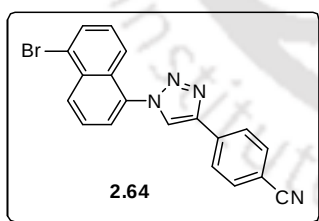
Using the general procedure, starting from 0.045 g of 1-azido-5-bromonaphthalene **2.55** and 0.040 g of **2.57**, 0.060 g of the title compound **2.63** was isolated as a pink solid (85%). IR (KBr): 1617, 1597, 1498, 1352, 802, 780, 532 cm^{-1} ;



^1H NMR (CDCl_3 ; 400 MHz) δ 2.99 (6H, s), 6.79 (2H, d, $J = 8.8$ Hz), 7.34 (1H, t, $J = 8.0$ Hz), 7.67 (3H, m), 7.79 (2H, d, $J = 9.2$ Hz), 7.86 (2H, d, $J = 6.8$ Hz), 7.98 (1H, s), 8.44 (1H, dd, $J = 1.4, 7.6$ Hz); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 40.6, 112.7, 118.2, 121.1, 122.8, 123.3, 124.6, 126.5, 127.0, 128.2, 129.7, 130.2, 131.4, 132.9, 134.4, 148.6, 150.8; HRMS calcd. for $\text{C}_{20}\text{H}_{17}\text{N}_4\text{Br}$ ($[\text{M}+\text{H}]^+$) 393.0715, found 393.0717.

2.10.4.6. Synthesis of 4-(1-(5-bromonaphthalen-1-yl)-1H-1,2,3-triazol-4-yl) benzonitrile (2.64)

Using the general procedure, starting from 0.024 g of 1-azido-5-bromonaphthalene **2.55** and 0.018 g of **2.58**, 0.02 g of the title compound **2.64** was



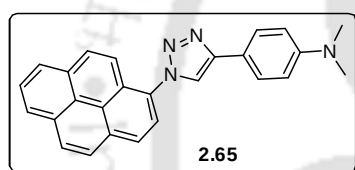
isolated as a reddish pink solid (56%). IR (KBr): 2227, 1602, 1591, 1497, 839, 773, 555 cm^{-1} ; ^1H NMR (CDCl_3 ; 400 MHz) δ 7.38 (1H, t, $J = 8.0$ Hz), 7.57 (1H, d, $J = 8.8$ Hz), 7.68 (1H, t, $J = 6.6$ Hz), 7.72–7.76 (3H, m), 7.89 (1H, d, $J = 7.6$ Hz), 8.05 (2H, d, $J = 8.4$ Hz), 8.21 (1H, s), 8.50 (1H, d, $J = 8.4$ Hz); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 112.2, 118.9, 122.4, 123.7, 124.8, 126.5, 128.5, 130.1, 130.4, 131.7, 133.1, 134.7, 146.3; HRMS calcd. for $\text{C}_{19}\text{H}_{11}\text{N}_4\text{Br}$ ($[\text{M}+\text{H}]^+$) 375.0245, found 375.0248.

Crystallographic description of 2.64: Crystal dimension (mm): 0.52 x 0.24 x 0.18. $\text{C}_{19}\text{H}_{11}\text{N}_4\text{Br}$, Mr = 375.22; Triclinic, space group P-1; a = 10.989(2) Å, b = 11.956(2) Å, c = 14.877(4) Å; $\alpha = 105.234(5)^\circ$, $\beta = 94.855(5)^\circ$, $\gamma = 116.184(4)^\circ$, V =

1646.5(6) Å³; Z = 4; ρ_{cal} = 1.514 Mg/m³; μ (mm⁻¹) = 2.503; F (000) = 752; Refinement method = Full-matrix least-squares on F²; Final R indices [I > 2σ_I] R1 = 0.0642, wR2 = 0.1012, R indices (all data) R1 = 0.0642, wR2 = 0.1169; goodness of fit = 0.990.

2.10.4.7. Synthesis of N, N-dimethyl-4-(1-(pyren-1-yl)-1H-1,2,3-triazol-4-yl)benzenamine (2.65)

Using the general procedure, starting from 0.025 g of 1-azidopyrene (2.56) and 0.022 g of 2.57, 0.024 g of the title compound 2.65 was isolated as a light pink solid (68% yield). IR (KBr): 1617, 1567, 1503, 1460, 1351, 819 cm⁻¹; ¹H NMR (CDCl₃;



400 MHz) δ 3.03 (6H, s), 6.84 (2H, d, J = 8.0 Hz), 7.87 (2H, d, J = 8.4 Hz), 7.97 (2H, d, J = 9.2 Hz), 8.06 – 8.15 (4H, m), 8.18 (1H, s), 8.20 – 8.29 (3H, m); ¹³C NMR (CDCl₃; 100 MHz) δ 40.6, 112.7, 118.6, 121.4,

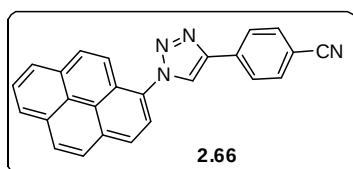
123.5, 124.3, 124.9, 125.2, 126.1, 126.5, 126.9, 127.1, 128.9, 129.7, 130.8, 131.3, 132.3, 148.6, 150.8; HRMS calcd. for C₂₆H₂₀N₄ ([M+H]⁺) 389.1766, found 389.1768.

Crystallographic description of 2.65: Crystal dimension (mm): 0.48 x 0.32 x 0.20. C₂₆H₂₀N₄, Mr = 388.46; Monoclinic, space group P2(1)/c; a = 5.7090(2) Å, b = 16.6315(5) Å, c = 20.8904(7) Å; α = 90.00°, β = 95.408(2)°, γ = 90.00°, V = 1974.70(11) Å³; Z = 4; ρ_{cal} = 1.307 Mg/m³; μ (mm⁻¹) = 0.079; F (000) = 816; Refinement method = Full-matrix least-squares on F²; Final R indices [I > 2σ_I] R1 = 0.0427, wR2 = 0.1016, R indices (all data) R1 = 0.1062, wR2 = 0.1170; goodness of fit = 0.982.

2.10.4.8. Synthesis of 4-(1-(pyren-1-yl)-1H-1,2,3-triazol-4-yl)benzonitrile (2.66)

Using the general procedure, starting from 0.010 g of 1-azidopyrene (2.56) and 0.008 g of 2.58, 0.010 g of the title compound 2.66 was isolated as a brown solid (66% yield). IR (KBr): 2225, 1614, 1489, 1461, 845 cm⁻¹; ¹H NMR (CDCl₃; 400

MHz) δ 7.77 (2H, d, $J = 7.6$ Hz), 7.88 (1H, d, $J = 8.4$ Hz), 8.08 – 8.29 (10H, m), 8.33



(1H, s); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 112.0, 118.9, 121.0, 123.5, 124.0, 124.3, 124.9, 125.3, 126.5, 126.9, 127.1, 129.4, 130.2, 130.8, 131.3, 132.7, 133.1, 134.9, 146.3; HRMS calcd. for $\text{C}_{25}\text{H}_{14}\text{N}_4$ ($[\text{M}+\text{H}]^+$) 371.1297,

found 371.1302.

2.10.5. UV-Visible and Fluorescence Measurements

All the UV–visible spectra of the compounds (10 μM) were measured in different solvents using Perkin- Elmer Lambda 25 UV-Visible spectrophotometer with a cell of 1 cm path length. Fluorescence spectra were obtained using Fluoromax-4 fluorescence spectrophotometer at 25 $^\circ\text{C}$ using 1 cm path length cell. All the solvents used in the solution preparation are of spectroscopic grade, purchase from Spectrochem, India. All the compounds were recrystallized twice, dried and used for photophysical study. The fluorescence quantum yields (Φ) were determined using quinine sulphate as a reference with the known Φ (0.54) in 0.1 molar solution in sulphuric acid.

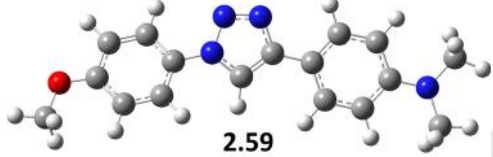
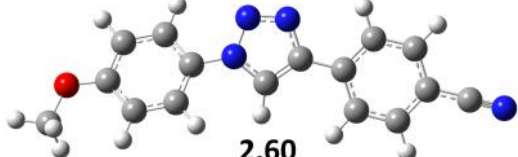
2.10.6. Fluorescence Lifetime Measurements

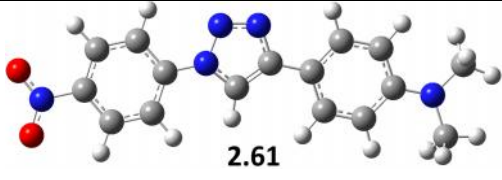
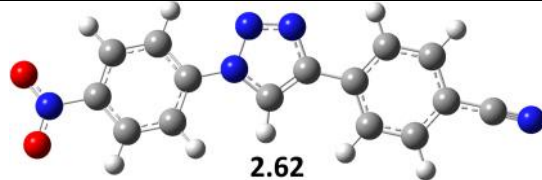
Fluorescence lifetimes were measured with the use of a system that employs microchannel plate photomultiplier as detector. Edinburgh 290 nm (for **2.60**) and 308 nm Pulsed LED (for **2.59**) and 375 nm (for **2.64**, **2.65** and **2.66**) Laser Diode were used for excitation. The fluorescence decay was analyzed by reconvolution (for **2.59**-**2.60**) and tail fitting (for **2.64**, **2.65** and **2.66**) method using in built software. All experiments were performed at 298 K.

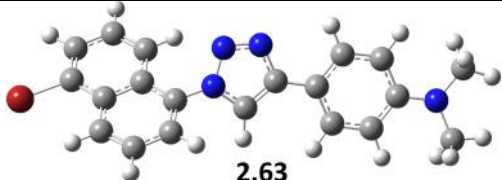
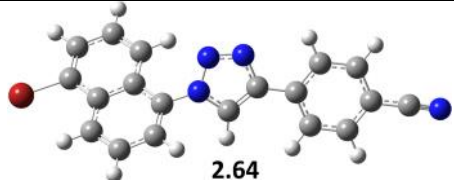
2.10.7. Theoretical Calculation

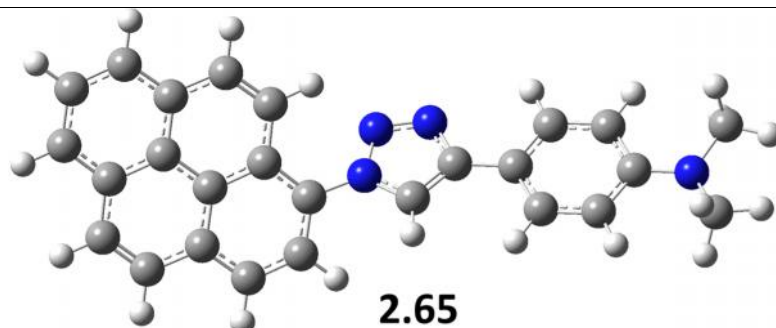
The ground state structures of the fluorophores were optimized using density functional theory (DFT) with B3LYP functional and 6-31G (d) basis set with Gaussian 03 program package.²⁹ The excited state related calculations were carried out with the time dependent density functional theory (TD-DFT) with the optimized structure of the ground state [B3LYP/6-31G(d)]. There were no imaginary frequencies observed in frequency analysis for all the calculated structures; therefore each calculated structure was a local energy minimum.

2.10.7.1. B3LYP/6-31G* Optimized Structure, Energy and Cartesian Coordinates of the Synthesized Compounds

 2.59				 2.60			
E(RB+HF-LYP) = -952.82911387 a.u.				E(RB+HF-LYP) = -911.13732175 a.u.			
0 1				0 1			
C	4.725917	1.187122	0.326362	C	-4.19743	-1.13479	0.372918
C	3.362144	0.893029	0.394203	C	-2.82742	-0.86765	0.456252
C	2.891579	-0.3628	0.014812	C	-2.32089	0.353092	0.01331
C	3.793623	-1.34447	-0.41896	C	-3.18677	1.329804	-0.49868
C	5.149006	-1.05995	-0.47151	C	-4.54861	1.073214	-0.56848
C	5.627844	0.208764	-0.10651	C	-5.06513	-0.16164	-0.13979
H	5.067281	2.170633	0.626857	H	-4.5689	-2.09008	0.724961
H	2.673411	1.645857	0.764589	H	-2.16524	-1.61248	0.888058
H	3.418157	-2.3198	-0.7054	H	-2.78445	2.279439	-0.83384
H	5.862437	-1.80694	-0.80419	H	-5.235	1.816423	-0.96201
O	6.975967	0.383681	-0.20663	O	-6.41347	-0.3132	-0.26024
C	7.519447	1.645018	0.150717	C	-7.00608	-1.53862	0.156555
H	8.595983	1.562359	-0.00806	H	-8.07416	-1.43005	-0.03707
H	7.120395	2.449727	-0.48098	H	-6.61395	-2.38604	-0.42048
H	7.325782	1.883504	1.204889	H	-6.84474	-1.71379	1.22798
N	1.503204	-0.66006	0.065232	N	-0.92284	0.619841	0.083342
C	0.438001	0.178461	-0.06696	C	0.12007	-0.2353	-0.07723
C	-0.67452	-0.63224	0.068515	C	1.251025	0.544737	0.09932
H	0.555616	1.229215	-0.27574	H	-0.02347	-1.27419	-0.32895
N	1.074755	-1.93376	0.272319	N	-0.46608	1.874685	0.345602
N	-0.22515	-1.91408	0.271733	N	0.831961	1.826065	0.351716
C	-2.10156	-0.30789	0.01452	C	2.672646	0.190818	0.040798
C	-3.06861	-1.31936	0.123142	C	3.651742	1.187506	0.206832
C	-2.5626	1.007447	-0.13993	C	3.089571	-1.13442	-0.17943
C	-4.42659	-1.03541	0.074838	C	5.004367	0.87101	0.153241
H	-2.73873	-2.34508	0.25272	H	3.336317	2.211185	0.377949
C	-3.91822	1.306864	-0.19153	C	4.440024	-1.45979	-0.23561
H	-1.85137	1.826966	-0.21422	H	2.354012	-1.92394	-0.30688
C	-4.89423	0.288252	-0.0968	C	5.411658	-0.45686	-0.0694
H	-5.1282	-1.8553	0.172145	H	5.752431	1.647206	0.282443
H	-4.21456	2.342926	-0.30362	H	4.75	-2.48614	-0.40635
N	-6.25217	0.574264	-0.17805	C	6.805447	-0.78807	-0.12644
C	-7.21734	-0.46091	0.150603	N	7.936891	-1.05919	-0.17336
H	-7.12088	-0.82401	1.186655				
H	-8.22574	-0.06296	0.019876				
H	-7.11344	-1.32214	-0.52003				
C	-6.69064	1.95817	-0.1374				
H	-6.26844	2.52839	-0.97358				
H	-7.77775	1.991464	-0.23413				
H	-6.41169	2.470622	0.79791				

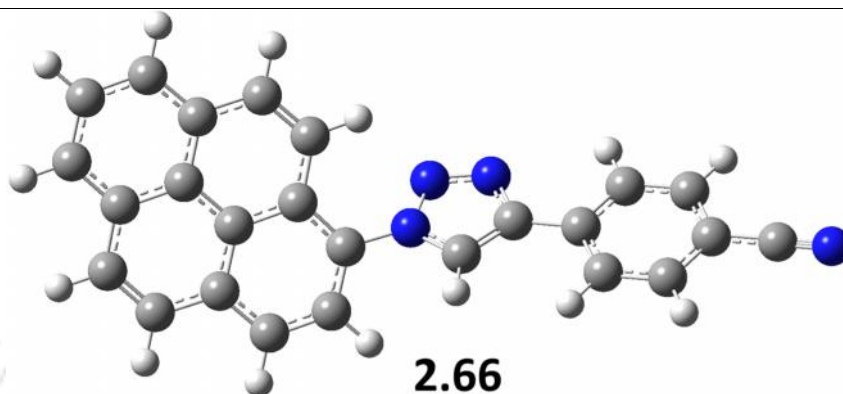
 2.61 E(RB+HF-LYP) = -1042.84449265 a.u.				 2.62 E(RB+HF-LYP) = -1001.11803497 a.u.			
O 1				O 1			
C	4.397126	1.329605	0.190432	C	-3.86329	-1.29455	0.273519
C	3.038655	1.031681	0.220196	C	-2.49909	-1.02326	0.319403
C	2.606995	-0.29024	0.033753	C	-2.03726	0.271572	0.047155
C	3.540716	-1.31912	-0.16961	C	-2.93722	1.303675	-0.25426
C	4.898339	-1.02327	-0.1908	C	-4.30148	1.035972	-0.28864
C	5.313395	0.298645	-0.01514	C	-4.74797	-0.26073	-0.03004
H	4.749882	2.343968	0.332985	H	-4.24404	-2.28737	0.480814
H	2.325377	1.827401	0.40392	H	-1.80716	-1.81496	0.58584
H	3.192482	-2.33515	-0.30856	H	-2.56217	2.299546	-0.4569
H	5.635004	-1.80204	-0.3481	H	-5.01689	1.815909	-0.52053
N	1.228146	-0.59813	0.051873	N	-0.64791	0.553052	0.080316
C	0.144401	0.231366	-0.03514	C	0.414134	-0.29302	-0.03282
C	-0.95494	-0.60137	0.044635	C	1.52843	0.516675	0.077182
H	0.235879	1.296087	-0.17241	H	0.298073	-1.35042	-0.2074
N	0.812313	-1.89216	0.179946	N	-0.20767	1.834816	0.252758
N	-0.48132	-1.89014	0.17673	N	1.085011	1.807289	0.251122
C	-2.38685	-0.301	0.003121	C	2.957356	0.190308	0.026992
C	-3.3388	-1.33343	0.045901	C	3.913852	1.216221	0.130321
C	-2.86806	1.015794	-0.07319	C	3.400021	-1.13594	-0.12313
C	-4.70231	-1.06772	0.007265	C	5.273033	0.926179	0.083531
H	-2.99855	-2.36195	0.114842	H	3.578735	2.241209	0.247341
C	-4.22864	1.297646	-0.11344	C	4.756934	-1.4347	-0.17118
H	-2.17133	1.850997	-0.0939	H	2.681883	-1.94755	-0.20173
C	-5.19156	0.258462	-0.08676	C	5.706723	-0.40284	-0.06819
H	-5.38961	-1.90428	0.050106	H	6.00461	1.724179	0.163717
H	-4.53903	2.334513	-0.16383	H	5.088249	-2.46187	-0.28716
N	-6.54971	0.527894	-0.15711	N	-6.19128	-0.54643	-0.07587
C	-7.50595	-0.54245	0.082267	O	-6.95071	0.386603	-0.33868
H	-8.51752	-0.14868	-0.03428	O	-6.55249	-1.70239	0.150854
H	-7.38073	-1.35241	-0.6462	C	7.107395	-0.70665	-0.11685
H	-7.41563	-0.97176	1.093356	N	8.243974	-0.95529	-0.1568
C	-7.01425	1.902251	-0.05276				
H	-6.59568	2.520998	-0.85566				
H	-8.10081	1.920167	-0.15708				
H	-6.75156	2.366532	0.912045				
N	6.747459	0.612602	-0.04526				
O	7.535654	-0.31818	-0.22342				
O	7.081088	1.789922	0.109045				

 2.63				 2.64			
E(RB+HF-LYP) = -3563.05064402 a.u.				E(RB+HF-LYP) = -3521.32665934 a.u.			
0 1				0 1			
C	4.658372	-1.82779	-0.69674	C	4.097486	-1.8989	-0.51686
C	4.851844	-0.51677	-0.33486	C	4.309406	-0.5624	-0.27881
C	3.777957	0.32404	0.080442	C	3.245805	0.326433	0.055992
C	2.462379	-0.26031	0.122085	C	1.923005	-0.23457	0.149739
C	2.2828	-1.61537	-0.26314	C	1.724919	-1.61706	-0.10689
C	3.35678	-2.37522	-0.66299	C	2.789151	-2.42431	-0.43293
H	4.944902	2.115046	0.39681	H	4.435583	2.123547	0.207922
H	5.500939	-2.4354	-1.00786	H	4.931598	-2.54431	-0.76883
C	3.949484	1.688271	0.433859	C	3.434709	1.715046	0.284007
C	1.380298	0.578714	0.538026	C	0.855262	0.655448	0.485612
H	1.292538	-2.04959	-0.21838	H	0.729822	-2.03442	-0.02313
H	3.210945	-3.41092	-0.9553	H	2.630452	-3.48102	-0.62629
C	1.582646	1.900129	0.875848	C	1.072393	1.998703	0.704626
C	2.876122	2.457911	0.815847	C	2.373254	2.53163	0.595523
H	0.741176	2.500208	1.207425	H	0.239049	2.637266	0.980586
H	3.021137	3.499156	1.087486	H	2.532795	3.590826	0.772134
Br	6.639399	0.17387	-0.40856	Br	6.103864	0.094681	-0.41385
N	0.04877	0.071139	0.584542	N	-0.48577	0.170476	0.575247
C	-1.07441	0.634658	0.056558	C	-1.59055	0.669288	-0.03841
C	-2.11122	-0.18917	0.453688	C	-2.63875	-0.0986	0.434615
H	-1.03019	1.521057	-0.55567	H	-1.52907	1.478078	-0.74906
N	-0.27631	-1.04955	1.291179	N	-0.83143	-0.85968	1.403743
N	-1.56284	-1.20215	1.203397	N	-2.11459	-1.01769	1.308882
C	-3.54907	-0.1093	0.187969	C	-4.07192	-0.04706	0.134724
C	-4.11211	0.934129	-0.56104	C	-4.60568	0.921881	-0.73194
C	-4.42352	-1.0857	0.689766	C	-4.94236	-0.98177	0.722111
C	-5.47761	1.005218	-0.80519	C	-5.96607	0.960154	-1.00786
H	-3.47602	1.719655	-0.96269	H	-3.95379	1.656815	-1.19557
C	-5.79031	-1.0278	0.454345	C	-6.30392	-0.95006	0.452347
H	-4.0151	-1.89984	1.279799	H	-4.53274	-1.72969	1.39199
C	-6.35976	0.016072	-0.31165	C	-6.82926	0.022058	-0.41657
H	-5.85634	1.839811	-1.38295	H	-6.36905	1.712384	-1.67822
H	-6.41822	-1.80491	0.873613	H	-6.96934	-1.6749	0.91016
N	-7.72276	0.06378	-0.57536	C	-8.23364	0.058902	-0.69809
C	-8.61568	-0.84212	0.127138	N	-9.37394	0.090144	-0.92823
H	-8.59453	-0.70333	1.220153				
H	-9.63766	-0.67559	-0.21919				
H	-8.3618	-1.88729	-0.08576				
C	-8.29026	1.257925	-1.17684				
H	-7.84286	1.453832	-2.15869				
H	-9.36086	1.106965	-1.3289				
H	-8.15355	2.15795	-0.55541				



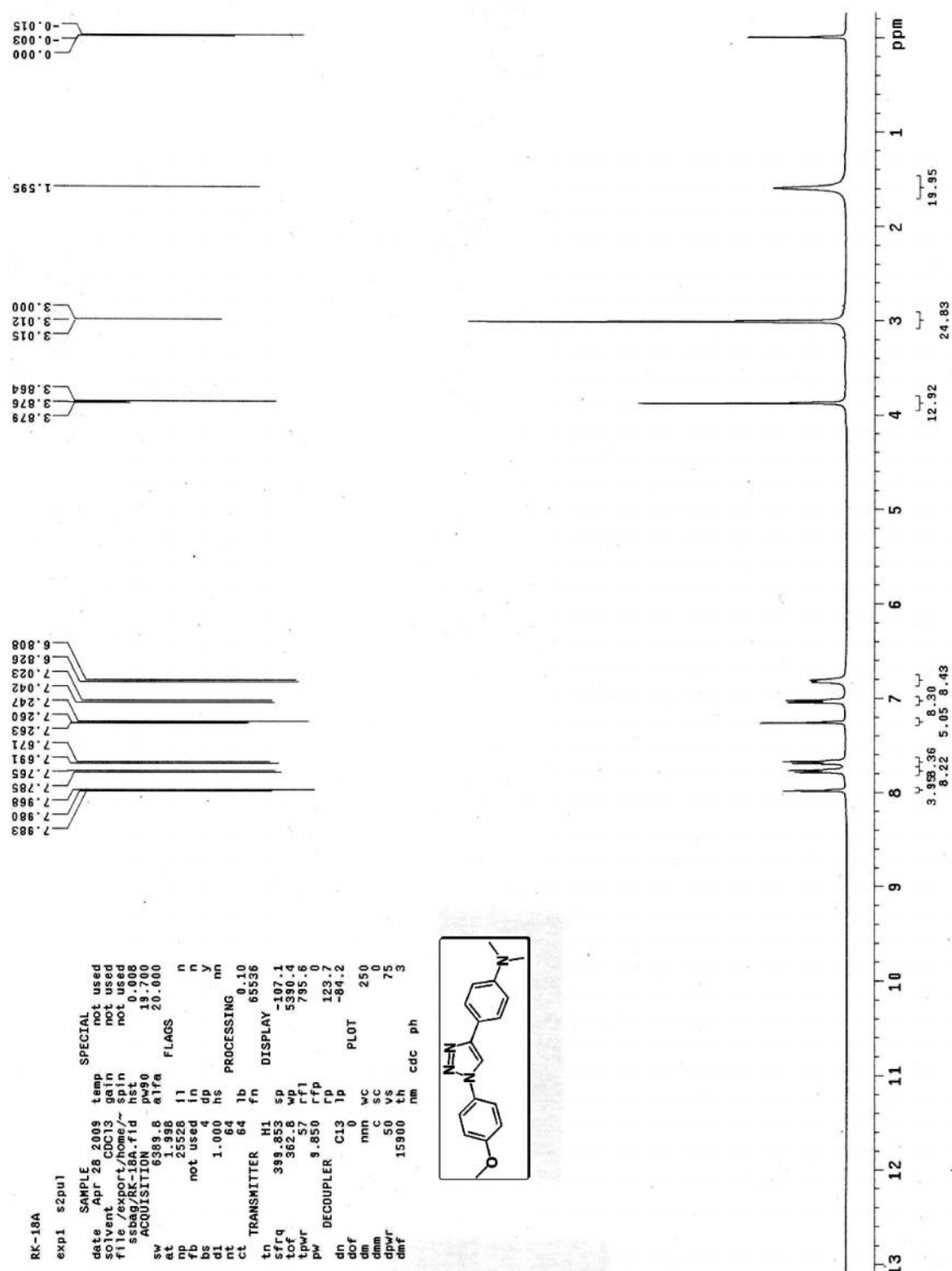
$E(\text{RB+HF-LYP}) = -1221.82734290 \text{ a.u.}$

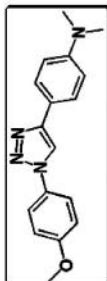
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C	7.030426	-1.92046	0.771853	N	-0.05828 0.240068 -0.60068
C	7.186576	-0.54295	0.629216	N	-0.3594 -0.78451 -1.44918
C	6.083272	0.281219	0.355629	N	-1.64351 -0.97179 -1.3904
C	4.789245	-0.31022	0.221374	C	-1.19431 0.708624 -0.01153
C	4.639517	-1.72128	0.373418	H	-1.16993 1.507452 0.712213
C	5.77201	-2.50536	0.64683	C	-2.21374 -0.07534 -0.51926
C	6.211435	1.704239	0.208302	C	-3.65391 -0.05638 -0.2542
C	3.651192	0.506371	-0.05916	C	-4.23993 0.877129 0.612948
C	3.81562	1.919935	-0.19092	C	-5.60663 0.887966 0.861255
C	5.128111	2.486251	-0.05023	C	-6.46895 -0.04241 0.236412
C	2.689552	2.713423	-0.45663	C	-5.8733 -0.99042 -0.62787
H	2.806981	3.788229	-0.56598	C	-4.50555 -0.98973 -0.86518
C	1.434161	2.137908	-0.59936	H	-3.61966 1.616654 1.114208
C	1.263528	0.756945	-0.46989	H	-6.00155 1.627886 1.547039
C	2.357875	-0.09363	-0.19522	H	-6.48135 -1.73892 -1.12171
C	2.237027	-1.51643	-0.02482	H	-4.07676 -1.72774 -1.53544
C	3.32685	-2.28628	0.245618	N	-7.84119 -0.01881 0.454179
H	3.213305	-3.36051	0.369012	C	-8.37983 0.821775 1.509042
H	1.261797	-1.97247	-0.13814	H	-9.4674 0.725792 1.522202
H	7.198976	2.147428	0.31078	H	-7.99794 0.554742 2.507952
H	7.895975	-2.54241	0.983124	H	-8.14507 1.877803 1.32887
H	8.170692	-0.09201	0.730488	C	-8.65832 -1.11631 -0.03432
H	5.655482	-3.58026	0.760658	H	-9.70431 -0.91721 0.207495
H	5.237558	3.562674	-0.15692	H	-8.58332 -1.2064 -1.12446
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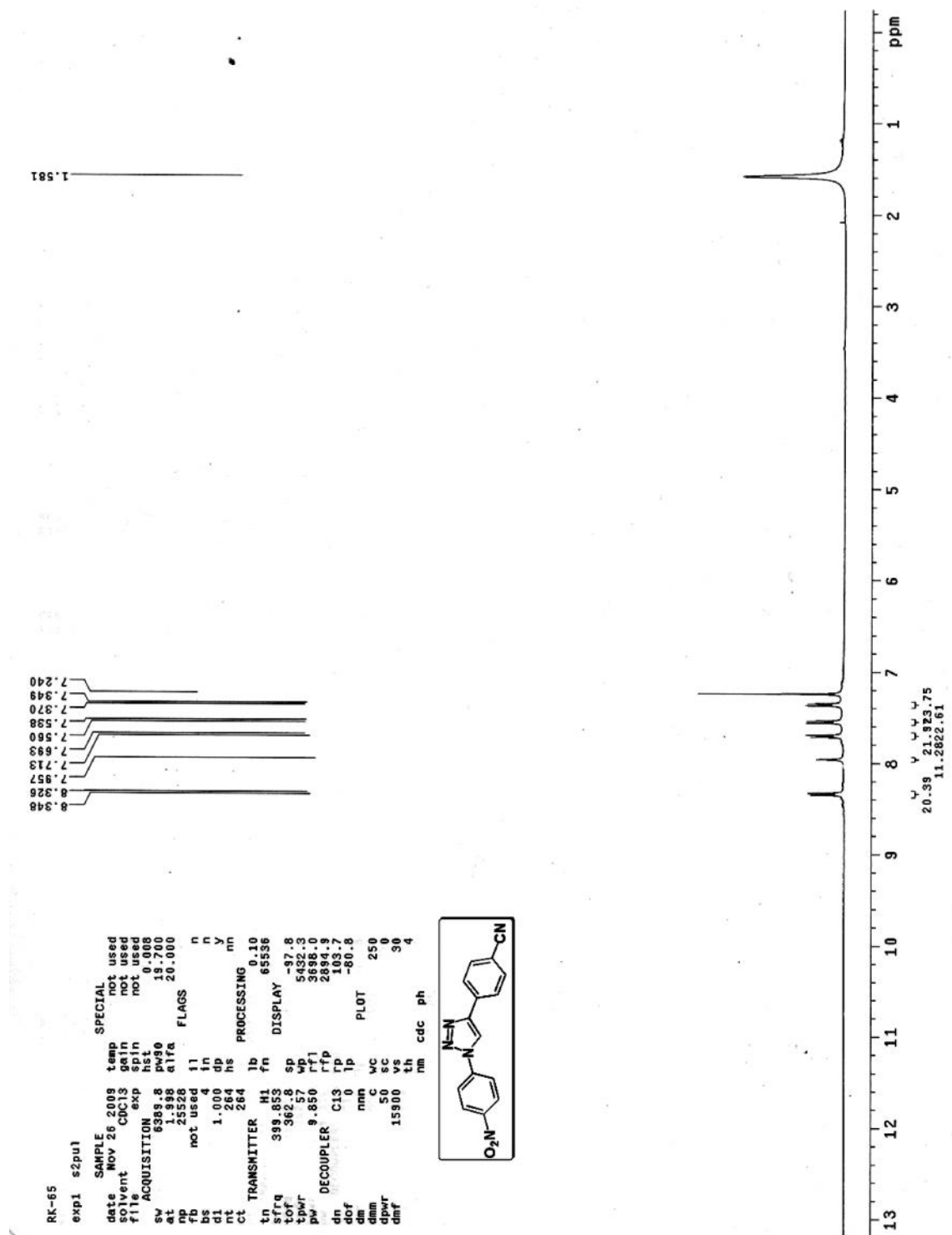


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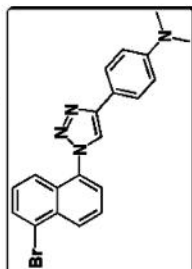
0 1							
C	-6.4707	-1.99616	-0.64633	H	-5.07601	-3.63589	-0.54179
C	-6.6439	-0.61485	-0.5803	H	-4.7463	3.551975	-0.02162
C	-5.55111	0.236744	-0.35269	H	-0.06711	2.840186	0.680421
C	-4.25051	-0.33058	-0.18618	N	0.58893	0.318026	0.583943
C	-4.0831	-1.74585	-0.25833	N	0.916443	-0.59786	1.543181
C	-5.20544	-2.55787	-0.48805	N	2.193991	-0.80558	1.459612
C	-5.6964	1.664028	-0.28361	C	1.698574	0.694437	-0.10248
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C	-3.30345	1.931436	0.099901	C	2.731573	-0.03175	0.462125
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C	-2.18678	2.752988	0.317619	C	4.701379	0.727413	-0.88467
H	-2.31732	3.830496	0.367242	C	6.057568	0.685197	-1.17969
C	-0.92336	2.201612	0.485686	C	6.909765	-0.15849	-0.44697
C	-0.74079	0.818103	0.432503	C	6.377534	-0.95469	0.582166
C	-1.82298	-0.06143	0.21334	C	5.020256	-0.90686	0.870099
C	-1.68377	-1.48981	0.128083	H	4.057161	1.385231	-1.46104
C	-2.76415	-2.28721	-0.09685	H	6.465811	1.301008	-1.97454
H	-2.63806	-3.36537	-0.15633	H	7.034558	-1.60651	1.148969
H	-0.70345	-1.92851	0.266491	H	4.604677	-1.51935	1.662586
H	-6.68926	2.088871	-0.40898	C	8.309671	-0.20471	-0.74827
H	-7.32823	-2.63933	-0.82325	N	9.446566	-0.24074	-0.99432
H	-7.63346	-0.18265	-0.70634				

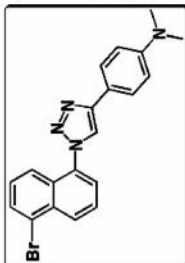


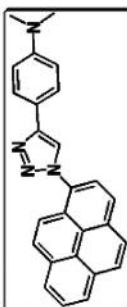


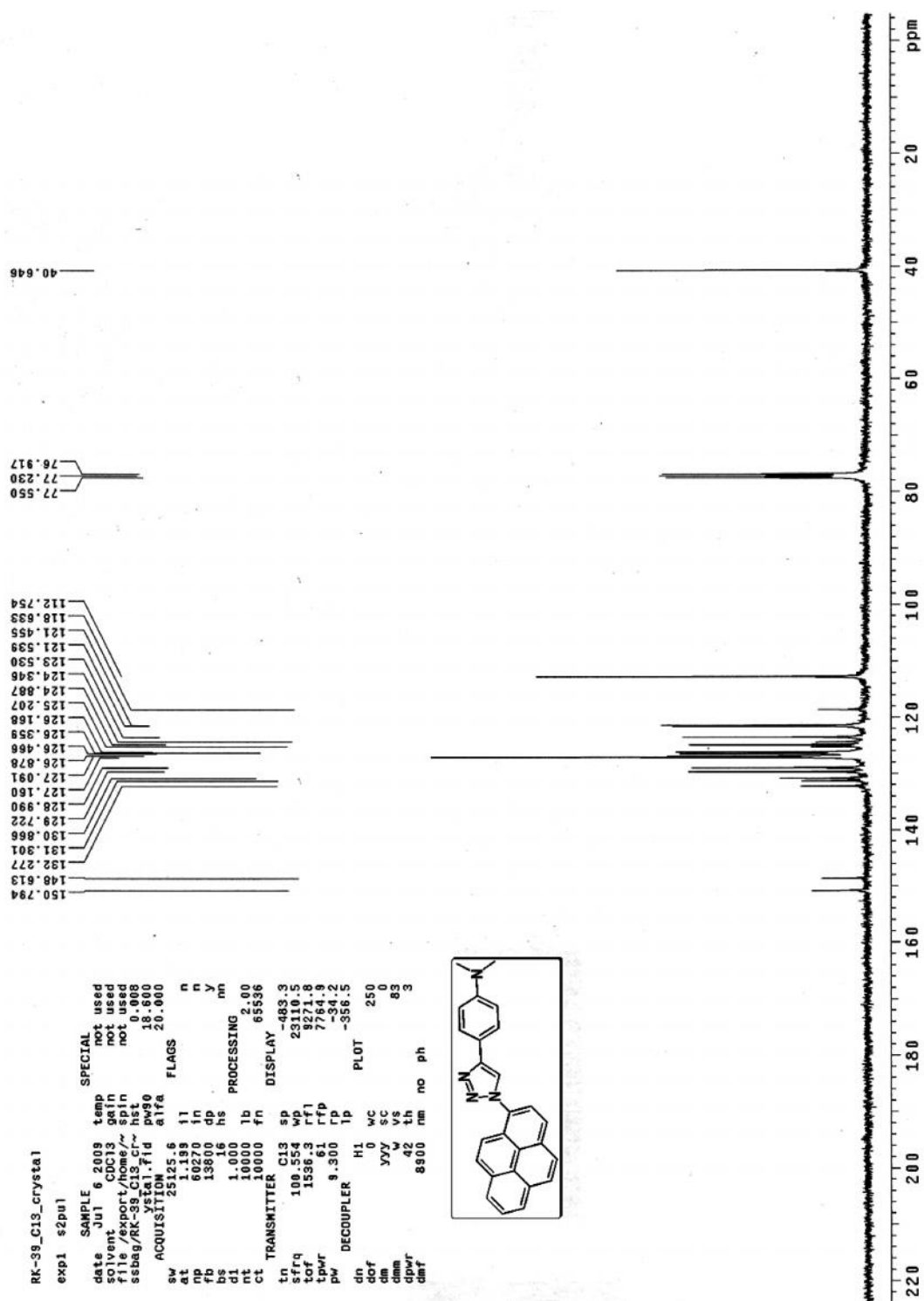












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Chapter 3

**A MODIFIED SONOGASHIRA
COUPLING PROTOCOL
TO THE SYNTHESIS OF FLUORESCENT
SMALL MOLECULES**

3.1. Introduction

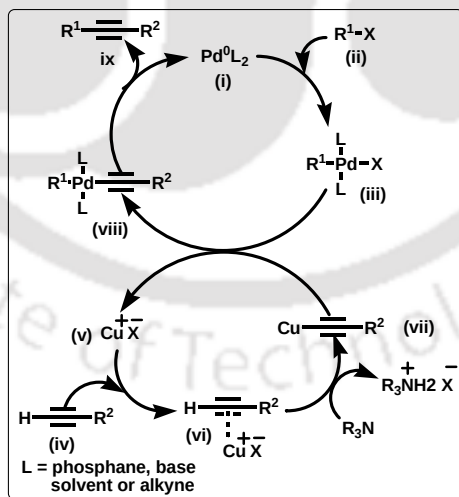
The design and synthesis of π -conjugated organic molecules are important for their various applications in diverse areas ranging from molecular scaffolding, natural products and pharmaceuticals, biological applications in designing fluorescent probes to molecular organic materials.¹ Moreover, alkynes are an attractive functional group for further derivatization in many synthetic transformations of biological importance.¹ Since its introduction in 1975, Sonogashira coupling, because of its mild reaction condition, attract much attention and became the most popular, widely used practical tool for the generation of several terminal and internal π -conjugated acetylenic compounds.^{1,2} Although, Pd(0)/CuI catalyzed Sonogashira cross coupling reaction of terminal alkynes with sp^2 -C halides for the synthesis of conjugated acetylenes is a reliable, simple, and overall excellent method, it suffers from several shortcomings, such as

- (a) the generation of undesired homocoupled products³ of terminal alkynes in the presence of air,
- (b) the requirement of specific substrates and the skillful tailoring of reaction conditions,
- (c) poor coupling under mild reaction conditions with unactivated halides,
- (d) the requirement of large amounts of a catalyst, and
- (e) low catalyst turnover.⁴

Because of its importance in the ever evolving field of synthetic acetylenic chemistry, especially, in the designing of conjugated fluorophores there has been a tremendous research effort over the course of the last decade to overcome these limitations;⁴ however, no single protocol could overcome the aforementioned shortcomings, especially the problem of homocoupling. Therefore, overcoming these limitations, especially the problem of the homocoupling side reaction, remains a challenge for researchers.

3.2. The Sonogashira Cross Coupling Reaction

Palladium-catalyzed sp²-sp coupling reactions between aryl or alkenyl halides or triflates and terminal alkynes have been independently reported by three groups, Heck,^{5a} Cassar^{5b} and Sonogashira^{2a} in 1975. The introduction of sp²-sp coupling reactions by Heck and Cassar was an extension of Heck reaction to terminal acetylenes. On the other hand, the last one introduced by Sonogashira was on the basis of development of Cu(I) catalyst in Pd-catalyzed cross-coupling of sp²-C halides with terminal acetylenes. This can be considered as an application of Pd-catalysts to Stephens–Castro reaction.^{5c} Both the Heck's and Cassar's procedures generally required high temperature (up to 100 °C). However, the addition of catalytic amount of CuI which was introduced by Sonogashira and Hagihara, greatly accelerates the reaction rate at room temperature. Since then, the Sonogashira coupling or Sonogashira-Hagihara protocol, became the most popular and practical tool for the synthesis of terminal or internal π -conjugated alkynyl derivatives (Scheme 3.1).

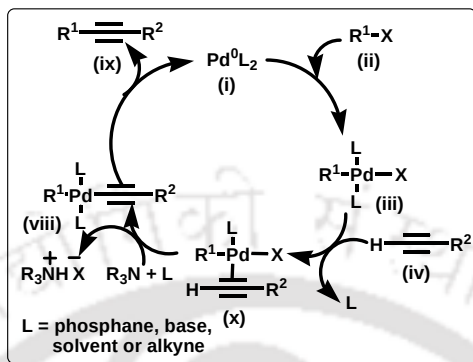


Scheme 3.1: Plausible mechanism of Sonogashira coupling.

The exact mechanism of the Sonogashira coupling is still not well understood, although it is generally supposed to take place through two independent cycles,

“palladium cycle” and “copper cycle”. The palladium cycle starts from catalytically activated species $\text{Pd}(0)\text{L}_2$ (**i**, stabilize by ligand like phosphane) which reacts with aryl halide (**ii**) to form $[\text{Pd}^{\text{II}}\text{L}_2\text{R}_1\text{X}]$ complex (**iii**) through oxidative addition (**Scheme 3.1**). On the other hand in copper cycle the terminal alkyne (**iv**) in presence of a base reacts with $\text{Cu}(\text{I})$ to form copper acetylide $[\text{R}_2\text{-C}\equiv\text{C-Cu}]$ complex (**vii**). Then the trans-metallation occurs between $[\text{Pd}^{\text{II}}\text{L}_2\text{R}_1\text{X}]$ and $[\text{R}_2\text{-C}\equiv\text{C-Cu}]$ complex (**iii** and **vii**, respectively) to form $[\text{R}_1\text{Pd}^{\text{II}}\text{L}_2\text{C}\equiv\text{C-R}_2]$ complex (**viii**) which in turn followed by reductive elimination forms the desired cross-coupled product $[\text{R}_1\text{C}\equiv\text{CR}_2]$ (**ix**).

Though the catalytic amount of $\text{Cu}(\text{I})$ salt greatly improved the reaction rate and condition of the Sonogashira coupling, however, its addition has some problem, such as the formation of homocoupled side product of the terminal alkyne. The copper acetylides, formed under the reaction conditions generate homocoupled products of the terminal alkyne as a result of popular Glaser coupling^{5d-e} along with the main reaction product in presence of oxidizing agents or air which is very difficult to remove from the reaction vessel completely. The formation of this homocoupled side product is mainly a big problem to the chemist as the terminal alkynes are very expensive in most of the cases and also the overall yield of the desired cross-coupled product decreases. Thus, to avoid the side reaction, significant effort has been paid to develop Cu-free Sonogashira coupling. However, all these Cu free protocol use excess of amine or in some cases the amine acting as solvent, which in turn reduce the homocoupled product to some extent. The mechanism of Cu free Sonogashira coupling is shown in **Scheme 3.2**.



Scheme 3.2: Plausible mechanism of Cu free Sonogashira coupling.

3.3. Modifications of Sonogashira Coupling

Several modifications of Sonogashira coupling have been reported throughout the past decades by various research groups to overcome the major shortcomings.⁴ As a result of tremendous research efforts in last decade, several developments have been done in Pd(0)-catalyzed coupling systems and many of the strategies were developed to overcome few of such problems. However, the problems still-standing needing improvement of Sonogashira cross coupling are

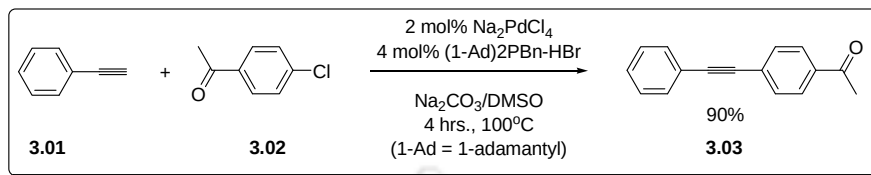
- (a) development of a catalyst for the coupling with unactivated aryl halides such as aryl chlorides,
- (b) improvement of turn over numbers (TON) for a catalyst, and
- (c) suppression of the homocoupled side reactions for terminal acetylenes.

Here, in the following few sections, a brief account of some important modifications are presented. Our focus will be more on those modifications which address diminishing the undesired homocoupled side product in the Sonogashira cross coupling reaction.

3.3.1. Modifications of Pd-Catalyst Addressing Coupling with Unactivated Aryl Halides

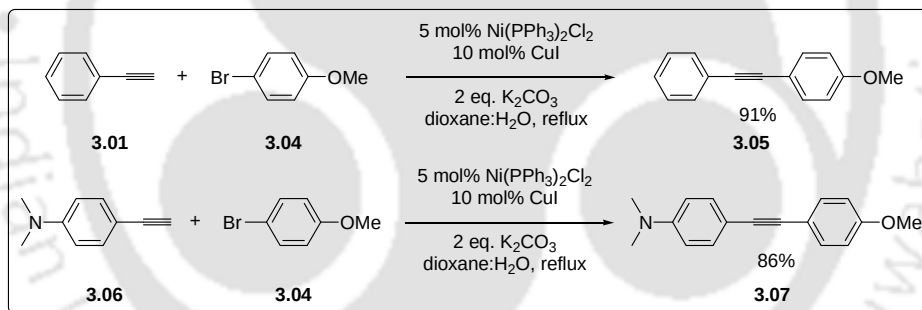
As was discussed in the introduction, the Sonogashira reaction is generally performed using a palladium-phosphine ligand complex as catalyst in the presence of a catalytic amount of a Cu(I) salt and an amine as a base or as a solvent under homogeneous conditions. The traditionally used catalysts are $\text{Pd}(\text{PPh}_3)_4$, with the more stable and soluble $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ being the most common, have been employed. Most frequently, rather high loadings of palladium (usually up to 5 mol %) and larger amounts of the copper(I) salt are required when these palladium species are employed for doing reaction with activated aryl halides. The most of the traditional catalysts or the reactions conditions do not offer any activation of unactivated halides as aryl chlorides, thus, boosting up the search for a more active catalyst for simpler, milder, and more effective reaction conditions to be applied for effective coupling to proceed even with unactivated aryl halides.

Thus, Plenio *et al.*, have reported a versatile catalyst for the Sonogashira coupling reaction of aryl chloride with terminal alkynes.^{6a} They have used $\text{Na}_2[\text{PdCl}_4]/\text{PR}_3/\text{CuI}$ ($\text{PR}_3 = (1\text{-Ad})_2\text{PBn}$, P^tBu_3) catalyst/co-catalyst system for the Sonogashira coupling of various aryl chlorides and alkynes. In their modification they focused on changing the phosphane ligand, PPh_3 , by $(1\text{-Ad})_2\text{PBn}$ ($1\text{-Ad} = 1\text{-adamantyl}$, $\text{Bn} = \text{benzyl}$) and P^tBu_3 . The adamantyl-substituted phosphane ligands and the respective phosphonium salts $(1\text{-Ad})_2\text{PBn}\cdot\text{HBr}$ and $\text{P}^t\text{Bu}_3\cdot\text{HBF}_4$ are insensitive towards oxidation and become deprotonated at the beginning of the coupling reactions. Thus, they have shown that the aryl chloride **3.02** upon reaction with the phenyl acetylene **3.01** in presence of 2 mol% Na_2PdCl_4 , 4 mol% $(1\text{-Ad})_2\text{PBn}\cdot\text{HBr}$, 1.5 mol% CuI and base Na_2CO_3 in DMSO solvent afforded the corresponding coupled product **3.03** in very good yield (**Scheme 3.3**).



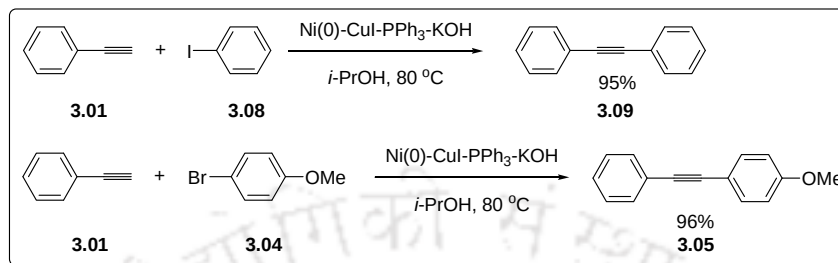
Scheme 3.3: Sodium salt of palladium chloride mediated Sonogashira coupling.

A Pd-free Sonogashira cross coupling reaction has been developed by Beletskaya *et al.*^{6b} They have used $\text{Ni}(\text{PPh}_3)_2\text{Cl}_2$ as a catalyst instead of catalytic system $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$, PPh_3 . Thus, phenyl acetylene, **3.01** reacts with 4-bromo-anisole, **3.04** in presence of 5 mol% $\text{Ni}(\text{PPh}_3)_2\text{Cl}_2$, 10 mol% CuI and 2 equivalent K_2CO_3 in 3:1 dioxane-water mixture under reflux condition to afford the corresponding crosscoupling product **3.05** in very high yield (**Scheme 3.4**).



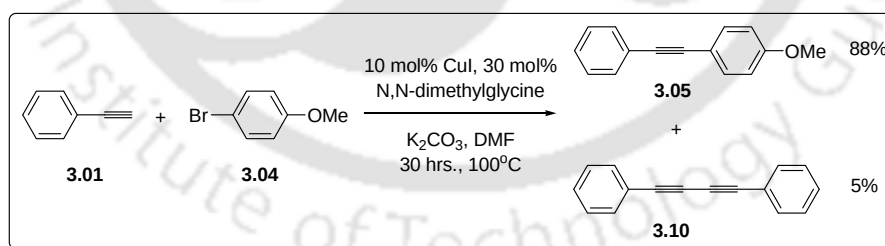
Scheme 3.4: $\text{Ni}(\text{PPh}_3)_2\text{Cl}_2$ catalysed Sonogashira coupling.

Later on Wang *et al.*,^{6c} have modified the $\text{Ni}(\text{PPh}_3)_2\text{Cl}_2$ catalyst by using ultrafine nickel(0) powder as an efficient catalyst for the same reaction (**Scheme 3.5**). It has been observed that aryl, alkenyl iodide and aryl bromide react with terminal alkynes in the presence of CuI , PPh_3 , KOH and ultrafine particle $\text{Ni}(0)$ to form the corresponding crosscoupling products with high yields.



Scheme 3.5: Ultrafine Ni(0) catalysed Sonogashira coupling.

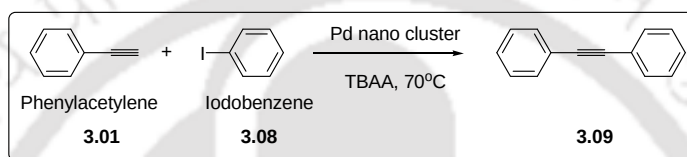
A palladium and phosphine free Cu(I) catalyzed Sonogashira coupling reaction have been reported by Ma *et al.*^{6d} In their strategy they have used *N,N*-dimethylglycine hydrochloride salt as an efficient catalyst. Thus, Phenyl acetylene **3.01** reacts with 4-bromo-anisole **3.04** in presence of 10 mol% CuI, 30 mol% *N,N*-dimethylglycine hydrochloride and K₂CO₃ to form the corresponding coupled product **3.05** (**Scheme 3.6**). Apart from the desired crosscoupled product (**3.05**), an undesired alkyne-alkyne homocoupled product (**3.10**) was formed in the same reaction mixture. The beauty of this reaction is that the catalyst system is cheap and easily removable by simple washing, however, the formation of alkyne-alkyne homocoupled product still remains as a problem to be solved.



Scheme 3.6: Pd(0)/phosphine free Cu(I) catalyzed Sonogashira coupling.

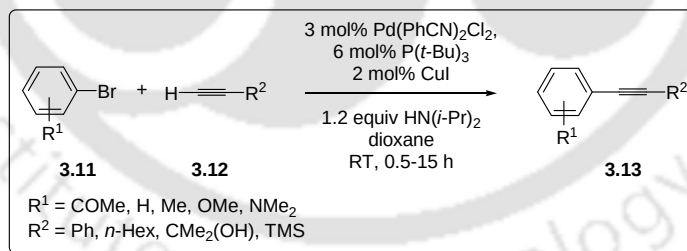
Use of Pd-nanoclusters as catalyst for C-C coupling protocol has been developed by Rothenberg *et al.*,^{6e} (**Scheme 3.7**). In this particular, the leached Pd species formed from the Pd nanocluster is the effective catalytic species for the reaction to occur. To perform the reaction they used a special U-tube permeation cell membrane reactor

consisting of two stainless steel compartments separated by a membrane. One side of the reactor contains the Pd clusters (15 nm) which cannot pass through the membrane; on the other side it contains the reaction mixture without catalyst. Only the leached Pd species diffused through the membrane and goes to the reaction mixture. Thus, this protocol contains a tedious reaction setup which is very difficult to maintain and use for the large scale synthesis.



Scheme 3.7: Pd(0)-nanocluster mediated Sonogashira coupling.

Fu *et al.*,^{6f} have reported a $\text{Pd}(\text{PhCN})_2\text{Cl}_2/\text{P}(t\text{-Bu})_3$ catalysed Sonogashira cross coupling reaction of aryl bromides with terminal alkynes (**Scheme 3.8**). Among various tested phosphine ligands like PPh_3 , $\text{P}(o\text{-tolyl})_3$, dppf , PCy_3 and $\text{P}(t\text{-Bu})_3$, the maximum yield was observed when the bulky electron rich phosphine, $\text{P}(t\text{-Bu})_3$ was used.

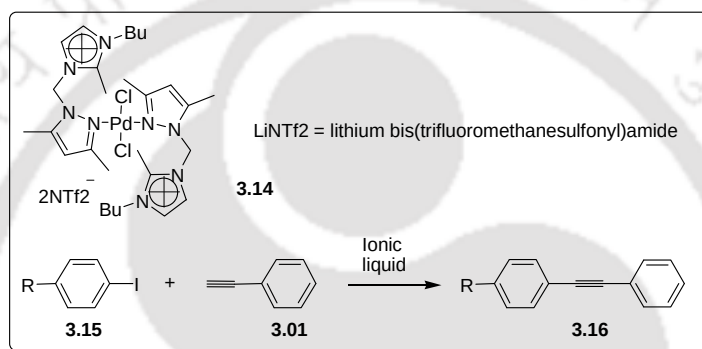


Scheme 3.8: Pd(II)/ $\text{P}(t\text{-Bu})_3$ catalyzed Sonogashira coupling.

3.3.1. Modifications Addressing Improvement of Turn Over Numbers for a Catalyst

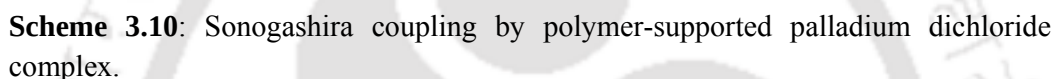
Shreeve *et al.*, have developed an ionic liquid which can serve both as solvent and as ligand for Sonogashira coupling.^{7a} Thus, they carried out the Sonogashira reaction using 1.0 mmol aryl iodides (3.15), 1.0 mmol phenylacetylene (3.01), 1.5 mmol

piperidine, and 1.0 mol% palladium(II) complex (**3.14**) dissolved in ionic liquid 1-(3,5-dimethylpyrazolylmethylene)-3-butyl-2-methylimidazolium bis(trifluoromethanesulfonyl)amide (**Scheme 3.9**). The corresponding cross-coupled products (**3.16**) were obtained with high yields. Glaser-type homocoupling products were not detected with this catalytic solution. They recycled the catalytic solution six times with different reactants without significant loss of catalytic activity.



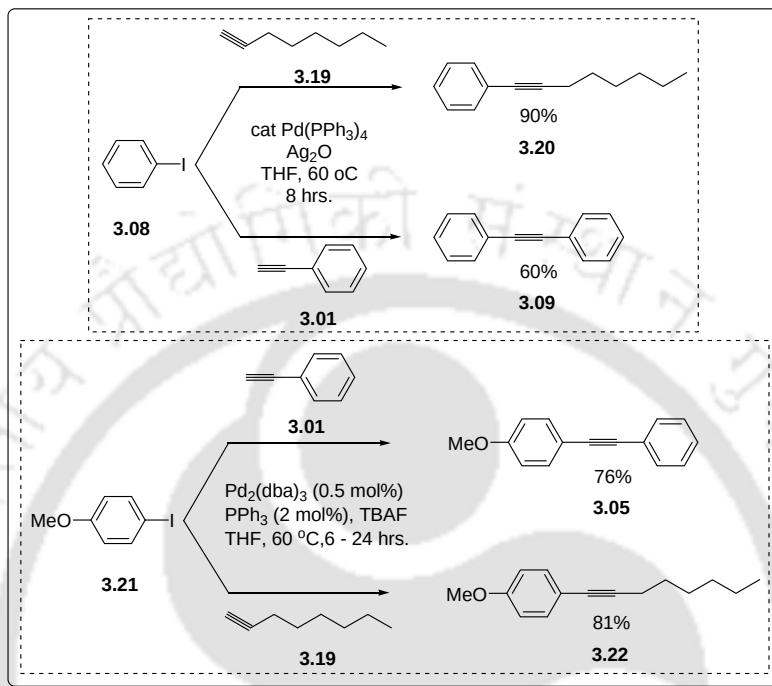
Scheme 3.9: Sonogashira coupling in ionic liquid.

Najera *et al.* have performed the cross-coupling of activated and deactivated iodides and bromides with phenylacetylene and triisopropylsilylacetylene with pyrrolidine (2 equiv) as base and TBAB as additive in water under reflux (**Scheme 3.10**).^{7b} For aryl iodides they have used 0.1 mol % of Pd with the polymeric **3.17** or the monomeric **3.18** catalysts, whereas for aryl bromides 0.2 mol% of Pd has been used to achieve better conversions. The catalyst could be recycled five more times without appreciable loss of catalytic activity.



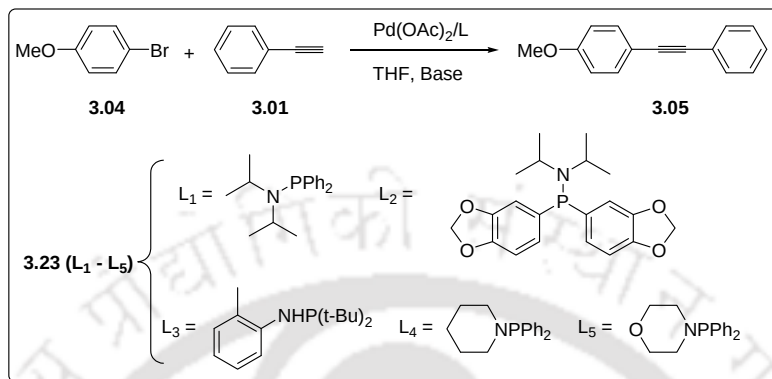
3.3.3.1. Cu-Free Sonogashira Coupling: Reduction of Homocoupling

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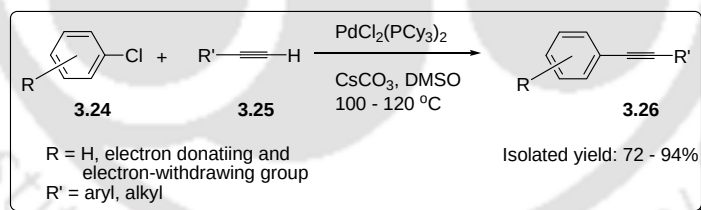
Scheme 3.11: Cu-free (Ag_2O or TBAF catalyzed) Sonogashira coupling.

A copper free Sonogashira reaction employing aminophosphines as ligands and $\text{Pd}(0)$ as catalyst has been developed by Zhang *et al.*^{8b} Thus, *p*-bromoanisole and phenylacetylene reacted together in presence of $\text{Pd}(\text{OAc})_2$, Et_3N and ligand (L_1) in THF solvent, afforded the corresponding crosscoupled product (**Scheme 3.12**). They found that L_1 is the most effective ligand for this reaction in comparison with others (L_2 , L_3 , L_4 , and L_5) in various solvents like THF, DMF and CH_3CN .



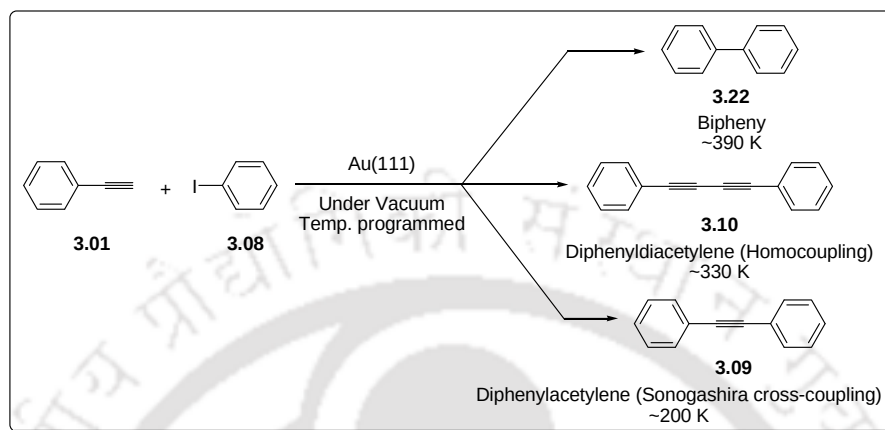
Scheme 3.12: Cu-free Sonogashira coupling using aminophosphines as ligand.

Another Cu-free version of Sonogashira crosscoupling reaction in presence of Cs_2CO_3 as a base, and $\text{PdCl}_2(\text{PCy}_3)_2$ as an efficient high activity catalyst has been reported by Hua *et al.*^{8c} Thus, with this catalytic system electron-rich, neutral, and electron deficient aryl chlorides have been observed to react with various terminal alkynes in DMSO at 100-120 °C to afford the desired crosscoupled product (**Scheme 3.13**).



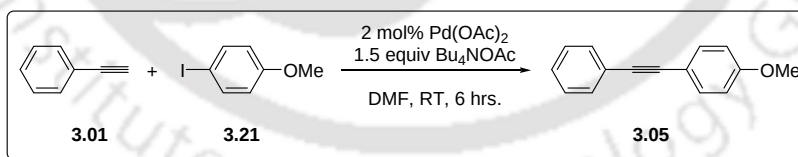
Scheme 3.13: Cu-free Sonogashira coupling mediated by Pd(II)/ Cs_2CO_3 .

In 2010, Kanuru *et al.*,^{8d} have reported a temperature programmed reaction where phenylacetylene and iodobenzene react on an extended gold surface under vacuum to form biphenyl, diphenyldiacetylene and diphenylacetylene (**Scheme 3.14**). The reaction is fully temperature controlled, thus, at 309 K biphenyl is formed, at 330 K diphenyldiacetylene is formed and at 200 K diphenylacetylene, the Sonogashira crosscoupled product is formed. Although the formation of products is temperature controlled, yet it is difficult to handle for large scale synthesis.



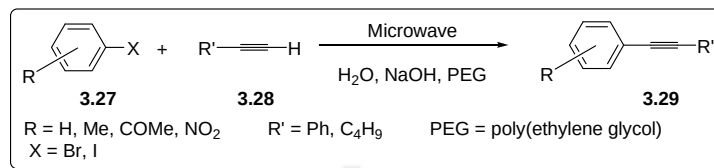
Scheme 3.14: Sonogashira coupling on gold surface under vacuum and programmable temperature.

An efficient ligand, copper and amine free palladium catalyzed Sonogashira reaction of aryl iodides and bromides with terminal alkynes has been reported by Verkade *et al.*,^{8e} (**Scheme 3.15**). Success of this reaction comes with the use of tetrabutylammonium acetate as the base. Thus, *p*-iodoanisole (3.21) reacted with phenyl acetylene (3.01) in presence of 2 mol% Pd(OAc)₂ and 1.5 equiv. of tetrabutylammonium acetate in DMF at room temperature affording the crosscoupled product (3.05).



Scheme 3.15: Ligand, amine and Cu-free Sonogashira coupling protocol.

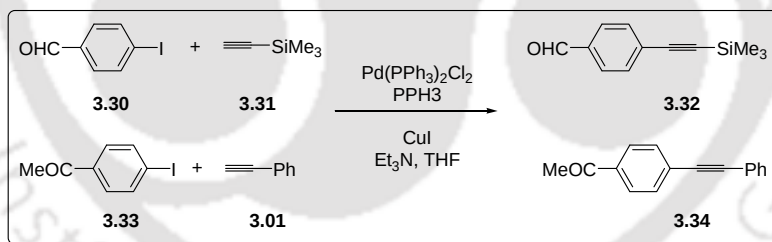
The transition metal free Sonogashira coupling reaction has been reported, for the first time, by Leadbeater *et al.*^{8f} Thus, under microwave condition, aryl halides (3.27), bromides and iodides react with terminal alkynes (3.28) in presence of water as a solvent, poly(ethylene glycol) as a phase-transfer agent, and sodium hydroxide as a base to form the corresponding crosscoupled products (3.29, **Scheme 3.16**).



Scheme 3.16: Cu-free and microwave assisted Sonogashira coupling reaction.

3.3.3.2. Slow Addition of Alkyne: Reduction of Homocoupling

A new method to diminish the alkyne-alkyne homocoupled product in Sonogashira coupling reaction have been reported by Krause *et al.*^{8g} The new technique of this reaction was the slow addition of alkyne into the reaction mixture to control the formation of homocoupled product by keeping the low concentration of alkyne in the reaction mixture. It was the first time where dilution technique was used in Sonogashira reaction to diminish the formation of homocoupled side product. Using this technique they were able to diminish the homocoupled product up to <5% (**Scheme 3.17**).

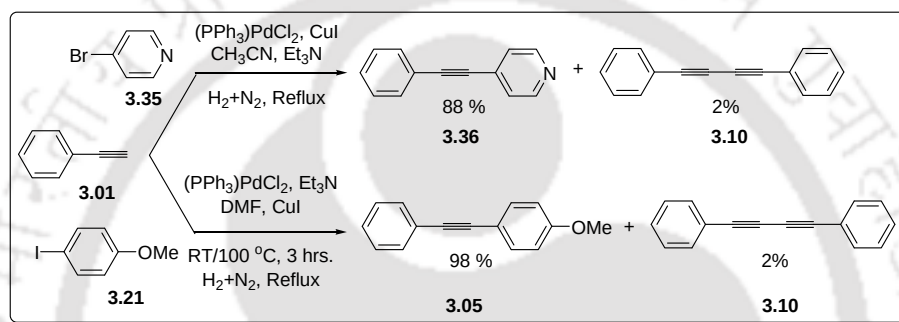


Scheme 3.17: Slow addition of alkyne in Sonogashira coupling protocol.

3.3.3.3. Generating a Reductive Atmosphere by Hydrogen Gas: Reduction of Homocoupling

Ho *et al.*,^{8h} have recently developed a new protocol to reduce the undesired side product formed by alkyne-alkyne homocoupling reaction in Sonogashira coupling. They were able to reduce the homocoupled product to about 2% using a reductive atmosphere created by continuous flow of hydrogen gas diluted with nitrogen into the reaction mixture (**Scheme 3.18**). Thus, terminal arylalkynes, diarylalkynes with

electron donating or withdrawing substituents and arylpyridylalkynes with donor substituents have been synthesized using this protocol. Although these protocols succeed to prevent the formation of homocoupled product, the continuous flow of hydrogen gas in reaction mixture is very critical to handle and expensive. Moreover, having explosive hydrogen gas in the laboratory raise the question of safety and thereby its success.



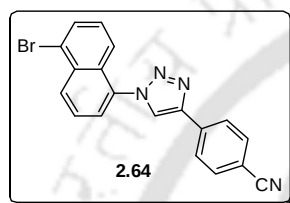
Scheme 3.18: Hydrogen atmosphere mediated Sonogashira coupling protocol.

3.4. Background

Therefore, from the above reported literatures, it is clear that the homocoupling side reaction in Sonogashira coupling is especially a major problem when the terminal acetylene is difficult to obtain or expensive. As per the mechanism of the reaction, it is obvious that in many cases, a considerable amount of dimer was formed in reality at the expense of crosscoupling product. Although most of the reactions are performed in inert atmosphere, the reaction setup is not completely devoid of oxygen and as long as the oxygen remains in the reaction mixture, it may oxidize the nascent Pd(0) to generate Pd(II) and or Cu(I) to Cu(II), which in turn may promote homocoupling of terminal alkynes in CuI co-catalyzed original Sonogashira coupling.^{2b,4e-g,9d} Therefore, in the journey of searching for an improved method, only few attempts, like slow addition of the acetylene and use of phase-transfer conditions, have been taken using original coupling reagents to afford reduced homocoupled undesired side product. Only recently, one method has been shown that the presence of a reductive

atmosphere formed by difficult-to-handle hydrogen gas can diminish homocoupling.^{8h} However, to get success with this method the system must be degassed and back-filled with reducing gas mixture. Moreover, handling explosive H₂ gas in the laboratory raise the question of safety and thereby its success.

Despite several modifications,⁹⁻¹⁰ no special steps have been taken to develop a modified version of the Cu(I) co-catalyst system so as to reduce the homocoupling



reaction and facilitate a high yield of desired product; however, the reaction rate can be accelerated and applied to a wider range of reaction conditions. As a part of our ongoing research effort for installing the fluorescence response to a non-fluorescent precursor *via* “click” reaction¹¹ (discussed in **Chapter 2**), we envisaged that Sonogashira coupling would enable us to realize more conjugated highly emissive fluorophores. As for example, the compound **2.64** was synthesized *via* click-reaction and the study of photophysical properties was discussed in **Chapter 2**. In brief, this fluorophore showed a very low solvatochromicity and faint emission under usual experimental condition. We, therefore, envisaged that this fluorophore might show high solvatochromicity and strong emission if we could increase the conjugation. As it contains an active C–Br bond, we thought that extending the conjugation via an attachment of a donor or an acceptor unit containing a terminal or an internal alkyne by Sonogashira coupling would endow the desired fluorescence property to this molecule. Keeping this idea in mind, we were about to carry out the classical Sonogashira reaction. However, as a part of our ongoing research efforts on click reaction¹¹, we were curious to know whether the same click reagents can serve as a source of reactive Cu(I) for Sonogashira coupling as well as can diminish the formation of undesired homocoupled side product by maintaining a reductive atmosphere.

3.5. Objective

With this above background and aim we framed our objectives as bellow.

- (a) To test whether the same click reagents can serve as a source of reactive Cu(I) for Sonogashira coupling,
- (b) We wanted to know whether Na-ascorbate can maintain a reducing atmosphere in this system,¹² and
- (c) To know whether the system would be more effective to reduce the incidence of homocoupling (alkyne-alkyne coupling) in comparison to CuI or other reported modified Sonogashira methods^{4,9,10}
- (d) To develop a click-reagent version of Sonogashira coupling protocol to generate conjugated molecules with interesting photophysical properties.
- (e) To extend the conjugation of and to show improved fluorescence emission property of the previously reported click fluorophore(2.64).

Thus, we have chosen the following reaction scheme, the aryl halides and the alkynes to carry out and establish our conceptual click-reagent version of Sonogashira coupling strategy (**Figure 3.1**). We were successful in developing a “click-reagent version of Sonogashira coupling” protocol under mild reaction conditions without any requirement of skillful reaction tailoring.

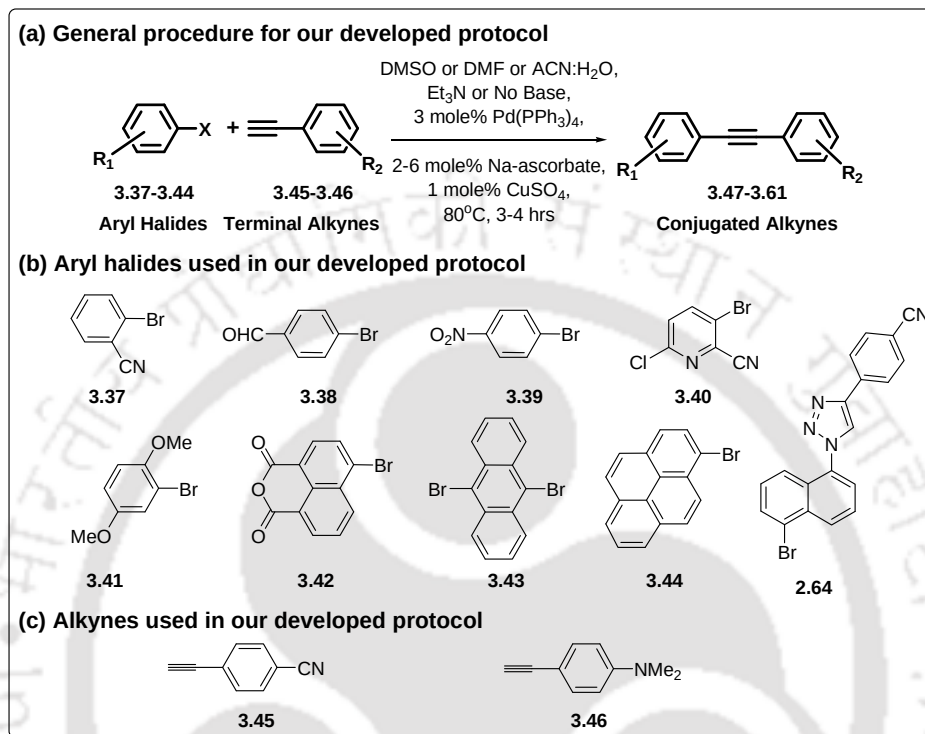


Figure 3.1: (a) Schematic representation of the general procedure. (b) The aryl halides and (c) alkynes that were used in our developed click-reagent version of Sonogashira coupling protocol.

The logic behind the use of click-reagent in our strategy is:

- to generate active Cu(I) in situ,
- to halt the oxidation of the generated Cu(I) into Cu(II), and
- to remove excessive air/O₂ from the reaction setup, which may damage nascent Pd(0) catalyst which in turn might promote homocoupling side reaction.

Sodium ascorbate can reduce oxygen to form water in the vicinity of nascent Pd(0),^{4e-g,9d} which reduces the oxygen concentration in the reaction; hence, the incidence of undesired oxidative homocoupling can drastically be diminished. Therefore, to maintain a constant reducing environment, we used six times more Na-ascorbate than Cu(II). Therefore, the most probable roles of the click-reagents in the

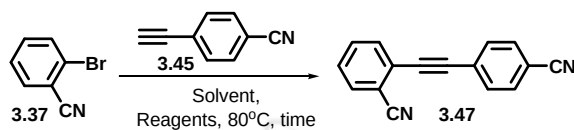
Sonogashira coupling procedure include the in situ provision of active Cu(I) co-catalyst, maintenance of a constant reducing atmosphere in the vicinity of nascent Pd(0), and achievement of the possible acceleration of the reductive elimination step, which would result in a high yield of a desired product.

3.6. Results and Discussion

We are now going to elaborate our developed click-reagent version of Sonogashira coupling protocol, which results in either a tremendous decrease in or no undesired homocoupling side product leading to an enhancement in the yield of the desired heterocoupled product within a short period of time.

3.6.1. Optimization of Reaction Condition

To establish the optimum reaction conditions for our protocol, we have investigated the possibility of coupling with varying catalytic concentrations. Thus, as is shown in **Table 3.1**, we carried out a reaction of aryl bromide **3.37** with alkyne **3.45** in various molar ratios of ascorbate, base, and Pd(0) in DMF. Therefore, we have carried out the coupling reaction using a CuSO₄/Na-ascorbate couple in a 1:6 mole% ratio as a source of active Cu(I) (1 mole%), Pd(0) (3 mole%) in a 1:1 DMF/Et₃N solvent mixture at 80 °C, and other possible molar ratios. All of the reactions were found to be completed within 4 hr. The products were isolated to purity with moderate to excellent yields *via* column chromatography and then characterized. We have also tested our methodology in the absence of base or copper, as well as in both copper- and base-free conditions; however, a low yield of the products (22-49%, **Table 3.1**) was obtained by applying these conditions. Finally, we observed that a minimum of 20 equivalents of base is required to achieve good yields; therefore, a catalytic composition of Pd(0):CuSO₄:ascorbate in a ratio of 3:1:6 mole% was identified to be the optimum yielding condition for our protocol (**Table 3.1**).

Table 3.1: Optimization of the reaction condition.

Solvents	Reagents (mole %)			Base (Et ₃ N)	Time (hrs.)	Yield (%) ^[c]
	Pd(0)	CR1 ^[a]	CR2 ^[b]			
DMF	3	1	6	excess	4	84
	3	1	6	20 eqv.	4	80
	3	1	6	10 eqv.	4	70
	3	1	6	5 eqv.	4	54
	3	1	6	2 eqv.	4	53
	3	1	6	---	4	20
	3	1	5	excess	4	71
	3	1	4	excess	4	56
	3	1	3	excess	4	48
	3	1	2	excess	4	40
	3	0	6	excess	4.5	49
	3	0	6	---	12	22
	1	1	6	excess	5	34
DMF/ H ₂ O (1:8)	3	1	6	excess	5	71
CH ₃ CN/ H ₂ O (1:1)	3	1	6	excess	4	82
DMF, Open air	3	1	6	excess	4	40
MW, Open air	3.5	1	6	excess	3.5 min.	90

^[a]CR1 is click-reagent 1, which is CuSO₄. ^[b]CR2 is click-reagent 2, which is sodium ascorbate. ^[c]yields consist of isolated pure compounds. For all of the reactions, the conversion was 100%.

3.6.2. Exploration of our Optimized Protocol

To test the feasibility and facilitate the generalization of our method, we used optimum yielding conditions and carried out the reactions of a series of simple aryl

bromides with two terminal alkynes (**Figure 3.1**) for the synthesis of several donor-acceptor-containing conjugated alkynes; one terminal alkyne containing electron-withdrawing substituents (**3.45**, -CN), whereas the other has an electron-donating group (**3.46**, -NMe₂). The products were isolated to purity at good to excellent yields *via* column chromatography and characterized by NMR, mass spectrometry, and, in a few cases, by X-ray crystallography (**Table 3.2** and **Figure 3.2**). We have observed that irrespective of the aryl halides, no homocoupled products with the electron-deficient alkyne **3.45** were formed; however, in a few cases, a negligible amount (1-6%) of homocoupled products with the electron-rich alkyne **3.46** was observed (**Table 3.2**). Here, it is important to mention that for 4-bromonaphthalic anhydride (entry **3.56**, **Table 3.2**), due to its poor solubility in DMF or DMSO, the resultant product was isolated to purity *via* filtration and recrystallization from 1:1 toluene-ethanol mixture. Therein, a loss of product resulted in a low yield of 65%. Also, in the case of dibromoanthracene, wherein the reaction was carried out in 1:1:1 toluene:THF:water, an appreciable amount of un-reacted starting material remained, which resulted in a moderate yield (72% for **3.58**) of the product; however, most importantly, no homocoupling was observed in both of these cases. In the case of pyridine halide **3.40**, the yield of the desired product, **3.52** was 61%; however, along with **3.52**, a biscoupled product **3.53** was obtained at a 25% yield after being isolated (**Figure 3.2**). This result, though it is only one example, indicates that our click-reagent version of Sonogashira coupling procedure proceeds even when using aryl chloride. The compound **3.62** (**Figure 3.2**) was synthesized from **2.64** (a bromo substituted triazole compound) by Sonogashira coupling with terminal alkyne (**3.46**) *via* our developed protocol with a yield of 60%.

Table 3.2: The synthesis of conjugated alkynes *via* the developed protocol.

Entry ^[a]	Aryl halides ^[b]	Alkyne ^[c]	Products ^[d]	Yield (%) ^[e]	Alkyne dimer (%)
1	3.37	3.45	3.47	86 (60%, 12 hrs.) ^{13a-b}	---
		3.46	3.48	86	3
2	3.38	3.45	3.49	50	---
3	3.39	3.45	3.50	91	---
		3.46	3.51	86	6
4^[f]	3.40	3.46	3.52	61	6
5	3.41	3.45	3.54	72	---
		3.46	3.55	71	2
6	3.42	3.45	3.56	65	---
		3.46	3.57	86	---
7^[g]	3.43	3.45	3.58	72	---
		3.46	3.59	68 (50%, 24 hrs.) ^{13b}	1
8	3.44	3.45	3.60	97	---
		3.46	3.61	92 (63%, 12 hrs.) ^{13c}	---
9	2.64	3.46	3.62	60	6

^[a] The reactions were performed in DMSO (for entry **1**), under MW condition (for entry **2**), in DMF (for entry **3-6**), in 1:1:1 toluene:THF:water (for entry **7**; conversion was 65%), and in DMF (for entry **8**). ^[b-c] See **Figure 3.1** and ^[d] See **Figure 3.2**. ^[e] Yields are of isolated pure compounds; in parentheses: yields of reported compounds using the original Sonogashira condition. ^[f] Along with **3.52**, a biscoupled product **3.53** was obtained in 25% yield. ^[g] **3.58** and **3.59** are monocoupled products.

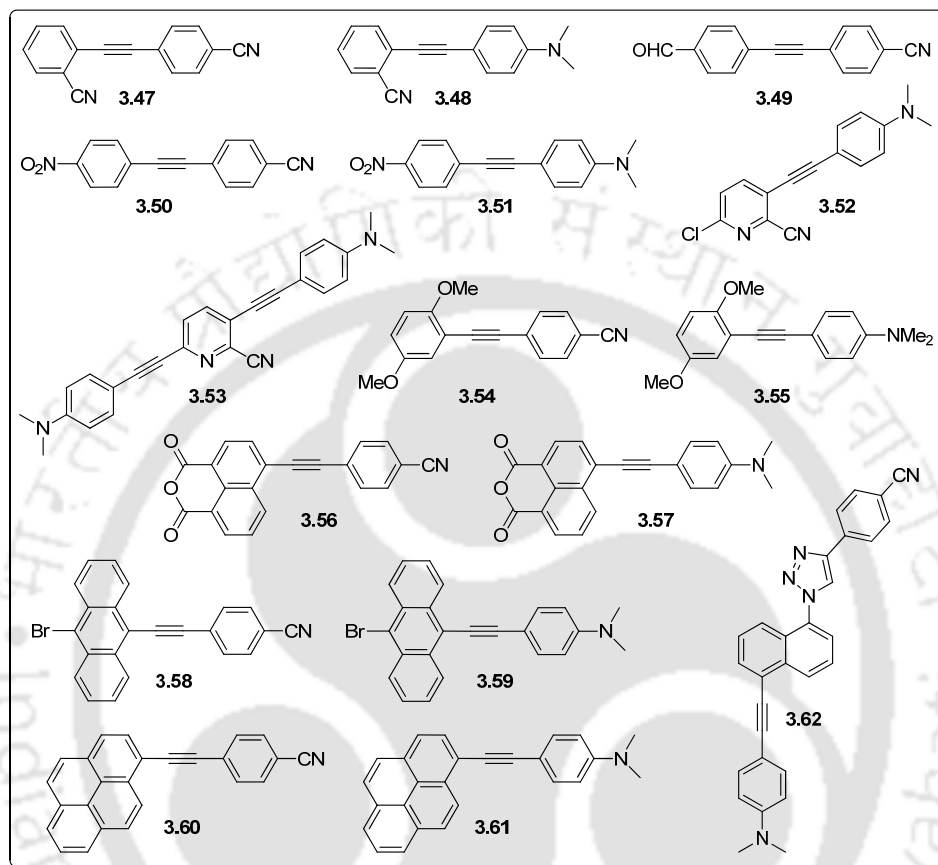


Figure 3.2: The structures of the compounds synthesized *via* our developed protocol.

3.6.3. Versatility of Our Method: Comparison between Our Method and Original Sonogashira Coupling

In comparison to the CuI co-catalyzed classical Sonogashira reaction, the yields of the cross coupled desired products were found to have tremendously increased at the expense of the quenched incidence of homocoupling *via* our protocol. We have tested our methodology in DMSO and also under microwave irradiation (3.5 minutes, 200 W) in both DMF and in DMSO. In both the cases, we observed no homocoupling side product, and the yields of the desired products were very good and comparable. From the examples listed in **Table 3.2**, it is clear that our methodology is highly efficient

with a variety of substrates, and the yields are very good to excellent, irrespective of the nature of the halides or alkynes that were utilized. Furthermore, in comparison to the reported low to moderate yields of compounds **3.47** and **3.61** (**Table 3.2**), which were synthesized *via* CuI co-catalyzed classical Sonogashira coupling,¹³ a tremendous increase in yields were observed *via* our protocol, which corroborates the success of our developed methodology.

3.6.4. Green Solvent/Air Compatibility of Our Developed Protocol

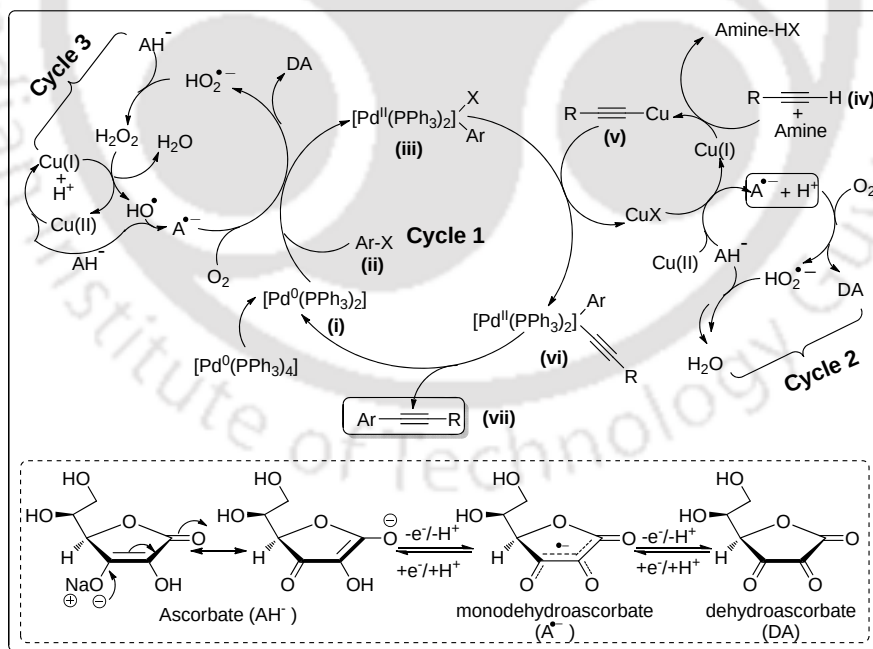
Next, we explored the capability of our methodology in the context of green solvents, such as water; however, because of the insolubility of the terminal alkynes or aryl halides in pure water, we performed the investigated reaction in 1:1:1 toluene:THF:water using aryl halide **3.43** (**Table 3.2**), in CH₃CN-water and DMF-water using aryl halide **3.37** as the starting halide (**Table 3.1**). Again, we found that the reaction proceeded smoothly with a high yield. These findings demonstrate that this methodology can be used in the context of water-soluble alkynes and aromatic halides. Therefore, the present click-reagent version of the cross-coupling protocol appears to be compatible with a several different kinds of substrates and green solvent systems, such as water.

As we used excess of sodium ascorbate to maintain an excellent reducing atmosphere in the reaction mixture to carry out the smooth reaction, we thought that it would be worthwhile to verify our methodology in open air without applying an inert atmosphere. Thus, we carried out a reaction of halide **3.34** with alkyne **3.45**, without degassing with N₂ gas in DMF at 80 °C and also separately under microwave irradiation. We obtained a very good yield of our desired product with no homocoupling. In these cases, all of the reactants and reagents were taken together; only a solution of 1 mole% of CuSO₄ in DMF was added at the end, and the reaction vessel was maintained in open atmosphere (**Table 3.1**). This observation proves the air-insensitivity of our protocol and thereby proves its versatility and practicality. Most importantly, in all cases throughout our investigation, the reducing agent,

sodium ascorbate, was observed not to adversely impact susceptible functional groups, such as $-\text{CHO}$, $-\text{NO}_2$, $-\text{CN}$, or anhydride.

3.6.5. Most Probable Reaction Mechanism

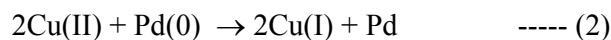
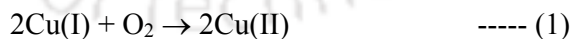
Scheme 3.19 depicted the most probable mechanism of our developed protocol. Thus, the reaction starts with the oxidative addition of aryl halide (**ii**) with $\text{Pd}(0)$ (**i**) to form $[\text{ArPd}^{\text{II}}(\text{PPh}_3)_2\text{X}]$ complex (**iii**). In the mean time ascorbate (AH^-) reduces $\text{Cu}(\text{II})$ to $\text{Cu}(\text{I})$ and become oxidized to monodehydroascorbate ($\text{A}^{\bullet-}$). The nascent $\text{Cu}(\text{I})$, thus generated, then reacts with terminal alkyne (**iv**) in the presence of amine base to form copper acetylide $[\text{R}-\text{C}\equiv\text{C}-\text{Cu}]$ complex (**v**). Then the trans-metallation occurs between $[\text{ArPd}^{\text{II}}(\text{PPh}_3)_2\text{X}]$ and $[\text{R}-\text{C}\equiv\text{C}-\text{Cu}]$ complex (**iii** and **v**, respectively) to form $[\text{ArPd}^{\text{II}}(\text{PPh}_3)_2\text{C}\equiv\text{C}-\text{R}]$ complex (**vi**) which in turn followed by reductive elimination forms the desired cross-coupled product $[\text{Ar}-\text{C}\equiv\text{C}-\text{R}]$ (**vii**) (**Scheme 3.19**, **Cycle 1**).



Scheme 3.19: The most probable reaction course of the developed protocol which show the roles of the click-reagents.

Our notion was that the air or oxygen (present in the reaction vessel) at the very inception of the reaction, may oxidize Pd(0) to Pd(II) leading to the decrease in amount of Pd(0) in the reaction as well as start the formation of homocoupled product catalyzed by the generated Pd(II). Here, Na-ascorbate has two roles- first, to generate nascent Cu(I) from Cu(II) and second, to remove the oxygen from the reaction medium. After reducing the Cu(II) to Cu(I), Na-ascorbate gets oxidized to monodehydroascorbate ($A^{\bullet-}$) which in turn reduces oxygen in presence of H^+ to $HO_2^{\bullet-}$ and again is oxidized to dehydroascorbate (DA). The $HO_2^{\bullet-}$ in presence of ascorbate (AH^-) forms H_2O_2 (**Scheme 3.19, Cycle 2**). H_2O_2 is then reduced to H_2O in presence of Cu(I) which is ultimately oxidized to Cu(II) (**Scheme 3.19, Cycle 3**). Again Cu(II) is reduced to Cu(I) by ascorbate and by this way the cycle is continued. Therefore, the **cycle 2** mainly controls the generation of nascent Cu(I) from Cu(II) as well as the reduction of any trace of O_2 to H_2O . The **cycle 3** is mainly responsible for reduction of O_2 in the vicinity of Pd(0), thereby restricting any damage of Pd(0) to Pd(II) at the start of the reaction.

The competing homocoupling is due to Pd(II) and/or Cu(II) can be explained as bellow. In competing scenario, Cu(I) may oxidize to give Cu(II) in presence of air or oxygen. Therefore, in analogy to Wacker oxidation, an intercepting Cu(I)/Cu(II) cycle fueled by oxygen may ultimately oxidize Pd(0) to Pd(II). Thus, Cu may well have a dual role in the alkyne homocoupling reaction, first being utilized to mediate alkynyl transfer to Pd(II) *via* transmetalation from a Cu-alkynyl species, and second in the redox sequence as is shown below.



Therefore, the reaction leads to a generation of unwanted homocoupled by-product in the Sonogashira coupling in the initial activation step.^{2b,4e-g,9d}

However, one may argue that Prof. Fairlamb in 2003¹⁴ has looked at internal homocoupling of alkynes and found that an oxidant is not required. This can easily be

resolved and clarified in the context of our findings by following ways. Fairlamb *et al.*,¹⁴ in 2003 have reported that in absence of aryl halide the terminal alkyne undergoes dimerization in absence of air or any oxidant *via* reductive elimination of dialkynylpalladium(II). However, that time they were investigating the mechanism. Later on, they have reported^{4f} that an oxidant is a must for the Pd-Cu catalyzed homocoupling of alkynes. In this report^{4f} of 2005 they have clearly written regarding their previous report¹⁴ – “However, although an added oxidant was not required such as iodine or chloroacetone, the question of whether air was promoting the homocoupling was unresolved.” In their report^{4f} of 2005 they showed conclusive evidence that stoichiometric quantities of air are required to get homocoupling under the previously reported¹⁴ conditions.

3.6.6. Spectral Characterization of Some Synthesized Alkynes

The ¹H NMR study of the compound **3.44** showed that the two methyl protons of -NMe₂ group appeared as singlet at 2.98 ppm. The two aromatic ortho hydrogens with respect to -NMe₂ group appeared as doublet at 6.63 ppm, and other two hydrogens of the same benzene ring appeared as doublet at 7.46 ppm. The two aromatic hydrogens, ortho and para to -CN group appeared as multiplet within the range of 7.48-7.55 ppm, and other two aromatic hydrogens, ortho and para to -C≡C- of the cyanophenyl unit appeared each as separate doublets at 7.60 and 7.30 ppm respectively. The IR spectra showed two distinct peaks for two triple bonds at 2204 cm⁻¹ (-C≡N) and 2173 cm⁻¹ (-C≡C-). Finally, the single crystal X-ray structure (ORTEP diagram is shown in **Figure 3.3**) corroborated the assigned structure.

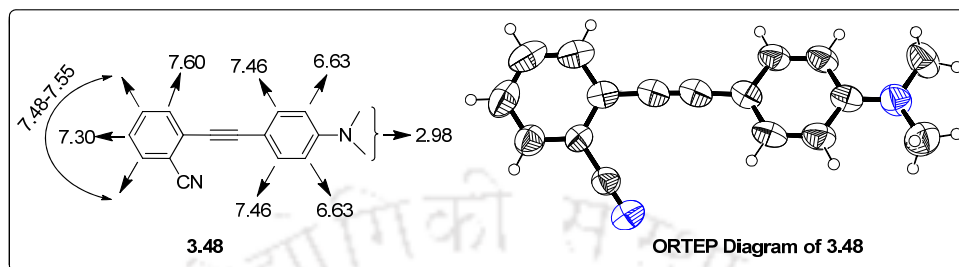


Figure 3.3: ^1H -NMR assignment and the ORTEP diagram with 50% thermal ellipsoid of **3.48**

Figure 3.4 showed the ^1H NMR characterization of compound **3.61**. Thus, the ^1H NMR spectrum of the compound **3.61** showed that the six protons of two methyl of $-\text{NMe}_2$ group appeared as singlet at 3.02 ppm. The two aromatic ortho hydrogens with respect to $-\text{NMe}_2$ group appeared as doublet at 6.76 ppm, and other two hydrogens of the same benzene ring resonated as doublet at 7.60 ppm. The aromatic hydrogens of pyrene ring near $-\text{C}\equiv\text{C}-$ appeared as doublet at 8.68 ppm and other hydrogens of pyrene ring resonated within the range of 7.98–8.20 ppm and appeared as multiplet. The IR spectra showed the peak corresponding to $-\text{C}\equiv\text{C}-$ at 2182 cm^{-1} . The X-ray single crystal structure (ORTEP diagram is shown in **Figure 3.4**) finally corroborated the assigned structure.

