

Smart Applications of Tunable Colours from Stimuli-Sensitive Molecules

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by

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Dedicated to my **beloved parents** for their unwavering support, endless love, and consistent encouragement throughout the years.





Declaration

I hereby declare that the matter embodied in this thesis is a result of investigations carried out by me in the Department of Chemistry, Indian Institute of Technology Guwahati, Guwahati, Assam, India, under the supervision of Prof. Debapratim Das. 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.

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To whom it may concern

This is to certify that the thesis entitled “**Smart Applications of Tunable Colours from Stimuli-Sensitive Molecules**” submitted by Priyam Das (Roll. No. 206122026) for the award of PhD degree to IIT Guwahati, is absolutely based on his own research work and neither this thesis nor any part of it has been submitted for any degree/diploma or any academic award anywhere before.

(Dr. Debapratim Das)



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Abstract

The thesis entitled “**Smart Applications of Tunable Colours from Stimuli-Sensitive Molecules**” explores techniques and approaches for implementing stimuli-responsive colours obtained from organic dyes, emissive molecules and luminescent assemblies in designing modern applications with unconventional properties.

Chapter 1 introduces the importance of stimuli-responsive chemical colours in building smart optical materials, control over stimuli and the progress accomplished so far in the field of advanced optical materials based on colours.

Chapter 2 demonstrates the application of a host-guest-based green phosphorescent probe for the qualitative and quantitative detection of a clinically important bioanalyte, Heparin and its antidote, Protamine Sulphate.

Chapter 3 deals with time-encoded information encryption achieved with a broad-spectrum transitory emission produced from the temporal regulation of a pH-responsive supramolecular assembly.

Chapter 4 focuses on emulating transient lights, as seen in bioluminescence, through the temporal control of a pH-dependent assembly with multi-colour emissions and subsequent applications in time-locked encryption strategies with anti-counterfeiting properties.

Chapter 5 highlights the significance of parallel time domains in time-controlled information encryption strategies, accomplished through the temporal variation in colours attained with multi-stimuli-responsive common organic dyes.



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List of Abbreviations

NMR	Nuclear Magnetic Resonance
ESI-MS	Electrospray Ionization Mass Spectrometry
MALDI	Matrix-Assisted Laser Desorption/Ionization
HPLC	High-Performance Liquid Chromatography
UV-Vis	Ultraviolet-Visible Spectroscopy
FESEM	Field Emission Scanning Electron Microscopy
FETEM	Field Emission Transmission Electron Microscopy
TCSPC	Time-Correlated Single Photon Counting
DLS	Dynamic Light Scattering
λ_{max}	The wavelength in the absorption spectrum where the absorbance is maximum
λ_{em}	The wavelength in the emission spectrum where the emission is maximum
DCM	Dichloromethane
THF	Tetrahydrofuran
EtOH	Ethanol
MeOH	Methanol
EtOAc	Ethyl Acetate
ACN	Acetonitrile
DMSO	Dimethyl Sulfoxide
DMF	N,N-Dimethylformamide
DIPEA	N,N-Diisopropylethylamine
TEA	Triethylamine
HOMO	Highest Occupied Molecular Orbital
LUMO	Lowest Unoccupied Molecular Orbital
FRET	Fluorescence Energy Resonance Transfer
ISC	Inter System Crossing
AIE	Aggregation Induced Emission
RIM	Restriction of Intramolecular Motion
ACQ	Aggregation Caused Quenching
NR	Nile Red
ANS	8-Anilino-1-naphthalenesulfonic acid
ThT	Thioflavin-T
TPE	Tetraphenylethylene