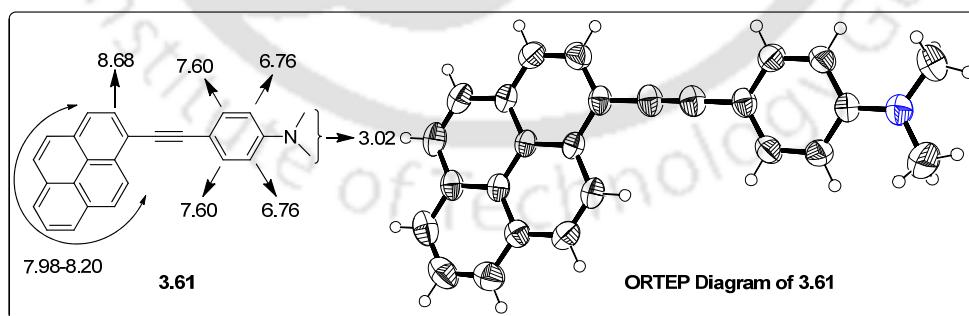


Figure 3.4: ^1H -NMR assignment and the ORTEP diagram with 50% thermal ellipsoid of **3.61**

3.6.7. Characterization by X-Ray Crystallography

X-ray intensity data were collected at 296 K with Mo K α radiation ($\lambda = 0.71073$ Å) using a Bruker Nonius SMART CCD diffractometer equipped with graphite monochromator and Apex CD camera. The SMART software was used for data collection and for indexing the reflections and determining the unit cell parameters; the collected data were integrated using SAINT software.¹⁵ The structures were solved by direct methods and refined by full-matrix least-squares calculations using SHELXTL software. All the non-H atoms were refined in the anisotropic approximation against F^2 of all reflections. The single crystals were collected from chloroform-ethylacetate mixture at room temperature for each case.

Table 3.3: Crystal data of the representative compounds.

Compounds	3.48	3.61
Data		
Z	4	4
Molecular Formula	C ₁₇ H ₁₄ N ₂	C ₂₆ H ₁₉ N
Temperature (K)	296	296
Space Group	Monoclinic, P 21/c	Monoclinic, P 21/c
a (Å)	8.9096(7)	8.5572(4)
b (Å)	7.9765(5)	7.3756(4)
c (Å)	20.3426(15)	29.6144(14)
α (degree)	90	90
β degree)	102.602(5)	99.560(3)
γ (degree)	90	90
M _r	246.30	345.42
V (Å ³)	1410.87(18)	1843.14(16)
μ (mm ⁻¹)	0.069	0.072
F(000)	520.0	728.0
R(reflections)	0.0535(1763)	0.0432(2740)
wR2(reflections)	0.1575(3498)	0.0791(4580)
Correction method	Multi Scan	Multi Scan

3.6.8. Study of Photophysical Properties

After establishing the click-reagent version of Sonogashira coupling protocol and synthesizing conjugated small organic molecules, we, next, turned our attention to explore the photophysical behavior of few fluorophores. Finally, we studied the photophysical properties of some of our synthesized fluorophores. Thus, in this preliminary study, the absorption spectra of the donor-acceptor acetylenes (for example, **3.51**, **3.53**, **3.56**, **3.57**, **3.60** and **3.62**) were characterized by an intense, broad, and low-energy transition, which derives from the intramolecular charge-transfer transition (ICT) that results from excitation from the donor to the acceptor group. The observed absorption band was solvatochromic. Furthermore, the molecules exhibited a structured emission in low polar solvents, such as hexane, dioxane etc.; however, as the solvent polarity was increased, intense, broad, and structureless emissions at longer wavelength regions were increasingly observed; i.e., the molecules exhibited a highly solvatochromic emission from the ICT state.

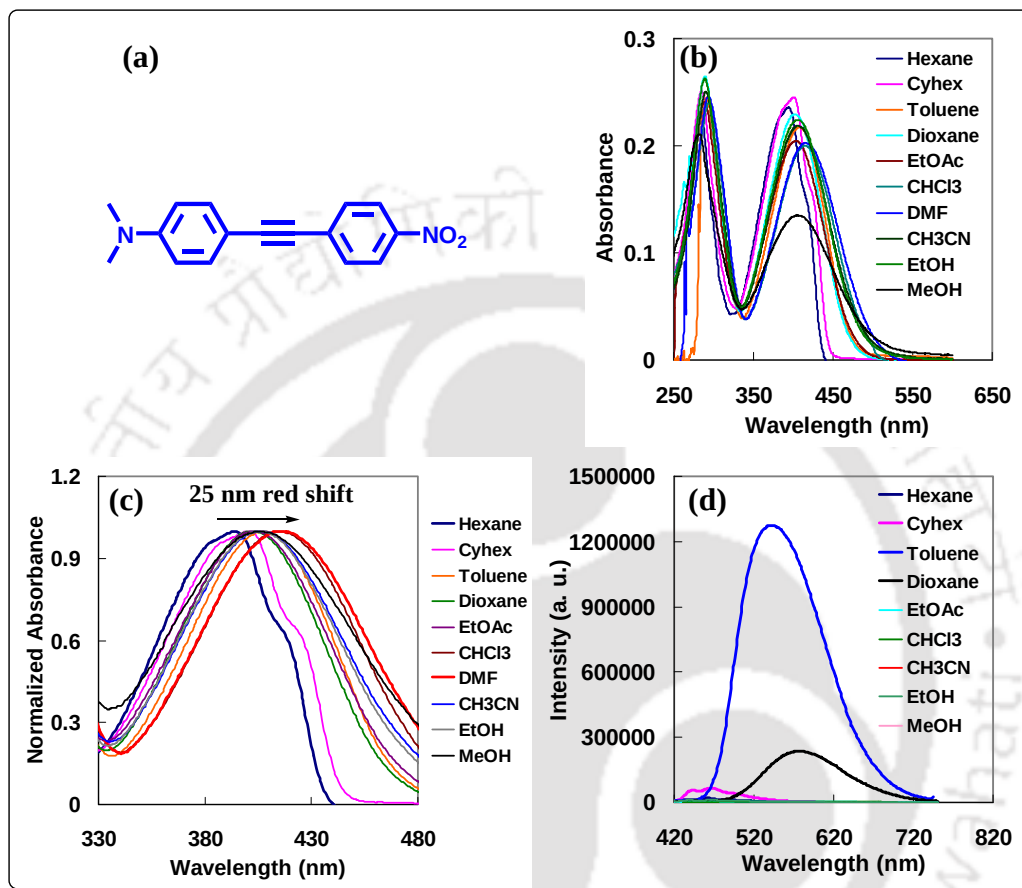


Figure 3.5: (a) The structure of fluorophore, (b) UV-visible, (c) normalized UV-visible and (d) fluorescence spectra of **3.51** in different solvents.

In particular, the UV-visible spectra of the fluorophore **3.51** showed a long wavelength absorption which is unambiguously assigned as charge transfer (CT) band. Thus, with increase in polarity a red shifted absorption maxima was observed. We found 25 nm red shift of absorption maxima (**Figure 3.5c**) when compared between nonpolar hexane and polar DMF. A study of fluorescence emission revealed that in non-polar solvents, such as toluene and dioxane the fluorophore showed a high intense emission band centered at 545 and 580 nm, respectively (**Figure 3.5d**). These emission bands originated from an ICT state and thus possess high solvatochromicity. Thus, in least polar solvents, like hexane and cyclohexane its emission maxima lie

below 500 nm, while as the solvent polarity increases up to dioxane, emission exhibited structureless long wavelength band. The intensity decreases as the polarity of the solvent increases and we did not observe any emission in solvents more polar than dioxane indicating a strong radiationless decay due to solvent-solute interaction.¹⁶

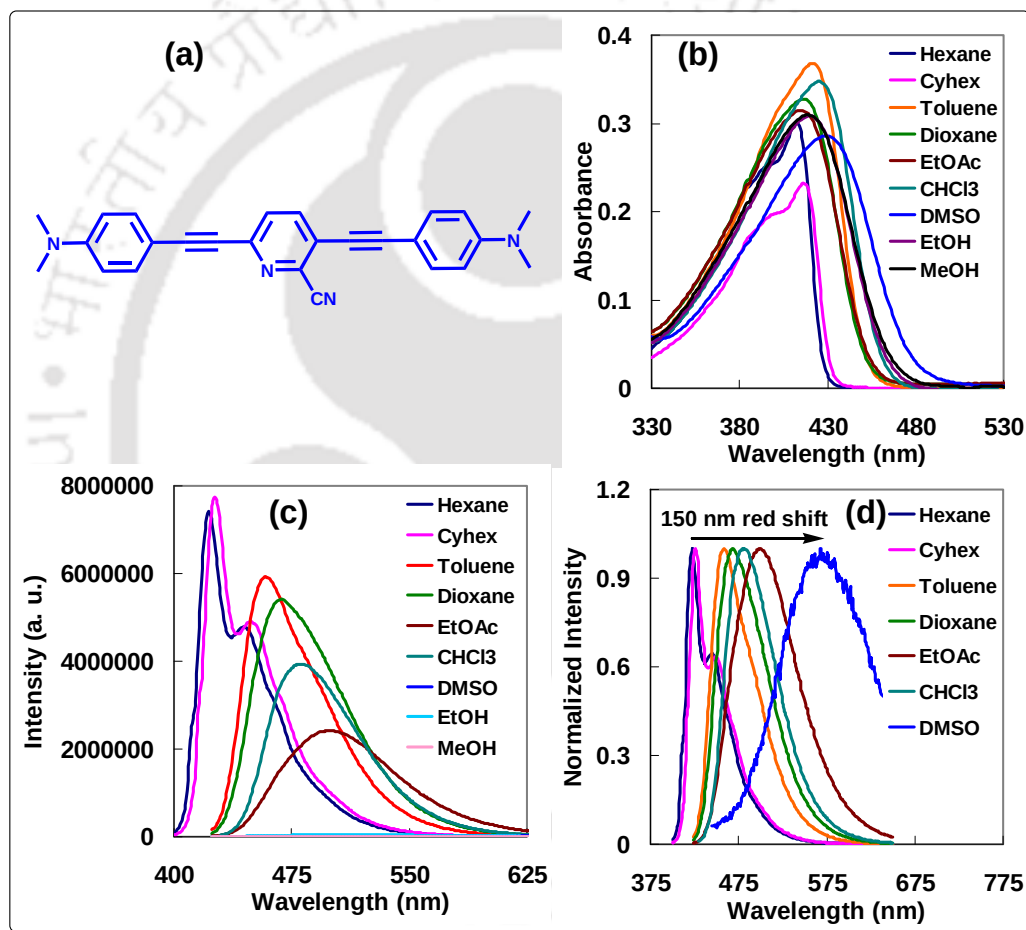


Figure 3.6: (a) The structure of fluorophore, (b) UV-visible (c) fluorescence and (d) normalized fluorescence spectra of **3.53** in different solvents.

For the case of fluorophore **3.53**, we observed 20 nm red shift of UV-visible absorption maximum (**Figure 3.6b**) when compared between nonpolar hexane to polar DMSO. The emission spectra (**Figure 3.6c**) of this fluorophore also showed 150

nm red shifted emission as the solvent polarity increased from hexane to DMSO with decreasing intensity. These observations clearly indicate that the fluorophore is highly solvatochromic and the excited state is stabilized by solvent polarity. However, the decrease in intensity of the fluorescence emission indicates a quenching of fluorescence due to dipolar interaction between the fluorophore and the solvent.

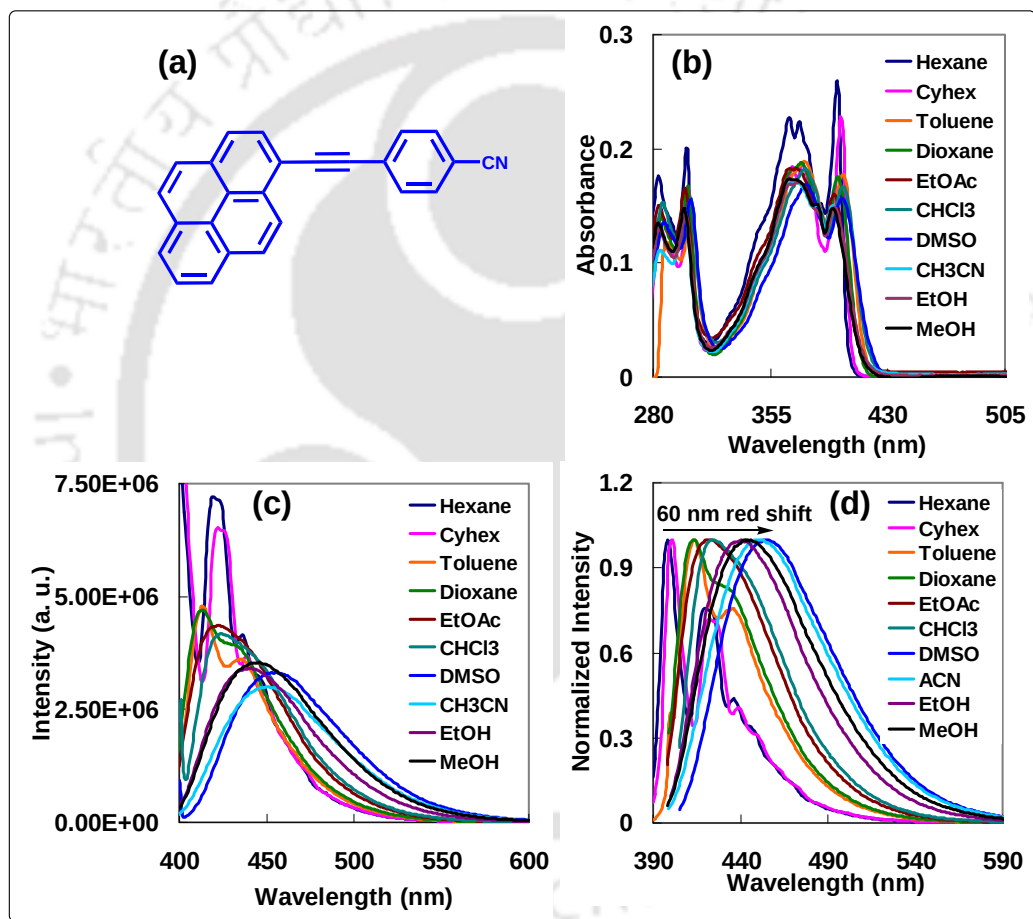


Figure 3.7: (a) The structure of fluorophore, (b) UV-visible, (c) fluorescence and (d) normalized fluorescence spectra of **3.60** in different solvents.

Next, we have studied the photophysical properties of another representative compound, **3.60** where pyrene is conjugated with benzonitrile *via* an acetylinic linker. Thus, in UV-visible spectra, no sharp change in absorption maxima was observed

with increase in solvent polarity (**Figure 3.7b**). However, the fluorescence spectra exhibited a structured emission in low polar solvents, such as hexane, dioxane *etc.* As the solvent polarity was increased, an intense, broad, and structureless emission at longer wavelength region was observed with decreasing intensity (**Figure 3.7c**). We found a 60 nm red shift in emission wavelength when compared between hexane and DMSO (**Figure 3.7c**). Thus, the above observation clearly indicates that the molecule exhibited solvatochromic emission.

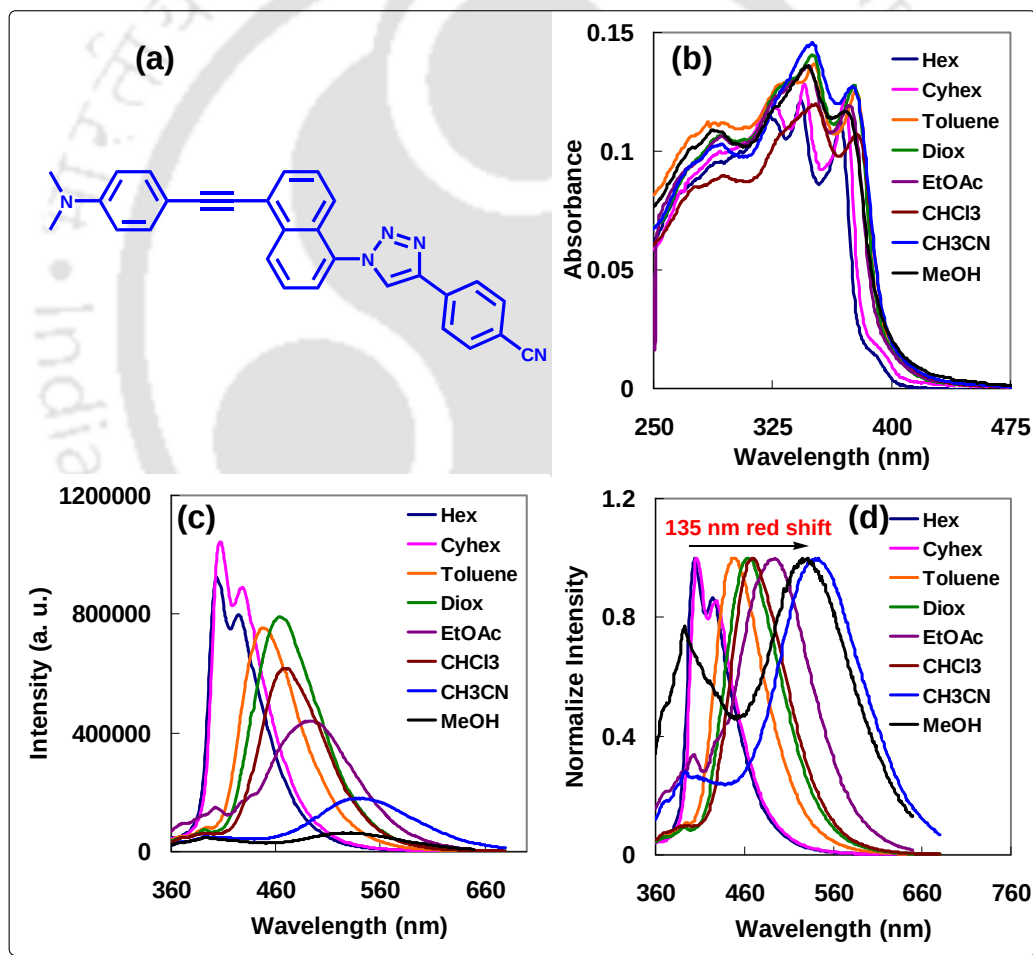


Figure 3.8: (a) The structure of fluorophore, (b) UV-visible, (c) fluorescence and (d) normalized fluorescence spectra of **3.62** in different solvents.

The fluorophore **3.62** was synthesized *via* our developed click-reagent version Sonogashira coupling protocol starting from our click chemistry generated fluorophore **2.54** (**Chapter 2**) to extend the conjugation and to get interesting photophysical properties. Thus, we next, turned our attention to study the photophysical property. An examination of photophysical properties revealed that the fluorophore showed very good and improved absorption/emission behaviour upon conjugation compared to its parent fluorophore (**2.64**) containing a bromine atom. From the UV-Visible spectra of the compound **3.62**, it is clear that the absorption was not much affected by the solvent polarity (**Figure 3.8b**). However, the emission behavior of the fluorophore was found to be highly affected by the solvent polarity. Thus, in low polar solvents a sharp, high intense structured band was observed; a broad, long wavelength emission with decreasing intensity was observed with increase in solvent polarity (**Figure 3.8c**). We found a 135 nm red shift in emission maxima when compared between hexane and acetonitrile (**Figure 3.8d**). These observations clearly indicate the high solvatochromic nature of the fluorophore and are of highly ICT character.

The study of the photophysical properties of some of the representative compounds synthesized *via* our developed protocols suggests that these fluorophores can be utilized in monitoring change in local microenvironment in biological world. Thus, our methodology can be utilized for the synthesis of π -conjugated fluorescent molecules of interesting photophysical properties.

3.7. Conclusion

In conclusion, we have developed a click-reagent version of Sonogashira coupling methodology that is high yielding, tolerant to a variety of substrates and functional groups, compatible with green solvents, and air-insensitive. We have exploited the known reducing behavior of sodium ascorbate to generate an active Cu(I) co-catalyst from CuSO₄, and we have maintained a constant reducing atmosphere to control and minimize undesired oxidative homocoupling, thereby tremendously increasing the incidence of desired cross-coupling. This is the first example of the use of click-reagents in Sonogashira coupling that yield no or drastically diminished amounts of undesired homocoupled products. Our developed protocol is also air-insensitive and effective under microwave conditions. Thus, we hope this methodology will find widespread applications in chemistry, material science, and chemical biology for the synthesis of desirable conjugated fluorescent alkynes.

Our synthesized fluorophores are highly solvatochromic as is revealed from the preliminary photophysical studies. These fluorophore therefore, may find applications in chemistry, biology, or material sciences.

3.8. Experimental Section

3.8.1. General Experimental

Organic extracts were dried over anhydrous sodium sulphate. Solvents were removed in a rotary evaporator under reduced pressure. Silica gel (60-120 mesh size) was used for the column chromatography. Reactions were monitored by TLC on silica gel 60 F254 (0.25mm). ^1H NMR spectra were measured with Varian 400 (400 MHz) and ^{13}C NMR spectra were measured with Varian 400 (100 MHz) spectrometer. Coupling constants (J value) were reported in hertz. The chemical shifts were shown in ppm downfield from tetramethylsilane, using residual chloroform ($\delta = 7.24$ in ^1H NMR, $\delta = 77.23$ in ^{13}C NMR), dimethyl sulfoxide ($\delta = 2.48$ in ^1H NMR, $\delta = 39.5$ in ^{13}C NMR), as an internal standard. Mass spectra were recorded using WATERS MS system, Q-tof premier and data analyzed using Mass Lynx 4.1. Melting points were recorded on Buchi B-545 melting point apparatus and are uncorrected. IR spectra were recorded in KBr on a Perkin Elmer Spectrum one FT-IR spectrometer.

3.8.2. Crystallographic Description

X-ray intensity data were collected at 296 K with Mo $K\alpha$ radiation ($\lambda = 0.71073$ Å) using a Bruker Nonius SMART CCD diffractometer equipped with graphite monochromator and Apex CD camera. The SMART software was used for data collection and for indexing the reflections and determining the unit cell parameters; the collected data were integrated using SAINT software.¹⁵ The structures were solved by direct methods and refined by full-matrix least-squares calculations using SHELXTL software. All the non-H atoms were refined in the anisotropic approximation against F^2 of all reflections. The single crystals were collected from chloroform-ethylacetate mixture at room temperature for all cases.

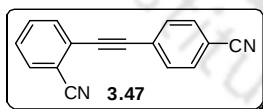
3.8.3. General Procedure for Click-Reagent Version of Sonogashira Coupling

To a solution of aryl halide in 1: 1 DMF- Et_3N under N_2 atmosphere, $\text{Pd}(\text{PPh}_3)_4$ followed by terminal alkyne was added. After 5 minutes of stirring under N_2

atmosphere, 6 mole% of a solution of sodium ascorbate in DMF was added followed by addition of 1 mole% of a solution of CuSO₄ in DMF. The reaction mixture was stirred for 4 hrs at 80 °C. After completion of the reaction monitored by TLC, the reaction mixture was extracted with ethyl acetate, washed with ammonium chloride, brine and dried over anhydrous Na₂SO₄. The products were isolated pure by column chromatography and characterized by IR, NMR, and mass spectrometry. All the products were isolated with very good to excellent yield ranging from 70-97% in DMF at 80 °C within 4 hrs only. The synthesis and the characterization data are described below. The compounds **3.47**,¹ **3.59**,² and **3.61**³ are known compounds, and their spectral data are in good agreement with those reported in literature. Melting point of **3.47** is not reported and the same are in accord with the literature values for the case of compound **3.59** and **3.61**. Full characterization data of unknown as well as of known compounds are also reported as was observed from our analysis.

3.8.4. Synthesis and Characterization

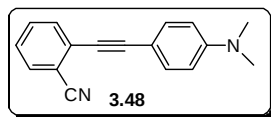
3.8.4.1. Synthesis of 2-(2-(4-(cyano)phenyl)ethynyl)benzonitrile^{13a-b} (3.47): Using the general procedure, starting from 0.05 g (0.275 mmol) of 2-bromobenzonitrile **3.37** and 0.042 g (0.330 mmol) of 4-ethynylbenzonitrile **3.45**, 0.054 g (0.237 mmol) of the title compound was isolated as a white solid. 86% yield; m.p. 133-135°C; IR (KBr):



3065, 2231, 1605, 1504, 1479, 840, 768 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 7.46 (1H, t, J = 7.2 Hz), 7.59 (1H, t, J = 7.6 Hz), 7.63–7.70 (6H, m); ¹³C NMR (CDCl₃; 100 MHz) δ 89.6, 93.9, 112.7, 115.9, 117.5, 118.5, 126.3, 127.0, 129.4, 132.3, 132.5, 132.6, 132.7, 133.0; ESI-TOF-MS m/z 229 [M+H]⁺; HRMS calcd. for C₁₆H₉N₂ ([M+H]⁺) 229.0765, found 229.0760.

3.8.4.2. Synthesis of 2-(2-(4-(*N,N*-dimethylamino)phenyl)ethynyl)benzonitrile (3.48): Using the general procedure, starting from 0.06 g (0.3296 mmol) of 2-bromobenzonitrile **3.37** and 0.057 g (0.3955 mmol) of 4-ethynyl-*N,N*-

dimethylbenzenamine **3.46**, 0.070 g (0.2845 mmol) of the title compound was isolated



as a lime solid. 86% yield; m.p. 125-126°C; IR (KBr):

3060, 2902, 2817, 2204, 2173, 1603, 1590, 1520, 1365,

1143, 815, 755 cm^{-1} ; ^1H NMR (CDCl_3 ; 400 MHz) δ 2.98

(6H, s), 6.63 (2H, d, $J = 8.8$ Hz), 7.30 (1H, ddd, $J = 1.2, 7.6$ Hz), 7.46 (2H, d, $J = 9.2$

Hz), 7.49–7.55 (2H, m), 7.60 (1H, dd, $J = 0.4, 8.0$ Hz); ^{13}C NMR (CDCl_3 ; 100 MHz)

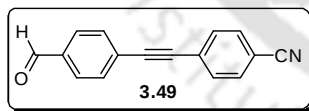
δ 40.2, 84.5, 98.3, 108.6, 111.8, 114.7, 118.1, 127.3, 128.4, 131.7, 132.4, 132.7,

133.4, 133.8, 150.8; ESI-TOF-MS m/z 247 $[\text{M}+\text{H}]^+$; HRMS calcd. for $\text{C}_{17}\text{H}_{15}\text{N}_2$

($[\text{M}+\text{H}]^+$) 247.1235, found 247.1230.

Crystallographic description of 3.48: Crystal dimension (mm): 0.42 x 0.35 x 0.28. $\text{C}_{17}\text{H}_{15}\text{N}_2$, Mr = 246.30; Monoclinic, space group P2(1)/c; $a = 8.9096(7)$ Å, $b = 7.9765(5)$ Å, $c = 20.3426(15)$ Å; $\alpha = 90.00^\circ$, $\beta = 102.602(5)^\circ$, $\gamma = 90.00^\circ$, $V = 1410.87(18)$ Å³; $Z = 4$; $\rho_{\text{cal}} = 1.160$ Mg/m³; $\mu(\text{mm}^{-1}) = 0.069$; $F(000) = 520$; Refinement method = Full-matrix least-squares on F^2 ; Final R indices $[I > 2\sigma_I]$ R1 = 0.0535, wR2 = 0.1325, R indices (all data) R1 = 0.1136, wR2 = 0.1575; goodness of fit = 1.026.

3.8.4.3. Synthesis of 4-(2-(4-formylphenyl)ethynyl)benzonitrile (3.49):



solution of 4-bromobenzaldehyde **3.38** (0.07 g, 0.3783

mmol) in 1: 1 DMF- Et_3N under N_2 atmosphere,

$\text{Pd}(\text{PPh}_3)_4$ followed by 4-ethynylbenzonitrile **3.45** (0.058

g, 0.4566 mmol) was added. After 2 minutes of stirring under N_2 atmosphere, 6

mole% of a solution of sodium ascorbate in DMF was added followed by addition of

1 mole% of a solution of CuSO_4 in DMF. Then, the reaction mixture was kept under

microwave radiation (200 Watt, 5 minutes, 80 °C). The completion of the reaction

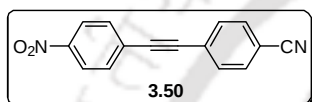
was monitored by TLC. The pure product (0.044 g, 0.1889 mmol) was isolated by

column chromatography. 50% yield; ^1H NMR (CDCl_3 ; 400 MHz) δ 7.52 (2H, d, $J =$

8.4 Hz), 7.63 (2H, d, $J = 6.0$ Hz), 7.68 (2H, d, $J = 8.4$ Hz), 7.88 (2H, d, $J = 8.4$ Hz),

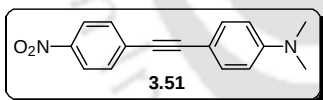
10.02 (1H, s); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 90.7, 91.9, 112.3, 118.2, 127.0, 129.7, 131.0, 132.0, 132.4, 132.7, 135.1, 191.0; ESI-TOF-MS m/z 232 $[\text{M}+\text{H}]^+$.

3.8.4.4. Synthesis of 4-(2-(4-nitrophenyl)ethynyl)benzonitrile (3.50): Using the general procedure, starting from 0.070 g (0.3465 mmol) of 1-bromo-4-nitrobenzene **3.39** and 0.053 g (0.4173 mmol) of 4-ethynylbenzonitrile **3.45**, 0.078 g (0.3145 mmol) of the title compound was isolated as a lime solid. 91% yield; m.p. 212-214°C;



IR (KBr): 3075, 2226, 1604, 1592, 1511, 1339, 836 cm^{-1} ; ^1H NMR (CDCl_3 ; 400 MHz) δ 7.61–7.68 (6H, m), 8.23 (2H, d, J = 8.8 Hz); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 91.4, 92.5, 112.7, 118.4, 123.9, 127.1, 129.2, 132.4, 132.5, 132.7, 147.7; ESI-TOF-MS m/z 249 $[\text{M}+\text{H}]^+$; HRMS calcd. for $\text{C}_{15}\text{H}_9\text{N}_2\text{O}_2$ ($[\text{M}+\text{H}]^+$) 249.0664, found 249.0667.

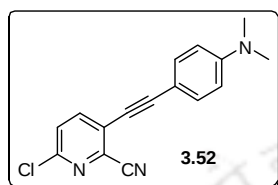
3.8.4.5. Synthesis of *N,N*-dimethyl-4-(2-(4-nitrophenyl)ethynyl)benzenamine (3.51): Using the general procedure, starting from 0.100 g (0.4950 mmol) of 1-bromo-4-nitrobenzene **3.39** and 0.0790 g (0.5448 mmol) of 4-ethynyl-*N,N*-



dimethylbenzenamine **3.46**, 0.1135 g (0.4267 mmol) of the title compound was isolated as a red solid. 86% yield; m.p. 209-212°C; IR (KBr): 3095, 2909, 2208, 1609, 1586, 1529, 1508, 1371, 1336, 1104, 820 cm^{-1} ; ^1H NMR (CDCl_3 ; 400 MHz) δ 3.00 (6H, s), 6.65 (2H, d, J = 8.8 Hz), 7.41 (2H, d, J = 8.4 Hz), 7.57 (2H, d, J = 9.2 Hz), 8.16 (2H, d, J = 8.4 Hz); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 40.3, 86.6, 97.3, 111.9, 123.8, 128.7, 128.8, 131.8, 132.1, 132.2, 132.3, 133.4, 133.8, 146.4, 150.9; ESI-TOF-MS m/z 267 $[\text{M}+\text{H}]^+$; HRMS calcd. for $\text{C}_{16}\text{H}_{15}\text{N}_2\text{O}_2$ ($[\text{M}+\text{H}]^+$) 267.1134 found 267.1130.

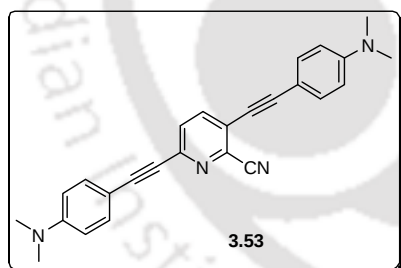
3.8.4.6. Synthesis of 6-chloro-3-(2-(4-(*N,N*-dimethylamino)phenyl)ethynyl)picolinonitrile (3.52): Using the general procedure, starting from 0.05 g (0.2299 mmol) of 3-bromo-6-chloropicolinonitrile **3.40** and 0.04

g (0.2758 mmol) of 4-ethynyl-*N,N*-dimethylbenzenamine **3.46**, 0.0393 g (0.1396 mmol) of the title compound was isolated as a yellow solid. 61% yield; m.p. 142-



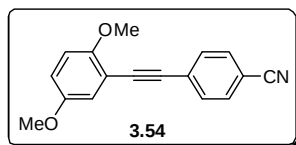
145°C; IR (KBr): 2923, 2853, 2193, 1606, 1560, 1527, 1369, 1156, 1023, 808 cm^{-1} ; ^1H NMR (CDCl_3 ; 400 MHz) δ 3.01 (6H, s), 6.64 (2H, d, $J = 8.8$ Hz), 7.42–7.47 (3H, m), 7.77 (1H, d, $J = 8.8$ Hz); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 40.2, 81.3, 103.3, 107.4, 111.8, 115.5, 125.6, 127.6, 130.4, 133.7, 140.9, 149.4, 151.3; ESI-TOF-MS m/z 282 $[\text{M}+\text{H}]^+$; HRMS calcd. for $\text{C}_{16}\text{H}_{13}\text{N}_3\text{Cl}$ ($[\text{M}+\text{H}]^+$) 282.0798, found 282.0789.

From the same reaction mixture we were able to isolate 0.0224 g (0.0574 mmol) of dihalide (Br and Cl) substituted compound (**3,6-bis(2-(4-(*N,N*-dimethylamino)phenyl)ethynyl)picolinonitrile, 3.53**) as a yellow solid. 25% yield; m.p. 210-212°C; IR (KBr): 2922, 2852, 2201, 1604, 1530, 1447, 1365, 1157, 816 cm^{-1} ; ^1H NMR (CDCl_3 ; 400 MHz) δ 2.99 (12H, d, $J = 1.6$ Hz), 6.63 (4H, d, $J = 8.8$ Hz),



7.45 (2H, d, $J = 3.2$ Hz), 7.47 (2H, d, $J = 3.2$ Hz), 7.50 (1H, d, $J = 8.4$ Hz), 7.73 (1H, d, $J = 8.4$ Hz); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 40.3, 82.7, 86.7, 95.5, 102.8, 107.9, 108.0, 109.9, 111.9, 116.2, 124.1, 128.8, 133.7, 133.8, 135.5, 138.4, 142.9, 151.1, 151.2; ESI-TOF-MS m/z 391 $[\text{M}+\text{H}]^+$; HRMS calcd. for $\text{C}_{26}\text{H}_{23}\text{N}_4$ ($[\text{M}+\text{H}]^+$) 391.1922, found 391.1930.

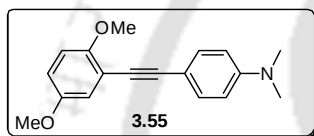
3.8.4.7. Synthesis of 4-(2-(2,5-dimethoxyphenyl)ethynyl)benzonitrile (3.54): Using the general procedure, starting from 0.030 g (0.1382 mmol) of 2-bromo-1,4-dimethoxybenzene **3.41** and 0.021 g (0.1653 mmol) of 4-ethynylbenzonitrile **3.45**, 0.026 g (0.0988 mmol) of the title compound was isolated as a white semisolid. 72% yield; IR (KBr): 2924, 2853, 2227, 1602, 1494, 1458, 1261, 1021, 840, 805, 699 cm^{-1} ; ^1H NMR (CDCl_3 ; 400 MHz) δ 3.77 (3H, s), 3.86 (3H, s), 6.84 (1H, s), 6.88 (1H, d, J



= 2.8 Hz), 7.01 (1H, d, J = 3.2 Hz), 7.60 (4H, m); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 56.0, 56.6, 90.5, 91.8, 111.5, 112.0, 112.3, 116.9, 118.4, 118.8, 128.6, 132.2, 132.3, 133.2, 153.5, 154.9; ESI-TOF-MS m/z 264 $[\text{M}+\text{H}]^+$;

HRMS calcd. for $\text{C}_{17}\text{H}_{14}\text{NO}_2$ ($[\text{M}+\text{H}]^+$) 264.1024, found 264.1030.

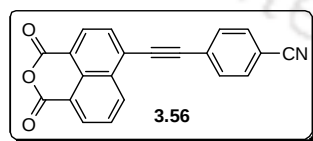
3.8.4.8. Synthesis of 4-(2-(2,5-dimethoxyphenyl)ethynyl)-*N,N*-dimethylbenzenamine (3.55): Using the general procedure, starting from 0.050 g (0.2304 mmol) of 2-bromo-1,4-dimethoxybenzene **3.41** and 0.0367 g (0.2534 mmol)



of 4-ethynyl-*N,N*-dimethylbenzenamine **3.46**, 0.046 g (0.1637 mmol) of the title compound was isolated as a lime semisolid. 71% yield; IR (KBr): 2924, 2853, 2830,

2207, 1603, 1521, 1499, 1356, 1225, 1197, 1042, 821, 712 cm^{-1} ; ^1H NMR (CDCl_3 ; 400 MHz) δ 2.97 (6H, s), 3.76 (3H, s), 3.85 (3H, s), 6.66 (2H, d, J = 7.6 Hz), 6.79 (2H, d, J = 1.6 Hz), 7.01 (1H, d, J = 3.6 Hz), 7.42 (2H, d, J = 8.8 Hz); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 41.4, 55.9, 56.7, 83.7, 94.9, 110.5, 111.9, 112.4, 114.2, 115.1, 118.1, 124.5, 124.6, 133.0, 150.3, 153.5, 154.4; ESI-TOF-MS m/z 282 $[\text{M}+\text{H}]^+$; HRMS calcd. for $\text{C}_{18}\text{H}_{20}\text{NO}_2$ ($[\text{M}+\text{H}]^+$) 282.1494, found 282.1488.

3.8.4.9. Synthesis of 4-(2-(4-(cyano)phenyl)ethynyl)-1,8-naphthalic anhydride (3.56): Using the general procedure, starting from 0.08 g (0.2888 mmol) of 4-bromo-

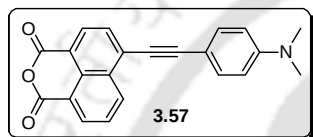


1,8-naphthalic anhydride **3.42** and 0.0404 g (0.3176 mmol) of 4-ethynylbenzonitrile **3.45**, 0.061 g (0.1888 mmol) of the title compound was isolated pure by

filtration followed by washing with water and ethanol as a yellow solid. 65% yield; m.p. $>312^\circ\text{C}$ (it became black); IR (KBr): 2223, 1778, 1758, 1601, 1588, 1043, 832 cm^{-1} ; ^1H NMR (CDCl_3 ; 400 MHz) δ 7.46–7.52 (4H, m), 7.67 (1H, t, J = 7.6 Hz), 7.77 (1H, d, J = 7.6 Hz), 8.30 (1H, d, J = 8.0 Hz), 8.38 (1H, d, J = 6.8 Hz), 8.51 (1H, d, J =

8.0 Hz); ESI-TOF-MS m/z 324 $[M+H]^+$; HRMS calcd. for $C_{21}H_{10}NO_3$ ($[M+H]^+$) 324.0660, found 324.0653.

3.8.4.10. Synthesis of 4-(2-(4-(*N,N*-dimethylamino)phenyl)ethynyl)-1,8-naphthalic anhydride (3.57): Using the general procedure, starting from 0.06 g (0.2166 mmol) of 4-bromo-1,8-naphthalic anhydride **3.42** and 0.0345 g (0.2382 mmol) of 4-ethynyl-*N,N*-dimethylbenzenamine **3.46**, 0.0635 g (0.1862 mmol) of the title compound was



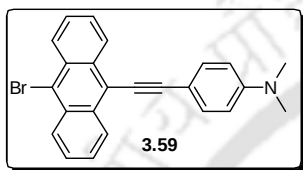
isolated as a red solid. 86% yield; m.p. 288–292°C; IR (KBr): 2917, 2862, 2188, 1752, 1730, 1604, 1585, 1575, 1524, 1371, 1169, 1010, 781 cm^{-1} ; 1H NMR ($CDCl_3$; 400 MHz) δ 3.06 (6H, s), 6.82 (2H, bs), 7.55 (2H, d, $J = 7.6$ Hz), 7.84–7.92 (2H, m), 8.53 (1H, d, $J = 7.6$ Hz), 8.64 (1H, d, $J = 7.2$ Hz), 8.81 (1H, d, $J = 8.0$ Hz); ^{13}C NMR ($CDCl_3$; 100 MHz) δ 40.1, 85.2, 103.8, 107.9, 110.6, 111.7, 112.1, 112.8, 118.9, 125.3, 127.5, 129.5, 130.5, 132.8, 133.5, 134.2, 135.0, 153.4, 160.4, 160.8; ESI-TOF-MS m/z 342 $[M+H]^+$; HRMS calcd. for $C_{22}H_{16}NO_3$ ($[M+H]^+$) 342.1130, found 342.1125.

3.8.4.11. Synthesis of 4-(2-(9-bromoanthracen-10-yl)ethynyl)benzonitrile (3.58): Using the general procedure, starting from 0.070 g (0.2083 mmol) of 9,10-dibromoanthracene **3.43** and 0.0265 g (0.2083 mmol) of 4-ethynylbenzonitrile **3.45**,



0.040 g (0.1190 mmol) 9,10-dibromoanthracene was isolated as unreacted and 0.0245 g (0.0641 mmol) of the title compound was isolated as a yellow solid. 72% yield; m.p. 214–215°C; IR (KBr): 2222, 2198, 1620, 1602, 1502, 837, 752 cm^{-1} ; 1H NMR ($CDCl_3$; 400 MHz) δ 7.62–7.64 (4H, m), 7.70 (2H, dd, $J = 1.6, 8.2$ Hz), 7.80 (2H, dd, $J = 1.2, 8.0$ Hz), 8.55–8.60 (4H, m); ^{13}C NMR ($CDCl_3$; 100 MHz) δ 90.6, 100.0, 112.0, 118.7, 127.0, 127.5, 127.8, 128.4, 128.7, 129.1, 130.5, 132.2, 132.5, 133.4, 142.5; ESI-TOF-MS m/z 383, 385 $[M+H]^+$; HRMS calcd. for $C_{23}H_{12}N$ ($[M-Br]$) 302.0970, found 302.0960.

3.8.4.12. Synthesis of 4-(2-(9-bromoanthracen-10-yl)ethynyl)-*N,N*-dimethylbenzenamine^{13c} (3.59): Using the general procedure, starting from 0.060 g (0.1785 mmol) of 9,10-dibromoanthracene **3.43** and 0.0258 g (0.1785 mmol) of 4-ethynyl-*N,N*-dimethylbenzenamine **3.46**, 0.016 g (0.0476 mmol) 9,10-dibromoanthracene was isolated as unreacted and 0.0305 g (0.0763 mmol) of the title



compound was isolated as a yellow solid. 68% yield; m.p. 234-236°C (reported 236 °C)^{13c}; IR (KBr): 2924, 2854, 2192, 1634, 1605, 1520, 1348, 1188, 811, 752 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 3.03 (6H, s), 6.73 (2H, d, J = 8.4 Hz), 7.56–7.64 (6H, m), 8.53 (2H, d, J = 8.0 Hz), 8.70 (2H, d, J = 8.8 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 40.5, 84.5, 96.2, 103.8, 112.3, 123.0, 126.6, 127.3, 127.6, 127.8, 128.3, 130.6, 132.9, 133.1, 152.8; ESI-TOF-MS m/z 401, 403 [M+H]⁺; HRMS calcd. for C₂₄H₁₉BrN ([M+H]⁺) 400.0701, found 400.0697.

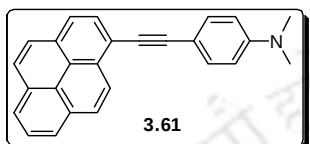
3.8.4.13. Synthesis of 4-(2-(pyren-1-yl)ethynyl)benzonitrile (3.60): Using the general procedure, starting from 0.055 g (0.1957 mmol) of 1-bromopyrene **3.44** and 0.0273 g (0.2152 mmol) of 4-ethynylbenzonitrile **3.45**, 0.062 g (0.1896 mmol) of the



title compound was isolated as a yellow solid. 97% yield; m.p. 201-203°C; IR (KBr): 3037, 2229, 2202, 1604, 1512, 1497, 854, 825, 718, cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 7.65 (2H, d, J = 8.0 Hz), 7.72 (2H, d, J = 8.4 Hz), 8.01–8.04 (2H, m), 8.09-8.12 (2H, m), 8.16–8.24 (4H, m), 8.56 (1H, d, J = 9.2 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 93.4, 93.6, 111.6, 116.7, 118.8, 124.4, 124.7, 124.8, 125.4, 126.2, 126.6, 127.4, 128.6, 128.9, 130.0, 131.2, 131.4, 132.2, 132.3; ESI-TOF-MS m/z 328 [M+H]⁺; HRMS calcd. for C₂₅H₁₄N ([M+H]⁺) 328.1126, found 328.1130.

3.8.4.14. Synthesis of *N,N*-dimethyl-4-(2-(pyren-1-yl)ethynyl)benzenamine^{13d} (3.61): Using the general procedure, starting from 0.055 g (0.1957 mmol) of 1-bromopyrene **3.44** and 0.0312 g (0.2152 mmol) of 4-ethynyl-*N,N*-

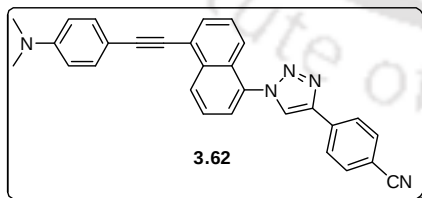
dimethylbenzenamine **3.46**, 0.062 g (0.1797 mmol) of the title compound was isolated as a bright yellow solid. 92% yield; m.p. 168-169 °C (reported 169 °C)^{9d}; IR (KBr): 3035, 2922, 2853, 2196, 2182, 1606, 1594, 1520, 1355, 1152, 845, 819, 711 cm⁻¹; ¹H



NMR (CDCl₃; 400 MHz) δ 3.02 (6H, s), 6.76 (2H, d, J = 7.6 Hz), 7.60 (2H, d, J = 8.8 Hz), 7.98–8.10 (4H, m), 8.14–8.20 (4H, m), 8.68 (1H, d, J = 8.8 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 40.6, 87.0, 96.8, 112.4, 119.1, 124.7, 124.8, 125.4, 125.5, 126.0, 126.3, 127.5, 127.9, 128.2, 129.5, 130.9, 131.4, 131.6, 131.7, 133.0, 150.3; ESI-TOF-MS m/z 346 [M+H]⁺; HRMS calcd. for C₂₆H₂₀N ([M+H]⁺) 346.1595, found 346.1589.

Crystallographic description of 3.61: Crystal dimension (mm): 0.30 x 0.25 x 0.20. C₂₆H₁₉N, Mr = 345.42; Monoclinic, space group P2(1)/c; a = 8.5572(4) Å, b = 7.3756(4) Å, c = 29.6144(14) Å; α = 90.00°, β = 99.560(3)°, γ = 90.00°, V = 1843.14(16) Å³; Z = 4; ρ_{cal} = 1.245 Mg/m³; μ (mm⁻¹) = 0.072; $F(000)$ = 728; Refinement method = Full-matrix least-squares on F^2 ; Final R indices [$I > 2\sigma_I$] R1 = 0.0432, wR2 = 0.0735, R indices (all data) R1 = 0.1205, wR2 = 0.0791; goodness of fit = 1.061.

3.8.4.15. Synthesis of 4-(1-(5-((4-(dimethylamino)phenyl)ethynyl)naphthalen-1-yl)-1H-1,2,3-triazol-4-yl)benzonitrile (3.62): Using the general procedure, starting from 0.04 g (0.1069 mmol) of 4-(1-(5-bromonaphthalen-1-yl)-1H-1,2,3-triazol-4-yl)



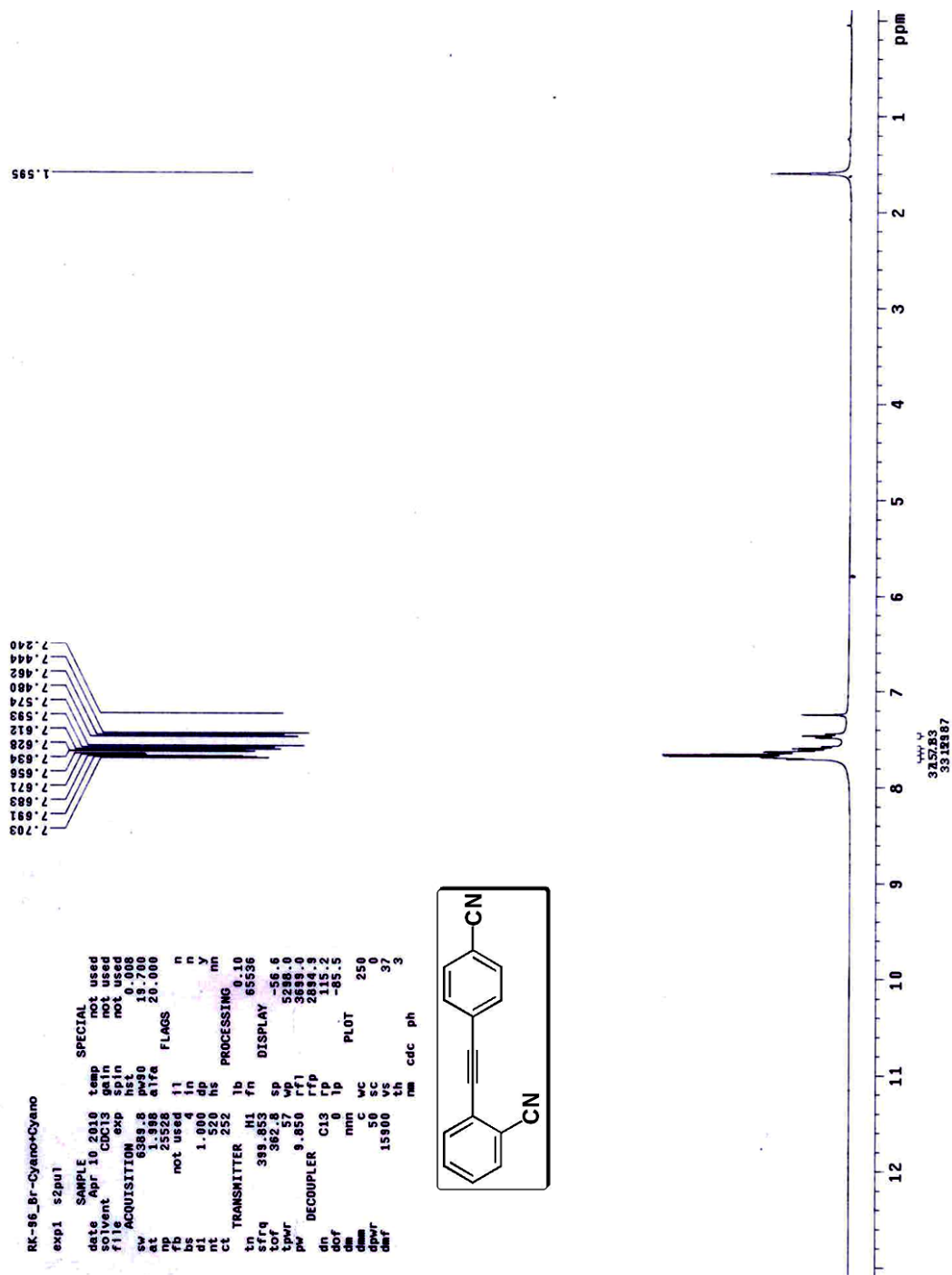
benzonitrile (**2.64**) and 0.019 g (0.1310 mmol) of 4-ethynyl-*N,N*-dimethylbenzenamine **3.46**, 0.028 g (0.0637 mmol) of the title compound was isolated as a bright yellow solid. 60% yield;

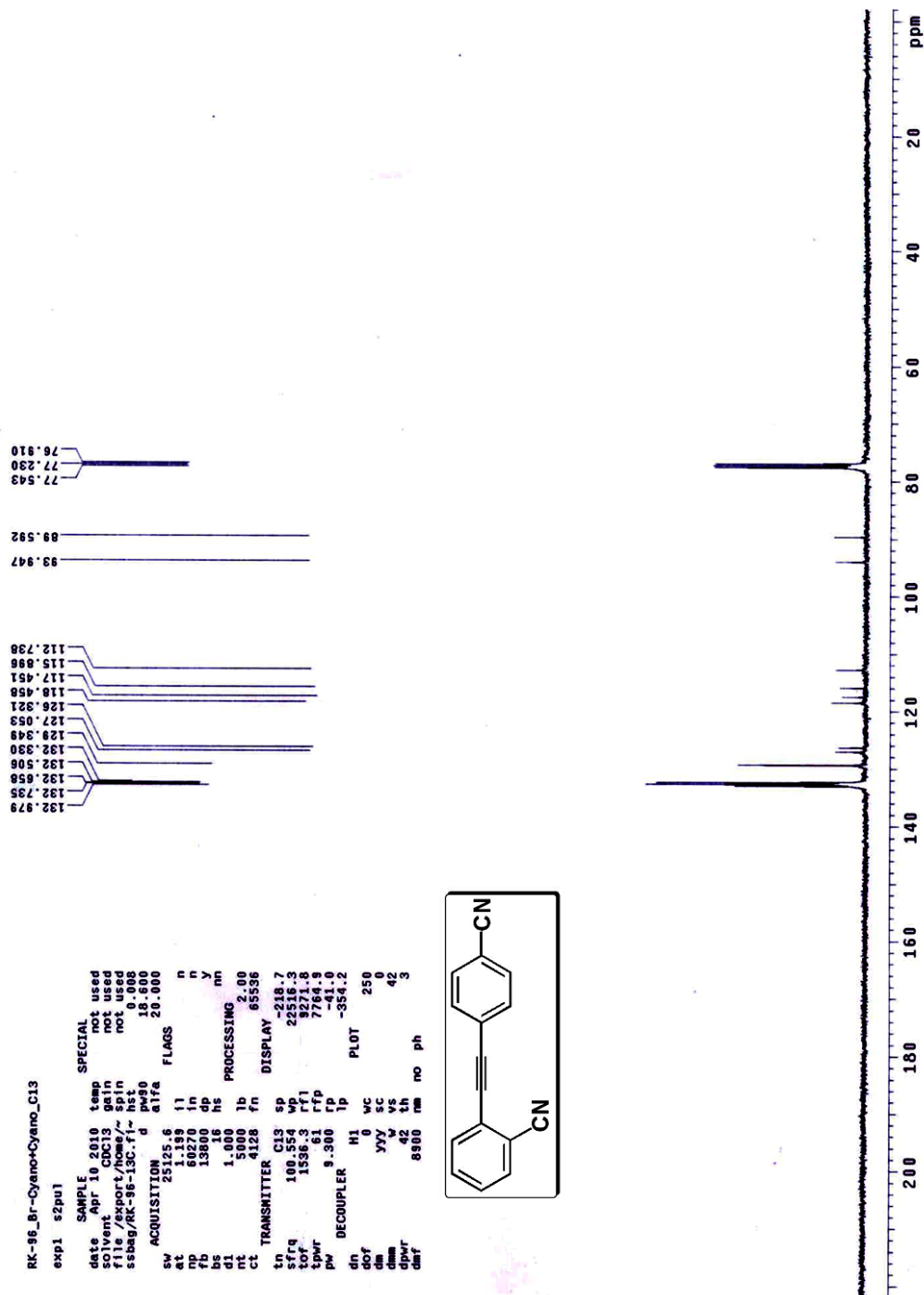
¹H NMR (CDCl₃; 400 MHz) δ 3.00 (6H, s), 6.70 (2H, d, J = 8.8 Hz), 7.38 (2H, d, J = 8.8 Hz), 7.47–7.53 (4H, m), 7.74–7.80 (4H, m), 8.06 (2H, d, J = 8.4 Hz), 8.23 (1H, s); ¹³C NMR (CDCl₃; 100 MHz) δ 41.1, 91.3, 92.4, 111.5, 112.0, 112.4, 118.5, 121.0,

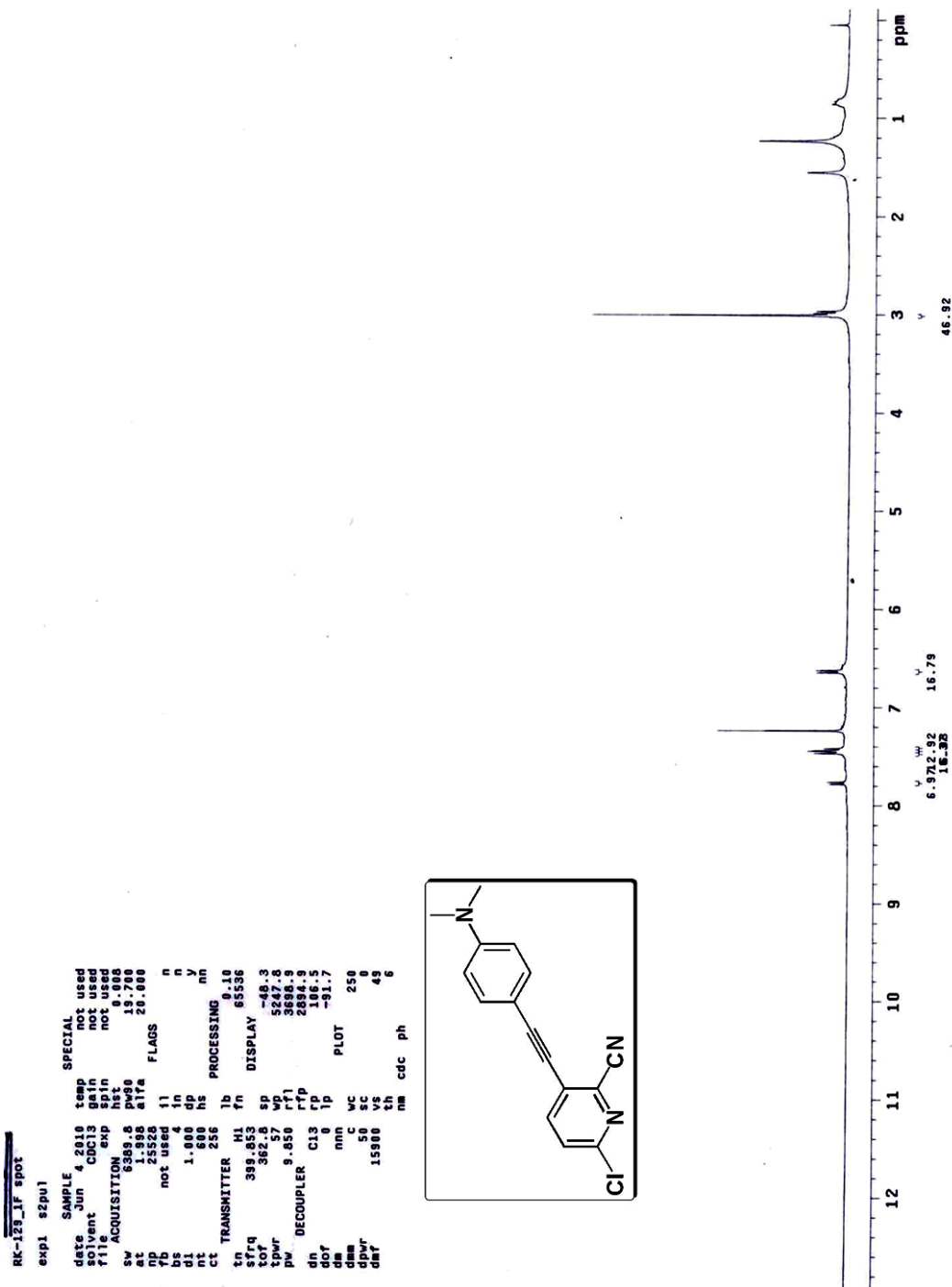
126.1, 126.2, 127.1, 128.0, 131.5, 131.6, 132.5, 135.1, 137.9, 138.0, 148.0, 148.5;
ESI-TOF-MS m/z 440 $[M+H]^+$.

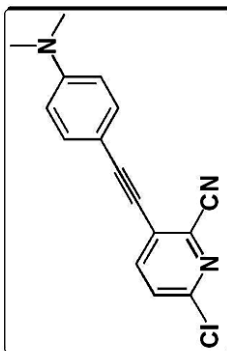
3.8.5. UV-Visible and Fluorescence Measurements

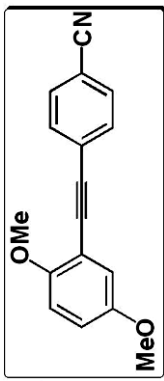
All the *UV* –visible spectra of the compounds (10 μ M) were measured in different solvents using Perkin- Elmer Lambda 25 UV-Visible spectrophotometer with a cell of 1 cm path length at 298 K. Fluorescence spectra were obtained using Fluoromax-4 fluorescence spectrophotometer at 25 °C using 1 cm path length cell at 298 K. All the solvents used in the solution preparation are of spectroscopic grade, purchase from Spectrochem, India. All the compounds were recrystallized twice, dried and used for photophysical study.

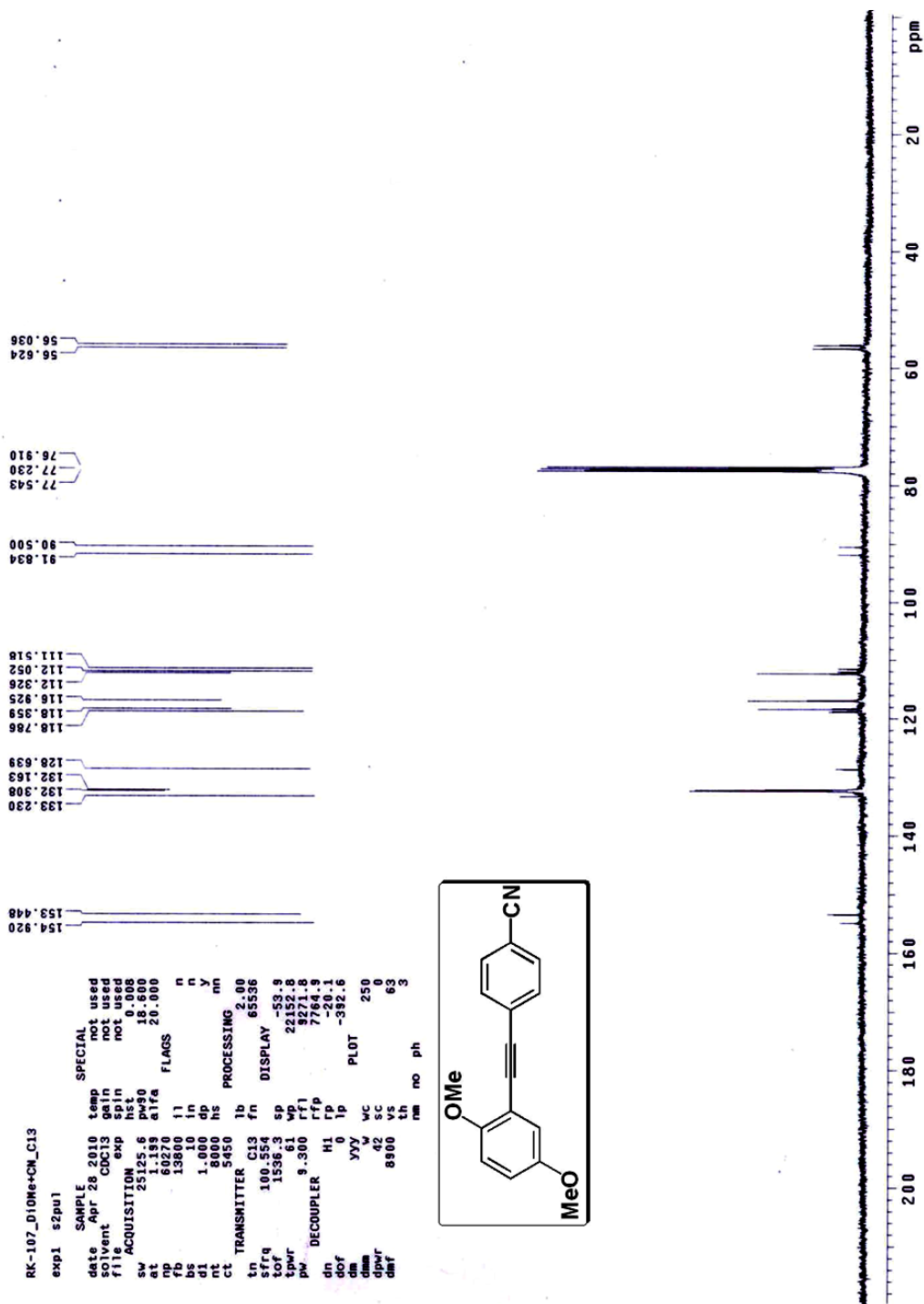


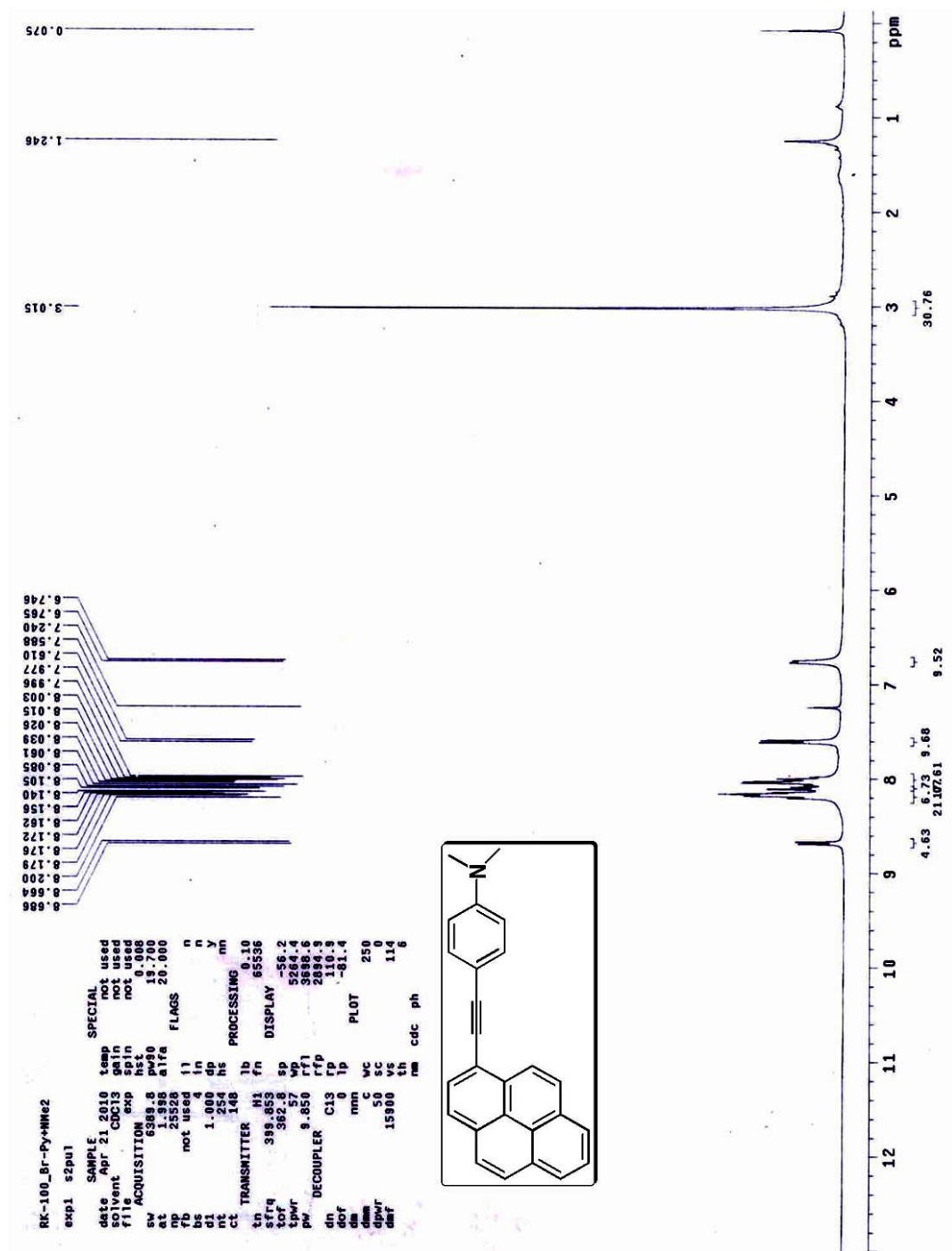


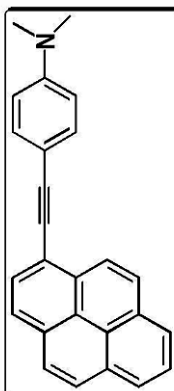












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Chapter 4

**PROBING BIOMOLECULAR
MICROENVIRONMENT
WITH SMALL FLUORESCENT
MOLECULE**

4.1. Introduction

Monitoring various structures, functions and dynamics of biological macromolecules such as DNA, protein, and their surroundings with an ideal microenvironment sensitive fluorescence probe is an emerging research area in biological science and engineering for understanding biological events associated with interbiomolecular interactions.¹ Fluorescence based detection technique has received special attention in many areas² because of its high sensitivity, low background noise and ease of operation. When a fluorescent probe makes complexes with biomacromolecules such as DNA and protein or comes into interaction, the fluorophore's emission property may be modulated such as fluorescence can be enhanced/quenched and/or emission wavelength can be shifted to red/blue region thereby enabling visual observations of the biomacromolecular species. Several fluorescent bioprobes have been reported based on the fluorophore's properties like intercalation, stacking, and/or groove binding for DNA detection with a generation of a distinct fluorescence signal.³ On the other hand, protein binding abilities of several chromophores also have been studied *via* a distinct fluorescence signal generation.⁴ However, many of the explored fluorophores are found to suffer from self quenching, short emission wavelength, or not suitable for use in buffers solutions; thus makes them inefficient biosensors.⁵ Therefore, there is a need to design probes which is low or zero emitting in buffer but upon interaction with the biomolecules, it fluoresce drastically. Thus, the development of new fluorescent probes for sensing biomacromolecules is a highly demanding and desirable goal to the chemists, biochemists and biologists.

4.2. Background

Fluorescence based techniques for monitoring the structural and conformational changes of protein biomolecules was first explored by Weber in 1953 with the use of organic fluorophore.^{6a} Since then, organic fluorophores have attracted attention of

biophysical chemists to be used for understanding the biomolecular structures, dynamics and biological events associated with interbiomolecular interactions. Then, Newton used organic fluorophores to study the interaction of bacterial membrane with polymyxin.^{6b} Azzi *et al.*, extended the study of fluorescent probe based technique towards mitochondrial membranes and they found an excellent probe 1-anilinonaphthalene-8-sulfonic acid (ANS) to respond to the phenomena of energy coupling.^{6c} They further studied the ANS's fluorescence changes associated with energy coupling upon its structural modifications.^{6d} The membrane polarity dependent fluorescence changes of ANS has been studied by Brocklehurst *et al.*^{6e} **Figure 4.1** shows chemical structures of some of the fluorophores used in the study of biomolecules, calf thymus DNA (ct-DNA) and bovine serum albumin (BSA).

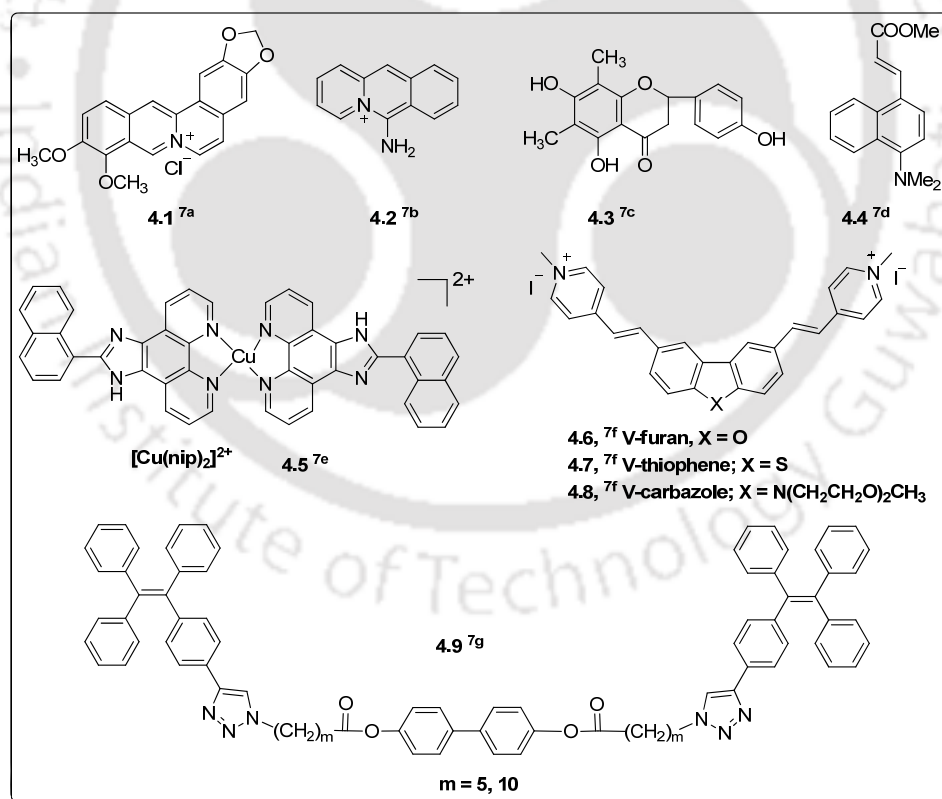


Figure 4.1: Examples of some fluorophore used in the studies on the interactions with ct-DNA and BSA protein.

As a result of tremendous research efforts, several fluorescent probes have been developed and many are commercially available to monitor various structures, functions and dynamics of biological molecules. However, many of the explored fluorophores are not suitable to study biomolecules may be because of low quantum yields, short wavelength emissive, insensitive to microenvironment or suffer from a self quenching. Therefore, there is a need to have probes with improved photophysical property like high solvatochromicity, long wavelength emission and high quantum yield with high stability. Toward this end, as a part of our ongoing research efforts to design solvatochromic fluorescent molecules *via* click reaction, we have synthesized several triazolyl fluorophores including triazolyl pyrene (^{TNDMB}Py) as a dual emissive, highly solvatochromic fluorophore⁸ and their photophysical properties have been evaluated and presented in detail in **Chapter 2**. Out of several synthesized click fluorophores, the triazolyl pyrene has attracted our attention much because of its highly interesting photophysical properties such as, it exhibits,

- (a) highly intense fluorescence emission with high quantum yield,
- (b) large Stokes's shifted emission,
- (c) high solvatochromicity,
- (d) intramolecular charge transfer (ICT) band at long wavelength (visible) region,
- (e) low emission only from ICT state in buffer solution.

Being interested by the interesting photophysical behaviour, we thought that it would be worthwhile to exploit this fluorophore's emission property for the study of possible interaction with biomacromolecules.

4.3. Objective

The logic and idea behind choosing triazolyl pyrene (^{TNDMB}Py) for probing biological molecules are as follows: triazole functionality (a) is metabolically inert, (b) possesses similar physicochemical (dipole-moment, polarizability and size) properties as that of an amide bond and thus acts as trans-amide mimetics, (c) is capable of hydrogen-bonding *via* triazolyl nitrogens, thus, (d) they could interact with

DNA and protein in different forms *via* H-bonding, stacking and electrostatic interactions, (e) is more than just passive linker; it therefore, can readily associate with biological targets, through hydrogen bonding and dipole interactions. Thus, we envisaged that the pseudo aromatic 1,2,3-triazole residue of the probe may significantly enhance the interaction ability *via* stacking/H-bonding/electrostatic interaction with the amino acid residues in a protein and/or with the aromatic bases in DNA; thereby allow one to gather information on the protein's/DNA's microenvironment. Our probe consists of a *N,N*-dimethyl aminophenyl donor linked triazole functionality which serves as an electron donor to effectively modulate the emission response of electronically coupled pyrene as well as an interaction site for biomolecules. This idea along with the intense emission of the probe in low polar media compared to buffer drew our attention to exploit it in the detection of ct-DNA and BSA protein which are easily available biomolecules with widespread applications and biological importance.³⁻⁴ Thus, we found that our probe interact strongly with both the biomolecules with high association constants with generation of enhanced fluorescence signal in the visible region. Therefore, our probe can be used as an efficient fluorescent light-up biosensor for the detection of DNA and protein.

The absorption and emission spectra of our designed probe, ^{TN}DMB^{Py} in different solvents have been elaborately discussed in **Chapter 2**. In brief the absorption spectra of the probe were not markedly affected by the change in solvent polarity; however the emission spectra were strongly affected by the change in solvent polarity. The molecule showed a dual emission behavior centered at 383 nm, a locally emission (LE) band and 466-544 nm, internal charge transfer (ICT) band (**Figure 4.2**). In high polar solvents (acetonitrile, methanol) the probe showed a low intense LE (local emission) band which is characteristic of structured band of pyrene. On the other hand, in ether, dioxane, toluene, chloroform and ethyl acetate, a dual emission was observed with strong red shifted pattern of the ICT (internal charge transfer) band as the solvent polarity is increased from toluene (466 nm) to ethyl acetate (544 nm).

Switching between these two states depends on the nature and polarity of the solvents. Here, in this chapter, first, we have studied absorbance and fluorescence behaviour at neutral pH in phosphate buffer. Thus, it showed a long wavelength emissive band at around 520 nm with less intensity compared to 10% water-dioxane solvent system (**Figure 4.2**). Therefore, the interesting fluorescence property of triazolyl pyrene in different media, particularly its intense emission in low polar media compared to buffer drew our attention to exploit it in the study of possible interaction with ct-DNA and BSA protein.

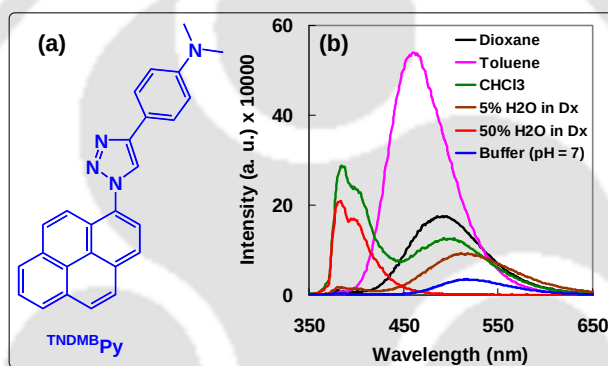


Figure 4.2: (a) Chemical structure of ^{TNDMB}Py and (b) emission behavior of ^{TNDMB}Py in various media.

With this idea in mind, we have exploited ^{TNDMB}Py probe to study the interaction with ct-DNA and BSA. We found that our probe interact strongly with both the biomacromolecules with an association constant in the order of $\sim 10^4 \text{ M}^{-1}$ with a generation of enhanced fluorescence signal in the long wavelength region. Therefore, our probe can be used as a fluorescence light-up probe for the detection of DNA and protein. The details of our observations are presented in this chapter in two sub chapters-(a) **Chapter-4A: STUDIES ON THE INTERACTION OF SMART FLUORESCENT PROBE, TRIAZOLYL PYRENE (^{TNDMB}Py), WITH CALF THYMUS DNA** which describes the interaction ability and photophysical response of the fluorescent probe with Calf Thymus DNA (ct-DNA), and (b) **Chapter-4B: STUDIES ON THE INTERACTION**

OF SMART FLUORESCENT PROBE, TRIAZOLYL PYRENE (^{TN^{DMB}}Py), WITH BOVIN SERUM ALBUMIN which deals with the photophysical response associated with the binding events of the same probe upon interaction with protein's microenvironment. Each chapter contains their individual introduction, results and discussion and experimental section.



Chapter 4A

**STUDIES ON THE INTERACTION OF
SMART FLUORESCENT PROBE,
TRIAZOLYLPYRENE (^{TNDMB}Py), WITH
CALF THYMUS DNA**

4A.1. Introduction

Considerable attention has been focused on new DNA-binding and modifying agents from natural products to wholly synthetic design as probes of DNA structure and as potential chemotherapeutic agents. The application of these molecules necessitates a precise understanding of the structural details and the mode of interaction of the molecules with the target double-helical DNA. In general, DNA-binding agents tend to interact noncovalently with the host DNA molecule through two modes: (i) **Threading Intercalation**⁹: in a groove-bound fashion stabilized by a mixture of hydrophobic, electrostatic, and hydrogen-bonding interactions and (ii) **Classical Intercalation**¹⁰: through an intercalative association in which a planar, heteroaromatic moiety slides between the DNA base pairs.

Groove binding/intercalative interaction, the most commonly studied, is the noncovalent stacking interaction resulting from the insertion of a planar heterocyclic aromatic ring between the base pairs of the DNA double helix. These structural modifications can lead to functional changes, often to inhibition of transcription and replication and DNA repair processes, which make intercalators potent mutagens. There is much interest in the ability of intercalators to inhibit nucleic acid synthesis *in vivo*, leading to activity as mutagens, antibiotics, antibacterials and antitumor agents.¹¹

4A.2. Structural Features of DNA

Double helix DNA consist of two single strand DNA antiparallel to each other, stabilized by a combination of hydrogen bonding interactions between the two specific DNA bases and $\pi - \pi$ stacking interactions of the DNA bases. The hydrogen bonding interactions between the Watson-Crick base pairs is the strongest interaction to stabilize a double helical DNA. However, $\pi - \pi$ stacking interactions also play a vital role in stabilizing the double helix DNA.

The chemical structures and the spatial dispositions of the Watson-Crick base pairs shows that there are two possible hydrogen bonding interactions between A – T

(Adenine – Thymine) base pairs and three possible hydrogen bonding interactions between G – C (Guanine – Cytosine) base pair (**Figure 4A.1**). The sugar and phosphate backbone of the paired strands define the helical grooves of DNA. The biologically relevant B-form DNA duplex exhibits a shallow, wide major groove and a deep, narrow minor groove.¹² The sequence of a DNA determines the molecular surface of the grooves which decides the mode of recognition by small molecules and proteins.

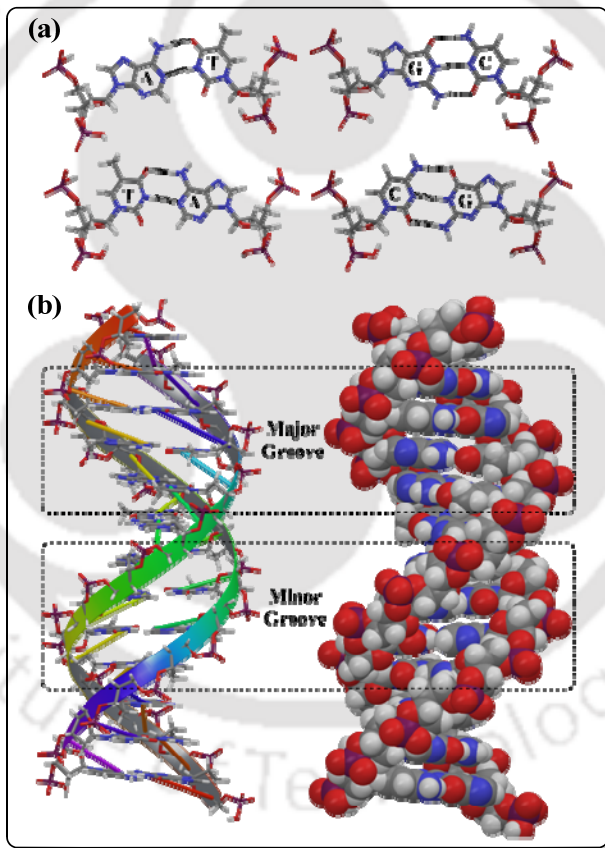


Figure 4A.1: (a) Chemical structure and the spatial arrangement of Watson-Crick base pairing and (b) double helical structure of DNA shows the major and minor groove.

4A.2.1. Various Binding Modes of DNA Interaction

Molecular recognition of B-DNA can take place in five distinct ways,

- (a) major groove recognition;
- (b) minor groove recognition;
- (c) sugar-phosphate backbone binding;
- (d) intercalation between the base pairs;
- (e) covalent binding or metal-coordination to the bases.

These DNA recognition modes were investigated in the 1960s and since then the research in DNA recognition field has grown up vastly. **Figure 4A.2** illustrates the various modes of binding of small molecules/ligand to DNA.

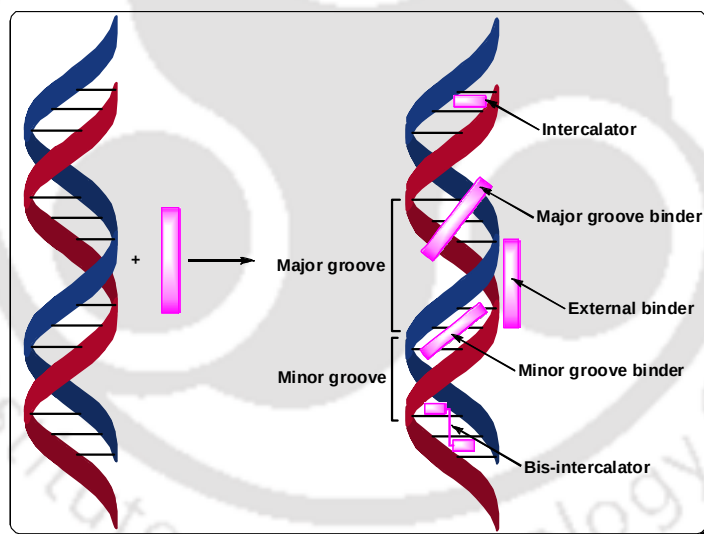


Figure 4A.2: Schematic diagram showing the different binding modes of ligand to DNA.

4A.2.2. Small Molecules-DNA Interaction: Groove Binding

The natural products like Hoechst 33258,^{13a} netropsin,^{13b} distamycin,^{13c} etc., have received special attention for their specific binding in minor groove due to their modular nature, reduced size, and diminished conformational freedom. These are also well-known examples of small molecules with antibiotic, antiviral and antitumor

activities. These molecules specifically bind to the DNA minor groove and have a preference towards AT-rich over GC-rich sequences.¹⁴ Various experimental and theoretical analyses of DNA-small molecule complexes have brought much information about the binding modes and their corresponding binding energies. Thus, the design and synthesis of new sequence-specific and sequence-selective DNA binding small molecules has received special attention during past few decades due to their ability to act as nucleic acid conformational probes, antitumor, anticancer drugs *etc.* The binding of small molecules with DNA is driven through a combination of electrostatic, van der Waals and hydrogen bonding interactions with phosphate backbone and stacked Watson-Crick base pairs. X-Ray crystal structure of various DNA-small molecule complexes has shown that the molecules to show potential binding ability should be flat and crescent-shaped as well as the molecule must possess suitable binding sites. **Figure 4A.3** shows chemical structures of some of the DNA groove binders.

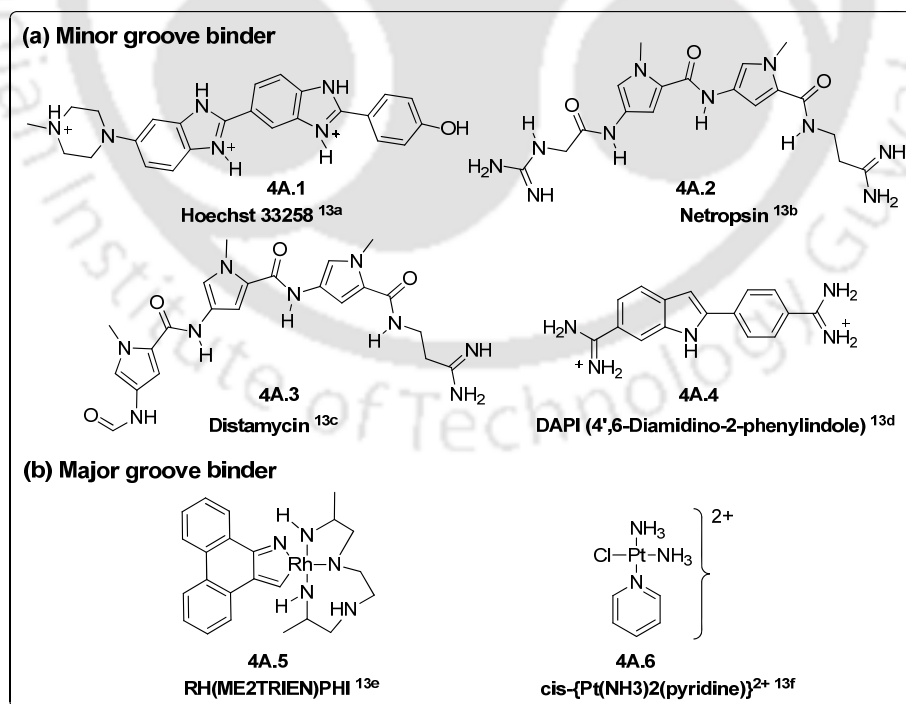


Figure 4A.3: Examples of DNA groove binders.

Figure 4A.4 and **4A.4** show the minor groove and major groove, respectively, binding mode of some of the groove binding agents.

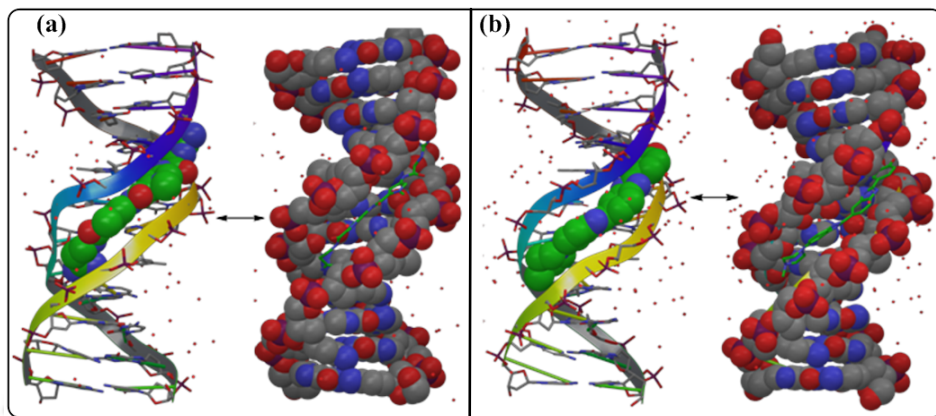


Figure 4A.4: Example of DNA minor groove binding. (a) Molecular structure of the Netropsin – 5'-d(CGCGATATCGCG)-3' complex (PDB ID: **1VTJ**) and (b) molecular structure of the Hoechst 33258 – 5'-d (CGCGAATTCGCG)-3' complex (PDB ID: **1DNH**).

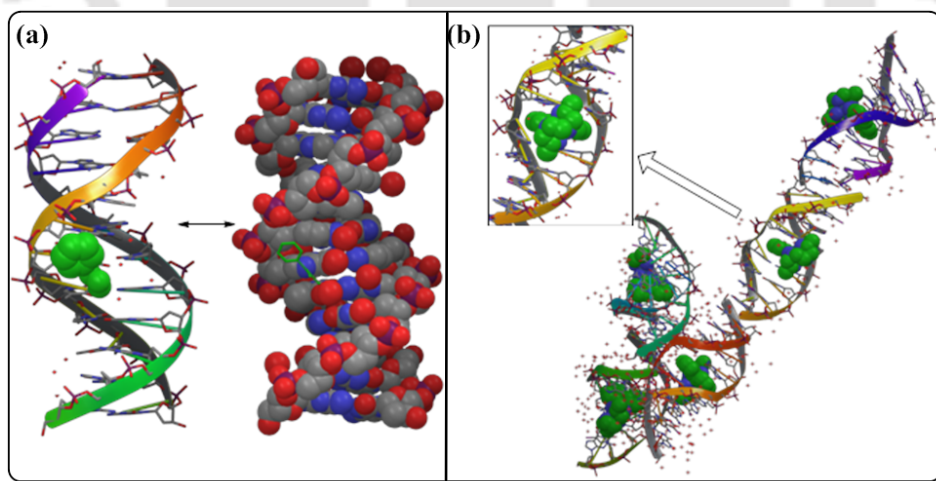


Figure 4A.5: Example of DNA major groove binding. (a) X-Ray crystal structure of cis-diammine(pyridine)chloroplatinum(II) bound to deoxyguanosine in a dodecamer duplex(PDB ID: **3CO3**) and (b) X-Ray crystal structure of rhodium complex, a major groove recognition and intercalation into DNA (PDB ID: **454D**).

4A.2.3. Small Molecules-DNA Interaction: Intercalator

Intercalative interactions between DNA and planar polycyclic aromatic organic intercalators,¹⁵⁻¹⁶ such as 9-Aminoacridine (9-AA) derivatives,^{16a-b} ethidiumbromide,^{16c-d} thiazole orange (TO)^{16e}, and benzo[*a*]pyrene (BP)^{16f} have been thoroughly studied. Studies of bulky intercalators are rare. Bis-intercalators have also been reported, such as bisnaphthalimide, which exhibits antitumor activity. A tetraintercalator has been reported by Iverson, *et al.*¹⁷ Some of the potent polycyclic aromatic intercalators are shown in **Figure 4A.6** as representative examples.

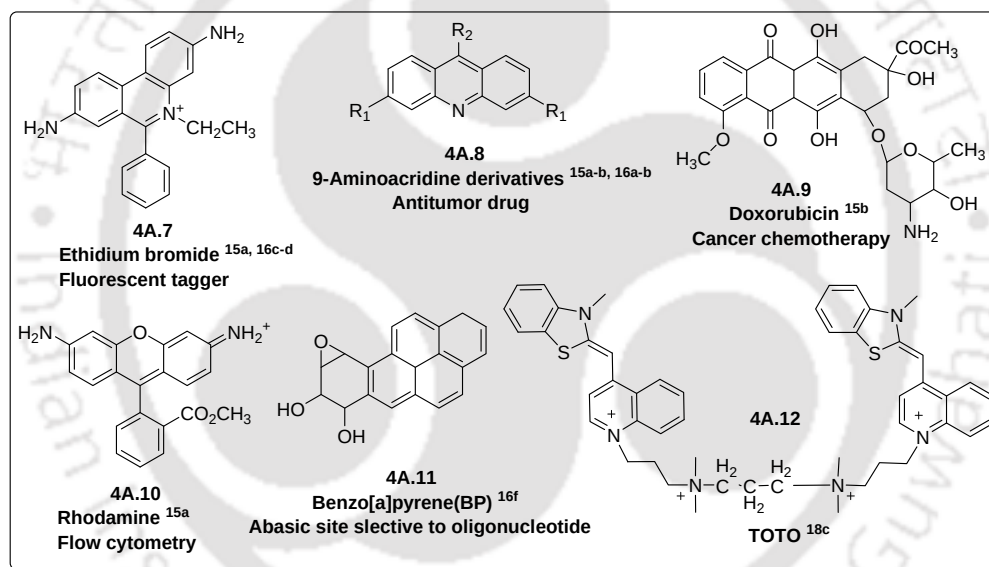


Figure 4A.6: Examples of some polycyclic aromatic organic intercalators.

Most of the naturally occurring intercalators contain rigid planar aromatic groups. It has been found that some intercalators are site selective, *i.e.*, they prefer to bind within a specific DNA sequences. Such types of intercalating compounds are mostly used in clinical diagnosis. When a molecule contains two planar aromatic groups/units attached with a flexible long side chain, can simultaneously be inserted into the DNA base stack and can interact with both the units. Such types of intercalators are called bisintercalators. As for examples bis-naphthalimide,^{18a} 1,1'-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)bis[4-(3-methyl-2,3-dihydrobenzo-1,3-thiazolyl-2-

methylidene)quinolinium] tetraiodide (TOTO)^{18b} etc., are well studied bisintercalators. Bisintercalating molecules have also received considerable attention in the bioorganic and drug development area of research.¹⁸ **Figure 4A.7** shows the mode of intercalative interaction of two bisintercalators.

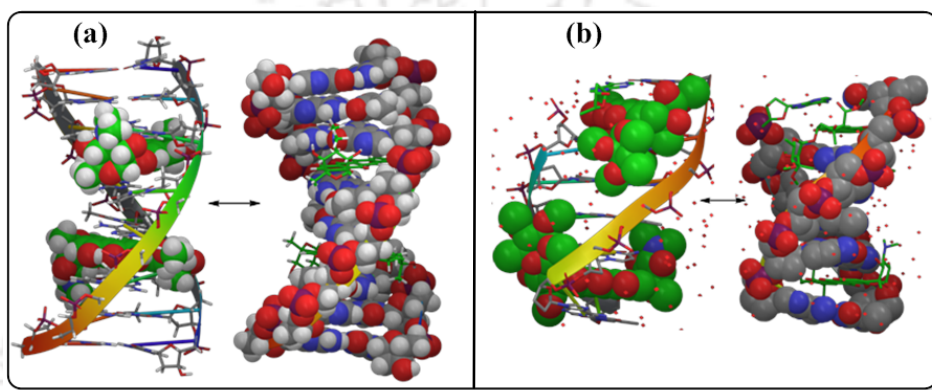


Figure 4A.7: Example of DNA – ligand intercalation. (a) The solution structure of a DNA complex with fluorescent bisintercalator TOTO (PDB ID: **108D**) and (b) X-Ray crystal structure of 5'-d(CGTACG)-3' complexes with bis(9-aminoacridine-4-carboxamide) derivative (PDB ID: **2GB9**).

Metal complexes that involve in intercalative interactions with DNA are widely used as potent anticancer, antitumor drugs. Lippard *et al.*,¹⁹ have shown that square-planar platinum(II) complexes containing heterocyclic aromatic ligands, such as terpyridine (terpy), etc., bind to DNA duplexes *via* intercalation (**4A.13-14**, **Figure 4A.8**).¹⁹ Metallointercalation has been extended Barton and other groups using octahedral complexes of Ru, Rh, etc., containing multi-heterocyclic aromatic ligands such as phen, dppz, chrysi, etc., (**4A.15-16**, **Figure 4A.8**).²⁰ Because of the proper shape, symmetry and functionalities responsible for interaction with DNA, octahedral metallointercalators allow the targeting of specific site of DNA.

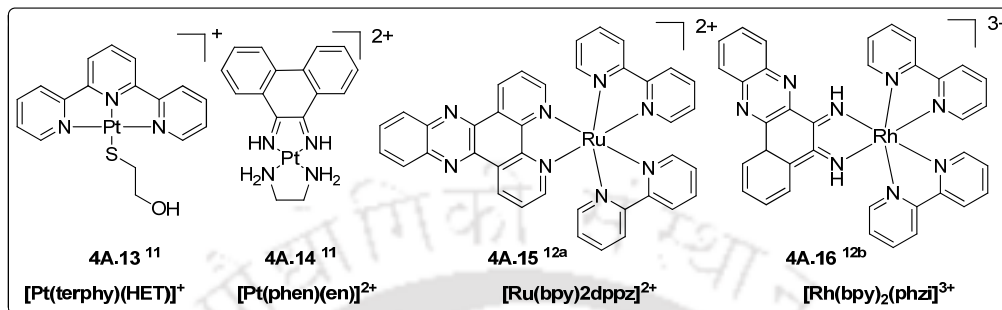


Figure 4A.8: Examples of some metallointercalators.

Microenvironment sensitive fluorescent molecule when interact with DNA, the emission properties of the molecule changes, the fluorescent intensity is either enhanced or quenched and /or the emission wavelength is shifted either to a long wavelength region or to a short wavelength region. Thus, the environment sensitive fluorescent small molecules would be a better choice to study the drug-DNA interaction. Hoechst 33258,^{21a} ethidium bromide^{21b,c} etc., are well-known examples of DNA binding small molecules whose emission properties enhances upon binding with DNA. These two compounds are mostly used as a model dye to investigate the binding modes (groove or intercalator) of other small fluorescent molecules. Several fluorescent small molecules have been reported to monitor the change of local microenvironments in DNA. However, many of them are found to suffer from a self-quenching, so, the development of new fluorescent small molecules or bioprobes is of immense importance.

4A.3. Background: Small Molecule Probes of Calf Thymus DNA (ct-DNA)

Calf thymus DNA (ct-DNA) is a polymer and has relatively low protein content with a highly polymerized skeleton.²² It is one of the most important and useful DNA model to study the properties, functions and binding events of small molecules/drugs with DNA. The model study with ct-DNA can give an important idea about the

functions and binding events of the designed small molecules/drugs. A large number of research works in this regard have been reported in literature. Few of them are discussed below.

Zsila *et al.*,^{23a} have reported an interaction between the non-toxic, cancer chemopreventive agent curcumin (**4A.17**, **Figure 4A.9**) and both natural (ct-DNA) and synthetic DNA duplex with the help of circular dichroism and UV-Visible spectroscopy. Molecular docking studies supported the possible minor groove binding interaction.

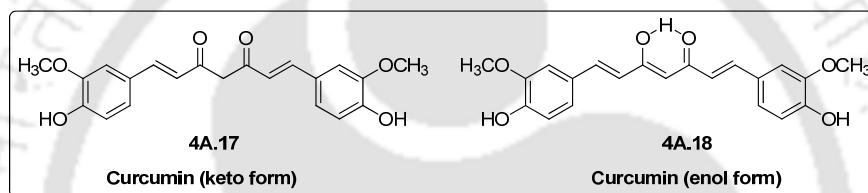


Figure 4A.9: Example of DNA recognition by small molecule, curcumin.

Fluorescence switch on probes, tetraphenylethylene derivatives (**4A.19-22**, **Figure 4A.10**) have been synthesized and exploited for the detection of ct-DNA by Tang *et al.*^{23b} Thus, the fluorescence intensity of the tetraphenylethylene derivatives have been found to increase upon binding with ct-DNA. The enhancement of fluorescence intensity has been explained on the basis of restriction of intra-molecular rotations of the dyes in the aggregates and in DNA complexes.

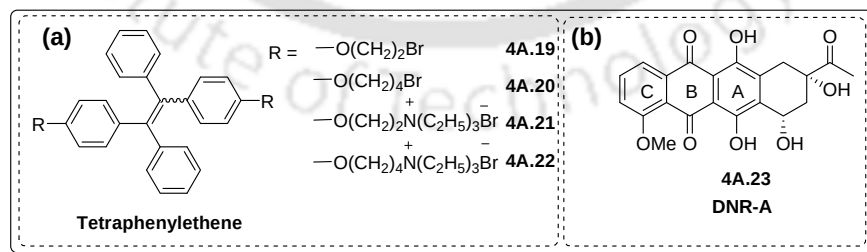


Figure 4A.10: Example of DNA recognition small molecules.

Cui *et al.*,^{23c} have studied the interaction of aglycon of daunorubicin (DNR-A, **4A.23**, **Figure 4A.10**) with calf thymus DNA by UV-visible absorption spectra and fluorescence spectroscopy. They observed a hypochromic effect in the absorption spectra and a decreased in fluorescence intensity with $K_{SV} = 6.203 \times 10^4 \text{ L mol}^{-1}$ of **4A.23** in the presence of ct-DNA.

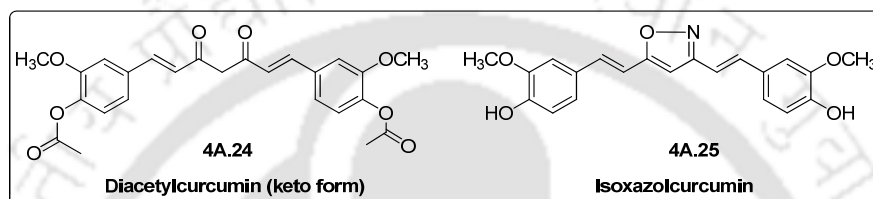


Figure 4A.11: Curcumin derivatives, DAC and IOC as ct-DNA binding agents.

The potent biological activity frequently exhibited by many naturally occurring small molecules have attracted attention for studying the interaction between these molecules and DNA to understand the origin of their biological activity and the dynamics of interaction with DNA or other biological macromolecules. As for example, Dasgupta *et al.*,^{23d} have reported the interaction of diacetylcurcumin (DAC, **4A.24**, **Figure 4A.11**), a synthetic derivative of natural non-toxic curcumin with ct-DNA spectroscopically. They observed fluorescence quenching effect of DAC in presence of ct-DNA with a high binding constant, $K_{sv} = 3.97 \pm 0.31 \times 10^5 \text{ M}^{-1}$. From the circular dichroism and dye displacement studies, they proposed the possible minor groove binding of DAC with ct-DNA. Later on, they^{23e} investigated the interaction of isoxazolcurcumin (IOC, **4A.25**, **Figure 4A.11**), with ct-DNA spectroscopically. For this case they observed fluorescence enhancement of isoxazolcurcumin in presence of ct-DNA with a binding constant $K = 6.3 \times 10^3 \text{ M}^{-1}$. Similar to curcumin, the circular dichroism and dye displacement studies showed the possible minor groove binding of IOC with ct-DNA.

4A.4. Objective

With the growing interest in DNA binding agents, like, groove binders intercallators, and reported limited number of switch on long wavelength emissive biomolecular probe, it is thus pertinent to develop new designer molecules and study the binding interaction with DNA to reveal how the compound may be further modified to enhance its binding activity or to provide guidelines for the development of more potent DNA binding agents with novel photophysical properties. Microenvironment sensitive small fluorescent molecules exhibit modulated fluorescence response (modulation in intensity and/or shifting of wavelength) upon interaction with DNA as the dielectric property inside and outside a DNA duplex is very different. Therefore, the polarity sensitive fluorescent small molecule would be a choice of molecule for probing biomolecular microenvironment and studying drug-DNA interaction. Therefore, many small molecular probes have been designed and utilized for studying their interaction behavior with ct-DNA as was discussed previously. However, many of them are found to suffer from either quenching of emission property in presence of DNA or a short wavelength emission with low solvatochromaticity. As a part of our ongoing research efforts for synthesizing fluorescent molecules, we became interested to explore one of our click chemistry derived fluorophore, triazolylpyrene. Therefore, with the above background and being interested by the interesting emission property of the triazolylpyrene, especially the low intensity emission in buffer, we thought that it would be worthwhile to exploit this molecule in studying interaction with ct-DNA.

We envisaged that the *N,N*-dimethyl aminophenyl triazole part of our probe may involve in intercalative stacking interaction with ct-DNA and the pyrene part bind to the groove (**Figure 4A.12**). If it is so, the pyrene would face more polar microenvironment and the binding event will be assessed by a strong fluorescence signal generation at its long wavelength emission band. With this aim and concept, we framed our objective to study spectroscopically the interaction of ^{TNDB}Py with ct-DNA as pointed out below.

- (a) Study of UV-visible/fluorescence photophysical properties of the probe in presence of ct-DNA.
- (b) Study of thermal stability of ct-DNA in presence of the probe.
- (c) Determination of association constant.
- (d) Evaluation of binding mode- groove binding vs. intercalation.
- (e) Study of time resolved fluorescence anisotropy and macromodel calculation to support observed binding mode.

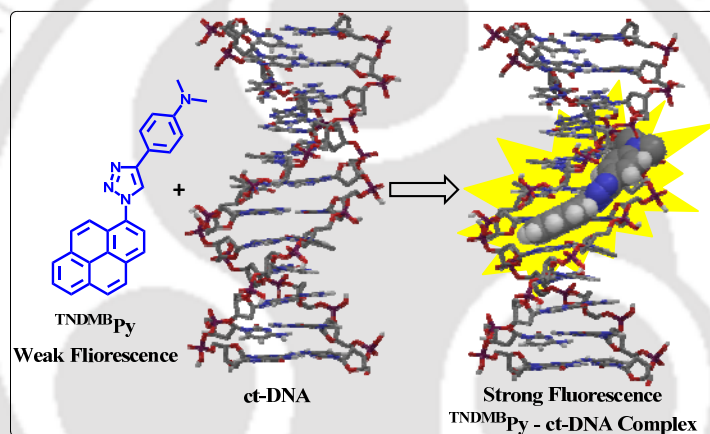


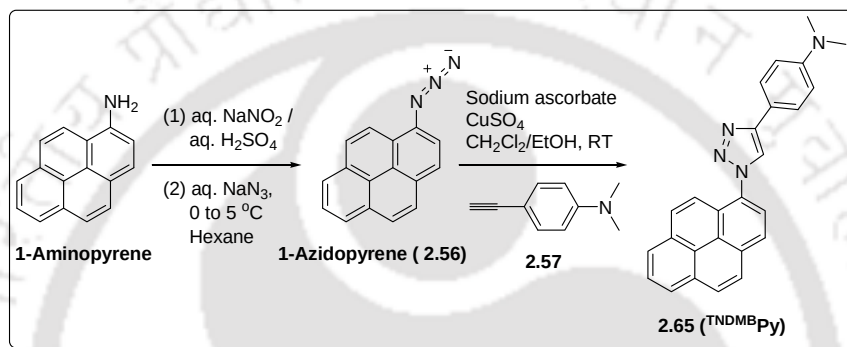
Figure 4A.12: Graphical representation of binding interaction of ^{TNDMB}Py with ct-DNA with a generation of enhanced fluorescence signal.

4A.5. Results and Discussions

4A.5.1. Synthesis of the Probe Triazolyl Pyrene

The synthesis of the probe (^{TNDMB}Py) was achieved following our reported click chemistry protocol (discussed in **Chapter 2**).⁸ Thus, 1-aminopyrene was converted to its corresponding 1-azidopyrene (**2.56**) *via* reaction with NaNO₂/aq. H₂SO₄ to diazonium salt followed by reaction with NaN₃ at 0 °C. Then the azide **2.56** was reacted with terminal alkyne (**2.57**) under click condition *i.e.*, in presence of sodium ascorbate and CuSO₄ in a mixture of CH₂Cl₂-EtOH solvent at room temperature to

form the product triazolyl pyrene **2.65**, ^{TNDBM}Py (**Scheme 4A.1**). The pure product was isolated by column chromatography and characterized by NMR, mass spectra and compared with our reported result. After isolation of the pure compound from column chromatography, we have recrystallized twice in chloroform-ethylacetate solvent mixture and very pure crystals were dried and used for further study with ct-DNA.



Scheme 4A.1: Synthesis of fluorescent probe, ^{TNDBM}Py.

To examine the biomolecule sensing ability of our probe, first, we have investigated the interaction behaviour with ct-DNA by UV-visible and fluorescence spectroscopy in aqueous phosphate buffer (pH 7.0) at 298 K.

4A.5.2. UV-Visible and Thermal Denaturation Study

From the study of UV-Visible absorption of the probe in sodium phosphate buffer at pH 7 it is clear that the absorption maxima of triazolyl pyrene, ^{TNDBM}Py located at 355 nm. As the ct-DNA concentration was increased gradually to a solution of the probe, the absorption band showed a clear hyperchromicity with a minimal blue shift in absorption wavelength (**Figure 4A.13a**). This observation indicated a probable external groove binding characteristics of the probe.^{23d-e,24}

The thermal denaturation experiment of ct-DNA in presence of probe, ^{TNDBM}Py (**Figure 4A.13a** and **Table 4A.1**) was also carried out. Thus, it was observed that the melting temperature (*T_m*) of ct-DNA slightly decreases in presence of the probe,

indicating a little destabilization of ct-DNA upon binding with the probe which is possibly because of steric clash near the groove position.

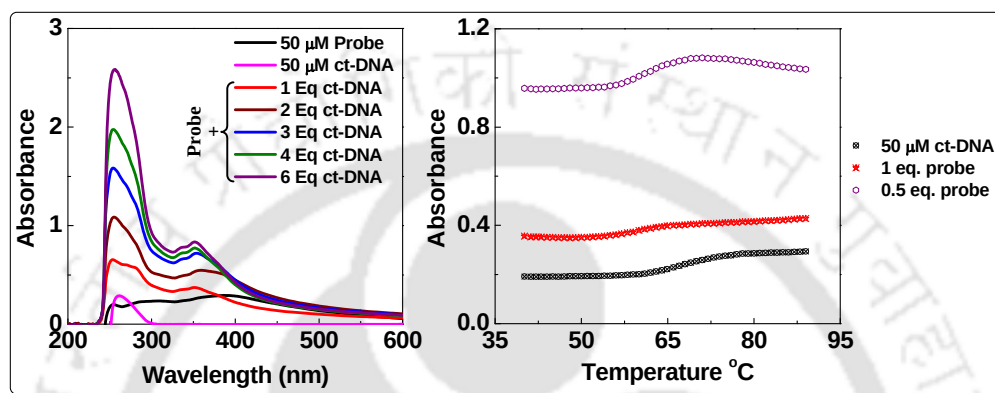


Figure 4A.13: (a) UV-visible spectra of ^{TNDMB}Py in presence of increasing ct-DNA concentration at 298K. [^{TNDMB}Py] = 50 μM and [ct-DNA] = 50, 100, 150, 200, 250 and 300 μM. (b) Thermal denaturation study of ct-DNA in presence of ^{TNDMB}Py. [^{TNDMB}Py] = 50 μM and [ct-DNA] = 50, 100 μM.

Table 4A.1: T_m values of ct-DNA in presence of ^{TNDMB}Py.

Entry	ct-DNA : ^{TNDMB} Py	T_m (°C)
1	50 μM ct-DNA only	67.74
2	1 : 1	61.20
3	2 : 1	61.04

4A.5.3. Study of Fluorescence Photophysical Properties in Presence of ct-DNA

Next we have investigated the fluorescence photophysical property of the probe in presence of various concentration of ct-DNA. Thus, from the fluorescence titration spectra it was observed that upon addition of increasing concentration of ct-DNA, the fluorescence intensity ($\lambda_{em} = 525$ nm) of triazolyl pyrene was significantly increased and reached maximum at 300 μM concentration of ct-DNA compared to 50 μM probe concentration (**Figure 4A.14a**), indicating a well-defined binding of the probe with

ct-DNA. The enhancement of fluorescence intensity was also evident from the appearance of bright green colouration of a 1:6 probe-ct-DNA mixture illuminated at 254 nm under a transilluminator (**Figure 4A.14b**).

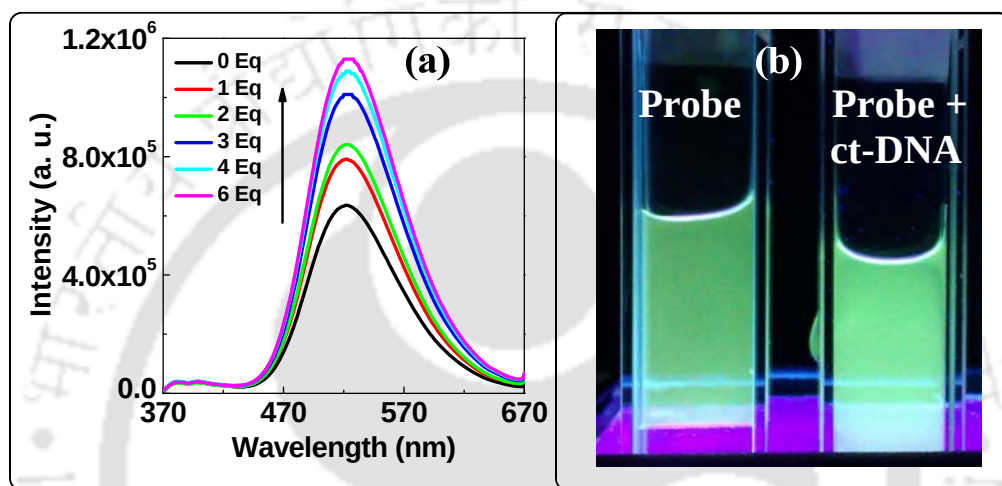


Figure 4A.14 (a) Fluorescence spectra ($\lambda_{\text{ex}} = 345 \text{ nm}$) of TNDMB-Py in presence of increasing ct-DNA concentration at 298K. (b) Fluorescence image under UV-light (254 nm) of only probe (50 μM) and in presence of ct-DNA (6 Eqv.).

4A.5.3.1. Benesi-Hildebrand Plot for Evaluation of Binding Constant

The association constant of TNDMB-Py with ct-DNA was determined by a Benesi-Hildebrand plot (**Figure 4A.15**) using the following equation 1,

$$\frac{1}{(I - I_0)} = \frac{1}{(I_\infty - I_0)} + \frac{1}{(I_\infty - I_0)K[\text{ct-DNA}]} \quad \dots\dots\dots (1)$$

where I_0 , I and I_∞ are the emission intensities of TNDMB-Py in the absence of ct-DNA, in the presence of an intermediate and at infinite concentration of ct-DNA, respectively. From the slope of the $1/(I - I_0)$ vs. $1/[\text{ct-DNA}]$ plot, the binding constant K was determined which was found to be $7.2 \times 10^3 \text{ M}^{-1}$. Next, the free energy of binding (ΔG) was calculated using the equation 2, which was found to be $\Delta G = -5.24 \text{ kcal/mol}$.

$$\Delta G = -RT \ln K \dots\dots\dots (2)$$

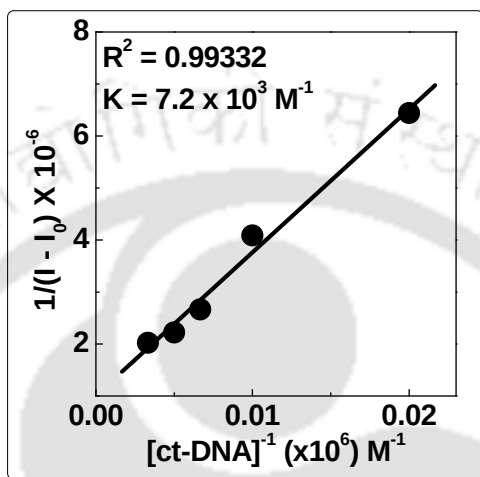


Figure 4A.15: Benesi–Hildebrand plot of ^{TNDMB}Py in presence of increasing ct-DNA concentration. [^{TNDMB}Py] = 50 μM and [ct-DNA] = 50, 100, 150, 200, 250 and 300 μM.

4A.5.4. Study of Circular Dichroism (CD) Spectroscopy

The CD spectra of a B-form DNA exhibits two bands in the UV region; a positive band at around 260 – 280 nm and a negative band at around 245 nm.²⁵ The position and amplitudes of the CD band differ markedly with sequence and conformational properties of DNA as well as with the interaction of external/remote chromophore. The ct-DNA in the B-form conformation also shows two CD bands in the UV region. The positive band at around 278 nm is due to the DNA base stacking and the negative band at around 245 nm is due to polynucleotide helicity.²⁶ Increase in molar ellipticity of the positive band in presence of an external molecule suggest the intercalative binding mode of interaction. The stacking interaction between the intercalator and the DNA bases enhances the molar ellipticity of the positive band.²⁷ As the probe, ^{TNDMB}Py has no chiral centre, so it will not exhibit any band in the CD-spectra.

Figure 4A.16 shows the CD spectra of ct-DNA in absence and presence of ^{TN}DMBPy. A slight decrease or negligible change of the positive band was observed in presence of ^{TN}DMBPy, which supports the non-intercalative binding mode of the probe. Overall, the CD spectral measurement in presence of the probe revealed an unperturbed B-form DNA conformation of ct-DNA. This result indicated that the probe possibly bind to a groove of ct-DNA (**Figure 4A.19**).^{23e,24b}

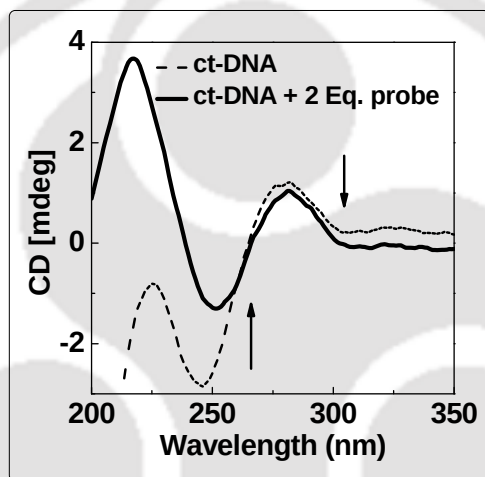


Figure 4A.16: CD spectra of ct-DNA in absence and presence of ^{TN}DMBPy at 298 K. [ct-DNA] = 50 μ M and [probe] = 100 μ M.

4A.5.5. Evaluation of Binding Mode of the Probe with ct-DNA by a Dye Displacement Study

To investigate the binding mode of the probe with ct-DNA, whether it is an intercalator or a groove binder, both the ethidium bromide (EB) and hoechst 33258 displacement experiments were carried out. Ethidium bromide is a well-known example of an intercalating agent.^{21b,c} In general, the fluorescence intensity of EB would increase when it binds with DNA because of intercalation. In a competing scenario, if our probe, ^{TN}DMBPy intercalates with ct-DNA, then it would be a competitive binding agent with EB and the fluorescence intensity of EB-ct-DNA complex would decrease with increase in probe concentration. However, we did not

observe any significant fluorescence change of EB-ct-DNA complex upon increase in $^{TNDMB}Py$ concentration (**Figure 4A.17a**). Thus, this observation indicates that $^{TNDMB}Py$ is not an intercalator but may be a groove binder.²⁸

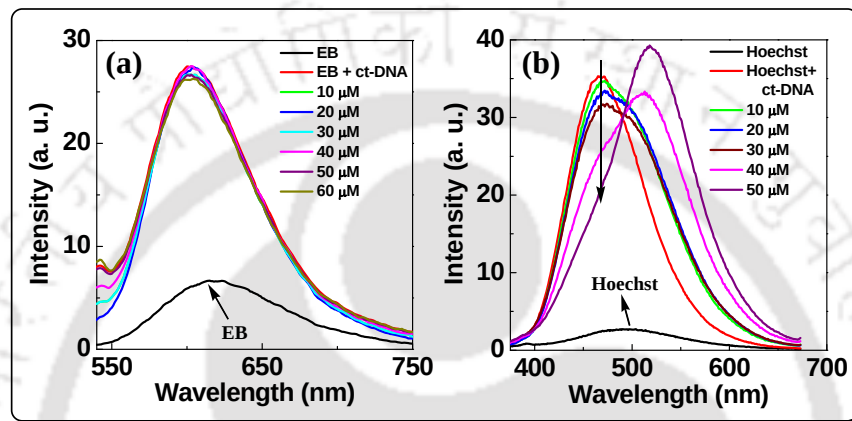


Figure 4A.17: (a) Emission spectra ($\lambda_{ex} \approx 480$ nm) of EB (black line at bottom), EB-ct-DNA complex and EB-ct-DNA complex mixed with $^{TNDMB}Py$. [EB] = 10 μM , [ct-DNA] = 50 μM respectively and [$^{TNDMB}Py$] = 10, 20, 30, 40, 50 and 60 μM . (b) Emission spectra ($\lambda_{ex} \approx 340$ nm) of hoechst (black line at bottom), hoechst-ct-DNA complex and hoechst-ct-DNA complex mixed with $^{TNDMB}Py$. [Hoechst] = 10 μM , [ct-DNA] = 50 μM and [$^{TNDMB}Py$] = 10, 20, 30, 40 and 50 μM .

Further to prove that $^{TNDMB}Py$ is a groove binder, fluorescence titration experiment with hoechst 33258^{23d} was carried out. Hoechst 33258 is a well known minor groove binding agent to double-helical DNA.^{21a} Like EB, the fluorescence intensity of hoechst 33258, in general, increases upon binding with DNA (**Figure 4A.17b**). The crystal structure of hoechst 33258-DNA complex shows that hoechst 33258 binds in the minor groove of DNA. If $^{TNDMB}Py$ binds in the minor groove, then again it would be a competitive binding agent of hoechst 33258 and it would replace hoechst 33258 and thus the fluorescence intensity of hoechst 33258-ct-DNA complex would decrease with increase in $^{TNDMB}Py$ concentration. Unlike the case with EB, we observed a significant decrease in fluorescence intensity ($\lambda_{em} = 468$ nm) of hoechst

33258 of hoechst 33258-ct-DNA complex with increase in ^{TN}DMB^{Py} concentration (**Figure 4A.17b**), indicating clearly that ^{TN}DMB^{Py} is likely to be a minor groove binder of ct-DNA.²⁹ As the excitation wavelength ($\lambda_{\text{ex}} \approx 340$) of both hoechst 33258 and ^{TN}DMB^{Py} are almost same, so we observed another emission band ($\lambda_{\text{em}} \approx 520$) dominates over the parent hoechst's emission band ($\lambda_{\text{em}} \approx 468$) in high concentration of ^{TN}DMB^{Py} (40 to 50 μM). The appearance of this band is due to the binding of ^{TN}DMB^{Py} with ct-DNA though increase in concentration of the probe may play a role.

4A.5.6. Study of Fluorescence Anisotropy to Support Groove Binding Event

Fluorescence anisotropy is a powerful tool in biochemical research and medical testing. When a fluorophore is excited with a polarized light the emission of the fluorophore is also polarized. The extent of polarization of the emission is described in terms of anisotropy (r). Small molecules are weakly polarized due to the rapid tumbling motion in aqueous media. However, when they intercalate to the helix of DNA, their rotational motion would be restricted and therefore the fluorescence polarization would increase. On the other hand, the binding of small molecules to the phosphate backbone or to the DNA grooves would not result in any enhancement of fluorescence polarization.^{30a} To get knowledge about the binding event of ^{TN}DMB^{Py} with ct-DNA steady state anisotropy experiment was also carried out. Thus, we observed an insignificant change of fluorescence anisotropy or the polarization of ^{TN}DMB^{Py}-ct-DNA complex in comparison to free ^{TN}DMB^{Py} in phosphate buffer (**Figure 4A.18**).³⁰ Thus, the groove binding event of the probe was further supported by this experiment.

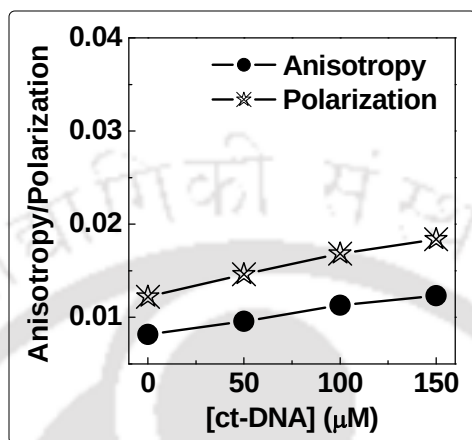


Figure 4A.18: Fluorescence anisotropy and polarization change of ^{TNDMB}Py in presence of various concentrations of ct-DNA. [^{TNDMB}Py] = 50 μM and [ct-DNA] = 50, 100 and 150 μM. $\lambda_{\text{ex}} = 345$ and $\lambda_{\text{max}}^{\text{fl}} = 524$ nm.

4A.5.7. Macromodel Calculation in Support of Minor Groove Binding Event

To support minor groove binding event, we have carried out Macromodel calculation using Schrodinger Macromodel Maestro, version 9.0.³¹ For the optimization we have taken the DNA sequence [5'-d(*CP*GP*CP*GP*AP*AP*TP*TP*CP*GP*CP*G)-3', (PDB Id: 1DNH)] where hoechst 33258 dye enter into the minor groove. Thus, Amber* optimized geometry of our probe triazolylpyrene with the model DNA sequence (**Figure 4A.19**) showed a similar kind of binding event like hoechst 33258 (**Figure 4A.5b**) which supports our experimental observation of minor groove binding event of ^{TNDMB}Py.

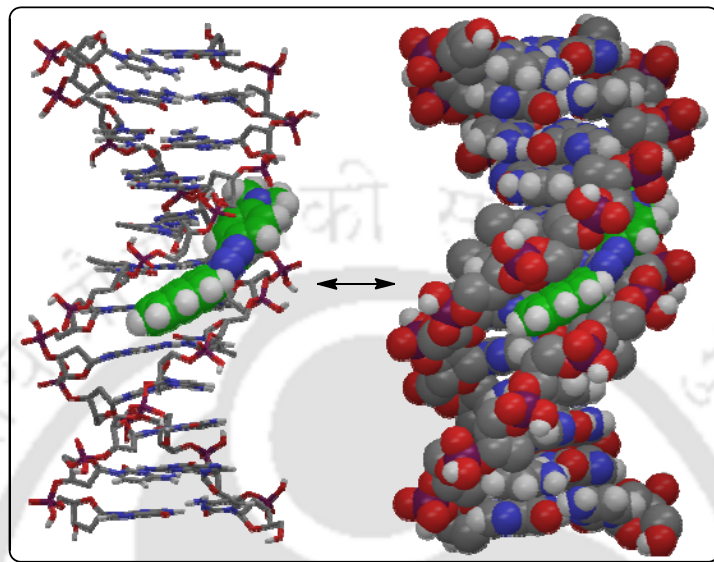


Figure 4A.19: Amber* energy minimized geometry of ^{TNDMPy} with DNA, showing the minor groove binding of the probe ^{TNDMPy}. The DNA sequence was 5'-d(*CP*GP*CP*GP*AP*AP*TP*TP*CP*GP*CP*G)-3', (**PDB Id: 1DNH**).

4A.6. Conclusion

In conclusion, we have successfully shown, for the first time, that the click chemistry derived triazolyl pyrene fluorophore serves as a versatile fluorescent light-up probe for DNA detection in aqueous media. Thus, the probe triazolylpyrene is efficient in sensing DNA biomolecule and its microenvironment *via* the generation of enhanced fluorescence intensity. The binding constant of the probe to ct-DNA was found to be $7.2 \times 10^3 \text{ M}^{-1}$ with free energy of binding, $\Delta G = -5.24 \text{ kcal/mol}$. The probe binds efficiently to the minor groove of ct-DNA is evident from a fluorescence anisotropy study as well as a study from Macromodel. The minor groove binding to ct-DNA was further confirmed by dye (ethidium bromide and hoechst 33258) displacement study. All the spectral evidences open up the possibilities for using our probe, triazolylpyrene, as a highly efficient fluorescent light-up probe for DNA detection. As the DNA minor groove binders are important in consideration of drug-DNA interaction, our probe might find application in pharmaceutical sciences.

4A.7. Experimental Section

4A.7.1. Materials

Calf thymus DNA was purchased from Sigma (USA) and used without further purification. Na_2HPO_4 and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (for preparation of phosphate buffer) were purchased from Merck, India. Water was obtained from a Milli-Q purification system. All experiments were performed with freshly prepared solutions. The probe molecule was synthesized and purified according to the procedure described in **Chapter 2**. We recrystallised the column purified material in chloroform-ethylacetate solvent mixture twice, dried under vacuum, again characterized and used for study.

4A.7.2. Preparation of ct-DNA Solution

The purity of ct-DNA was checked by UV-visible spectroscopy by measuring the ratio of absorbances at 260 nm to 280 nm which was found to be 1.9 indicating that the ct-DNA is sufficiently free of protein.³² The concentration of ct-DNA was measured from UV-visible spectra using molar absorption coefficient (ϵ) of $6600 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm.

4A.7.3. Photophysical Study

4A.7.3.1. UV-Visible Study

The UV-Visible absorbance measurements were performed using Shimadzu UV-2550 UV-Visible spectrophotometer with a cell of 1 cm path length at 298 K. All the UV-Visible studies were carried out in 5 mM phosphate buffer of pH 7.0 containing 5 mM NaCl solution at 298 K. 2-3 % DMF was used to solubilize the probe. The measurements were taken in absorbance mode and the absorbance values of the sample solutions were measured in the wavelength regime of 200–600 nm. All the experiments were carried out by freshly prepared sample solutions.

4A.7.3.2. Fluorescence Study

All fluorescence and steady state anisotropy experiments were performed using a Fluoromax 4 spectrophotometer with a cell of 1 cm path length at 298 K. All the fluorescence studies were carried out in 5 mM phosphate buffer of pH 7.0 containing 5 mM NaCl solution at 298 K. 2-3 % DMF was used to solubilize the probe. The excitation wavelength for probe (^{TN}DMBPy) was set at 343 nm, and emission spectra were measured in the wavelength regime of 350–675.

4A.7.3.3. Melting Temperature (T_m) Measurements

The melting temperature (T_m) of the sample solutions (ct-DNA with probe) were measured in 5 mM sodium phosphate buffer (pH 7.0) containing 5 mM sodium chloride and 1 mM Na₂EDTA. Absorbance vs. temperature profiles were measured at 260 nm using a Shimadzu UV-2550 spectrophotometer equipped with a peltier temperature controller using 1 cm path length cell. The absorbance of the samples was monitored at 260 nm from 20 to 90 °C with a heating rate of 1 °C/min. From these profiles, first derivatives were calculated to determine T_m values.

4A.7.4. Theoretical Study

The Macromodel calculation of the probe with a model DNA (**PDB Id: 1DNH**) was carried out by Maestro, version 9.0 with AMBER* force field in water.³¹

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Chapter 4B

**STUDIES ON THE INTERACTION OF
SMART FLUORESCENT PROBE,
TRIAZOLYL PYRENE (^{TNDMB}Py), WITH
BOVIN SERUM ALBUMIN**

4B.1. Introduction

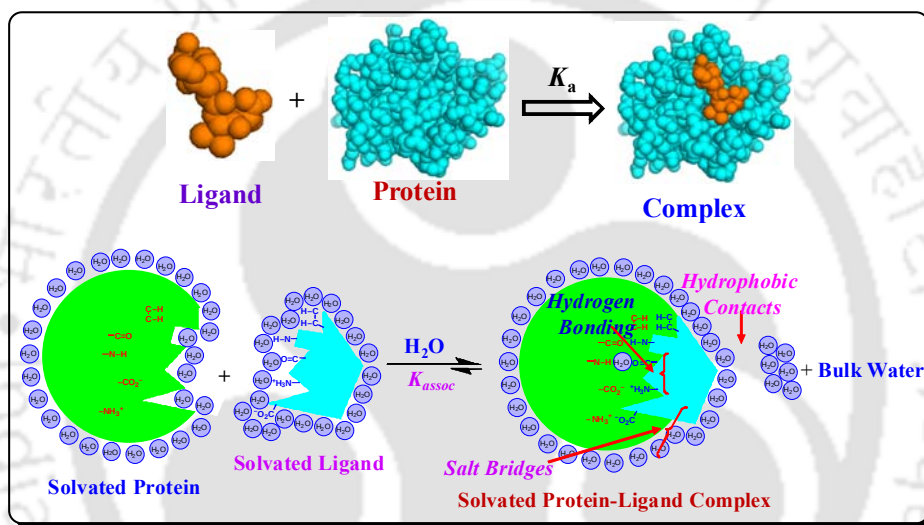
Protein-drug interaction has been found to play a significant role in pharmacology.¹ Understanding the nature of drug-protein interactions or small molecule-protein interaction is of paramount importance for the development of new chemotherapeutics for several diseases and has led the foundation of chemical biology. Thus, with the completion of human genome project, the newer concept of drug discovery research mainly focuses on the molecular design and proteomics. Because of the abundant nature and biological roles they play,² serum albumins, both bovine serum albumin (BSA) and human serum albumin (HAS), have been widely used as models to gain insight into protein-small molecules binding events and to understand primarily the structure, dynamics and functions that ultimately help in designing newer small molecular drug candidates for biological and clinical applications. Therefore, studies on the complexation properties of proteins with synthetic small to large molecules in aqueous media has got extensive research interest, in recent years, because of their great importance in a variety of chemical and biological processes.

4B.2. Background

4B.2.1. Protein Ligand Interaction

Protein-ligand interaction is in general controlled by a complex array of intermolecular interactions. Such interactions depend both on the specific interactions in the binding site as well as non-specific forces outside the binding pocket. The complexity of interactions between proteins and flexible target molecules, including other proteins, nucleic acids and small molecules is often determined by the considerable flexibility of the protein binding sites and by the structural rearrangements of the bound molecule. The process of forming a complex between a small molecule and a protein is a complex equilibrium process (**Scheme 4B.1**). A solvated ligand likely exists as an equilibrium mixture of several conformers.

Similarly the solvated protein also exists as several conformers in equilibrium. To form a protein-ligand complex the solvent molecules that occupy the binding site are displaced by the ligand. Tight complexes will result when the protein-ligand interactions are significantly stronger than the interactions of the protein alone and ligand alone with solvent molecules. **Scheme 4B.1** represents the typical protein ligand complexation phenomenon.



Scheme 4B.1: Schematic representation of protein-ligand complexation.

4B.2.2. Bovine Serum Albumin (BSA): Widely Used Model Protein

Serum albumins, the major soluble and transport proteins³ found in blood plasma, have many physiological functions, for example, they serve as transporters for a variety of important compounds like fatty acids, bilirubin, bile salts, lecithin nutrients, steroids, certain metal ions, hormones and a variety of therapeutic drugs.⁴ Extensive research has been carried out to investigate the structure, properties, functions of serum albumins and their interactions with other proteins, drugs and bioactive small molecules. Among the various serum albumins, bovine serum albumin (BSA) is one of the most studied model proteins, because of its easy availability and structural homology with human serum albumin (HSA).⁵ Although the crystal structure of BSA

is not well established, the homology of various binding pockets with HAS was established through various investigations. The sequence of BSA exhibits 75% identity and 87% similarity with HSA. It is roughly a heart shaped protein, and has three domains I, II and III. Each domain is divided into two sub domains IA, IB; IIA, IIB; and IIIA, IIIB. The specific physiological activity of the ligands upon binding with serum albumin originates from the presence of two major and structurally selective binding sites, namely site-I and site-II. Site-I is situated in the hydrophobic core of sub domain IIA, whereas site-II is in the sub domain IIIA.⁶ The extensive studies on the drug binding event reveal that site-I is dominated by strong hydrophobic interactions while the interaction at site-II involve a combination of hydrophobic, hydrogen bonding and electrostatic interactions.⁷ The ligand bounded at site-I is located in the vicinity of tryptophan, which can serve as an efficient energy donor. **Figure 4B.1** shows the structures of BSA and HAS and the positions of their various homologous domain and sub domain.

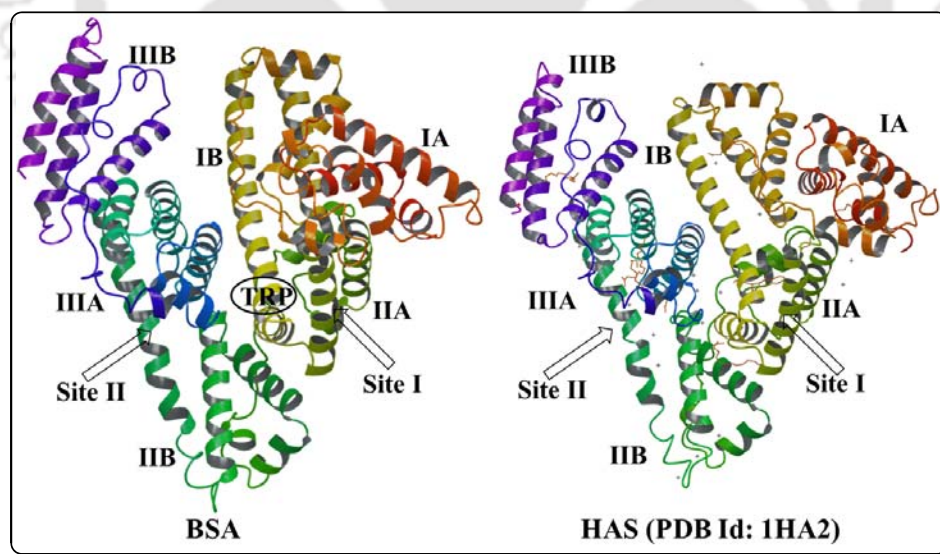


Figure 4B.1: Structure of BSA and HAS and their various homologous domain.

4B.2.3. Some Reported Small Molecule Probes of BSA

As was discussed earlier, bovine serum albumin (BSA) is one of the most transport proteins³ found in the blood plasma. BSA is capable of binding, transporting and delivering an extraordinarily diverse range of endogenous and exogenous compounds like fatty acids, nutrients, steroids, certain metal ions, hormones and a variety of therapeutic drugs⁸ in the bloodstream to their target organs.⁹ To get insight into the structure and binding event of BSA with the drugs and small molecules, fluorescence based studies have been widely used since the past decades. Thus, through the years, proteins binding ability of several small molecules and drugs, chromophores have been studied *via* photophysical properties with a distinct fluorescence signal generation.¹⁰

Thus, Tang *et al.*,^{11a} have studied the interaction of tetraphenylethylene derivatives (**4B.1-4B.4**, **Figure 4B.2**) with BSA by fluorescence technique. Fluorescence spectroscopic investigation showed that the emission intensity of the tetraphenylethylene derivatives increased upon binding with BSA. They proposed that the enhancement of fluorescence is due to restriction of intra-molecular rotations of the dyes in the aggregates and complexes.

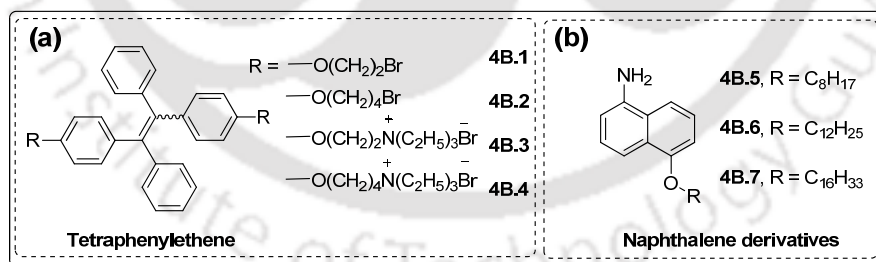


Figure 4B.2: (a) Tetraphenylethylene and (b) naphthalene derivatives as fluorescent probes of BSA.

Novel amphiphilic fluorescent probes have been designed and synthesized (**4B.5-4B.7**, **Figure 4B.2**) for selective detection of BSA *via* non-covalent interactions with enhancement of fluorescence signal by Das and co-workers.^{11b} The intrinsic weak fluorescence of the probes in an aqueous medium was found to be increased

dramatically with addition of BSA, while responses to various other proteins/enzymes were negligible within the same experimental condition.

Guchhait *et al.*,^{11c} investigated the binding interaction of a flexible charge transfer fluorophore, 5-(4-dimethylamino-phenyl)-penta-2,4-dienitrile (DMAPPDN, **4B.8**, **Figure 4B.3**) with BSA *via* spectroscopic investigation as well as theoretical study. From the fluorescence experiment it was observed that the fluorescence intensity of DMAPPDN increased with a 78 nm of blue shift of emission wavelength in presence of increased concentration of BSA in aqueous buffer solution. The binding constant of the DMAPPDN-BSA complex has been calculated and found to be $8.821 \pm 0.01 \times 10^3 \text{ M}^{-1}$ with free energy of binding $-5.359 \text{ kcal mol}^{-1}$.

Squaraine derivatives, such as, bis(2,4,6-trihydroxyphenyl) squaraine, bis(3,5-dibromo-2,4,6-trihydroxyphenyl) squaraine and bis(3,5-diiodo-2,4,6-trihydroxyphenyl) squaraine (**4B.9-11**, respectively; **Figure 4B.3**) have been found to be effective fluorescent probe of both BSA and HAS. Thus, Ramaiah *et al.*,^{11d} have reported the interaction behaviour of some squaraine dyes with serum albumins by studying photophysical properties. The fluorescence spectroscopic investigations revealed that in presence of serum albumins the emission intensities of the dyes increased strongly. They inferred the interaction event as a strong binding of the probe within the hydrophobic pocket of the proteins leading to an enhancement of fluorescence intensity.

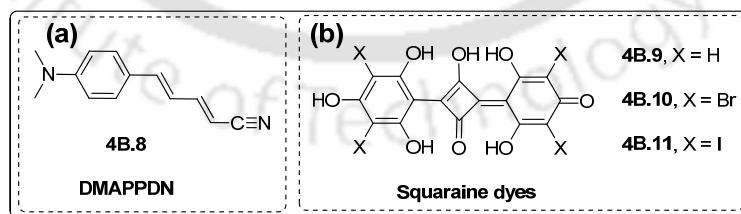


Figure 4B.3: Examples of protein recognition small molecule probes for BSA.

Curcumin, a natural product, is found in the rhizome of *Curcuma longa* and it possesses diverse pharmacological potencies such as effective antioxidant,

antidiabetic, anti-inflammatory and antimicrobial/antiviral properties.^{11e-g} The compound is also considered as a cancer chemo-preventive agent. Thus, because of the potent activities, the isoxazole and diacetyl derivatives of curcumin have got attention in studying the interactions with BSA. As for example, Dasgupta *et al.*,^{11h} have reported the interaction of curcumin derivatives, diacetylcurcumin and isoxazolecurcumin (**4B.12-13**, **Figure 4B.4**) with BSA on the basis of FT-IR, absorption and circular dichroism (CD) study. The absorbance of diacetylcurcumin and isoxazolecurcumin were found to decrease with increasing concentration of BSA. The observed binding constant was found in the range of 10^4 M^{-1} . Warfarin displacement study confirmed the binding of these two curcumin derivatives to the hydrophobic pocket of the subdomain IIA in BSA. This study with curcumin derivatives may provide promise in the design of many such potential fluorescent probes that could associate more strongly with serum albumins.

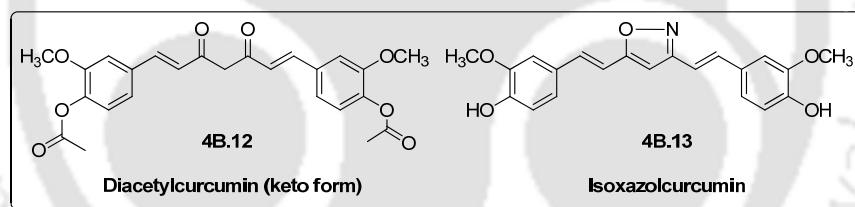


Figure 4B.4: Chemical structure of diacetylcurcumin and isoxazolecurcumin.

4B.3. Objective

With this background and the observed binding events with an enhanced fluorescence signal of our designed dual fluorescent probe, triazolyl pyrene (^{TNDBM}Py) with calf thymus DNA motivated us to study its interaction behaviour with BSA. We envisaged that binding event would result in an enhanced fluorescence signal generation upon binding to the hydrophobic pocket of BSA (**Figure 4B.5**). Thus we framed our objective to study the following:

- (a) Study of UV-Visible and fluorescence photophysical properties of the probe in presence of BSA.

- (b) Determination of binding constant.
- (c) Study of binding mode by steady state and time resolved fluorescence anisotropy experiments.
- (d) Study of energy transfer between the donor tryptophan (TRP) unit of BSA and acceptor probe by calculation of Förster distance.
- (e) Finally, a docking study can substantiate the experimental observation.

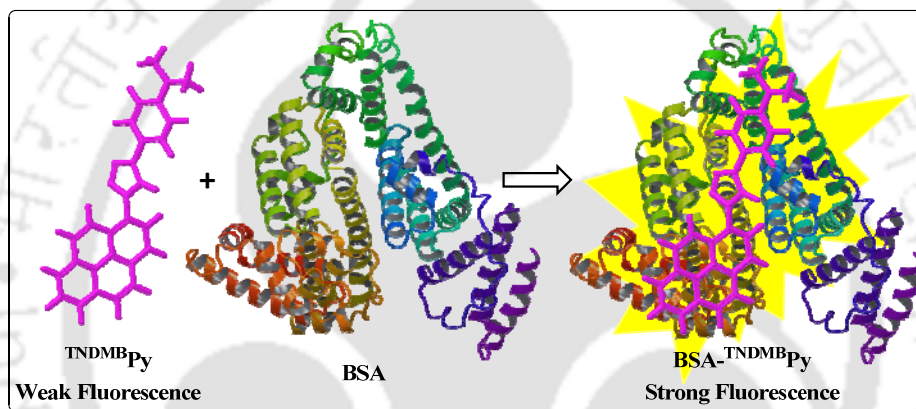


Figure 4B.5: Graphical representation of binding interaction of ^{TNDMB}Py with BSA.

4B.4. Results and Discussion

To examine the protein sensing ability of our probe, first, we have investigated the interaction behaviour of our probe with BSA protein by UV-visible and fluorescence spectroscopy in aqueous phosphate buffer (pH 7.0) at 298 K.

4B.4.1. UV-Visible and Fluorescence Study

The UV-visible and fluorescence spectra of ^{TNDMB}Py in aqueous phosphate buffer of varying BSA concentrations were recorded at physiological pH of 7 at 298 K. Thus, upon addition of increasing amount of BSA to the probe's solution, we observed a hyperchromicity with a very little (7 nm) blue shift of the absorbance band as is clear from the UV-visible spectra (**Figure 4B.6a**). To get further insight into the

binding properties and to investigate the protein sensing ability of the probe, we then investigated the fluorescence changes upon addition of various concentrations of BSA to the solution of the probe upon excitation at 345 nm. Thus, with increasing concentration of BSA, both the fluorescence intensity and quantum yield of the fluorophore, ^{TN}DMBPy increased with a blue shift of emission maxima by 41 nm (Figure 4B.6b and 4B.7a). The fluorescence enhancement was maximized upon addition of 25 μ M (2.5 equiv.) of BSA with respect to 10 μ M of probe. It is the probe's intrinsic property that a blue shift of emission maxima have been observed on moving from the high polar solvent to low polar one as was revealed from the fluorescence photophysical property of the probe. Thus, our result on fluorescence emission of the probe upon interaction with BSA indicated a clear binding of the probe in more hydrophobic pocket of BSA which is also evident from the intrinsic emission behaviour of the probe in various solvents of varying dielectric constants.^{12,13,14} The summary of photophysical properties of the probe, ^{TN}DMBPy in presence of various concentrations of BSA is shown in Table 4B.1.

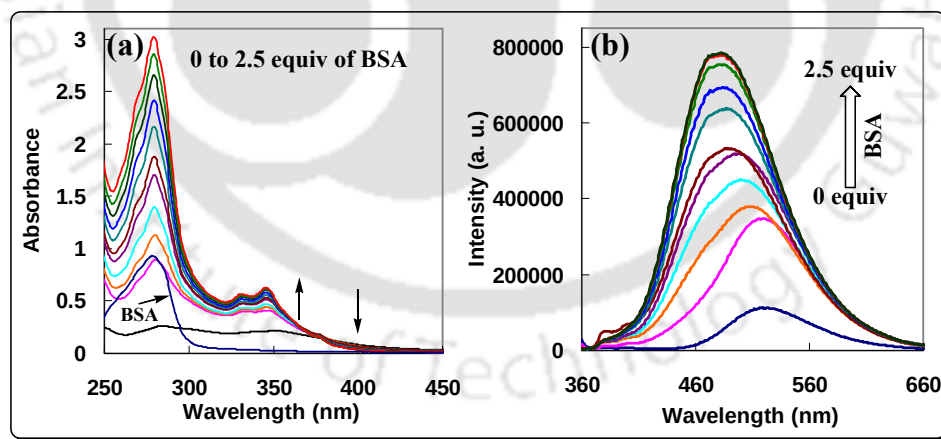


Figure 4B.6: (a) UV-visible and (b) fluorescence spectra ($\lambda_{\text{ex}} = 345$ nm) of ^{TN}DMBPy in presence of increasing BSA concentration at 298K. [^{TN}DMBPy] = 20 μ M and [BSA] = 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 μ M.

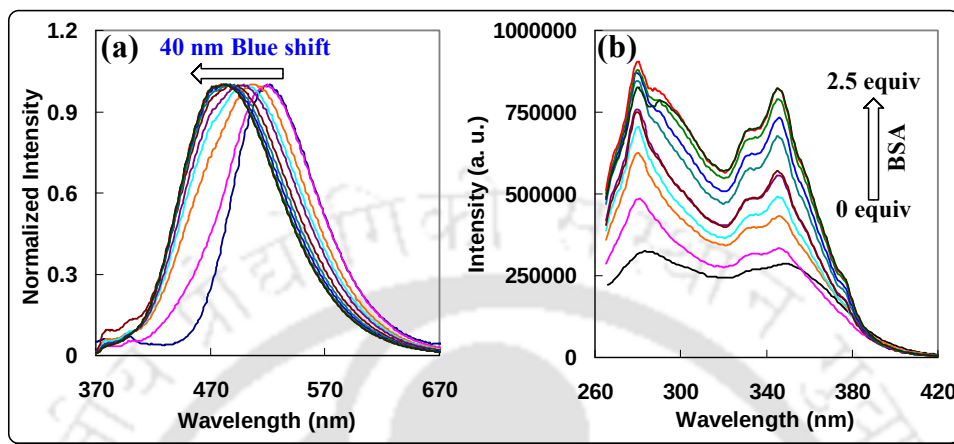


Figure 4B.7: (a) Normalized emission spectra ($\lambda_{\text{ex}} = 345 \text{ nm}$) and (b) fluorescence excitation spectra of TNDMB-Py in presence of increasing BSA concentration at 298K. $[\text{TNDMB-Py}] = 20 \text{ }\mu\text{M}$ and $[\text{BSA}] = 0, 5, 10, 15, 20, 25, 30, 35, 40, 45$ and $50 \text{ }\mu\text{M}$.

Table 4B.1: Summary of photophysical properties of TNDMB-Py in presence of various concentrations of BSA

BSA : TNDMB-Py	$\lambda_{\text{abs}}^{\text{max}}$ (nm)	$\lambda_{\text{em}}^{\text{max}}$ (nm)	Φ
0 : 1	352	524	0.027
0.25 : 1	348	522	0.055
0.5 : 1	348	511	0.057
0.75 : 1	347	504	0.064
1 : 1	347	500	0.067
1.25 : 1	346	494	0.067
1.5 : 1	346	490	0.073
1.75 : 1	345	488	0.075
2 : 1	345	485	0.077
2.25 : 1	345	483	0.077
2.5 : 1	345	483	0.077

The blue shift of emission wavelength was also clear from a change of colour of probe solution from yellowish green to bright blue colour of probe-BSA solution when irradiated at 254 nm under a transilluminator (**Figure 4B.8**).

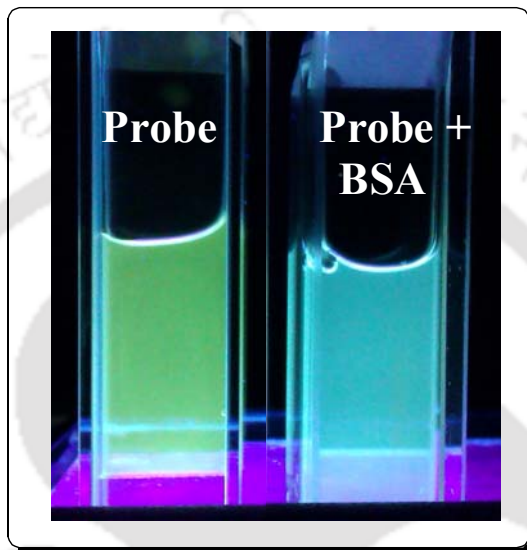


Figure 4B.8: Fluorescence image under UV-transilluminator (254 nm) of only probe (50 mM) and in presence of BSA (2.5 eqv.).

We have also studied the absorption and emission of tryptophan (TRP) of BSA titrated with various concentration of the probe which showed an interesting emission properties. Thus, with increasing concentration of the probe, the emission intensity of the peak corresponding to BSA decreased while intensity of the emission corresponding to ^{TNDB}Py naturally increased (**Figure 4B.9b**). The UV-visible spectra showed an increase in of BSA absorbance as the concentration of the probe was increased (**Figure 4B.9a**). The excitation spectra monitored at BSA's and probe's emission maxima are shown in **Figure 4B.9c** and **Figure 4B.9d** respectively.

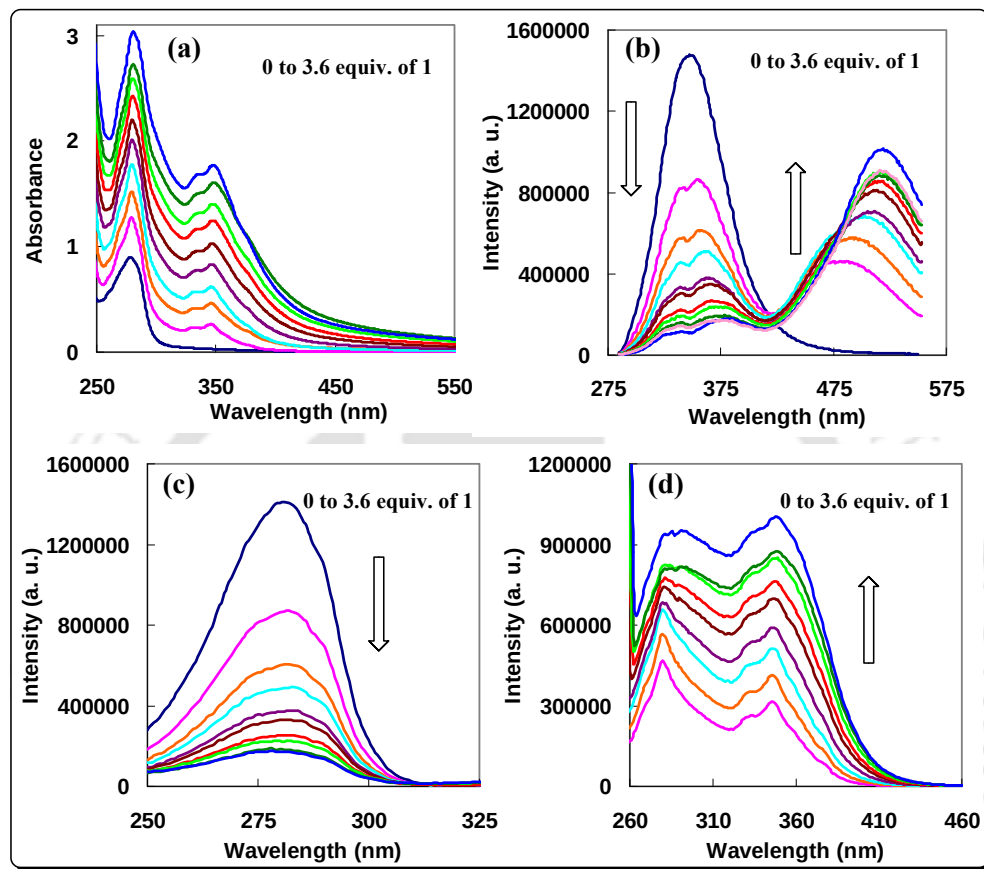


Figure 4B.9: (a) UV-visible and (b) fluorescence spectra ($\lambda_{\text{ex}} = 280 \text{ nm}$) of BSA in presence of increasing TNDMBPy concentration at 298K. Fluorescence excitation spectra of BSA in presence of increasing TNDMBPy concentration monitoring at (c) BSA's emission peak ($\lambda_{\text{max}}^{\text{fl}} = 350 - 375 \text{ nm}$) and (d) TNDMBPy 's emission spectra ($\lambda_{\text{max}}^{\text{fl}} = 521 - 484 \text{ nm}$) at 298 K. $[\text{BSA}] = 20 \mu\text{M}$ and $[\text{TNDMBPy}] = 0, 8, 16, 24, 32, 40, 48, 56, 64$ and $72 \mu\text{M}$.

4B.4.2. Determination of Protein–Probe Binding Constant

The binding constant (K) of the protein-probe complex was determined spectroscopically by well-known Benesi-Hildebrand plot (**Figure 4B.10**) using the following equation 1,

$$\frac{1}{(I - I_0)} = \frac{1}{(I_\infty - I_0)} + \frac{1}{(I_\infty - I_0)K[BSA]} \quad \dots\dots\dots (1)$$

where I_0 , I and I_∞ are the emission intensities of ^{TN}DMB^BPy in the absence of BSA, and in the presence of intermediate and at an infinite concentration of BSA, respectively. From the slope (linear region's data points) of the plot of $1/(I - I_0)$ vs. $1/[BSA]$ the binding constant K was determined, which was found to be $5.1 \times 10^4 \text{ M}^{-1}$. Next, the free energy of binding (ΔG) was calculated using equation 2, which was found to be $\Delta G = -6.41 \text{ kcal/mol}$.

$$\Delta G = -RT \ln K \quad \dots\dots\dots (2)$$

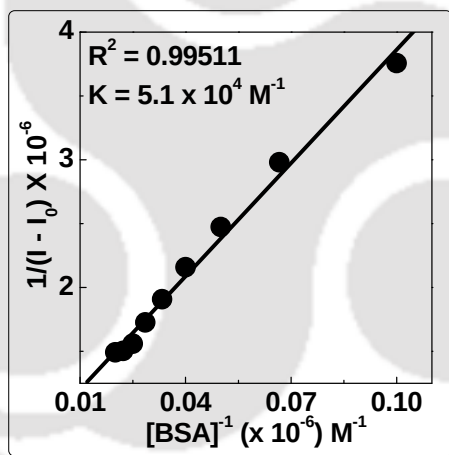


Figure 4B.10: Benesi–Hildebrand plot of ^{TN}DMB^BPy in presence of increasing concentration of BSA at 298K.

4B.4.3. Circular Dichroism (CD) Study

CD experiment was also carried out to get insight into the structural perturbation of BSA if at all in presence of the probe. BSA exhibits two negative bands in the far UV region at 208 and 222 nm characteristics of α -helices. The α -helix content of free BSA is within the range observed in other studies.¹¹ Thus, an investigation of CD spectra of BSA in presence and absence of the probe also revealed the same band

position. However, slight increase in % of α -helicity in presence of the probe was observed that can be attributed to conformational adjustments on complex formation (**Figure 4B.11**).^{11h} No induced CD was found by adding BSA to the probe from 300 to 500 nm under the experimental condition.

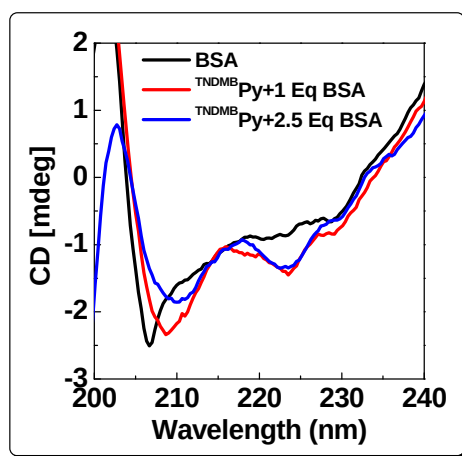


Figure 4B.11: CD spectra of BSA in presence and absence of probe.

4B.4.4. Steady State Anisotropy and Time Resolved Fluorescence Anisotropy Study

The steady state fluorescence anisotropy study can explain the nature of surrounding environment of the probe and its interaction with protein in a much clear way than the steady state fluorescence. Experimentally, the anisotropy/polarization is determined from measurements of fluorescence intensities parallel and perpendicular with respect to the plane of linearly polarized excitation light, and is expressed in terms of fluorescence anisotropy (r) or polarization (P). In aqueous medium the small molecules are weakly polarized due to the rapid tumbling motion. However, when they bind with protein, the rotational motion of the probe molecules would restrict by the binding protein and thereby the fluorescence polarization would increase. Thus, upon binding with BSA the probe moves from the free aqueous environment to a rigid protein environment. The anisotropy experiment also reflects our expected result.

With increasing concentration of BSA the free motion of the probe is restricted by rigid environment of the protein and thus anisotropy values increases. Thus, the protein bound probe experiences a more rigid environment compared to its free aqueous environment and we observed a change in anisotropy value (r_0) from 0.018 in free buffer to 0.201 in 2.5 equivalent of BSA. At the same time, the polarization value changes from 0.027 in free buffer to 0.273 in 2.5 equivalent of BSA (**Figure 4B.12a**). The time resolved fluorescence anisotropy decay analysis was also carried out, which showed the increase in anisotropy, $r(t)$ from ~ 0 in free buffer to ~ 0.21 in 40 μM of BSA (**Figure 4B.12b**). At the same time the anisotropy decay time increases from 1.99 ns in free buffer to 23.7 ns in 40 μM of BSA (**Table 4B.2**). Thus, from above result it is evident that the probe is involved in tight binding inside the hydrophobic pocket of BSA and experiences a highly restricted rotational motion.¹⁵

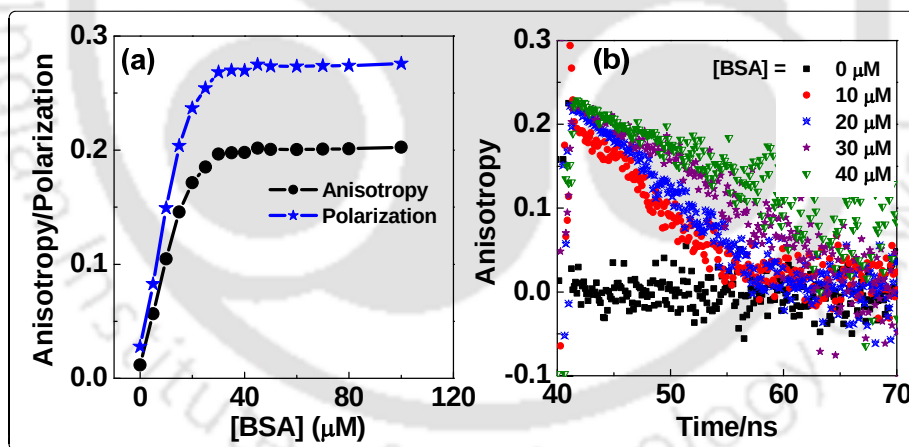


Figure 4B.12: (a) Fluorescence anisotropy and polarization change of TNDMBPy in presence of various concentrations of BSA at 298K. $[\text{TNDMBPy}] = 20 \mu\text{M}$ and $[\text{BSA}] = 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80$ and $100 \mu\text{M}$. $\lambda_{\text{ex}} = 345$ and $\lambda_{\text{max}}^{\text{fl}} = 524, 522, 511, 504, 500, 494, 490, 488, 485, 483, 483, 483, 483, 483$ and 483 nm . (b) Time resolved fluorescence anisotropy decay of TNDMBPy in presence of various concentrations of BSA. $[\text{TNDMBPy}] = 20 \mu\text{M}$ and $[\text{BSA}] = 0, 10, 20, 30, 40 \mu\text{M}$. $\lambda_{\text{ex}} = 375 \text{ nm}$ lasers and $\lambda_{\text{max}}^{\text{fl}} = 524, 511, 500, 490, 485 \text{ nm}$.

Table 4B.2: Time resolved anisotropy decay time of ^{TN^{DMB}}Py in presence of various concentrations of BSA.

[BSA] (μM)	$r_0^{[a]}$	τ_I (ns)
0	0.011	1.99
10	0.105	6.99
20	0.171	9.36
30	0.196	18.38
40	0.198	23.79

^[a]Steady state anisotropy value**4B.4.5. Fluorescence Resonance Energy Transfer (FRET) and Förster Distance**

The lipophilic nature of the probe and the results of fluorescence anisotropy indicated that it would preferentially bind to the hydrophobic sub-domain of BSA, thus might involve in energy transfer with Trp (Tryptophan) unit.¹⁴ The possibility of energy transfer process from Trp to the probe molecule is satisfied primarily by the spectral overlap between the fluorescence emission spectrum of BSA and the UV-visible absorption spectrum of the probe (**Figure 4B.13a**). Thus, upon excitation at donor wavelength (280 nm of Trp of BSA), we observed quenching of fluorescence of BSA and enhancement of fluorescence of the acceptor probe at long wavelength (490-514 nm). This result indicated the occurrence of a FRET process (**Figure 4B.13b**).

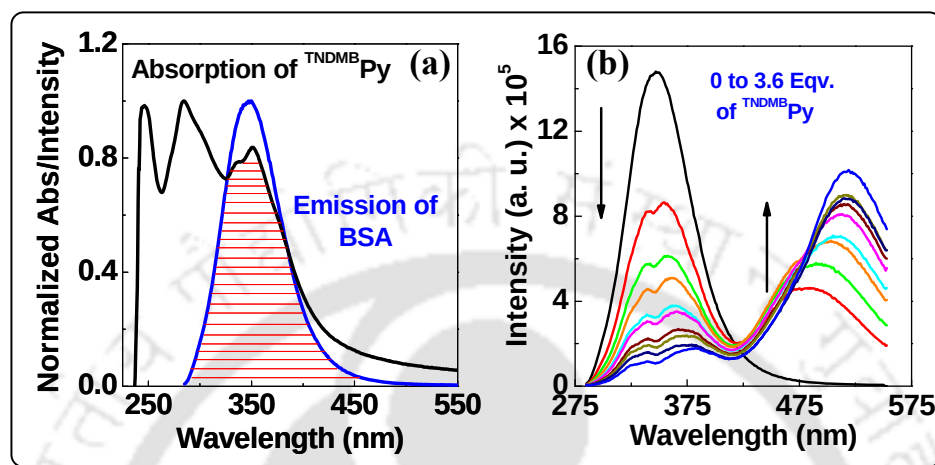


Figure 4B.13: (a) Overlap spectra of BSA's emission and ^{TNDMBPy}'s absorption and the concentrations were 20 μM each. (b) Fluorescence spectra ($\lambda_{\text{ex}} = 280 \text{ nm}$) of BSA in presence of increasing ^{TNDMBPy} concentration at 298 K. [BSA] = 20 μM and [^{TNDMBPy}] = 0, 8, 16, 24, 32, 40, 48, 56, 64 and 72 μM .

The fluorescence resonance energy transfer (FRET) process occurs when the emission spectra of one molecule (say, protein), called the donor, overlap with the absorption spectra of another molecule (say, probe), called the acceptor and the donor and acceptor are within the characteristic Förster distance. The efficiency of energy transfer, E , is calculated using the equation 3,

$$E = 1 - \frac{I}{I_0} = \frac{R_0^6}{R_0^6 + r^6} \dots\dots\dots (3)$$

where I and I_0 are the fluorescence intensities of the donor in the presence and absence of acceptor, r is the distance between the donor and acceptor and R_0 is the critical distance when the energy transfer efficiency is 50% .

The Förster distance R_0 (Å) was calculated by the following equation 4,¹⁶

$$R_0 = 0.21 [\kappa^2 n^{-4} \Phi_f J(\lambda)]^{1/6} \dots\dots\dots (4)$$

where κ^2 is the orientation, n is the refractive index of the medium, Φ_f is the quantum yield of the donor in the absence of acceptor, $J(\lambda)$ is the overlap integral of the

fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor given by the following equation 5,

$$J = \frac{\int_0^\infty F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda}{\int_0^\infty F_D(\lambda) d\lambda} \dots\dots\dots (5)$$

where $F_D(\lambda)$ is the fluorescence intensity of the donor in the wavelength range λ to $\lambda + \Delta \lambda$ with the total intensity normalized to unity. $\varepsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor as a function of wavelength (λ).

The efficiency of energy transfer (E) from donor to acceptor was calculated using the equation 3, and it was found that there was 62.3% efficiency of energy transfer from donor (tryptophan) to acceptor (^{TNDMB}Py). Using the values of $\kappa^2 = 2/3$, $n = 1.33$, $\Phi_f = 0.084$, and the obtained overlap integral, $J(\lambda) = 1.3255 \times 10^{14}$ the Förster distance (R_0) and r values were calculated which were found to be $R_0 = 24.37 \text{ \AA}$ and $r = 22.41 \text{ \AA}$.

4B.4.6. Molecular Docking Study

Docking calculations were carried out using DockingServer¹⁷ (Bikadi, Hazai, 2009). The amino acid sequence of BSA protein was observed from the NCBI website, <http://www.ncbi.nlm.nih.gov/protein/CAA76847.1>. The 3D model of the BSA protein was built using the 3D structure 1AO6 chain 'A' as template using the ESyPred3D¹⁸ web server. This template shares 72.4% identities with the BSA sequence. Following is the BSA sequence which was used to generate the 3D model.

```
>gi|3336842|emb|CAA76847.1| bovine serum albumin [Bos taurus]
MKWVTFISLLLLFSSAYSARGVFRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQ
YLQQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLR
ETYGDMADCCEKQEPERNECFLSHKDDSPDLPKLPDPNTLCDEFKADEKKF
WGKYL YEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETM
REKVL TSSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTD
LTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCI
```


AEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYA
VSVLLRLAKEYEATLEECCA KDDPHACYSTVFDKLKHLVDEPQNLIKQNC DQ
FEKLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMP
CTEDYLSLILNRLCVLHEKTPVSEKVT KCCTESLVNRRPCFSALTPDETYVPK
AFDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQLKTVMENFVA
FVDKCCAADDKEACFAVEGPKLVVSTQTALA

Among the various conformation of docking result of the ^{TN}DMBPy with BSA, the energy minimized docking pose with rank-1 (**Table 4B.3**) is shown in **Figure 4B.14**. The minimum energy conformer showed that the ligand binds to the hydrophobic pocket of sub-domain IIA in site-I. From the result it was also observed that the ligand molecule is surrounded by the hydrophobic amino acid side chain. Thus, it is concluded that the interaction between ^{TN}DMBPy and BSA is strongly hydrophobic in nature. It was also observed that the molecule is located in the vicinity of tryptophan (Trp-237). Thus this result supports the possible occurrence of FRET process between Trp (BSA) and the probe, ^{TN}DMBPy. The free energy of binding (ΔG) of ^{TN}DMBPy-BSA complex obtained from the docking calculation is $-7.72 \text{ kcal mol}^{-1}$ which is close to our experimental value of $-6.41 \text{ kcal mol}^{-1}$ calculated from the binding constant value (K) obtained from Benesi–Hildebrand plot.

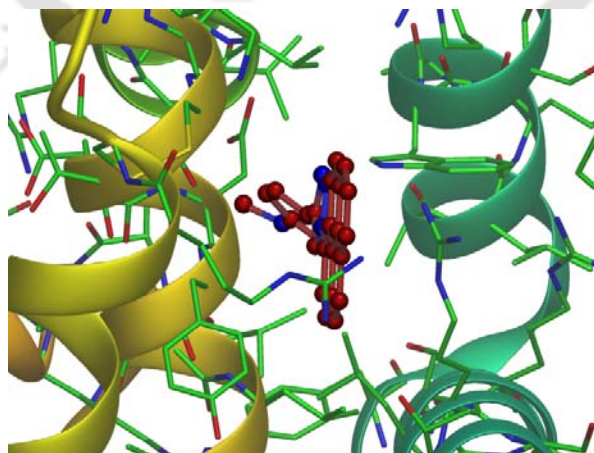
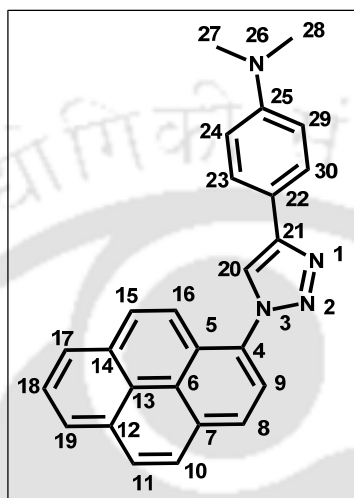


Figure 4B.14: Docking pose of ^{TN}DMBPy in presence of BSA.

Table 4B.3: Docking result of ^{TNDMB}Py-BSA complex obtained from DockingServer

Rank	Binding energy (kcal mol ⁻¹)	K _i (mM)	Total Intermolecular Energy (kcal mol ⁻¹)
1	-7.72	0.002	-8.84
2	-6.07	0.035	-6.98
3	-6.01	0.039	-7.24
4	-0.68	318.88	-1.62
5	-0.02	969.85	-1.14

Table 4B.4 shows the different types of interaction (polar, hydrophobic, $\pi-\pi$ etc.) of ^{TNDMB}Py with BSA obtained from docking calculation.

Table 4B.4: Various interactions of ^{TN}DMB^{Py} with BSA obtained from DockingServer.

Polar	Hydrophobic	$\pi - \pi$	Other	
N2- ARG218	C3- LEU221	C12-TRP237	C23-THR214	C25- SER367
N3- ARG218	C12- LEU221	C14-TRP237	C6- ARG218	C17- ARG459
N1- TRP237	C13- LEU221	C13- RP237	C16-ARG218	C19- ARG459
N1- ASP474	C26- TRP237	C7- TYR475	C1- ARG218	C20- ARG459
	C26- LEU370	C16-TYR475	C7- ARG218	C15- ASP474
	C6- LEU478	C23- TYR475	C5- ARG218	N2- ASP474
	C16- LEU478		C8- ARG218	N3- ASP474
	C25- LEU504		C15- ARG218	N1- ASP474
	C26- LEU504		C21- ARG218	C2- ASP474
			C2- TRP237	C4- ASP474
			C3- TRP237	C10- ASP474
			C12- TRP237	C10- SER477
			C13- TRP237	C11- SER477
			C10- SER367	C14- SER477
			C11- SER367	N4- LEU504

4B.5. Conclusion

In conclusion, we have successfully shown, for the first time, that the click chemistry derived triazolyl fluorophore serves as a versatile fluorescent light-up probe for protein detection in the aqueous media. Thus, the probe triazolylpyrene is efficient in sensing biomolecules and their micro environment *via* the generation of enhanced fluorescence intensity. Slight blue shift of the absorption band (7 nm) and blue shift of emission maxima (41 nm) of the fluorophore with enhancement of intensity upon addition of BSA indicate spontaneous complexation with a high binding constant, $K = 5.1 \times 10^4 \text{ M}^{-1}$. Steady state and time resolved anisotropy of the probe in the presence of BSA infer tight binding of the probe in the restricted hydrophobic protein interior. FRET and molecular docking studies suggest efficient binding and proper orientation of the probe within the hydrophobic cavity of sub domain IIA (site-I). The experimental free energy of binding ($\Delta G = -6.41 \text{ kcal/mol.}$) is in close agreement with the calculated free energy of binding ($\Delta G = -7.72 \text{ kcal/mol.}$) from molecular docking calculation. Overall, our study and both the experimental and theoretical results open up the possibilities for using our probe as a highly efficient fluorescent light-up bioprobe for further applications.

4B.6. Experimental Section

4B.6.1. Materials

BSA, Na_2HPO_4 and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (for preparation of phosphate buffer) were purchased from Merck, India and used without further purification. Water was obtained from a Milli-Q purification system. All experiments were performed with freshly prepared solutions. The probe molecules was synthesized and purified according to the procedure describe in **Chapter 2**. We recrystallized the column purified material in chloroform-ethylacetate solvent mixture twice, dried under vacuum, again characterized and used for the study.

4B.6.2. Preparation of BSA Solution

The BSA solution was prepared in Milli-Q water and the concentration was measured from UV-visible spectra using molar absorption coefficient (ϵ) of $43,800 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm.¹⁹

4B.6.3. Photophysical Study

4B.6.3.1. UV-Visible Study

The UV–Visible absorbance measurements were performed using Shimadzu UV-2550 UV–Visible spectrophotometer with a cell of 1 cm path length at 298 K. All the UV-Visible studies were carried out in 5 mM phosphate buffer of pH 7.0 containing 5 mM NaCl solution at 298 K. 2-3 % DMF was used to solubilize the probe. The measurements were taken in absorbance mode and the absorbance values of the sample solutions were measured in the wavelength regime of 200–600 nm. All the experiments were carried out with freshly prepared sample solutions.

4B.6.3.2. Fluorescence Study

All fluorescence and steady state anisotropy experiments were performed using a Fluoromax 4 spectrophotometer with a cell of 1 cm path length at 298 K. All the

fluorescence studies were carried out in 5 mM phosphate buffer of pH 7.0 containing 5 mM NaCl solution at 298 K. 2-3 % DMF was used to solubilize the probe. The excitation wavelength for probe (^{TNDB}Py) was set at 343 nm, and emission spectra were measured in the wavelength regime of 350–675 nm. Steady state anisotropy of the solutions was measured using Fluoromax 4 spectrophotometer. Time resolved fluorescence anisotropy decay were measured using Life Space-II (Edinburgh Instruments) time resolved fluorescence spectrophotometer. The fluorescence quantum yields (Φ) were determined using quinine sulphate as a reference with the known Φ (0.54) in 0.1 molar solution in sulphuric acid.

4B.6.4. Theoretical Study

Docking calculations were carried out using DockingServer¹⁷ (Bikadi, Hazai, 2009). The amino acid sequence of BSA protein was observed from the NCBI website, <http://www.ncbi.nlm.nih.gov/protein/CAA76847.1>. The 3D model of the BSA protein was built using the 3D structure 1AO6 chain 'A' as template using the ESyPred3D¹⁸ web server. This template shares 72.4% identities with the BSA sequence. Following is the BSA sequence which was used to generate the 3D model.

>gi|3336842|emb|CAA76847.1| bovine serum albumin [Bos taurus]

MKVVTFISLLLLFSSAYSRGVFRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQ
YLQQCPFDEHVKLVLNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLR
ETYGDMADCCEKQEPERNECFLSHKDDSPDLPKLPDPNTLCDEFKADEKKF
WGKYLVEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETM
REKVLTSARQLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTD
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Chapter 5

**FLUORIMETRIC SENSING OF
MICELLER MICROENVIRONMENT
AND Cu²⁺ ION WITH SMART
FLUORESCENT PROBE, TRIAZOLYL
PYRENE (^{TNDMB}Py)**

5.1. Introduction

The study of cell membrane and the role it plays in living cells is necessary to understand cellular function. It is also important to study drug-membrane interaction with a model system which is a real mimic of cell membrane. Because micelles are amphiphilic molecules, like lipids, some of the same rules governing lipid behaviour may also apply to the micelle. Therefore, micelles have been used widely as a model of biomembrane to acquire information about structure, microenvironmental properties and function. Thus, probing of micellar microenvironment with fluorescence sensor molecules or ions is of paramount importance to know the structure and to modulate the charge distribution on the interface and thereby to correlate those with real membrane system. With the introduction of a proper fluorescent probe that is very sensitive to microenvironmental dielectric properties and viscosity, one can study the process of micellisation and also the effect of interfacial electric field on the probe thereby enabling correlation with the biomembrane.

On the other hand, fluorescence chemosensors for sensing of metal ions have attracted attention due to their high sensitivity and selectivity towards analytes and easy handling in solution phase.¹ Therefore, the design and synthesis of fluorescent chemosensors for some physiologically essential trace metal ions like Cu^{2+} and some hazardous metal species present in water like Pb^{2+} , As^{3+} , etc., are currently an active research target for biological² and environmental applications. Cu^{2+} ion has attracted considerable attention since many years as this metal ion is not only a significant environmental pollutant^{3a-c} but also the third most abundant and an essential trace element^{3b-d}. As a result of extensive research efforts several fluorescent probes have been developed for the detection of Cu^{2+} ion from organic and aqueous media.⁴ Efforts have also been put forth for the same inside the cell *via* fluorescence imaging technique.

In general, sensing/detection of metal ions require a designed fluorophore with a metal anchoring unit. Nitrogenous ligands are, therefore, in many cases found to be

utilized as binding sites for the selective recognition of soft metal ions. In this respect “click triazole” might be an excellent choice. In the present day of chemistry the “click triazole” has been frequently utilised as spacer between two functional units or as a linker in the areas of functional materials, biology, polymer science, drug discovery, *etc.* However, its potential as a metal coordination platform has been explored comparatively less and there are very few examples of fluorescent triazole probe used for sensing metal ions with enhancement of fluorescence intensity. More importantly there are no reports of sensing Cu^{2+} ion utilizing a triazolyl fluorophore.⁵ Thus, there is a need for design of triazole based light up fluorescent sensor for trace metal ions like Cu^{2+} . Some examples of fluorescent probes used for metal ion sensing and for sensing of micellar microenvironment are shown in **Figure 5.1**.

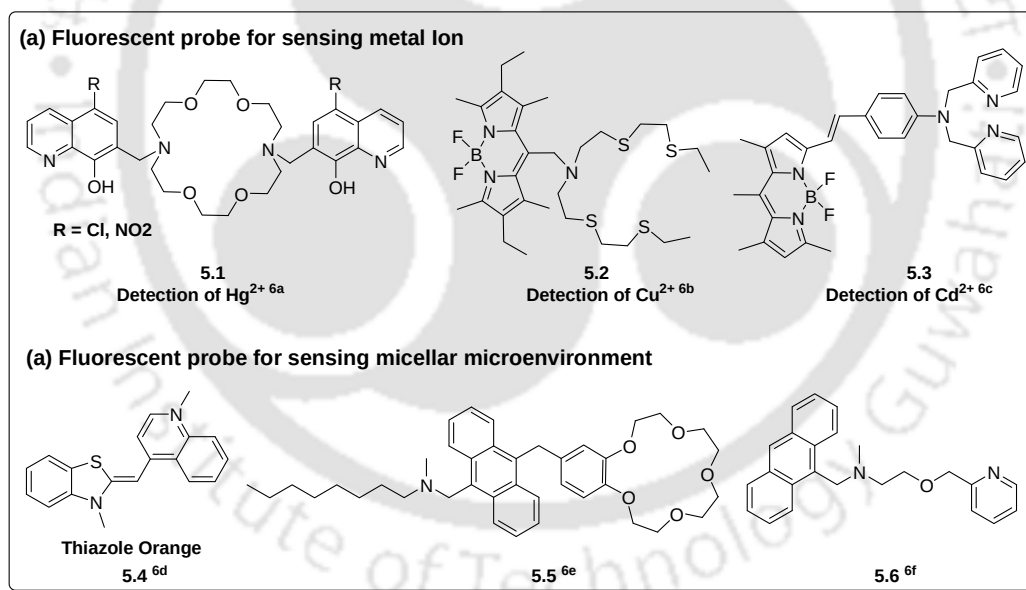


Figure 5.1: Some examples of fluorescent probes used for metal ion sensing and for sensing of micellar microenvironment.

The design and synthesis of new fluorescence sensors for various analytes such as, metal ions, biomolecules, micelle *etc.*, in a traditional way is cost effective and time consuming. However, rather than synthesizing a number of fluorophores for sensing

of different analytes, if one can utilize a single fluorophore whose emission property/sensing ability can be so modulated as to get a distinct fluorescence signal in presence of different analytes *via* a strong interaction, thereby serving the purpose of sensing multiple analytes, will be a great achievement.

Recently, enhancement of the sensing ability of a fluorescent chemosensor by noncovalent interaction with surfactant assemblies has got a considerable research interest.⁷ The micelles are well known for the encapsulation of various fluorescent active components like fluorophores in their nano structure cavity; thereby enabling enhanced communication between an analyte and the fluorophore.⁸ The fluorophore's emission properties inside the micellar nano core is tuned and is reflected in the distinct fluorescence signal while binding the analyte. Thus, the surfactants can improve the sensory ability of the fluorophore. Therefore, this property of surfactants has recently been exploited for detection of various analytes like metal ions, such as Cu^{2+} ion inside the micellar nanocore with the help of a fluorescent probe.

5.2. Objective

With this above mentioned background and knowledge and the success on the studies on interaction with BSA and ct-DNA with a switch-on fluorescence response, we thought that it would be worthwhile to explore the possibility of utilizing our designed “smart” dual-fluorescent probe, triazolyl pyrene ($^{\text{TNDMB}}$ Py) in probing of (a) micellar microenvironment and (b) in sensing Cu^{2+} ions in organic and micellar media. We envisaged that formation of self-assembled complex with both the micelle and metal ions (*e.g.*, Cu^{2+}) would result in a remarkable change in the fluorescence emission intensity of both the LE band (monomer band) and the ICT band of triazolyl pyrene. Thus, sensing micellar microenvironment and the Cu^{2+} ion can be achieved either by monitoring LE emission or ICT emission or both the LE/ICT emission and the wavelength shifting of ICT band ($\lambda_{em}^{\text{ICT}}$).

The absorption and emission spectra of triazolyl pyrene, ^{TNDBM}Py, in different solvents and in neutral pH in phosphate buffer have been elaborately discussed in **Chapter 2** and **4** respectively. Here, we want to exploit the interesting photophysical behavior of the probe, ^{TNDBM}Py in sensing micellar microenvironment and Cu²⁺ ion, the third most abundant essential trace element among the various common metal ions. We also want to exploit the sensing of Cu²⁺ ion in SDS micellar environment.

Therefore, this chapter is divided into two sub chapter, (a) **Chapter-5A: PROBING MICELLER MICROENVIRONMENT WITH SMART FLUORESCENT PROBE, TRIAZOLYL PYRENE (^{TNDBM}Py)** which describes the interaction and photophysical behavior of our previously synthesized fluorescent probe with micelle, and (b) **Chapter-5B: SENSING Cu²⁺ ION WITH SMART FLUORESCENT PROBE, TRIAZOLYL PYRENE (^{TNDBM}Py)** which describes the sensing capability of our synthesized probe towards Cu²⁺ ion. Each chapter contains their individual introduction, results and discussion and experimental section.

Chapter 5A

**PROBING MICELLER
MICROENVIRONMENT WITH SMART
FLUORESCENT PROBE, TRIAZOLYL
PYRENE (^{TNDMB}Py)**

5A.1. Introduction

Surfactants are amphiphilic molecules with one polar moiety, called hydrophilic head and a nonpolar moiety, called hydrophobic tail (**Figure 5A.1a**). The surfactant head can be cationic, anionic, zwitterionic, or nonionic and the surfactant tail is a long chain hydrocarbon residue. Cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), 4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol (Triton X-100), and 1,2-dioctanoyl phosphatidylcholine are typical examples of cationic, anionic, nonionic and zwitterionic surfactants, respectively (**Figure 5A.1b**).

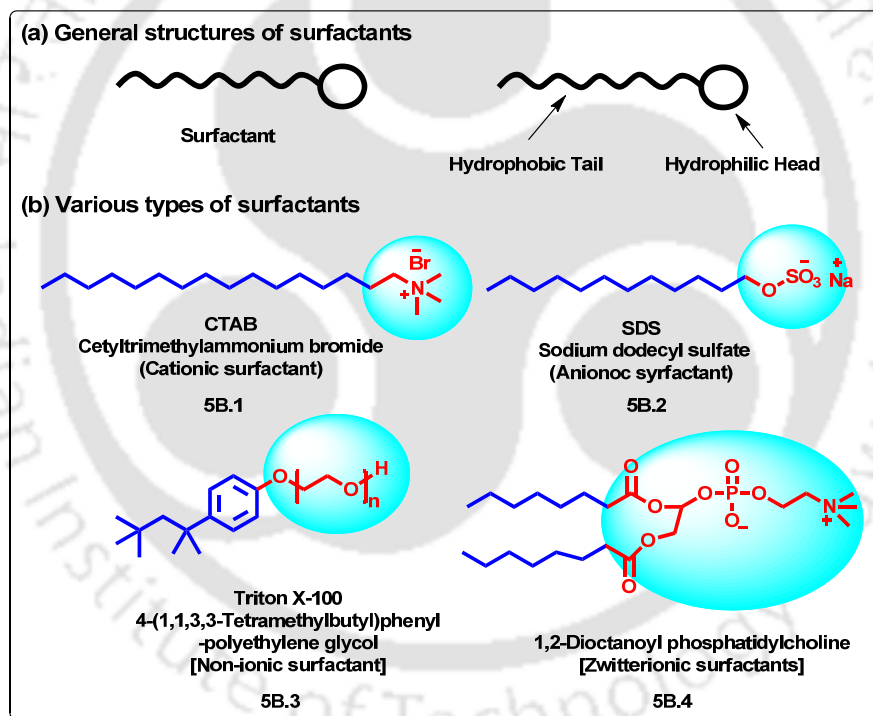


Figure 5A.1: (a) General structure of surfactant. (b) Examples of cationic (CTAB), anionic (SDS), nonionic (Triton X-100) and zwitterionic (1,2-dioctanoyl phosphatidylcholine) surfactants.

Surfactants play an important role as cleaning, wetting, dispersing, emulsifying, foaming and anti-foaming agents in many practical applications and products, including: detergents, paints, inks, adhesives, deinking of recycled papers, in flotation,

washing and enzymatic processes, Agrochemical formulations, nanomaterial-quantum dot coatings, cosmetics, firefighting, in biochemistry and biotechnology to solubilize drug candidates and in many areas of research.

5A.1.1. The Micelle

Micelle is an aggregation of surfactant molecules dispersed in liquid. **Figure 5A.2** illustrates the typical reversible monomer-micelle thermodynamic equilibrium of surfactant molecule to micelle formation. The size of the micellar aggregates is usually 1–10 nm, and the aggregation number, i.e., the number of the surfactant molecules per micelle, ranges from 20 to 200. The concentration at which surfactants start forming micelle is called the "critical micelle concentration (CMC)". With the aid of micelles, surfactants are used as emulsifiers, dispersants, bubbles, antifoams, and detergents. Depending on the nature of the supramolecular assemblies, micelles are classified as (a) normal micelle or (b) inverted micelle.

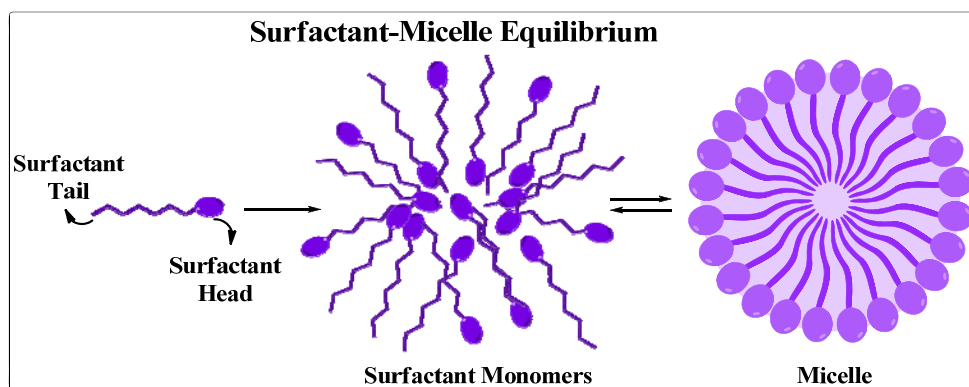


Figure 5A.2: Schematic illustration of the reversible surfactant-micelle thermodynamic equilibrium.

5A.1.1.1. Normal or Aqueous Micelle

In general in case of normal micelle, the core of a micelle is essentially dry and consists of the hydrophobic tails while the hydrophilic heads with charged group resides outside the core into the bulk water. The Stern layer surrounding the core

comprises of the polar head groups bound with counterions and water molecules (**Figure 5A.3a**). Between the Stern layer and the bulk water there is a diffuse layer, termed the Guoy–Chapman (GC) layer (**Figure 5A.3a**).⁹ The GC layer contains the free counterions and water molecules (**Figure 5A.3a**).

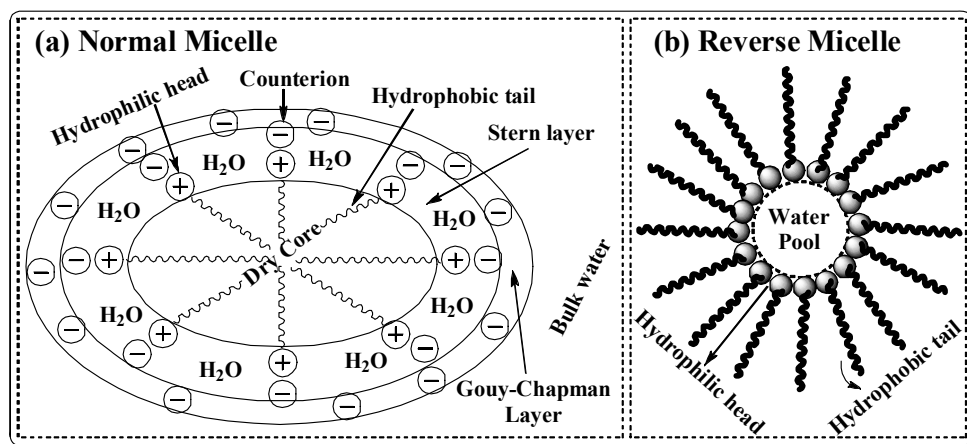


Figure 5A.3: Structures of (a) normal and (b) reverse micelles.

5A.1.1.2. Inverse or reverse micelles

In nonpolar solvents, the molecular association of the surfactant monomers occurs in an inverted manner with the polar head groups collected together in an inner core and the hydrocarbon tails spread out into the nonpolar oil phase (**Figure 5A.3b**). Hence, these aggregates are called reverse or inverted micelles. A distinguishing feature of these reverse micelles is their ability to solubilize fairly large amounts of water inside the polar core. The structures of reverse micelles depend on the amount of surfactants/cosurfactants/ water present in the multi system.^{10a} The function of co-surfactants is to reduce the repulsion between the polar head groups.^{10b} The degree of hydration in reverse micelles can be controlled by gradual addition of water to the surfactants in organic solvent and hence varying the water/surfactant molar ratio R .^{10c-f} Two types of water molecules inside the reverse micelles are found *viz.* bound water and free water. When water molecules are near the interfacial region of reverse micelles, called as bound water, they hydrate the polar head group of molecule. On

the other hand, when water molecules remain inside the core of water pool called as core water or free water. By changing the water to surfactant molar ratio ($R = [\text{H}_2\text{O}]/[\text{Surfactant}]$), the size of water pool in a reverse micelles changes. As the size of the water pool entrapped by the surfactant increases, the microviscosity of the solubilized water decreases and its polarity increases. Addition of water also dramatically increases^{10g} the average aggregation number of reverse micelles, with little dependence on the hydrocarbon solvent used or the surfactant concentration.^{10h} With increasing micellar size, its polydispersity increases. Extensive research has been done in order to understand the effect of surfactant-water interaction on the structure and dynamics of encapsulated water molecules.¹¹

Small-angle X-ray and neutron-scattering (SANS) studies have given much information on the structure of a variety of micelles.¹² These studies revealed that the thickness of the Stern layer for cationic cetyltrimethylammonium bromide (CTAB) and anionic sodium dodecyl sulfate (SDS) micelle is 6–9 Å, whereas for non ionic Triton X-100 (TX-100) micelles, the thickness of palisade layer is about 20 Å. The overall radii of CTAB and SDS micelles are about 50 and 30 Å, respectively. Whereas the radii of the dry, hydrophobic core of TX-100 are 25–27 Å, and thus the overall radius of the TX-100 micelle is about 51 Å.

In the past few decades considerable efforts have been devoted to investigate the structure, functions, and biological applications of the micelle. Among the various methods available, fluorescent based technique by using a suitable environment sensitive fluorescent probe is one of the most important, easier and efficient technique to determine the structure of micelle and its function. Fluorescence properties of a polarity sensitive probe are highly sensitive towards its surrounding environments. Thus, when a probe comes in contact with surfactant molecules, its emission intensity may be either enhanced or decreased and/or the position of emission wavelength (λ_{max}^f) may be shifted from its original band position either to a shorter or to a longer wavelength region. Among the various fluorescent probes, charge transfer fluorescent

probes being by far the most polarity sensitive probes are very important and efficient to probe micellar microenvironment, thereby enabling the determination of structure and function of micelle *via* the generation of a distinct fluorescence signal.

5A.2. Importance and Use of Micelle

Surfactants play a vital role in both fundamental and applied sciences. As is discussed above micelles are colloidal-sized clusters formed in solutions from the surfactant molecules. Micelles are used in several applications; few of such applications in brief are presented below.

5A.2.1. Micelles as Drug Carriers

Micelle exhibits important significance in pharmaceutical sciences because of their ability to increase the solubility of sparingly soluble drug candidates in water.^{13a} Because of the drug solubilizing property, a numerous drug delivery and drug targeting systems have been studied in micelle to minimize drug degradation and loss, to prevent harmful side effects, and to increase drug bioavailability.^{13b-f} Micelle is an association colloid that is formed by a spontaneous process of self-assembling and is thermodynamically more stable towards both dissociation and aggregation.^{13g} Thus, the utilization of micelles as drug carriers is more suitable than other drug carriers like polymers and/or liposomes which being the lyophobic colloids are quite unstable and frequently form large aggregates.

Extensive research works have shown that hydrophilic drugs can be adsorbed on the surface of the micelle.^{13a} Drugs with intermediate solubility generally are located in intermediate positions within the micelle such as between the hydrophilic head groups of micelles^{13b} and in the palisade layer between the hydrophilic groups and the first few carbon atoms of the hydrophobic group.^{13c} The completely insoluble hydrophobic drugs may be carried by the inner core *i.e.*, in the hydrophobic core of the micelle.^{13d,14} It is worthwhile to mention that the drugs solubilizing capacity of surfactants depends on chemical structure of the surfactant, chemical structure of the

drug, temperature, pH, ionic strength, *etc.* It has been established that the hydrophobic drugs can better be solubilized by nonionic surfactants than ionic surfactants, because of their low critical micelle concentration (CMC) values. However, there is no established relationship between the degree of solubilization of a polar drug and the chemical structure of the surfactant.

5A.2.2. Micelles as Model of Biological System

Besides the drug solubilizing and drug carrier property, micelle is also an important target of biophysical researchers. The study of cell membranes and of the roles it plays in living cells contributes significantly to the understanding of cellular function. Biological membranes have been shown to consist of lipids, phospholipids being among the most common, in association with proteins and glycoproteins that are organized in a bilayer structure (**Figure 5A.4**), resulting in a fluid lipid matrix of varying composition and fluidity.^{15a}

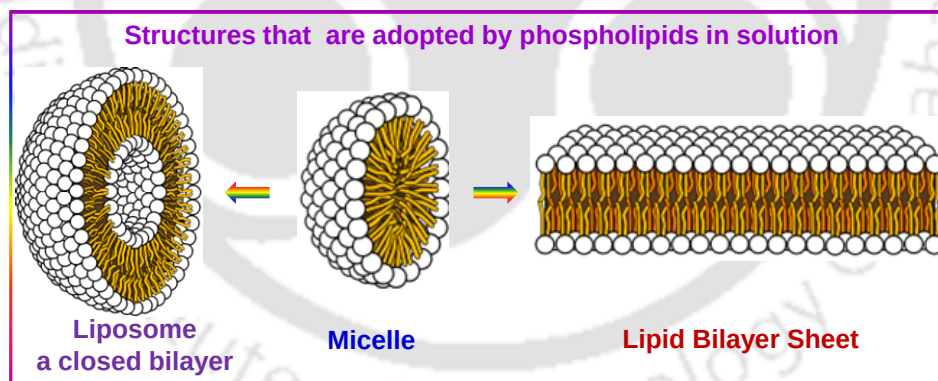


Figure 5A.4: Structural mimetic of micelle and liposome/lipid bilayer.

Many biological processes occur at membrane surface or within their hydrophobic moiety. Owing to the ionic head groups of the lipids, the surface of biological membranes frequently presents a net charge, giving rise to different binding properties of charged and uncharged forms of molecules such as drugs.^{15b-c} In this sense, the relationship between the binding properties of a drug and its active form, as well as its

membrane location, interaction with membrane, deserves considerable attention among various related research groups. Despite the efforts aiming at the understanding of drugs mechanism of action at the molecular level, demonstrated by the number of studies on the interaction of drugs with biological membranes, more studies involving model systems are necessary.^{15d-e}

Surfactants have a far-ranging use in membrane studies. Because surfactants are amphiphilic molecules, like lipids, some of the same rules governing lipid behavior also apply to the surfactants. Among the membrane models utilized, micellar system can be considered as an interesting alternative to study the interactions of different compounds with membranes because of the relative simplicity of this system, and therefore has been used with this purpose.^{15f-g}

5A.2.3. Micelles in Reaction Catalysis

Reactions behaviour observed at surfactant interfaces are expected to be more representative of many biological reactions than are reactions studied in dilute aqueous solutions.^{16a} In this sense, micellar catalysis of reactions is important because of its parallelism with enzymes behavior. Catalysis by both normal micelles and reversed micelles is possible. In normal micelles in aqueous medium, enhanced reaction of the solubilized substrate generally occurs at the micelle-water interface; in reversed micelles in non-polar medium this reaction occurs deep in the inner core.^{16b}

Micelle formation is essential for the absorption of fat-soluble vitamins and complicated lipids within the human body. Bile salts formed in the liver and secreted by the gall bladder allow micelles of fatty acids to form. This allows the absorption of complicated lipids (*e.g.*, lecithin) and lipid soluble vitamins (A, D, E and K) within the micelle by the small intestine.

Considering the importance of micellar systems in the pharmaceutical field and the many applications that it presents, micelles have widely been used as a model of biomembrane to acquire information about structure, microenvironment properties and functions. Thus, probing of micellar microenvironment with fluorescence sensor

molecules or ions is of paramount importance to know the structure and to modulate the charge distribution on the interface and thereby to correlate those with real membrane system. With the introduction of a proper fluorescent probe that is very sensitive to microenvironmental dielectric properties and viscosity, one can study the process of micellisation and also the effect of interfacial electric field on the probe thereby enabling correlation with the biomembrane. Several studies on micelle have been reported out of various research efforts till the date. We are here presenting a few of such important studies addressing fluorescence probing of micellar microenvironment.

5A.3. Background: Fluorescence Probes of Micelle

A substantial amount of research has been carried out to understand the photophysics and the photochemistry of molecules in micellar solution.^{17a-g} Incorporation of the molecular probe into the aqueous micelles effectively reveals parameter such as CMC, roughness of the micelle surfaces, polarity, viscosity and degree of water penetration into these surfactant aggregations. Most of the probes have been chosen for their particular affinity for one or two of the principal micellar region: the hydrocarbon core formed from surfactants tails, the interface composed primarily of the amphiphilic head groups, or surrounding aqueous solvent phase. Selectively targeting of specific micellar domains has been precisely achieved with the fluorophore such as polycyclic aromatic hydrocarbons such as pyrene^{17h} with ionic derivatives of aromatic chromophores such as 2-pyrazoline, 4-(2-pyridylazo) resorcinol *etc.*^{9c-g} However, the probes such as 6-propiorly-2-(dimethylamino)naphalene (prodan) is soluble in an extensive range of media, suggesting the possibility of the simultaneous partitioning of the probe into nearly all the regions of the micelle.¹⁷ⁱ

Fluorescence technique has also been used to characterize the structure of the reverse micelle.¹⁸ Probes employed exhibit a particular affinity for one or more than one of the micellar regions: the water pool, the micellar interface, or the surrounding

hydrocarbon solvent phase.^{10h} For example, 1, 8-anilidonaphthalene sulphonate, pyrenesulphonic acid, rhodamine B, 2,6-toluidinonaphthalenesulphonic acid, auramine and pyranine partition in the aqueous micellar core.¹⁹ Indoleacetate ions distribute between the micellar interface and water pools.^{20a} 1, 2-Dimethylindole remains predominately in the hydrocarbon phase,^{20b} while pyrene^{20c} and indole^{20b} molecules partition into both the hydrocarbon continuous phase and the micellar interfacial microphase, whereas the microenvironment for prodan^{20d} include an inner “free” water pool, a bound water region, the micellare phase and the surroundings hydrocarbon solvent phase.

Shirota *et al.*,^{21a} have studied the microenvironment of sodium dodecyl sulfate (SDS) micelle solutions by fluorescent probes coumarin 102 (C102) and coumarin 153 (C153) (**5A.5** and **5A.6**, **Figure 5A.5**). Thus, they observed that both the absorption and fluorescence maxima of C102 shift dramatically at the SDS concentration of 8.1 mM, which is the critical micelle concentration for SDS in water. The absorption maximum shifts to the longer wavelength and the fluorescence maximum shifts to the shorter wavelength when the SDS concentration is beyond the critical micelle concentration. However, both the absorption and fluorescence maxima of C102 in SDS micelle solutions with SDS concentrations of ≥ 16.2 mM show only a small shift in wavelength.

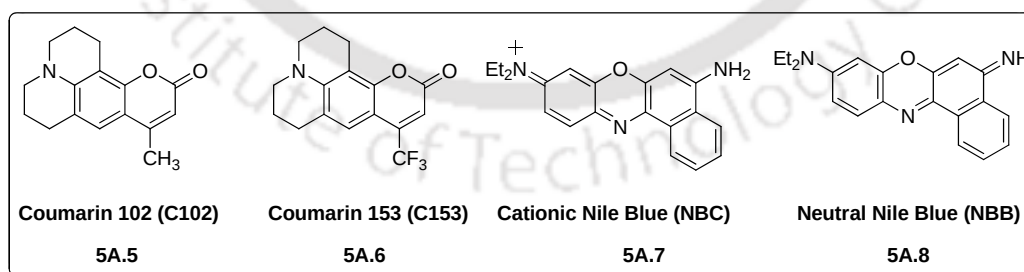


Figure 5A.5: Examples of fluorescent probes of micellar microenvironment.

Ground state prototropic equilibrium of the Nile Blue dye (**5A.7** and **5A.8**, **Figure 5A.5**) has been studied in Triton-X 100/benzene–hexane reverse micellar system by

Das *et al.*^{21b} They observed from the absorption spectrum that in absence of water, the deprotonated form of the dye is predominant. Addition of water produces the protonated form. However, at highest water loading, the equilibrium is still shifted towards the deprotonated form. In fluorescence spectra they observed that in neat TX-100, the emission peak of Nile Blue appeared at 587 nm, whereas in TX-100 reverse micelles the emission peak was blue shifted to 570 nm for $R = 0$ ($R = [\text{H}_2\text{O}]/[\text{Surfactant}]$), which finally red shifts to 587 nm when $R = 10$. Also, the emission intensity increases slightly at $R = 4$ and then decreases at $R = 10$.

Probing of micellar microenvironment by a donor acceptor fluorescent probe, ethyl ester of *N,N*-dimethylaminonaphthyl- (acrylic)-acid (EDMANA) (**5A.9**, **Figure 5A.6**) have recently been reported by Guchhait *et al.*^{21c} Thus, they investigated the photophysics of donor acceptor charge transfer molecule EDMANA inside the anionic (SDS), cationic (CTAB) and non-ionic (Triton-X 100) micellar nanocages by using steady state and time resolved spectroscopy. They also showed that this polarity sensitive probe serves as an excellent reporter for micellar microenvironments. The internal charge transfer (ICT) emission was much more modulated by the micelles media than the local emission (LE).

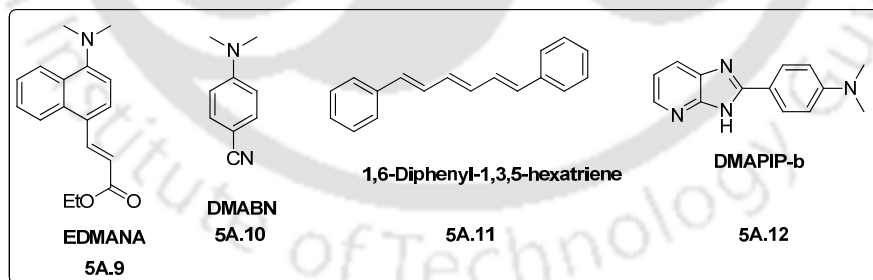


Figure 5A.6: Donor-acceptor fluorophore and other fluorescent molecules probe of micellar microenvironment.

Chattopadhyay *et al.*,^{21d} studied the twisted intramolecular charge transfer (TICT) of *p*-*N,N*-dimethylaminobenzonitrile (DMABN) (**5A.10**, **Figure 5A.6**) in the photoexcited state in cationic (CTAB) and anionic (SDS) micellar solutions. They

found that the TICT process is affected by the surface charge of the micellar aggregates. They also established that the probe (DMABN) resides within the micellar environment keeping the dimethylamino group at the micellar periphery and the other end deep in the micellar core on the basis of a comparative study of the TICT and the locally excited (LE) emission.

A novel method of determination of the critical micellization concentrations (CMC) of surfactants using fluorescence depolarization of a fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (**5A.11**, **Figure 5A.6**) has been developed by Burt *et al.*^{21e} They measured the fluorescence anisotropies of Triton X-100, sodium dodecyl sulfate, and sodium cholate using this probe and found that fluorescence anisotropy decreased with increasing surfactant concentrations below the CMC and leveled off above the CMC.

Krishnamoorthy *et al.*,^{21f} studied the photophysical behavior of 2-(4'-*N,N*-dimethylaminophenyl) imidazo[4,5-*b*]pyridine (DMAPIP-*b*) (**5A.12**, **Figure 5A.6**) in cationic (CTAB), anionic (SDS) and nonionic (TX-100) micelles using steady state and time resolved fluorescence techniques. In fluorescence study, they observed both normal and TICT emission in SDS and TX- 100 but only normal emission in CTAB. They suggested this difference in behavior of the fluorophore is due to varying extent of hydrogen bonding experienced by it in different micelles.

5A.4. Objective

The importance of micelles in various research fields and a knowledge of profound literature of fluorescent probes along with our success in fluorescence switch-on probing of ct-DNA and BSA, we thought that it would be worthwhile to explore the possibility of utilising our designed “smart” dual-fluorescent probe, triazolyl pyrene (^{TNDB}Py) in probing of micellar microenvironment. We envisaged that formation of self-assembled complex with the micelle would result in a significant change in the fluorescence emission intensity of either of the LE band (monomer band) or the ICT band or both of the bands of triazolyl pyrene. Thus,

sensing micellar microenvironment can be achieved by evaluating spectral characteristics of our probe.

Keeping this view in our mind, we have framed our objective as below:

- (a) To investigate the photophysical property of triazolyl pyrene (^{TNDBM}Py), in presence of various surfactants (cationic, anionic and non-ionic) of varying concentration.
- (b) As we know that, the reverse micelle system is a real mimic of cell membrane, we, thus also interested in studying the photophysical properties of ^{TNDBM}Py in reverse micelle of TX-100 in benzene-hexane (3:7) and water system.

5A.5. Results and Discussions

5A.5.1. Study of Photophysical Properties

The synthesis of the probe (^{TNDBM}Py) was achieved following our reported click chemistry protocol (already has discussed in **Chapter 2**) with very high yield.²² After characterizing and recrystallization which afforded very pure compound we then turned our attention to study the photophysical properties of the probe in various media.

Previously we have studied in detail the photophysical properties of triazolyl pyrene in presence of various solvents and solvent mixtures with varying dielectric constant (**Chapter 2**). In brief the absorption spectrum of the probe did not markedly differ with the solvent polarity; however the emission spectrum strongly differed with the solvent polarity. The molecule showed a dual emission behavior centered at 383 nm, a locally emission (LE) band and 466-544 nm, internal charge transfer (ICT) band (**Figure 5A.7**). In high polar solvents (acetonitrile, methanol) the probe showed a low intense LE (local emission) band, characteristic structured band of pyrene. In ether, dioxane, toluene, chloroform and ethyl acetate a dual emission was observed with strong red shift pattern of the ICT (internal charge transfer) band as the solvent

polarity is increased from toluene (466 nm) to ethyl acetate (544 nm). Our probe consists of a *N,N*-dimethyl aminophenyl donor linked triazole functional which serves as an efficient electron donor and a pyrene functional which acts as an electron acceptor. Thus, the probe consists of an efficient charge transfer property.

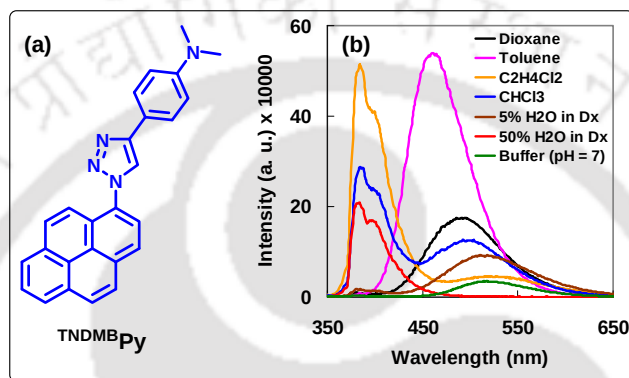


Figure 5A.7: (a) Structure of triazolylpyrene probe and (b) emissions behavior of the probe in different solvents.

Here, the photophysical properties of probe were studied in presence of cationic (CTAB), anionic (SDS) and non-ionic (Triton X-100) surfactant of varying concentration. Apart from these three surfactants, the similar study was done in presence of reverse micelle (TX-100 in 3:1 benzene-hexane and water system) also.

5A.5.1.1. Study of UV-Visible Spectra

The absorption spectra of $^{TNDMB}Py$ in aqueous solutions of varying surfactant concentration, CTAB, SDS (**Figure 5A.8**) and TX-100 and in reverse micelle (**Figure 5A.9**) were recorded keeping the fixed probe concentration presentation in **Figure 5A.8** and **5A.9**, respectively. From these figures it is clear that the absorbance of the probe is increased with increase in surfactant concentrations even beyond the critical micellisation concentrations of all the surfactants. However, the absorption wavelength maxima, λ_{max}^{abs} remains unaltered and centered at around 344 nm in all the cases. The increase in absorbance in each surfactant solution with increase in

surfactant concentration is attributed to the enhanced solubilization of the probe ($^{TNDMB}Py$) in surfactant solutions.

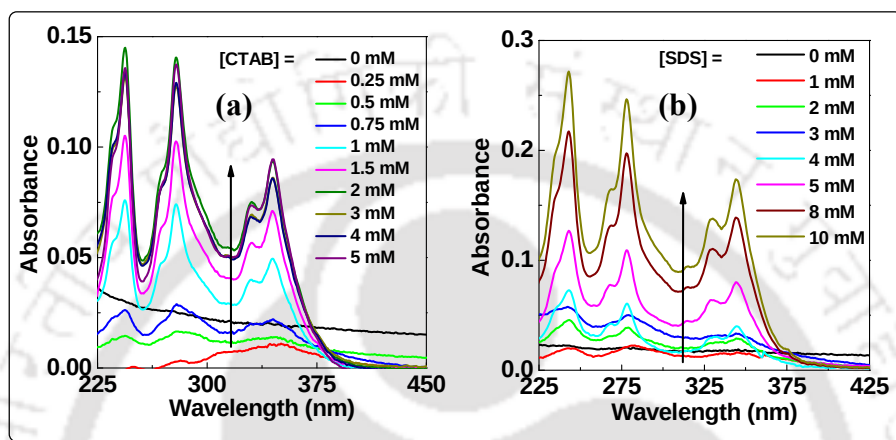


Figure 5A.8: UV-visible spectra of $^{TNDMB}Py$ in aqueous solution of varying surfactant concentration of (a) CTAB and (b) SDS. [$^{TNDMB}Py$] = 10 μ M.

However, in reverse micellar system we observed no regular change in absorbance value, with increase in molar ratio of water.

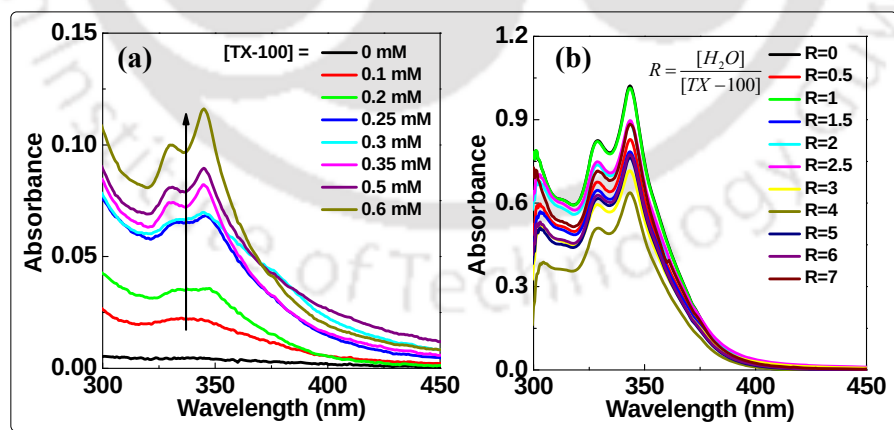


Figure 5A.9: UV-visible spectra of $^{TNDMB}Py$ in (a) aqueous solution of varying TX-100 concentration and (b) in reverse micelle of TX-100 in benzene-hexane (3:7) and water mixture. [$^{TNDMB}Py$] = 10 μ M.

5A.5.1.2. Study of Fluorescence Spectra

The steady state fluorescence techniques have been widely used to study the micellar microenvironment and the mechanism of micelle formation with the help of a suitable fluorescent probe. As our designed probe, ^{TNDBM}Py consists of a donor (*N,N*-dimethyl aminophenyl linked with triazole) as well as acceptor (pyrene) groups linked *via* a triazole unit, so there may be a possibility of charge transfer in the excited state, which would be affected by the polarity of the surrounding environment. The charge transfer phenomenon of the probe, ^{TNDBM}Py in dioxane-water system was well studied and discussed in **Chapter-2**. A dual fluorescence emission (LE, local emission and ICT, internal charge transfer) was observed in dioxane-water solvent system. With increase of % of water content, a red shifted ICT band with decreased intensity was observed. It has been well studied that the dual fluorescence appears from the equilibrium between two states, LE and ICT in the excited state. The short wavelength emission band arises from the locally excited (LE) state and the long wavelength emission band arises from the newly generated ICT state having higher dipole moment than the LE state. The ICT state is stabilized by the solvent polarity and thus the energy gap between ground and excited state decreases and we observe a long wavelength emission, called ICT band.

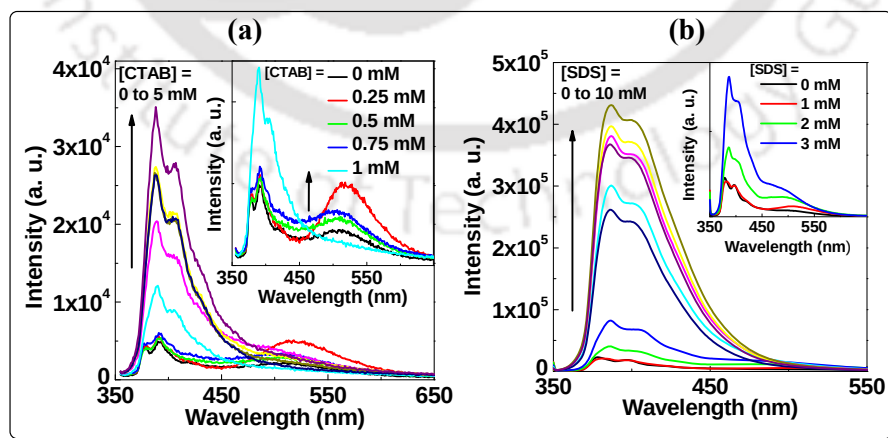


Figure 5A.10: Fluorescence spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of ^{TNDBM}Py in aqueous solution of varying surfactant concentration of (a) CTAB and (b) SDS. [^{TNDBM}Py] = 10 μM .

The steady-state fluorescence spectra of $^{TNDMB}Py$ in all three surfactant solutions, CTAB, SDS and TX-100 as well as in reverse micelle were recorded. In ionic surfactant solution a dual emission (LE and ICT) was observed at low surfactant concentration (insets **Figure 5A.10**). The ICT band showed a blue shifting pattern with enhanced intensity that disappeared as the concentration of surfactant increases (> 1 mM for CTAB and > 3 mM for SDS, **Figure 5A.10a** and **Figure 5A.10b** respectively). Thus, this observation suggest that microenvironment around the probe, $^{TNDMB}Py$ is different from the pure aqueous phase. The spectral shifts and the enhancement of the fluorescence intensities of ICT band at low surfactant concentration and gradual increase of the fluorescence intensities of LE band with the increase in surfactant concentration can be explained in terms of binding of this probe to a less polar site of the micelle.

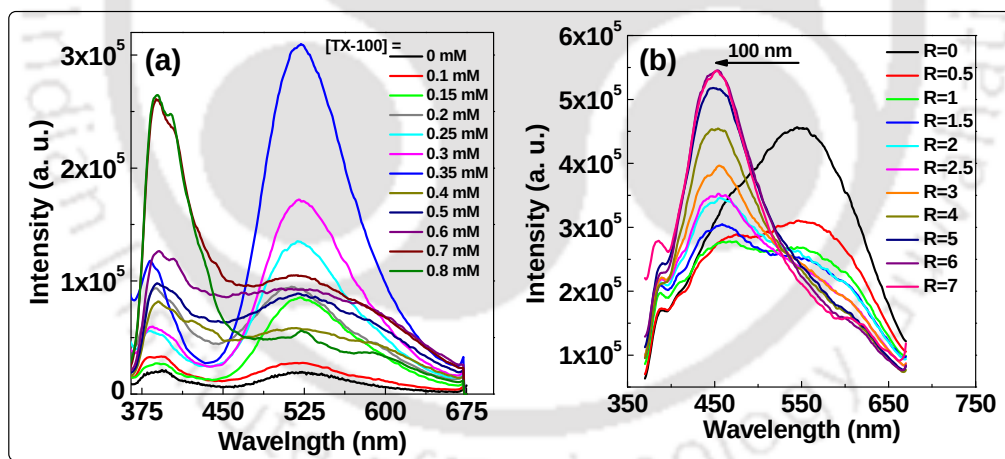


Figure 5A.11: Fluorescence spectra ($\lambda_{ex} = 343$ nm) of $^{TNDMB}Py$ (a) in aqueous solution of varying concentration of TX-100 surfactant and (b) in reverse micelle of TX-100 in benzene-hexane (3:7) and water mixture. R is molar ratio of water to TX-100.

In nonionic surfactant solution, Triton X-100 and in reverse micelle of Triton X-100 in benzene-hexane and water system, we observed an interesting emission

behavior of the probe ^{TNDBM}Py. In low TX-100 concentration (up to 0.35 mM), the fluorophore showed dual emission with dominant ICT emission over LE emission. However, at high concentration the LE band predominates over ICT band (**Figure 5A.11a**).

In reverse micelle we observed a blue shifted pattern of the ICT band with no emission from LE state as the molar concentration of water is increased (**Figure 5A.11b**). We found 100 nm blue shifting when we moved from R = 0 to R = 7, where R is the molar ratio of water to TX-100 ($R = [H_2O]/[TX-100]$). The blue shifting of ICT band is probably because of movement of the probe from hydrophilic core to more hydrophobic core (surface) of the reverse micelle. Thus, from the steady-state fluorescence study it was observed that the ICT band of the probe, ^{TNDBM}Py was more influenced by the micellar environment in comparison to the LE band.

5A.5.1.3. Time Resolved Fluorescence Study

Fluorescence lifetime of a molecule is very sensitive towards its local environment. Thus this study can nicely explain the location and distribution of the probe in micellar microenvironment. The time resolved fluorescence spectra of ^{TNDBM}Py were recorded in all three surfactant solutions, CTAB, SDS and TX-100 as well as in reverse micelle of Triton X-100 in benzene-hexane and water system.

The fluorescent life time (τ) was expressed using the following two equations.

$$a_n = \frac{B_n}{\sum_{i=1}^N B_i} \dots\dots\dots (1)$$

where a is the fluorescence lifetime coefficient and B is the pre-exponential factor. The mean lifetime was calculated by the following equation

$$\langle \tau \rangle = \frac{\sum a_i \tau_i^2}{\sum a_i \tau_i} \dots\dots\dots (2)$$

where τ_i and a_i are the fluorescence lifetime and its coefficient of the i^{th} component respectively

From the time resolved fluorescence study, it was observed that the decay time (τ) of ^{TNDBM}Py increases with increasing surfactant concentration for all three surfactants (CTAB, SDS and TX-100). These results showed a good correlation with the steady state fluorescence study, where we observed an increase in fluorescence intensity with increase in surfactant concentration. In SDS surfactant, a bi-exponential decay with increasing mean lifetime ($\langle\tau\rangle$) of ^{TNDBM}Py was observed as the concentration of SDS was increased (Table 5A.1 and Figure 5A.12).

Table 5A.1: Summary of time resolved fluorescence of ^{TNDBM}Py in presence of SDS, monitored at LE band.

[SDS] (mM)	λ_{em} (nm)	τ_1 (ns)	τ_2 (ns)	$\langle\tau\rangle$ (ns)
1	400	0.74 (77.3%)	8.54 (22.7%)	6.76
2	400	2.07 (60.8%)	16.7 (39.2%)	14.3
3	400	2.10 (53.1%)	16.5 (46.9%)	14.7
4	400	2.68 (37.5%)	21.5 (62.5%)	20.2
5	400	2.23 (27.1%)	24.7 (72.9%)	24.0

The mean lifetime increases from 6.76 ns in 1mM SDS to 24.0 ns in 5mM SDS concentration. The increase in decay time and the fluorescence intensity is probably because of substantial decrease in non-radiative decay in the micellar environment. From the bi-exponential fitting we observed that τ_1 and τ_2 increases (monitoring wavelength was LE band) with increasing surfactant concentration, however, the abundance of first component (a_1) decreases and the second component (a_2) increases with increasing surfactant concentration.

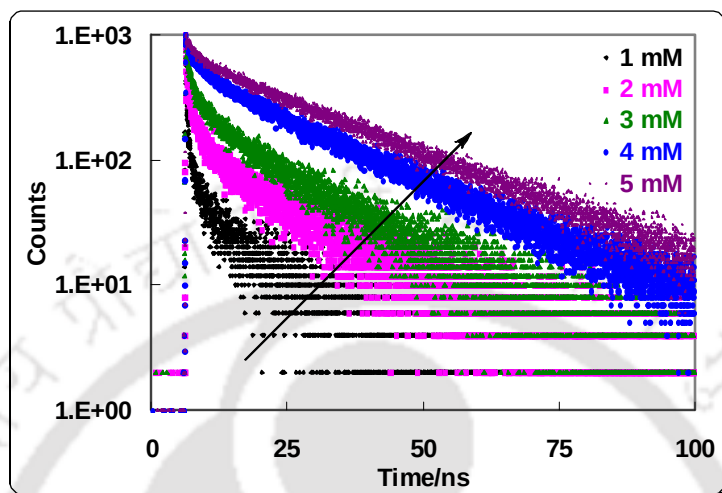


Figure 5A.12: Time resolved fluorescence spectra of ^{TNDBM}Py in presence of SDS (λ_{em} = LE band).

Time resolved fluorescence was also measured by monitoring the ICT band (515 – 480 nm) in SDS surfactant solution (**Figure 5A.13** and **Table 5A.2**). A bi-exponential fitting of the decay curve revealed that as SDS concentration increases from 1 mM to 3 mM, the mean lifetime decreases. This result also correlates well with the steady state fluorescence study, where we observed no ICT band at high SDS (> 3 mM) concentration.

Table 5A.2: Time resolved fluorescence summary of ^{TNDBM}Py in presence of SDS monitored at ICT band.

[SDS] (mM)	$\lambda_{em(ICT)}$ (nm)	τ_1 (ns)	τ_2 (ns)	$\langle\tau\rangle$ (ns)
1	515	4.2 (60.46%)	25.34 (39.54%)	21.07
2	500	3.26 (64.64%)	19.22 (35.36%)	15.44
3	480	2.57 (70.95%)	19.85 (29.05%)	15.70

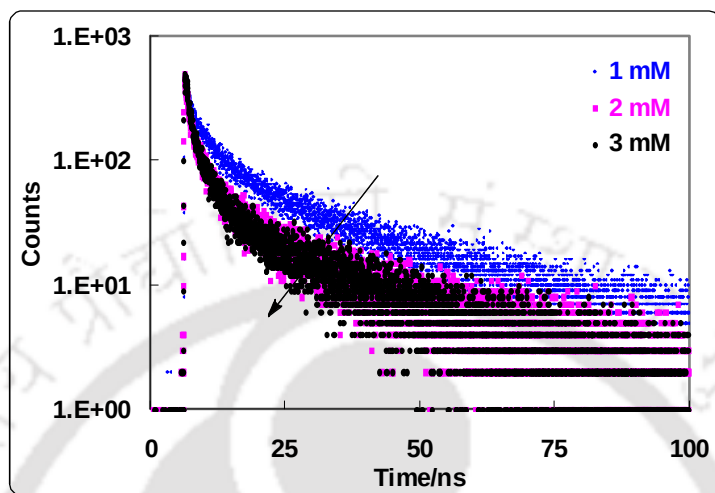


Figure 5A.13: Time resolved fluorescence spectra of $^{TNDMB}Py$ in presence of SDS ($\lambda_{em} = \text{ICT band}$).

Next, we have measured the lifetime of the probe in presence of cationic surfactant, CTAB solution. The lifetime data were fitted to a biexponential decay fitting curve. Thus, similar to the case of SDS surfactant, a bi-exponential decay with increasing mean lifetime ($\langle \tau \rangle$) of $^{TNDMB}Py$ was observed as the concentration of CTAB was increased (**Table 5A.3** and **Figure 5A.14**). The mean lifetime increases from 0.53 ns in water to 6.41 ns in 1.5 mM CTAB concentration. The increase in decay time and the fluorescence intensity is probably because of substantial decrease of non-radiative decay in the micellar environment.

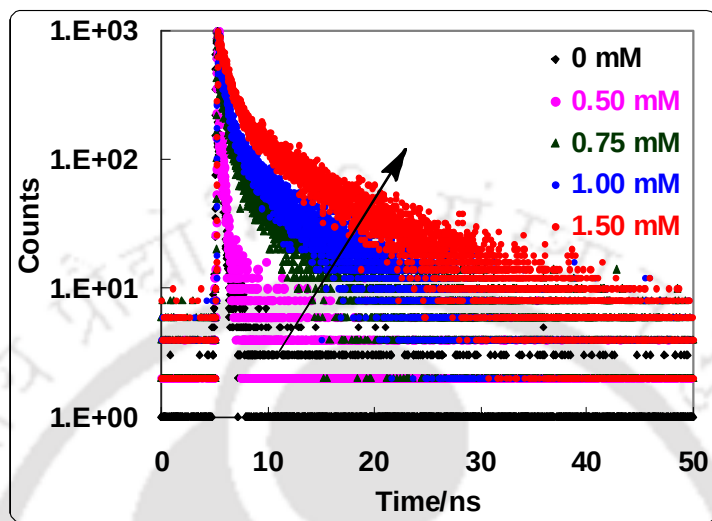


Figure 5A.14: Time resolved fluorescence spectra of ^{TNDBM}Py in presence of CTAB (λ_{em} = LE band).

Table 5A.3: Time resolved fluorescence summary of ^{TNDBM}Py in presence of CTAB, monitored at LE band.

[CTAB] (mM)	λ_{em} (nm)	τ_1 (ns)	τ_2 (ns)	$\langle\tau\rangle$ (ns)
0	400	0.24 (90.8%)	1.15 (9.2%)	0.53
0.5	400	0.35 (93.5%)	1.97 (6.5%)	0.80
0.75	400	0.80 (78.7%)	5.93 (21.3%)	4.22
1	400	0.86 (74.9%)	6.56 (25.1%)	4.94
1.5	400	0.99 (70.2%)	7.99 (29.8%)	6.41

After measuring the time resolved fluorescence of the probe in presence of ionic surfactants we next investigated the same in presence of neutral surfactant. Thus, we observed a similar result in presence of non-ionic surfactant as that was observed in the case of ionic surfactant. The decay time of ^{TNDBM}Py was increased with increase in concentration of TX-100 up to 0.35 mM, and then it started decreasing as the concentration was increased beyond 0.35 mM (Table 5A.4 and Figure 5A.15). Our

result on the fluorescence response of the probe upon changing the concentration of neutral surfactant showed a very good correlation with the steady state fluorescence study where an increase in intensity was observed till the concentration of TX-100 increased to 0.35 mM and it decreased upon further increase in concentration of TX-100. We observed a bi-exponential fitting with increase in mean decay time from 28.1 ns in 0.1 mM TX-100 to 50.7 ns in 0.35 mM TX-100. Further increase in concentration of TX-100 showed a decrease in decay time of the probe (45.1 ns in 0.5 mM TX-100, **Table 5A.4**).

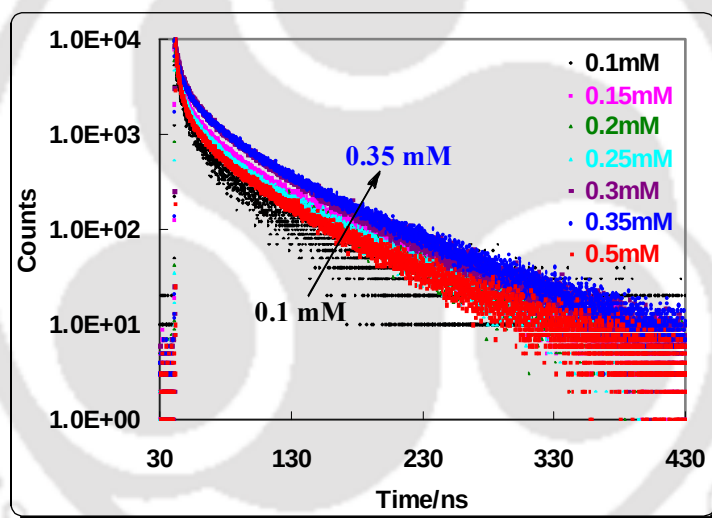


Figure 5A.15: Time resolved fluorescence spectra of TNDMB Py in presence of TX-100 (λ_{em} = ICT band).

Table 5A.4: Time resolved fluorescence summary of ^{TNDBM}Py in presence of TX-100, monitored at ICT band.

[TX-100] (mM)	λ_{em} (nm)	τ_1 (ns)	τ_2 (ns)	$\langle\tau\rangle$ (ns)
0.1	520	4.02 (84.9%)	41.3 (15.1%)	28.1
0.15	520	10.6 (64.7%)	56.2 (35.3%)	44.5
0.2	520	10.2 (67.3%)	54.1 (32.7%)	41.8
0.25	520	11.0 (68.4%)	57.2 (31.6%)	43.6
0.3	520	11.7 (63.8%)	57.5 (36.2%)	45.4
0.35	520	15.0 (62.5%)	64.5 (37.5%)	50.7
0.4	520	13.6 (55.5%)	60.0 (44.5%)	49.8
0.5	520	11.6 (63.1%)	56.9 (36.9%)	45.1
0.7	520	8.95 (70.5%)	51.1 (29.5%)	38.7

After studying the photophysical behaviours of the probe in anionic, cationic and neutral surfactant solution, we next turn our attention to study the time resolved fluorescence behaviour of our probe in presence of TX-100 in benzene-hexane-water system where reverse micelle formation takes place. Thus, we observed a multi exponential fitting with slight change in the decay time in this reverse micellar system (Figure 5A.16 and Table 5A.5).

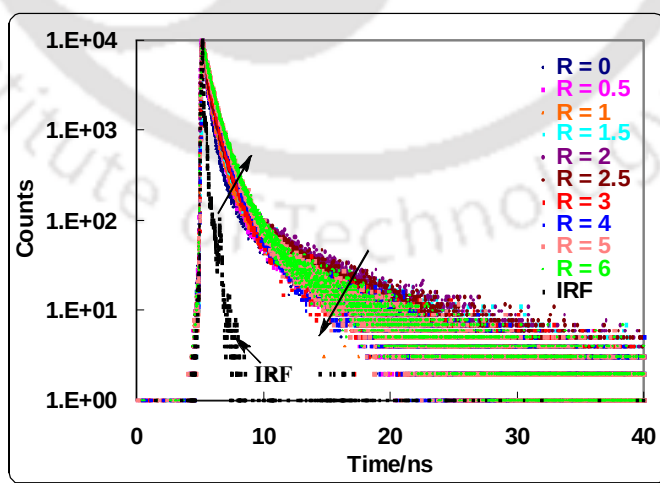
**Figure 5A.16:** Time resolved fluorescence spectra of ^{TNDBM}Py in reverse micelle (λ_{em} = ICT band).

Table 5A.5: Time resolved fluorescence summary of ^{TNDBM}Py in presence of reverse micelle, monitored at ICT band.

R ^[a]	λ_{em} (nm)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	$\langle\tau\rangle$ (ns)
0	460	0.266 (72.81%)	0.700 (25.55%)	4.35 (1.64%)	1.10
0.5	460	0.269 (60.66%)	0.766 (37.86%)	4.50 (1.48%)	1.09
1	460	0.276 (61.20%)	0.764 (37.25%)	4.57 (1.56%)	1.12
1.5	455	0.298 (61.10%)	0.820 (37.32%)	5.15 (1.57%)	1.27
2	450	0.339 (66.16%)	0.912 (32.09%)	5.64 (1.75%)	1.46
2.5	450	0.366 (68.98%)	0.955 (29.54%)	5.73 (1.49%)	1.37
3	450	0.384 (63.86%)	0.909 (35.08%)	5.18 (1.06%)	1.08
4	450	0.474 (66.95%)	1.02 (31.93%)	4.96 (1.12%)	1.09
5	450	0.485 (63.89%)	1.05 (36.06%)	5.11 (0.05%)	0.81
6	450	0.502 (61.91%)	1.07 (37.02%)	5.28 (1.07%)	1.15

^[a]R = [H₂O]/[TX-100]

Thus, a triexponential decay fitting with very short decay time of the first two major components and a long decay time ($\tau_3 = 4.35$ ns) with least abundant (1.64%) third component was observed when $R = 0$. The decay time of the third component increases from 4.35 ns to 5.73 ns upon increase in $R = 0$ to $R = 2.5$ and beyond $R = 2.5$, it decreased. However, the decay time of first two components showed gradual increase with increase in molar ratio of water (**Table 5A.5**).

5A.5.2. Determination of Critical Micellization Concentration (CMC)

Surfactant molecules aggregate in liquid to form micelle in solution. At which concentration, the surfactant molecules formed micelles is called the critical micelle concentration (CMC). The microenvironments above and below the CMC are different. From the steady-state fluorescence study we observed that the fluorescence intensity of the probe, ^{TNDBM}Py increases with increase in surfactant concentration for the case of all surfactant solution (SDS, CTAB and TX-100). After a certain concentration limit the fluorescence intensity drastically jumped to a higher value and

again smoothly increases. Thus, the CMC value of all the three surfactants, CTAB, SDS and TX-100 were calculated with the help of steady-state fluorescence spectra of TNDMB Py. The plots of fluorescence Intensity vs. [surfactant] are shown in the following **Figure 5A.17**. The experimental CMC values of three surfactants obtained from the plots were 0.7 mM for CTAB, 6.5 mM for SDS and 0.25 mM for TX-100 are close and comparable with the literature reported values of 0.94 mM for CTAB, 7.80 mM for SDS and 0.26 mM for TX-100 (**Table 5A.6**).

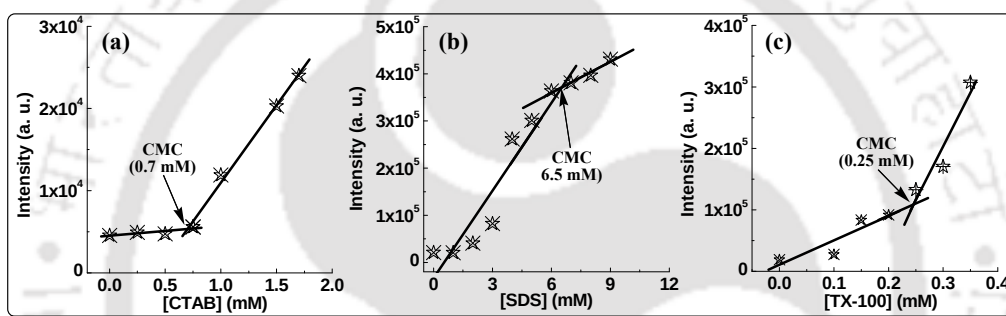


Figure 5A.17: Plots of fluorescence intensity vs. (a) [CTAB], (b) [SDS] and (c) [TX-100].

Table 5A.6: Summary table of obtained CMC and binding constant values.

Medium	CMC (mM) (Experimental)	CMC (mM) (Reported) ²⁵	Binding Constant (K_{eq}) (M^{-1})
CTAB	0.70	0.94	4.28×10^4
SDS	6.50	7.80	2.77×10^5
TX-100	0.25	0.26	1.6×10^6

5A.5.3. Determination of Binding Constant

The fluorescence intensity of the probe in aqueous and in micellar media is far different. Thus, the binding constant (K) of the probe with micelle was calculated from the fluorescence intensity values in micelle media. We used the following

relationship demonstrated by Almgren *et al.*,²³ to calculate the binding constants (K_s) of the probe in various micelles.

$$\frac{(I_\infty - I_0)}{(I_t - I_0)} = 1 + (K[M])^{-1} \dots \dots \dots (3)$$

where I_∞ , I_0 , and I_t are the fluorescence intensities of the probe under complete micellization, in the absence of micellization, and in presence of intermediate amounts of surfactant, respectively. $[M]$ is the micelle concentration, which can be expressed by the following relationship

$$[M] = \frac{(S - CMC)}{N} \dots \dots \dots (4)$$

where S is the surfactant concentration, CMC is the critical micelle concentration and N is the aggregation number of the micelle. The N values used in the calculation of $[M]$ are 60 for CTAB, 62 for SDS, and 143 for Triton X-100.²⁴ The plots of $(I_\infty - I_0)/(I_t - I_0)$ vs. $[M]^{-1}$ for all the three micelles, CTAB, SDS and TX-100 are shown in **Figure 5A.18 a-c** respectively.

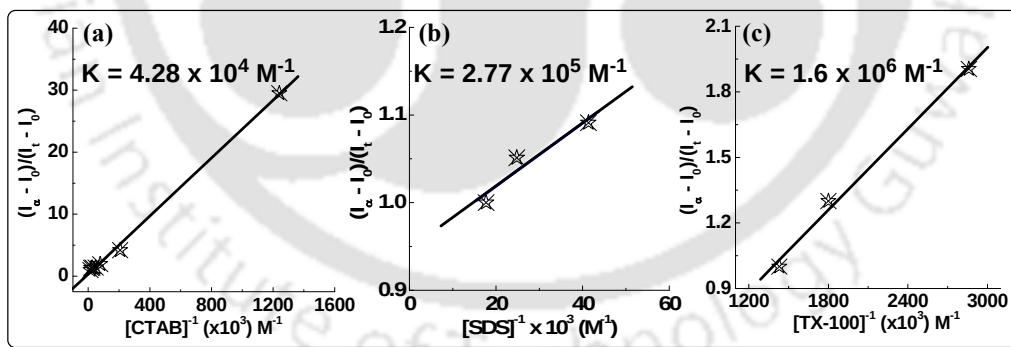


Figure 5A.18: Plots of $(I_\infty - I_0)/(I_t - I_0)$ vs. $[Surfactant]^{-1}$ of ^{TNDB}Py for (a) CTAB, (b) SDS and (c) TX-100.

The calculated binding constants of the probe in various micelles are summarized in **Table 5A.6**. Thus, we have seen that the binding constant of the probe with cationic, anionic and non-ionic micelle follow the order CTAB<SDS<TX-100.

5A.6. Conclusion

In conclusion, we have investigated the photophysics of donor acceptor charge transfer probe, ^{TNDBM}Py inside the micellar microenvironment. In ionic surfactant solution the probe showed a dual emission (LE and ICT) at low surfactant concentration. The ICT band showed a blue shifting pattern with enhanced intensity that disappeared as the concentration of surfactant increases (> 1 mM for CTAB and > 3 mM for SDS). At low concentration of non-ionic surfactant TX-100, the probe showed dual emission with dominant ICT emission over LE emission. However, at high concentration of TX-100, the LE band predominates over ICT band. In reverse micelle a blue shifted pattern of the ICT band with no LE band was observed with increase in molar concentration of water. The experimental CMC values of surfactant from the fluorescence intensity vs. [surfactant] plot are in good agreement with the literature reported values. The binding constant of the probe with cationic, anionic and non-ionic micelle follow the order CTAB<SDS<TX-100. Thus, we have successfully demonstrated that our probe, triazolyl pyrene (^{TNDBM}Py) is an efficient fluorescence switch-on probe of micellar microenvironment. Therefore, our probe might find application in probing of membrane in biological systems which expectedly would generate a distinct fluorescence signal for such interaction events and shed light to the environment around it inside the cell.

5A.7. Experimental Section

5A.7.1. Materials

CTAB, SDS, benzene and hexane were purchased from Merck, India. Triton X-100 was purchased from SRL, India. Water was obtained from a Milli-Q purification system. All experiments were performed with freshly prepared solutions in Milli-Q water. The probe molecule was synthesized and purified according to the procedure described in **Chapter 2**. We recrystallised the column purified material in chloroform-ethylacetate solvent mixture twice, dried under vacuum, again characterized and used for study.

5A.7.2. Methods

5A.7.2.1. Preparation of Probe-Surfactant Solutions

The various concentrations of a particular surfactant solution were prepared by using Milli-Q water. Then the surfactant solutions of various concentrations were added to the sample vials containing probe. The sample solutions were mixed well before the experiment.

5A.7.2.2. UV-Visible Study

The UV-Visible absorbance measurements were performed using Shimadzu 2550 UV/vis spectrophotometer with a cell of 1 cm path length at 298 K. The probe with surfactant solutions were mixed well before doing the experiment. The measurements were taken in absorbance mode. The absorbance values of the sample solutions were measured in the wavelength regime of 200–600 nm.

5A.7.2.3. Fluorescence Study

All fluorescence studies were performed using a Fluoromax 4 spectrophotometer with a cell of 1 cm path length at room temperature. The excitation wavelength for probe (^{TNDMB}Py) was set at 343 nm, and emission spectra were measured in the wavelength regime of 350–675 nm. The probe with surfactant solutions were mixed

well before doing the experiment. Fluorescence lifetimes were measured by Edinburgh instrument, Life Space II with Edinburgh 375 nm Laser source for excitation. The fluorescence decay was analyzed by tail fitting method using in built software. All the experiments were carried out at 298 K.



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Chapter 5B

**SENSING Cu²⁺ ION WITH SMART
FLUORESCENT PROBE, TRIAZOLYL
PYRENE (^{TN}DMBPy)**

5B.1. Introduction

Much effort has been put forth to the design and synthesis of fluorescent chemosensors usable for the sensing and recognition of environmentally and biologically important species.¹ A chemosensor is a compound that renders a significant change in electrical, electronic, magnetic, or optical signals when it binds to or comes into interaction with a specific guest counterpart. In general, chemical sensing involves the interaction between a target molecule *i.e.* an analyte and a receptor called chemosensor that responds by generation of an easily detectable signal.² Most chemosensors involve in a binding event or a chemical reaction to change the emission characteristics of an appended fluorophore *i.e.* reporter linked with the recognition unit through a spacer.^{1c} The Fluoroionophores, fluorescent chemosensors for ions, consist of an ion recognition unit (ionophore) and a fluorogenic unit (fluorophore) linked together through a proper spacer. As a result of tremendous research effort, many chemosensors have been synthesized for the purpose of detection of biologically and environmentally important analytes such as biologically important metal ions, biological molecules *etc.*

The design of highly sensitive and selective fluorescent sensors capable of detecting transition-metal ions, especially Cu^{2+} ion, has attracted considerable attention since many years as this metal ion is not only a significant environmental pollutant^{3a-c} but also the third most abundant and an essential trace element^{3b-d} in human biology.^{1c,3} Thus, considerable efforts have been devoted to the development of Cu^{2+} ion selective fluorescence chemosensors.³⁻⁵ As a result of tremendous research effort, there are many reports of “turn-off” fluorescent probes for Cu^{2+} ion which are generally less sensitive, and offers limited resolution.⁴ On the other hand, the “turn-on” fluorescence sensors are highly sensitive and offer high resolution. However, the design of such probes is a challenging task as being a paramagnetic metal ion Cu^{2+} often leads to the quenching of the fluorescence of the probe; thus limiting the abundance of “turn-on” fluorescence sensor for Cu^{2+} ion.⁵ Only in recent years, few examples of “turn-on” sensors have appeared; however, many of them

need special and complex designed architecture to provide metal coordinating platform. Therefore, there is a need to design cost effective, less cumbersome, simple, new and novel turn-on fluorescent probes to detect Cu^{2+} ion.

Among the “turn-on” fluorescent probes, solvatochromic fluorophores with internal charge transfer (ICT) character or excimer forming ability with increasing or decreasing intensities are more prominent and highly quantified as efficient sensor. However, such types of fluorescent probes for detection/sensing of Cu^{2+} ion are rare. A very few examples are available where fluorescence intensity of the excimer band changes with the change in concentration of Cu^{2+} ion.^{5g-l}

To improve the fluorescence intensity enhancement of the receptor upon binding of Cu^{2+} ion, one needs to carefully design the receptor molecule containing a fluorophore so that the responsible mechanism for fluorescence quenching is maximized in the fluorophore of the receptor in unbound state, whereas it is minimized in the metal-bound state of the receptor. For the selective recognition of soft, heavy metal ions, nitrogen binding sites might be a choice, as is well exemplified with classical azacrown ethers. In this respect “click triazole” might also be an excellent choice.⁶ Rich electron density and soft binding site of the triazole’s nitrogen would be a promising choice to bind with or recognize the Cu^{2+} ion. Since the very inception of the philosophy of “click chemistry” in 2001, it has attracted attention of scientific world of diverse range of research field. Whereas the “click triazole” has been frequently utilized as spacer between two functional units or as a linker in the areas of functional materials, biology, polymer science, and drug discovery, *etc.*,⁶ its potential as a metal coordination platform with a generation of fluorescence signal has been explored to some extent only in recent years.⁷ Therefore, it is observed that in addition to the synthetic simplicity and modular nature of the click reaction, the resulting 1,2,3-triazoles are able to perform a variety of important roles in sensing.⁸ They may: (1) contribute to the binding of the target analyte, (2) act as a linker between the binding site and the reporter, or (3) contribute to the reporter, usually as a part of a conjugated fluorophore. However, in many of the cases, the triazole ligated

fluoroionophoric sensory systems often need complex designed supramolecular architecture for accommodating the metal ions inside the cavity of such astonishing structure. It is also important to mention that in almost all the cases triazole play merely as a spacer. There are also a very few reports of fluorescence sensor signaled by an enhancement of fluorescence intensity of the fluorophore upon coordination with the triazole unit of the fluoroionophore. Thus, there is a high need for design of simple and efficient triazole based “light-up” fluorescent sensor for trace metal ions like Cu^{2+} . However, at present very few triazole based chemosensors for the detection of Cu^{2+} are reported in literature.

Pyrene is a well-known example for the efficient excimer formation. The intensity ratio of the excimer to monomer emission ($I_{\text{Exci}}/I_{\text{Mono}}$) of the pyrene derivative is a valuable informative parameter in sensory application. However, the ratio depends upon the structure of the pyrene derivative and the surrounding environment and thus has been widely useful in biological, polymeric and sensory materials chemistry.^{9,10}

5B.2. Background

From the above discussion in previous section, it is clear that fluorescent molecules, whose emission spectra and quantum yields are markedly sensitive to solvent polarity, are widely used as reporter probes for investigating chemical, biochemical, and biological phenomena including metal ion sensing. Of various types of chemosensors, fluorescent chemosensors have several advantages over other methods given their sensitivity, specificity, and real-time monitoring with fast response time. Recently, the development of fluorescent chemosensors capable of selective recognition and sensing of metal ions is one of the most challenging fields in organic and supramolecular chemistry.^{10,11} Therefore, a large number of sensory systems have been developed since many years for the detection of metal ions of both environmental and biological importance.

The journey of chemosensor started in early 1980s. Thus, in 1980 Tsien *et al.*, first reported the synthesis of fluorescent calcium indicators.^{12,13} These indicators were

based on calcium ion chelate receptors, and linked to aromatic rings or chromophores. At that time organic chemists generally referred to these molecular indicators as “sensors”. In 1993 Czarnik *et al.*, first introduced the concept of “Chemosensor”.^{14,15} and they defined it as: “A compound of abiotic origin that complexes to an analyte reversibly with a concomitant fluorescent signal transduction” and it constitutes only the active transduction unit of a sensor.^{14,15} Since then tremendous research has been carried out for the design and synthesis of fluorescent chemosensor.¹⁶⁻²⁰ However, a few effective sensors without the loss of sensitivity in the target system are currently available. Thus, the design and synthesis of fluorescent chemosensors without the loss of sensitivity in functional device or in presence of target analytes is still a challenging research field.

Fluorescent chemosensors for the detection of environmentally as well as biologically hazardous metal ions and some physiological essential trace elements are highly desirable. Transition and heavy-metal ions play important roles in living systems and their high level of concentration are hazardous for the environment.²¹ Heavy metal ions like mercury, lead, *etc.*, and metalloids like arsenic present in the soil, water and air are highly toxic for our ecosystem. Similarly, the essential trace elements are responsible for biochemical process of the living system and their deficiency causes various diseases. Thus, the design and synthesis of environmentally and user friendly chemosensor for the detection of essential trace elements and the ecological hazardous elements are of high importance.

In particular, detection of Cu^{2+} ion by a fluorescence sensor has got much attention in recent years because Cu^{2+} is the third most abundant essential trace element after iron and zinc in the human body. Copper ions play a very important role in various physiological processes. However, their abnormal levels are toxic for physiological system and can cause oxidative stress and neurological disorders, including Alzheimer's, Parkinson's, Menke's, and Wilson's diseases.²² Thus, it is very important to detect copper ions. In the past few years, a number of techniques have been developed to detect copper ions. Among them fluorescence based detection

has taken the leading role in the detection area because of its high sensitivity, selectivity, and ease of operation. Thus, the design and synthesis of fluorescent probes for Cu^{2+} ion has received special attention. However, most of the synthesized fluorophores which are reported are fluorescence “turn-off” sensors.^{5,23} Although “turn-on” probes have high sensitivity due to the lack of background signal, a major limitation of intensity-based probes is that variations in the sample environment may influence the fluorescence intensity measurements. Ratiometric fluorescent probes are of good choice to overcome this problem. These probes allow the measurement of fluorescent intensities at two wavelengths, one increases and other decreases. They permit signal rationing and thus increase the dynamic range and provide built-in correction for environmental effects.²⁴ Therefore, during the past few years a number of ratiometric probes have also been synthesized and reported in literature for Zn^{2+} ,²⁵ Ag^+ ,²⁶ and Pb^{2+} .²⁷ However, as the Cu^{2+} ion is a fluorescence quencher, because of its paramagnetic nature, very few ratiometric fluorescent sensors for Cu^{2+} have been found in the literature.

A few examples of some of the reported chemosensors for the detection of Cu^{2+} ion are presented below.

5B.2.1. Fluorescence Turn-Off Sensor for Cu^{2+} Ion

For most of the reported Cu^{2+} fluorescent sensors, it is found that the binding of the Cu^{2+} ion causes a fluorescence quenching due to its paramagnetic nature. As a result of various research efforts a large number of fluorescence turn-off sensors for Cu^{2+} ion have been developed and reported. As for example Xie *et al.*,^{28a} have reported *N*-pyrenylacetamide-substituted sugar-aza-crown ethers derivatives as fluorescent chemosensors of Cu^{2+} ion (5B.1 and 5B.2, **Figure 5B.1**). The compound 5B.1 and 5B.2 exhibited fluorescence characteristic of pyrene monomer and excimer emission and both compounds showed fluorescence quenching upon addition of Cu^{2+} ion.

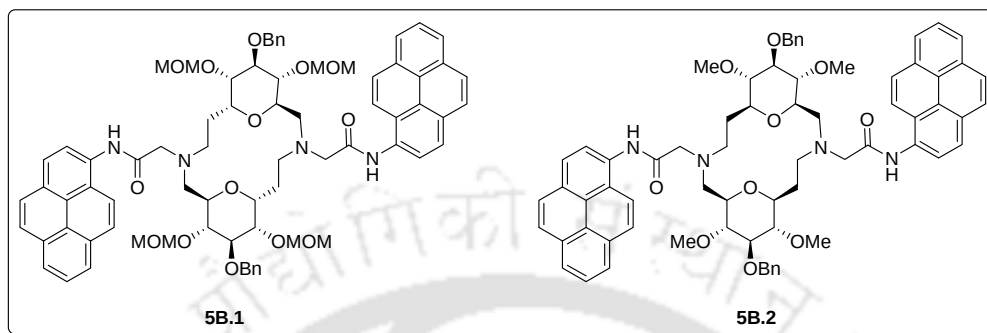


Figure 5B.1: Fluorescence turn-off Cu^{2+} sensors based on sugar-aza-crown ether.

A chiral perazamacrocyclic fluorescent sensor (**5B.3a**, **Figure 5B.2**), which showed fluorescent turn-off property in presence of Cu^{2+} ion among other metal ions has been developed by Zhu *et al.*^{28b}

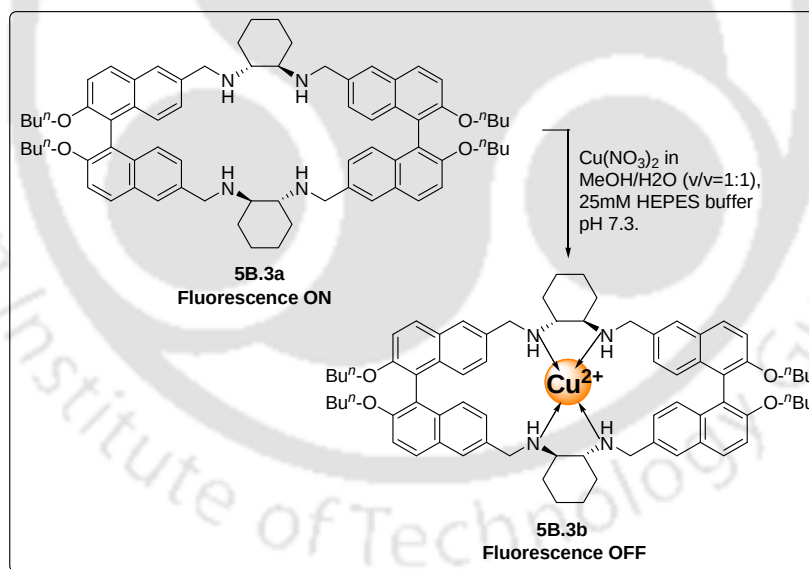


Figure 5B.2: Chiral fluorescence turn-off Cu^{2+} sensor developed by Zhu *et al.*^{28b}

A coumarin-based fluorogenic probe was exploited as a fluorescent chemosensor for Cu^{2+} ion by Lee *et al.*, (**5B.4a**, **Figure 5B.3**).^{28c} Thus the compound **5B.4a** showed fluorescent quenching effect in aqueous buffer solution (HEPES:DMSO = 9:1, v/v)

upon addition of Cu^{2+} ion. They also claimed that **5B.4a** could be one of the most important sensing probes for the detection of Cu^{2+} ion in living cells.

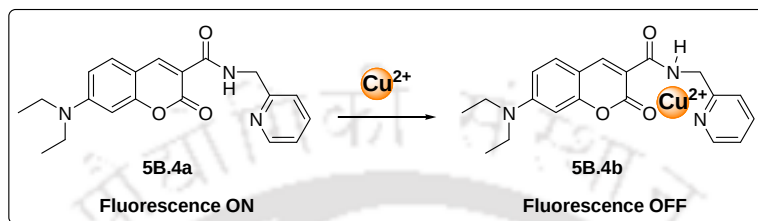


Figure 5B.3: Coumarin-based fluorescence turn-off probe for Cu^{2+} ion.

5B.2.2. Fluorescence Switch-On Sensor for Cu^{2+} Ion

Although Cu^{2+} ion causes a fluorescence quenching of many of the fluorophore, there exists a few reports of sensors where the binding of the Cu^{2+} ion causes an increase in the fluorescence intensity of the probe.²⁹ Here, in this section, a few of such switch-on fluorophorescent sensor for Cu^{2+} ion are presented in very brief.

Yoon *et al.*,^{30a} have reported a fluorescence “off-on” chemosensor and chemodosimeter for Cu^{2+} ion (**Figure 5B.4**). Thus, compound **5B.5** showed selective and large chelation enhanced fluorescence effects with Pb^{2+} and Cu^{2+} among the various other metal ions tested. On the other hand, compound **5B.6** is a fluorescent chemodosimeter which effectively recognized Cu^{2+} ion *via* a selective hydrolysis of the acetyl group.

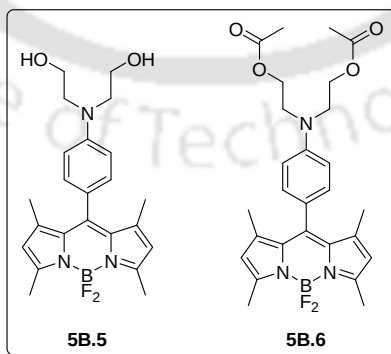


Figure 5B.4: Cu^{2+} chemo- and chemodosimeter sensor.

Penttiptycene-bispyrenyl system has been developed by Yang *et al.*,¹⁰¹ as a fluorescent chemosensor for metal ions (**5B.7**, **Figure 5B.5**). Thus, in presence of Cu^{2+} ion, compound **5B.7** showed a blue shifted pattern along with an enhancement of intensity of the pyrene excimer emission. This new signaling mode of pyrene probes results from the formation of a static pyrene excimer from its sandwich-like dynamic excimer.

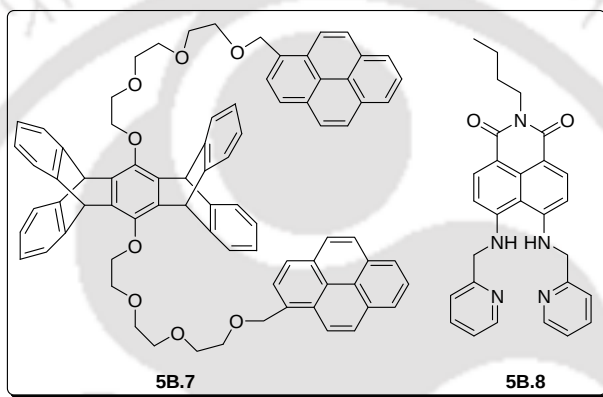


Figure 5B.5: Pyrene, naphthalimide fluorophore based fluorescence switch-on Cu^{2+} ion sensors developed by Yang *et al.*,¹⁰¹ (**5B.7**), and Qian *et al.*,^{30b} (**5B.8**).

Qian *et al.*,^{30b} have reported a ratiometric and selective fluorescent sensor **5B.8**, *N*-butyl-4,5-di[(pyridin-2-ylmethyl)amino]-1,8-naphthalimide, for Cu^{2+} ion (**Figure 5B.5**). In aqueous ethanol solution, compound **5B.8** formed a 1:1 metal-ligand complex in the presence of Cu^{2+} ion, which exhibited a strong and increase in fluorescence emission centered at 475 nm at the expense of the fluorescence emission centered at 525 nm from an internal charge transfer (ICT) state. Later on, they^{30c} modified the structure of **5B.8** and developed a colorimetric and ratiometric fluorescence chemosensor, **5B.9a** which showed a large red-shifted in emission in presence of Cu^{2+} ion (**Figure 5B.6**). The compound **5B.9a** displayed color change from primrose yellow to pink and the red-shift of emission wavelength from green to red in presence of Cu^{2+} ion through the deprotonation of the two secondary amines conjugated to the naphthalene ring.

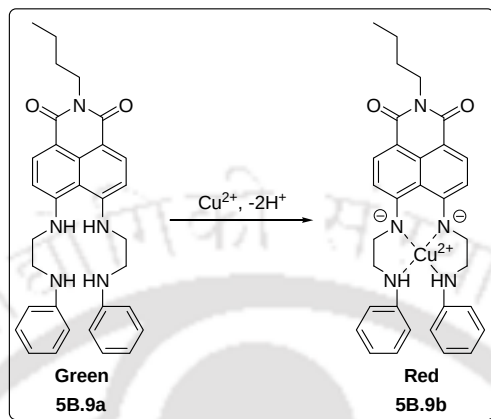


Figure 5B.6: Cu^{2+} sensors developed by Qian *et al.*^{30c}

A new pyrene based probe, 1,4-bis(1-pyrenyl)-2,3-diaza-1,3-butadiene (**5B.10**, **Figure 5B.7**), which selectively senses Hg^{2+} and Cu^{2+} through the enhancement of the fluorescence with the red shift of the excimer emission has been developed by Molina *et al.*^{30d} Later on, they reported another fluorescent probe, 2-aza-1,3-butadiene derivatives^{30e} containing an anthracene or pyrene unit (**5B.11**, **Figure 5B.7**). Compound **5B.11** containing a pyrene unit detects Cu^{2+} via an enhancement of fluorescence intensity in aqueous environment.

Shiraishi *et al.*,^{30f} have reported a new rhodamine derivative containing an ethylenediamine-*N,N*-diacetic acid moiety and exploited in sensing Cu^{2+} ion (**5B.12**, **Figure 5B.7**). Thus, the compound **5B.12** showed strong green fluorescence in presence of Cu^{2+} ion, while with other metal ions it showed very weak orange fluorescence. Kim *et al.*,^{10m} have reported a monopyrenylalkylamine derivative (**5B.13**, **Figure 5B.7**) which exhibited an excimer emission at 455 nm along with a weak monomer emission at 375 nm in presence of Cu^{2+} ion in 1:1 (v/v) $\text{H}_2\text{O}/\text{CH}_3\text{CN}$. A coumarin-based colorimetric chemosensor has also been developed by Wang *et al.*,^{30g} (**5B.14**, **Figure 5B.7**), which exhibited good sensitivity and selectivity for the Cu^{2+} ion over other tested cations such as Zn^{2+} , Cd^{2+} , Pb^{2+} , Co^{2+} , Fe^{2+} , Ni^{2+} , Ag^+ , and alkali and alkaline earth metal cations in aqueous solution. Further, they investigated

the sensitivity of **5B.14** on paper-made test kits which turned yellowish-orange to red in aqueous solution containing Cu^{2+} ion.

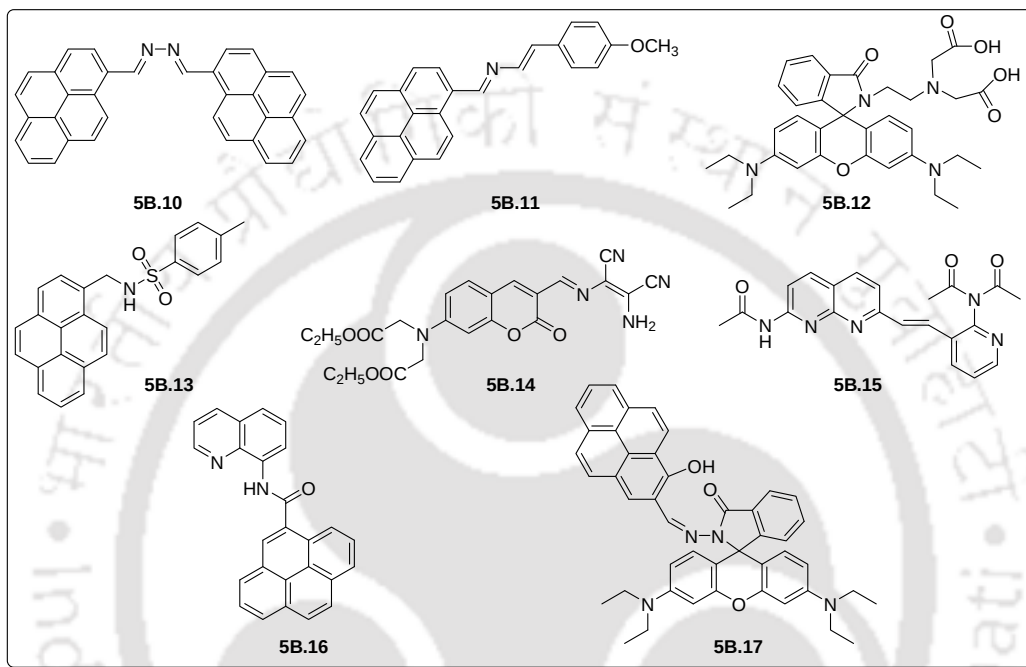


Figure 5B.7: Example of some light-up probe for the detection of Cu^{2+} ion.

A 1,8-naphthyridine-based fluorescent chemodosimeter **5B.15** (**Figure 5B.7**), for the selective detection of Zn^{2+} and Cu^{2+} ions has been reported by Wei *et al.*^{30h} Thus, compound **5B.15** exhibited a dual emissive behavior in presence of Zn^{2+} ion and a fluorescent “on-off” behavior upon addition of Cu^{2+} ion. Ham *et al.*,¹⁰ⁿ have reported a sensor containing a pyrene derivative, **5B.16** (**Figure 5B.7**), for Cu^{2+} ion. Thus, in CH_3CN compound **5B.16** exhibited a Cu^{2+} ion induced strong static excimer emission at 460 nm, along with a weak monomer emission at 388 nm. Yoon *et al.*,³⁰ⁱ reported a rhodamine-based (**5B.17**, **Figure 5B.7**) ratiometric and fluorescent “off-on” chemosensor for Cu^{2+} ion. Thus, compound **5B.17** displayed a selective and chelation enhanced ratiometric fluorescence change and colorimetric change with Cu^{2+} ion among the metal ions examined.

Recently, 1,4-disubstituted triazole has become an increasingly common motif in chemical sensors. Although, most of the applications of these click-derived triazoles are found in ligation strategy in biological world, they are, now a days, explored in chemosensory application because of their ability to bind both cations and anions via the triazolyl nitrogens. Thus, there is a growing number of reports in the literature related to triazole-based systems involving azamacrocycles, calixarenes, rotaxanes, cyclodextrine, DNA, nanoparticles etc. that can potentially be converted into effective sensors by appending a fluorophoric reporter units. However, in almost all the cases triazoles are acting as a mere linker to a fluorophore. Very few examples are there where triazoles are utilized as a metal coordination platform in a sensory system. **Figure 5B.8** illustrates chemical structures of some of the triazole based sensory systems which have effectively been utilized for detection of several metal cations including very few examples for Cu^{2+} ions. In most of the cases, triazol units act merely as a linker unit of fluorophore-receptor systems. However, very few examples appeared in the literature wherein triazole units really act as metal ion coordinating platform, as for an example compound 5B.22.

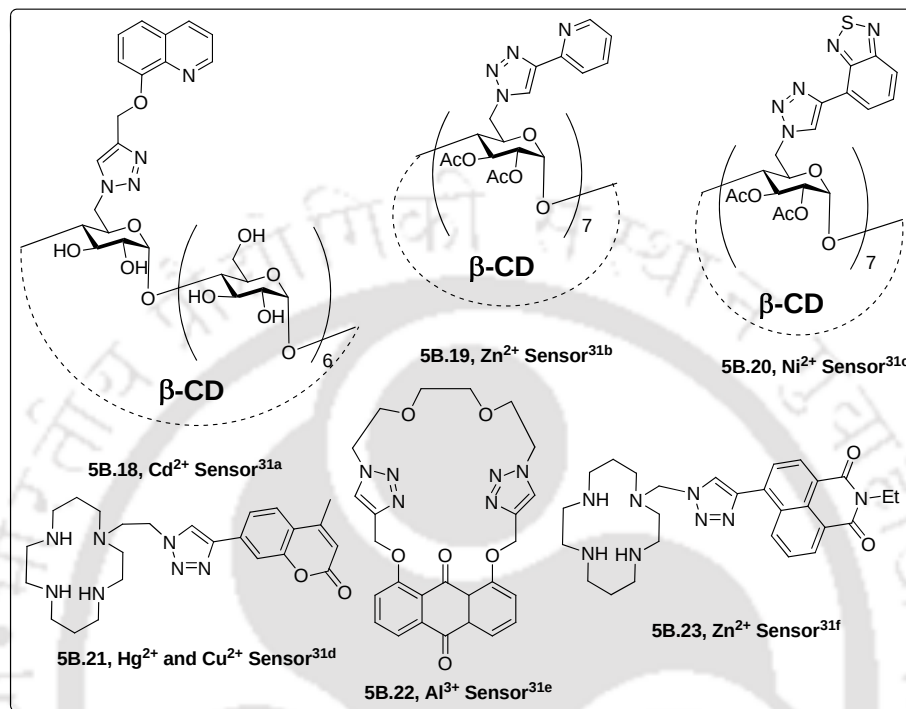


Figure 5B.8: Example of some triazole based chemosensor.

However, there are no reports of Cu^{2+} ion sensory system, to the best of our knowledge, wherein triazole units act really as coordinating platform for Cu^{2+} ion.

5B.3. Objective

With this above literature background of fluorophoric systems for Cu^{2+} ion detection and the lack of triazole based metal coordinating sensory system, in particular, for Cu^{2+} ion as well as our success in probing micellar microenvironment with our efficient triazolyl pyrene smart fluorescent probe, we thought that it would be worthwhile to exploit this probe as a sensor for Cu^{2+} ion. Therefore, with this aim and idea we framed our objective as below:

- Study of metal sensing ability of the probe triazolyl pyrene ($^{\text{TNDMB}}$ Py), by UV-visible and fluorescence spectroscopy.

- (b) Study of selectivity in sensing Cu^{2+} ion.
- (c) We envisaged that our probe might form excimer by Cu^{2+} ion coordination through two triazolyl nitrogen of two monomer probes. Thus, we wanted to study the possibility of excimer fluorescence theoretically which might support our observation of excimer emission if at all.
- (d) Our probe was found to be efficient switch-on fluorescent probe of micellar microenvironment. Micellar systems are known to modulate fluorescence property of a fluorophore; thereby enhancing the sensing ability of a probe for an analyte inside the micellar nanocore. Therefore, we thought that it would be interesting if our probe can be utilized in detection of Cu^{2+} ion inside the micellar nanocore. Thus, we are also interested to test our probe's ability in this event.

5B.4. Results and Discussion

The synthesis of the probe ($^{\text{TNDMB}}$ Py) was achieved following our reported click chemistry protocol (already has discussed in **Chapter 2**) with very high yield.³² After synthesizing our designed probe **5B.27** ($^{\text{TNDMB}}$ Py), and having the very pure probe in hand by recrystallization, we turned our attention to study the photophysical properties of the probe in presence of various metal cations in organic and in aqueous micellar environment.

5B.4.1. Study of Photophysical Properties

We have previously reported (in **Chapter 2**) the fluorescence behavior of the probe in different solvents of varying polarity. The probe, triazolylpyrene showed an interesting fluorescence property with high solvatochromicity and quantum yield. In brief the molecule shows a dual emission behaviour centered at 383 nm, a locally emission (LE) band and 466-544 nm, internal charge transfer (ICT, depending on solvent polarity) band. Switching between these two states depends on the nature and polarity of the solvent. **Figure 5B.9** shows the emission behavior of the probe **5B.27**

($^{\text{TNDMB}}$ Py) in various polar solvents. In particular in acetonitrile solution and in buffer solution of pH 7.0, it shows only a weak single emission band at around 525 nm originating from ICT state. The emission from LE state in these two solvents is negligible.

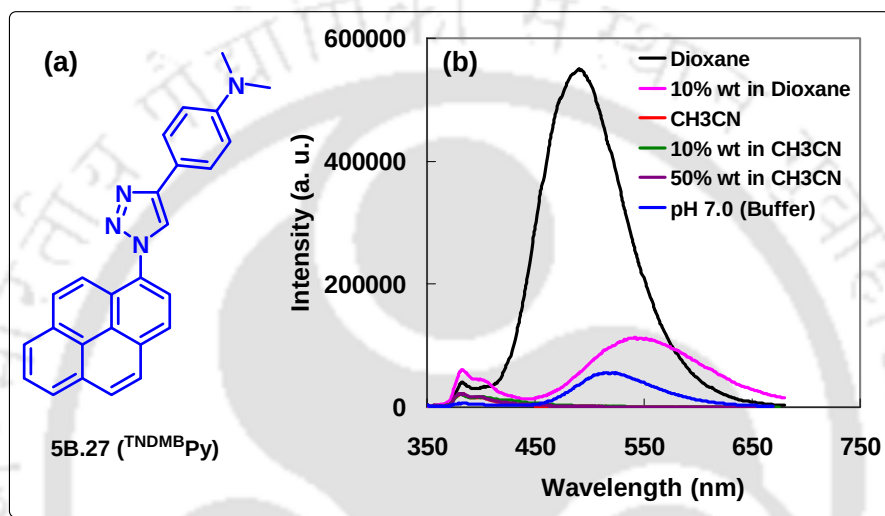


Figure 5B.9: (a) Structure of triazolylpyrene ($^{\text{TNDMB}}$ Py) probe. (b) Emissions behavior of the probe in different solvents.

5B.4.1.1. UV-Visible Study

After evaluating the probe's fluorescence emission in acetonitrile and in buffer, we next explored the sensing capability of $^{\text{TNDMB}}$ Py towards Cu^{2+} ion in acetonitrile in presence of other metal ions like, Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Co^{2+} , Ni^{2+} , Cu^+ , Zn^{2+} , Ag^+ ions. To do so, we added the various concentration of each metal solution into the probe's solution of fixed concentration and then photophysical properties were studied. Thus, from the UV-visible absorption it was observed that the Cu^{2+} ion induces a change in absorption spectra (**Figure 5B.10a-b**), while other metals have negligible/inconsistent effect which implied that only the Cu^{2+} ion interact significantly strongly in a well-defined manner with the probe under the experimental condition which is also evident from the generation of a bright blue colour of the solution observable under day light (**Figure 5B.11a**). We also observed that with

increasing Cu^{2+} ion concentration the absorbance of the probe ($^{\text{TNDMB}}$ Py) increases consistently (**Figure 5B.10b**); however, for other metal ions the absorbance remains unaltered or inconsistent change was observed (**Figure 5B.22-30**, UV-visible spectra under section 5B.4.4).

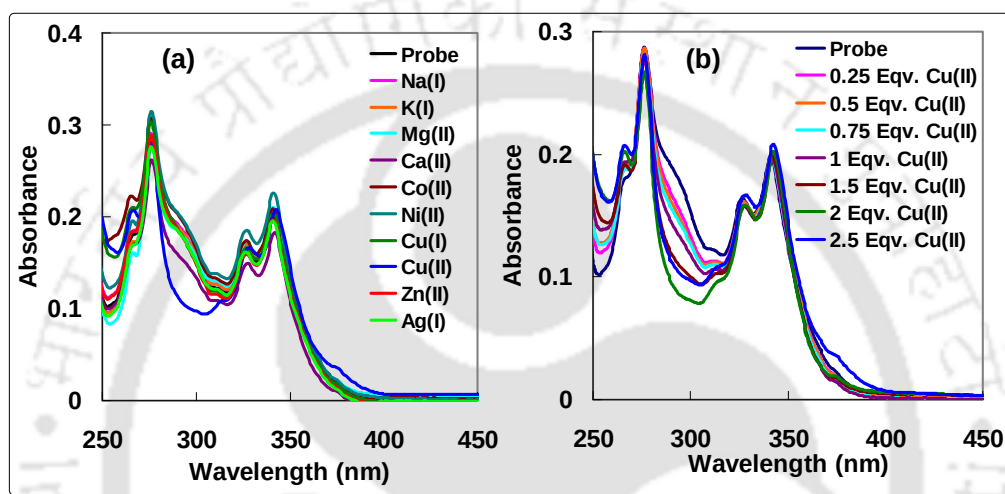


Figure 5B.10: (a) UV-vis spectra of probe ($^{\text{TNDMB}}$ Py) (10 μM) in presence of various metal ions (2.5 equiv.). (b) UV-vis spectra of probe ($^{\text{TNDMB}}$ Py) (10 μM) in presence of increasing Cu^{2+} concentration.

5B.4.1.1.1. Job's Plot from UV-Visible Study

To quantify the stoichiometry between probe ($^{\text{TNDMB}}$ Py) and the Cu^{2+} ion, the absorption Job's plot measurement was carried out. For this purpose, an equal concentration of probe ($^{\text{TNDMB}}$ Py) and Cu^{2+} ion solutions separately were prepared in UV-grade acetonitrile solvent. Then, the probe and the Cu^{2+} ion solutions were mixed in different fraction of volume maintaining the total volume of mixture 3 mL. All the solutions were mixed well and kept for some time in room temperature. Then the absorbance spectra of the solutions of different composition of probe and Cu^{2+} ion were recorded. To calculate the probe- Cu^{2+} complexation ratio, $[\text{probe-Cu}^{2+}]$ vs. X_{probe} were plotted, where $[\text{probe-Cu}^{2+}] = \Delta A/A_0 \times [\text{probe}]$, $\{\Delta A = (A - A_0)\}$; and X_{probe} is the mole fraction of probe, $\{X_{\text{probe}} = [\text{probe}]/([\text{probe}] + [\text{Cu}^{2+}])\}$. The corresponding

X_{probe} value where the concentration of probe- Cu^{2+} complex, $[\text{probe-Cu}^{2+}]$ was maximum was taken as probe-to-metal stoichiometry. The Job's plot (**Figure 5B.11b**) showed the point of maximum at the mole fraction of ~ 0.66 of the probe, which clearly indicated a 2:1 stoichiometry of the probe to Cu^{2+} ion in the complex.

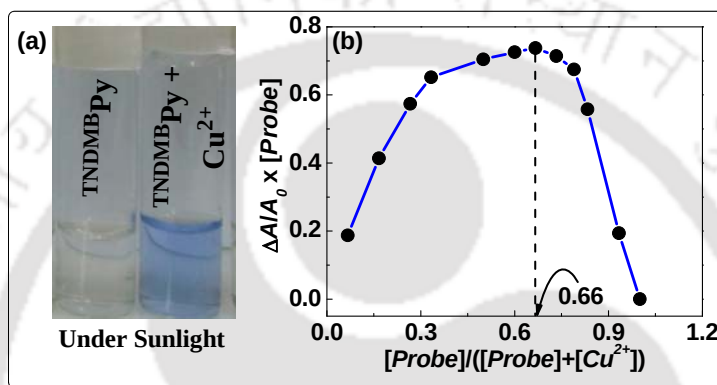


Figure 5B.11: (a) Day light colour of the probe's solution mixed with 2.5 equivalent of Cu^{2+} ion in CH_3CN . (b) Absorption Job's plot of probe, $^{\text{TNDMB}}$ Py in presence of Cu^{2+} ion in CH_3CN . $[^{\text{TNDMB}}\text{Py}] = 30 \mu\text{M}$ and $[\text{Cu}^{2+}] = 75 \mu\text{M}$.

5B.4.1.2. Fluorescence Study

Next, the fluorescence study of the probe, $^{\text{TNDMB}}$ Py in presence and absence of various metal ions of different concentration was carried out. We observed a remarkable change in the emission behavior of the probe in presence of Cu^{2+} ion only among the other metal ions like, Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Co^{2+} , Ni^{2+} , Cu^+ , Zn^{2+} , Ag^+ ions tested (**Figure 5B.12a**). The probe $^{\text{TNDMB}}$ Py exhibited only a faint LE emission or negligible monomer band ($\lambda_{\text{em}} = 383 \text{ nm}$) in acetonitrile. Upon addition of Cu^{2+} ion the fluorescence intensity of the monomer band drastically increased. With gradual increasing intensity of monomer band, a newly generated excimer band ($\lambda_{\text{em}} = 466 \text{ nm}$) with increasing intensity was also observed with increasing concentration of Cu^{2+} ion. The intensity of both the monomer (I_M) and excimer (I_E) bands and the quantum yields were also found to be increased with $[\text{Cu}^{2+}]$ and reached maxima when the

Cu^{2+} ion concentration was 2.5 equivalent of that of the probe, triazolyl pyrene (**Figure 5B.12b**, **Table 5B.1**). This observation clearly suggested that there is a strong complexation between the probe and the Cu^{2+} ion. This result was also clear from the deep blue colour of the probe's solution mixed with 2.5 equivalent of Cu^{2+} ion when irradiated at 254 nm under a transilluminator (**Figure 5B.12c**). However, as the Cu^{2+} ion concentration exceeded 25 μM (2.5 equiv.), quenching of both the monomer as well excimer emission was observed. Therefore, maximum emission intensity of the probe was observed at 2.5 equiv of Cu^{2+} ion only over the other studied metal ions, indicating a well-defined binding of Cu^{2+} ion to the functional triazole units of the probe forming a $^{\text{TNDMB}}\text{Py}-\text{Cu}^{2+}$ complex.

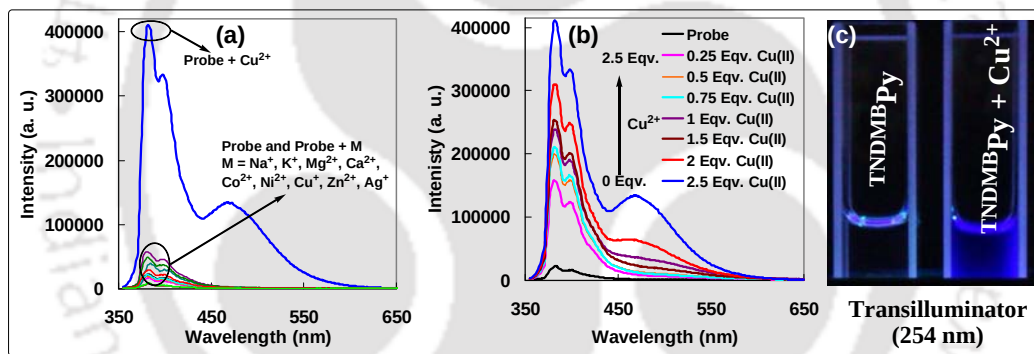


Figure 5B.12: (a) Fluorescence spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of probe ($^{\text{TNDMB}}\text{Py}$) ($10 \mu\text{M}$) in presence of various metal ions (2.5 equiv.). (b) Fluorescence spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of probe ($^{\text{TNDMB}}\text{Py}$) ($10 \mu\text{M}$) in presence of increasing Cu^{2+} concentration. (c) Colour of the probe's solution mixed with 2.5 equivalent of Cu^{2+} ion under a transilluminator when irradiated at 254 nm. [$^{\text{TNDMB}}\text{Py}$] = $30 \mu\text{M}$ and [Cu^{2+}] = $75 \mu\text{M}$.

Table 5B.1: Photophysical summary of $^{\text{TNDMB}}$ Py with $[\text{Cu}^{2+}]$ ion in CH_3CN .

Cu^{2+} (Eqv.)	$\lambda_{\text{max}}^{\text{abs}}$ (nm)	Abs	$\lambda_{\text{mono}}^{\text{fl}}$ / $\lambda_{\text{exci}}^{\text{fl}}$ (nm)	Φ
0.00	276/343	0.287/0.191	383	0.003
0.25	276/343	0.286/0.194	383	0.020
0.50	276/343	0.286/0.197	383	0.026
0.75	276/343	0.279/0.193	383	0.026
1.00	276/343	0.276/0.195	383/466	0.035
1.50	276/343	0.267/0.197	383/466	0.038
2.00	276/343	0.267/0.202	383/466	0.049
2.50	276/343	0.281/0.208	383/466	0.073

The fluorescence change of the probe ($^{\text{TNDMB}}$ Py, 10 μM) in presence and absence of other metal ions was also investigated to test the specificity of the probe in sensing Cu^{2+} ion. Thus, unlike Cu^{2+} ion, only a negligible/inconsistent change in the monomer emission was observed in presence of various concentrations of other metal ions. Then, we plotted the emission intensity of the probe (10 μM) in presence of various metal ions of fixed concentration (25 μM) (**Figure 5B.13**) and compared the fluorescence intensity change $((I - I_0)/I_0 \times 100\%)$ of the probe in presence of various metal ions. Thus, from the bar diagram it is clear that about 1800 fold enhancement of fluorescence intensity was observed upon addition of Cu^{2+} ion compared to other metal cations.

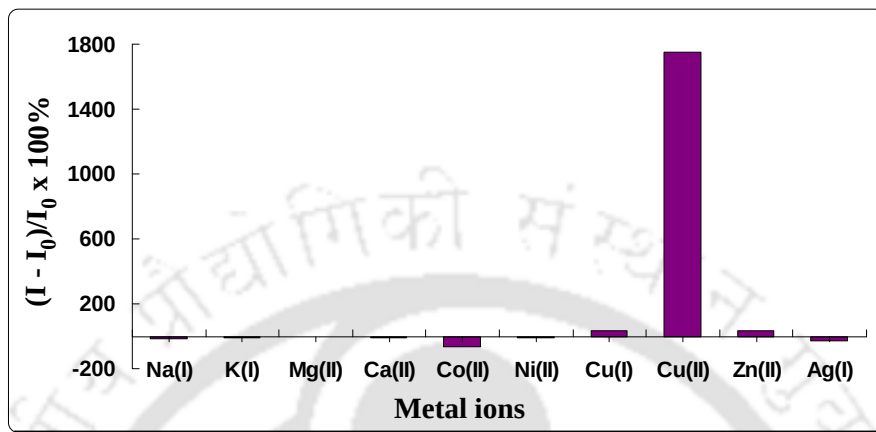


Figure 5B.13: Fluorescence ($\lambda_{\text{ex}} = 343 \text{ nm}$) intensity changes ($(I - I_0)/I_0 \times 100\%$) of probe ($^{\text{TNDMB}}$ Py) ($10 \mu\text{M}$) at 298 K upon addition of various metal ions (2.5 equiv). I_0 is fluorescent emission intensity at 383 nm of free host and I is the fluorescent intensity after adding metal ions.

The photophysical properties of the probe $^{\text{TNDMB}}$ Py in presence of Cu^{2+} ion of different concentration in CH_3CN are summarized in **Table 5B.1**. Next, we evaluated the I_{Excimer} to I_{Monomer} ratio of the probe ($10 \mu\text{M}$) which was found to gradually increase with increasing Cu^{2+} concentration and reached maxima at 2.5 equiv. of Cu^{2+} concentration with respect to the probe. **Figure 5B.14** shows the plot of I_{Excimer} to I_{Monomer} and I_{Monomer} to I_{Excimer} ratio of $^{\text{TNDMB}}$ Py in presence of increasing Cu^{2+} concentration. The ratio of I_{Excimer} to I_{Monomer} depends upon the $[\text{Cu}^{2+}]$, thus the ratio can be utilized for tentative measurement of $[\text{Cu}^{2+}]$ present in a solution.

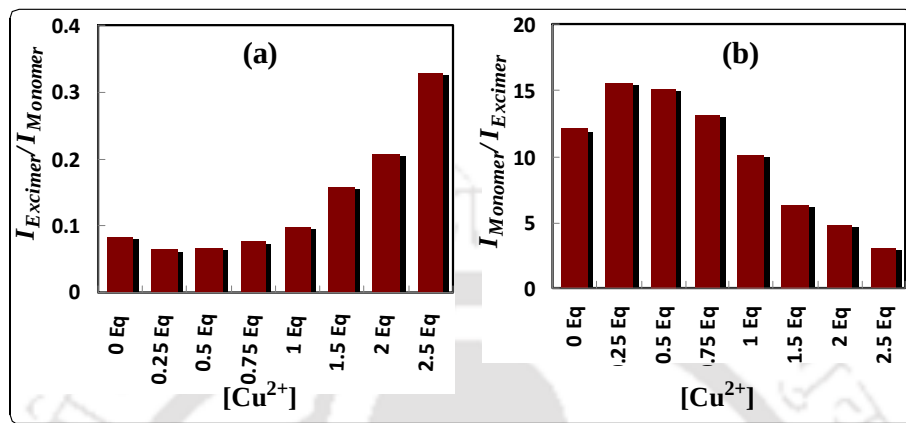


Figure 5B.14: Plot of (a) I_{Excimer} to I_{Monomer} and (b) I_{Monomer} to I_{Excimer} ratio of $^{\text{TNDMB}}$ Py in presence of various concentration of Cu^{2+} ion in CH_3CN .

5B.4.1.2.1. Fluorescence Job's Plot

To determine the stoichiometry of the probe- Cu^{2+} complexation and to support our earlier observation of 2:1 complexation from UV-visible study, we have studied fluorescence Job's plot using the fluorescence intensity change of LE band in absence and presence of various concentrations (0 to 20 equiv.) of Cu^{2+} ions in CH_3CN . Thus, ΔI vs. X_{probe} was plotted for the various concentrations of Cu^{2+} ions, where $\Delta I = I - I_0$ (I_0 and I are the fluorescence intensities of $^{\text{TNDMB}}$ Py in absence and presence of Cu^{2+} ions) and X_{probe} is the mole fraction of probe, $X_{\text{probe}} = [\text{probe}]/([\text{probe}] + [\text{Cu}^{2+}])$. The corresponding X_{probe} value at ΔI value maxima was taken as probe-to-metal stoichiometry, which was found to be 0.66 (**Figure 5B.15**). This observation clearly indicated a 2:1 stoichiometry of the probe to Cu^{2+} ion complexation and supporting our observation from UV-visible absorption study (**Figure 5B.11b**).

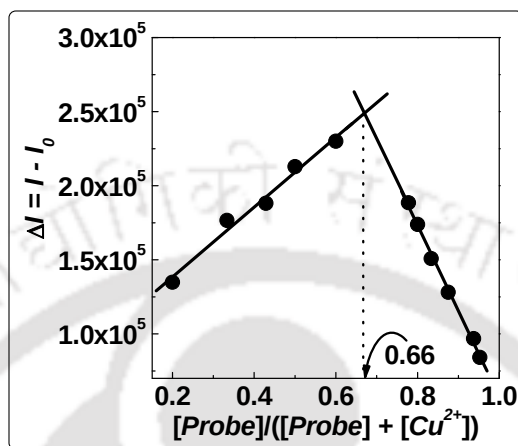


Figure 5B.15: Fluorescence Job's plot of probe, $^{\text{TNDMB}}$ Py in presence of Cu^{2+} ion in CH_3CN .

5B.4.1.2.2. Determination of Binding Constant from Fluorescence

After determining and confirming the 2:1 probe- Cu^{2+} complexation we, next, turn our attention to evaluate the binding constant by fluorescence spectroscopy. Thus, to determine binding constant we have constructed the well-known Benesi-Hildebrand plot using the following equation.

$$\frac{1}{(I - I_0)} = \frac{1}{(I_\infty - I_0)} + \frac{1}{(I_\infty - I_0)K[\text{Cu}^{2+}]} \dots\dots\dots (1)$$

where I_0 , I and I_∞ are the emission intensities of $^{\text{TNDMB}}$ Py in the absence of Cu^{2+} ion, at intermediate and at infinite concentration (where maximum intensity was observed) of Cu^{2+} ion respectively. To determine the binding constant, $1/(I - I_0)$ vs. $1/[\text{Cu}^{2+}]$ was plotted (**Figure 5B.16**). A linear fit was fitted by taking the points of linear region and from the slope value the binding constant was calculated, which was found to be $2.23 \times 10^5 \text{ M}^{-1}$ indicating a strong complexation between Cu^{2+} with probe $^{\text{TNDMB}}$ Py.

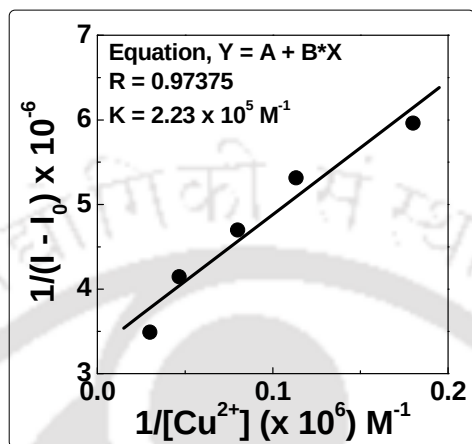


Figure 5B.16: Benesi-Hildebrand plot of the monomer band of $^{\text{TNDMB}}$ Py with Cu^{2+} .

5B.4.1.2.3. Support of 2:1 Complexation by Mass Spectral Analysis

To support the 2:1 probe- Cu^{2+} ion complexation we have carried out a mass spectral analysis of the solution of the probe containing Cu^{2+} ion. Thus, the ESI-Mass spectrum analysis revealed the formation of 2:1 probe-to-metal complex (**Figure 5B.17**). Thus, we observed a peak at $m/z = 841.65$ which is in good accord with the calculated mass for $[(^{\text{TNDMB}}\text{Py})_2\text{Cu-H}]^+$ (841.50; $\text{C}_{52}\text{H}_{41}\text{N}_8\text{Cu}$). This result again supports that one Cu^{2+} ion is coordinated by two triazole-Ns of two probes, $^{\text{TNDMB}}$ Py. Further support of 2:1 stoichiometry of probe- Cu^{2+} ion complex came from the theoretical study discussed later in section 5B.4.3.

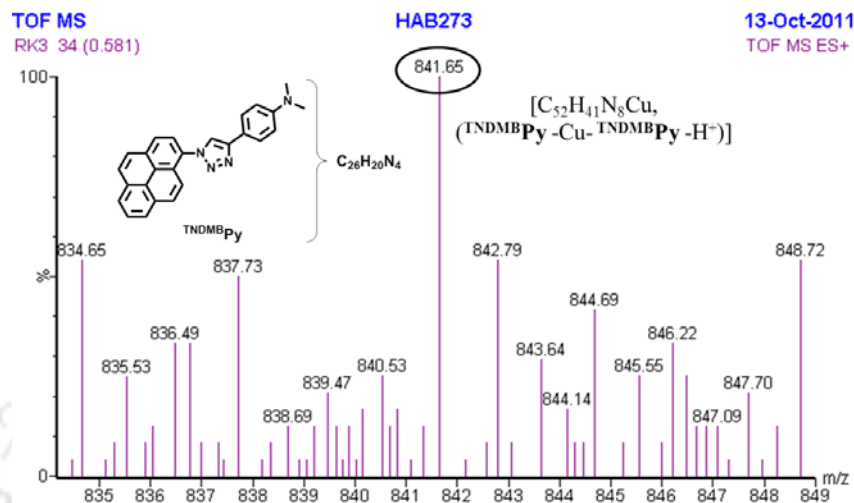


Figure 5B.17: ESI-MS of the complex $[\text{TNDMBPy-Cu}^{2+}-\text{TNDMBPy}]$.

5B.4.1.2.4. Test for Selectivity in Sensing of Cu^{2+} Ion

Competition experiments were carried out to explore the utility of our probe as an ion-selective switch-on fluorescence chemosensor for Cu^{2+} ion. Thus, to do so, the fluorescence spectra (**Figure 5B.18a**) of the probe (10 μM) were taken in presence of Cu^{2+} ion (20 μM) along with other background metal ions in 50 equiv. excess concentration each. Although the fluorescence property of the probe with Cu^{2+} ion in presence of other background metal ions was negligibly changed, but the ratios of excimer-monomer and/or vice versa of $^{\text{TNDMB}}$ Py with Cu^{2+} ion in absence and presence of other metal ions almost remained same (**Figure 5B.18b-c**). Thus, this result indicated that the probe could be used as a potential Cu^{2+} ion selective turn-on fluorescence sensor.

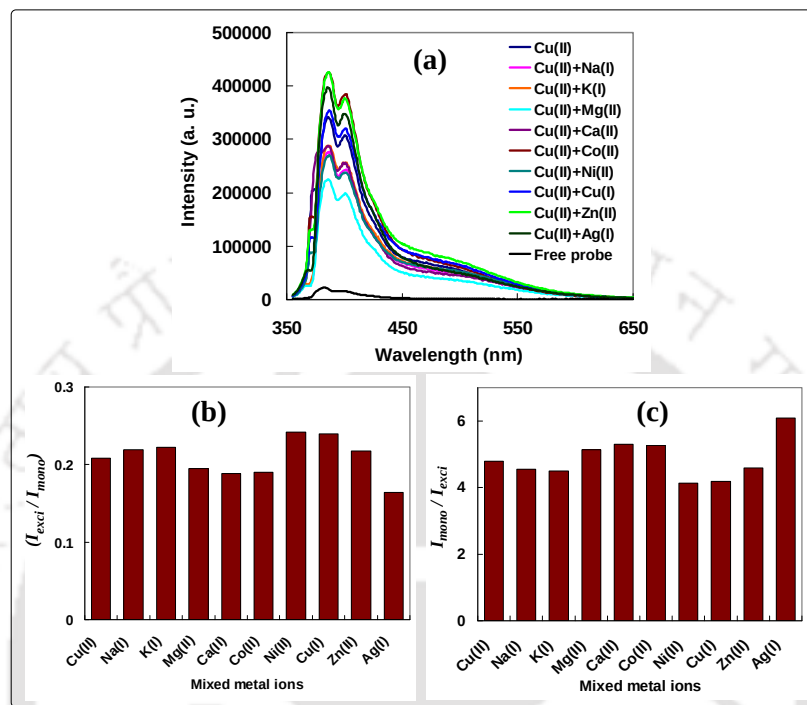


Figure 5B.18: (a) Emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of $^{\text{TNDMB}}$ Py (10 μM) with Cu^{2+} ion (2 equiv.) in absence and presence of other metal ions (50 equiv.). (b) I_{466}/I_{383} and (c) I_{383}/I_{466} ratios of fluorescence intensity of $^{\text{TNDMB}}$ Py (10 μM) with Cu^{2+} ion (2 equiv.) in absence and presence of other metal ions (50 equiv.).

5B.4.1.2.5. Determination of the Detection Limit

We have next calculated the detection limit of the fluorescent probe $^{\text{TNDMB}}$ Py towards Cu^{2+} ion by plotting $(I_{\text{min}} - I)/(I_{\text{min}} - I_{\text{max}})$ vs. $\log[\text{Cu}^{2+}]$. The linear fit was drawn by taking the five points (2.5 μM to 15 μM) of the linear region.³³ The extended line where it crossed the ordinate axis is the detection limit which was found to be 177 nM. Thus, our probe can be utilized for the detection of the submillimolar concentration range of Cu^{2+} ions found in many chemical and biological systems as its detection limit was calculated to be $1.77 \times 10^{-8} \text{ mol L}^{-1}$ for Cu^{2+} ion (**Figure 5B.19**).³³

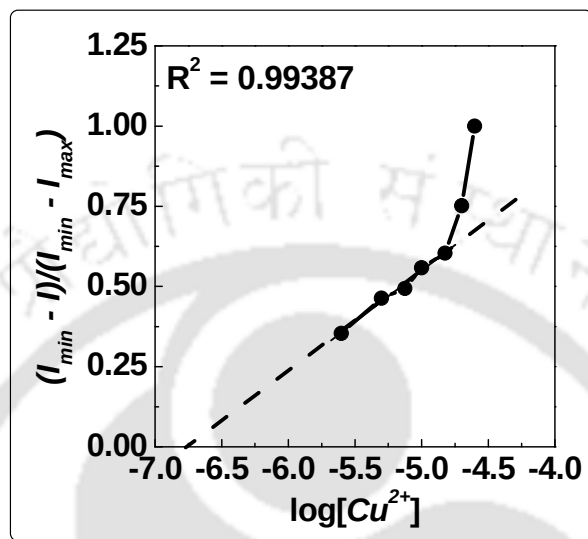


Figure 5B.19: Plot of $(I_{\min} - I)/(I_{\min} - I_{\max})$ vs. $\log[\text{Cu}^{2+}]$ of $^{\text{TNDMB}}$ Py for monomer band.

5B.4.2. Explanation of Switch-On Fluorescence

The “on-off” fluorescence response in the excimer as well as in the monomer emission of the probe might be attributed to the formation of stable complex between the probe’s triazole moieties and Cu^{2+} ion, and the subsequent blocking of the communication of the acceptor pyrene unit from the donor triazole moiety.³⁴ The low fluorescence intensity of the probe in CH_3CN in the absence of Cu^{2+} ion may be attributed to radiationless channel from the $n\text{-}\pi^*$ state of the emission of the triazolyl pyrene by the nitrogenous donor triazole of the probe. When Cu^{2+} ion coordinates with the triazole’s nitrogen, the energy of the $n\text{-}\pi^*$ state would be raised so that the $\pi\text{-}\pi^*$ state of the emission of the triazolyl pyrene becomes the lowest excited state, leading to a substantial increase in the fluorescence intensity. As the complexation with one molecule of the probe started, to satisfy the coordination of Cu^{2+} ion, another molecule of the probe comes closer to coordinate with the same Cu^{2+} ion, thus, bringing two pyrene units of two probes closer in face resulting in the appearance of intermolecular excimer emission. Upon further increase in copper concentration up to

2.5 equivalents, complete formation of the complex occurs resulting in maximum intensity of both the monomer and the excimer emission. However, when the concentration of Cu^{2+} ion exceeds 25 μM , emissions from both the monomer and excimer bands started quenching due to paramagnetic Cu^{2+} ion excess of which now involve in the cation- π interactions between two closely faced pyrene moieties.³⁵

5B.4.3. Support of 2:1 Complexation/Excimer Emission by Theoretical Study

As both the Job's plot and ESI-MS supported the formation of 2:1 ligand-to-metal complexation between $^{\text{TNDMB}}$ Py and Cu^{2+} ion, the energy optimization calculations were also carried out using B3LYP/3-21G* level of theory with Gaussian 03 package³⁶ to further support the possible 2:1 complexation leading to excimer emission *via* π - π interaction between two closely faced pyrene units of two probes in the complex. Thus, LANL2DZ effective core potential (ECP) was employed for Cu^{2+} ion and in other cases the 3-21G* basis sets were used for the calculation. Various geometries of the dimers of $^{\text{TNDMB}}$ Py with Cu^{2+} ion were used for DFT calculations to support both the 2:1 complexation and possible excimer formation between two pyrenyl units of two molecules of probe *via* π - π stacking interaction. The predicted optimized geometry of the $^{\text{TNDMB}}$ Py- Cu^{2+} complex is shown to be coordinated with the two nitrogens of the two triazoles of two triazolylpyrenes with least energy compared to other possible coordination through other nitrogen of the triazole functionalities (**Figure 5B.20**). The two Cu – N bond distances were found to be 1.898 Å (N2 – Cu) and 1.897 Å (N3 – Cu) which are comparable with the critical Cu – N bond distance of any crystal structure of Cu-complex with nitrogenous ligands.³⁷ Thus, the coordination through triazoles rigidify the complex, which brings two pyrene moieties closer. As a result, excimer formation is conformationally permitted *via* π - π stacking interaction between these two closely faced pyrenes in the complex which was exactly observed from our experimental excimer emission.

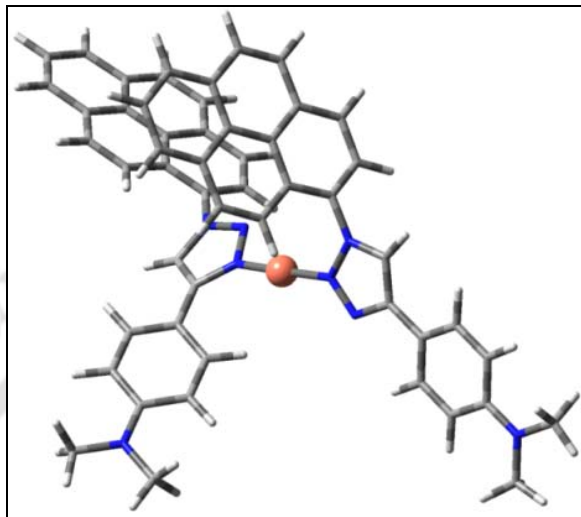


Figure 5B.20: B3LYP/3-21G* energy minimized structure of $^{\text{TNDMB}}$ Py. Cu^{2+} complex by DFT calculation. The LANL2DZ basis set was used for the Cu^{2+} ion.

5B.4.4. UV-Visible and Fluorescence Spectra of $^{\text{TNDMB}}$ Py in Presence of Metal ions rather than Cu^{2+} ion

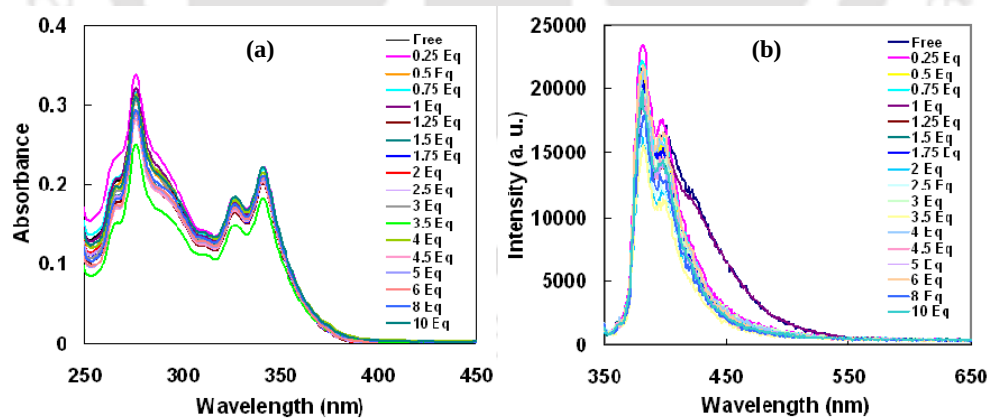


Figure 5B.21: (a) UV-vis, (b) emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of $^{\text{TNDMB}}$ Py ($10 \mu\text{M}$) in presence of Na^+ ion.

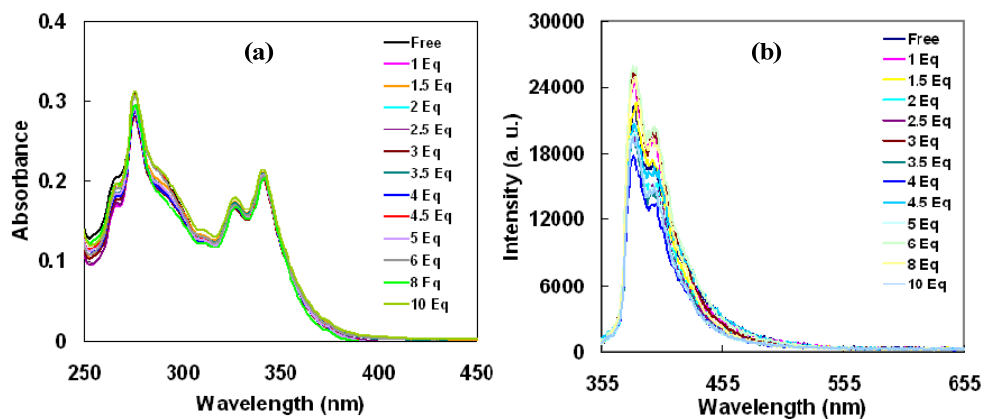


Figure 5B.22: (a) UV-vis, (b) emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of $^{\text{TNDMB}}$ Py (10 μM) in presence of K^+ ion.

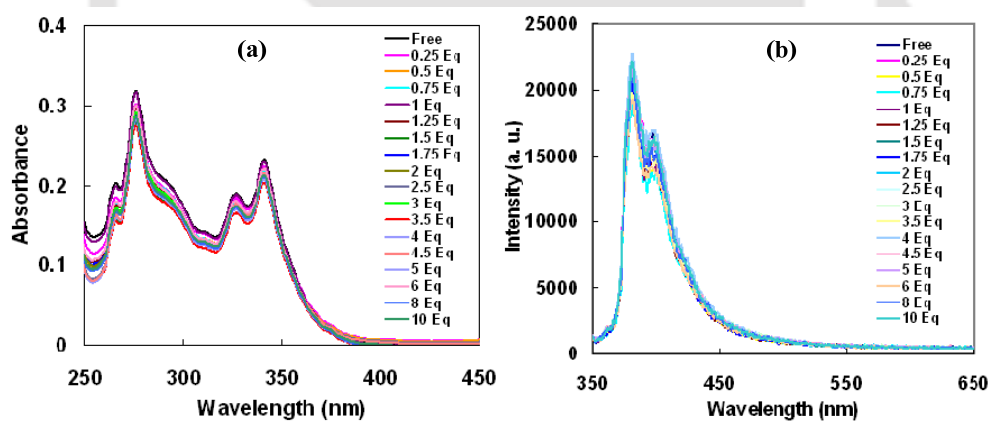


Figure 5B.23: (a) UV-vis, (b) emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of $^{\text{TNDMB}}$ Py (10 μM) in presence of Mg^{2+} ion.

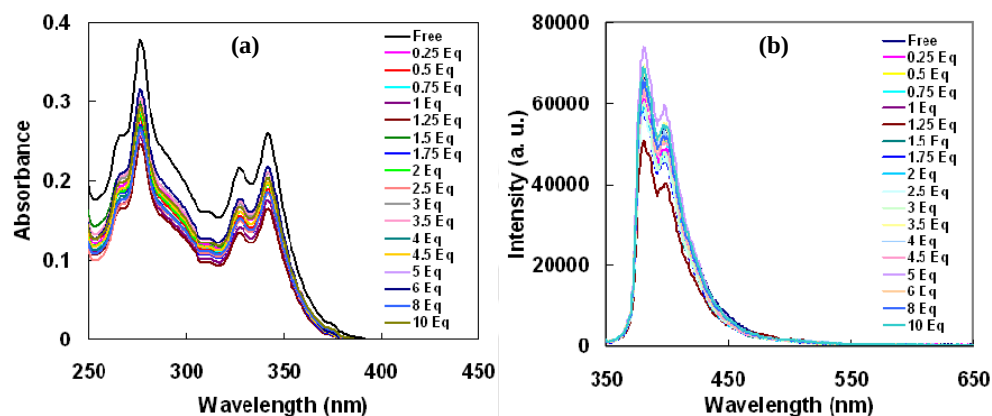


Figure 5B.24: (a) UV-vis, (b) emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of $^{\text{TNDMB}}$ Py (10 μM) in presence of Ca^{2+} ion.

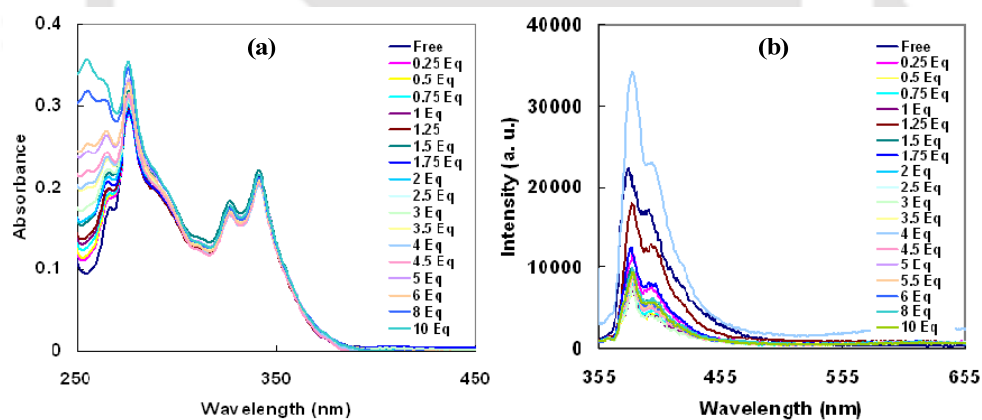


Figure 5B.25: (a) UV-vis, (b) emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of $^{\text{TNDMB}}$ Py (10 μM) in presence of Co^{2+} ion.

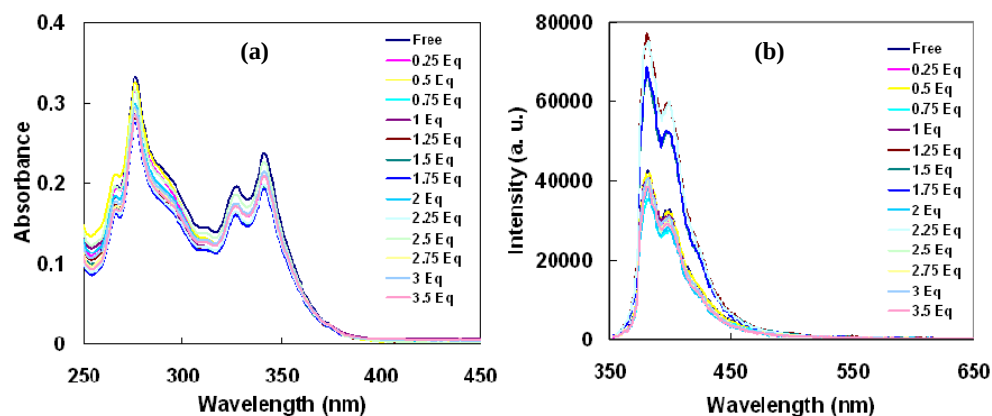


Figure 5B.26: (a) UV-vis, (b) emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of $^{\text{TNDMB}}$ Py (10 μM) in presence of Ni^{2+} ion.

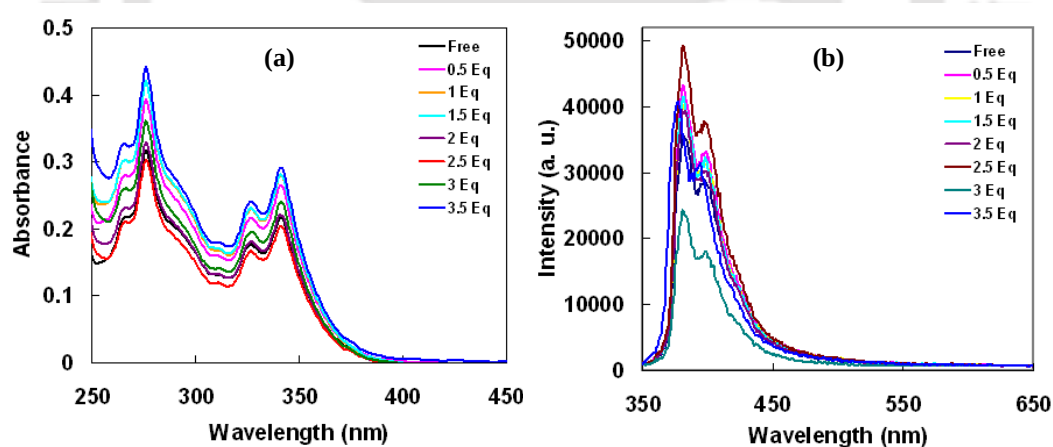


Figure 5B.27: (a) UV-vis, (b) emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of $^{\text{TNDMB}}$ Py (10 μM) in presence of Cu^{+} ion.

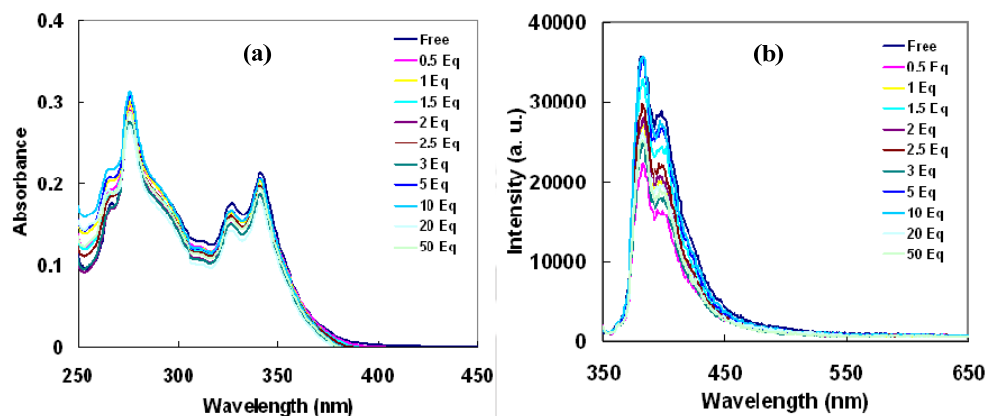


Figure 5B.28: (a) UV-vis, (b) emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of $^{\text{TNDMB}}$ Py (10 μM) in presence of Zn^{2+} ion.

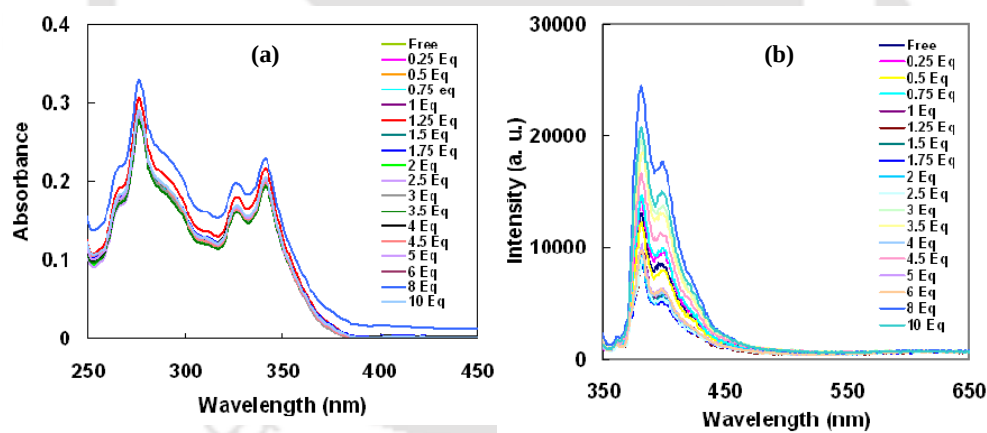


Figure 5B.29: (a) UV-vis, (b) emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of $^{\text{TNDMB}}$ Py (10 μM) in presence of Ag^{+} ion.

5B.5. Application of the Probe in Sensing SDS Micelle-Encapsulated- Cu^{2+} Ion

After exploring the photophysical properties of $^{\text{TNDMB}}$ Py in micelle, and sensing ability of the probe toward Cu^{2+} ion, we have further extended our study towards the detection of Cu^{2+} ion in micelle by using the same probe. As was discussed earlier, fluorescence chemosensors are becoming increasingly popular due to their high sensitivity and selectivity for metal ions.³⁸ However, in the detection of trace metal ions in water and in biological systems, most of these methods suffer from several shortcomings, like poor selectivity and sensitivity in various pH conditions. Therefore, a traditional approach needs to play to design new fluorescent sensors mostly by modifying available dye molecules which again is expensive as well as time consuming. However, rather than synthesizing a number of fluorophores for sensing of different analytes, if one can utilize a single fluorophore whose emission property/sensing ability can be so modulated as to get a distinct fluorescence signal in presence of different analytes *via* a strong interaction, thereby serving the purpose of sensing multiple analytes, would be a great achievement. Therefore, modern chemosensor research focuses on tuning the sensor efficiency of nature friendly probes by a simple way such as *via* noncovalent interaction with a third molecules/molecular assembly rather than synthesizing new water soluble sensor molecule. Thus, there has been considerable interest in the possibility to enhance the sensing capability of a designed chemosensor by noncovalent interaction with molecular assemblies such as surfactant molecules which on micellisation behave and widely used as the simplest mimic of biomembranes and also are used in biosensory application. Surfactant micelle is also known to encapsulate the active components such as fluorophore, quencher *etc.*, and various analytes such as protein, metal ions, *etc.*,³⁹ in micellar nano-core leading to enhanced communication between the active species and helps to improve the sensory capability of fluorophore. Recently, several studies have been reported that have taken this advantage of micelle in enhancing

sensing ability of a fluorophore to design new class of fluorescent chemosensory system with enhanced selectivity and sensitivity.⁴⁰

Cu^{2+} ion being physiologically important trace element, detection of it in cell or at least in a membrane model is recently an interesting research area. Thus, we wanted to explore the possibility of tuning of Cu^{2+} ion sensing capability of our probe, triazolyl pyrene in biomimicking anionic micellar nanocore at various pH. It was previously observed that the interaction of Cu^{2+} ion with triazolylpyrene in organic media is signaled by an enhancement of fluorescence intensity of both the monomer and excimer band of the probe, thus, helps detection of Cu^{2+} ion. Also, in the presence of SDS micellar environment it showed a fluorescence switch –on response with binding constant of $2.77 \times 10^5 \text{ M}^{-1}$. Thus, an examination of detecting Cu^{2+} in micelle by the probe resulted in an observation of a significant enhancement of fluorescence switch-on (sensing) efficiency of the probe in presence of SDS micelle at pH ~ 8 . Thus, one can exploit this fluorophore and the technique of enhancement of sensing capability by surfactant *via* non-covalent interaction to measure the concentration of the essential trace metal ions in biological environments. Keeping this in mind, we have examined the modulation of sensing efficiency of our probe triazolylpyrene to its maximum level toward sensing of Cu^{2+} ion simply by altering its environment using anionic surfactant at various pH.

5B.5.1. Fluorescence Response in SDS Micelle in Presence of Cu^{2+} Ion

Therefore, we have studied the fluorescence properties of the probe in anionic micelle in presence of Cu^{2+} ion within two pH window. Here, micelle act as a receptor unit and fluorophore, triazolyl pyrene acts as a transducer that translates the binding events of the analyte, Cu^{2+} ion with the receptor system in a readable manner through a change in emission pattern. At first we have studied the fluorescence response of the probe in SDS micelle at various pH in absence of Cu^{2+} ion. Thus, we observed only a single emission band at around 390 nm generated from LE state at pH 8. In neutral pH

7, exclusively local emission band was observed centered at around 390 nm. However, at acidic pH 2, a dual emission band, LE/monomer at 390 nm and excimer at 470 nm appeared (**Figure 5B.30a**).

Next, we studied the fluorescence response at different pH in SDS micelle in presence of various concentration of Cu^{2+} ion. Thus, fluorescence titration experiment in SDS micelle showed a quenching of emission of the long wavelength emissive band with unaltered monomer emission upon addition of Cu^{2+} ion in acidic pH with a Stern Volmer constant, $K_{\text{SV}} = 3.8 \times 10^2 \text{ M}^{-1}$ (**Figure 5B.30b**). The quenching was continued up to addition of 450 μM of Cu^{2+} ion. In neutral medium of SDS micelle, the probe showed only a monomer emission. Like, in acidic pH, here also we observed a quenching events upon addition of Cu^{2+} ion with a Stern Volmer constant, $K_{\text{SV}} = 1.1 \times 10^4 \text{ M}^{-1}$ (**Figure 5B.31a**). Thus, we have seen that the Stern Volmer constant is greater in neutral medium than in acidic medium. This indicated that the probe is more sensitive towards SDS micelle-encapsulated- Cu^{2+} ion in neutral medium than in acidic medium.

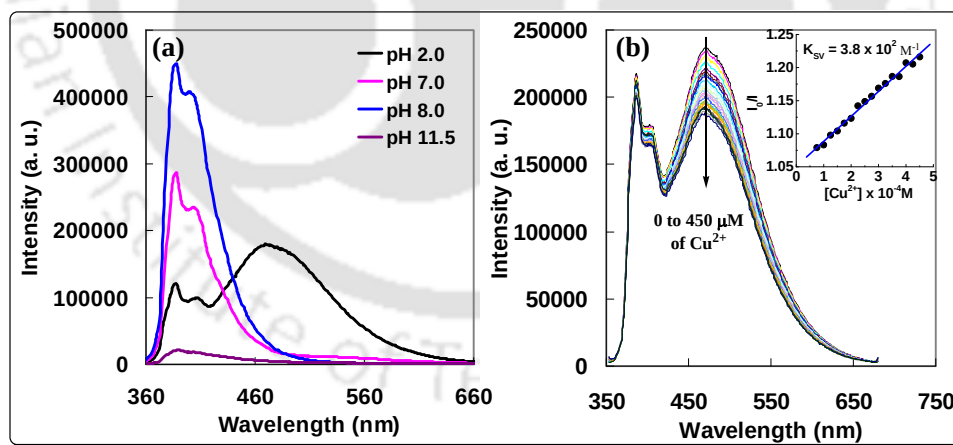


Figure 5B.30: (a) Emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of triazolyl pyrene (10 μM) in SDS micelle at different pH. (b) Emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of triazolyl pyrene (10 μM) in presence of Cu^{2+} ion in aqueous SDS micelle at pH 2. Inset in (b) is the Stern Volmer plot.

We have also studied the fluorescence response of the probe in micelle in presence of various concentration of Cu^{2+} ion at basic pH 8. Thus, in basic pH we observed only the monomer emission and an increased concentration of Cu^{2+} ion has led to an enhancement of the fluorescence intensity with a binding constant, $K = 3.65 \times 10^3 \text{ M}^{-1}$ (**Figure 5B.31b**). Thus, our results demonstrated the practical utility of our probe in detecting Cu^{2+} ion efficiently in aqueous micelle environment.

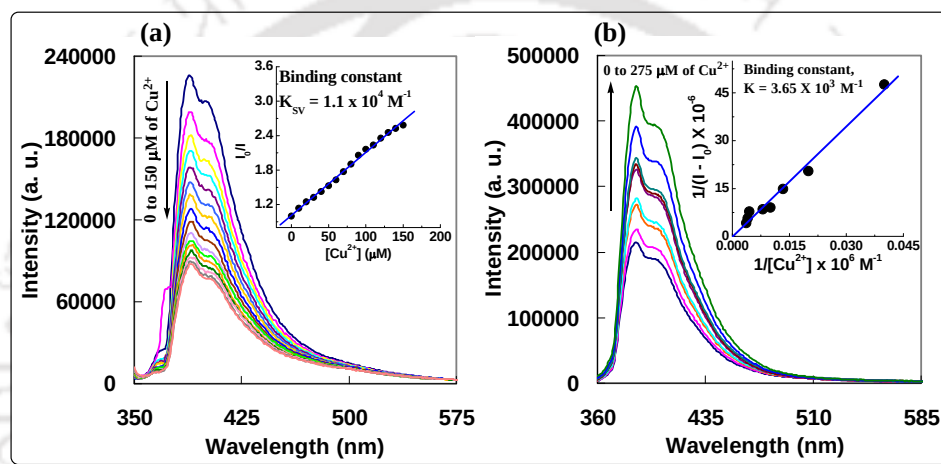


Figure 5B.31: Emission spectra ($\lambda_{\text{ex}} = 343 \text{ nm}$) of triazolyl pyrene (10 μM) in presence of Cu^{2+} ion (a) in neutral aqueous media of SDS micelle and (b) in aqueous SDS micelle at pH 8. Inset in (a) is the Stern Volmer plot and (b) is the Benesi-Hildebrand plot.

5B.5.2. Probable Mechanism

The mechanism of the change in fluorescence behavior of the probe in SDS micelle at different pH in presence of Cu^{2+} ion can probably be explained by following ways. Due to low polarity in the micellar region, ionic analytes like Cu^{2+} may not be available in the inner core of the micelle. It is expected to be available either in the bulk aqueous phase or in the micelle water interface depending on the charge of the analytes. Again, the availability of the positively charged analyte would depend on the surface charge of the micellar units and it is expected to be remarkably greater in the periphery of the anionic micellar system with negative surface charge

and vice versa. Thus, the scenario where a probe is located in the micellar core, there should not be any appreciable communication between fluorophore and Cu^{2+} because of the unavailability of Cu^{2+} at micellar core. But if the fluorophore is located in the micelle–water interface then Cu^{2+} -induction on the fluorescence of the fluorophore is supposed to be remarkably greater than that in pure aqueous medium. Thus, modulation of activity of the fluorescence sensor in presence of a fixed surfactant concentration can be rationalized by considering two competing process:

- (a) interaction between the anionic micellar Stern layer and the triazolyl pyrene probe at various pH,
- (b) availability of the probe molecules towards the metal ion at the micellar interface.

The first factor leads to an increase in the local concentration of the probe molecule in the vicinity of SDS micelle interface. But the second factor governs probability of the interaction between probe and metal ion at the micellar interface. Thus, close proximity of the fluorophore and the metal ions in micellar aggregates ensures effective communication between the active components responsible for change in fluorescence response.

In short, the following fluorescence response was observed. Thus, in acidic pH the probe showed an excimer emission centered at around 470 nm. Upon gradual addition of Cu^{2+} ion a fluorescence quenching of the excimer band was observed. In aqueous medium of SDS solution the probe showed only a local emission at around 390 nm with no excimer emission. Upon addition of Cu^{2+} ion a fluorescence quenching of the LE band was observed. On the contrary, unlike in acidic and aqueous media, an enhanced fluorescence signal centered at 390 nm was observed at basic pH 8 upon addition of Cu^{2+} ion to the probe's solution in SDS micelle. In this case, only a single emission from the LE state was observed which is supported by the intrinsic property of the probe (which showed the single emission band in solvents/media of intermediate polarity) and the emission response of the probe in SDS micelle at pH 8 without Cu^{2+} ion (**Figure 5B.30a**).

The binding constant value (K) of the probe with Cu^{2+} in SDS micelle was found to be higher at pH~8 compared to that at pH~2. This suggests that availability of the probe towards interaction with the Cu^{2+} in SDS micellar interface is higher at pH~8 compared to pH~2. In acidic pH, the $-\text{NMe}_2$ group and the triazolyl nitrogen of the probe can exist in protonated form (at pH~2), which is not possible at pH~8. Therefore, the approachability of bivalent metal cation, Cu^{2+} towards the coordinating site (triazole-Ns) of the probe in probe-SDS micellar aggregates would possibly be higher at pH~8 compared to pH~2. Thus, both the availability of the probe at the micellar interface and the approachability of the Cu^{2+} ion towards the coordinating site (triazole-Ns) of the probe at the micellar interface are higher at pH~8 compared to pH~2. Therefore, at pH~8, close proximity of the probe's triazolyl moiety and Cu^{2+} in the probe-SDS micellar aggregates ensures effective coordination by Cu^{2+} with the lone pairs of nitrogens of triazole unit subsequently blocking the communication between the acceptor pyrene unit and the donor triazole moiety. Thus, when Cu^{2+} ion coordinates with the triazole's nitrogen, the energy of the $n-\pi^*$ state would be raised so that the $\pi-\pi^*$ state of the emission of the triazolyl pyrene becomes the lowest excited state, leading to the substantial increase in the fluorescence intensity of the LE emission of the probe. This is the situation what exactly has been observed at pH 8. **Figure 5B.32a** showed the most probable situation.

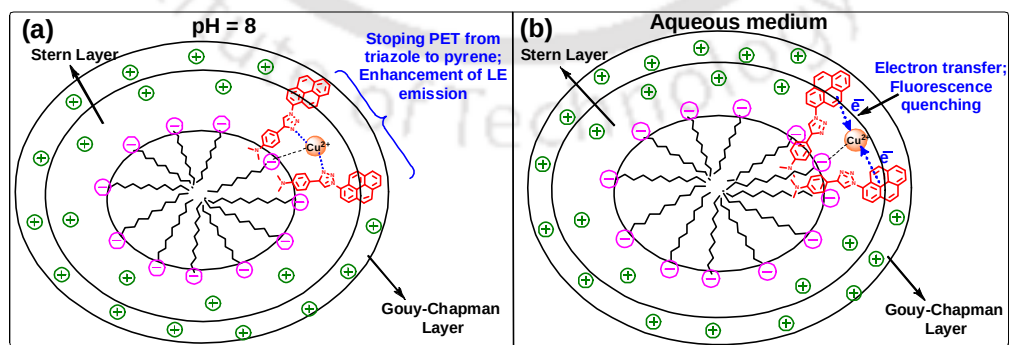


Figure 5B.32: Possible mechanism of the interaction of Cu^{2+} ion with the probe in SDS micelle at pH 8(a) and in neutral aqueous media (b).

In neutral aqueous medium of SDS solution the probe showed only a single emission originating from the LE state at around 390 nm with no excimer emission. Upon addition of Cu^{2+} ion a fluorescence quenching of the LE band was observed. This can be explained if we consider an electron transfer process from the pyrene unit to the Cu^{2+} ion in micelle-probe interface (**Figure 5B.32b**). On the other hand, in acidic pH (pH 2), the complex formation between the probe and the Cu^{2+} ion is not possible due to the protonation of $-\text{NMe}_2$ as well as triazolyl nitrogen of the probe in Stern layer of the SDS micelle. Therefore, there might be a possibility of interaction between the two pyrene units of the two probe molecules *via* a strong π -stacking interaction leading to an excimer emission which is again because of possible molecular aggregation of the probe at pH 2. However, increasing the concentration of Cu^{2+} ion, both the LE and the excimer emission started quenching due to paramagnetic nature of the Cu^{2+} ion and the cation- π interaction between two closely faced pyrene moieties and Cu^{2+} ion. The possible electron transfer process that probably occurs between pyrene unit of the probe and the Cu^{2+} ion leading to fluorescence quenching of the excimer emission is depicted in **Figure 5B.33**.

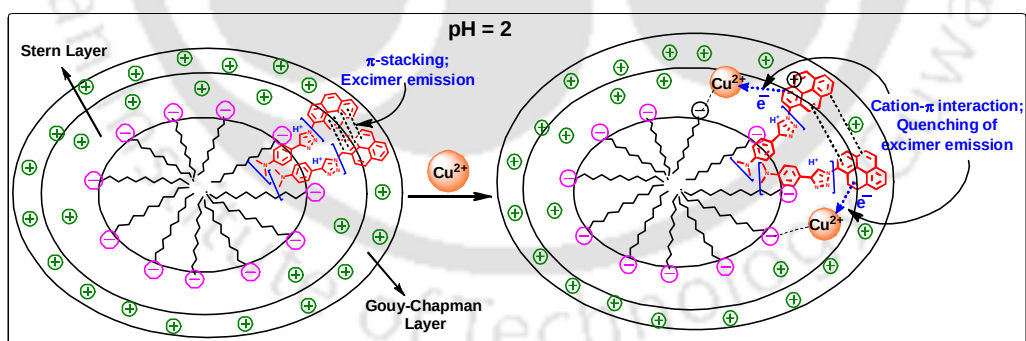


Figure 5B.33: Possible mechanism of the interaction of Cu^{2+} ion with probe in SDS micelle at pH 2.

5B.6. Conclusion

In conclusion, we have developed a new and smart fluorescence switch-on chemosensor for Cu²⁺ ion which showed a remarkably enhanced fluorescence intensity and a high sensitivity and selectivity. Our smart fluorescent probe show a 2:1 probe-metal complexation with high binding constant which is investigated both by absorption and fluorescence measurements. Computational studies showed a closely faced conformation of the two pyrene units in the complex thus the excimer formation is possible. The successful use of triazolylpyrene as a Cu²⁺ ion selective fluoroionophore with low detection limit of 177 nM, may open the way for the design of new, efficient, and simple triazole-based fluorescence light-up probe for metal ion detection.

We have also shown that the probe is capable of sensing Cu²⁺ ion inside the nanocore of micelle with a change in fluorescence pattern that is selective to the pH of the system. In particular, at pH 8, the probe detects the Cu²⁺ ion in SDS micelle with a large enhancement of fluorescence intensity of the monomer emission band. All the results indicate that our probe could be used as an efficient Cu²⁺ selective fluorescence light-up probe, and may find applications in chemical and biological systems.

5B.7. Experimental Section

5B.7.1. Materials

SDS, metal salts, NaOH, and HCl were purchased from Merck, India. Water was obtained from a Milli-Q purification system. All experiments were performed with freshly prepared solutions. The probe molecule was synthesized and purified according to the procedure described in **Chapter 2**. We recrystallised the column purified material in chloroform-ethylacetate solvent mixture twice, dried under vacuum, again characterized and used for study.

5B.7.2. Methods

5B.7.2.1. UV–Visible Study

The UV–visible absorbance measurements were performed using Shimadzu 2550 UV/vis spectrophotometer with a cell of 1 cm path length at 298 K. First, the samples were dissolved in minimum quantity of solvent, and then various metal ions of different concentration were added maintaining the total volume of 3 mL of the mixtures (10 μM probe and 0, 5, 10, 15, 20, 25 *etc.* μM metal ions respectively) and finally the *UV* –visible spectra in presence of various interfering metal ions were measured. The concentration of the probe ($^{\text{TNDMB}}$ Py) was fixed, 10 μM for all the experiments. The measurements were taken in absorbance mode. The absorbance values of the sample solutions were measured in the wavelength regime of 200–600 nm.

5B.7.2.2. Fluorescence Study

All fluorescence studies were performed using a Fluoromax 4 spectrophotometer with a cell of 1 cm path length at room temperature. All the sample solutions for fluorescence spectra measurement were prepared as described in UV-visible measurement experiments. The excitation wavelength for probe ($^{\text{TNDMB}}$ Py) was set at 343 nm, and emission spectra were measured in the wavelength regime of 350–675 nm. The slit widths for both excitation and emission were set at 2 nm. The

fluorescence quantum yields (Φ) were determined using quinine sulphate as a reference with the known Φ (0.54) in 0.1 molar solution of sulphuric acid. For the fluorescence study in the presence of micelle, surfactant (SDS) solution was prepared in MQ water. The concentration of SDS was 10 mM, above micellar concentration to make sure the formation of micelle in the experimental conditions. pH of the solutions was adjusted by dilute HCl and or dilute NaOH. The probe's solutions in SDS surfactant (10 mM) were mixed well and then Cu^{2+} ion (solution of $[\text{Cu}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}]$ in MQ water) of different concentrations were added maintaining the final volume 3 mL of the mixtures (10 μM probe in 10 mM SDS solution and 0, 10, 25, 50, 75, 100, 125 *etc.*, μM Cu^{2+} ions respectively). Then, again all the solutions were mixed well before taking the fluorescence spectra. All the experiments were carried out at 25 °C.

5B.7.2.3. Job's Plot

To quantify the stoichiometry between probe ($^{\text{TNDMB}}$ Py) and the Cu^{2+} ion, the absorption Job's plot measurement was carried out. For this purpose, an equal concentration of probe ($^{\text{TNDMB}}$ Py) and Cu^{2+} ion solutions each were prepared in UV-grade acetonitrile solvent. Then, the probe and the Cu^{2+} ion solutions were mixed in different fractions of volume maintaining the total volume of mixture 3 mL. All the solutions were mixed well and kept for some time in room temperature. Then the absorbance spectra of the solutions of different composition of probe and Cu^{2+} ion were recorded. To calculate the probe- Cu^{2+} complexation ratio, $[\text{probe-Cu}^{2+}]$ vs. X_{probe} were plotted, where $[\text{probe-Cu}^{2+}] = \Delta A/A_0 \times [\text{probe}]$, $\{\Delta A = (A - A_0)\}$, and X_{probe} is the mole fraction of probe, $\{X_{\text{probe}} = [\text{probe}]/([\text{probe}] + [\text{Cu}^{2+}])\}$. The corresponding X_{probe} value where the concentration of probe- Cu^{2+} complex, $[\text{probe-Cu}^{2+}]$ was maximum was taken as probe-to-metal stoichiometry.

The fluorescence Job's plot was plotted using the fluorescence intensity change of LE band in absence and presence of various concentrations (0 to 20 equiv.) of Cu^{2+} ions in CH_3CN . Thus, ΔI vs. X_{probe} was plotted for the various concentrations of Cu^{2+} ions, where $\Delta I = I - I_0$ (I_0 and I are the fluorescence intensities of $^{\text{TNDMB}}$ Py in absence and presence of Cu^{2+} ions) and X_{probe} is the mole fraction of probe, $X_{\text{probe}} =$

$[\text{probe}]/([\text{probe}] + [\text{Cu}^{2+}])$. The corresponding X_{probe} value at ΔI value maxima was taken as probe-to-metal stoichiometry.

5B.7.2.4. Benesi-Hildebrand Plot

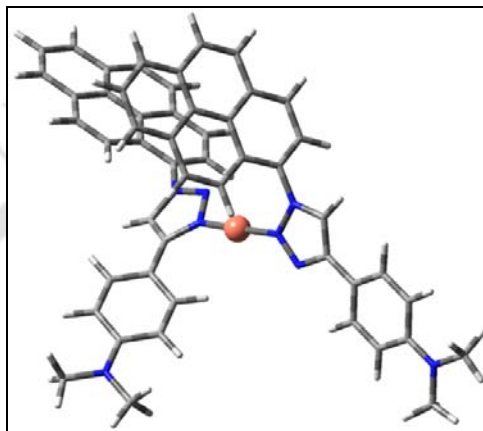
The binding constant of the Cu^{2+} ion with probe, $^{\text{TNDMB}}$ Py was determined from the well-known Benesi-Hildebrand plot using the following equation,

$$\frac{1}{(I - I_0)} = \frac{1}{(I_\infty - I_0)} + \frac{1}{(I_\infty - I_0)K[\text{Cu}^{2+}]}$$

where I_0 , I and I_∞ are the emission intensities of $^{\text{TNDMB}}$ Py in the absence of, at intermediate and at infinite concentration (where maximum intensity was observed) of Cu^{2+} ion respectively. To determine the binding constant, $1/(I - I_0)$ vs. $1/[\text{Cu}^{2+}]$ was plotted. A linear fit was fitted by taking the points of linear region and from the slope value the binding constant was calculated.

5B.7.2.5. Determination of Detection Limit

To measure the detection limit of the fluorescent probe (5B.21, $^{\text{TNDMB}}$ Py) towards Cu^{2+} ion, the intensity data were normalized between the minimum intensity (Cu^{2+} ion free) and the maximum intensity (25 μM Cu^{2+} ion) and $(I_{\min} - I)/(I_{\min} - I_{\max})$ vs. $\log[\text{Cu}^{2+}]$ was plotted. A linear fit was fitted by taking the five intermediate points (2.5 μM to 15 μM) of the linear region. The linear line where it crossed the ordinate axis was taken as detection limit.

**5B.7.3. Cartesian Co-ordinates of B3LYP/3-21G* Energy Minimized Structure
of $^{\text{TNDMB}}$ Py. Cu^{2+} Complex** (LANL2DZ basis set was used for the Cu^{2+} ion)

E(UB+HF-LYP) = -2625.96196719 a.u.

2 2			
N	0.56908000	1.14276400	-0.70243700
N	2.66532400	1.10615400	-1.30839800
N	1.47729900	0.37796800	-1.37849000
N	-1.50230000	6.44281900	2.60668200
C	3.82905600	0.62970800	-1.99517700
C	1.15404900	2.30480700	-0.22157800
C	0.45480900	3.34291200	0.51878200
C	2.48291100	2.27431700	-0.62126800
H	3.25828000	3.00971100	-0.51538500
C	5.08465400	0.58530400	-1.34433300
C	3.67825000	0.23139600	-3.32568200
H	2.69637200	0.27116800	-3.77731400
C	6.06290100	-0.20258100	-3.48941600
C	6.21852900	0.17727900	-2.11915400
C	7.51284000	0.14350900	-1.52042300
C	1.14499000	4.13776800	1.45729200
H	2.19131200	3.94101800	1.66099500
C	0.50877700	5.15023700	2.14737300
H	1.07481400	5.73032800	2.86120200
C	-1.56087600	4.62201900	0.98993400
H	-2.60471900	4.80605400	0.78183800
C	-0.91176200	3.61216000	0.30483000
H	-1.46100800	3.04775800	-0.44105300

C	-0.87092500	5.43310700	1.93571000
C	5.27847600	0.89187600	0.04523800
H	4.41951900	1.13437200	0.65723100
C	4.77631000	-0.18352400	-4.06289800
H	4.65082700	-0.47720400	-5.09793400
C	8.64952200	-0.24348900	-2.29483900
C	7.68008500	0.49213200	-0.14686100
C	9.91906400	-0.25986000	-1.68566600
H	10.78239700	-0.54882100	-2.27403100
C	8.46045700	-0.60699300	-3.66939200
H	9.32796300	-0.89971100	-4.24974000
C	6.51895700	0.84657200	0.61365400
H	6.64246200	1.07108400	1.66726000
C	10.07281100	0.08817500	-0.34421700
H	11.05730300	0.06889800	0.10599000
C	7.22051400	-0.59234300	-4.24089100
H	7.08984300	-0.87409700	-5.27910800
C	8.96854900	0.45739700	0.42107200
H	9.09421900	0.72136100	1.46498500
C	-2.93224800	6.72876600	2.36306200
H	-3.10489500	6.99374600	1.31346200
H	-3.23131100	7.57005400	2.98692500
H	-3.55386600	5.86353800	2.62140200
C	-0.76091600	7.28448300	3.57200300
H	-0.35440100	6.67700400	4.38858700
H	-1.44632300	8.01843500	3.99393600
H	0.06010200	7.81623000	3.07756500
N	-4.00631300	-0.03771700	-0.90068100
N	-3.01554500	-1.92142200	-0.22760000
N	-2.82041800	-0.55276500	-0.53667500
N	-10.36559500	-0.10542500	-2.20928000
C	-1.91466000	-2.76350900	0.15850700
C	-4.95192000	-1.04084400	-0.84141000
C	-6.34396000	-0.81530800	-1.18344800
C	-4.32140100	-2.21691500	-0.41673600
H	-4.71266200	-3.20038100	-0.23189800
C	-1.31770000	-2.58711200	1.42489200
C	-1.46760900	-3.73343000	-0.74344100
H	-1.94999100	-3.82753400	-1.70866800
C	0.23737300	-4.43370300	0.83909600
C	-0.21946800	-3.44076100	1.76350200
C	0.41237300	-3.31017400	3.03587000
C	-7.30848900	-1.84018200	-1.08641100

H	-7.02296100	-2.82771200	-0.74404800
C	-8.62759100	-1.61356100	-1.41918600
H	-9.33526400	-2.42442400	-1.33013600
C	-8.07849200	0.69526200	-1.96925900
H	-8.35934500	1.67998400	-2.31285600
C	-6.76390900	0.45572000	-1.63153500
H	-6.03169200	1.24849500	-1.71321000
C	-9.06219400	-0.33386200	-1.87537300
C	-1.77063600	-1.62034400	2.39038400
H	-2.62035800	-0.99435200	2.14880700
C	-0.40551200	-4.56067000	-0.40656200
H	-0.06255100	-5.31089600	-1.10856300
C	1.50287000	-4.16543100	3.38252300
C	-0.04877800	-2.33494700	3.97210200
C	2.10403400	-4.02632000	4.64506300
H	2.92816100	-4.67785600	4.91231700
C	1.94224000	-5.14810600	2.42874200
H	2.76843000	-5.79488900	2.70166900
C	-1.16001200	-1.50314500	3.60371800
H	-1.52085000	-0.77918100	4.32620500
C	1.64673200	-3.07082500	5.55239600
H	2.11863800	-2.98426700	6.52327300
C	1.33872500	-5.27746800	1.21476300
H	1.67607600	-6.02667500	0.50811900
C	0.58241600	-2.23292300	5.22365300
H	0.22735000	-1.49881300	5.93810400
C	-10.80216800	1.22782700	-2.68774500
H	-10.61458100	1.99188000	-1.92555400
H	-11.87094400	1.19236000	-2.89246500
H	-10.27540900	1.49875300	-3.60924800
C	-11.37561200	-1.18616800	-2.10850600
H	-11.11333900	-2.02262000	-2.76543300
H	-12.34236800	-0.79005400	-2.41497800
H	-11.45375700	-1.54651300	-1.07704800
Cu	-1.13209600	0.31647400	-0.54791700

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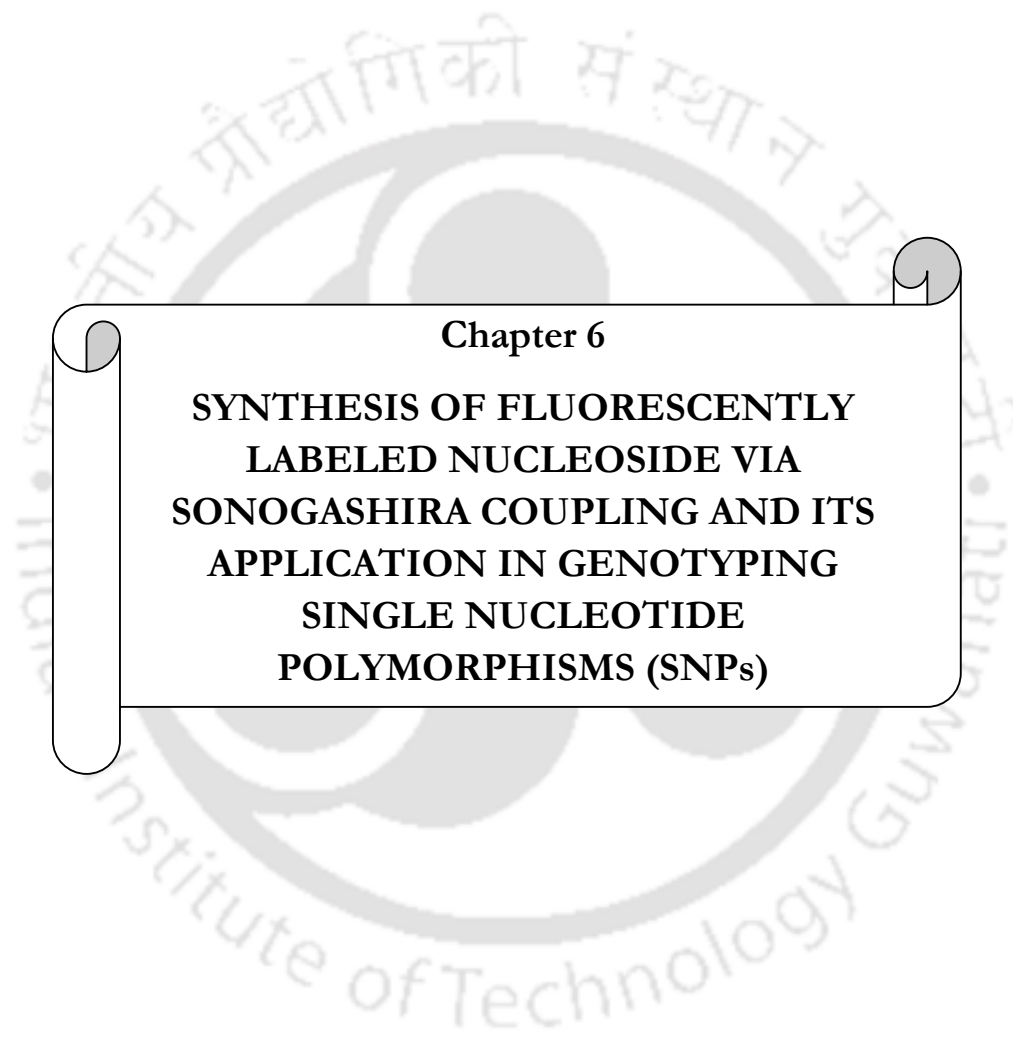
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Chapter 6

SYNTHESIS OF FLUORESCENTLY LABELED NUCLEOSIDE VIA SONOGASHIRA COUPLING AND ITS APPLICATION IN GENOTYPING SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs)

6.1. Introduction

DNA is most widely thought of as the central storehouse of genetic information in each cell and obviously that is a major function. However, it serves several other biological functions, like polyfunctional switch for control of gene expression as well. Thus, chemists and biomedical researchers have used the DNA structure as a starting point of design of many new molecules with new functions such as to act as a ligand for nucleic acids¹ or as sensors,² and probes³ for genetic analysis.

The completion of human genome project has set the stage to the biomedical researchers of all over the world to find out the variations in the human genome through the genetic analysis.⁴ Genetic variations affect the human health largely.⁵ In particular, genetic variations account for some of the differences between individuals, such as eye and hair color, height *etc.* However, they may also predispose some people to disease and explain why some peoples respond better to certain drugs than others.⁶ Therefore, detecting genetic differences between individuals and determining their impact on human health are fundamental in genomic research. Thus, it is worthwhile to identify the DNA sequence variants conferring susceptibility or resistance to common human diseases with improved methods on a genome wide scale. The mapping of genetic variations would lead to the understanding of the role of mutations in simple as well as complex genetic diseases. Among all other genetic variations, single nucleotide polymorphism (SNP), a single base-pair mutation that occurs at a specific site in a DNA sequence, and present in at least 1% of the population, is the most common one.⁷ Scientists think that the SNP is the key enabler in the realization of the concept of personalized medicine, the target of recent research efforts of all biomedical researchers throughout the world.⁸

Therefore, because of their significant biological and clinical importance, recently, there has been a growing interest in single nucleotide polymorphisms (SNPs) typing.⁹ A great deal of efforts has been paid by various research groups all over the world for the development of SNP genotyping technologies since last few years. As a result, a lot of different SNP typing protocols have become available for us however; there is

no single chemistry, platform, and protocol that meet all research needs.¹⁰ The technology, which will offer high sensitivity, reproducibility, accuracy, less laborious, less time consuming, and low cost of genotyping SNPs will be the better alternative than the presently available methods. The presently available methods generally consider the difference in hybridization efficiency or the difference in enzymatic recognition between the nucleotide probes.¹¹

The single base discrimination in nearly all reported methods is achieved directly or indirectly on the basis of difference in hybridization efficiency between matched and mismatched target DNA and probe DNA. However, as far as the detection relies on the hybridization events, such DNA probes suffer from shortcomings in their selectivity. The differences in the hybridization efficiency are varied with sequence context and often very small for the detection of a single base mismatch in long target DNA. In order to realize high sensitivity, the hybridization and washing conditions are carefully selected to minimize undesirable responses from the mismatched hybridization probes. From these points of view, the alternative probes that do not rely on the hybridization events are highly desirable.¹²

Fluorescence techniques, now a days, find widespread application in biochemical and biophysical studies of macromolecules, specially, in the studies of nucleic acids, fluorescence spectroscopy provides an important tool for the detection and probing of structure, dynamics and interactions. Fluorescence based detection schemes are now used routinely in numerous applications, such as DNA sequencing,¹³ in situ genetic analysis through techniques like fluorescence in situ hybridization (FISH),¹⁴ and high throughput screening (HTS).¹⁵ However, there is a concomitant need to develop methods for the rapid and routine analysis of specific nucleic acid sequences so that this information can be understood and interpreted easily. In recent development for the detection of single base alterations, the methods that utilize fluorescence-labeled short oligonucleotide probes to reveal the presence of complementary sequences offer an attractive alternative.

6.2. Solvatochromic Fluorophores Used in Nucleic Acid Detection Research

The natural nucleobases only absorb light in ultraviolet region and not responsive to the visible light or cannot be detected by highly sensitive fluorescence detection techniques as the intrinsic fluorescence of the naturally occurring nucleotide bases in DNA and RNA is extremely weak. These bases exhibit very short fluorescent decay times, generally in the range of a few picoseconds, and do not provide much structural information since signals are normally averaged over all bases in the oligonucleotide sequence. Thus, the lack of naturally occurring fluorescent bases has spurred the development of new fluorescent nucleosides which will be the probe for DNA analysis. These nucleosides can be so designed as to get optimized fluorescence properties (i.e. higher quantum yields and longer lifetimes).

6.3. Designing Criteria for Modified Fluorescent Nucleosides

For probing DNA structure, the ideal fluorescent nucleoside should possess the following characteristic features:

- (a) High molar extinction coefficients, high emission intensity, high fluorescence brightness factor ($\epsilon\Phi$), and long excitation and emission wavelengths, high quantum yields which is sensitive to its local microenvironment, large Stokes shift, compatible size, hydrophobicity/hydrophilicity, and stability;
- (b) Should be amenable to phosphoramidite preparation for incorporation into oligo-nucleotides by solid-phase synthesis;
- (c) Should not disrupt duplex formation and should mimic one of the regular nucleosides. In general, maintaining Watson-Crick and Hoogsteen base pairing hydrogen bonding is an important aspect in the design and synthesis of fluorescent nucleotide base analogs;
- (d) Should behave as a regular nucleoside in its interaction with proteins and enzymes, and

- (e) Should be capable of being converted to the triphosphate and be incorporated into DNA with high efficiency by current commercial polymerases.

6.4. Efforts Toward the Design of Fluorescent Nucleosides for DNA Detection

The lack of naturally occurring fluorescent nucleobases has prompted the design and synthesis of new nonnatural fluorescent nucleosides or nucleoside analogues for probing local microenvironmental change around DNA and genetic analysis. These nucleosides can be so designed as to get optimized fluorescence properties such as, higher quantum yields, longer lifetimes, high solvatochromicity and that can be incorporated into an oligonucleotide using standard automated synthetic methods. The fluorescence properties, from DNA and RNA molecules, thus can be observed without any competing background signals mainly by two ways:

- (a) incorporation of unnatural nucleoside into an oligonucleotide sequence and/or
- (b) incorporation of fluorophore into the natural bases *i.e.*, by synthesizing fluorescently labeled bases.

We will present here a brief review of recent literature focusing covalent cross-linking of fluorophores to generate fluorescent nucleosides usable for DNA detection.

6.5. Fluorescently Labeled Nucleosides

As was discussed earlier, one of the most important strategy to install the fluorescence property into the natural nucleosides for biochemical applications, is the attachment of a fluorophore covalently through a rigid carbon linker producing what is called is fluorescently labeled nucleosides. Oligonucleotides containing such labeled nucleosides can widely be used in a variety of genomic assays including gene quantitation, allelic discrimination, expression analysis, and SNP typing. Many assays use such fluorescently labeled oligonucleotide probes in which a biochemical event (*e.g.*, hybridization) causes an increase in fluorescence intensity. The fluorescently labeled nucleobases, in contrast, are attached through a carbon linker to

oligonucleotides and can move more freely in ways that may be independent of the overall complex. Such intrinsic motions of a dye can complicate the interpretation of some fluorescence measurements, yet a rigid carbon linker will have the capability to restrict this motion to some extent and these fluorophore are so designed as to achieve intercalation or groove binding properties.

Since several years Saito and coworkers¹⁶ are engaged in designing covalently labeled nucleobases popularly known as base-discriminating fluorescent (BDF) nucleosides for SNPs typing in homogeneous solution. The fluorescently labeled nucleobases were synthesized by attaching the fluorophores on to the natural nucleobases through a carbon linker *via* a Sonogashira coupling protocol. The labeled nucleosides upon incorporation into an oligonucleotide are capable of detecting the change of microenvironment within the DNA and can report the presence of opposite base in a target DNA *via* a generation of an enhanced fluorescence signal.

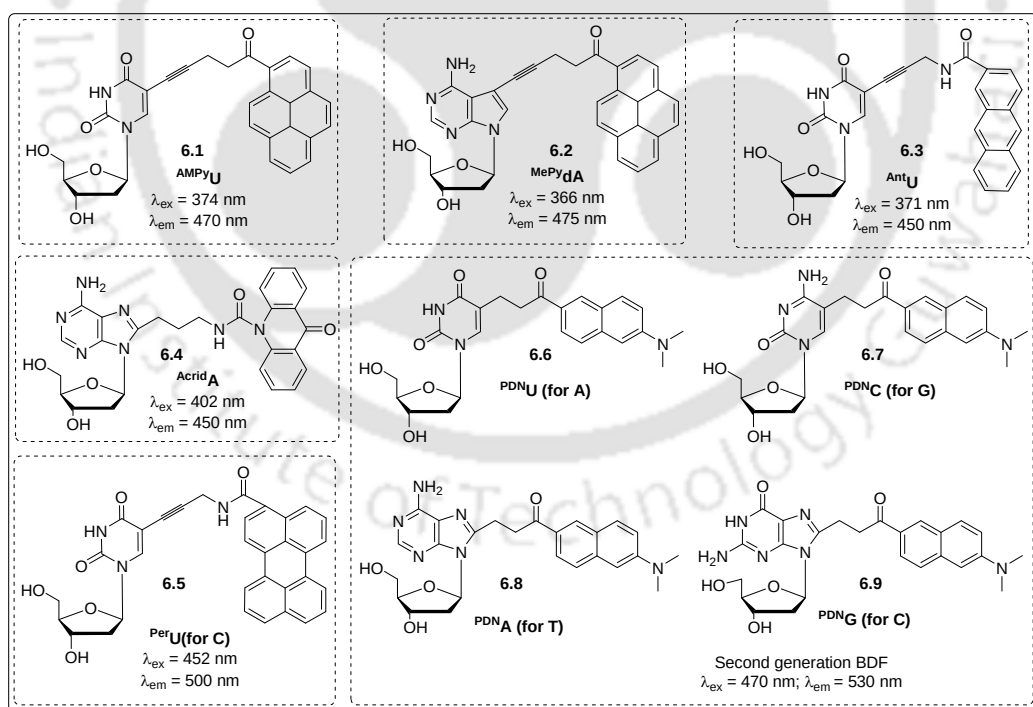


Figure 6.1: Few examples of covalently conjugated fluorescent nucleosides

Figure 6.1 shows some examples of fluorescently labeled nucleosides used for DNA detection. The examples shown here and many other fluorescently labeled nucleosides have been synthesised *via* utilising classical Sonogashira coupling protocols. We will now describe in brief a few of such reported fluorescent nucleosides mostly of which were synthesised *via* the Sonogashira coupling protocol, their photophysical properties, and applications in DNA detection.

6.5.1. Pyrene Labeled Nucleoside

Looking at the very high solvatochromic photophysical properties of pyrenecarbonyl derivatives like pyrene-1-carboxyaldehyde, in 2004, Saito *et al.*,^{17a} have reported pyrene labeled base-discriminating fluorescent (BDF) nucleosides ^{Py}U and ^{Py}C (**6.10** and **6.11** respectively, **Figure 6.2**). The synthesis of the nucleosides involves a Sonogashira coupling strategy. They have incorporated the BDF nucleosides into the short oligonucleotide and studied the photophysical properties of the labeled probes upon hybridization with target complementary oligonucleotides (ODNs). The designed probes containing ^{Py}U and ^{Py}C selectively detect **A** and **G** bases, respectively, of target ODNs opposite to the labeled bases of probe ODNs *via* generation of enhanced fluorescence signals. Such sharp change in fluorescence intensity is mainly because of the difference in polarity of the microenvironments near pyrenecarboxamide moieties of BDF bases, ^{Py}U and ^{Py}C. The difference in polarity was also supported by a macromodel study for the duplex containing a ^{Py}U/A, ^{Py}C/G base pairs, respectively.

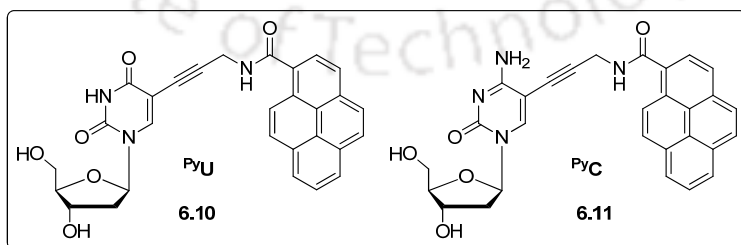


Figure 6.2: Chemical structure of BDF nucleosides ^{Py}U and ^{Py}C.

Later on, they^{17b} have developed other pyrene-labeled BDF nucleosides, ^{AMPy}U (6.1, **Figure 6.3**) which exhibits polarity sensitive strong fluorescence emission at long wavelength region. They incorporated the BDF nucleosides into short oligonucleotide and studied the photophysical properties of the probe upon hybridization with complementary target ODNs. The designed probe labeled with fluorescent nucleoside, ^{AMPy}U, selectively emit fluorescence only when the base opposite to BDF nucleoside is adenine and thus, act as effective reporter probe for homogeneous SNP typing (**Figure 6.3**).

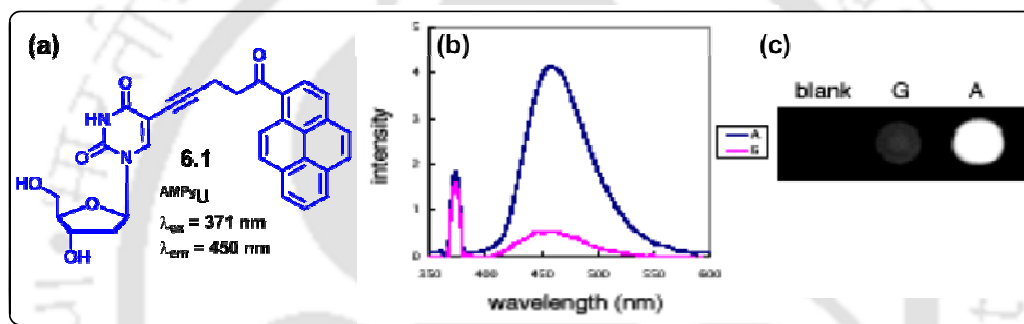
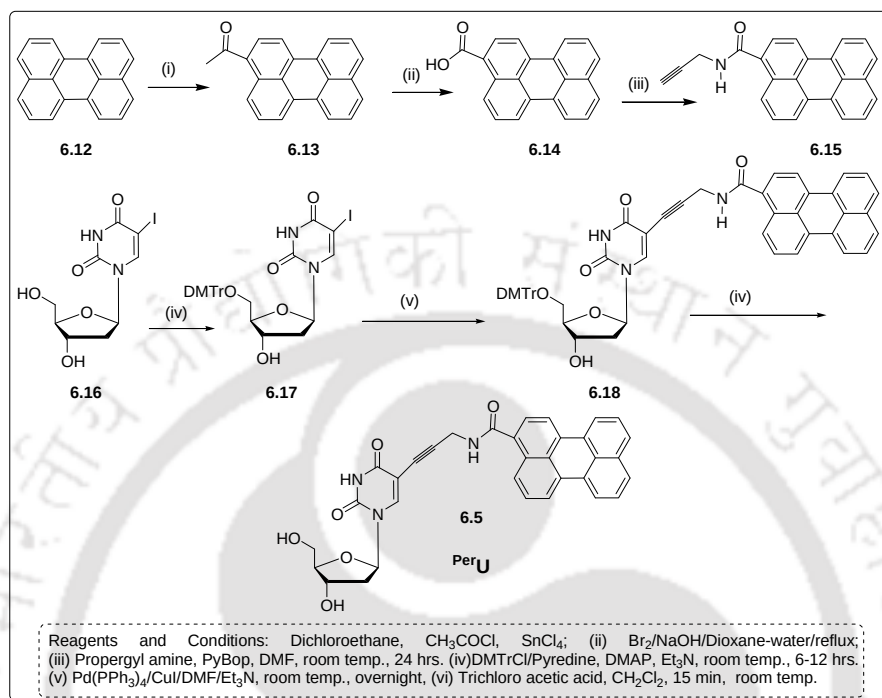


Figure 6.3: (a) Chemical structure of ^{AMPy}U, (b) fluorescence spectra of ODN (^{AMPy}U) hybridized with ODN (A or G) and (c) Corresponding fluorescence image (taken from reference^{17b}).

6.5.2. Perylene Labeled Nucleoside (^{Per}U)

The previously described labeled nucleosides emit at around 400-450 nm of wavelength which restrict to some extent their use in cell genotyping or in microarray based DNA detection techniques. Bag *et al.*,¹⁸ have synthesized a perylene labeled nucleoside, ^{Per}U (6.12) for detection of SNP *via* Sonogashira coupling protocol (**Scheme 6.1**).



Scheme 6.1: Synthesis of perylene labeled oligonucleotide probe.

Thus, they have synthesized perylene carboxamide containing nucleoside $^{\text{Per}}\text{U}$ bearing a rigid acetylenic linker *via* Sonogashira coupling of 5-iodo-2'-deoxyuridine and the corresponding perylene carboxamide. Nucleoside $^{\text{Per}}\text{U}$ was ultimately incorporated into two oligonucleotide sequences ODN **1** and **2**, differ only by flanking bases near the labeled nucleoside [ODN **1** contains C (G–C rich sequence), while **2** contains T (A–T rich sequence) as the flanking bases] *via* standard DNA synthesis protocol. Their fluorescence photophysical study revealed that the $^{\text{Per}}\text{U}$ -labeled oligonucleotide probes are capable of detecting mismatched cytosine base (C) from the corresponding complementary target ODNs, selectively, at wavelength 500 nm *via* an enhancement of fluorescence signal. Their detection is highly important as it does not depend on the sequence of the probe. The fluorescence enhancement was observed only when the base opposite to $^{\text{Per}}\text{U}$ was cytidine. Thus, they observed fluorescence enhancement in case of mismatched duplex ($^{\text{Per}}\text{U}$ -C) while in matched duplex ($^{\text{Per}}\text{U}$ -A) they observed a decrease in fluorescence intensity.

The beauty of the ^{Per}U labeled probe lies on the sequence independent discrimination of cytosine (C) base from adenosine (A) base of target ODNs *via* an enhanced fluorescence signal generation. Thus, the probe might find application as an efficient probe for detection of several other SNPs in any sequence context.

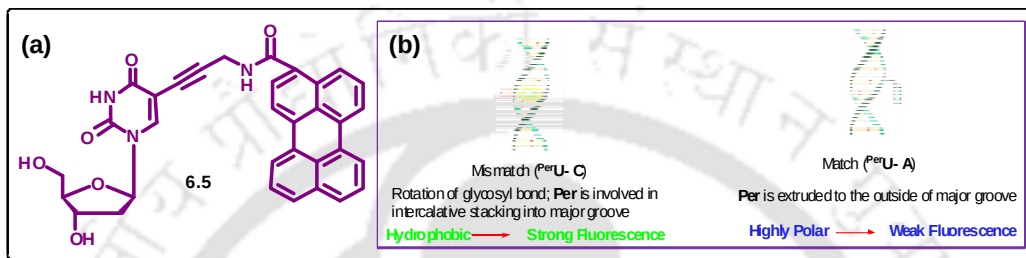


Figure 6.4: (a) Chemical structure of ^{Per}U and (b) schematic diagram of the working principle of perylene labeled nucleoside (^{Per}U).

For matched case the perylene chromophore resides outside of major groove and faced more polar aqueous environment and thus fluorescence quenching was observed. However, for mismatched case the perylene chromophore involve in intercalative stacking interaction (because of the lack of Watson-Crick base pair) within the duplex and faced more hydrophobic organic microenvironment and thus fluorescence enhancement was observed (**Figure 6.4**). This concept is completely opposite to the case of pyrene chromophore which upon hybridization with mismatched target oligonucleotide, showed only weak fluorescence.^{17a} Thus, the use of mismatch-detection concept will be a promising research effort in giving rise to a new powerful tool for the detection of SNP and in designing quencher-free molecular beacon.¹⁹

6.5.3. PRODAN Labeled Fluorescent Nucleosides

Okamoto *et al.*,²⁰ have reported a novel solvatochromic fluorophore PRODAN as a microenvironment-sensitive probe. They further developed PRODAN labeled fluorescent nucleosides, ^{PDN}A , ^{PDN}G , ^{PDN}C , and ^{PDN}T (**Figure 6.5**) where PRODAN fluorophore was attached at pyrimidine C-5 or purine C-8 position. These fluorescent

nucleosides are highly sensitive to the solvent polarizability; thus, these are very useful for the mapping of the local dielectric properties around DNA.

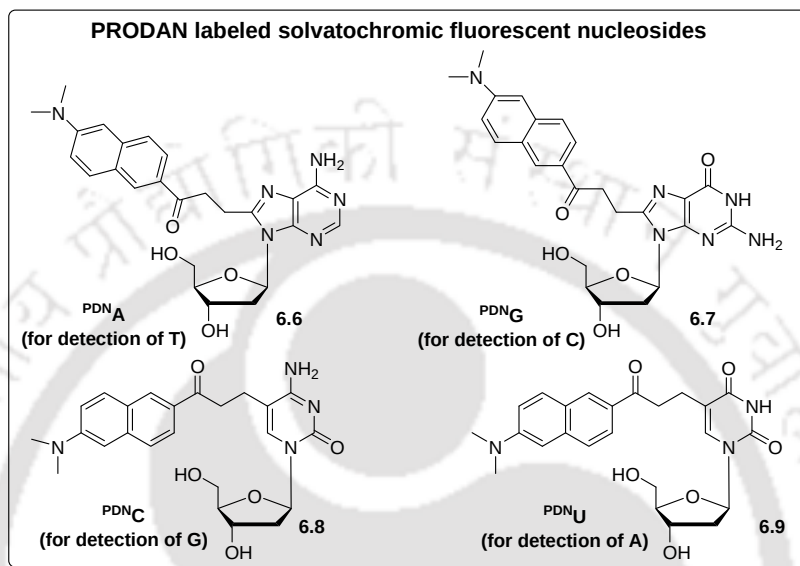


Figure 6.5: PRODAN labeled fluorescent nucleosides.

The examination of photophysical properties of ^{PDN}U nucleoside in different solvents revealed that the fluorescence excitation spectra shifted depending on the nature of the solvent which was attributed to specific interactions, such as hydrogen bonding, between ^{PDN}U and solvent molecules. It showed highly Stoke's shifted emission with the change in solvent polarity. ^{PDN}U in protic solvents showed a larger solvatochromic shift, suggesting the existence of specific solute–solvent interactions, such as hydrogen bonding. Such solvatochromic behavior of ^{PDN}U made possible fluorometric detection of the microstructural changes in DNA through incorporation of ^{PDN}U into DNA and subsequent monitoring of the photochemical behavior.

Thus, upon hybridization of the probe oligonucleotide containing ^{PDN}U with the fully matched target DNA, an enhanced emission intensity was observed when excited at 450 nm, whereas the fluorescence emission of the single-stranded probe and the duplexes containing single mismatched bases were suppressed. The fluorescence behavior of probe ODN containing ^{PDN}U suggested that the effect of the

nature of the neighboring base pair on the photochemical behavior of ^{PDN}U is not significant. Similarly ^{PDN}C labeled oligonucleotide probe emits strongly when hybridized with its perfectly matched complementary strand, allowing the detection of G base of a target opposite of ^{PDN}C of the probe ODN.

6.5.4. Alkynylpyrene and Fluorene Labeled Fluorescent Nucleosides

Kim and co-workers have designed the Quencher-Free Molecular Beacons²¹ (QF-MBs, **Figure 6.6**) containing fluorescent nucleoside analogues at the middle of the loop of the hairpin MB. They have demonstrated that the resulting fluorescent oligonucleotides function as quencher-free MBs and were able to recognize base mismatched oligonucleotides. Their design was based on the rationale that substitutions at the C-5 position of uridine and the C-8 position of adenine do not perturb the stability and hybridization to form DNA duplexes. The absorption spectra of the single-base-mismatched duplexes, ODN1/ODN2 [N = G, C, T] were found to be slightly red-shifted relative to that of the fully matched duplex ODN1/ODN 2 [N = A] which suggested that the fluorene units in the single base mismatched duplexes presumably interact with one or more of the nucleobases.

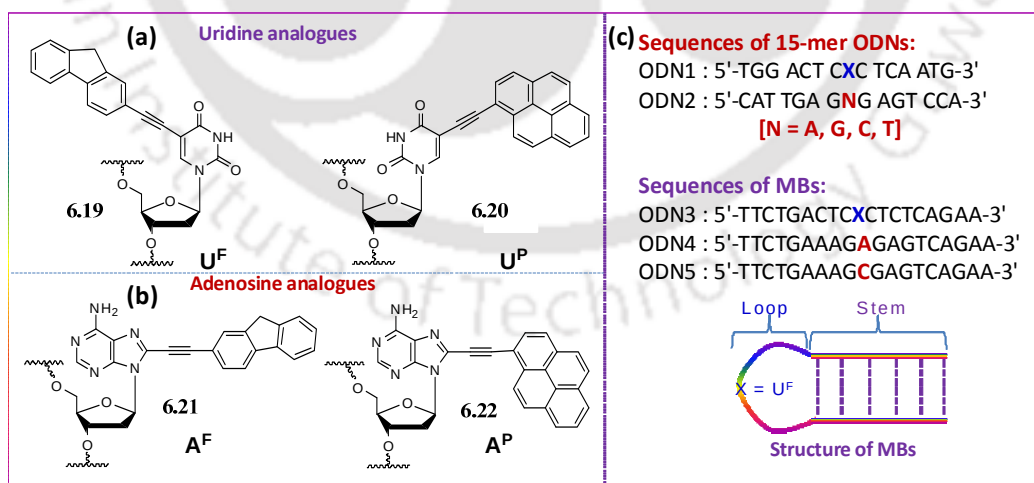


Figure 6.6: Kim's fluorescent nucleosides, X^F and X^P (X = A and U) and quencher-free MBs.

On the basis of these observations, Kim designed the oligonucleotide model system as a new type of MB that consists of a six-base-pair stem and a seven-base loop sequence possessing a fluorine labeled deoxyuridine core (**Figure 6.6c**).^{21a} Thus, the hybridization of hairpin ODN **3** with its matched complementary ODN **4** and single base mismatched ODN **5** led them to recognize, fluorimetrically, of a single (A/C) base mismatch with a total discrimination factor of 14.7. Thus their MBs are useful probes that can distinguish between the target and one base-mismatched (A/C) DNA sequences with a fluorescence signal.

Similarly, they have synthesized pyrene labeled deoxyuridine **U^P** and deoxyadenosine **A^P** which were subsequently incorporated into oligonucleotide sequences for DNA detection (**Figure 6.6a-b**).^{21b} From their fluorescence experiment it was inferred that similar to the fluorene-labeled ODNs, the pyrene-labeled ODNs containing **U^P** and/or **A^P** are also sensitive probes that discriminate between perfect and single base-mismatched base pairing through changes in fluorescence intensity. They have also exploited ethynylpyrene-labeled deoxyadenosine and deoxyuridine (**A^P** and **U^P**) nucleosides to the design of a probe for monitoring the B-to-Z DNA transition depending on different salt concentration.^{21c} For that purpose, they have designed fluorescent G, C-alternating oligonucleotides containing **A^P** and **U^P** at dangling positions. They have shown that the oligonucleotides adopted a Z-DNA conformation at high salt concentration while at low salt concentration they adopted B-DNA conformation. The transition from B-DNA to Z-DNA was thus detected with the generation of a strong fluorescence signal.

6.5.5. Fluorescent Metallonucleosides for the Design of “Metallobeacons”

Joshi and Tor have utilized nucleosides containing a 1,10-phenanthroline moiety to co-ordinate Ru(II) and Os(II) cations as reporters and quenchers in ‘metallobeacons’ (**Figure 6.7**).²² While the Ru(II) nucleoside exhibits a long lived excited state and a relatively high emission quantum efficiency in phosphate buffer pH 7.0, the Os (II)-containing nucleoside is quite non-emissive in an aqueous

environment. Thus, they have used both the metallo-nucleosides and used them as donor-acceptor pair in a designed “metallobeacon” for sensing fully matched target DNA and discriminating single base mismatched DNA *via* a drastic change in fluorescence intensity from the Ru(II) containing fluorescent nucleoside.

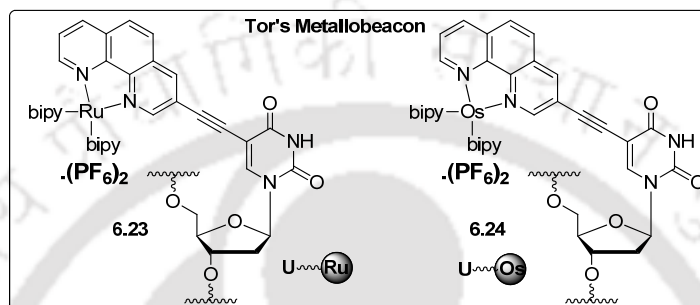


Figure 6.7: Tor's fluorescent metallo nucleosides and the metallobeacon.

6.5.6. Fluorescent Nucleosides for the Design of “HyBeacon” Probe

Molecular Beacons²³ immobilized on an array have the potential to probe unlabelled nucleic acids, allowing the fluorescently-labelled oligonucleotide (i.e., the Molecular Beacon) to be reused. Tom Brown and others have developed fluorescent “HyBeacon” probe format for genetic analysis utilizing commercially available fluorophore fluorescein. In their design, fluorescein is positioned in the major or minor groove by conjugation to either the base or the 2'-position of ribose (**Figure 6.8**).^{24a-c}

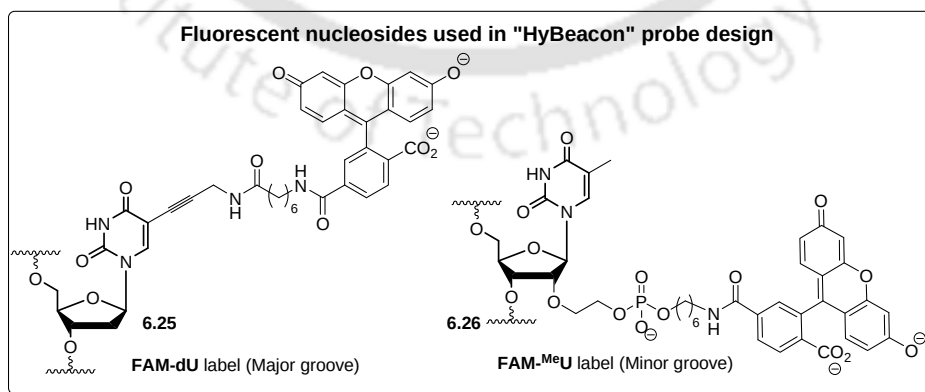


Figure 6.8: Brown's fluorescein labeled fluorescent nucleosides.

HyBeacon probes are single stranded oligonucleotides containing two or more internal fluorophores. They exhibit higher fluorescence intensity when hybridized to their complementary strand compared to the corresponding single stranded form. A strong interaction between the dyes, and with the nucleobases, which is absent in their duplex state but present in their single stranded form, resulted in a low or quenched fluorescence intensity to the single stranded HyBeacon probe. HyBeacon probes have been widely used in DNA sequence detection, allelic discrimination, human STR analysis, and in the analysis of human genetic polymorphisms.

There is a great deal of interest in developing new synthetic methods to construct chemically modified oligonucleotides (ODNs) for biological and nanotechnological applications. Since the very inception of the click chemistry philosophy in 2001, it quickly has attracted the attention of nucleic acids researchers to offer and adopt new ideas in this field of research to create “functional” nucleic acids. Thus, click chemistry, in brief, has got much recent attention in the field of nucleic acid research for various purposes as pointed out below:

- (a) In oligonucleotide labeling with fluorophores and carbohydrates.
- (b) In oligonucleotide immobilization on glass substrates.
- (c) In oligonucleotide metallization to achieve enhanced conductivity of DNA nanostructures, thereby enabling them to be used as molecular wires, nano- and microscale assemblies which have potential uses as nanoelectronic devices.
- (d) In oligonucleotide cross-linking.
- (e) In artificial DNA: oligonucleotide base and backbone analogues.
- (f) In oligonucleotide ligation, cyclisation and catenane formation.
- (g) In conjugation of peptides to DNA and PNA.

Few applications of click chemistry in nucleic acids research were given in **Chapter 2**. It is a vast and rapidly growing field and beyond the scope of the thesis to

picture in details. Therefore, here, we want to depict only one example of fluorescent oligonucleotide probes derived via the click chemistry to design “HyBeacon” probes for genetic analysis. Recently, Brown *et al.*, have utilized click chemistry protocol to the design and synthesis of triazolyl fluorophores labeled oligonucleotides, “HyBeacon” probes, for genetic analysis.^{24d-e} Thus, they have synthesized several alkynylated fluorophores to link with the azides of azide labelled oligonucleotides postsynthetically. In their strategy they also have explored the simple and efficient methods of introducing multiple azide groups into oligonucleotides in order to carry out ‘reverse click labeling’ with fluorescent reporter groups. They have utilised these triazolylfluorescent labelled 2'-uridine derivatives in designing HyBeacon probes. Their study revealed that the fluorescent labels attached to the 2'-position of the ribose sugar of thymidine do not prevent base pairing and the approach is highly important and versatile and can be used to introduce fluorophores onto any thymidine nucleotide in a DNA sequence, and for multiple labeling.

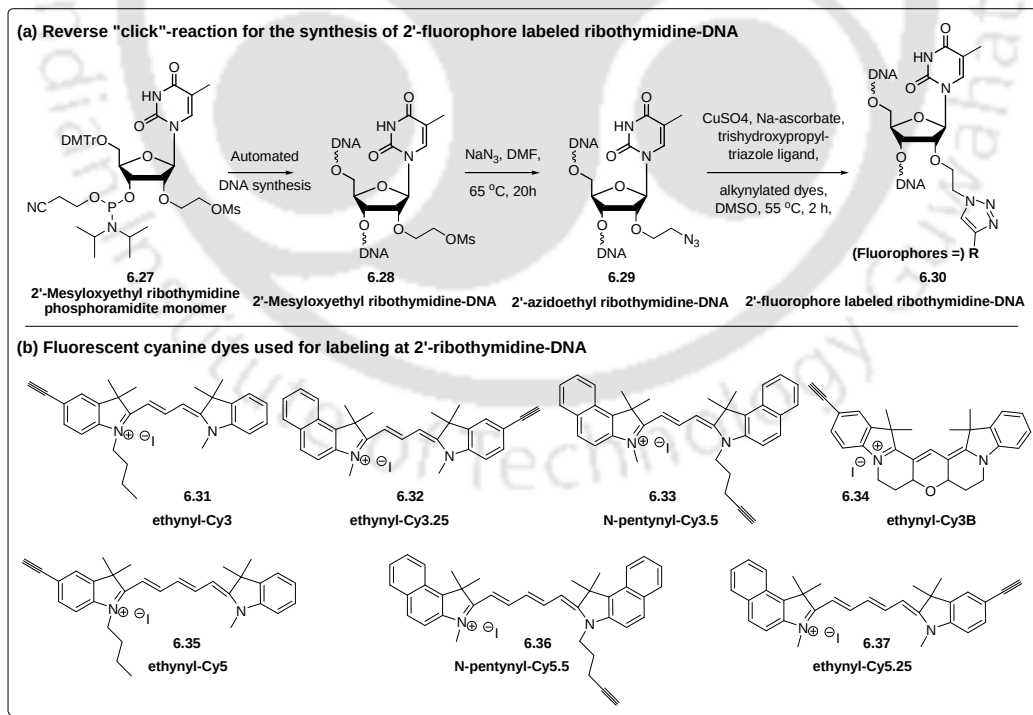


Figure 6.9: (a) Synthesis of “Click”-derived fluorescent nucleosides labelled oligonucleotides. (b) The structures of Cy-dyes used for labelling.

As shown in **Figure 6.9**, Brown *et al.* have used their click-methodology to synthesize a series of Cyanine (Cy)-Dye labeled HyBeacon probes.^{24d} They have studied fluorescence melting upon hybridisation of HyBeacons with the wild type (WT) target or mutant type (MT) target to form duplexes. Their study revealed that when the optimum dye separation was either 5 or 7 nucleotides, the HyBeacons gave the best melting curves. It was also concluded that the large difference in emission intensity between single and double stranded oligonucleotides was observed in case of doubly labeled HyBeacon labelled with Cy5 and Cy3B. Thus they envisaged that such probes could be useful for in vivo imaging of RNA targets.

6.6. Objective

From the above discussion it is clear that several fluorescently labeled nucleosides have been designed for the purpose of making fluorescently labeled probe for DNA detection utilizing various protocols and fluorescence techniques. Many other chemistry, platforms and protocols for DNA detection have recently been developed. As for example, MagSNiPer probe,^{25a} DigiTag2 assay,^{25b} Peptide nucleic acids (PNA) based probes,^{25c-d} were developed and reported for efficient genetic analysis. Ultrasensitive DNA detection using photonic crystals also was recently reported.^{25e} Gold nanoparticles modified with locked nucleic acid (LNA) system was developed and found that they form stronger duplexes with a single stranded DNA target and offer better discrimination against single base pair mismatches than analogous DNA probes. The LNA nanoparticle probes have also been used to detect double stranded DNA through triplex formation and it enabled in simultaneous detection and identification of multiple DNA targets.^{25f}

Therefore, a vast majority of SNPs detection methodologies are available in literature. However, no single protocol is available that meets all research need; because some protocols need strict hybridization condition, involve washing and drying steps, some are of high cost, time consuming, need sophisticated

instrumentation technique, or operationally not simple. So, there is a high need for searching an alternative method for homogeneous SNPs typing.

With this background and aim in mind we wanted to devise an alternative to the presently available methods for SNPs genotyping and therefore, we framed our objective as follows:

- (a) Design and synthesis of fluorescently labeled nucleoside *via* Sonogashira coupling.
- (b) Study of solvatochromic photophysical properties.
- (c) Incorporation of that nucleoside into short oligonucleotide sequences.
- (d) Study of photophysical properties of single stranded fluorescent oligonucleotide probes and their various duplexes formed upon hybridization with complementary fully matched and single/double base mismatched oligonucleotide sequences. Thus, we have chosen pyrene based fluorophore to be conjugated to 2'-deoxyuridine at its C-5 position (**Figure 6.10**).

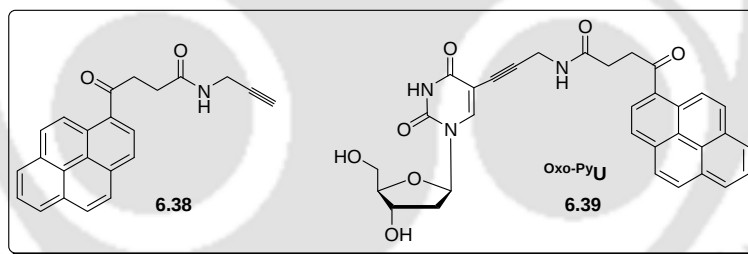


Figure 6.10: Our target fluorophore and the fluorescent nucleoside

- (e) The DNA sequences we wanted to synthesise are: (i) ODN **1**: 5'-d(CGCAAC^{Oxo-Py}UCAACGC)-3'-containing G/C flanking base pair; (ii) ODN **2**: 5'-d(CGCAAT^{Oxo-Py}UTAACGC)-3'-containing A/T flanking base pair. This sequence context was so chosen as to test the sequence dependency of the probe in the detection of target DNA.
- (f) We also thought that there could be a possibility of detecting two consecutive opposite bases from the target strand if we could incorporate consecutive two ^{Oxo-Py}U in a probe either by efficient monomer emission or *via* an excimer

emission depending on the geometric disposition of the two pyrene units. With this view, we also wanted to synthesize a third DNA probe sequence (iii) ODN 3: 5'-d(CGCAAT^{Oxo-Py}U^{Oxo-Py}UTAACGC)-3' containing two consecutive fluorescent nucleoside, ^{Oxo-Py}U.

- (g) All the ODNs were designed to use for homogeneous detection of SNPs based on labeling the bases by solvofluorochromic molecules capable of interacting directly and specifically with the DNA duplex, such as intercalators, DNA groove binders *etc.*

In the design of fluorescently labeled nucleoside we focused on the following design criteria to achieve a solvatochromic fluorescent nucleoside which would be able to monitor the local microenvironmental change around a DNA.

1. The fluorophore should be dielectric sensitive.
2. Emission of the fluorophore should lie in the visible region.
3. Emission must possess high quantum yield in highly polar solvent.
4. Emission and absorption intensities should be so that high fluorescence brightness ($\epsilon \cdot \Phi$) would be observed.

Therefore looking at the strong fluorescence emission and high quantum yield of pyrene chromophore, we have chosen γ -oxo-pyrenecarboxamide to be linked with 2'-deoxy uridine at the C-5 position *via* an acetylenic linker employing Sonogashira coupling protocol to generate the solvatochromic fluorescent nucleoside.

6.6.1. Our Concept of DNA Detection: Just-Mix and Read Strategy

Our concept of DNA detection using fluorescently labeled oligonucleotide probes is based on the fluorescence change of the labeled nucleoside base itself in response to the natural DNA bases on a complementary strand, not on whether the probe is hybridized or not as is illustrated in **Figure 6.11**. We hypothesized that upon binding

with its fully matched complementary sequence, the labeled nucleoside has to reside outside the groove exposing itself to the more polar aqueous microenvironment and as a result strong fluorescence signal would be observed. On the contrary, because of lack of Watson-Crick base pairing in the mispaired position in the mismatched duplexes, the fluorophore would remain inside the duplex facing highly hydrophobic environment thereby exhibits very weak fluorescence. Thus, if we are able to utilize our novel concept of detecting DNA with our designed γ -oxo-pyrene labeled fluorescent nucleoside, ^{Oxo-Py}U, it would be highly promising research effort in giving rise to new powerful tool for the detection of SNPs. The merits of homogeneous assay using our “Just-Mix & Read Strategy” are as follows:

- (a) Fluorescence labeling of targets is not required.
- (b) Hybridization condition is very simple- just mixing.
- (c) No strict hybridization condition or annealing step is required to make duplex.
- (d) No washing, drying or centrifugation step.

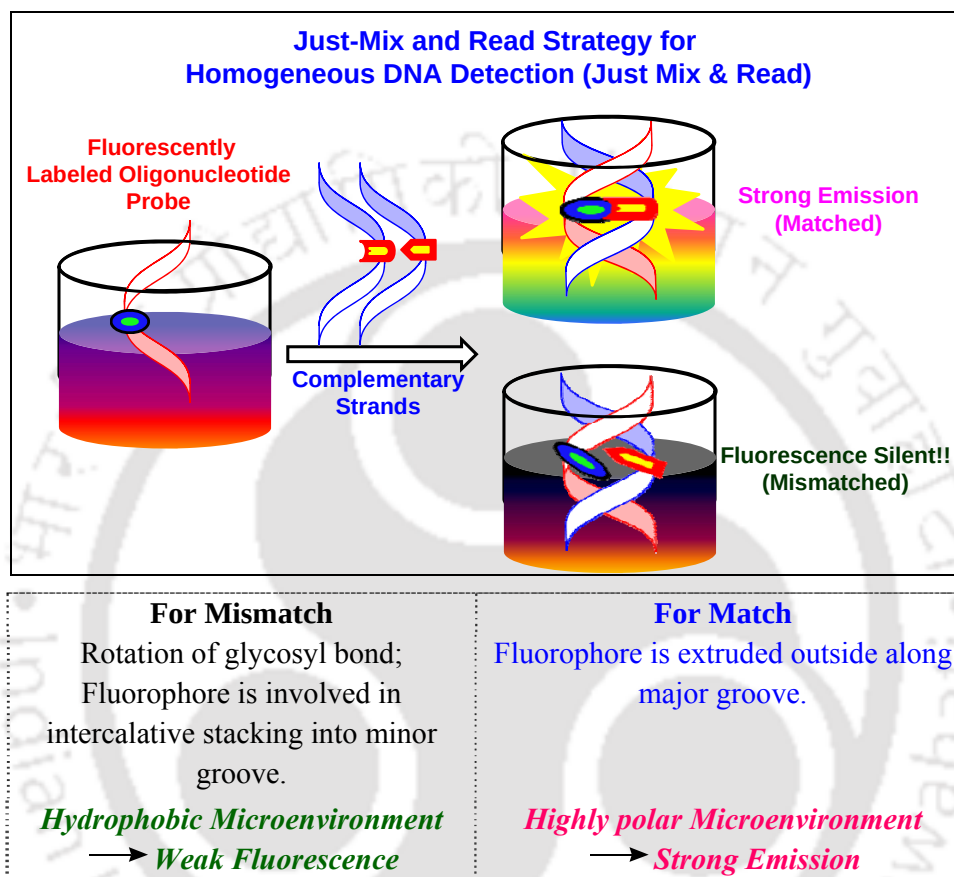


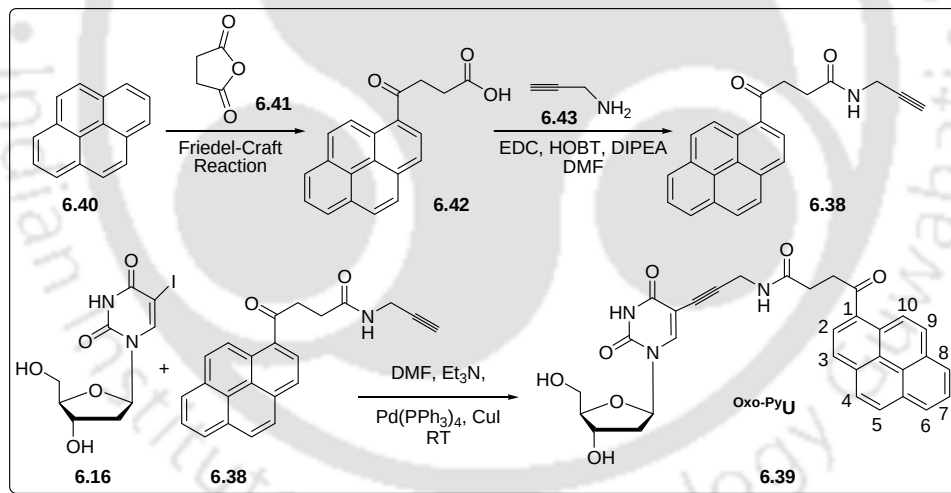
Figure 6.11: Graphical presentation of our concept of homogeneous DNA detection.

6.7. Results and Discussion

With this design concept we have synthesized the fluorophore with acetylenic linker and the conjugated nucleoside *via* a Pd(0)-mediated classical Sonogashira coupling protocol.

6.7.1. Synthesis of Fluorescent Nucleoside

Synthesis of the fluorescent nucleoside started with a Friedel-Craft reaction. Thus, the γ -oxo-pyrene butyric acid (**6.42**) was synthesized from commercially available pyrene (**6.40**) and succinic anhydride (**6.41**) *via* a Friedel-Craft acylation. Then, the γ -oxo-pyrene butyric acid was converted to its corresponding amide, *N*-propargyl-1- γ -oxo-pyrenebutyric acid amide (**6.38**) by reaction with propargyl amine (**6.43**) in presence of EDC, HOBT, and base DIPEA in DMF. The fluorescent nucleoside (**6.39**) was synthesized *via* a classical Sonogashira cross-coupling²⁶ reaction of *N*-propargyl-1- γ -oxo-pyrenebutyric acid amide (**6.38**) with 5-iodo-2'-deoxyuridine (**6.16**) in presence of $\text{Pd}(\text{PPh}_3)_4$, CuI, and base Et_3N in DMF under nitrogen atmosphere at room temperature (**Scheme 6.2**). The final product was isolated pure by column chromatography and characterized by NMR and mass spectrometry.



Scheme 6.2: Synthesis of fluorescent nucleoside **6.39**.

6.7.2. Spectral Characterization of Fluorescent Nucleoside (^{Oxo-PyU})

The ^1H NMR study of the fluorescent nucleoside **6.39** showed that the 1'-H, 2'-H, 3'-H, 4'-H and 5'-H of the sugar unit resonated at 6.12, 2.12, 3.48, 4.23 and 3.36 ppm, respectively and each appeared as multiplet in their respective field of resonance. The aromatic $-\text{N}_3\text{-H}$ and $-\text{C}_6\text{-H}$ protons of the uracil base resonated at 11.68 ppm, and

8.14 ppm, respectively. The amide –N–H proton appeared as doublet at 4.13 ppm. The –CH₂ proton attached to –C≡C– appeared at 5.26 ppm as a doublet. The –CH₂– protons next to amide bond of pyrene butyric acid part appeared as triplet at 2.50 ppm. The other –CH₂– protons close to pyrene carbonyl unit appeared as multiplet at 3.80 ppm. The aromatic C₂–H and C₃–H protons of pyrene unit resonated as doublet at 8.76, and 8.58 ppm, respectively. The other aromatic protons of pyrene unit appeared within the range of 8.14–8.41 ppm as multiplet (**Figure 6.12**).

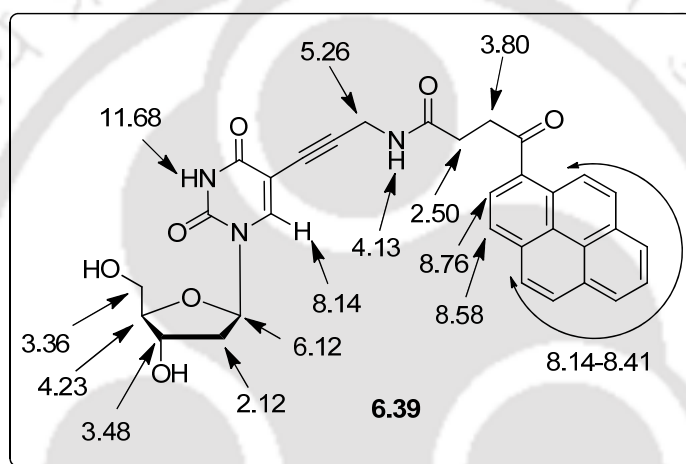


Figure 6.12: ¹H-NMR assignment of **6.39**.

6.7.3. Study of Photophysical Properties of Fluorescent Nucleoside

After synthesizing the nucleoside **6.39**, we studied the photophysical properties in different solvents of varying polarity. Thus the UV-visible spectra showed a red shifted pattern with decreasing absorbance as the polarity of the solvent increases (**Figure 6.13a**). We observed a 20 nm red shift from DMSO ($\lambda_{max}^{abs} = 345$ nm) to trifluoroethanol (TFE, $\lambda_{max}^{abs} = 355$ nm), the most polar organic solvent tested.

Next, we have measured the fluorescence photophysical properties of the nucleoside. Most interestingly, we observed that, both the fluorescence intensity and the wavelength were markedly affected by solvent polarity (**Figure 6.13b**). Thus, in DMSO, nucleoside **6.39** displayed a very weak emission with a maximum at 396 nm ($\Phi = 0.015$), while in TFE, the most polar organic solvent tested, the nucleoside **6.39**

showed a strong emission ($\Phi = 0.22$) at around 467 nm that decayed deeply into the visible region (>565 nm). We have also calculated fluorescence brightness factor (F.B. = $\epsilon \cdot \Phi$) which showed an 11-fold increase in TFE in comparison with that was observed in DMSO (**Figure 6.13b**, **Table 6.1**). We observed that with increase in solvent polarity both the quantum yield and the shift in emission wavelength maxima (λ_{max}^f) were increased ($\Delta \lambda_{max}^f = 70$ nm from DMSO to TFE). Thus, the oxo-pyrene-labeled nucleoside showed highly polarity sensitive emission behaviour. **Table 6.1** shows the summary of the photophysical property of the nucleoside **6.39** in different solvents.

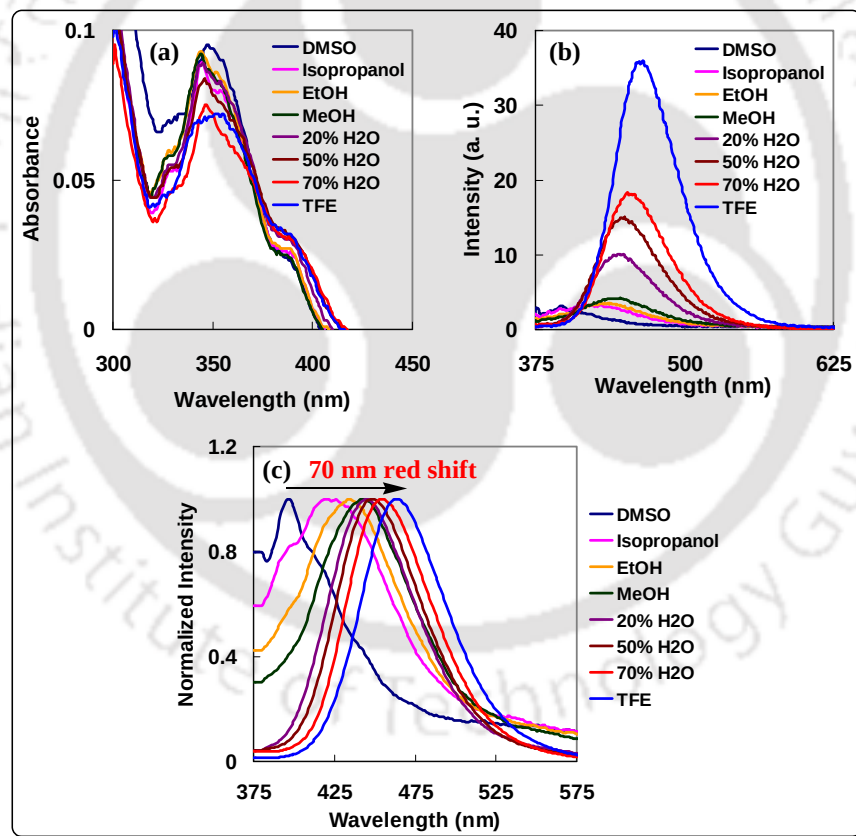


Figure 6.13: (a) UV-visible, (b) fluorescence and (c) normalized fluorescence spectra of nucleoside **6.39** in different solvents ($\lambda_{ex} = \lambda_{max} = 345 - 355$ nm). Water titration was done in EtOH-H₂O mixture.

Table 6.1: Summary of photophysical property of the nucleoside **6.39** in different solvents.

Solvents	ϵ (mol ⁻¹ cm ⁻¹)	λ_{em}^{max} (nm)	Φ^{27}	$\epsilon \cdot \Phi$
DMSO	9500	396	0.015	142.5
Isopropanol	9700	417	0.021	203.3
EtOH	9300	438	0.023	216.7
MeOH	9200	446	0.027	244.7
20% H ₂ O in EtOH	8900	447	0.050	442.3
50% H ₂ O in EtOH	8400	450	0.082	688.8
70% H ₂ O in EtOH	7500	452	0.117	750.0
TFE	7200	467	0.22	1584.0

We have also studied the fluorescence behaviour of nucleoside **6.39** in EtOH titrated with water (**Figure 6.14**). We observed a red shifted emission behaviour with increasing intensity as the polarity of the solvent mixture increases. Thus, in pure EtOH, the emission was around 438 nm ($\Phi = 0.023$) which was shifted to 452 nm and decayed deeply into visible region, 580-600 nm, when the ratio of water and ethanol was 70 : 30 ($\Phi = 0.12$). We observed 30 nm red shifting from absolute EtOH to 95% water in EtOH (**Figure 6.14b**). The maximum intensity was observed at 70% water in EtOH, further increasing of water content has led to the decrease in the emission intensity (**Figure 6.14a**). This is because of decreasing solubility of the nucleoside at high % water in EtOH-water solvent mixture.

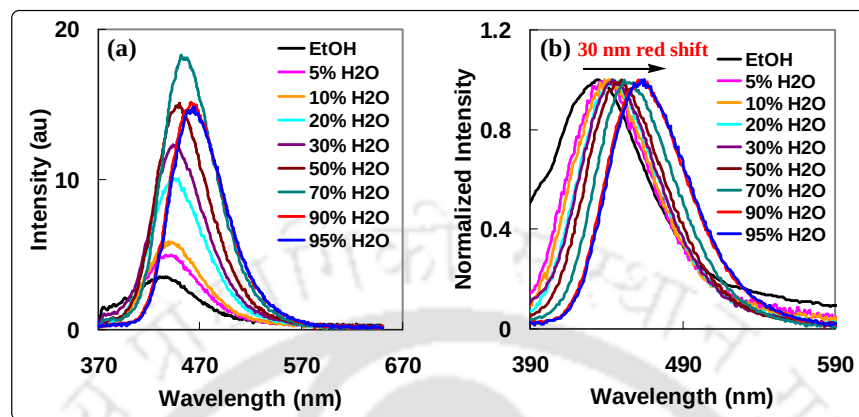
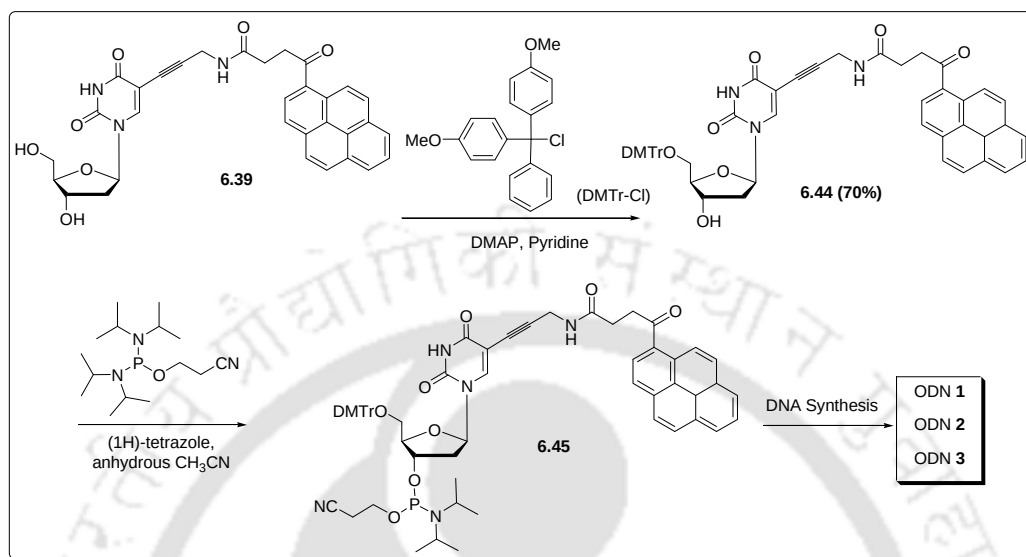


Figure 6.14: (a) Fluorescence spectra and (b) normalized fluorescence spectra of nucleoside **6.39** in EtOH titrated with water ($\lambda_{\text{ex}} = \lambda_{\text{max}} = 345\text{-}350\text{ nm}$).

Therefore, our study on the photophysical properties of the fluorescent nucleoside revealed that the nucleoside is highly solvatochromic and could potentially be used for sensing DNA microenvironment.

6.7.4. Synthesis of the Oligonucleotide Probe

Being interested by the photophysical properties of the fluorescently labeled nucleoside (**6.39**), we next incorporated the nucleoside into short oligonucleotide sequences. To do the oligonucleotide synthesis, we first protected the 5'-OH of the nucleoside (**6.39**) with 4,4'-dimethoxytrityl group by reaction with 4,4'-dimethoxytrityl chloride in presence of catalytic amount of *N,N*-dimethylamino pyridine (DMAP) in dry pyridine solvent. The 5'-DMTr protected nucleoside **6.44** was isolated pure by a silica gel column chromatography using 20:1 methanol-chloroform solvent mixture and characterized by NMR and mass spectrometry. Next, the DMTr protected nucleoside (**6.44**) was converted to its phosphoramidite derivative (**6.45**) by reaction with 2-cyanoethyl-*N,N,N',N'*-tetraisopropyl-diphosphoramidite in presence of (1*H*)-tetrazole in anhydrous acetonitrile under nitrogen atmosphere. The phosphoramidite derivative (**6.45**) so produced was used for the DNA synthesis without further purification (**Scheme 6.3**).



Scheme 6.3: Synthesis of fluorescently labeled ODNs.

Finally, we generated three oligonucleotide sequences, ODN **1**, **2** and **3**, containing the fluorescent nucleoside, **6.39**, at the middle of each strand *via* standard DNA synthesis protocol using an automated DNA/RNA synthesizer. The synthesized ODNs were purified by reversed phase HPLC and characterized by MALDI-TOF mass spectrometry (**Table 6.2**). The 13-mer ODN **1** and **2** contained singly labeled ^{Oxo-Py}U and differed only by flanking bases near the labeled nucleoside (ODN **1** contained C, while ODN **2** contained T as the flanking bases), while 14-mer ODN **3** was labeled with two consecutive ^{Oxo-Py}U with flanking bases T. As was mentioned earlier, while ODN **1** and ODN **2** were synthesized to test the sequence dependency in DNA detection, the ODN **3** was synthesized with an expectation of detecting consecutive AA bases of a target DNA either from a monomer emission or from a possible excimer emission from the pyrene containing fluorescent nucleoside.

Table 6.2: Synthesized fluorescently labeled oligonucleotide sequences.

ODNs	Sequences
1	5'-d(CGCAAC ^{Oxo-Py} UCAACGC)-3'
2	5'-d(CGCAAT ^{Oxo-Py} UTAACGC)-3'
3	5'-d(CGCAAT ^{Oxo-Py} U ^{Oxo-Py} UTAACGC)-3'

6.7.5. Characterization of the Synthesized ODNs

Synthesized ODNs were purified by reverse phase HPLC on a 5-ODS-H column (10×150 mm, elution with 50 mM ammonium formate buffer (AF), pH 7.0, linear gradient over 45 min from 3% to 40% acetonitrile at a flow rate 2.0 ml/min). The ODNs purified by HPLC were characterized by MALDI-TOF mass spectrometry. The calculated masses of the ODNs are in good agreement with the experimentally found masses. **Table 6.3** shows the calculated MALDI-TOF masses and experimentally found MALDI-TOF mass data of the synthesized ODNs.

Table 6.3: MALDI-TOF mass spectral data for the synthesized ODNs.

ODNs	MALDI-TOF mass calcd [M+H] ⁺	MALDI-TOF mass found [M+H] ⁺
ODN 1	3957.68	3956.97
ODN 2	3927.66	3928.47
ODN 3	4185.93	4186.72

6.7.6. DNA Detection by Measuring Photophysical Properties

6.7.6.1. UV-Visible Study

After synthesizing the ODNs, the UV-visible absorption properties of the single strand ODNs as well as their various duplex ODNs formed by hybridization with various complementary ODNs were studied. **Table 6.4** shows the oligonucleotide sequences used for the photophysical studies. Thus, it was observed that the UV-

absorptions of the single strand ODNs and the duplex ODNs were almost similar for all the three modified ODNs. **Figure 6.15** shows the UV-visible spectra of the single strand oligonucleotides, ODN 1-3 and their various duplexes formed by hybridization with various complimentary ODNs.

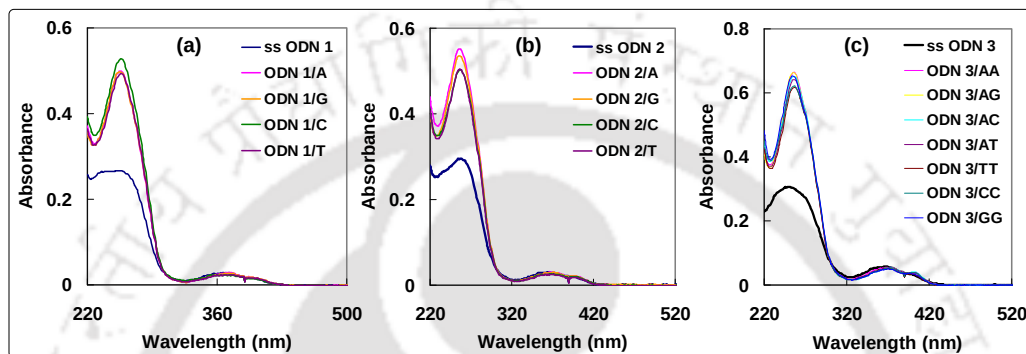


Figure 6.15: UV-visible spectra of (a) ss ODN 1 and ODN 1 hybridized with ODN 7 (N = A, G, C and T), (b) ss ODN 2 and ODN 2 hybridized with ODN 8 (N = A, G, C and T) and (c) ss ODN 3 and ODN 3 hybridized with ODN 9 (N = AA, AG, AC, AT, TT, CC and GG) at 298 K. Final duplex concentration was 2.5 μ M, [buffer] (sodium phosphate) = 50 mM, pH = 7.0, [NaCl] = 0.1 M; “ss” denotes a single-stranded probe.

Table 6.4: Oligonucleotide sequences used for the photophysical studies.

ODNs	Sequences
1	5'-d(CGCAAC ^{Oxo-Py} U CAACGC)-3'
2	5'-d(CGCAAT ^{Oxo-Py} U TAACGC)-3'
3	5'-d(CGCAAT ^{Oxo-Py} U ^{Oxo-Py} U TAACGC)-3'
4	5'-d(CGCAAC T CAACGC)-3'
5	5'-d(CGCAAT T TAACGC)-3'
6	5'-d(CGCAAT TT TAACGC)-3'
7	5'-d(GCGTTG N GTTGCG)-3' [N = A, G, C, T]
8	5'-d(GCGTTA N ATTGCG)-3' [N = A, G, C, T]
9	5'-d(GCGTTA NN ATTGCG)-3' [NN = AA, AG, AC, AT, TT, CC, GG]

6.7.6.2. Fluorescence Study

Being interested by the solvent polarity dependent fluorescence property of the fluorescent nucleoside (**6.39**, ^{Oxo-Py}U), we thought that ^{Oxo-Py}U would be usable for monitoring the change in DNA microenvironment. Therefore, we studied the fluorescence properties of single stranded **ODN 1**, (5'-CGCAAC ^{Oxo-Py}U CAACGC-3') and **ODN 2**, (5'-CGCAAT ^{Oxo-Py}U TAACGC-3') in the presence and absence of their various complementary strands (**Figure 6.16-18**). As revealed from the fluorescence spectra (**Figure 6.16b** and **Table 6.5**) it was observed that the fluorescence of **ODN 1** with flanking G/C base pair was completely quenched when hybridized to perfectly matched or single base mismatched complementary sequences, **ODN 7** [N = A, G, C, T]. This is so obvious and a positive result of our expectation that the flanking G/C base pair is responsible for the quenching of ^{Oxo-Py}U fluorescence. Thus, ^{Oxo-Py}U could potentially be useful in designing G-quenched molecular beacon as well.²⁸

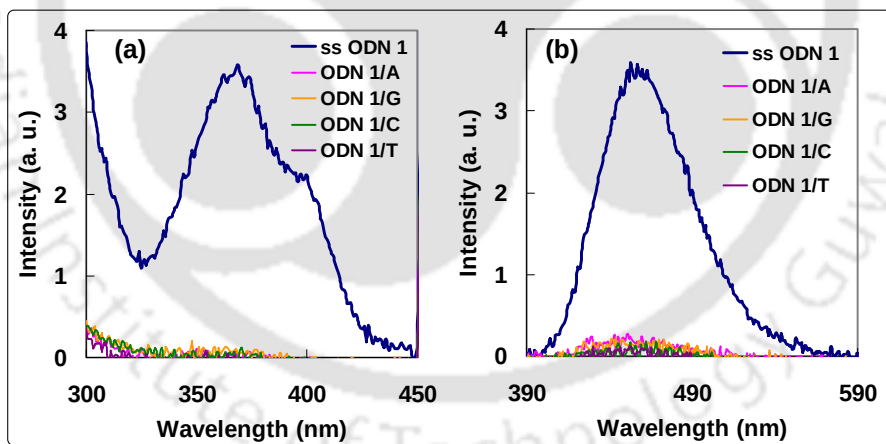


Figure 6.16: (a) Fluorescence excitation and (b) fluorescence emission spectra of **ODN 1** hybridized with 2.5 μ M **ODN 7** (N = A, full much), **ODN 7** (N = G, C, T), and single-stranded **ODN 1** at 298 K. Excitation wavelength was at 369 nm. Final duplex concentration was 2.5 μ M, [buffer] (sodium phosphate) = 50 mM, pH = 7.0, [NaCl] = 0.1 M; “ss” denotes a single-stranded probe.

On the other hand, ODN 2, (5'-CGCAAT ^{Oxo-Py}U TAACGC-3'), upon hybridization with perfectly matched sequence, ODN 8 [N = A] showed a very strong emission at 465 nm (**Figure 6.17b**). However, a fluorescence quenching was observed in case of the duplex ODN 2/8 [N = G] where mismatched base G is opposite to the labeled nucleoside in the complementary target strand. Thus, this observation again indicated that the fluorescence of ^{Oxo-Py}U is tremendously quenched by neighboring G-base and can allow us to detect matched base A (Adenine) with an intense fluorescence signal.²⁹ The strong fluorescence emission for perfectly matched duplex ODN 2/8 [N = A] can be explained if we consider the more hydrophilic microenvironment faced by oxo-pyrene chromophore outside the groove. As the opposite base is matched base A, the fluorophore has to extrude outside the groove and thus faced to the more aqueous microenvironment, resulting in an enhancement of fluorescence (**Figure 6.17b** and **Table 6.5**). The excitation spectra, fluorescence spectra of single strand probe ODN 2 and its various duplexes are shown in **Figure 6.17**. **Figure 6.17c** represents separately the emission intensity difference between the fully matched duplex, ODN 2/ODN 8 (N = A), and single G base mismatched duplex ODN 2/ODN 8 (N = G). Thus it is clear that the probe ODN 2 can be utilized for the discrimination of A/G allele *via* the generation of a distinct fluorescence signal.

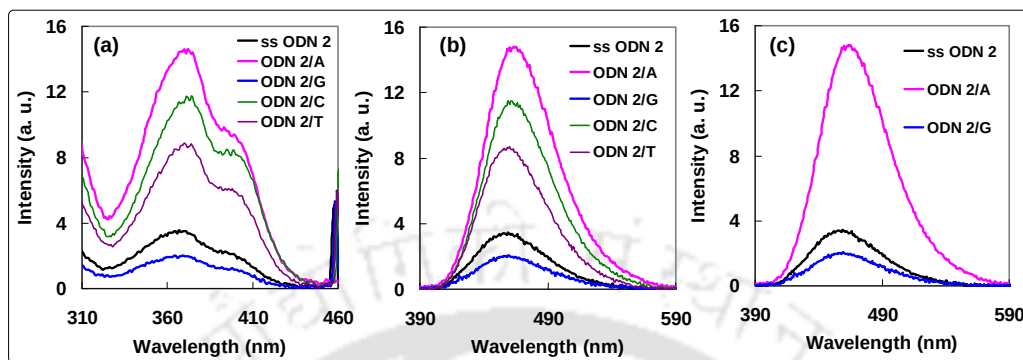


Figure 6.17: (a) Fluorescence excitation and (b) fluorescence emission spectra of ODN 2 hybridized with 2.5 μM ODN 8 ($N = A$, full much), ODN 8 ($N = G, C, T$), and single-stranded ODN 2 at 298 K. Excitation wavelength was at 369 nm, Final duplex concentration was 2.5 μM , [buffer] (sodium phosphate) = 50 mM, pH = 7.0, [NaCl] = 0.1 M; “ss” denotes a single-stranded probe. (c) Fluorescence emission of ss ODN 2 and the duplex ODN 2/ODN 8 ($N = A$) and ODN 2/ODN 8 ($N = G$) under same experimental condition.

As ODN 2 showed a highly A-selective fluorescence emission, we thought that there could be a possibility of detecting opposite two consecutive $-AA-$ sequence from the target strand if we incorporate two consecutive Oxo-PyU in the probe either by an enhanced monomer emission or *via* an excimer emission depending on the geometrical disposition of the two oxo-pyrene rings. Therefore, we prepared a doubly Oxo-PyU -labeled probe, ODN 3 [5'-d(CGCAAT Oxo-PyU Oxo-PyU TAACGC)-3'] and measured the fluorescence of the duplexes formed *via* hybridization with complementary strands ODN 9 [{5'-d(GCGTTA NN ATTGCG)-3'}; NN = AA, AG, GG]. Unfortunately, in any case we could not observe an excimer fluorescence. However, the detection of $-AA-$ bases was perfectly accomplished by a strong monomer emission at 467 nm ($\Phi = 0.15$) with a long tail in the visible region (>567 nm) together with a high signal to noise ratio and a large fluorescence brightness factor (F.B. = 3284). In contrast, the emission of the single stranded ODN 3 and the duplexes ODN 3/9 [NN = AG] and ODN 3/9 [NN = GG] was strongly

suppressed (**Figure 6.18b** and **Table 6.5**). Therefore, ODN 3 can act as a useful probe which emits strong fluorescence with a high AA selectivity.

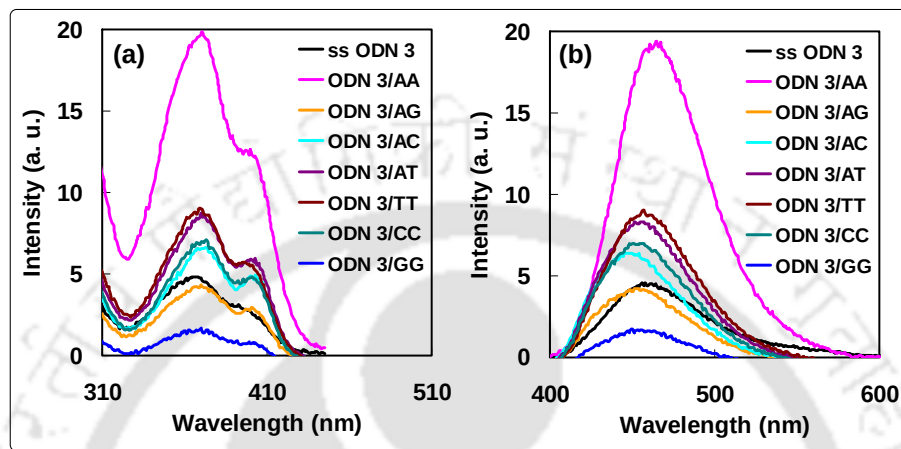


Figure 6.18: (a) Fluorescence excitation and (b) fluorescence emission spectra of ODN 3 hybridized with 2.5 μM ODN 9 ($N = \text{AA}$, full much), ODN 9 ($N = \text{AG}$, AC , AT , TT , CC , GG), and single-stranded ODN 3 at 298 K. Excitation wavelength was at 369 nm. Final duplex concentration was 2.5 μM , [buffer] (sodium phosphate) = 50 mM, pH = 7.0, [NaCl] = 0.1 M; “ss” denotes a single-stranded probe.

Table 6.5: Summary of photophysical properties of various ODNs.

Duplexes	ϵ_{max} ($\text{mol}^{-1}\text{cm}^{-1}$)	Φ^{27}	$\epsilon \cdot \Phi$
ODN 1	11,200	0.054	605
ODN 1/7 [$N = \text{A}$]	11,200	0.002	22
ODN 1/7 [$N = \text{G}$]	10,800	0.001	11
ODN 2	11,600	0.046	543
ODN 2/8 [$N = \text{A}$]	12,000	0.216	2640
ODN 2/8 [$N = \text{G}$]	11,200	0.030	336
ODN 3	22,648	0.036	815
ODN 3/9 [$\text{NN} = \text{AA}$]	21,896	0.150	3284
ODN 3/9 [$\text{NN} = \text{AG}$]	21,492	0.025	550
ODN 3/9 [$\text{NN} = \text{GG}$]	19,696	0.007	153

Thus, the results of study of fluorescence summarizes that the fluorescence of ODN 1 was quenched by flanking G/C base pair (**Figure 6.19a**). The ODN 2 showed

maximum fluorescence intensity when the opposite base of the labeled nucleoside was A (adenine). It also showed fluorescence quenching when the opposite base of the labeled nucleoside was G (**Figure 6.17c**). Finally, the doubly labeled ODN **3** showed maximum fluorescence intensity when the bases of the target DNA opposite of two consecutive ^{Oxo-Py}U of probe ODN –AA–. The doubly labeled probe also showed quantified fluorescence quenching when the bases of target ODN opposite of two consecutive ^{Oxo-Py}U of probe ODN were –GG– (**Figure 6.19b**).

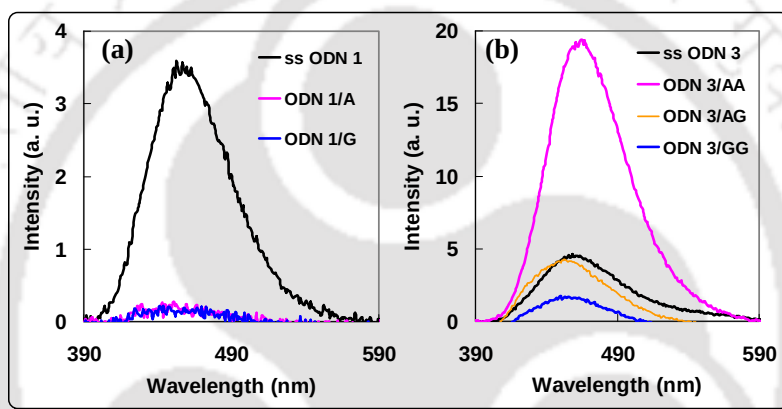


Figure 6.19: Fluorescence emission spectra of (a) ODN **1** hybridized with ODN **7** ($N = A$, full match and G) and ss ODN **1** and (b) ODN **3** hybridized with ODN **9** ($N = AA$, full match and AG, GG) and ss ODN **3** at 298 K. Excitation wavelength was at 369 nm. Final duplex concentration was 2.5 μM , [buffer] (sodium phosphate) = 50 mM, pH = 7.0, [NaCl] = 0.1 M; “ss” denotes a single-stranded probe.

6.7.6.3. Evaluation of Thermal Stability of the Duplexes

The thermal stability of the duplex was also measured *via* thermal denaturation study using UV-visible spectroscopy (**Table 6.6**). Thus, we observed that all the three matched duplexes with modified ODNs (ODN **1/7** [$N = A$]; ODN **2/8** [$N = A$]; and ODN **3/9** [$N = AA$]) are more stable than unmodified ODNs (ODN **4/7** [$N = A$]; ODN **5/8** [$N = A$]; ODN **6/9** [$N = AA$]). The thermal melting behavior showed that the matched duplex ODN **1/7** [$N = A$] is more stable ($T_m = 70.9\text{ }^\circ\text{C}$) than unmodified duplex ODN **4/7** [$N = A$] ($T_m = 59.8\text{ }^\circ\text{C}$). Similarly, the matched duplex ODN **2/8**

[N = A] is more stable ($T_m = 64.1$ °C) than unmodified duplex ODN 5/8 [N = A] ($T_m = 56.1$ °C). Duplex with doubly labeled ODN (ODN 3/9 [NN = AA], $T_m = 64.1$ °C) also showed more stability than the unmodified ODN (ODN 6/9 [NN = AA], $T_m = 61.1$ °C). Thus, the thermal stability study implies that fluorophore resides along the major groove and involved in stacking type interaction and hence bringing more stability of the matched duplexes.

Table 6.6: Thermal melting temperatures of duplex ODNs.

Duplexes	T_m (°C)
ODN 1/7 [N = A]	70.9
ODN 1/7 [N = G]	60.7
ODN 4/7 [N = A]	59.8
ODN 2/8 [N = A]	64.1
ODN 2/8 [N = G]	52.5
ODN 5/8 [N = A]	56.1
ODN 3/9 [NN = AA]	64.1
ODN 3/9 [NN = AG]	58.4
ODN 3/9 [NN = GG]	53.2
ODN 6/9 [NN = AA]	61.1

6.7.7. Molecular Modeling Study

To understand the spatial disposition of the fluorophore within the duplex in a better way, we have carried out the molecular modeling study of the duplexes (fluorescently labeled ODNs and the complementary ODNs) by MacroModel calculation (Maestro vs. 9.0) using AMBER* force field in water.³⁰ Thus, the optimized structure of duplex ODN 2/8 [N = A] showed that the fluorophore resides along the major groove and thus it would experience more hydrophilic environment (**Figure 6.20a**). As a result we would expect strong fluorescence emission. On the other hand as the fluorophore resides along the major groove, it involved in stacking type interaction and hence brings more stability of the duplex (ODN 2/8 [N = A]). Both molecular modeling and photophysical studies strongly support our concept and

expected result. For the duplex ODN **3/9** [**N** = AA] we expected excimer emission by two consecutive ^{Oxo-Py}U. However, we have not observed any excimer emission from the duplex ODN **3/9** [**N** = AA] in fluorescence spectra. To understand the fact of no excimer emission by the former duplex we have carefully examined the molecular modeling results. Thus, from the optimized structure it was found that the two pyrene rings are far apart and positioned parallel and in a cofacial configuration to each other, thereby inhibiting an excimer formation (**Figure 6.20b-c**).³¹

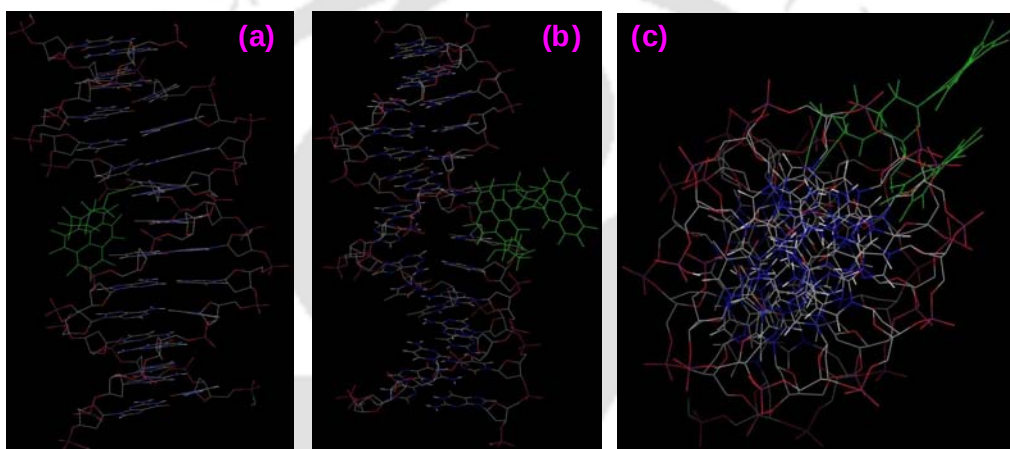


Figure 6.20: AMBER* energy minimized conformation of (a) duplex ODN **2/8** [**N** = A] (Side view); (b, c) duplex ODN **3/9** [**NN** = AA] (side and top view, respectively) in water.

6.8. Conclusion

In conclusion, we have successfully synthesized a fluorescently labeled nucleoside (^{Oxo-Py}U) which showed a highly solvatochromic fluorescence emission with high quantum yield in high polar solvent. Further, we have incorporated the fluorescently labeled nucleoside into short oligonucleotide sequences and generated fluorescently labeled oligonucleotide probes containing singly and doubly ^{Oxo-Py}U label and investigated their fluorescence emission properties in presence of complementary target ODNs. Our investigations on the fluorescence emission properties of the fluorescent DNA probes containing singly and doubly labeled ^{Oxo-Py}U in the presence of various complementary strands, revealed that the fluorescence of ^{Oxo-Py}U is strongly quenched by neighboring G-base and hence can be applied for designing quencher free molecular beacon. We have also shown that the probes are highly specific for the detection of matched base A of a target DNA opposite to the labeled base of probe DNA *via* the enhancement of fluorescence signal. Furthermore, the doubly labeled probe is also capable for the detection of opposite matched consecutive AA bases of target ODN with a strong fluorescence signal generation, high signal to noise ratio and a large fluorescence brightness factor. Therefore, the doubly labeled ODN which exhibits unique fluorescence property depending on the number of adenines on the complementary strand, would be effective for the detection of consecutive –AA– sequences located at a specific site of the target DNA. The singly and doubly labelled probes also could be useful for detection of A/G or AA/GG allele, respectively as revealed from their fluorescence behaviour.

6.9. Experimental Section

6.9.1. General

^1H NMR spectra (400 MHz) and ^{13}C NMR spectra (100 MHz) were measured with Bruker Avance 400F spectrometer. Coupling constant (J value) is reported in hertz. The chemical shifts are shown in ppm downfield from tetramethylsilane, using residual chloroform ($\delta = 7.24$ in ^1H NMR, $\delta = 77.23$ in ^{13}C NMR) dimethyl sulfoxide ($\delta = 2.5$ in ^1H NMR, $\delta = 39.5$ in ^{13}C NMR), and methanol ($\delta = 3.30$ in ^1H NMR, $\delta = 49.0$ in ^{13}C NMR), as an internal standard. FAB masses were recorded on a JEOL JMS HX-110A spectrometer.

The reagents for DNA synthesis were purchased from Glen Research. Mass spectra of oligodeoxynucleotides were determined with a MALDI-TOF MS (Shimadzu AXIMA-LNR, acceleration voltage 20 kV, positive mode) with 2',3',4'-trihydroxyacetophenone as a matrix. Calf intestinal alkaline phosphatase (Promega), *Crotalus adamanteus* venom phosphodiesterase I (USB), and *Penicillium citrinum* nuclease P1 (Roche) were used for the enzymatic digestion of ODNs. All aqueous solutions utilized purified water (Millipore, Milli-Q sp UF). Reversed-phase HPLC was performed on CHEMCOBOND 5-ODS-H columns (10×150 mm, 4.6×150 mm) with a JASCO Chromatograph, Model PU-2080, using a UV detector, Model UV-2075 plus, at 254 nm.

6.9.2. Synthesis and Characterization

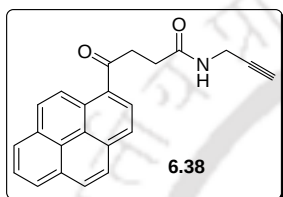
6.9.2.1. γ -oxo-pyrenebutyric acid (6.42)

A solution of 1.0 gm. (4.94 mmol) of pyrene and 0.55 gm. (5.43 mmol) of succinic anhydride in 25-30 ml of nitrobenzene was prepared in a 100 ml round bottom flask and stirred at 0°C . After one hour 0.7 gm. (5.94 mmol) of aluminum chloride was added slowly in the mixture. Stirring was continued at 0°C for 5-6 hours more and the solution was allowed to stand for twelve hours and come to room temperature. Then concentrated hydrochloric acid was added at 0°C . The crude

material was filtered under vacuum and purified by recrystallization from alcohol. The crystalline material was used for the next step.

6.9.2.2. *N*-Propargyl-1- γ -oxo-pyrenebutyric acid amide (**6.38**)

A solution of 1- γ -oxo-pyrenebutyric acid (**3**, 613.0 mg, 2.03 mmol), EDC (346.6 mg, 2.23 mmol) and HOBT (301.3 mg, 2.23 mmol) in anhydrous DMF (10 mL) was



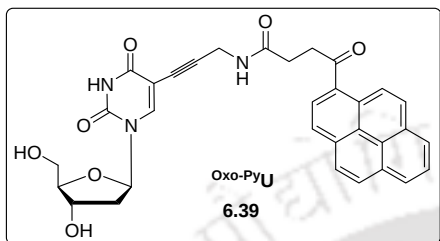
stirred at room temperature. After 15 min, propargylamine (0.142 mL, 2.23 mmol) and *i*-Pr₂NEt (387 μ L, 2.23 mmol) were added, and the mixture was stirred at room temperature for 6 h. The resulting mixture was concentrated

in vacuo and diluted with ethyl acetate. This solution was washed with sat. NH₄Cl solution and brine, dried over Na₂SO₄, filtered and evaporated. The crude product was purified by silica gel column chromatography (chloroform–methanol = 30:1) to yield **6.38** (585 mg, 85%) as a yellow solid: ¹H NMR (CDCl₃, 400 MHz) δ = 2.25 (1H, t, *J* = 2.6 Hz), 2.80 (2H, t, *J* = 6.5 Hz), 3.60 (2H, t, *J* = 6.5 Hz), 4.12 (2H, dd, *J* = 5.2, 2.6 Hz), 6.18 (1H, br), 8.06–8.02 (2H, m), 8.24–8.13 (5H, m), 8.40 (1H, d, *J* = 8.0 Hz), 8.92 (1H, d, *J* = 9.4 Hz); ¹³C NMR (CDCl₃, 100 MHz) δ = 29.4, 30.6, 37.4, 71.6, 79.5, 124.0, 124.2, 124.7, 124.9, 126.0, 126.3, 126.3, 126.4, 127.0, 129.4, 129.6, 129.7, 130.4, 131.0, 131.6, 133.9, 171.8, 203.2; FABMS, *m/z* 339 (*M*⁺), HRMS calcd. for C₂₃H₁₇NO₂ (*M*⁺) 339.1259, found 339.1260.

6.9.2.3. 5-[3-(1- γ -oxo-pyrenecarboxamido)propynyl]-2'-deoxyuridine (**6.39**).

To a solution of 5-iodo-2'-deoxyuridine (**6.16**, 95.6 mg, 0.270 mmol), **6.38** (91.6 mg, 0.270 mmol), and triethylamine (41.0 mg, 0.405 mmol) in 3.0 mL of anhydrous DMF, 62.4 mg (0.054 mmol) tetrakis(triphenylphosphine)palladium(0) and 10.3 mg (0.054 mmol) copper(I) iodide were added under nitrogen atmosphere. The mixture was stirred at room temperature for 12 h. The resulting mixture was concentrated in vacuo and the residue was purified by silica gel column chromatography (CHCl₃/MeOH = 20:1) to yield **6.39** (60%, 91.6 mg) as a pale yellow

solid: ^1H NMR (DMSO- d_6 , 400 MHz) δ = 2.12 (2H, br m), 2.50 (2H, t, J = 1.8 Hz), 3.36 (2H, br), 3.49 (1H, m), 3.6 (2H, m), 3.80 (2H, br), 4.13 (1H, br), 4.23 (1H, br),

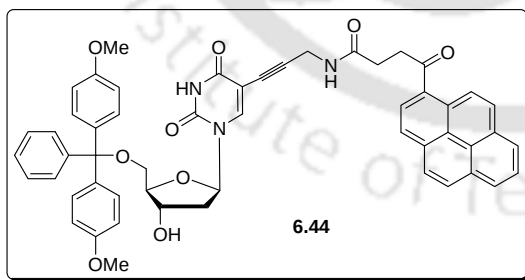


5.26 (2H, d, J = 4.0), 6.12 (1H, m), 8.14 (1H, s), 8.15–8.25 (2H, m), 8.27–8.41 (5H, m), 8.58 (1H, d, J = 8.1 Hz), 8.76 (1H, d, J = 9.4 Hz), 11.68 (1H, d, J = 8.0 Hz); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ = 28.7, 29.5, 37.0,

40.0, 60.9, 70.2, 74.3, 84.6, 87.5, 89.6, 98.1, 123.4, 123.9, 124.4, 124.5, 125.9, 126.3, 126.4, 126.7, 127.1, 128.0, 129.0, 129.2, 129.9, 130.6, 132.7, 132.9, 143.6, 149.4, 161.6, 170.9, 203.5; FABMS, m/z 566 ($[\text{M}+\text{H}]^+$), HRMS calcd for $\text{C}_{32}\text{H}_{27}\text{N}_3\text{O}_7$ ($[\text{M}+\text{H}]^+$) 566.1927, found 566.1929.

6.9.2.4. 5-[3-(1- γ -Oxopyrenecarboxamido) propynyl]-5'- O -(4,4'-dimethoxytrityl)-2'-deoxyuridine (**6.44**)

A solution of **6.39** (85.0 mg, 0.150 mmol), 4-dimethylaminopyridine (catalytic amount), and 4,4'-dimethoxytrityl chloride (56.0 mg, 0.165 mmol) in dry pyridine (3.0 mL) was stirred at room temperature for 16 h. After concentration of the solution to dryness, the residue was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH}$ = 20:1) to yield **6.44** (70%, 91.1 mg) as a pale yellow solid: ^1H NMR (CDCl_3 , 400 MHz) δ = 2.20 (1H, ddd, J = 13.3, 6.3, 3.3 Hz), 2.66 (4H, m), 2.40 (1H,



m), 3.32–3.21 (1H, m), 3.36 (2H, t, J = 6.8 Hz), 3.663 (3H, s), 3.88 (1H, t, J = 5.1 Hz), 4.03 (1H, m), 4.45 (1H, t, J = 2.8 Hz), 5.83 (2H, br), 6.27 (1H, t, J = 6.6 Hz), 6.75 (4H, dd, J = 8.9, 3.7 Hz), 7.27–7.19 (9H, m), 7.36 (3H, m),

7.96–7.92 (3H, m), 8.06–8.02 (3H, m), 8.10 (1H, s), 8.14–8.12 (2H, m), 8.27 (1H, d, J = 8.1), 8.80 (1H, d, J = 9.4); ^{13}C NMR (CDCl_3 , 100 MHz) δ = 10.2, 30.1, 30.2, 37.3, 41.7, 45.8, 55.2, 63.6, 70.5, 71.9, 74.6, 77.3, 85.7, 86.5, 86.9, 89.0, 99.4, 113.3, 124.0, 124.7, 125.9, 126.2, 126.3, 126.4, 127.0, 127.9, 128.0, 129.2, 129.4, 129.5, 129.9,

130.0, 130.4, 131.0, 131.8, 135.5, 143.0, 144.6, 149.9, 158.5, 158.5, 163.0, 171.4, 203.0, 203.2; FABMS, m/z 868 (M^+), HRMS calcd. for $C_{53}H_{45}N_3O_9$ (M^+) 867.3155, found 867.3159.

6.9.2.5. 5-[3-(1- γ -oxo-pyrenecarboxamido)propynyl]-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyuridine 3'-*O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (6.45)

To a solution of **6.44** (52.0 mg, 0.060 mmol) and 1H-tetrazole (5.0 mg, 0.078 mmol) in anhydrous CH_3CN , 24.8 μL (0.078 mmol) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite was added under nitrogen atmosphere. The mixture was stirred at room temperature for 1-2 h. Then the mixture was filtered off and used for oligodeoxynucleotide synthesis without further purification.

6.9.3. UV-Visible and Fluorescence Study of the Nucleoside

The UV-visible spectra of the nucleoside (**6.39**, 10 μM) in different solvents were recorded using Shimadzu UV-2550 UV-visible spectrophotometer at 298 K using 1 cm path length cell. The Fluorescence spectra of the nucleoside (**6.39**, 10 μM) in different solvents were obtained using a Shimadzu RF-5300PC spectrophotometer at 298 K using 1 cm path length cell. All the solvents used in the solution preparation are of spectroscopic grade, purchase from Spectrochem, India. The fluorescent nucleoside was recrystallized twice from chloroform-methanol solvent mixture, dried and used for photophysical study. The fluorescence quantum yields (Φ) were determined using 9,10-diphenylanthracene as a reference with the known Φ (0.95) in ethanol.²⁷

6.9.4. ODNs Synthesis and Characterization

All the reagents for DNA synthesis were purchased from Glen Research. ODNs were synthesized by a conventional phosphoramidite method by using an Applied Biosystems 3400 DNA/RNA synthesizer. Synthesized ODNs were purified by reverse phase HPLC on a 5-ODS-H column (10 $\times$ 150 mm) Elution was done with 50 mM

ammonium formate buffer (AF), pH 7.0, with a linear gradient over 45 min from 3% to 40% acetonitrile at a flow rate of 2.0 ml/min. The ODNs purified by HPLC were characterized by MALDI-TOF MS (**Table 6.7**).

Table 6.7: Synthesized fluorescently labeled ODNs and their MALDI-TOF MS.

ODNs	Sequences	MALDI-TOF mass calcd. $[M+H]^+$	MALDI-TOF mass found $[M+H]^+$
1	5'-d(CGCAAC ^{Oxo-Py} U CAACGC)-3'	3957.68	3956.97
2	5'-d(CGCAAT ^{Oxo-Py} U TAACGC)-3'	3927.66	3928.47
3	5'-d(CGCAAT ^{Oxo-Py} U ^{Oxo-Py} PyUTAACGC)-3'	4185.93	4186.72

6.9.5. Concentration Determination of the ODNs

The concentration of the ODNs was determined by enzymatic degradation method and analyzed by HPLC in order to verify their compositions. Thus, ODNs containing modified nucleotides were fully digested with calf intestine alkaline phosphatase (50 U/ml), and P1 nuclease (50 U/ml) at 37 °C for 12 h. Digested solutions were analyzed by HPLC on a CHEMCOBOND 5-ODS-H column (4.6×150 mm, elution with a solvent mixture of 50 mM ammonium formate (AF) buffer, pH 7.0, linear gradient over 20 min from 0% to 20% acetonitrile at a flow rate of 1.0 mL/min. The concentration of each DNA was determined by comparing a given peak area with those of 0.1 mM standard solution containing dA, dC, dG, and dT.

6.9.6. UV-Visible and Fluorescence Study with ODNs

All the solutions were prepared in MQ water. The UV-visible and fluorescence spectra of the ss-ODNs and duplex ODNs (fluorescently labeled ODNs and complementary ODNs) were obtained by Shimadzu UV-2550 UV-visible and Shimadzu RF-5300PC fluorescence spectrophotometer respectively at 298 K using 1 cm path length cell. All the UV-visible and fluorescence spectra of the ss-ODNs

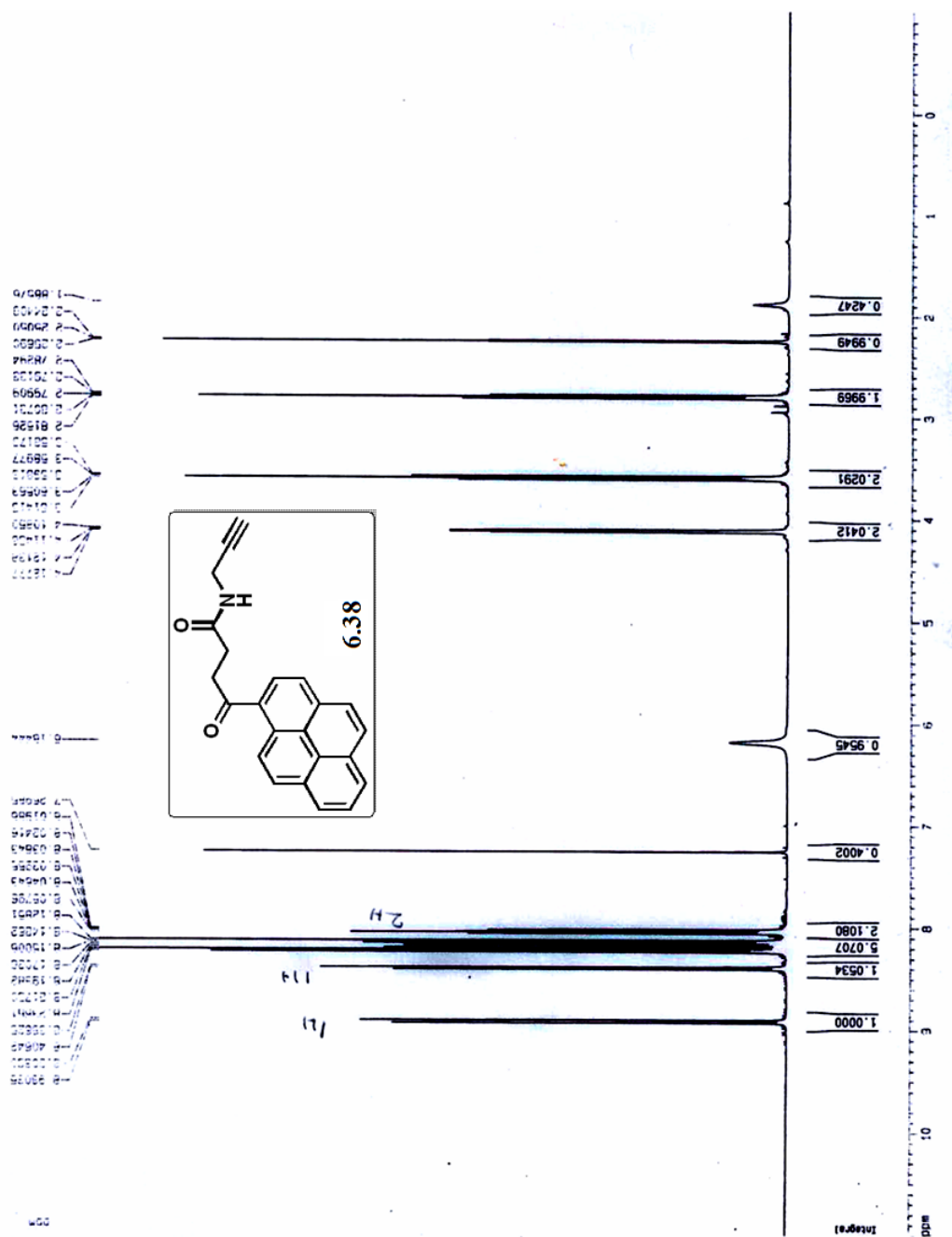
(2.5 μM , final concentration) and duplex ODNs (2.5 μM , final concentration) were measured in 50 mM sodium phosphate buffers (pH 7.0) containing 100 mM sodium chloride. For fluorescence study the excitation bandwidth was 1.5 nm and the emission bandwidth was 1.5 nm. The fluorescence quantum yields (Φ) were determined using 9,10-diphenylanthracene as a reference with the known Φ (0.95) in ethanol.²⁷

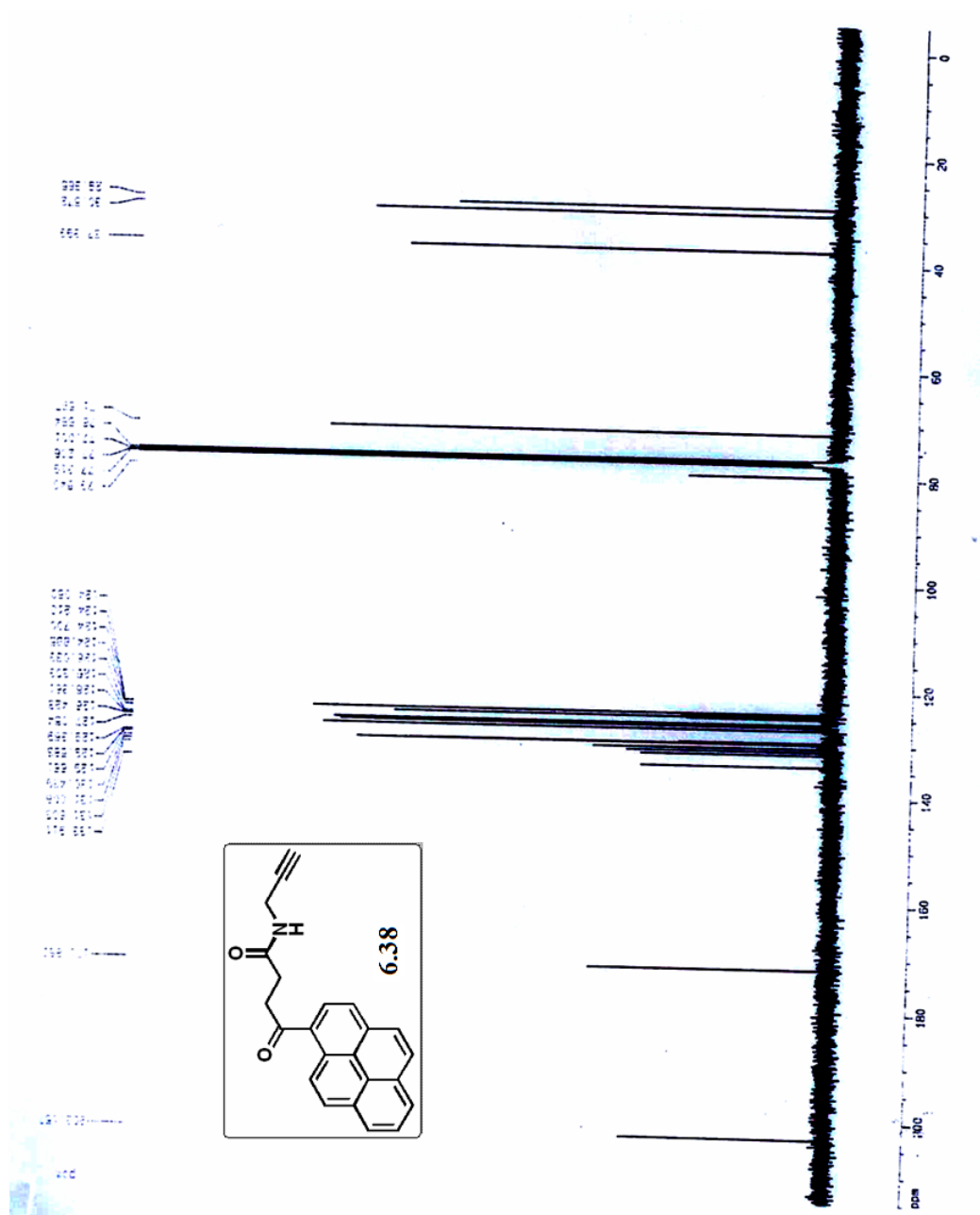
6.9.7. Melting temperature (T_m) measurements

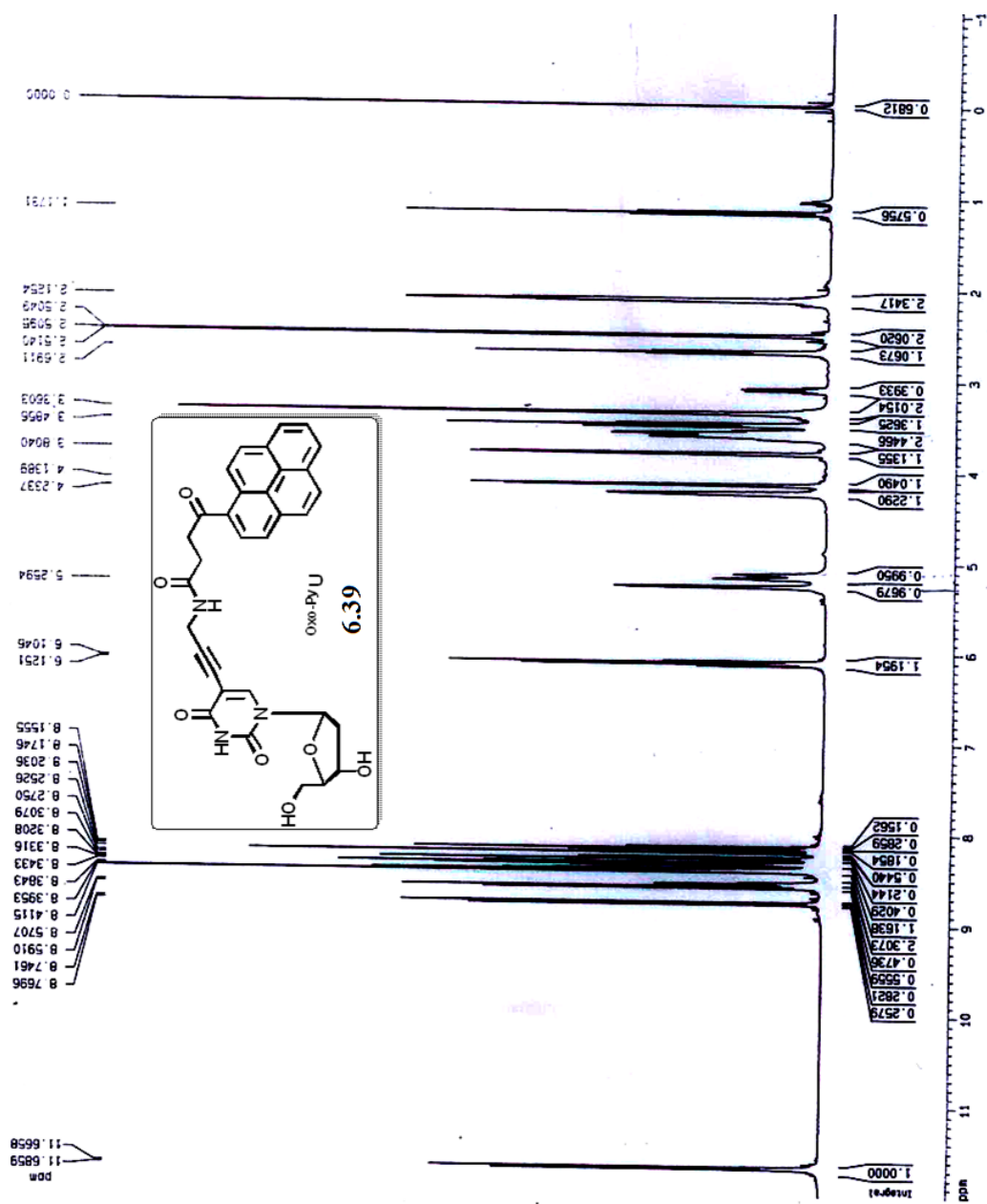
All melting temperatures (T_m s) of the ODNs (2.5 μM , final duplex concentration) were measured in 50 mM sodium phosphate buffers (pH 7.0) containing 100 mM sodium chloride. Absorbance vs. temperature profiles were measured at 260 nm using a Shimadzu UV-2550 spectrophotometer equipped with a peltier temperature controller using 1 cm path length cell. The absorbance of the samples was monitored at 260 nm from 4 to 90 $^{\circ}\text{C}$ with a heating rate of 1 $^{\circ}\text{C}/\text{min}$. From these profiles, first derivatives were calculated to determine T_m values.

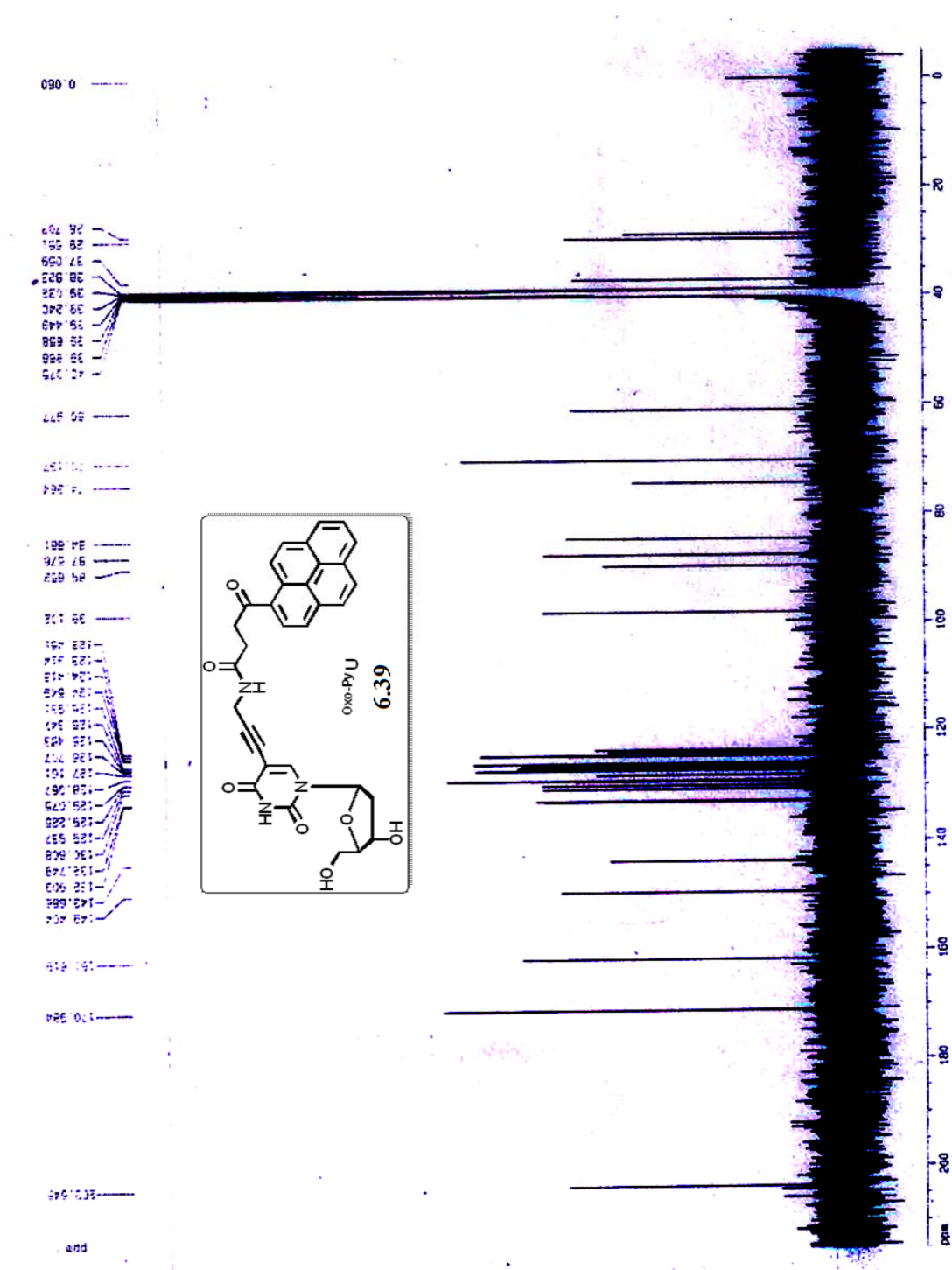
6.9.8. Theoretical Study

Molecular modeling study of the duplexes (fluorescently labeled ODNs and the complementary ODNs) were carried out by MacroModel (Maestro vs. 9.0) using AMBER* force field in water.³⁰









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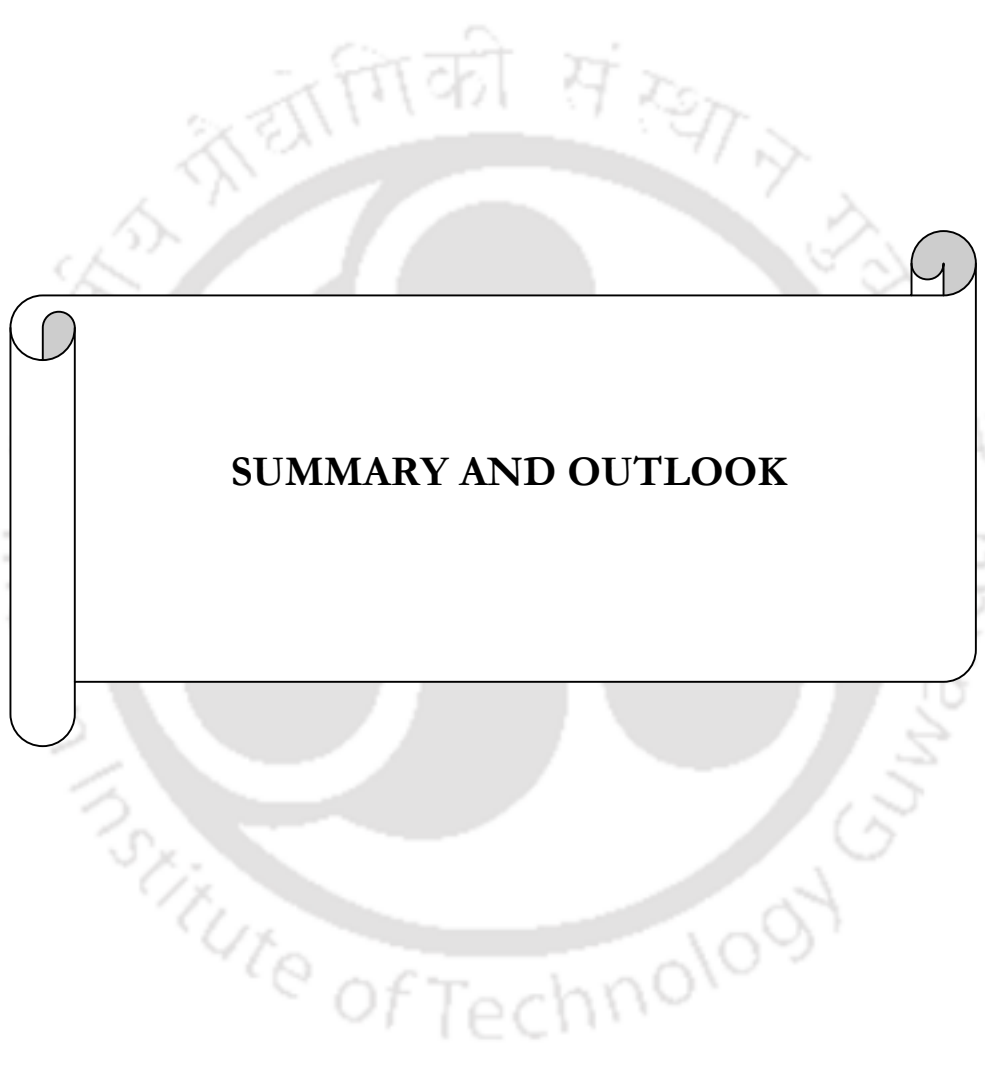
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SUMMARY AND OUTLOOK

Summary and Outlook

Major findings and the future outlook of the **present investigations** described in this dissertation have been summarized below:

In this dissertation we have shown the use of two important reactions, click-chemistry and Sonogashira coupling to the synthesis of fluorescent small organic molecules and fluorescent nucleoside.

Thus in **Chapter 2**, we have introduced the use of click chemistry to the synthesis of triazolyl based donor-acceptor (D-T-A or A-D-T) fluorescent small organic molecules. The synthesized fluorophores show different solvatochromicity and/or intramolecular charge transfer (ICT). We observed that some of the synthesized fluorophores showed purely ICT character while emission from some of them arose from LE state. However, one of the fluorophore showed both ICT and LE emission. As the ICT to LE intensity ratio can be employed as a sensing index, the dual emissive fluorophore can be utilized in designing molecular recognition systems. The synthesized fluorophore can be incorporated into DNA for biophysical study for investigation inter-biomolecular interaction. Thus, our investigation on the photophysical properties of click chemistry derived fluorophores is of immense importance for the development of new fluorophores with predetermined photophysical properties that may find a wide range of applications in chemistry, biology, or material sciences.

Chapter 3 described the development of a click-reagent version of Sonogashira coupling methodology to the synthesis of alkynyl conjugated fluorescent small molecules. This methodology is high yielding, reducing the alkyne-alkyne homocoupling, tolerant to a variety of substrates and functional groups, compatible with green solvents, and air-insensitive. Our developed protocol is also air-insensitive and effective under microwave conditions. Thus, we hope this methodology will find widespread applications in chemistry, material science, and chemical biology for the synthesis of desirable conjugated fluorescent alkynes. In a recent article toward the

total synthesis of (–)-Quinocarcin (Hiroaki Ohno *et al. Angew. Chem. Int. Ed.* **2012**, *51*, 9169 –9172) our methodology was used in one of the crucial step. The authors quoted that “...the use of CuSO₄/sodium ascorbate was vital to prevent the generation of undesired homocoupling products of the terminal alkyne...” This and few more citations prove the impact of our methodology. We hope that this methodology will have great impact on the synthetic chemistry. Moreover, we showed that our synthesized fluorophores are highly solvatochromic as is revealed from the preliminary photophysical studies and can be incorporated into DNA for biophysical study for investigation inter-biomolecular interaction. Thus, the fluorophores synthesized applying our methodology might find applications in chemistry, biology, and material sciences.

In **Chapter 4** we have shown the studies on the interaction of biomolecular microenvironment with our synthesized fluorophore, triazolylpyrene (^{TNDMB}Py, **Chapter 2**). In particular, we have exploited the interesting emissive property of triazolyl pyrene to study its interaction with (a) Calf Thymus DNA (ct-DNA), and (b) Bovin Serum Albumin (BSA) spectroscopically. The probe bound efficiently to the minor groove of ct-DNA and the hydrophobic pocket of BSA protein with the generation enhanced fluorescence signal. Furthermore, because the groove binding event in DNA and strong interaction with BSA are important in consideration of drug-DNA/drug-protein interaction, our probe might find application in studying such events in pharmaceutical sciences. The probe can also be used as label-free fluorescent light up sensing and discrimination of DNA base mismatches which is reflected in our published work. Thus, the probe would be very useful and might find future applications for the detection of and targeting DNA lesions with a less laborious and cost-effective label-free way and also the sensing of G-quadruplex DNA.

Chapter 5 described the fluorimetric sensing of micellar microenvironment and Cu²⁺ ion with smart fluorescent probe, triazolylpyrene (^{TNDMB}Py). Thus, our probe might find application in probing of membrane in biological systems which

expectedly would generate a distinct fluorescence signal for such interaction events and shed light to the environment around it inside the cell. The successful use of triazolylpyrene as a Cu^{2+} ion selective probe with low detection limit of 177 nM, may open the way for the design of new, efficient, and simple triazole-based fluorescence light-up probe for metal ion detection. We have also shown that the probe is capable of sensing Cu^{2+} ion inside the nano-core of micelle with a change in fluorescence pattern that is selective to the pH of the system. All the results indicate that our probe could be used as an efficient Cu^{2+} selective fluorescence off-on probe in organic and aqueous solvents, and may find applications for sensing other heavy metal ions and Cu^{2+} ion in biological system.

Finally, **Chapter 6** introduced the successful synthesis of a highly solvatochromic fluorescently labelled nucleoside (Oxo-PyU) via classical Sonogashira coupling. Further, we have incorporated the fluorescently labeled nucleoside into short oligonucleotide sequences and generated fluorescently labeled oligonucleotide probes containing singly and doubly Oxo-PyU label and investigated their fluorescence emission properties in presence of complementary target ODNs. Our investigations on the fluorescence emission properties of the fluorescent DNA probes containing singly and doubly labelled Oxo-PyU in the presence of various complementary strands, revealed that the fluorescence of Oxo-PyU is strongly quenched by neighbouring G-base and hence can be applied for designing quencher free molecular beacon. We have also shown that the probes are highly specific for the detection of matched base A of a target DNA opposite to the labelled base of probe DNA *via* the enhancement of fluorescence signal. Furthermore, the doubly labelled probe is also capable for the detection of opposite matched consecutive AA bases of target ODN with a strong fluorescence signal generation, high signal to noise ratio and a large fluorescence brightness factor. Therefore, the doubly labelled ODN which exhibits unique fluorescence property depending on the number of adenines on the complementary strand, would be effective for the detection of consecutive –AA– sequences located at a specific site of the target DNA. The singly and doubly labelled probes also could be

useful for detection of A/G or AA/GG allele, respectively as revealed from their fluorescence behaviour. Moreover, incorporation of this probe into a long T-base sequence could be done for possible future use in DNA ink technology.



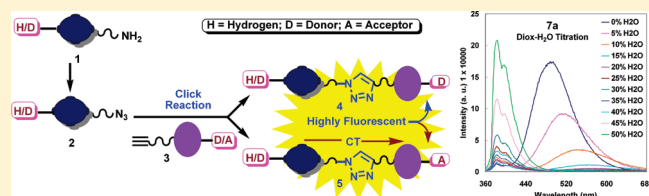
Installation/Modulation of the Emission Response via Click Reaction

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Supporting Information

ABSTRACT: We have demonstrated the installation of a fluorescence property into a nonfluorescent precursor and modulation of an emission response of a pyrene fluorophore via click reaction. The synthesized fluorophores show different solvatochromicity and/or intramolecular charge transfer (ICT) feature as is revealed from the UV–visible, fluorescence photophysical properties of these fluorophores, and DFT/TDDFT calculation. We observed that some of the synthesized fluorophores showed purely ICT character while emission from some of them arose from the LE state. A structureless and solvent polarity-sensitive dual emission behavior was observed for one of the triazolyipyrene fluorophores that contains an electron-donating $-NMe_2$ substituent (fluorophore, **7a**). Conversely, triazolyipyrene with an electron-withdrawing $-CN$ group (fluorophore, **7b**) showed a solvent polarity-independent vibronic emission. The effect of ICT on the photophysical properties of these fluorophores was studied by fluorescence emission spectra and DFT/TDDFT calculations. Fluorescence lifetimes were also measured in different solvents. All of our findings revealed the delicate interplay of structure and emission properties and thus having broader general utility. As the CT to LE intensity ratio can be employed as a sensing index, the dual emissive fluorophore can be utilized in designing the molecular recognition system too. We envisage that our investigation is of importance for the development of new fluorophores with predetermined photophysical properties that may find a wide range of applications in chemistry, biology, and material sciences.



INTRODUCTION

Fluorescent organic molecules have been well-known for a long time for probing biomolecular structure, properties, and functions.¹ However, the photophysical properties of many of the fluorophores sometimes could not be interrelated with their structure due to the deficiency of distinct rationale for their design. Moreover, many of the fluorophores are neither long wavelength emissive nor solvatochromic and hence, disabling their use in cell or in sensing the change in microenvironment in the biological world. Therefore, the design of microenvironment sensitive as well as long wavelength emissive organic fluorophores with a predictable photophysical property in relation to their designed structures via a simple and versatile method is highly desirable for such biochemical applications.¹ The excellent regioselectivity, simplicity along with the versatility of the reaction conditions, render “click chemistry” advantageous for synthesizing small organic fluorescent molecules and for achieving the selective coupling of small fluorogenic molecules within a complex biological environment, which enhances greatly the efficiency of biological studies aimed at understanding the natural biological processes via a signal generation.^{1,2}

Only recently, have a few numbers of click fluorescent molecules been reported wherein the intrinsic fluorescence property of the well-explored fluorophores is tuned.^{1c,3} However, the research efforts toward the installation of the fluorescence property into a nonfluorescent precursor and/or the modulation of the emission response of a weakly fluorescent molecule via “click chemistry” and thereby the synthesis of new solvatochromic click-fluorophores has

been given limited attention. Here, we want to report our design toward the installation of an emission property into a nonfluorescent precursor on one hand and the modulation/enhancement of an emission response of a weakly fluorescent molecule via click chemistry on the other hand (Figure 1). The main importance of our work is that we utilized readily available nonfluorescent starting materials of very low cost and installed an emission property via triazole formation. The synthesized fluorophores are solvofluorochromic and thus can be used for applications in chemistry, material science, and other related areas of interest.^{1e,2b,3}

RESULTS AND DISCUSSION

In our design donor and acceptor units are linked via a triazole moiety so as to allow a charge transfer process that leads to an installation of a fluorescence emission onto a nonfluorescent precursor (Figure 1). We have also focused on the design of a triazoly-linked intrinsic fluorophore for modulation of its emission response. For this purpose we have chosen a versatile fluorophore, pyrene, which shows featured excimer/monomer emission and long fluorescence lifetime. However, the pyrene fluorophore suffers from short emission wavelength, which makes it difficult to use for applications such as in vivo fluorescence imaging. Therefore, preparation of long wavelength emissive and solvatochromic pyrene derivatives is of immense importance.^{4–6} Thus, we have designed pyrene-based

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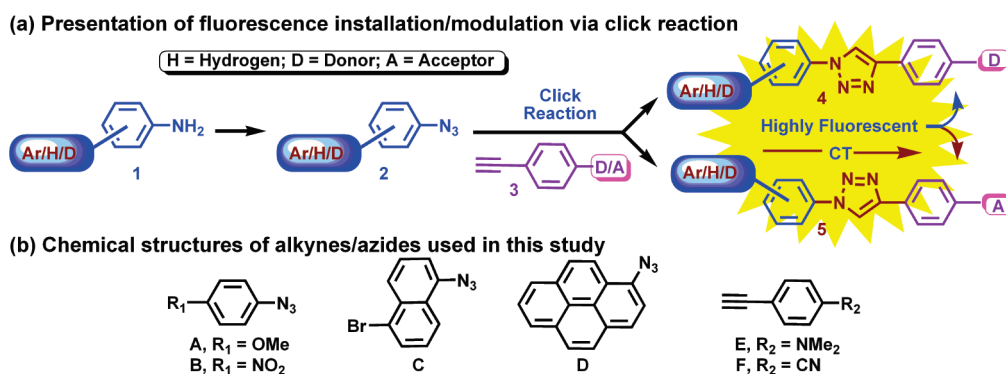
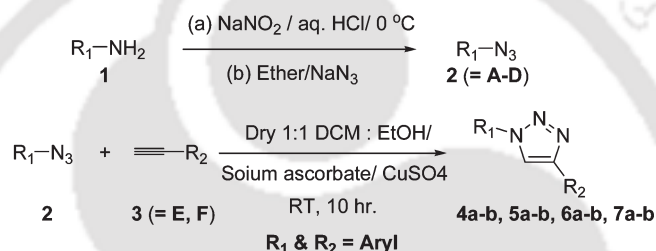


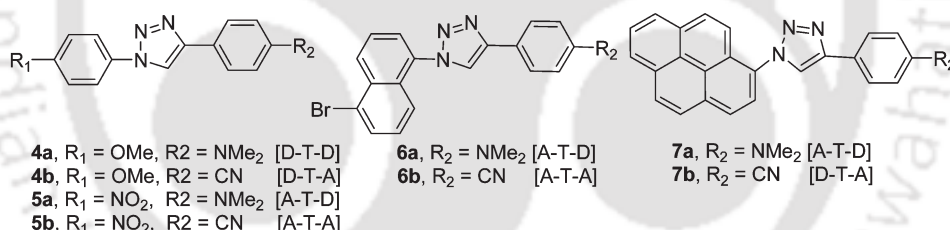
Figure 1. (a) Schematic presentation of the fluorescence installation/modulation via click reaction and (b) the chemical structures of the azides and alkynes used in this study.

Scheme 1. (a) General Procedure for the Synthesis of Aromatic Azides and Click Fluorophores and (b) Chemical Structures of the Synthesized Fluorophores

(a) General procedure for the synthesis of aromatic azides and click fluorophores



(b) Chemical structures of the synthesized click fluorophores



fluorophores keeping in mind that the electron-donating property of the triazolyl ring may effectively modulate the emission response of an electronically coupled pyrene (Figure 1).

Synthesis. The synthesis was accomplished (Scheme 1) via a modified click reaction protocol² and the products obtained in moderate to high yields were characterized well by NMR, mass, and in some cases single crystal X-ray analysis. Thus, to a 1:1 dichloromethane:ethanol solution of aryl azides (A–D, Figure 1; synthesized via a diazotization reaction of the corresponding aryl amines and subsequent treatment with sodium azide), 1.5 equiv of alkyne (E and F, Figure 1) was added. After 5 min of degassing with N₂ and stirring, a solution of sodium ascorbate (6 mol %) followed by a solution of CuSO₄ in water (1 mol %) was added to the reaction mixture. After completion of the reaction monitored by TLC, the reaction mixture was evaporated to dryness, partitioned between water and ethyl acetate. The organic layer was washed with water and brine, then dried over Na₂SO₄. The final product was obtained in purity by column chromatography and characterized. While compounds 4a, b (Figures 2, 3), 5a, b (see SI, Figure S9, S10), and 6a, b (see SI, Figure S11, and Figure 4, respectively) are designed to show an installation of a fluorescence response into

nonfluorescent precursor amines or alkynes, compounds 7a,b (Figure 5, 6) have been synthesized to show the modulation of an emission response of pyrene (Figure 1 and Scheme 1).

Studies of Photophysical Properties. Having all the products in hand, we have studied, first, their photophysical properties in different solvents of varying dielectric constants. The aim of this study is to explore the effect of solvent polarity on the fluorophores' photophysical properties and to correlate these effects to their structure.

Whereas solvent polarity has only a minor influence on the absorptive properties of almost all the fluorophores, it drastically affects their emission properties. We have observed that all the products showed an increase in fluorescence intensity over their parent amine or the alkyne precursors. It has also been found that the responses of some of the products are highly sensitive toward a solvent's dielectric constant leading to a red-shifted emission. In general, the fluorophore of the type D(donor)-T(triazole)-A(acceptor) wherein the precursor amine and alkyne contain a donor –OMe group and an acceptor –CN group, respectively, as in the case of 4b, showed a structureless emission with a red shift (Figure 3b). Also, the quantum yield is increased as the solvent

polarity is increased (Table 1). These results infer an intramolecular charge transfer (ICT) feature of the lowest-lying excited state,⁷ which is supported by a solvatochromicity test (Figure 3c).

Table 1. Fluorescence Photophysical Properties of the Fluorophores^a

entry	solvents	Φ_f	τ_1 [ns]	τ_2 [ns]	$\langle\tau\rangle$ [ns]	k_f [10 ⁸ s ⁻¹]	k_{nr} [10 ⁸ s ⁻¹]
4a	dioxane	0.052	0.78	2.71	0.847	0.79	11.01
	DMSO	0.308	0.41		0.41	7.5	17
	EtOH	0.093	7.08		7.08	0.13	1.28
	15% H ₂ O	0.094	8.26		8.26	0.11	1.88
4b	dioxane	0.010	0.04	3.06	0.06	1.63	165.0
	DMSO	0.619	3.06		3.06	2.02	1.24
	MeOH	0.46	1.75		1.75	2.6	3.08
	50% H ₂ O	0.623	3.45		3.45	1.8	1.09
7a	hexane	0.180	0.81	2.50	0.986	1.82	8.31
	toluene	0.202	1.59	3.26	2.463	0.82	3.23
	dioxane	0.076	2.95	6.57	3.256	0.23	2.83
	EtOAc	0.022	3.87		3.87	0.13	2.44
	CHCl ₃	0.059	2.62		2.62	0.40	3.40
	MeOH	0.003	1.87	14.30	8.988	0.003	1.1
	10% H ₂ O	0.019	1.70	5.47	2.285	0.09	4.28
7b	hexane	0.071	1.40	8.71	6.655	0.10	1.39
	DMSO	0.39	16.49		16.49	0.23	0.37
	MeOH	0.13	14.99		14.99	0.08	0.58

^a Excitation at λ_{ex} nm and recorded at λ_{max} . $\langle\tau\rangle$, k_f and k_{nr} are weighted means from the biexponential fits: $\langle\tau\rangle = 1/(\alpha_1/\tau_1 + \alpha_2/\tau_2)$, $k_f = \Phi_f/\langle\tau\rangle$, and $k_{nr} = (1 - \Phi_f)/\langle\tau\rangle$.^{7c,g}

Table 2. The Dipole Moment and Excitation Energy Values of 4a, 4b, and 7a

$\Delta\mu$ in debye	4a ^a	4b ^a	7a ^a
$\Delta\mu^b = (\mu_e - \mu_g)$	24	21	21
excitation energy (calcd) in eV	4.55	4.14	3.54
excitation energy (exptl) in eV	4.27	4.35	3.61

^a Onsager radii for 4a, 4b, and 7a are 6.6, 7.2, and 6.3 Å, respectively (obtained from B3LYP/6-31G* optimized geometry). ^b Calculated from eq 2 and slope from the plots of $\Delta\tilde{\nu}$ values against Δf .

We have observed structureless, broad, and strong solvent polarity dependent dominant emission from the fluorophore 4a (a D-T-D system) (Figure 2b). Thus, 4a showed a 122 nm red-shifted emission when compared between the solvent hexane (almost zero intensity) and MeOH. Upon titration of a dioxane solution of 4a by water a red shift of 129 nm was observed in 50% water (Figure 2c). These features indicate an emission from an ICT state.^{7,8} Fluorescence quantum yields are also found to be increased with the increase in solvent polarity (Table 1). This clearly indicates a highly polar emissive state. However, it is decreased in MeOH and ethanol, which may be due to a protic solvent–solute interaction.^{7a,9}

Fluorophore 6b showed a comparatively less solvent polarity sensitive emission that is centered at around 406 nm. A spectral shift of only 23 nm is observed with a decrease in intensity as we move from hexane to MeOH. This result is clearly an indication of an ICT emission (Figure 4).

Compounds 5a,b and 6a showed only faint emission under the condition used for others. However, at large slit width, the following behaviors were observed where quantum yields could not be calculated. Thus, fluorophore 5b (A-T-A system) showed a solvent polarity independent structured emission (see SI, Figure S10). A highly solvent polarity dependent emission was exhibited by fluorophore 5a, an A-T-D system. Thus, as the solvent polarity increased, a shift of 56 nm from cyclohexane to MeOH with decreased intensity was observed, which is characteristic of ICT emission (see the SI, Figure S9). Fluorophore 6a exhibited a dual behavior similar to that of 7a in low polar solvents, but a single emission centered at 357 nm appeared in high polar solvents like ACN, DMSO, and MeOH. In low polar solvents like hexane, cyclohexane, ether, 1,4-dioxane, toluene, chloroform, and ethyl acetate, the second band at 399 nm in hexane shifted gradually with a decrease in intensity as the polarity increases and reaches 537 nm in ethyl acetate, which again inferred an emission from ICT state (see the SI, Figure S11).

We have observed an interesting fluorescence photophysical property exhibited by the fluorophore 7a (an A-T-D system), a triazolyl modified pyrene. Thus, the fluorophore 7a showed a strong structureless emission centered at 415 nm in hexane and cyclohexane. However, in ether, dioxane, toluene, CHCl₃, and EtOAc, a dual emission behavior has been observed with a strong red-shifted pattern of the ICT band as the solvent polarity is increased from toluene (466 nm) to EtOAc (544 nm) (Figure 5b).

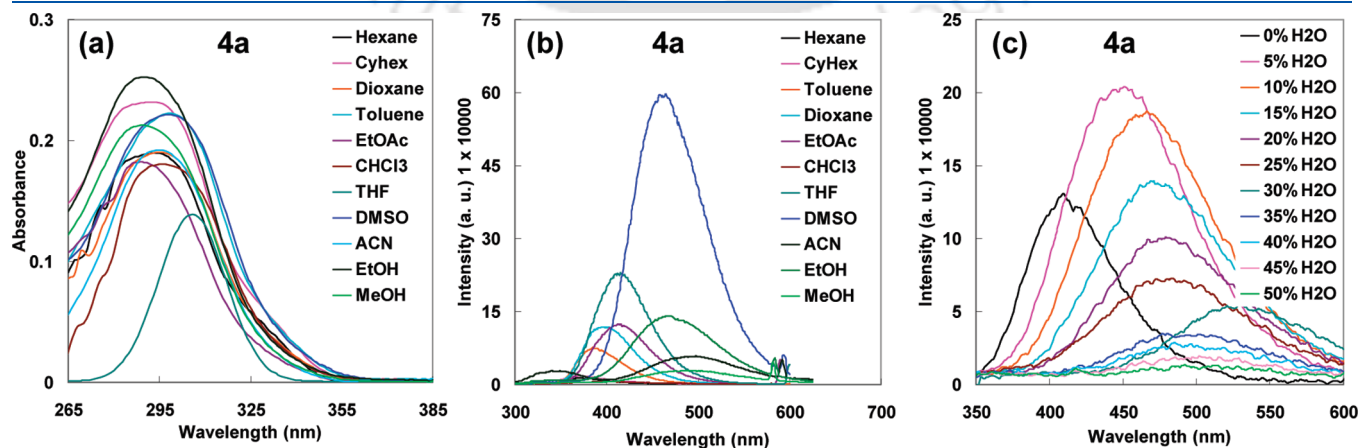


Figure 2. UV–visible (a) and fluorescence spectra (b) of 4a in different solvents and fluorescence in dioxane–water solvent system (c) (10 μ M, λ_{ex} = λ_{max} of each solvent).

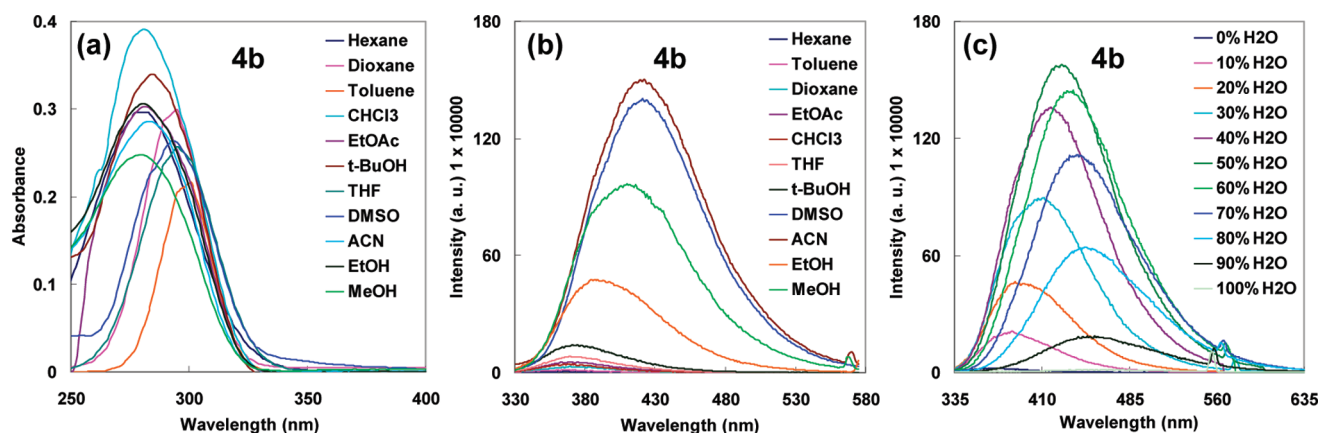


Figure 3. UV–visible (a) and fluorescence spectra (b) of **4b** in different solvents and fluorescence in dioxane–water solvent system (c) (10 μ M, rt; $\lambda_{\text{ex}} = \lambda_{\text{max}}$ of each solvent).

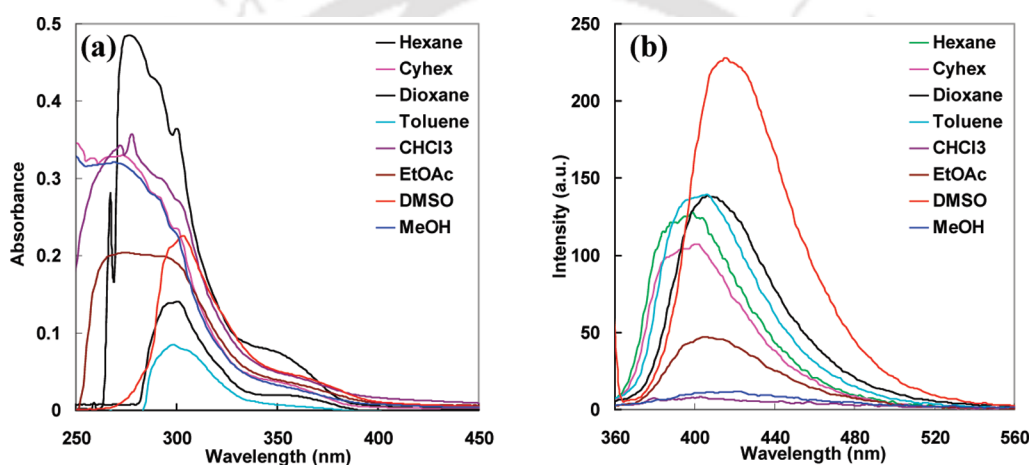


Figure 4. UV–visible (a) and fluorescence spectra (b) of **6b** in different solvents (10 μ M, rt; $\lambda_{\text{ex}} = \lambda_{\text{max}}$ of each solvent).

In highly polar solvents like ACN and MeOH, a structured band characteristic of a pyrene emission with a very low intensity has been observed that is also supported by a solvatochromicity test (Figure 5c). Thus, in pure dioxane, the ICT emission is highly dominated over the LE emission and as the percent of H₂O is increased, the intensity of the LE emission is also going to increase. However, in 50% H₂O in dioxane, we have observed a pure LE emission (Figure 5c). Compared to pyrene-amine, **7a** showed about 100 nm of red-shifted emission in polar solvents. On the other hand, in the case of the fluorophore **7b** (D-T-A system), which contains an electron-withdrawing –CN substituent, the emission is centered at 400 nm, and the vibronic structure of a pyrene emission is maintained, i.e., the emission originates from a purely LE state (Figure 6b).^{7c,10} The fluorescence quantum yield of **7b** is increased as the solvent polarity is increased with no solvatochromicity (see the SI, Table S5) while it is lower in a polar solvent than that in a nonpolar solvent in the case of **7a** (Table 1, and see Table S5 in the SI). These results infer an ICT feature of the lowest-lying excited state in **7a** (with the pyrene as an electron acceptor) but not in **7b**.^{7,8,10}

Therefore, our study clearly shows that a “click” reaction between an azide and an alkyne sufficiently installs a fluorescence emission property into a nonfluorescent precursor and modulates the emission response of a pyrene fluorophore. It is important to note that

these fluorophores show a direct trend between the fluorescence intensities and solvent dielectric constants and thus have a broader general utility (see the SI, Figures S5–12). Examination of the band-shape and fluorescence quantum yields of the fluorophores reveals the delicate interplay of structure and emissive states. Thus, compounds **4a**, **4b**, and **6b** showed a highly emissive, pure ICT state as is indicated by a high quantum yield, broad band-shape, and red-shifted pattern as the solvent polarity is increased. The solvent polarity independent LE emission of **7b** suggests the emitting state as a nonpolar ¹ π – π^* state. The dual emission exhibited by **7a** indicates the presence of a mixed LE and CT state. Switching between these two states depends on the structure and the solvent polarity.^{8,10e}

To get an insight into the different solvatochromic behaviors of **4a**, **4b** and **7a**, **7b**, the spectral dependency on solvent polarity was studied on the basis of the Lipert–Mataga model.¹¹ The Lipert–Mataga polarity parameter (Δf) has been considered as the measure of the polarity of different solvents and solvent mixtures used and was calculated by using the following eq 1.^{11–14}

$$\Delta f = \frac{\epsilon - 1}{2\epsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \quad (1)$$

Thus, the absorption ($\lambda_{\text{max}}^{\text{abs}}$) and fluorescence ($\lambda_{\text{max}}^{\text{fl}}$) maxima of the more intense fluorophores **4a**, **4b** and **7a** were measured in different solvents and solvent mixtures along with the polarity

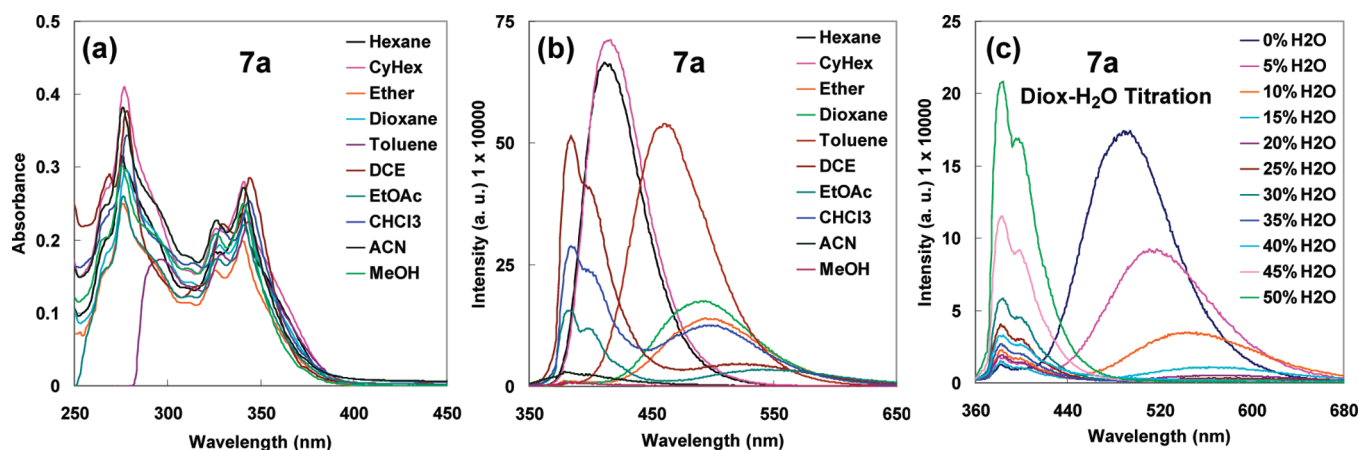


Figure 5. UV–visible (a) and fluorescence spectra (b) of **7a** in different solvents and fluorescence in dioxane–water solvent system (c) (10 μ M, rt; $\lambda_{\text{ex}} = \lambda_{\text{max}}$ of each solvent) showing a dual behavior.

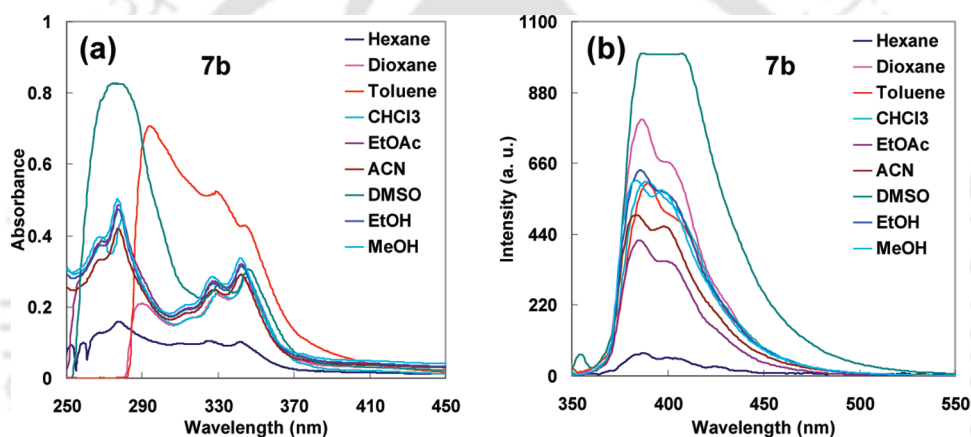


Figure 6. UV–visible (a) and fluorescence spectra (b) of **7b** in different solvents (10 μ M, rt; $\lambda_{\text{ex}} = \lambda_{\text{max}}$ of each solvent).

parameter Δf of the different solvent systems calculated with eq 1 (see the SI, Table S3). As is revealed from the UV–visible and fluorescence spectra (Figures 2 and 3 for **4a,b** and Figures 5 and 6 for **7a,b**, respectively), the $\lambda_{\text{max}}^{\text{fl}}$ values gradually undergo a red shift when the solvent polarity increases for all the fluorophores. However, the red shift of $\lambda_{\text{max}}^{\text{fl}}$ is much larger than the shift in $\lambda_{\text{max}}^{\text{abs}}$ values, suggesting the fluorescent states of all the fluorophores to be more polar in nature than their ground states.^{7c,10g,11b,11c,14b,15–17} In particular, fluorophore **4a,b** showed a slight red shift with Δf in the lower solvent polarity region ($\Delta f < 0.265$) but at the higher solvent polarity region the $\lambda_{\text{max}}^{\text{abs}}$ values showed a small blue shift (see the SI, Table S3). However, as the solutions of **4a,b** in dioxane were titrated with water, a normal red shift was observed for **4a** while a blue shift was the result for fluorophore **4b**. The $\lambda_{\text{max}}^{\text{fl}}$ values gradually undergo a more red shift when the solvent polarity increases for **4a,b**. In the case of fluorophore **7a**, the $\lambda_{\text{max}}^{\text{abs}}$ values showed a very small red shift in low polar solvents and a blue shift in high polar solvents; however, in the dioxane–water solvent system the $\lambda_{\text{max}}^{\text{abs}}$ remains almost unchanged. A similar behavior was observed for fluorophore **7b**. However, the $\lambda_{\text{max}}^{\text{fl}}$ of the ICT band shifted much more to the red for **7a** while an unchanged but increased intensity of the $\lambda_{\text{max}}^{\text{fl}}$ was observed for **7b** as the solvent polarity is increased.

Next, we examined the correlations of the absorption and fluorescence maxima ($\tilde{\nu}_{\text{max}}^{\text{abs}}$ and $\tilde{\nu}_{\text{max}}^{\text{fl}}$, respectively, in cm^{-1}) of

the fluorophore **4a,b** and **7a**, respectively, with the solvent polarity function Δf (Figure 7a–c). Thus, from Figure 7b and c it is seen that for the fluorophore **4b**, and **7a**, the $\tilde{\nu}_{\text{abs}}$ values apparently correlate linearly with Δf , suggesting that the ground state of these fluorophores is moderately polar in nature. For the fluorophore **4a**, a smaller correlation is observed for the $\tilde{\nu}_{\text{abs}}$ vs Δf plot (Figure 7a). However, following the UV–visible spectra (Figure 2a and 3a for **4a** and **4b**, respectively), which is quite similar to that of the **4b**, we expect that the ground state of the **4a** is also of polarity similar to that of the **4b**.

However, as is shown in Figure 7a–c, the $\tilde{\nu}_{\text{fl}}$ values for all the fluorophores show a very good linear correlation with Δf for the whole range of the solvent polarity tested. This observation, therefore, suggests that for all the fluorophores the nature of the fluorescent states remains essentially unchanged in all the solvents used. However, the spectral feature changes significantly due to the solvent-induced modulation. The reasonably high slopes of the $\tilde{\nu}_{\text{fl}}$ vs Δf plots for all the fluorophores further suggest that the fluorescent states of these fluorophores are highly polar in nature, most possibly of intramolecular charge transfer (ICT) character.^{7,8,10,15–17}

In different solvents, the absorption and fluorescence spectra involve the electronic transitions between the same two ground and excited electronic states, and the Stokes' shift ($\Delta\tilde{\nu}$) is expected to follow a linear relation with Δf , as suggested by the Lippert and

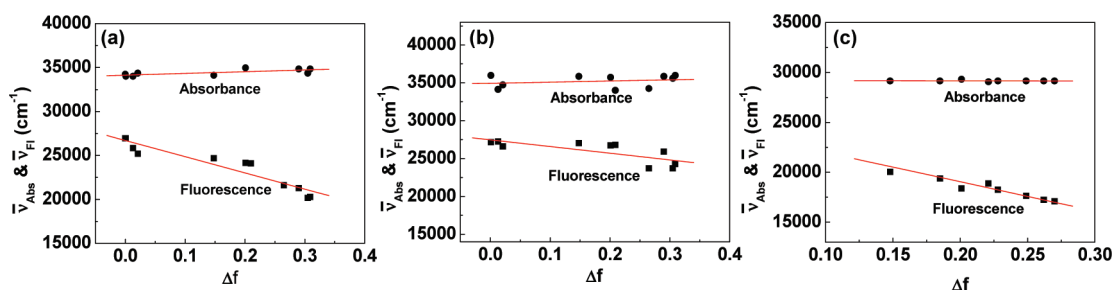


Figure 7. Plots of $\tilde{\nu}_{\text{fl}}$ and $\tilde{\nu}_{\text{abs}}$ values against Δf for fluorophore 4a (a), 4b (b), and 7a (c) ($\tilde{\nu}_{\text{fl(ICT)}}$ and $\tilde{\nu}_{\text{abs}}$ values) in different solvents.

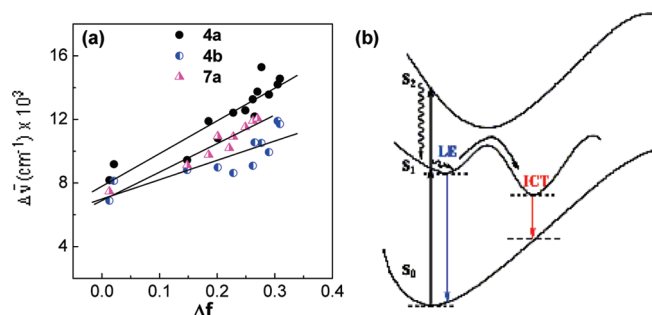


Figure 8. (a) A plot of $\Delta\tilde{\nu}$ vs Δf of 4a, 4b, and 7a, and (b) proposed photophysical process for the dual emission.

Mataga eq 2^{12–14}

$$\Delta\tilde{\nu} = \Delta\tilde{\nu}_0 + \frac{2(\mu_e - \mu_g)^2}{hc^2 r^2} \Delta f \quad (2)$$

where μ_e and μ_g are the excited (fluorescent) state and the ground state dipole moments of the fluorophore, h is Planck's constant, c is the velocity of light, and r is the Onsager radius of the dipole–solvent interaction sphere. The $\Delta\tilde{\nu}$ values for 4a, 4b, and 7a (for the long wavelength emissive band) in different solvents were calculated (in cm^{-1}) (see the SI, Table S2) and the $\Delta\tilde{\nu}$ was plotted against the solvent polarity Δf following eq 2. Thus, it is seen from the plot that the $\Delta\tilde{\nu}$ (Figure 8a) values correlate linearly well with the Δf values for all the solvents studied. Linear correlation with large slopes of the $\Delta\tilde{\nu}$ vs Δf plots suggests that the fluorescence states are highly polar in nature for all the fluorophores. Considering the Onsager radius r values for the fluorophores given in the footnote of Table 2 (obtained from geometry optimized structures at the B3LYP/6-31G* level¹⁸), the $(\mu_e - \mu_g)$ values for 4a, b and 7a are estimated by using the slopes of the respective $\Delta\tilde{\nu}$ vs Δf plots and are found to be about 24.0, 21.0, and 21.0 D, respectively. Substantially high values of $(\mu_e - \mu_g)$ thus obtained from the plot (Figure 8a and Table 2) suggest that the fluorescence states of all these fluorophores are of strong ICT character, which was inferred earlier. The $(\mu_e - \mu_g)$ value for 4a is somewhat higher than that of the 4b and 7a, which indicates a higher ICT character of 4a compared to others. Comparing the spectral behaviors and all the experimental results, it is clear that the ICT state is developed from the LE state in the case of 7a (Figure 8b).

Theoretical Calculation. To investigate the relation between the polarity-dependent emission and the ICT feature, we have carried out TDDFT calculations using the Gaussian 03 program package¹⁸ (see the SI, Figures S16–23 and Table S6). Thus, as revealed from the overlapping of HOMO–LUMO as well as the transition oscillator strength, the electronic transitions of $S_0 \rightarrow S_1$

are fully allowed for all the fluorophores (Figure 9, and in the SI Figures S16–23, and Table S6). This means that reverse transition, i.e., $S_0 \leftarrow S_1$, is also fully allowed indicating the potential fluorophoric nature of the molecules. The emissive state of 7b and 5b is basically a locally excited (LE) state as is suggested by the HOMO–LUMO distributions. For 4b (Figure 9b), 5a, and 6b, however, the emissive state is characterized with more significant electron redistribution, i.e., ICT feature. Calculated results supported our above explanation.¹⁹ Calculated excitation energies for the transition of $S_0 \rightarrow S_1$ of 7b and 5b are 355 and 358 nm (in vacuum), respectively. These values are close to the experimental result of 342 and 278 nm (in hexane), respectively. The excitation energies for the same transition of 4b, 5a, and 6b are 299, 547, and 318 nm (in vacuum), respectively. These values are close to the experimental result of 293 (in toluene), 290 (in dioxane), and 300 nm (in dioxane), respectively. These calculations rationalized the explanation of ICT origin of the solvent polarity dependency of the fluorophores' emission (Figures S17, S18, and S21, respectively, in the SI). Fluorophore 7a and 6a showed dual emission, which again depends on solvent polarity, thus showing an emission from both LE and CT states. The excitation energies for the transition of $S_0 \rightarrow S_1$ of 4a, 7a, and 6a are 330, 447, and 419, respectively, which are closer to the experimental results (experimental: 294, 340, and 292 nm in hexane, respectively). Fluorophore 4a showed a strong ICT emission (Figure 9a) and 7a showed dual emission, which again depends on solvent polarity, thus showing an emission from both LE and CT states. For fluorophore 7a, the $S_0 \rightarrow S_1$ transition is excited at a much longer wavelength of 447 nm (exptl in hexane 340 nm) with an f value of 0.10. The transition $S_0 \rightarrow S_2$ (349 nm, in vacuum) is, however, closer to the experimental value with higher f value ($f = 0.39$) indicating that the S_2 state is populated well (Figure 9c and in the SI, Figure S22).

As revealed from the overlapping of HOMO–LUMO as well as the transition oscillator strength for all the fluorophores except for 6a all the electronic transitions of $S_0 \rightarrow S_1$ are allowed. This means that reverse transition, i.e., $S_0 \leftarrow S_1$, is also fully allowed indicating the potential fluorophoric nature of the molecules. For molecule 6a, however, the $S_0 \rightarrow S_1$ transition is excited at a much longer wavelength of 419 nm with an f value of 0.0651 (see the SI, Figure S20). This small f value indicated the very weakly fluorescent properties of 6a, which was true as we did not see any fluorescence for it under the same condition as for the other fluorophores. For 5a, however, the $S_0 \rightarrow S_1$ transition is excited at a much longer wavelength of 547 nm (2.27 eV, exptl in dioxane 290 nm) with an f value of 0.119 (see the SI, Figure S18).

The emissive state of 7b is basically a locally excited (LE) state as is suggested by the HOMO–LUMO distributions. For 4b and 6b, however, the emissive state is characterized with more

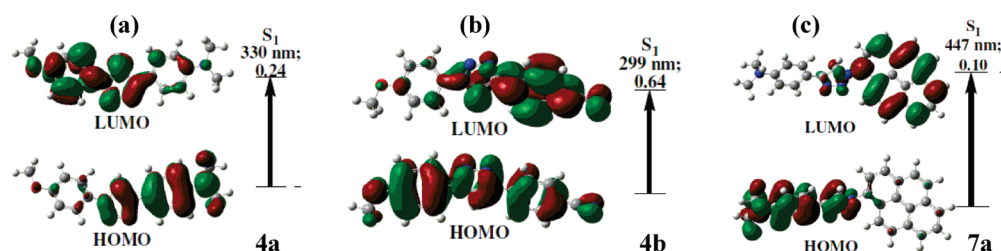


Figure 9. HOMO and LUMO of the representative fluorophores (a) 4a, (b) 4b, and (c) 7a.

Table 3. Fluorescence Lifetime Dependence on Wavelength of 7a

entry	solvents	Φ_f	λ [nm]	τ_1 [ns]	τ_2 [ns]	$\langle\tau\rangle$ [ns]	k_f [10^8 s $^{-1}$]	k_{nr} [10^8 s $^{-1}$]
7a	dioxane	0.001	406	3.78 (29.0%)	2.00 (71.0%)	2.317	0.33	3.98
		0.076	496	2.95 (82.7%)	6.57 (17.3%)	3.256	0.23	2.83
	EtOAc	0.030	385	3.27 (5.9%)	1.27 (94.1%)	1.305	0.39	7.26
		0.022	548	3.87 (100%)		3.87	0.13	2.44
	CHCl ₃	0.058	384	7.20 (100%)		7.20	0.14	1.24
		0.059	498	2.62 (100%)		2.62	0.40	3.40
	10% H ₂ O in dioxane	0.003	401	4.27 (11.7%)	2.56 (88.3%)	2.685	0.08	3.64
		0.019	577	1.70 (63.0%)	5.47 (37.0%)	2.285	0.09	4.28

significant electron redistribution, i.e., ICT feature. Calculated results support our above explanation (see the SI, Table S6).¹⁹ Fluorophore 4a showed a strong ICT emission and 7a showed a dual emission, which again depends on solvent polarity. Therefore, an emission from both the LE and ICT states has been observed in the case of 7a (see the SI, Table S6).

Fluorescence Lifetime Measurement. To interpret the photophysical properties in a more intuitive manner, the fluorescence lifetimes of 4a,b, 6b, and 7a,b were measured in different solvents. Thus, biexponential decay is observed in nonpolar dioxane or hexane and the lifetimes of the major components are less than or closer to 1.0 ns (Table 1). However, as the solvent polarity is increased, lifetimes are also increased. As for example, in dioxane, the major component (68%) of fluorophore 4b is short-lived ($\tau_1 = 0.04$ ns) and its relative contribution is increased to 100% in 1:1 dioxane:water solution ($\tau_1 = 3.45$ ns), which is an indication of a $^1\pi-\pi^*$ state. In protic polar solvent, like methanol, compared to DMSO, the lifetime of 4b is decreased, which may be because of the fluorescence quenching through the nonradiative pathway via hydrogen bonding.^{7a} The relative contribution of the longer lifetime component of 7a ($\tau_2 = 2.5$ ns) in hexane is increased from 27% to 100% as the solvent polarity is increased (in CHCl₃, $\tau_1 = 2.62$ ns). In methanol, the minor component showed a decay time τ_1 of 1.87 ns, for which a weak emission from LE state was observed. No ICT emission has been observed as the decay time of the major component ($\tau_1 = 14.30$ ns) is very large and the fluorophore followed a nonradiative pathway.

Lifetime data were also collected at two emission maxima for 7a in solvents displaying biexponential decays (Table 3). Thus, it has been observed that the values of τ_1 and τ_2 remain consistent, but the relative contributions of the two lifetimes vary according to the observed wavelength. As for example, for 7a, in dioxane, the relative contribution of the longer lifetime τ_1 is increased from 29% to 83% as the observed wavelength is increased from 406 to 496 nm. Similar behavior was observed in 10% water in dioxane solvent. As the intensity of the LE band is increased from dioxane to 10% water in dioxane solution, the lifetime is also increased

from 3.78 to 4.27 ns (monitoring wavelength is the LE band). This observation correlates with the k_{nr} value that is decreased from 3.98 to 3.64 ns. When the monitoring emission wavelength is the ICT band, we have observed that with decrease in intensity, the lifetime is also decreased from 2.95 ns to 1.70 ns, which again correlates with the increased k_{nr} value of 4.28 ns from 2.83 ns.

In conclusion, we have been able to install the fluorescence response into a nonfluorescent precursor and modulate the emission response of a pyrene fluorophore via click reaction. Our findings reveal the delicate interplay of structure and emission properties, thus having a broader general utility. As the ICT to LE intensity ratio can be employed as a sensing index, the dual emissive fluorophore can be utilized in designing molecular recognition systems. Our investigation on the photophysical properties of click chemistry derived fluorophores is of immense importance for the development of new fluorophores with predetermined photophysical properties that may find a wide range of applications in chemistry, biology, or material sciences. Currently, the exploration of the dual fluorescent probe as a metal ion sensor is under investigation.

EXPERIMENTAL SECTION

General Methods. All reactions were carried out under a nitrogen atmosphere in flame-dried glassware, using a nitrogen filled balloon. Organic extracts were dried over anhydrous sodium sulfate. Solvents were removed in a rotary evaporator under reduced pressure. Silica gel (60–120 mesh size) was used for the column chromatography. Reactions were monitored by TLC on silica gel 60 F254 (0.25 mm). ¹H NMR spectra were recorded at 400 MHz and ¹³C NMR spectra were recorded at 100 MHz. Coupling constants (*J* value) were reported in hertz. The chemical shifts were shown in ppm downfield from tetramethylsilane, using residual chloroform ($\delta = 7.24$ in ¹H NMR, $\delta = 77.23$ in ¹³C NMR), dimethyl sulfoxide ($\delta = 2.48$ in ¹H NMR, $\delta = 39.5$ in ¹³C NMR), as an internal standard. Mass spectra were recorded with an HR mass spectrometer and data analyzed by using the built-in software. IR spectra were recorded in KBr or neat on a FT-IR spectrometer.

General Procedure for the Preparation of Aryl Azides. An ice cold solution of sodium nitrite (3 equiv) in water was added dropwise

to a cold solution of aryl amine **1** (1 equiv) in water and concentrated hydrochloric acid at 0 °C over 7–10 min. The reaction mixture was slowly stirred for 1–2 min before an ice cold solution of sodium azide (6 equiv) in water was added dropwise at 0 °C over 10 min. The mixture was stirred for 15 min. The resulting mixture was extracted with ether. The organic layer was washed with water, followed by a brine solution, dried over anhydrous Na₂SO₄, and then concentrated to yield pure aryl azides **2** (average yield 60–90%). Formation of the product azide was confirmed from IR study (1-azido-4-methoxybenzene (A): yield 60%; IR: 2105 cm⁻¹; 1-azido-4-nitrobenzene (B): yield 62%; IR 2126 cm⁻¹; 1-azido-5-bromonaphthalene (C): yield 89%; IR 2134 cm⁻¹; 1-azido-pyrene (D): yield 75%; IR 2133 cm⁻¹) and the produced azides were then immediately used for the next step without further purification.

General Procedure for the [3 + 2] Cycloaddition of Azides and Terminal Alkynes. In a round-bottomed flask fitted with a septum, azide **2** (1.0 equiv) was dissolved by dry 1:1 dichloromethane and ethanol and degassed for 5 min with N₂. Then, alkyne **3** (1.5 equiv) was added and both the stirring and degassing were continued for the next 5 min. After that a 6 mol % sodium ascorbate was added. The reaction mixture was stirred for 5 min before 1 mol % powdered CuSO₄ was added. Degassing was continued for the next 10 min. The reaction was allowed to proceed to room temperature and monitored by TLC. After total consumption of the starting azide, the reaction mixture was evaporated completely to a solid material and partitioned between water and ethyl acetate. The organic layer was washed with water followed by brine solution, dried over Na₂SO₄, and then concentrated. The title triazolyl compounds were separated by column chromatography and characterized. The average yields were 50% to 85%.

Synthesis of 4-(1-(4-Methoxyphenyl)-1H-1,2,3-triazol-4-yl)-N,N-dimethylbenzenamine (4a). Using the general procedure, starting from 0.116 g of 1-azido-4-methoxybenzene **A** and 0.169 g of **E**, 0.137 g of the title compound **4a** was isolated as a white solid (60%). IR (KBr) 1618, 1593, 1518, 1503, 1354, 1250, 1040, 808 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 3.01 (6H, s), 3.87 (3H, s), 6.81 (2H, d, *J* = 7.2 Hz), 7.03 (2H, d, *J* = 7.6 Hz), 7.68 (2H, d, *J* = 8.0 Hz), 7.77 (2H, d, *J* = 8.0 Hz), 7.98 (1H, s); ¹³C NMR (CDCl₃; 100 MHz) δ 40.7, 55.8, 112.8, 114.9, 116.5, 118.9, 122.2, 126.9, 130.9, 148.9, 150.6, 159.8; HRMS calcd for C₁₇H₁₈N₄O ([M + H]⁺) 295.1559, found 295.1562.

Synthesis of 4-(1-(4-Methoxyphenyl)-1H-1,2,3-triazol-4-yl)benzonitrile (4b). Using the general procedure, starting from 0.116 g of 1-azido-4-methoxybenzene **A** and 0.148 g of **F**, 0.144 g of the title compound **4b** was isolated as a white solid (67% yield). IR (KBr) 2231, 1611, 1519, 1489, 1258, 1025, 825 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 3.87 (3H, s), 7.04 (2H, d, *J* = 8.8 Hz), 7.66 (2H, d, *J* = 8.8 Hz), 7.73 (2H, d, *J* = 8.0 Hz), 8.00 (2H, d, *J* = 8.0 Hz), 8.18 (1H, s); ¹³C NMR (CDCl₃; 100 MHz) δ 55.8, 111.8, 115.1, 118.9, 119.2, 122.4, 126.3, 130.3, 132.9, 134.9, 146.5, 160.3; HRMS calcd for C₁₆H₁₂N₄O ([M + H]⁺) 277.1089, found 277.1091.

Synthesis of N,N-Dimethyl-4-(1-(4-nitrophenyl)-1H-1,2,3-triazol-4-yl)benzenamine (5a). Using the general procedure, starting from 0.067 g of 1-azido-4-nitrobenzene **B** and 0.090 g of **E**, 0.050 g of the title compound **5a** was isolated as an orange solid (50%). IR (KBr) 1621, 1595, 1517, 1497, 1368, 1335, 853 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 3.00 (6H, s), 6.78 (2H, d, *J* = 6.8 Hz), 7.08 (2H, d, *J* = 8.8 Hz), 7.59 (2H, d, *J* = 8.8 Hz), 7.77 (1H, s), 8.28 (2H, d, *J* = 8.8 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 41.1, 113.5, 125.0, 125.5, 126.2, 129.9, 131.1, 133.6, 138.5, 141.9, 147.6; HRMS calcd for C₁₆H₁₅N₅O₂ ([M + H]⁺) 310.1304, found 310.1309.

Synthesis of 4-(1-(4-Nitrophenyl)-1H-1,2,3-triazol-4-yl)benzonitrile (5b). Using the general procedure, starting from 0.064 g of 1-azido-4-nitrobenzene **B** and 0.075 g of **F**, 0.017 g of the title compound **5b** was isolated as a yellow solid (51%). IR (KBr) 2228, 1612, 1594, 1521, 1490, 1366, 1338, 854 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 7.35 (2H, d, *J* = 8.4 Hz), 7.54 (2H, d, *J* = 8.8 Hz), 7.70 (2H, d, *J* = 8.0 Hz),

7.95 (1H, s), 8.33 (2H, d, *J* = 8.8 Hz); ¹³C NMR (CDCl₃ + *d*₆-DMSO; 100 MHz) δ 113.2, 117.4, 124.8, 125.3, 129.0, 130.1, 132.6, 134.4, 136.0, 140.3, 147.5; HRMS calcd for C₁₅H₉N₅O₂ ([M + H]⁺) 292.0834, found 292.0840.

Synthesis of 4-(1-(5-Bromonaphthalen-1-yl)-1H-1,2,3-triazol-4-yl)-N,N-dimethylbenzenamine (6a). Using the general procedure, starting from 0.045 g of 1-azido-5-bromonaphthalene **C** and 0.040 g of **E**, 0.060 g of the title compound **6a** was isolated as a pink solid (85%). IR (KBr) 1617, 1597, 1498, 1352, 802, 780, 532 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 2.99 (6H, s), 6.79 (2H, d, *J* = 8.8 Hz), 7.34 (1H, t, *J* = 8.0 Hz), 7.67 (3H, m), 7.79 (2H, d, *J* = 9.2 Hz), 7.86 (2H, d, *J* = 6.8 Hz), 7.98 (1H, s), 8.44 (1H, dd, *J* = 1.4, 7.6 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 40.6, 112.7, 118.2, 121.1, 122.8, 123.3, 124.6, 126.5, 127.0, 128.2, 129.7, 130.2, 131.4, 132.9, 134.4, 148.6, 150.8; HRMS calcd for C₂₀H₁₇N₄Br ([M + H]⁺) 393.0715, found 393.0717.

Synthesis of 4-(1-(5-Bromonaphthalen-1-yl)-1H-1,2,3-triazol-4-yl)benzonitrile (6b). Using the general procedure, starting from 0.024 g of 1-azido-5-bromonaphthalene **C** and 0.018 g of **F**, 0.02 g of the title compound **6b** was isolated as a reddish pink solid (56%). IR (KBr) 2227, 1602, 1591, 1497, 839, 773, 555 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 7.38 (1H, t, *J* = 8.0 Hz), 7.57 (1H, d, *J* = 8.8 Hz), 7.68 (1H, t, *J* = 6.6 Hz), 7.72 (1H, s), 7.76 (1H, d, *J* = 8 Hz), 7.89 (1H, d, *J* = 7.6 Hz), 8.05 (2H, d, *J* = 8.4 Hz), 8.21 (1H, s), 8.50 (1H, d, *J* = 8.4 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 112.2, 118.9, 122.4, 123.7, 124.8, 126.5, 128.5, 130.1, 130.4, 131.7, 133.1, 134.7, 146.3; HRMS calcd for C₁₉H₁₁N₄Br ([M + H]⁺) 375.0245, found 375.0248.

Synthesis of N,N-Dimethyl-4-(1-(pyren-1-yl)-1H-1,2,3-triazol-4-yl)benzenamine (7a). Using the general procedure, starting from 0.025 g of 1-azidopyrene (**D**) and 0.022 g of **E**, 0.024 g of the title compound **7a** was isolated as a light pink solid (68% yield). IR (KBr) 1617, 1567, 1503, 1460, 1351, 819 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 3.03 (6H, s), 6.84 (2H, d, *J* = 8.0 Hz), 7.87 (2H, d, *J* = 8.4 Hz), 7.97 (1H, d, *J* = 9.2 Hz), 8.06–8.20 (6H, m), 8.24–8.29 (3H, m); ¹³C NMR (CDCl₃; 100 MHz) δ 40.6, 112.7, 118.6, 121.4, 123.5, 124.3, 124.9, 125.2, 126.1, 126.5, 126.9, 127.1, 128.9, 129.7, 130.8, 131.3, 132.3, 148.6, 150.8; HRMS calcd for C₂₆H₂₀N₄ ([M + H]⁺) 389.1766, found 389.1768.

Synthesis of 4-(1-(Pyren-1-yl)-1H-1,2,3-triazol-4-yl)benzonitrile (7b). Using the general procedure, starting from 0.010 g of 1-azidopyrene (**D**) and 0.008 g of **F**, 0.010 g of the title compound **7b** was isolated as a brown solid (66% yield). IR (KBr) 2225, 1614, 1489, 1461, 845 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 7.77 (2H, d, *J* = 7.6 Hz), 7.88 (1H, d, *J* = 8.4 Hz), 8.08–8.29 (10H, m), 8.33 (1H, s); ¹³C NMR (CDCl₃; 100 MHz) δ 112.0, 118.9, 121.0, 123.5, 124.0, 124.3, 124.9, 125.3, 126.5, 126.9, 127.1, 129.4, 130.2, 130.8, 131.3, 132.7, 133.1, 134.9, 146.3; HRMS calcd for C₂₅H₁₄N₄ ([M + H]⁺) 371.1297, found 371.1302.

UV–Visible Measurements. All the UV–visible spectra of the compounds (10 μM) were measured in different solvents, using a UV–visible spectrophotometer with a cell of 1 cm path length.

Fluorescence Experiments. All the sample solutions were prepared as described in UV measurement experiments. Fluorescence spectra were obtained with use of a fluorescence spectrophotometer (for **4a**, **4b**, and **7a**) at 25 °C using a 1 cm path length cell. The fluorescence quantum yields (Φ_f) were determined with quinine sulfate as a reference with the known Φ_f (0.55) in 0.1 M solution in sulfuric acid.

Fluorescence Lifetime Measurements. Fluorescence lifetimes were measured with the use of a system that employs a microchannel plate photomultiplier as the detector. Edinburgh 290 nm (for **4b**) and 308 nm Pulsed LED (for **4a**) and 375 nm (for **6b**, **7a**, **7b**) Laser Diode were used for excitation. The fluorescence decay was analyzed by reconvolution (for **4a**, **b**) and tail fitting (for **6b**, **7a**, **7b**) method, using built-in software. All experiments were performed at 298 K.

Theoretical Calculation. The ground state structures of the fluorophores were optimized by using density functional theory (DFT)¹⁸ with B3LYP functional and 6-31G (d) basis set. The excited state related

calculations were carried out with the time dependent density functional theory (TD-DFT) with the optimized structure of the ground state (B3LYP/6-31G(d)). There are no imaginary frequencies in frequency analysis of all the calculated structures, therefore each calculated structure is a local energy minimum.

■ ASSOCIATED CONTENT

S Supporting Information. Full chemical structures, crystallographic data, photophysical spectra and lifetime measurement, Cartesian coordinates, TDDFT calculation, and $^1\text{H}/^{13}\text{C}$ NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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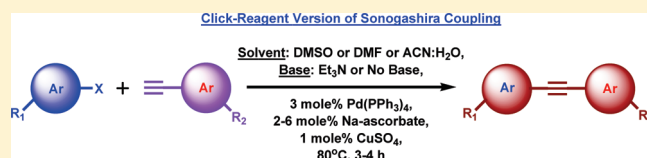
Click-Reagent Version of Sonogashira Coupling Protocol to Conjugated Fluorescent Alkynes with No or Reduced Homocoupling

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Supporting Information

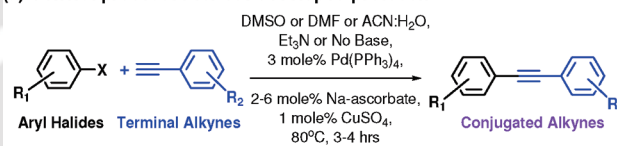
ABSTRACT: A click-reagent version of the Sonogashira-coupling protocol has been developed. Diarylalkynes with donor and/or acceptor substituents have been synthesized via this protocol at moderate to excellent yields and with no or drastically reduced quantities of undesired homocoupled side products. This protocol is green-solvent compatible, air-insensitive, and effective under microwave conditions.



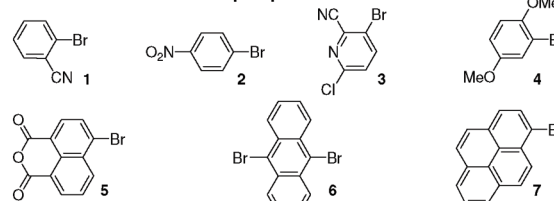
Since the discovery of Sonogashira coupling in 1975, this protocol has become a very widely used and practical tool for the generation of several terminal and internal π -conjugated acetylenic compounds that have widespread applications.^{1,2} Despite its reliability, simplicity, and overall applicability, Sonogashira coupling suffers from several shortcomings, such as (a) the generation of undesired homocoupled products³ of terminal alkynes in the presence of air, (b) the requirement of specific substrates and the skillful tailoring of reaction conditions, (c) poor coupling in the presence of mild conditions with unactivated halides, (d) the requirement of large amounts of a catalyst, and (e) low catalyst turnover.⁴ Thus, there has been a tremendous research effort over the course of the past decade to overcome these limitations;⁴ however, no single protocol has overcome the aforementioned shortcomings, especially the problem of homocoupling. Therefore, overcoming these limitations, especially the problem of the homocoupling side reaction, remains a challenge for researchers.

The presence of oxidizing agents or air in the reaction setup, both of which are difficult to completely remove, may oxidize nascent Pd(0) to Pd(II) and/or Cu(I) to Cu(II), which, in turn, may promote the homocoupling of terminal alkynes in CuI cocatalyzed original Sonogashira coupling.^{2b,4e-g,5d} Despite several modifications,^{5,6} no special steps have been taken to develop a modified version of the Cu(I) cocatalyst system so as to reduce the homocoupling reaction and facilitate a high yield of desired product; however, the reaction rate can be accelerated and applied to a wider range of reaction conditions. As a part of our ongoing research efforts toward installing a fluorescence response in a nonfluorescent precursor via a click reaction,⁷ we have envisioned that Sonogashira coupling could potentially enable us to discover more conjugated and highly emissive fluorophores. Therein, the specific questions we wanted to address are as follows: (a) Can the same click-reagents serve as a source of reactive Cu(I) for Sonogashira coupling, (b) can Na-ascorbate maintain a reducing atmosphere in this system,⁸ and, (c) if so, would it more effectively reduce the incidence of homocoupling in comparison to CuI or other reported

(a) General procedure for our developed protocol:



(b) Aryl halides used in our developed protocol:



(c) Alkynes used in our developed protocol:

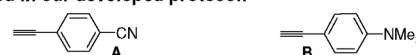


Figure 1. (a) Schematic representation of the general procedure. (b) The aryl halides and (c) alkynes that were used in our developed click-reagent version of Sonogashira coupling protocol.

modified methods?⁴⁻⁶ Immediate attempts were taken to answer our curiosities, and we were successful in developing a click-reagent version of Sonogashira coupling method (Figure 1) under mild reaction conditions without any requirement of skillful reaction tailoring.

The logic behind the use of click-reagent in our strategy is the following: (a) to generate active Cu(I) in situ, (b) halt the oxidation of the generated Cu(I) into Cu(II), and (c) remove excessive air/O₂ from the reaction setup, which may damage nascent Pd(0) catalyst. Sodium ascorbate can reduce oxygen to form water in the vicinity of nascent Pd(0),^{4e-g,5d} which reduces the oxygen concentration in the reaction; hence, the incidence of

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The diagram illustrates the Cu-catalyzed oxidative coupling of alkynes. The main cycle involves Cu(I) and Cu(II) species. Cu(I) reacts with an alkyne (R-C≡C-H) to form a Cu-alkyne complex. This complex then undergoes oxidative coupling with an aryl group (Ar-X) to form an aryl-substituted alkyne (Ar-C≡C-R). The Cu(II) species is regenerated to Cu(I) by a reductant (AH⁻), which is oxidized to A^{•-}. A side reaction shows A^{•-} reacting with O₂ to form DA and HO₂^{•-}. The HO₂^{•-} species is then oxidized to DA and H₂O by Cu(II), which is regenerated to Cu(I) by AH⁻. The overall reaction is summarized as: Ar-C≡C-H + R-C≡C-H → Ar-C≡C-R.

^a CR1 is click-reagent 1, which is CuSO₄. ^b CR2 is click-reagent 2, which is sodium ascorbate. ^c Yields consist of isolated pure compounds. For all of the reactions, the conversion was 100%.

$$\begin{array}{c}
 \text{R}_1\text{-C}_6\text{H}_4\text{-X} + \text{C}_6\text{H}_5\text{-R}_2 \\
 \text{1-7} \quad \text{A and/or B}
 \end{array}
 \xrightarrow[\text{6 mole\% Na-ascorbate, 1 mole\% CuSO}_4, 80^\circ\text{C, 3-4 h}]{\text{DMF or DMSO:Et}_3\text{N(1:1), 3 mole\% Pd(PPh}_3\text{)}_4}
 \begin{array}{c}
 \text{R}_1\text{-C}_6\text{H}_4\text{-C}\equiv\text{C-C}_6\text{H}_4\text{-R}_2 \\
 \text{1-7(A and/or B)}
 \end{array}$$

^a The reactions were performed in DMSO (for entry 1), in DMF (for entry 2-5), in 1:1:1 toluene:THF:water (for 6; conversion was 65%), and in DMF (for entry 7). ^b Figure 1. ^c SI, Figure S1. ^d Yields are of isolated pure compounds; yields in parentheses are of reported compounds using the original Sonogashira condition. ^e Along with **3B**, a biscoupled product **3B'** was obtained in 25% yield. ^f **6A–B** are mono-coupled products.

most probable roles of the click-reagents in the Sonogashira coupling procedure include the in situ provision of active Cu(I) cocatalyst, maintenance of a constant reducing atmosphere in the vicinity of nascent Pd(0), and achievement of the possible

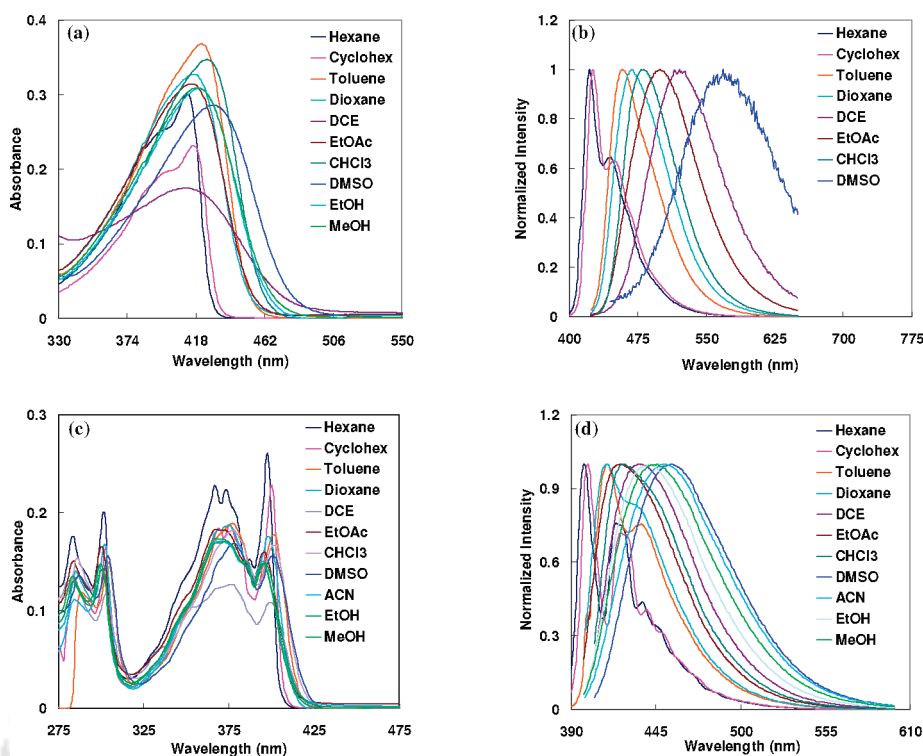


Figure 2. (a) UV–visible and (b) normalized fluorescence spectra of **3B'** and (c) UV–visible and (d) normalized fluorescence spectra of **7A** in different solvents.

acceleration of the reductive elimination step, which would result in a high yield of a desired product. Scheme 1 represents the most probable course of the reaction showing the roles of click-reagents.

In this particular article, we want to present our developed method, which was observed to result in either a tremendous decrease in or no undesired homocoupling and an enhancement in the yield of the desired heterocoupled product within a short period of time. To establish the optimum conditions of our protocol, we have investigated the possibility of coupling with varying catalytic concentrations. Thus, as is shown in Table 1, we carried out a reaction of aryl bromide **1** with alkyne **A** in various molar ratios of ascorbate, base, and Pd(0) in DMF. Thus, we have carried out the Sonogashira reaction using a CuSO₄/Na-ascorbate couple in a 1:6 mol % ratio as a source of active Cu(I) (1 mol %), Pd(0) (3 mol %) in a 1:1 DMF/Et₃N solvent at 80 °C, and other possible molar ratios. All of the reactions were found to be complete within 4 h. The products were isolated to purity with moderate to excellent yields via column chromatography and then characterized. We have also tested our methodology in the absence of base or copper, as well as in both copper- and base-free conditions; however, a low yield of the products (22–49%, Table 1) was obtained by applying these conditions. Finally, we observed that a minimum of 20 equiv of base is required to achieve good yields; therefore, a catalytic composition of Pd(0):CuSO₄:ascorbate in a ratio of 3:1:6 mol % was identified to be the optimum yielding condition (Table 1).

To test the feasibility and facilitate the generalization of our method, we used optimum yielding conditions and carried out the reactions of a series of simple aryl bromides with two terminal alkynes (Figure 1) for the synthesis of several donor–acceptor-containing conjugated alkynes; one terminal alkyne contains

electron-withdrawing substituents (**A**, –CN), whereas the other has an electron-donating group (**B**, –NMe₂). The products were isolated to purity at good to excellent yields via column chromatography and characterized by NMR, mass spectrometry, and, in a few cases, X-ray crystallography (Table 2 and Figure S1 in the SI). We have observed that irrespective of the aryl halides, no homocoupled products with the electron-deficient alkyne **A** were formed; however, in a few cases, a negligible amount (1–6%) of homocoupled products with the electron-rich alkyne **B** was observed (Table 2). Here, it is important to mention that for 4-bromonaphthalic anhydride (entry **5A**, Table 2), due to its poor solubility in DMF or DMSO, the resulting product was isolated to purity via filtration and recrystallization from 1:1 toluene–ethanol. Therein, a loss of product resulted in a low yield of 65%. Also, in the case of dibromoanthracene, where in the reaction was carried out in 1:1:1 toluene:THF:water, an appreciable amount of unreacted starting material remained, which resulted in a moderate yield (72% for **6A**) of the product; however, most importantly, no homocoupling was observed in both of these cases. In the case of pyridine halide **3**, the yield of the desired product, **3B**, was 61%; however, along with **3B**, biscoupled product **3B'** was obtained at a 25% yield after being isolated (SI, Figure S1). This result, though it is only one example, indicates that our click-reagent version of the Sonogashira coupling procedure proceeds even when using aryl chloride.

In comparison to the CuI cocatalyzed reaction, the yields of the cross-coupled desired products were found to have tremendously increased at the expense of the quenched incidence; of homocoupling via our protocol. We have tested our methodology in DMSO and under microwave irradiation (3.5 min, 200 W) in both DMF and DMSO. In both cases, we observed no homocoupling, and the yields were very good and comparable. From the examples listed in Table 2, it is clear that our

methodology is highly efficient with a variety of substrates, and the yields are very good to excellent, irrespective of the nature of the halides or alkynes that were utilized. Furthermore, in comparison to the reported low to moderate yields of compounds **1A** and **7B** (Table 2), which were synthesized via CuI cocatalyzed original Sonogashira coupling,⁹ a tremendous increase in yields was observed via our protocol, which corroborates the success of our developed methodology.

Next, we explored the capability of our methodology in the context of green solvents, such as water; however, because of the insolubility of the terminal alkynes or aryl halides in pure water, we performed the investigated reaction in 1:1:1 toluene:THF:water using aryl halide **6** (Table 2), and in ACN–water and DMF–water using aryl halide **1** as the starting halide (Table 1). Again, we found that the reaction proceeded smoothly with a comparably high yield. These findings demonstrate that this methodology can be used in the context of water-soluble alkynes and aromatic halides. Therefore, the present click-reagent version of the cross-coupling protocol appears to be compatible with several different kinds of substrates and green solvent systems, such as water.

Because an excess of ascorbate was found to maintain an excellent reducing atmosphere for the reactions to be carried out, we thought that it would be worthwhile to verify our methodology in open air without applying an inert atmosphere. Thus, we carried out a reaction of halide **1** with alkyne **A**, without degassing with N₂ gas in DMF at 80 °C and also separately under microwave irradiation. We obtained a very good yield of our desired product with no homocoupling. In these cases, all of the reactants and reagents were taken together; only a solution of 1 mol % of CuSO₄ in DMF was added at the end, and the reaction vessel was maintained in open atmosphere (Table 1). This observation proves the air-insensitivity of our protocol and thereby proves its versatility and practicality. Most importantly, in all cases throughout our investigation, the reducing agent, sodium ascorbate, was observed to not adversely impact susceptible functional groups, such as NO₂, –CN, or anhydride.

Finally, we studied the fluorescence photophysical properties of two of our synthesized fluorophores. Thus, in this preliminary study, the absorption spectra of the donor–acceptor acetylenes (for example, **3B'** and **7A**) are characterized by an intense, broad, and low-energy transition, which derives from the intramolecular charge-transfer transition (ICT) that results from excitation from the donor to the acceptor group (SI, Figures S4–S6). The observed absorption band was solvatochromic, which is characteristic of a large dipole moment change between the ground and the excited states. Furthermore, the molecules exhibited a structured emission in low polar solvents, such as hexane, dioxane, etc.; however, as the solvent polarity was increased, intense, broad, and structureless emissions at longer wavelength regions were increasingly observed, i.e., the molecules exhibited a highly solvatochromic emission from the ICT state (Figure 2).

In conclusion, we have developed a click-reagent version of Sonogashira coupling methodology that is high yielding, tolerant to a variety of substrates and functional groups, compatible with green solvents, and air-insensitive. We have exploited the known reducing behavior of sodium ascorbate to generate an active Cu(I) cocatalyst from CuSO₄, and we have maintained a constant reducing atmosphere to control and minimize undesired oxidative homocoupling, thereby tremendously increasing the incidence of desired cross-coupling. This is the first example of the use of click-reagents in Sonogashira coupling that yield no

or drastically diminished amounts of undesired homocoupled products. Our developed protocol is also air-insensitive and effective under microwave conditions. Thus, we hope this methodology will find widespread applications in chemistry, material science, and chemical biology for the synthesis of desirable conjugated fluorescent alkynes.

EXPERIMENTAL SECTION

General Procedure for the Click-Reagent Version of Sonogashira Coupling. To a solution of aryl halide in 1:1 DMF–Et₃N under N₂, Pd(PPh₃)₄ followed by terminal alkyne was added. After 5 min of stirring under N₂ atmosphere, 6 mol % of a solution of sodium ascorbate in DMF was added followed by addition of 1 mol % of a solution of CuSO₄ in DMF. The reaction mixture was stirred for 4 h at 80 °C. After completion of the reaction monitored by TLC, the reaction mixture was extracted with ethyl acetate, washed with ammonium chloride and brine, and dried over anhydrous Na₂SO₄. The products were isolated pure by column chromatography and characterized by IR, NMR, and mass spectrometry. All the products were isolated with very good to excellent yield ranging from 70% to 97% in DMF at 80 °C within 4 h only. The synthesis and the characterization data are described below. The compounds **1A**,¹ **6B**,² and **7B**³ are known compounds, and their spectral data were in agreement with those reported in the literature. The melting point of **1A** is not reported and the melting points are in accord with the literature values for the case of compounds **6B** and **7B**. Full characterization data of unknown as well as of known compounds are also reported as was observed from our analysis.

Synthesis of 2-(2-(4-(cyano)phenyl)ethynyl)benzonitrile^{9a,b} (1A): Using the general procedure, starting from 0.05 g (0.275 mmol) of 2-bromobenzonitrile **1** and 0.042 g (0.330 mmol) of 4-ethynylbenzonitrile **A**, 0.054 g (0.237 mmol) of the title compound was isolated as a white solid. Yield 86%; mp 133–135 °C; IR (KBr) 3065, 2231, 1605, 1504, 1479, 840, 768 cm^{−1}; ¹H NMR (CDCl₃; 400 MHz) δ 7.46 (1H, t, *J* = 7.2 Hz), 7.59 (1H, t, *J* = 7.6 Hz), 7.63–7.70 (6H, m); ¹³C NMR (CDCl₃; 100 MHz) δ 89.6, 93.9, 112.7, 115.9, 117.5, 118.5, 126.3, 127.0, 129.4, 132.3, 132.5, 132.6, 132.7, 133.0; ESI-TOF-MS *m/z* 229 [M + H]⁺; HRMS calcd for C₁₆H₉N₂ ([M + H]⁺) 229.0765, found 229.0760.

Synthesis of 2-(2-(4-(*N,N*-dimethylamino)phenyl)ethynyl)benzonitrile (1B): Using the general procedure, starting from 0.06 g (0.3296 mmol) of 2-bromobenzonitrile **1** and 0.057 g (0.3955 mmol) of 4-ethynyl-*N,N*-dimethylbenzenamine **B**, 0.070 g (0.2845 mmol) of the title compound was isolated as a lime solid. Yield 86%; mp 125–126 °C; IR (KBr) 3060, 2902, 2817, 2204, 2173, 1603, 1590, 1520, 1365, 1143, 815, 755 cm^{−1}; ¹H NMR (CDCl₃; 400 MHz) δ 2.98 (6H, s), 6.63 (2H, d, *J* = 8.8 Hz), 7.30 (1H, ddd, *J* = 1.2, 7.6 Hz), 7.46 (2H, d, *J* = 9.2 Hz), 7.49–7.55 (2H, m), 7.60 (1H, dd, *J* = 0.4, 8.0 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 40.2, 84.5, 98.3, 108.6, 111.8, 114.7, 118.1, 127.3, 128.4, 131.7, 132.4, 132.7, 133.4, 133.8, 150.8; ESI-TOF-MS *m/z* 247 [M + H]⁺; HRMS calcd for C₁₇H₁₅N₂ ([M + H]⁺) 247.1235, found 247.1230.

Synthesis of 4-(2-(4-nitrophenyl)ethynyl)benzonitrile (2A): Using the general procedure, starting from 0.070 g (0.3465 mmol) of 1-bromo-4-nitrobenzene **2** and 0.053 g (0.4173 mmol) of 4-ethynylbenzonitrile **A**, 0.078 g (0.3145 mmol) of the title compound was isolated as a lime solid. Yield 91%; mp 212–214 °C; IR (KBr) 3075, 2226, 1604, 1592, 1511, 1339, 836 cm^{−1}; ¹H NMR (CDCl₃; 400 MHz) δ 7.61–7.68 (6H, m), 8.23 (2H, d, *J* = 8.8 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 91.4, 92.5, 112.7, 118.4, 123.9, 127.1, 129.2, 132.4, 132.5, 132.7, 147.7; ESI-TOF-MS *m/z* 249 [M + H]⁺; HRMS calcd for C₁₅H₉N₂O₂ ([M + H]⁺) 249.0664, found 249.0667.

Synthesis of *N,N*-dimethyl-4-(2-(4-nitrophenyl)ethynyl)benzenamine (2B): Using the general procedure, starting from 0.100 g (0.4950 mmol) of 1-bromo-4-nitrobenzene **2** and 0.0790 g

(0.5448 mmol) of 4-ethynyl-*N,N*-dimethylbenzenamine **B**, 0.1135 g (0.4267 mmol) of the title compound was isolated as a red solid. Yield 86%; mp 209–212 °C; IR (KBr) 3095, 2909, 2208, 1609, 1586, 1529, 1508, 1371, 1336, 1104, 820 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 3.00 (6H, s), 6.65 (2H, d, *J* = 8.8 Hz), 7.41 (2H, d, *J* = 8.4 Hz), 7.57 (2H, d, *J* = 9.2 Hz), 8.16 (2H, d, *J* = 8.4 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 40.3, 86.6, 97.3, 111.9, 123.8, 128.7, 128.8, 131.8, 132.1, 132.2, 132.3, 133.4, 133.8, 146.4, 150.9; ESI-TOF-MS *m/z* 267 [M + H]⁺; HRMS calcd for C₁₆H₁₅N₂O₂ ([M + H]⁺) 267.1134, found 267.1130.

Synthesis of 6-chloro-3-(2-(4-(*N,N*-dimethylamino)phenyl)ethynyl)picolinonitrile (3B): Using the general procedure, starting from 0.05 g (0.2299 mmol) of 3-bromo-6-chloropicolinonitrile **3** and 0.04 g (0.2758 mmol) of 4-ethynyl-*N,N*-dimethylbenzenamine **B**, 0.0393 g (0.1396 mmol) of the title compound was isolated as a yellow solid. Yield 61%; mp 142–145 °C; IR (KBr) 2923, 2853, 2193, 1606, 1560, 1527, 1369, 1156, 1023, 808 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 3.01 (6H, s), 6.64 (2H, d, *J* = 8.8 Hz), 7.42–7.47 (3H, m), 7.77 (1H, d, *J* = 8.8 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 40.2, 81.3, 103.3, 107.4, 111.8, 115.5, 125.6, 127.6, 130.4, 133.7, 140.9, 149.4, 151.3; ESI-TOF-MS *m/z* 282 [M + H]⁺; HRMS calcd for C₁₆H₁₃N₃Cl ([M + H]⁺) 282.0798, found 282.0789.

From the same reaction mixture we were able to isolate 0.0224 g (0.0574 mmol) of dihalide (Br and Cl) substituted compound, 3,6-bis(2-(4-(*N,N*-dimethylamino)phenyl)ethynyl)picolinonitrile, **3B'** as a yellow solid. Yield 25%; mp 210–212 °C; IR (KBr) 2922, 2852, 2201, 1604, 1530, 1447, 1365, 1157, 816 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 2.99 (12H, d, *J* = 1.6 Hz), 6.63 (4H, d, *J* = 8.8 Hz), 7.45 (2H, d, *J* = 3.2 Hz), 7.47 (2H, d, *J* = 3.2 Hz), 7.50 (1H, d, *J* = 8.4 Hz), 7.73 (1H, d, *J* = 8.4 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 40.3, 82.7, 86.7, 95.5, 102.8, 107.9, 108.0, 109.9, 111.9, 116.2, 124.1, 128.8, 133.7, 133.8, 135.5, 138.4, 142.9, 151.1, 151.2; ESI-TOF-MS *m/z* 391 [M + H]⁺; HRMS calcd for C₂₆H₂₃N₄ ([M + H]⁺) 391.1922, found 391.1930.

Synthesis of 4-(2-(2,5-dimethoxyphenyl)ethynyl)benzonitrile (4A): Using the general procedure, starting from 0.030 g (0.1382 mmol) of 2-bromo-1,4-dimethoxybenzene **4** and 0.021 g (0.1653 mmol) of 4-ethynylbenzonitrile **A**, 0.026 g (0.0988 mmol) of the title compound was isolated as a white semisolid. Yield 72%; IR (KBr) 2924, 2853, 2227, 1602, 1494, 1458, 1261, 1021, 840, 805, 699 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 3.77 (3H, s), 3.86 (3H, s), 6.84 (1H, s), 6.88 (1H, d, *J* = 2.8 Hz), 7.01 (1H, d, *J* = 3.2 Hz), 7.60 (4H, m); ¹³C NMR (CDCl₃; 100 MHz) δ 56.0, 56.6, 90.5, 91.8, 111.5, 112.0, 112.3, 116.9, 118.4, 118.8, 128.6, 132.2, 132.3, 133.2, 153.5, 154.9; ESI-TOF-MS *m/z* 264 [M + H]⁺; HRMS calcd for C₁₇H₁₄NO₂ ([M + H]⁺) 264.1024, found 264.1030.

Synthesis of 4-(2-(2,5-dimethoxyphenyl)ethynyl)-*N,N*-dimethylbenzenamine (4B): Using the general procedure, starting from 0.050 g (0.2304 mmol) of 2-bromo-1,4-dimethoxybenzene **4** and 0.0367 g (0.2534 mmol) of 4-ethynyl-*N,N*-dimethylbenzenamine **B**, 0.046 g (0.1637 mmol) of the title compound was isolated as a lime semisolid. Yield 71%; IR (KBr) 2924, 2853, 2830, 2207, 1603, 1521, 1499, 1356, 1225, 1197, 1042, 821, 712 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 2.97 (6H, s), 3.76 (3H, s), 3.85 (3H, s), 6.66 (2H, d, *J* = 7.6 Hz), 6.79 (2H, d, *J* = 1.6 Hz), 7.01 (1H, d, *J* = 3.6 Hz), 7.42 (2H, d, *J* = 8.8 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 41.4, 55.9, 56.7, 83.7, 94.9, 110.5, 111.9, 112.4, 114.2, 115.1, 118.1, 124.5, 124.6, 133.0, 150.3, 153.5, 154.4; ESI-TOF-MS *m/z* 282 [M + H]⁺; HRMS calcd for C₁₈H₂₀NO₂ ([M + H]⁺) 282.1494, found 282.1488.

Synthesis of 4-(2-(4-(cyano)phenyl)ethynyl)-1,8-naphthalic anhydride (5A): Using the general procedure, starting from 0.08 g (0.2888 mmol) of 4-bromo-1,8-naphthalicanhydride **5** and 0.0404 g (0.3176 mmol) of 4-ethynylbenzonitrile **A**, 0.061 g (0.1888 mmol) of the title compound was isolated pure by filtration followed by washing with water and ethanol as a yellow solid. Yield 65%; mp >312 °C (it became black); IR (KBr) 2223, 1778, 1758, 1601, 1588, 1043,

832 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 7.46–7.52 (4H, m), 7.67 (1H, t, *J* = 7.6 Hz), 7.77 (1H, d, *J* = 7.6 Hz), 8.30 (1H, d, *J* = 8.0 Hz), 8.38 (1H, d, *J* = 6.8 Hz), 8.51 (1H, d, *J* = 8.0 Hz); ESI-TOF-MS *m/z* 324 [M + H]⁺; HRMS calcd for C₂₁H₁₀NO₃ ([M + H]⁺) 324.0660, found 324.0653.

Synthesis of 4-(2-(4-(*N,N*-dimethylamino)phenyl)ethynyl)-1,8-naphthalic anhydride (5B): Using the general procedure, starting from 0.06 g (0.2166 mmol) of 4-bromo-1,8-naphthalicanhydride **5** and 0.0345 g (0.2382 mmol) of 4-ethynyl-*N,N*-dimethylbenzenamine **B**, 0.0635 g (0.1862 mmol) of the title compound was isolated as a red solid. Yield 86%; mp 288–292 °C; IR (KBr) 2917, 2862, 2188, 1752, 1730, 1604, 1585, 1575, 1524, 1371, 1169, 1010, 781 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 3.06 (6H, s), 6.82 (2H, bs), 7.55 (2H, d, *J* = 7.6 Hz), 7.84–7.92 (2H, m), 8.53 (1H, d, *J* = 7.6 Hz), 8.64 (1H, d, *J* = 7.2 Hz), 8.81 (1H, d, *J* = 8.0 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 40.1, 85.2, 103.8, 107.9, 110.6, 111.7, 112.1, 112.8, 118.9, 125.3, 127.5, 129.5, 130.5, 132.8, 133.5, 134.2, 135.0, 153.4, 160.4, 160.8; ESI-TOF-MS *m/z* 342 [M + H]⁺; HRMS calcd for C₂₂H₁₆NO₃ ([M + H]⁺) 342.1130, found 342.1125.

Synthesis of 4-(2-(9-bromoanthracen-10-yl)ethynyl)benzonitrile (6A): Using the general procedure, starting from 0.070 g (0.2083 mmol) of 9,10-dibromoanthracene **6** and 0.0265 g (0.2083 mmol) of 4-ethynylbenzonitrile **A**, 0.040 g (0.1190 mmol) of 9,10-dibromoanthracene was isolated as unreacted and 0.0245 g (0.0641 mmol) of the title compound was isolated as a yellow solid. Yield 72%; mp 214–215 °C; IR (KBr) 2222, 2198, 1620, 1602, 1502, 837, 752 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 7.62–7.64 (4H, m), 7.70 (2H, dd, *J* = 1.6, 8.2 Hz), 7.80 (2H, dd, *J* = 1.2, 8.0 Hz), 8.55–8.60 (4H, m); ¹³C NMR (CDCl₃; 100 MHz) δ 90.6, 100.0, 112.0, 118.7, 127.0, 127.5, 127.8, 128.4, 128.7, 129.1, 130.5, 132.2, 132.5, 133.4, 142.5; ESI-TOF-MS *m/z* 383, 385 [M + H]⁺; HRMS calcd for C₂₃H₁₂N ([M – Br]) 302.0970, found 302.0960.

Synthesis of 4-(2-(9-bromoanthracen-10-yl)ethynyl)-*N,N*-dimethylbenzenamine^{9c} (6B): Using the general procedure, starting from 0.060 g (0.1785 mmol) of 9,10-dibromoanthracene **6** and 0.0258 g (0.1785 mmol) of 4-ethynyl-*N,N*-dimethylbenzenamine **B**, 0.016 g (0.0476 mmol) of 9,10-dibromoanthracene was isolated as unreacted and 0.0305 g (0.0763 mmol) of the title compound was isolated as a yellow solid. Yield 68%; mp 234–236 °C (reported 236 °C);^{9c} IR (KBr) 2924, 2854, 2192, 1634, 1605, 1520, 1348, 1188, 811, 752 cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 3.03 (6H, s), 6.73 (2H, d, *J* = 8.4 Hz), 7.56–7.64 (6H, m), 8.53 (2H, d, *J* = 8.0 Hz), 8.70 (2H, d, *J* = 8.8 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 40.5, 84.5, 96.2, 103.8, 112.3, 123.0, 126.6, 127.3, 127.6, 127.8, 128.3, 130.6, 132.9, 133.1, 152.8; ESI-TOF-MS *m/z* 401, 403 [M + H]⁺; HRMS calcd for C₂₄H₁₉BrN ([M + H]⁺) 400.0701, found 400.0697.

Synthesis of 4-(2-(pyren-1-yl)ethynyl)benzonitrile (7A): Using the general procedure, starting from 0.055 g (0.1957 mmol) of 1-bromopyrene **7** and 0.0273 g (0.2152 mmol) of 4-ethynylbenzonitrile **A**, 0.062 g (0.1896 mmol) of the title compound was isolated as a yellow solid. Yield 97%; mp 201–203 °C; IR (KBr) 3037, 2229, 2202, 1604, 1512, 1497, 854, 825, 718, cm⁻¹; ¹H NMR (CDCl₃; 400 MHz) δ 7.65 (2H, d, *J* = 8.0 Hz), 7.72 (2H, d, *J* = 8.4 Hz), 8.01–8.04 (2H, m), 8.09–8.12 (2H, m), 8.16–8.24 (4H, m), 8.56 (1H, d, *J* = 9.2 Hz); ¹³C NMR (CDCl₃; 100 MHz) δ 93.4, 93.6, 111.6, 116.7, 118.8, 124.4, 124.7, 124.8, 125.4, 126.2, 126.6, 127.4, 128.6, 128.9, 130.0, 131.2, 131.4, 132.2, 132.3; ESI-TOF-MS *m/z* 328 [M + H]⁺; HRMS calcd for C₂₅H₁₄N ([M + H]⁺) 328.1126, found 328.1130.

Synthesis of *N,N*-dimethyl-4-(2-(pyren-1-yl)ethynyl)benzenamine^{9d} (7B): Using the general procedure, starting from 0.055 g (0.1957 mmol) of 1-bromopyrene **7** and 0.0312 g (0.2152 mmol) of 4-ethynyl-*N,N*-dimethylbenzenamine **B**, 0.062 g (0.1797 mmol) of the title compound was isolated as a bright yellow solid. Yield 92%; mp 168–169 °C (reported 169 °C);^{9d} IR (KBr) 3035, 2922, 2853, 2196,

2182, 1606, 1594, 1520, 1355, 1152, 845, 819, 711 cm^{-1} ; ^1H NMR (CDCl_3 ; 400 MHz) δ 3.02 (6H, s), 6.76 (2H, d, $J = 7.6$ Hz), 7.60 (2H, d, $J = 8.8$ Hz), 7.98–8.10 (4H, m), 8.14–8.20 (4H, m), 8.68 (1H, d, $J = 8.8$ Hz); ^{13}C NMR (CDCl_3 ; 100 MHz) δ 40.6, 87.0, 96.8, 112.4, 119.1, 124.7, 124.8, 125.4, 125.5, 126.0, 126.3, 127.5, 127.9, 128.2, 129.5, 130.9, 131.4, 131.6, 131.7, 133.0, 150.3; ESI-TOF-MS m/z 346 $[\text{M} + \text{H}]^+$; HRMS calcd for $\text{C}_{26}\text{H}_{20}\text{N}$ ($[\text{M} + \text{H}]^+$) 346.1595, found 346.1589.

■ ASSOCIATED CONTENT

S Supporting Information. General experimental details, crystallographic data, crystallographic information files (CIFs), copies of selected UV and fluorescence spectra, and copies of ^1H and ^{13}C NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Sensing of biomolecules and label-free discrimination of DNA containing a triple T–C/T–G mismatch pair with a fluorescence light-up probe, triazolylpyrene (^{TNDMB}Py)

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ABSTRACT

Binding to the minor groove of calf-thymus DNA (ct-DNA) and strong binding in hydrophobic pocket of bovine serum albumin (BSA), switched-on the fluorescence of smart fluorescent probe, triazolylpyrene (^{TNDMB}Py). Also, a novel label-free strategy was adopted to detect DNA base mismatch via the generation of distinct fluorescence signal at visible region utilizing the same probe

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Monitoring various structures, functions, and dynamics of biological molecules such as DNAs and proteins and their surroundings with an ideal microenvironment sensitive fluorescence probe is an emerging research area in biological science and engineering for understanding biological events associated with intermolecular interactions.¹ In such a probing scenario, when the fluorophore comes into interaction with biomolecules, the fluorophores' emission property may be so modulated as to enable visual observation of biomacromolecular species.^{1e–h} Several fluorescent bioprobes, thus, have been reported based on the fluorophores' properties like intercalation, stacking, and/or groove binding for DNA detection with the generation of enhanced fluorescence signal.² On the other hand, protein binding abilities of several chromophores also have been studied via enhanced fluorescence signal generation.³ However, many of the explored fluorophores were found to suffer from self quenching of emissions that makes them inefficient biosensors.⁴ Therefore, there is a need to design probes which would show low or zero emission in buffer but upon interaction with biomolecules it could fluoresce drastically.

As a part of our ongoing research efforts on the design of solvatochromic fluorescent molecules via click reaction, we, recently have reported triazolylpyrene as a dual emissive highly solvatochromic fluorophore (Fig. 1a).⁵ We have now observed that in buffer (2% DMF was added in each case to completely solubilize the

probe) of various pHs, it showed only a faint ICT emission ($\lambda_{em} = 525$ nm). We envisaged that the pseudo aromatic 1,2,3-triazole residue of the probe might significantly enhance the interaction ability via stacking/H-bonding/electrostatic interaction with the amino acid residues in a protein and/or with the aromatic bases in DNA. This would allow one to gather information on the protein's/DNA's microenvironments. Our probe consists of an *N,N*-dimethyl aminophenyl donor linked triazole functionality which served as an electron donor to effectively modulate the emission response of electronically coupled pyrene and also can provide an interaction site for biomolecules (Fig. 1a). This idea along with the intense emission of the probe in low polar media compared to buffer drew our attention to exploit it for the possible detection of ct-DNA and BSA protein which are easily available biomolecules with widespread applications and biological importance.^{2,3}

Herein, we report our probe triazolylpyrene as an efficient fluorescent light-up biosensor for the detection of DNA and protein.

Before going to study the interactions with DNA/protein, we wanted to establish that the fluorescence at 525 nm is only due to ICT emission. Thus, we subsequently studied the spectral parameters over a broad range of probe concentration. Figure 1 showed the absorption and fluorescence spectra of the probe over the concentration range of 10–70 μ M in a buffer of pH 7.0. The absorption, excitation, and fluorescence spectra did not change in band position. Moreover, the absorbance, and fluorescence intensities depend linearly on the concentrations of the probe in solution

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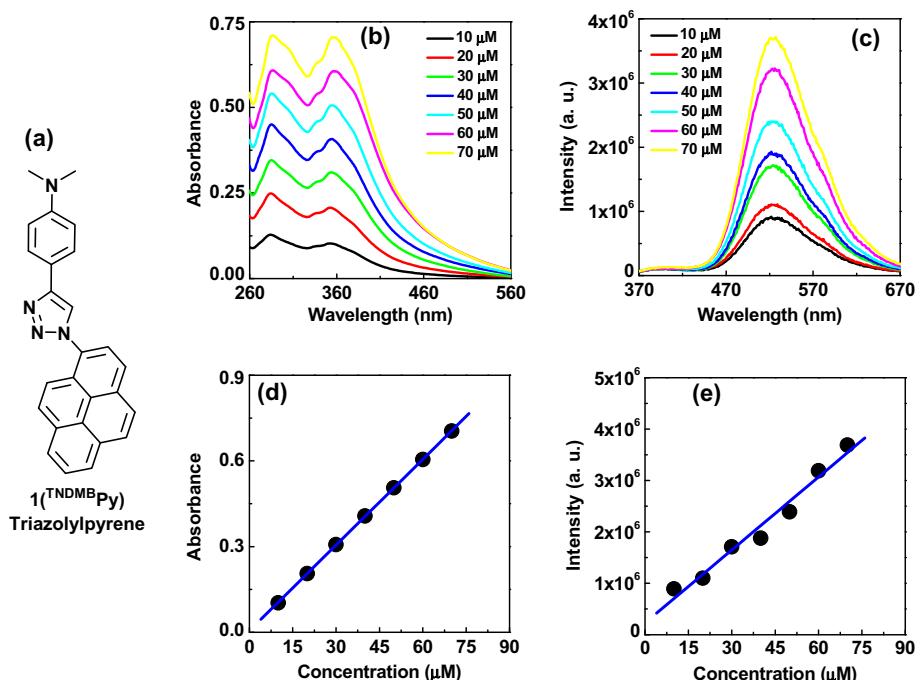


Figure 1. (a) Chemical structure of the probe **1** (TNDBMPy). UV–visible (b) and emission spectra (c) of various concentrations of **1** in phosphate buffer of pH 7.0. Plots of absorbance (d) and emission intensity (e) versus concentration of the probe ($\lambda_{\text{ex}} = 345$ nm).

(Fig. 1d and e) suggesting that the triazolylpyrene existed in a monomeric state and no aggregation formed. We have also studied the absorption of the probe of various concentrations in the presence of a fixed amount of biomolecule, BSA and plotted the absorbance versus concentration of the probe. The plot showed a good linear relationship (see [Supplementary data](#)) that would allow us to fit fluorescence titration curve data quantitatively to derive the association constants of probe–protein/DNA complex with no harm.

The strong emission at a long wavelength region (520 nm) in the presence of biomolecule in buffer again might be confused with the possibility of aggregation induced emission.⁶ However, our observation in emission behavior of a dioxane solution of the probe when titrated with water did not reflect any aggregation induced emission (see [Supplementary data](#)). Thus, increasing % of water led to a decrease in emission along with a red shift of the band at 520 nm of the probe. The band totally died down at 20% water and the LE band at 380 nm started appearing. The LE band intensity reached maximum at 70% water content. When a large amount (80% and 100%) of water, a poor solvent of the probe, was added to the dioxane solution (with the final concentration of the mixture being adjusted to 10 μM) the LE band intensity again started to decrease with a negligible appearance of only LE band in 100% water under identical measurement conditions. These results suggested that the emission was not from an aggregate state but from the monomeric probe (see [Supplementary data](#)).

We attributed further that the emission at 520 nm was due to the ICT emission. However, the structure-less emission at longer wavelength could also be a characteristic to the excimer emission of pyrene.⁷ Therefore, to establish the ICT emission we measured emission spectra of the probe of varying concentrations and plotted emission intensities versus concentrations at two different wavelengths of emission. If the emission at 520 nm was due to excimer emission, only this emission should increase compared with the emission at 380 nm (monomer emission) at the higher concentration; otherwise both the emissions would increase. Thus,

we checked this event and we could say that the long wavelength emission at around 520 nm was due to ICT emission and not due to the characteristic excimer emission of pyrene. The proof of our assignment came from a study of concentration dependent emission of both the bands in 10% water in dioxane solvent and evaluation of the changes in intensity of both the LE (~380 nm) and ICT (~520 nm) bands. This solvent system was so chosen as to get both the emissions prominently. However, in this solvent system, the ICT band was appearing at 570 nm and the LE band centered at 393 nm. If the emission at 570 nm was excimer emission, it should increase compared with the emission at 393 nm at higher concentrations. However, we observed that the intensity of both the emission bands increased linearly as the concentration of the probe increased supporting our assignment of the band at 570 as ICT emission (see [Supplementary data](#)).

After establishing the long wavelength emission band as ICT emission and linear dependency of absorption and emission to the probe concentration either in the absence or in the presence of biomolecule, we next turned our attention to exploit this fluorophore's emission property to the study of possible interaction with protein/DNA biomolecules.

To examine the biomolecule sensing ability of our probe, first, we investigated the interaction behavior with ct-DNA by UV–visible and fluorescence spectroscopy in a buffer (pH 7.0) at 25 °C. Thus, the absorption maxima of triazolyl pyrene located at 355 nm showed a clear hyperchromicity with a minimal shift in absorption wavelength as ct-DNA was added gradually. This indicated a probable external groove binding characteristics of our probe (see [Supplementary data](#)).⁸ From the fluorescence titration spectra it was evident that upon addition of increasing concentrations of ct-DNA, the fluorescence intensity ($\lambda_{\text{em}} = 525$ nm) of triazolyl pyrene significantly increased and reached to a maximum at 300 μM concentration of ct-DNA compared to 50 μM probe concentration. This result indicated a well-defined binding of the probe with ct-DNA (Fig. 2a). The association constant of triazolyl pyrene with ct-DNA was determined by a Benesi–Hildebrand plot (Fig. 2b) which was found to be $7.2 \times 10^3 \text{ M}^{-1}$ with free energy of

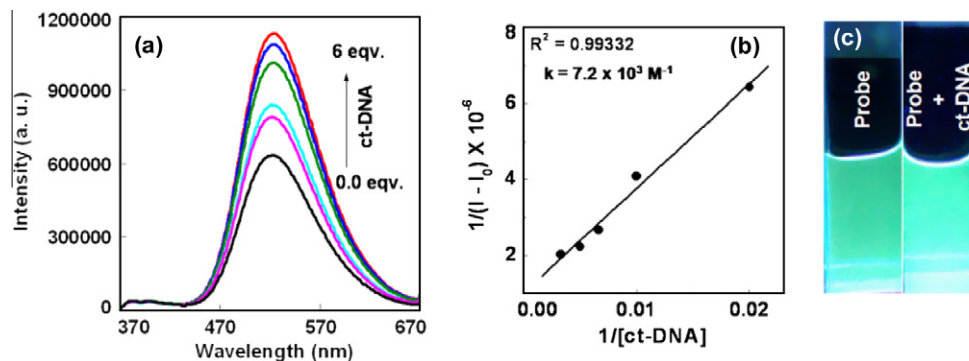


Figure 2. (a) Fluorescence titration of probe with various concentrations of ct-DNA. (b) Benesi–Hildebrand plot. (c) Fluorescence image under UV-transilluminator (254 nm) of only probe, in the presence of ct-DNA. All the experiments were carried out in 5 mM phosphate buffer, 5 mM NaCl, pH 7.0, rt, $\lambda_{\text{ex}} = 345 \text{ nm}$.

binding, $\Delta G = -5.24 \text{ kcal/mol}$. The enhancement of fluorescence emission was also clear from a change of color of the probe solution from that of ct-DNA-probe solution when irradiated at 254 nm under a transilluminator (Fig. 2c).

Thermal denaturation experiment indicated a little destabilization of ct-DNA upon binding with the probe possibly because of steric clash near the groove. However, CD spectral measurement in the presence of the probe revealed an unperturbed B-form DNA conformation of ct-DNA. These results indicated that the probe possibly bind to a groove of ct-DNA (see Supplementary data).⁸ To investigate the binding mode of the probe with ct-DNA, whether it is an intercalator or a groove binder, both the ethidium bromide (EB) and Hoechst 33258 displacement experiments were carried out. Ethidium bromide is a well-known example of an intercalating agent.⁹ Fluorescence intensity of EB increases when it binds with DNA because of intercalation. In a competing scenario, if our probe, **TNDMPy**, intercalates with ct-DNA, then it would be a competitive binding agent with EB and the fluorescence intensity of EB bound with ct-DNA would decrease with an increase in probe concentration. However, we did not observe any significant fluorescence change of EB-ct-DNA complex upon increase in probe concentration. Thus, this observation implied that the probe may be a groove binder (see Supplementary data).¹⁰ To establish the groove binding property of the probe, fluorescence titration experiment with Hoechst 33258 was carried out. Hoechst 33258 is a minor groove binding agent to double-helical DNA.¹¹ Like EB the fluorescence intensity of Hoechst 33258 increased upon binding with DNA. The crystal structure of Hoechst 33258 bound with DNA complex showed that Hoechst 33258 bound to a minor groove of DNA. If the probe binds in minor groove, then again it would be a competitive binding agent of Hoechst 33258 and it would replace Hoechst 33258. Thus, the fluorescence intensity of Hoechst 33258 bound with ct-DNA would decrease with an increase in probe concentration. Unlike with EB, we observed a significant decrease in fluorescence intensity ($\lambda_{\text{em}} = 468 \text{ nm}$) of hoechst 33258 of hoechst 33258-ct-DNA complex with an increase in probe concentration (Fig. 3a). The Multi Gaussian fitting spectra clearly shows the decrease in Hoechst emission with an increase in probe concentration (Fig. 3b). This result indicated that the probe was likely to be a minor groove binder of ct-DNA. As the excitation wavelengths ($\lambda_{\text{ex}} \approx 340 \text{ nm}$) of both hoechst 33258 and the probe were quite same, we observed another emission band ($\lambda_{\text{em}} \approx 520 \text{ nm}$) dominated over the parent hoechst's band ($\lambda_{\text{em}} \approx 468 \text{ nm}$) in high concentrations of the probe (40–50 μM). The appearance of this band was due to the binding of the probe with ct-DNA and possibly because of energy transfer between Hoechst and the probe triazolyl pyrene (see Supplementary data).^{11b} Hoechst had a significantly lower quantum yield when free in solution, in comparison to that when

bound to ct-DNA. Therefore, both resonance energy transfer and release of Hoechst from ct-DNA probably were the main cause of decrease in emission intensity of Hoechst. Finally, the competition between the probe and hoechst for association with ct-DNA indicated that the probe triazolyl pyrene bound to the DNA minor groove.¹² The groove binding event of the probe was further supported by an insignificant change of fluorescence anisotropy/polarization (Fig. 3b).¹³ Amber* optimized geometry of the model DNA-probe complex also supported the minor groove binding event of the probe (see Supplementary data).¹⁴

Motivated by the detection of ct-DNA with an enhanced fluorescence signal and to explore the versatility of our probe we next investigated the detection of protein, BSA spectroscopically.^{1,3} Thus, upon addition of increasing amounts of BSA to the probe's solution, we observed a hyperchromicity with very little (7 nm) blue shift of the absorbance band as was clear from the UV–vis. spectra (see Supplementary data). To get further insight into the binding properties and to investigate the protein sensing ability of the probe, we then investigated the fluorescence changes upon addition of various concentrations of BSA to the solution of the probe upon excitation at 344 nm. Thus, with increasing concentrations of BSA, both the fluorescence intensity and quantum yield of the fluorophore increased with a blue shift of 41 nm of emission maximum (Fig. 4a). The fluorescence enhancement was maximized upon addition of 2.5 equiv of BSA. This result indicated a clear binding of the probe in more hydrophobic pocket of BSA which was also evident from the intrinsic emission behavior of the probe in various solvents of varying dielectric constants (see Supplementary data).^{3,5,15} The binding constant of triazolyl pyrene with BSA was also determined fluorimetrically by a Benesi–Hildebrand plot which was found to be $5.1 \times 10^4 \text{ M}^{-1}$ with an experimental free energy of binding, $\Delta G = -6.41 \text{ kcal/mol}$ (Fig. 4b). The blue shift of emission wavelength was also clear from a change of color of probe solution from yellowish green to bright blue color of probe-BSA solution when irradiated at 254 nm under a transilluminator (Fig. 4c). An increase in the % α -helicity, as was indicated by CD spectra of BSA in the presence of our probe, could be attributed to conformational adjustments on complex formation (see Supplementary data).

The lipophilic nature of the probe and the above results indicated that it would preferentially bind to the hydrophobic sub-domain of BSA, thus might involve in energy transfer with Trp unit.¹⁵ The possibility of energy transfer process from Trp residue of BSA to the probe molecule was satisfied primarily by the spectral overlap between the fluorescence emission spectrum of BSA and the UV–visible absorption spectra of the probe (see Supplementary data). Thus, upon excitation at donor wavelength (280 nm of Trp of BSA), we observed quenching of fluorescence

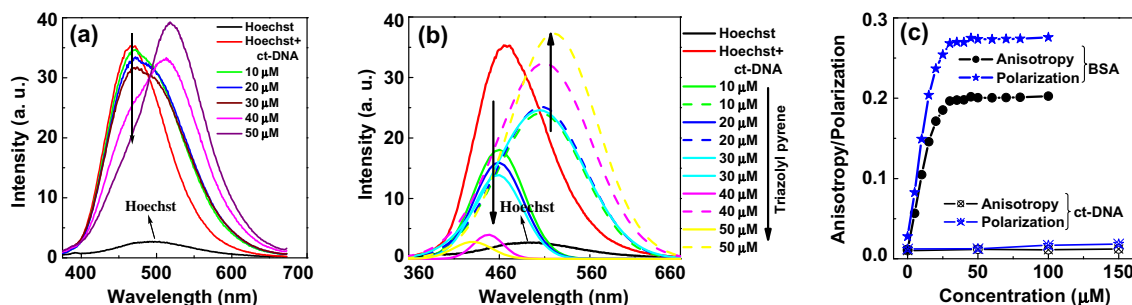


Figure 3. (a) Emission spectra of hoechst, hoechst-ct-DNA complex, and hoechst-ct-DNA complex titrated with various concentrations of TNDMPy ($\lambda_{\text{ex}} = 340 \text{ nm}$). (b) Multi Gaussian fitting of spectra 'a' to show clear decreases in Hoechst emission with an increase in $[\text{TNDMPy}]$. Solid lines and dotted lines are the contribution of Hoechst's and TNDMPy 's emission respectively in each concentration; $[\text{Hoechst}] = 10 \mu\text{M}$, $[\text{ct-DNA}] = 50 \mu\text{M}$ and $[\text{TNDMPy}] = 10, 20, 30, 40$, and $50 \mu\text{M}$. (c) Fluorescence anisotropy change of TNDMPy in the presence of various concentrations of ct-DNA and BSA. All the experiments were carried out in 5 mM phosphate buffer, 5 mM NaCl, pH 7.0, rt, $\lambda_{\text{ex}} = 345 \text{ nm}$.

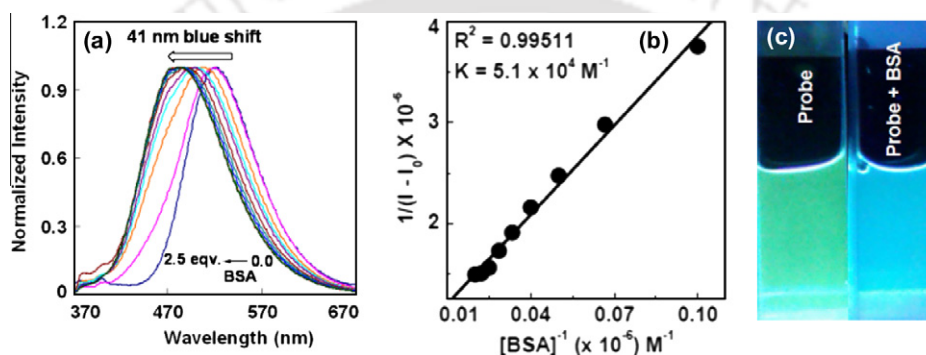


Figure 4. (a) Fluorescence titration of probe with various concentrations of BSA. (b) Benesi-Hildebrand plot. (c) Fluorescence image under UV-transilluminator (254 nm) of only probe, in the presence of BSA. All the experiments were carried out in 5 mM phosphate buffer, pH 7.0, rt, $\lambda_{\text{ex}} = 345 \text{ nm}$.

of BSA and enhancement of fluorescence of the acceptor probe at long wavelength (490–514 nm). This result indicated the occurrence of a FRET process (see [Supplementary data](#)). We calculated the Förster distance of energy transfer which was found to be $R_0 = 24.4 \text{ Å}$ with an energy transfer efficiency of 62.3%.¹⁵

The occurrence of FRET process was also supported by a molecular docking calculation which clearly showed that the probe was located in the vicinity of tryptophan (Trp-237) and remained surrounded by other hydrophobic amino acids of the hydrophobic pocket of sub-domain IIA in site-I of BSA (see [Supplementary data](#)). The free energy of binding (ΔG) of TNDMPy -BSA complex obtained from the docking calculation was found to be in (−7.72 kcal/mol) good agreement with our experimental value. That the probe involved in tight binding inside the hydrophobic pocket of BSA and experienced a highly restricted rotational motion, was further evident from an enhancement of fluorescence anisotropy of the probe from 0.018 in free aqueous buffer to 0.201 in the presence of 2.5 equiv of BSA (Fig. 3b).^{16,17} This large fluorescence enhancement in case of BSA is quite good for practical applications.

Therefore, it is clear from the above findings that the probe triazolylpyrene was efficient in sensing biomolecules via the generation of enhanced fluorescence intensity. The low fluorescence intensity of the probe in phosphate buffer in the absence of biomolecules was not because of poor solubility of the probe in buffer but might be attributed to radiationless channel assisted by intermolecular hydrogen bonding present in aqueous solution. However, in the presence of biomolecules the non-radiative channels were possibly blocked to a great extent and less effective as the probe bound more and more with protein's hydrophobic pocket leading to a fluorescence switch-on signal of enhanced intensity

and quantum yield. The same explanation possibly holds true in case of ct-DNA induced fluorescence enhancement of the probe where the probe experienced restricted radiationless channel inside the groove of ct-DNA.

To explore the versatility of our probe, we also studied the detection ability of short DNA duplex containing triple mismatched base pairs. DNA base mismatch is frequent among the most common forms of DNA lesions which when left unrepaired by the base mismatch repair machinery can lead to deleterious mutations that are dangerous to cellular survival and replication.¹⁸ Therefore, the detection and targeting of such specific small deformation in DNA are of paramount importance for the design of new diagnostics and chemotherapeutics. Several methods have been developed to detect mismatch DNA with fluorescent probe.¹⁹ However, conceptual design of label-free detection which we present here is a novel, simple, cost effective, and rapid strategy (Fig. 5).

Spectroscopic evidences of minor groove binding of the probe with ct-DNA and Amber* optimized geometry of the probe with a model DNA sequence (see [Supplementary data](#)) using MacroModel led us to think of the detection of short oligonucleotide duplex with our probe via just 'Mix & Read' strategy. Our concept relies on the groove binding of the probe assisted by strong interaction possibly via triazolyl nitrogens in the vicinity of a deformed/mismatched position of the DNA duplex. We envisaged that the triazolyl pyrene might involve in stacking interaction along the groove close to the deformed (mismatched) site (Fig. 6a). Such kind of groove binding and recognition of DNA base mismatch were also shown by imidazole-imidazole pair.^{19a} Because of such groove binding we expected that the probe would experience restricted radiationless channel inside the groove that would lead to the

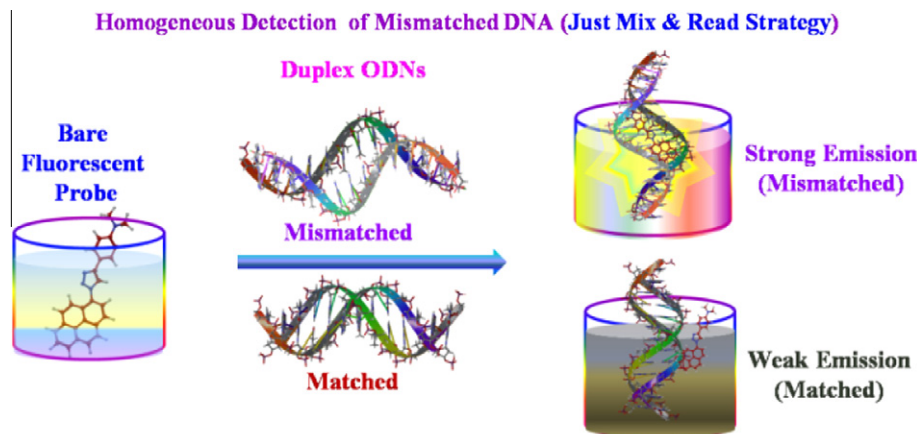


Figure 5. Schematic presentation of our concept of mismatch DNA detection.

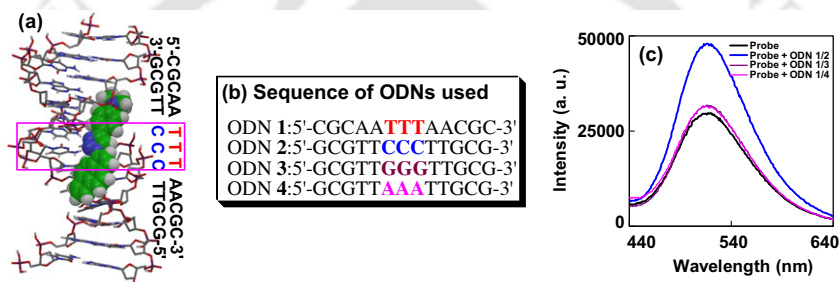


Figure 6. (a) Amber* optimized geometry of probe-mismatched DNA showing groove binding. (b) Sequences of ODNs used. (c) Fluorescence response of TNDMBPy in the presence of various mispaired ODNs at pH 7.0, 50 mM sodium phosphate, 0.1 M sodium chloride, room temperature. Excitation wavelength was, λ_{ex} = 345 nm. Concentration of each single strand and the probe, TNDMBPy was 2.5 μ M.

generation of enhanced fluorescence signal. Thus, we could detect deformed DNA duplex with a light-up fluorescence response from bare fluorescent probe, TNDMBPy.

We also surmised that depending on the sequence at the deformed (mismatched) site the probe might be able to generate a distinct fluorescence signal allowing one to possible discrimination of different mismatched duplex DNAs. Because of quenching nature of G-bases the fluorescence of probe in a triple T/G containing short DNA duplex might not show any enhancement. However, the fluorescence enhancement could be the result when the probe would bind to a triple T/C mismatch containing DNA. Therefore, we could discriminate T/C mismatch DNA from T/G mismatch DNA via the generation of a distinct and enhanced fluorescence signal (Fig. 5).

From the fluorescence spectra it was evident that an enhancement of fluorescence compared to probe's emission was observed upon addition of 2.5 μ M of probe in duplex ODN 1/2 containing triple T/C mispair (Fig. 6b). On the other hand, duplex ODN 1/3 containing triple T/G mispair or a fully matched duplex ODN 1/4 led to no change in emission of bare fluorescent probe, TNDMBPy. These results indicated that our probe was able to discriminate triple T/C mispair from a triple T/G mispair duplex via the generation of an enhanced and distinct fluorescence signal (Fig. 6c). Though the discrimination was not high, our preliminary result might be an inspiration to the design of such fluorescent small molecule to bring efficient discrimination. Currently we are focusing in this aspect.

In conclusion, we have successfully shown, for the first time, that the click chemistry derived triazolyl fluorophore serves as a versatile fluorescent light-up probe for protein and DNA detection

in aqueous media. The probe bound efficiently to the minor groove of ct-DNA and the hydrophobic pocket of BSA protein. These modes of binding were supported by fluorescence anisotropy experiment. Also, the probe was able to detect DNA base mismatch with a generation of distinct fluorescence signal. All the spectral evidences open up a multitude of possibilities for using our probe, triazolylpyrene as a fluorescent light-up bioprobe. Furthermore, because the groove binding event in DNA and strong interaction with BSA are important in consideration of drug-DNA/drug-protein interaction, our probe might find application in studying such events. Our preliminary study on the label-free fluorescent light-up sensing and discrimination of DNA base mismatches revealed that the probe would be very useful and might find future applications for the detection of and targeting DNA lesions with a less laborious and cost-effective label-free way. We are currently exploring a detailed study in the context of sensing G-quadruplex DNA with the same probe.

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Supplementary data

Supplementary data (photophysical spectra, docking/macro-model structures) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tetlet.2013.03.029>.

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Sensing of Micellar Microenvironment with Dual Fluorescent Probe, Triazolyipyrene (^{TNDMB}Py)

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Abstract We report a dual fluorescent triazolyipyrene (^{TNDMB}Py) as an efficient fluorescent light-up probe of various micellar microenvironments. The absorption spectra of ^{TNDMB}Py in an aqueous solution of varying surfactant concentration, CTAB, SDS and TX-100 showed that as the surfactant concentration was increased the absorbance increased with no shift in wavelength maxima. The increase of absorbance in each surfactant solution with increase in surfactant concentration was due to the enhanced solubilization of ^{TNDMB}Py in surfactant solutions. Our investigations based on steady state and time resolved fluorescence techniques showed that the probe reports the microenvironment of ionic surfactant solutions (CTAB and SDS) via dual emission (LE and ICT) at low surfactant concentration. The ICT band showed a blue shifting pattern with enhanced intensity that disappeared as the concentration of surfactant increases (> 1 mM for CTAB and > 3 mM for SDS). In non-ionic surfactant (Triton X-100) solution, the fluorophore showed dual emission with dominant ICT behaviour over LE emission at low concentration (up to 0.35 mM). In reverse micelle we observed a blue shifted ICT band with no LE band with increasing molar concentration of water. We found 100 nm blue shifting when we moved from R=0 to R=7, where R is the molar ratio of water to TX-100 (R=[H₂O]/[TX-100]). The blue shifting of ICT band is because of the movement of the probe from hydrophilic core to hydrophobic core (surface) of the reverse micelle. Thus from the steady-state fluorescence study it was observed that the

ICT band of the probe, ^{TNDMB}Py was more influenced by the micellar environment in comparison to the LE band. This difference in behaviour of the fluorophore is probably because of varying extent of hydrophobic/hydrogen bonding interactions experienced by the probe and its relative disposition inside the various micellar nanocores.

Keywords Triazolyipyrene probe · Micelle · Reverse micelle · Micropolarity · LE and ICT emission · Critical micellization concentration (CMC)

Introduction

The study of cell membrane and the role it plays in living cells is necessary to understand cellular function. It is also important to study drug-membrane interaction with a model system which is a real mimic of cell membrane. Micelles are colloidal-sized clusters formed in solutions from the surfactant molecules [1–14]. They are amphiphilic molecules, like lipids, thus, some of the same rules governing lipid behaviour may also apply to the micelle. Therefore, micelles have widely been used as a model of biomembrane to acquire information about structure, microenvironmental properties and function. Micelles play a vital role in both fundamental and applied sciences. Thus, micelles have been used in several applications such as (a) in accommodating and transporting insoluble or sparingly soluble drugs [15], (b) a model of biomembrane because of their similarity in microenvironment to that of proteins, enzymes, phospholipid bilayer and they mimic biomolecular surfaces and properties [16–19], (c) a model of enzyme's catalytic behavior [20, 21]. Many kinds of reverse micelles (consisting of a polar/aqueous inner core and a nonpolar outer surface) [8–14] are able to solubilize fairly large amount of water and hydrophilic molecules, such as proteins. Thus, they

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found application in biotechnology, in the mimicking of biological structures, in studying the relationship between environment and conformation/activity of functional biopolymers. Many drugs are extremely water-insoluble but may contain many polar moieties. Therefore, a micellar environment that is water insoluble but feature polar groups for dipole interaction or H-bonding can solubilise such drugs thereby micelle can be a carrier of drug candidates.

Therefore, the study of micelles and their role in chemistry, pharmacy, and biology is of paramount importance. Considering the importance of micellar systems in the pharmaceutical field and the many applications that it presents, micelles have widely been used as a model of biomembrane to acquire information about structure, microenvironment properties and functions. Thus, probing of micellar microenvironment with fluorescence sensor molecules or ions is of paramount importance to know the structure and to modulate the charge distribution on the interface and thereby to correlate those with real membrane system. With the introduction of a proper fluorescent probe that is very sensitive to microenvironmental dielectric properties and viscosity, one can study the process of micellisation and also the effect of interfacial electric field on the probe thereby enabling correlation with the biomembrane. In the past few decades considerable efforts have been devoted to investigate the structure, functions, and biological applications of the micelle. Among the various methods available, fluorescent based technique by using a suitable environment sensitive fluorescent probe is one of the most important, easier and efficient technique to determine the structure of micelle and its function. Fluorescence properties of a polarity sensitive probe are highly sensitive towards its surrounding environments. Thus, when a probe comes in contact with surfactant molecules, its emission intensity may either be enhanced or decreased and/or the position of emission wavelength (λ_{max}^f) may be shifted from its original band position either to a shorter or to a longer wavelength region. Among the various fluorescent probes, charge transfer fluorescent probes being by far the most polarity sensitive probes are very important and efficient to probe micellar microenvironment, thereby enabling the determination of structure and function of micelle via the generation of a distinct fluorescence signal.

A substantial amount of research have been carried out to understand the photophysics and the photochemistry of fluorescent molecules in micellar solution [22–28]. Incorporation of the molecular probe into the aqueous micelles effectively reveals parameter such as CMC, roughness of the micelle surfaces, polarity, viscosity and degree of water penetration into these surfactant aggregations. Most of the probes have been chosen for their particular affinity for one or two of the principal micellar region: the hydrocarbon core formed from surfactants tails, the interface composed primarily of the

amphiphilic head groups, or surrounding aqueous solvent phase. Many of the probes are soluble in an extensive range of media, suggesting their inability of probing of specific micellar domains [4, 5, 29, 30]. There are limited numbers of fluorophore that can selectively target specific micellar domains [31]. Therefore, there is need for development of probes with specificity of probing micellar microenvironment via the generation of enhanced fluorescence signal.

As a part of our ongoing search for a suitable environmentally sensitive fluorescent probe, we recently have developed triazolylpyrene (^{TNDMB}Py) as a dual fluorescent probe [32]. Our ongoing observations on fluorescence switch-on probing of ct-DNA, BSA and Cu²⁺ ion led us to explore the possibility of utilising our designed “smart” dual-fluorescent probe, triazolylpyrene (^{TNDMB}Py) in probing of micellar microenvironment. We envisaged that formation of self-assembled complex with the micelle would result in a significant change in the fluorescence emission intensity of either of the LE band (monomer band) or the ICT band or both of the bands of triazolylpyrene. Thus, sensing micellar microenvironment can be achieved by evaluating spectral characteristics of our probe. Keeping this view in our mind, we have framed our objective as follow: (a) to investigate the photophysical property of triazolylpyrene (^{TNDMB}Py), in presence of various surfactants (cationic, anionic and non-ionic) of varying concentration. (b) As we know that, the reverse micelle system is a real mimic of cell membrane, we, thus also interested in studying the photophysical properties of ^{TNDMB}Py in reverse micelle of TX-100 in benzene-hexane (3:7) and water system.

Results and Discussions

Study of Photophysical Properties

The synthesis of the probe (^{TNDMB}Py) was achieved following our reported click chemistry protocol with very high yield [32]. After characterizing and recrystallization which afforded very pure compound we then turned our attention to study the photophysical properties of the probe in various media. Previously we have reported in detailed the photophysical properties of triazolylpyrene in presence of various solvents and solvent mixtures with varying dielectric constant. In a summary the absorption spectrum of the probe did not markedly differ with the solvent polarity; however the emission spectrum strongly differed with the solvent polarity. The molecule showed a dual emission behavior centered at 383 nm, a locally emission (LE) band and 466–544 nm, internal charge transfer (ICT) band (Fig. 1). In high polar solvents (acetonitrile, methanol) the probe showed a low intense LE (local emission) band, characteristic structured band of pyrene. In ether, dioxane, toluene, chloroform and ethyl acetate a dual emission was

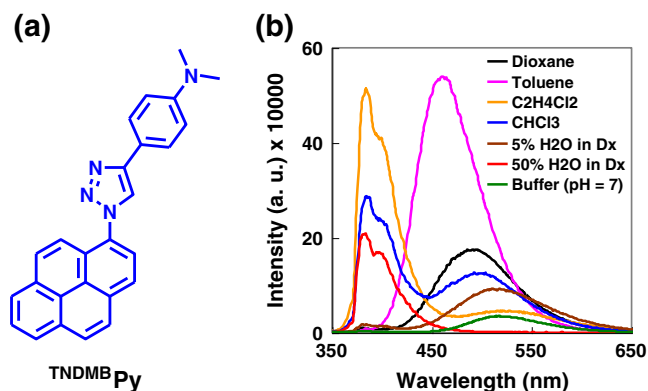


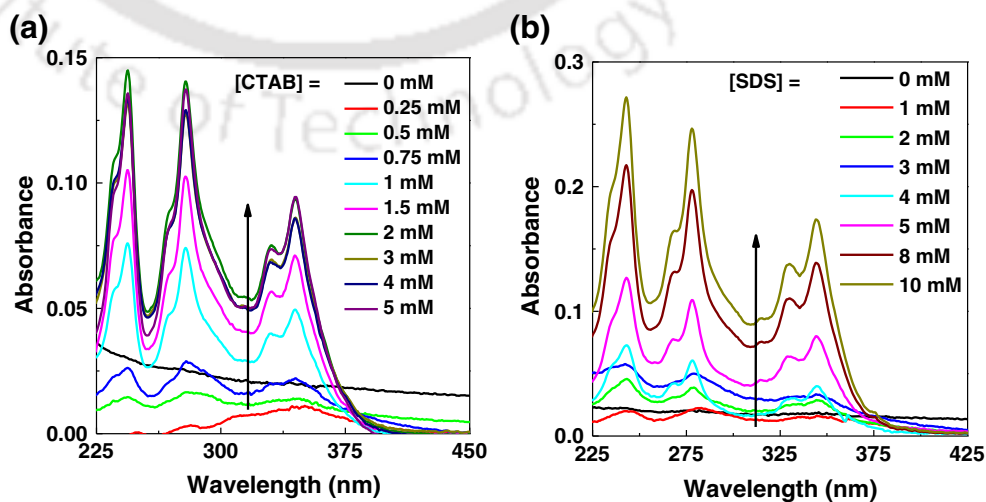
Fig. 1 **a** Structure of triazolypyrene probe and **b** emissions behavior of the probe in different solvents

observed along with a strong red shifted pattern of the ICT (internal charge transfer) band as the solvent polarity increased from toluene (466 nm) to ethyl acetate (544 nm) [32]. Our probe consists of a *N,N*-dimethyl aminophenyl donor linked triazole functional which serves as an efficient electron donor and a pyrene functional which acts as an electron acceptor. Thus, the probe consists of an efficient charge transfer property. A dioxane water titration revealed that the long wavelength emission band is not because of aggregation (see [Supplementary data](#)). Linear increasing relationship between absorbance and emissions at both the emission wavelengths and concentration of the probe suggested that the long wavelength emission band originated purely from an intermolecular charge transfer (ICT) state and not because of excimer emission (see [Supplementary data](#)).

Study of UV-Visible Spectra

Next, we have studied, the photophysical properties of probe in presence of cationic (CTAB), anionic (SDS) and non-ionic (Triton X-100) surfactant and in presence of reverse micelle (TX-100 in 3:7 benzene-hexane and water

Fig. 2 UV-visible spectra of TNDMBPy in aqueous solution of varying surfactant concentration of **(a)** CTAB and **(b)** SDS. $[TNDMBPy] = 10 \mu M$



system). Thus, the absorption spectra of TNDMBPy in aqueous solutions of varying surfactant concentration, CTAB, SDS (Fig. 2) and TX-100 and in reverse micelle (Fig. 3) were recorded keeping the concentration of the probe fixed. From these spectra it is clear that the absorbance of the probe is increased with increase in surfactant concentrations even beyond the critical micellization concentrations of all the surfactants. However, the absorption wavelength maxima, λ_{max}^{abs} remains unaltered and centered at around 344 nm in all the cases. The increase in absorbance in each surfactant solution with increase in surfactant concentration is attributed to the enhanced solubilization of the probe (TNDMBPy) in surfactant solutions.

However, in reverse micellar system we observed no regular change in absorbance value, with increase in molar ratio of water.

Study of Fluorescence Spectra

The steady state fluorescence techniques have been widely used to study the micellar microenvironment and the mechanism of micelle formation with the help of a suitable fluorescent probe. As our designed probe, TNDMBPy consists of a donor (*N,N*-dimethyl aminophenyl linked with triazole) as well as acceptor (pyrene) groups linked via a triazole unit, so there may be a possibility of charge transfer in the excited state, which would be affected by the polarity of the surrounding environment. The charge transfer phenomenon of the probe, TNDMBPy in dioxane-water system was well studied. A dual fluorescence emission (LE, local emission and ICT, internal charge transfer) was observed in dioxane-water solvent system. With increase of % of water content, a red shifted ICT band with decreased intensity was observed. It was studied that the dual fluorescence appears from the equilibrium between two states, LE and ICT in the excited state [32]. The short wavelength emission band arises from the locally excited (LE) state and the long

wavelength emission band arises from the newly generated ICT state having higher dipole moment than the LE state. The ICT state is stabilized by the solvent polarity and thus the energy gap between ground and excited state decreases and we observe a long wavelength emission, called ICT band.

The steady-state fluorescence spectra of ^{TN}DMBPy in all three surfactant solutions, CTAB, SDS and TX-100 as well as in reverse micelle were recorded. In ionic surfactant solution a dual emission (LE and ICT) was observed at low surfactant concentration (insets Fig. 4). The ICT band showed a blue shifting pattern with enhanced intensity that disappeared as the concentration of surfactant increases (> 1 mM for CTAB and > 3 mM for SDS, Fig. 4a and b respectively). Thus, this observation suggested that micro-environment around the probe, ^{TN}DMBPy is different from the pure aqueous phase. The spectral shifts and the enhancement of the fluorescence intensities of ICT band at low surfactant concentration and gradual increase of the fluorescence intensities of LE band with the increase in surfactant concentration can be explained in terms of binding of this probe to a less polar site of the micelle.

In nonionic surfactant solution, Triton X-100 and in reverse micelle of Triton X-100 in benzene-hexane and water system, we observed an interesting emission behavior of the probe ^{TN}DMBPy. Thus, in low TX-100 concentration (up to 0.35 mM), the fluorophore showed dual emission with dominant ICT emission over LE emission. However, at high concentration the LE band predominates over ICT band (Fig. 5a).

In reverse micelle we observed a blue shifted pattern of the ICT band with no emission from LE state as the molar concentration of water is increased (Fig. 5b). We found 100 nm blue shifting when we moved from R=0 to R=7, where R is the molar ratio of water to TX-100 ($R = [H_2O]/[TX-100]$). The blue shifting of ICT band is probably because of movement of the probe from hydrophilic core to more hydrophobic core (surface) of the reverse micelle. Thus, from the steady-state fluorescence study it was observed that the ICT band of the

probe, ^{TN}DMBPy was more influenced by the micellar environment in comparison to the LE band.

Time Resolved Fluorescence Study

Fluorescence lifetime of a molecule is very sensitive towards its local environment. Thus this study can nicely explain the location and distribution of the probe in micellar microenvironment. The time resolved fluorescence spectra of ^{TN}DMBPy were recorded in all three surfactant solutions, CTAB (Fig. 4c), SDS (Fig. 4d) and TX-100 (Fig. 5c) as well as in reverse micelle of Triton X-100 in benzene-hexane and water system (Fig. 5d).

The fluorescent life time (τ) was expressed using the following two equations.

$$a_n = \frac{B_n}{\sum_{i=1}^N B_i} \quad (1)$$

where a is the fluorescence lifetime coefficient and B is the pre-exponential factor. The mean lifetime was calculated by the following equation

$$\langle \tau \rangle = \frac{\sum a_i \tau_i^2}{\sum a_i \tau_i} \quad (2)$$

where τ_i and a_i are the fluorescence lifetime and its coefficient of the i^{th} component respectively

From the time resolved fluorescence study, it was observed that the decay time (τ) of ^{TN}DMBPy increases with increasing surfactant concentration for all three surfactants (CTAB, SDS and TX-100). These results showed a good correlation with the steady state fluorescence study, where we observed an increase in fluorescence intensity with increase in surfactant concentration. In SDS surfactant, a bi-exponential decay with increasing mean lifetime ($\langle \tau \rangle$) of ^{TN}DMBPy was observed as the concentration of SDS was increased (Table 1 and Fig. 4d).

Fig. 3 UV-visible spectra of ^{TN}DMBPy in **a** aqueous solution of varying TX-100 concentration and **b** in reverse micelle of TX-100 in benzene-hexane (3:7) and water mixture. [^{TN}DMBPy]=10 μ M

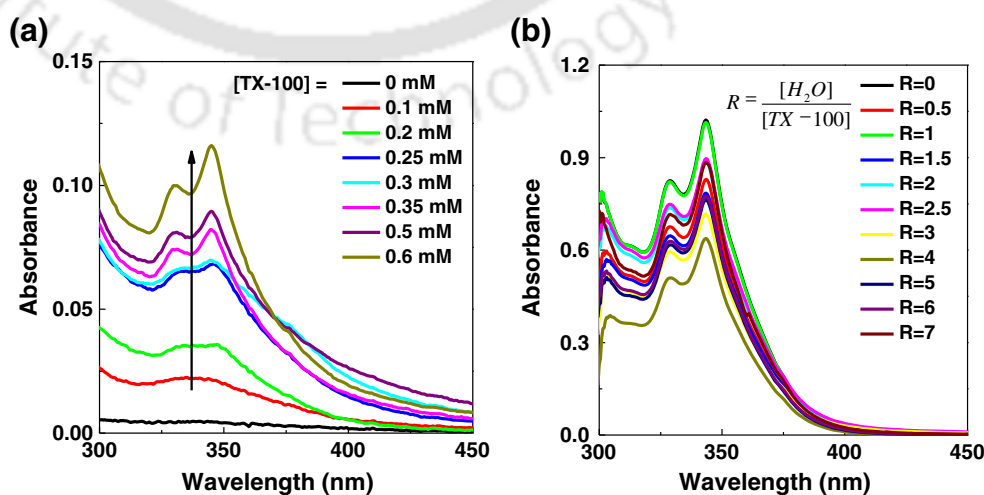


Fig. 4 Fluorescence spectra ($\lambda_{\text{ex}}=343$ nm) of TNDMBPy in aqueous solution of varying surfactant concentration of **a** CTAB and **b** SDS. $[\text{TNDMBPy}]=10$ μM . Time resolved fluorescence spectra of TNDMBPy in presence of **c** CTAB and **d** SDS ($\lambda_{\text{em}}=\text{LE}$ band)

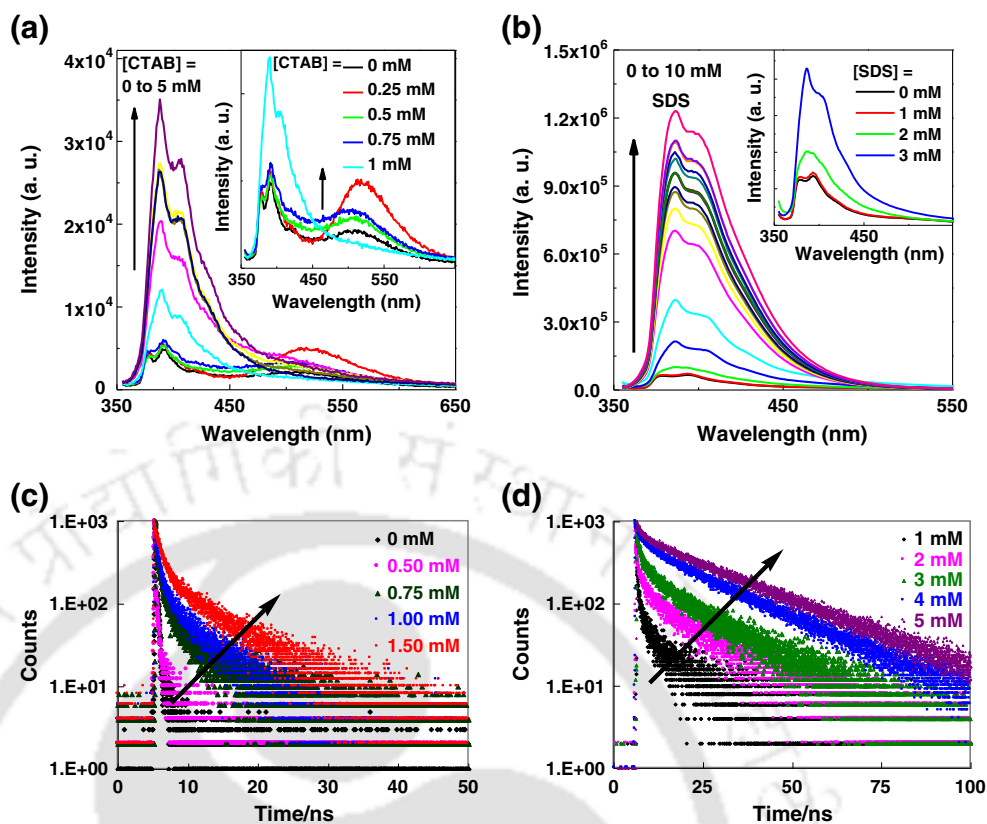


Fig. 5 Fluorescence spectra ($\lambda_{\text{ex}}=343$ nm) of TNDMBPy in aqueous solution of varying concentration of TX-100 surfactant and **b** in reverse micelle of TX-100 in benzene-hexane (3:7) and water mixture. R is molar ratio of water to TX-100. Time resolved fluorescence spectra of TNDMBPy in presence of **c** TX-100 surfactant and **d** in reverse micelle of TX-100 ($\lambda_{\text{em}}=\text{ICT}$ band)

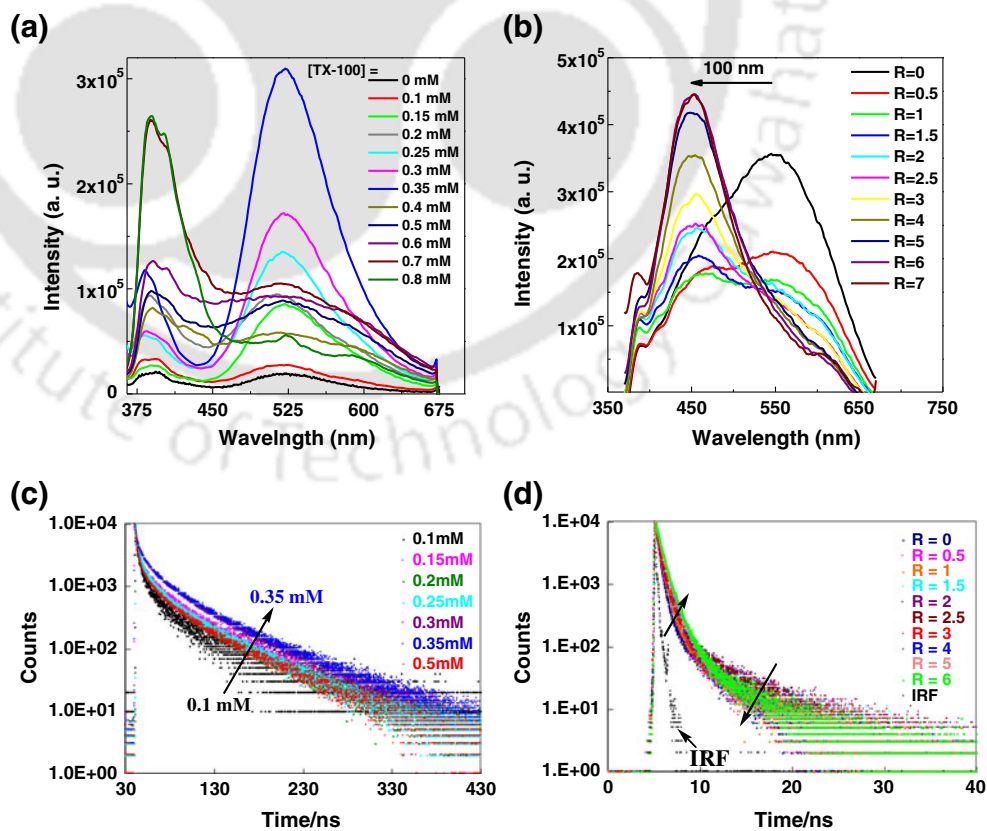


Table 1 Summary of time resolved fluorescence of ^{TN}DMBPy in presence of SDS, monitored at LE band (400 nm)

[SDS] (mM)	τ_1 (ns)	τ_2 (ns)	$\langle\tau\rangle$ (ns)	Amplitude	
				B ₁	B ₂
1	0.74 (77.3 %)	8.54 (22.7 %)	6.76	91.04	26.69
2	2.07 (60.8 %)	16.7 (39.2 %)	14.3	91.28	58.78
3	2.10 (53.1 %)	16.5 (46.9 %)	14.7	132.40	116.84
4	2.68 (37.5 %)	21.5 (62.5 %)	20.2	294.59	491.77
5	2.23 (27.1 %)	24.7 (72.9 %)	24.0	236.13	633.78

The mean lifetime increases from 6.76 ns in 1 mM SDS to 24.0 ns in 5 mM SDS concentration. The increase in decay time and the fluorescence intensity is probably because of substantial decrease in non-radiative decay in the micellar environment. From the bi-exponential fitting we observed that τ_1 and τ_2 increases (monitoring wavelength was LE band) with increasing surfactant concentration, however, the abundance of first component (a_1) decreases and the second component (a_2) increases with increasing surfactant concentration.

Time resolved fluorescence was also measured by monitoring the ICT band (515–480 nm) in SDS surfactant solution (Table 2 and see [Supplementary data](#)). A bi-exponential fitting of the decay curve revealed that as SDS concentration increases from 1 mM to 3 mM, the mean lifetime decreases. This result also correlates well with the steady state fluorescence study, where we observed no ICT band at high SDS (> 3 mM) concentration.

Next, we have measured the lifetime of the probe in presence of cationic surfactant, CTAB solution (Fig. 4c). The lifetime data were fitted to a biexponential decay fitting curve. Thus, similar to the case of SDS surfactant, a bi-exponential decay with increasing mean lifetime ($\langle\tau\rangle$) of ^{TN}DMBPy was observed as the concentration of CTAB was increased (Table 3 and Fig. 4c). The mean lifetime increases from 0.53 ns in water to 6.41 ns in 1.5 mM CTAB concentration. The increase in decay time and the fluorescence intensity is probably because of substantial decrease of non-radiative decay in the micellar environment.

After measuring the time resolved fluorescence of the probe in presence of ionic surfactants we next investigated the same in presence of neutral surfactant. Thus, we observed a similar result in presence of non-ionic surfactant as that was observed in the case of ionic

surfactant. The decay time of ^{TN}DMBPy was increased with increase in concentration of TX-100 up to 0.35 mM, and then it started decreasing as the concentration was increased beyond 0.35 mM (Fig. 5c, Table 4). Our result on the time resolved fluorescence response of the probe upon changing the concentration of neutral surfactant showed a very good correlation with the steady state fluorescence study where an increase in intensity was observed till the concentration of TX-100 increased to 0.35 mM and it decreased upon further increase in concentration of TX-100. We observed a bi-exponential fitting with increase in mean decay time from 28.1 ns in 0.1 mM TX-100 to 50.7 ns in 0.35 mM TX-100. Further increase in concentration of TX-100 showed a decrease in decay time of the probe (45.1 ns in 0.5 mM TX-100, Fig. 5c, Table 4).

After studying the photophysical behaviours of the probe in anionic, cationic and neutral surfactant solution, we next turn our attention to study the time resolved fluorescence behaviour of our probe in presence of TX-100 in benzene-hexane-water system where reverse micelle formation takes place. Thus, we observed a multi exponential fitting with slight change in the decay time in this reverse micellar system (Fig. 5d and Table 5).

Thus, a triexponential decay fitting with very short decay time of the first two major components and a long decay time ($\tau_3=4.35$ ns) with least abundant (1.64 %) third component was observed when $R=0$. The decay time of the third component increases from 4.35 ns to 5.73 ns upon increase in $R=0$ to $R=2.5$ and beyond $R=2.5$, it decreased. However, the decay time of first two components showed gradual increase with increase in molar ratio of water (Table 5).

Table 2 Time resolved fluorescence summary of ^{TN}DMBPy in presence of SDS monitored at ICT band

[SDS] (mM)	$\lambda_{em(ICT)}$ (nm)	τ_1 (ns)	τ_2 (ns)	$\langle\tau\rangle$ (ns)	Amplitude	
					B ₁	B ₂
1	515	4.2 (60.46 %)	25.34 (39.54 %)	21.07	122.44	80.07
2	500	3.26 (64.64 %)	19.22 (35.36 %)	15.44	80.75	44.17
3	480	2.57 (70.95 %)	19.85 (29.05 %)	15.70	95.51	39.10

Table 3 Time resolved fluorescence summary of ^{TN}DMBPy in presence of CTAB, monitored at LE band (400 nm)

[CTAB] (mM)	τ_1 (ns)	τ_2 (ns)	$\langle\tau\rangle$ (ns)	Amplitude	
				B ₁	B ₂
0	0.24 (90.8 %)	1.15 (9.2 %)	0.53	132.18	13.36
0.5	0.35 (93.5 %)	1.97 (6.5 %)	0.80	80.49	5.62
0.75	0.80 (78.7 %)	5.93 (21.3 %)	4.22	129.87	35.19
1	0.86 (74.9 %)	6.56 (25.1 %)	4.94	154.71	51.79
1.5	0.99 (70.2 %)	7.99 (29.8 %)	6.41	238.23	101.16

Determination of Critical Micellization Concentration (CMC)

Surfactant molecules aggregate in liquid to form micelle in solution. At which concentration, the surfactant molecules formed micelles is called the critical micelle concentration (CMC). The microenvironments above and below the CMC are different. From the steady-state fluorescence study we observed that the fluorescence intensity of the probe, ^{TN}DMBPy increases with increase in surfactant concentration for the case of all surfactant solution (SDS, CTAB and TX-100). After a certain concentration limit the fluorescence intensity drastically jumped to a higher value and again smoothly increases. Thus, the CMC value of all the three surfactants, CTAB, SDS and TX-100 were calculated with the help of steady-state fluorescence spectra of ^{TN}DMBPy. The plots of fluorescence Intensity vs. [surfactant] are shown in the following Fig. 6. The experimental CMC values of three surfactants obtained from the plots were 0.7 mM for CTAB, 6.5 mM for SDS and 0.26 mM for TX-100 are close and comparable with the literature reported values of 0.94 mM for CTAB, 7.80 mM for SDS and 0.26 mM for TX-100 (Table 6).

Determination of Binding Constant

The fluorescence intensity of the probe in aqueous and in micellar media is far different. Thus, the binding constant

(*K*) of the probe with micelle was calculated from the fluorescence intensity values in micelle media. We used the following relationship demonstrated by Almgren et al., [34] to calculate the binding constants (*K*s) of the probe in various micelles.

$$\frac{(I_{\infty} - I_0)}{(I_t - I_0)} = 1 + (K[M])^{-1} \quad (3)$$

where *I*_∞, *I*₀, and *I*_t are the fluorescence intensities of the probe under complete micellization, in the absence of micellization, and in presence of intermediate amounts of surfactant, respectively. [*M*] is the micelle concentration, which can be expressed by the following relationship

$$[M] = \frac{(S - CMC)}{N} \quad (4)$$

where *S* is the surfactant concentration, *CMC* is the critical micelle concentration and *N* is the aggregation number of the micelle. The *N* values used in the calculation of [*M*] are 60 for CTAB, 62 for SDS, and 143 for Triton X-100 [35]. The plots of (*I*_∞ - *I*₀)/(*I*_t - *I*₀) vs. [*M*]⁻¹ for all the three micelles, CTAB, SDS and TX-100 are shown in Fig. 7a-c respectively.

The calculated binding constants of the probe in various micelles are summarized in Table 6. Thus, we have seen that the binding constant of the probe with

Table 4 Time resolved fluorescence summary of ^{TN}DMBPy in presence of TX-100, monitored at ICT band (520 nm)

[TX-100] (mM)	τ_1 (ns)	τ_2 (ns)	$\langle\tau\rangle$ (ns)	Amplitude	
				B ₁	B ₂
0.1	4.02 (84.9 %)	41.3 (15.1 %)	28.1	572.23	101.50
0.15	10.6 (64.7 %)	56.2 (35.3 %)	44.5	1981.66	1082.10
0.2	10.2 (67.3 %)	54.1 (32.7 %)	41.8	1870.381	910.27
0.25	11.0 (68.4 %)	57.2 (31.6 %)	43.6	1924.27	889.86
0.3	11.7 (63.8 %)	57.5 (36.2 %)	45.4	2689.36	1525.56
0.35	15.0 (62.5 %)	64.5 (37.5 %)	50.7	2159.30	1293.09
0.4	13.6 (55.5 %)	60.0 (44.5 %)	49.8	2545.23	2037.48
0.5	11.6 (63.1 %)	56.9 (36.9 %)	45.1	1292.52	756.16
0.7	8.95 (70.5 %)	51.1 (29.5 %)	38.7	1002.11	419.59

Table 5 Time resolved fluorescence summary of ^{TN}DMBPy in presence of reverse micelle, monitored at ICT band

R ^[a]	λ_{em} (nm)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	$\langle\tau\rangle$ (ns)	Amplitude		
						B ₁	B ₂	B ₃
0	460	0.266 (72.81 %)	0.700 (25.55 %)	4.35 (1.64 %)	1.10	7054.78	2476.22	158.88
0.5	460	0.269 (60.66 %)	0.766 (37.86 %)	4.50 (1.48 %)	1.09	5937.20	3705.49	144.51
1	460	0.276 (61.20 %)	0.764 (37.25 %)	4.57 (1.56 %)	1.12	5898.99	3590.56	150.09
1.5	455	0.298 (61.10 %)	0.820 (37.32 %)	5.15 (1.57 %)	1.27	5874.53	3588.46	151.12
2	450	0.339 (66.16 %)	0.912 (32.09 %)	5.64 (1.75 %)	1.46	6193.60	3004.39	163.75
2.5	450	0.366 (68.98 %)	0.955 (29.54 %)	5.73 (1.49 %)	1.37	6420.28	2749.14	138.62
3	450	0.384 (63.86 %)	0.909 (35.08 %)	5.18 (1.06 %)	1.08	5937.21	3260.93	98.41
4	450	0.474 (66.95 %)	1.02 (31.93 %)	4.96 (1.12 %)	1.09	6327.04	3017.93	106.00
5	450	0.485 (63.89 %)	1.05 (36.06 %)	5.11 (0.05 %)	0.81	6019.18	3397.44	4.59
6	450	0.502 (61.91 %)	1.07 (37.02 %)	5.28 (1.07 %)	1.15	6005.99	3590.79	104.03

^[a] R = [H₂O]/[TX-100]

cationic, anionic and non-ionic micelle follow the order CTAB<SDS<TX-100.

The excited state dipole moment of triazolylpyrene was calculated to be 17.07 D [32]. During the course of charge transfer process from the donor *N,N*-dimethylaminophenyltriazol unit to the acceptor pyrene of the probe, the -NMe₂ group will carry more positive charge and the acceptor part will carry more negative charge. In case of SDS micelle the Stern layer contains negatively charged head groups and the Gouy-Chapman layer contains positively charged counter ions. Therefore, the probe more likely would reside in the hydrophobic core of the micelle exposing toward Stern layer. This is supported by quenching of monomer emission of the probe by Cu²⁺ as quencher in the SDS micellar environment [36]. In aqueous medium of SDS solution the probe showed only a local emission at around 390 nm with no ICT emission. Upon addition of Cu²⁺ ion a fluorescence quenching of the LE band was observed [36]. This can be explained if we

consider an electron transfer process from the pyrene unit to the Cu²⁺ ion in micelle-probe interface.

Conclusions

In conclusion, we have investigated the photophysical response of a donor acceptor charge transfer fluorescent probe, triazolylpyrene (^{TN}DMBPy) inside the micellar microenvironment. In ionic surfactant solution the probe showed a dual emission (LE and ICT) at low surfactant concentration. The ICT band showed a blue shifting pattern with enhanced intensity that disappeared as the concentration of surfactant increases (> 1 mM for CTAB and > 3 mM for SDS). At low concentration of non-ionic surfactant TX-100, the probe showed dual emission with dominant ICT emission over LE emission. However, at high concentration of TX-100, the LE band predominates over ICT band. In reverse micelle a blue shifted pattern of the ICT band with no LE band was observed with increase in molar concentration of water. Over all, the

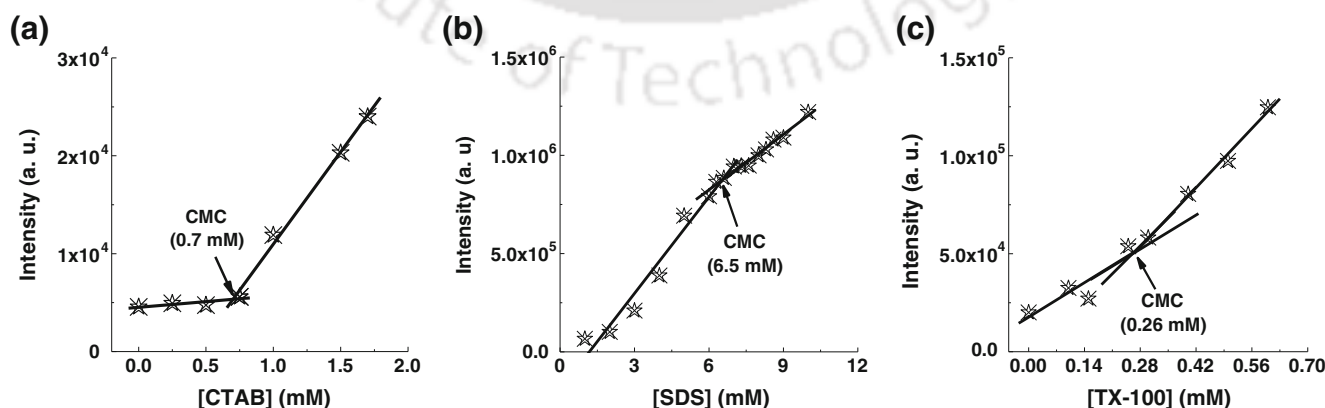


Fig. 6 Plots of fluorescence intensity vs. **a** [CTAB], **b** [SDS] and **c** [TX-100]

Table 6 Summary table for the CMC and binding constant values

Medium	CMC (mM) (Experimental)	CMC (mM) (Reported) [33]	Binding Constant (K_{eq}) (M^{-1})
CTAB	0.70	0.94	4.28×10^4
SDS	6.50	7.80	2.12×10^5
TX-100	0.26	0.26	6.84×10^5

ICT emission was found to be modulated much more by the media than the LE emission. The changes in polarity and strong interaction of the probe with micelles deactivate the non-radiative decay channels thereby increasing fluorescence intensity. The micelle probe interaction was studied spectroscopically. The experimental CMC values of surfactant from the fluorescence intensity vs. [surfactant] plot are in good agreement with the literature reported values. The binding constant of the probe with cationic, anionic and non-ionic micelle follow the order CTAB < SDS < TX-100. Thus, we have successfully demonstrated that our probe, triazolyipyrene ($^{TNDMB}Py$) is an efficient fluorescence switch-on probe of micellar microenvironment. Therefore, our probe might find application in probing of membrane in biological systems which expectedly would generate a distinct fluorescence signal for such interaction events and shed light to the environment around it inside the cell.

Materials and Methods

Materials

CTAB, SDS, benzene and hexane were purchased from Merck, India. Triton X-100 was purchased from SRL, India. Water was obtained from a Milli-Q purification system. All experiments were performed with freshly prepared solutions in Milli-Q water. The probe molecule was synthesized and

purified according to the reported procedure. We recrystallised the column purified material in chloroform-ethylacetate solvent mixture twice, dried under vacuum, again characterized and used for study.

Preparation of Probe-Surfactant Solutions

The various concentrations of a particular surfactant solution were prepared by using Milli-Q water. Then the surfactant solutions of various concentrations were added to the sample vials containing probe. The sample solutions were mixed well before the experiment.

UV-Visible Study

The UV-Visible absorbance measurements were performed using Shimadzu 2550 UV/vis spectrophotometer with a cell of 1 cm path length at 298 K. The probe with surfactant solutions were mixed well before doing the experiment. The measurements were taken in absorbance mode. The absorbance values of the sample solutions were measured in the wavelength regime of 200–600 nm.

Fluorescence Study

All fluorescence studies were performed using a Fluoromax 4 spectrophotometer with a cell of 1 cm path length at room temperature. The excitation wavelength for probe ($^{TNDMB}Py$) was set at 343 nm, and emission spectra were measured in the wavelength regime of 350–675 nm. The probe with surfactant solutions were mixed well before doing the experiment. Fluorescence lifetimes were measured by Edinburgh instrument, Life Space II with Edinburgh 375 nm Laser source for excitation. The fluorescence decay was analyzed by tail fitting method using in built software. All the experiments were carried out at 298 K.

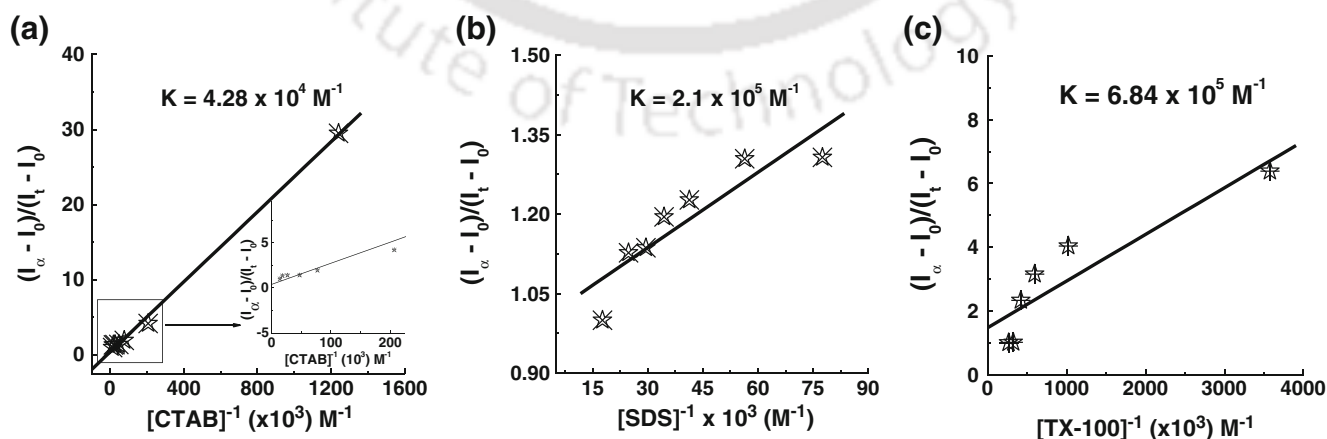


Fig. 7 Plots of $(I_{\infty} - I_0)/(I_t - I_0)$ vs. $[Surfactant]^{-1}$ of $^{TNDMB}Py$ for **a** CTAB, **b** SDS and **c** TX-100

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Fluorometric sensing of Cu^{2+} ion with smart fluorescence light-up probe, triazolylpyrene (TNDMPy)

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ABSTRACT

We report the detection of Cu^{2+} ion with fluorescence light-up probe, triazolylpyrene (TNDMPy). Thus, the probe showed a remarkable fluorescence enhancement of both of the monomer and excimer emissions and a 2:1 probe– Cu^{2+} complexation with high selectivity, high stability constant and low detection limit. Formation of the excimer was also rationalized on the basis of DFT calculation. Our probe is also capable of sensing SDS micelle-encapsulated- Cu^{2+} ion in aqueous media with high association constant, thus, showing its practical utility. Thus, our probe could be used as an efficient Cu^{2+} ion selective fluorescence light-up probe, and may find applications in chemical and biological systems.

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The design of highly selective and sensitive fluorescent sensors capable of detecting transition-metal ions, especially Cu^{2+} ion has attracted considerable attention since many years as this metal ion is not only a significant environmental pollutant^{1b–d} but also the third most abundant and an essential trace element^{1c–e} in human biology.¹ Thus, considerable efforts have been devoted to the development of Cu^{2+} ion selective fluorescent chemosensors.^{1–3} However, Cu^{2+} is a well-known paramagnetic ion and strongly quenches the fluorescence of the fluorophore near it via electron or energy transfer. Therefore, there are many reports of ‘turn-off’ fluorescent sensors for Cu^{2+} ions which are less sensitive, and offer limited resolution.² On the other hand, the ‘turn-on’ fluorescent sensors are highly sensitive and offer high resolution. However, the design of such probes is a challenging task as being a paramagnetic metal ion Cu^{2+} often leads to quenching of the fluorescence of the probe; thus limiting the abundance of ‘turn-on’ fluorescent sensor for Cu^{2+} ion.³ Only in recent years, a few examples of ‘turn-on’ sensors have appeared; however, many of them need special and complex designed architecture for coordination. Therefore, there is a high need to design cost effective, less cumbersome, simple, new and novel ‘turn-on’ fluorescent probes to detect Cu^{2+} ion.

Many of the fluoroionophore for cation recognition are composed of a cation recognition site with a fluorescent moiety.⁴ Pyrene is the most frequently used fluorescent unit appended along with a cation recognition unit due to its relatively efficient excimer formation ability and strong emission properties like high quantum

yield.⁵ Nitrogenous ligands in many cases are found to be utilized as binding sites for the selective recognition of metal ions. In this respect ‘click triazole’ might be an excellent choice.⁶ Whereas the ‘click triazole’ has been frequently utilized as a spacer between two functional units or as a linker in the areas of functional materials, biology, polymer science, drug discovery, etc.,⁶ its potential as a metal coordination platform with a generation of fluorescence signal has been explored comparatively less and are needed complex supramolecular architectures for recognition.⁷ Thus, there is a high need for the design of simple and efficient triazole based ‘light-up’ fluorescent sensor for trace metal ions like Cu^{2+} .

As a part of our ongoing research efforts toward the design of solvatochromic fluorescent small organic molecules via click reaction, we recently, have shown that the triazolyl units are capable of installation of fluorescence property into a non fluorescent precursor as well as electronic coupling between a triazole and a fluorophore give rise to a modulated emission response to the fluorophore.⁸ Thus, our reported triazolylpyrene showed an interesting fluorescence property with high solvatochromicity and quantum yield (Fig. 1a). In brief, the molecule shows a dual emission behaviour appearing at 383 nm (LE) and 466–544 nm (ICT, depending on solvent polarity). In particular, in CH_3CN it shows only a faint LE emission similar to the monomer emission of pyrene (Fig. 1b).

Therefore, owing to our previous result, we thought that it would be worthwhile to exploit this fluorophore’s emission property to the study of possible sensing of metal ions. We envisaged that the formation of self-assembled complex with metal ions through the coordination with triazolyl nitrogens would result in a remarkable change in the fluorescence emission intensity of the

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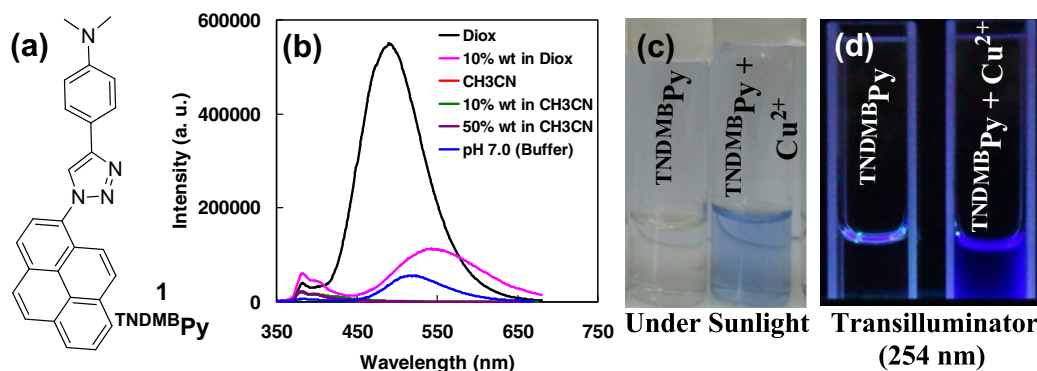


Figure 1. (a) Structure of triazolylpyrene probe. (b) Emission behaviour of the probe in different solvents. Colour of the probe's solution mixed with 2.5 equiv of Cu^{2+} ion (c) under day light and (d) under a transilluminator when irradiated at 254 nm.

LE band (monomer band) of triazolyl pyrene in acetonitrile. We also expected the possibility that the two pyrene units of two molecules of the probe might come closer to form an intermolecular excimer upon binding with Cu^{2+} ion. Thus, sensing of Cu^{2+} ion can be achieved by measuring the fluorescence response either from monomer or from excimer or both.

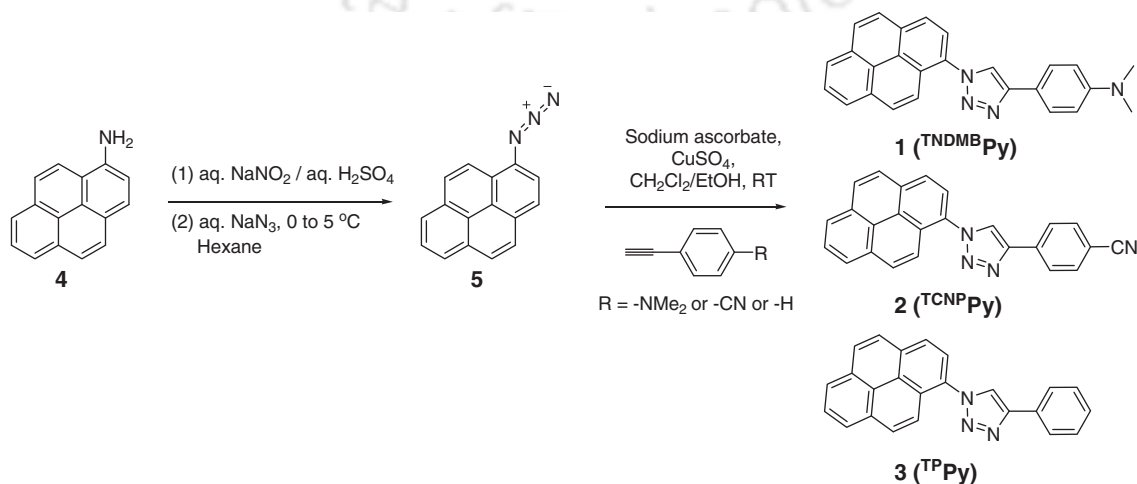
Therefore, in this report we want to present our newly designed smart fluorescent sensor triazolyl pyrene (**TNDMBPy**, **1**, Fig. 1a) in sensing of Cu^{2+} ion with a remarkable enhancement of fluorescence intensity. Our probe consists of a *N,N*-dimethyl aminophenyl donor linked triazole functionality which serves dual role: electron donating property of the triazolyl ring effectively modulates the emission response of an electronically coupled pyrene that is acting as an electron acceptor and the triazolyl nitrogens serve as a recognition and binding site for the metal ions. Sensor **TNDMBPy** displays a high sensitivity, and selectivity for Cu^{2+} among the metal ions examined with a switch-on fluorescence property.

The synthesis of the probe **1** was achieved following our reported click chemistry protocol with very high yield and is shown in Scheme 1.⁸ Thus, 1-aminopyrene was converted into the corresponding 1-azidopyrene via reaction with $\text{NaNO}_2/\text{aq. H}_2\text{SO}_4$ followed by reaction with NaN_3 at 0°C . Next, 1-azidopyrene was reacted with terminal *N,N*-dimethylaminophenyl acetylene under click reaction condition in the presence of sodium ascorbate and CuSO_4 in a mixture of CH_2Cl_2 –EtOH solvent at room temperature to form product **1**, **TNDMBPy** (Scheme 1). In a similar way probes **2–3** were also synthesized. After purification via silica-gel column

chromatography, recrystallization, and characterization by NMR and HRMS spectrometry, we turned our attention to study the photophysical properties in different solvents.

After getting the very pure compound **1** in hand, we turned our attention to study the photophysical properties in different solvents (see Supplementary data). To examine the optical sensor property of our probe for metal ions, the tested metal ions Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Co^{2+} , Ni^{2+} , Cu^+ , Cu^{2+} , Zn^{2+} and Ag^+ were added to the solution of **1**, 'respectively', and the photophysical properties were studied. Thus, from the UV–visible absorption it was observed that the Cu^{2+} ion induces a change in absorption spectra, while other metals have negligible/inconsistent effect which implies that only the Cu^{2+} ion interacts significantly in a well-defined manner with the probe under the experimental condition and is also evident from the generation of bright blue colour of the solution under day light (Fig. 1c). To investigate the binding property of the probe toward the Cu^{2+} ion, titration experiments were carried out which showed a considerable interaction between probe and the metal (see Supplementary data). A plot of the variation of the absorbance ($A_0 - A_1$) at 343 nm versus molar ratio of Cu^{2+} ion (X_M) revealed a clear formation of 2:1 probe– Cu^{2+} complex (see Supplementary data).

To get further insight into the binding properties and to investigate the chemosensor behaviour of the probe toward metal ions, we then investigated the fluorescence changes upon addition of various concentrations of different metal cations to the CH_3CN solution of the probe upon excitation at 343 nm. On addition of



Scheme 1. Synthesis of the probe triazolylpyrenes via click reaction.

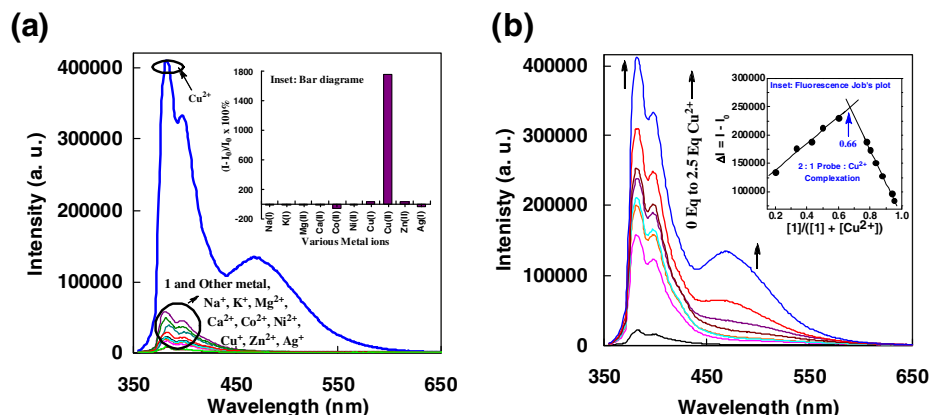


Figure 2. (a) Fluorescence responses of **1** (10 μ M) in CH_3CN upon the addition of (2.5 equiv) various metal ions; Inset: bar diagram. (b) Fluorescence titration of **1** (10 μ M) with various concentrations of $\text{Cu}(\text{ClO}_4)_2$ in CH_3CN ($\lambda_{\text{ex}} = 343$ nm); Inset: fluorescence Job's plot for the determination of stoichiometry of **1**– Cu^{2+} complex.

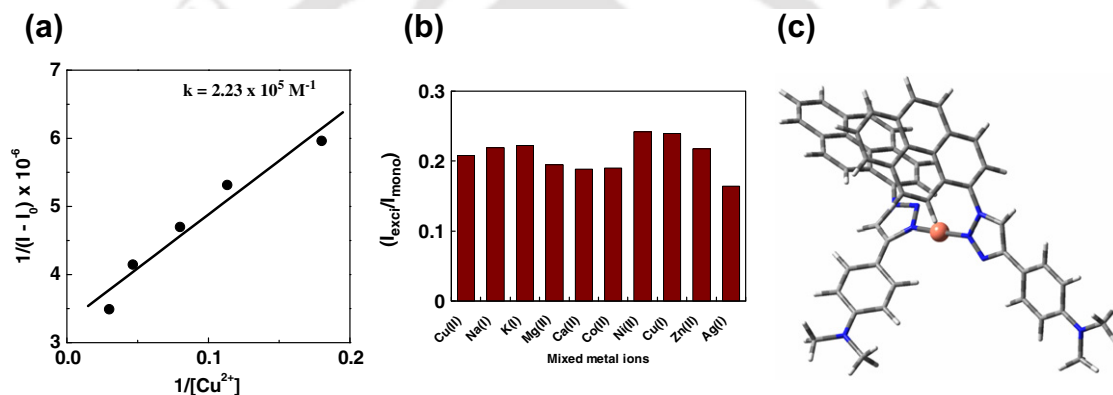


Figure 3. (a) Benesi–Hildebrand plot of the monomer band of **1** with Cu^{2+} (linear region) for the determination of stability constant. (b) Fluorescence response (ratio of fluorescence intensity at 466 and 384 nm) of **1** (10 μ M) in CH_3CN upon the addition of 2.0 equiv of Cu^{2+} ion in the presence of various background metal ions (50 equiv; $\lambda_{\text{ex}} = 343$ nm). (c) B3LYP/3-21G+ energy minimized structure of **1**– Cu^{2+} complex by DFT calculation. The LANL2DZ basis set was used for the Cu^{2+} cation.

various concentrations of metal ions other than Cu^{2+} , only a single monomer emission with no regular trend in fluorescence changes was observed (see [Supplementary data](#)). About 1800-fold enhancement of fluorescence intensity was observed upon addition of Cu^{2+} ion compared to other metal cations (Fig. 2a and see [Supplementary data](#)). As the concentration of $\text{Cu}(\text{II})$ perchlorate was increased, the monomer emission intensity increased remarkably along with the appearance of increasingly intense excimer emission at around 466 nm. The monomer intensities and the quantum yields of both the monomer and excimer bands were increased with the concentration of Cu^{2+} ion and reached maxima when the Cu^{2+} ion concentration was 2.5 equiv of that of the probe, triazolyl pyrene (Fig. 2b). This result was also clear from the deep blue colour of the probe's solution mixed with 2.5 equiv of Cu^{2+} ion when irradiated at 254 nm under a transilluminator (Fig. 1d). However, as the Cu^{2+} ion concentration exceeded 25 μM (2.5 equiv), quenching of both the monomer as well as excimer emission was observed (see [Supplementary data](#)). Therefore, the maximum emission intensity of the probe was observed at 2.5 equiv of Cu^{2+} ion concentration, indicating a well-defined binding of Cu^{2+} ion to the functional triazole units of the probe forming a **1**– Cu^{2+} complex.

To examine the stoichiometry of the **1**– Cu^{2+} complex, the mole ratio method for the fluorescence intensity versus the number of equivalents of Cu^{2+} ions added was plotted which clearly indicated a 2:1 stoichiometry (Inset of Fig. 2b) of the probe to Cu^{2+} ion in the

absorbances (see [Supplementary data](#)). The association constant of Cu^{2+} ion with triazolyl pyrene, was determined by Benesi–Hildebrand plot which comes as $2.23 \times 10^5 \text{ M}^{-1}$ indicating a strong and stable complexation (Fig. 3a). Furthermore, our probe can be utilized for the detection of the sub-millimole concentration range of Cu^{2+} ions found in many chemical and biological systems as its detection limit was calculated to be $1.77 \times 10^{-8} \text{ mol L}^{-1}$ for Cu^{2+} ion (see [Supplementary data](#)).⁹

The ESI-MS technique was employed to confirm the formation of the 2:1 probe– Cu^{2+} complex. Thus, we observed a peak at $m/z = 841.65$ which is in good accord with the calculated mass for $[(\text{TNDMPy})_2\text{Cu} - \text{H}]^+$ (841.50; $\text{C}_{52}\text{H}_{41}\text{N}_8\text{Cu}$, see [Supplementary data](#)). This result again supports that one Cu^{2+} ion is coordinated by two triazole-Ns of two probes, TNDMPy .

Competition experiments were carried out to explore further utility of our probe as an ion-selective switch-on fluorescent chemosensor for Cu^{2+} ion. Thus, it was observed that the tested different background metal ions (50 equiv) showed hardly any interference with the detection of Cu^{2+} ion (Cu^{2+} ion 20 μM , probe 10 μM), indicating that the probe could be used as a potential practical Cu^{2+} -selective turn-on fluorescent sensor (Fig. 3b).

The 'on-off' fluorescence response in the excimer as well as in the monomer emission of the probe might be attributed to the formation of stable complex between the probe's triazole moieties and Cu^{2+} ion, and the subsequent blocking of the communication of the acceptor pyrene unit from the donor triazole moiety.¹⁰ The low

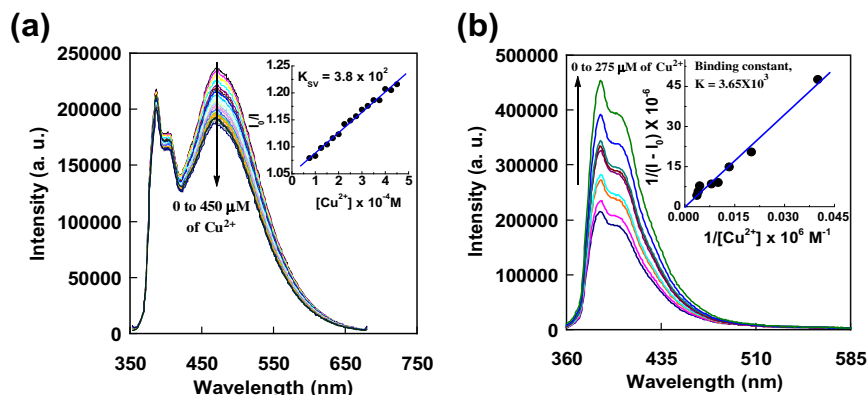


Figure 4. Emission behaviour of **1** in the presence of Cu²⁺ ion in SDS micelle at pH 2 (a), and at pH 8 (b).

fluorescence intensity of the probe in CH₃CN in the absence of Cu²⁺ ion may be attributed to radiationless channel from the n-π* state of the emission of the triazolypyrene by the nitrogenous donor triazole of the probe. When Cu²⁺ ion coordinates with the triazole's nitrogens, the energy of the n-π* state would be raised so that the π-π* state of the emission of the triazolypyrene becomes the lowest excited state, leading to a substantial increase in the fluorescence intensity. As the complexation with one molecule of the probes starts, to satisfy the coordination of Cu²⁺ ion, another molecule of the probe comes closer to coordinate with the same Cu²⁺ ion, thus, bringing two pyrene units of two probes closer in face resulting in the appearance of intermolecular excimer emission. With further increase in copper concentration up to 2.5 equiv, a complete formation of the complex has occurred resulting in maximum intensity of both the monomer and the excimer emission. However, when the concentration of Cu²⁺ ion exceeds 25 μM, emissions from both the monomer and excimer bands start quenching due to paramagnetic Cu²⁺ and the cation-π interactions between two closely faced pyrene moieties and Cu²⁺ ion.¹¹

To support our observations of both the 2:1 complexation and possible excimer formation between two pyrene units of two molecules of probe via π-π stacking interaction, we have carried out DFT calculations with B3LYP exchange functionals using the Gaussian 03 package.¹² For Cu²⁺ LANL2DZ effective core potential (ECP) was employed and in other cases the 3-21G* basis sets were used. The predicted optimized geometry of the **1**-Cu²⁺ complex (Fig. 3c) is shown to be coordinated with the two nitrogens of the two triazoles of two triazolypyrenes with least energy compared to other possible coordinations through other nitrogens of the triazole functionalities. The coordination through triazoles makes the complex rigid, thus, bringing the two pyrene moieties closer. As a result, the excimer formation is conformationally permitted via π-π stacking interaction between these two closely faced pyrenes in the complex which is exactly observed from experimental excimer emission.

The role of the donor dimethylamino phenyl substituent in selective sensing of Cu²⁺ was established by synthesizing other substituted phenyls, such as, fluorophore **2**⁸ (TCNP^{Py}) containing electron withdrawing substituents (-CN) and **3** (TP^{Py}) with no substitution at phenyl ring and studied their specificity/selectivity in sensing the same metal ion (see Supplementary data). These two fluorophores show only a single emission band at around 380 nm which is characteristic of pyrene vibronic structures of the monomer emission both in the presence and in the absence of any metal ions in acetonitrile.

We have examined the optical sensor property of our probes **2**–**3** for metal ions, the tested metal ions Na⁺, Mg²⁺, Ni²⁺ and Cu²⁺ were added to the solution of **2**–**3**, 'respectively', and the

photophysical properties were studied. Thus, from the metal titration experiments in the presence and/or absence of Cu²⁺ ions and other metal ions, we observed that both the latter synthesized fluorophores (**2**–**3**) did not induce any selective change in the absorbance or fluorescence emission properties of the probes. Also in a competitive scenario, in the presence of other background metal ions, the addition of Cu²⁺ ions to any of the probe's solution did not bring any change in fluorescence intensity of the probes. These observations suggested that the probes **2**–**3** are inefficient to interact significantly with any metal ions tested. These observations are totally opposite of that observed in case of probe **1** that showed a clear selectivity in sensing of Cu²⁺ ion.

The results can easily be explained if we consider the probable role of donor and/or acceptor substituents. Thus, in case of probe **1** the donor *N,N*-dimethylaminophenyl group is donating the electron which is accumulated within the nitrogens of triazole ring yielding to better coordinating nitrogens for Cu²⁺ ions. However, in case of probe **2**, the acceptor unit (-CN) is withdrawing the electron density from the triazole's nitrogens, therefore, the less electron dense triazole's nitrogens are unable to coordinate with metal ions. In case of probe **3** (the intermediate situation), also we observed no interaction with metal ions. Therefore, it is clear that the presence of donor *N,N*-dimethylaminophenyl unit is mainly responsible for Cu²⁺ ion coordination and detection with high selectivity and large fluorescence enhancement.

To explore the versatility of our probe, we have also studied its sensing capability of SDS micelle-encapsulated-Cu²⁺ ion within two pH windows. The study of cell membranes and the roles it play in living cells is necessary to understand the cellular function. Thus, it is important to study the drug-membrane interaction with a model system which is the real mimic of cell membrane. Because micelles are amphiphilic molecules, like lipids, some of the same rules governing lipid behaviour also apply to the micelle. Therefore, micelles have been used widely as a model of biomembrane to acquire information about structure, microenvironmental properties and function.¹³ Micelles are also known as a receptor system for various analytes like protein, metal ions, etc.¹⁴ Thus, probing of micellar microenvironment and sensing an analyte inside with fluorescent sensor molecules is an important research area to get insight into the cell membrane. While micelle acts as a receptor unit, fluorophore triazolypyrene acts as a transducer that translates the binding events of the analyte, Cu²⁺ ion with the receptor system in a readable manner through a change in the emission pattern. Thus, the fluorescence titration experiment with SDS micelle showed a quenching of emission of the long wavelength emissive band with unaltered monomer emission upon addition of Cu²⁺ ion in acidic pH (Fig. 4a). In neutral pH only monomer emission with a quenching event was observed (see Supplementary data).

At pH 8, only the monomer band appeared and an increased concentration of Cu^{2+} ion led to an enhancement of fluorescence intensity (Fig. 4b). Thus, our result demonstrated the practical utility of our probe in detecting Cu^{2+} ion in aqueous solution.

In conclusion, we have shown that our smart fluorescence switch-on probe **1** efficiently detects Cu^{2+} ion with remarkably enhanced fluorescence intensity, high sensitivity, selectivity, high binding constant and a low detection limit. We established that the presence of donor *N,N*-dimethylaminophenyl unit is important for Cu^{2+} ion detection with high selectivity and large fluorescence enhancement. We have also shown that the probe is capable of sensing Cu^{2+} ion inside the nanocore of micelle with a change in fluorescence pattern that is selective to the pH of the system. Thus, the probe is suitable for use both in organic as well as in aqueous environment showing its practical utility. The physical insight of the fluorescence pattern in the presence of Cu^{2+} ion has a high potential to either modify the existing micelle based fluorescent chemosensors or develop new fluorescent chemosensors. All the results indicate that our probe could be used as an efficient Cu^{2+} ion selective fluorescence light-up probe, and may find applications in chemical and biological systems. Because of the triazole's ability to strongly associate with biomolecules via H-bonding, electrostatic and stacking interaction, we are currently interested in investigating the probe's efficiency in sensing biomolecules such as ct-DNA and BSA protein.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tetlet.2012.08.074>.

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Singly and doubly labeled base-discriminating fluorescent oligonucleotide probes containing oxo-pyrene chromophore

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ABSTRACT

We have developed new oxo-pyrene labeled fluorescent nucleoside, **Oxo-PyU** which showed a strong fluorescence dependency on solvent polarity at long wavelength. The designed singly and doubly **Oxo-PyU** labeled fluorescent oligonucleotide probes were found highly efficient for the discrimination of A and consecutive AA bases of target DNA opposite to the labeled base via generation of enhanced fluorescence signal.

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Fluorescent oligonucleotide probes are powerful tools for structural studies of nucleic acids, sequencing, molecular diagnostics, and other applications relating genomics.¹ Oligonucleotide probes labeled with solvatochromic fluorophores are particularly useful for detecting the change in microenvironment around the nucleic acids and thus can be used for DNA detection and other applications like single nucleotide polymorphisms (SNPs) typing.¹ The concept of the labeled probe is based on the fluorescence change of the labeled base itself in response to the opposite bases on a complementary strand. As a part of our ongoing project in searching for long wavelength emissive and highly solvatochromic fluorescent nucleosides, we demonstrated a novel method for homogeneous fluorescence assay that allows a clear distinction of the base opposite to the labeled base by a drastic fluorescence change.² Such fluorescent nucleosides named as base-discriminating fluorescence (BDF) nucleosides have been used extensively for gene detection and SNP genotyping.^{1c,f,2,3}

Previously reported fluorescent probes containing BDF base **MPyU**- and **AMPyU** with oxo-pyrene chromophore were found to be useful for the discrimination of perfectly matched base A opposite to the BDF base in a complementary strand.^{3a} However, the preparation of nucleosides **MPyU**- and **AMPyU** needed rather complicated synthetic steps of low yield and the synthesis in large quantity was very difficult.^{3a} Therefore, the assay using these BDF bases are not practical. In contrast, a new fluorescent nucleoside **Oxo-PyU**

described here was very easily synthesized from readily available γ -oxo-pyrene butyric acid and the fluorescence emission was more solvent polarity sensitive with stronger fluorescence intensity and higher quantum yield (Fig. 1). Therefore, **Oxo-PyU** is more convenient and practically more useful for the preparation of oxo-pyrene labeled BDF probes as compared with previous **MPyU**- and **AMPyU** probes.^{3a}

Aiming a practical application of the BDF base, we thought that it would be worthwhile to tether oxo-pyrene chromophore to the oligonucleotide sequence and follow up the fluorescence behavior in the presence of its complementary target sequences. We also thought that there is a possibility of detecting two consecutive opposite bases from the target strand if we could incorporate two consecutive **Oxo-PyU** in the probe, either by an enhanced monomer emission or via excimer emission, depending on the geometrical disposition of the two pyrene rings. With this view in mind, we have synthesized oxo-pyrene labeled 2'-deoxyuridine, **Oxo-PyU**, which was incorporated into short oligonucleotides as one and two consecutive BDF bases.

Thus, we synthesized nucleoside **1** bearing a rigid acetylene linker via Sonogashira cross-coupling⁴ of 5-iodo-2'-deoxyuridine with γ -oxo-pyrene carboxamide **2** (Scheme 1). The synthesis of γ -oxo-pyrene butyric acid (**3**) was accomplished from commercially available pyrene and succinic anhydride via Friedel–Craft acylation. Nucleoside **1** was ultimately incorporated into three oligonucleotide sequences ODN **1**, **2** and **3** via standard DNA synthesis protocol and the ODNs were analyzed by MALDI-TOF mass spectrometry (Table 1). While 13-mer ODN **1** and **2** contained

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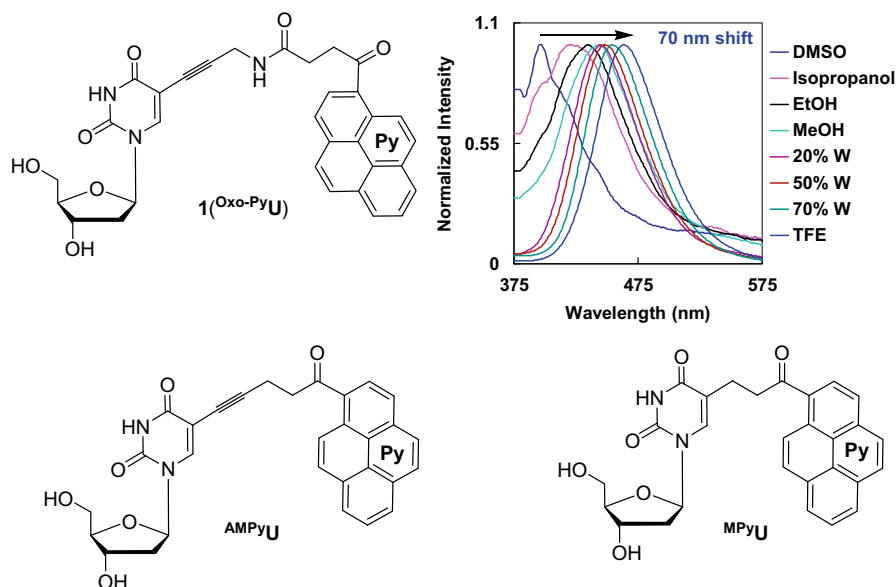
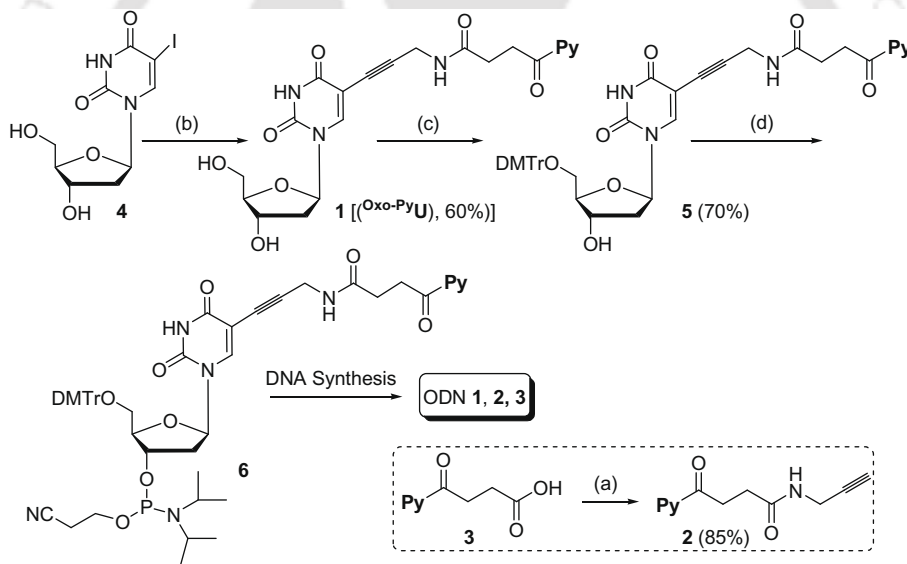


Figure 1. BDF nucleoside **1** (**Oxo-PyU**), its solvatochromicity and the structures of **AMPyU** and **MPyU**.



Scheme 1. Synthesis of BDF nucleoside **1** and ODNs.

Table 1
Oligonucleotide sequences used in this study

ODNs	Sequences
1	5'-d(CGCAAC ^{Oxo-Py} UACACG)-3'
2	5'-d(CGCAAT ^{Oxo-Py} UTAACG)-3'
3	5'-d(CGCAAT ^{Oxo-Py} ^{Oxo-Py} UTAACG)-3'
4	5'-d(CGCAAC T CAACG)-3'
5	5'-d(CGCAAT T TAACG)-3'
6	5'-d(CGCAAT TT TAACG)-3'
7	5'-d(GCGTTG N GTTGCG)-3' [N = A, G]
8	5'-d(GCGTTA N ATTGCG)-3' [N = A, G]
9	5'-d(GCGTTA NN ATTGCG)-3' [NN = AA, AG, GG]

singly labeled ^{Oxo-Py}U and differed only by flanking bases near the labeled nucleoside (ODN **1** contained C, while ODN **2** contained T as the flanking bases). 14-mer ODN **3** labeled with two consecutive ^{Oxo-Py}U was also prepared.

Photophysical properties of nucleoside **1** were then examined. We observed that with increasing solvent polarity a decreased and a red shifted patterns were observed in the UV absorption spectra of **1** ($\lambda_{\text{max}} = 345$ in DMSO; $\lambda_{\text{max}} = 355$ nm on TFE). Most interestingly, both the fluorescence intensity and the wavelength were markedly affected by solvent polarity (Fig. 1). In DMSO nucleoside **1** displayed a very weak emission with a maximum at 396 nm ($\Phi = 0.015$), while in TFE, the most polar organic solvent tested, **1** showed a strong emission ($\Phi = 0.22$) at around 467 nm and decayed deeply into the visible region (>565 nm). We have also calculated fluorescence brightness factor (F.B. = $\epsilon\Phi$) which showed an 11-fold increase in TFE in comparison with DMSO (Supplementary data, Fig. S1, Table S1). The fluorescence titration of a solution of **1** in EtOH with water revealed a similar observation. In pure EtOH, the emission was around 438 nm ($\Phi = 0.023$) which was shifted to 452 nm and decayed deeply into visible region, 580–600 nm, when the ratio of water and ethanol was 70:30 ($\Phi = 0.12$). As expected, the fluorescence of oxo-pyrene-labeled nucleoside

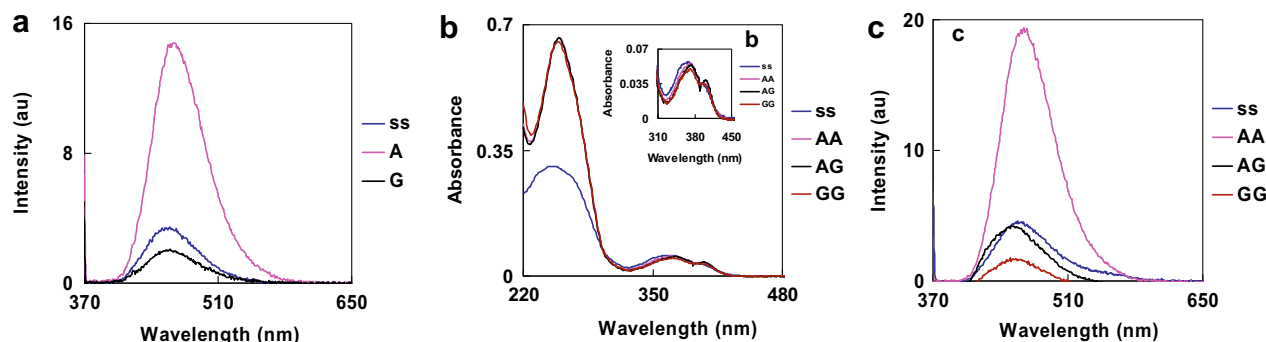


Figure 2. (a) Fluorescence of single strand ODN 2, (b) UV-vis and (c) fluorescence spectra of ODN 3 (2.5 μ M) and the different duplexes formed by hybridization with ODN 8 [N = A, G] and 9 [NN = AA, AG, GG], (2.5 μ M, 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, rt; λ_{ex} = 369 nm.).

showed a high sensitivity to the solvent polarity. It is noteworthy that the emission wavelength in 30% water is about 55 nm longer than that reported for previous pyrenecarboxamide (PyU)-labeled probes (397 nm in water)^{2c} and the solvatochromicity ($\Delta 70$ nm) was 20 nm larger than those of ^{MPyU} and ^{AMPyU} ($\Delta 50$ nm).^{3a}

Thus, the solvent polarity dependent fluorescence property of ^{Oxo-PyU} would be usable for monitoring the change in DNA microenvironment. Therefore, we examined the photophysical properties of single stranded ODN 1 (5'-CGCAAC ^{Oxo-PyU} CAACGC-3') and 2 (5'-CGCAAT ^{Oxo-PyU} TAACGC-3') in the presence and absence of their complementary strands. As revealed from the fluorescence spectra (Supplementary data, Fig. S2), it was observed that the fluorescence of ODN 1 with flanking GC base pair was completely quenched when hybridized to perfectly matched or single base mismatched complementary sequences ODN 7 [N = A, G]. This is so obvious and a positive result of our expectation that the flanking GC base pair is responsible for the quenching of ^{Oxo-PyU} fluorescence. Thus, ^{Oxo-PyU} could potentially be useful in designing G-quenched molecular beacon as well.⁵

On the other hand, ODN 2 (5'-CGCAAT ^{Oxo-PyU} TAACGC-3') upon hybridization with perfectly matched sequence ODN 8 [N = A] showed a very strong emission at 465 nm (Fig. 2a). A very weak fluorescence was observed in case of the duplex ODN 2/8 [N = G] where mismatched base G is opposite to the labeled nucleoside in the complementary strand. This observation again indicated that the fluorescence of ^{Oxo-PyU} is tremendously quenched by neighboring G-base and can allow us to detect matched base A with an intense fluorescence signal.^{3,6}

The strong fluorescence emission for perfectly matched duplex ODN 2/8 [N = A] can be explained if we consider the more hydrophilic microenvironment faced by oxo-pyrene chromophore outside the groove. As the opposite base is matched base A, the fluorophore has to extrude outside the groove and thus faced to more aqueous microenvironment, resulting in an enhancement of fluorescence (Fig. 2a). The thermal melting behaviors of the duplex ODNs also support our explanation. The thermal melting behavior showed that the matched duplex ODN 2/8 [N = A] is more stable (T_m = 64.1 $^{\circ}$ C) than unmodified duplex ODN 5/8 [N = A] (T_m = 56.1 $^{\circ}$ C). Thus, the fluorophore resides along the major groove and involved in stacking type interaction and hence bringing more stability. Molecular modeling study using AMBER[®] force field in water employing MacroModel vs. 9.0 also supported this explanation.

Recently, Yoshida et al., have reported a doubly labeled probe for efficient SNP typing using ^{PyU} and ^{AMPyU}.^{3b} Since ODN 2 showed a highly A-selective fluorescence emission, we thought that there could be a possibility of detecting opposite two consecutive AA sequence from the target strand if we incorporate two

emission or via an excimer emission depending on the geometrical disposition of the two oxo-pyrene rings. Therefore, we prepared a doubly ^{Oxo-PyU}-labeled probe, ODN 3 [5'-d(CGCAAT ^{Oxo-PyU} ^{Oxo-PyU} TAACGC)-3'] and measured the fluorescence of the duplexes formed via hybridization with complementary strands ODN 9 [5'-d(GCGTTA NN ATTGCG)-3']; NN = AA, AG, GG]. Unfortunately, in any case we could not observe an excimer fluorescence. However, the detection of -AA- bases was perfectly accomplished by a strong monomer emission at 467 nm (Φ = 0.15) with a long tail in the visible region (>567 nm) together with a high signal to noise ratio and a large fluorescence brightness factor (F.B. = 3284). In contrast, the emission of the single stranded ODN 3 and the duplexes ODN 3/9 [NN = AG] and ODN 3/9 [NN = GG] was strongly suppressed (Fig. 2b and Table 2). Therefore, ODN 3 can act as a useful probe which emits strong fluorescence with a high AA selectivity. To understand the fact of no excimer emission by two consecutive ^{Oxo-PyU} we have examined the molecular modeling study. From the optimized structure it was found that the pyrene rings are far apart and positioned parallel and in cofacial configuration to each other, thus inhibiting an excimer formation (Supplementary data, Fig. S5).⁸

In conclusion, we have investigated the fluorescence emission properties of fluorescent DNA probes containing singly and doubly labeled ^{Oxo-PyU} in the presence of complementary strands. We showed that the fluorescence of ^{Oxo-PyU} is strongly quenched by neighboring G-base. We have also shown that the probes are highly specific for the detection of matched base A. The doubly labeled ODN which exhibit unique fluorescence properties depending on the number of adenines on the complementary strands would be also useful for the detection of consecutive A sequence.

Table 2

Thermal melting temperatures and photophysical properties of ODNs

Duplexes	T_m ($^{\circ}$ C)	ϵ_{max} ($\text{M}^{-1} \text{cm}^{-1}$)	Φ^7	F.B.
ODN 1	—	11,200	0.054	605
ODN 1/7 [N = A]	70.9	11,200	0.002	22
ODN 1/7 [N = G]	60.7	10,800	0.001	11
ODN 4/7 [N = A]	59.8	—	—	—
ODN 2	—	11,600	0.046	543
ODN 2/8 [N = A]	64.1	12,000	0.216	2640
ODN 2/8 [N = G]	52.5	11,200	0.030	336
ODN 5/8 [N = A]	56.1	—	—	—
ODN 3	—	22,648	0.036	815
ODN 3/9 [NN = AA]	64.1	21,896	0.150	3284
ODN 3/9 [NN = AG]	58.4	21,492	0.025	550
ODN 3/9 [NN = GG]	53.2	19,696	0.007	153
ODN 6/9 [NN = AA]	61.1	—	—	—

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.04.063.

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- Spectroscopic data for compound 1:** ¹H NMR (DMSO-*d*₆, 400 MHz) δ = 11.68 (d, 1H, *J* = 8.0 Hz), 8.76 (d, 1H, *J* = 9.4 Hz), 8.58 (d, 1H, *J* = 8.1 Hz), 8.41–8.27 (m, 5H), 8.25–8.15 (m, 2H), 8.14 (s, 1H), 6.13 (t, 1H, *J* = 8.2 Hz), 5.26 (br, 1H), 5.16 (br, 1H), 4.23 (br, 1H), 4.14 (br, 1H), 3.80 (br, 1H), 3.6 (m, 2H), 3.49 (m, 1H), 3.36 (br, 2H), 2.69 (broad t, 1H), 2.50 (t, 2H, *J* = 1.8 Hz), 2.12 (br m, 2H); ¹³C NMR (DMSO-*d*₆, 100 MHz) δ = 203.5, 170.9, 161.6, 149.4, 143.6, 132.9, 132.7, 130.6, 129.9, 129.2, 129.0, 128.0, 127.1, 126.7, 126.4, 126.3, 125.9, 124.5, 124.4, 123.9, 123.4, 98.1, 89.6, 87.5, 84.6, 74.3, 70.2, 60.9, 40.0, 37.0, 29.5, 28.7; FABMS, *m/z* 566 ([M+H]⁺), HRMS calcd for C₃₂H₂₇N₃O₇ ([M+H]⁺) 566.1927, found 566.1929.