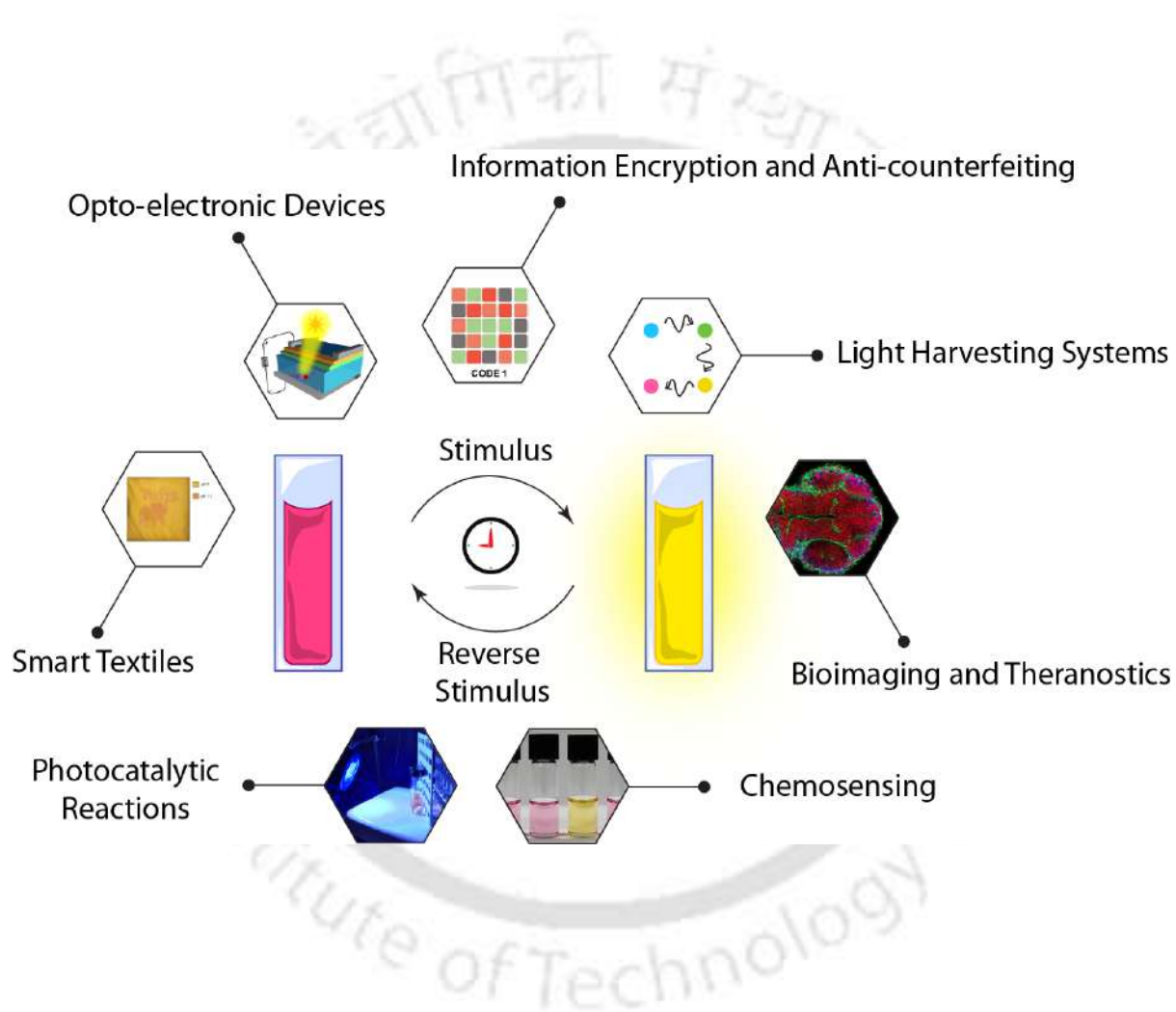
TPA	Triphenyl amine
DBT	4,7-dithien-2-yl-2,1,3-benzothiadiazole
NBD	Nitrobenzoxadiazole
SP	Spiropyran
MC	Merocyanine
BODIPY	Boron Dipyrromethene
NDI	Naphthalene Diimide
RhB	Rhodamine B
DAPI	4',6-diamidino-2-phenylindole
BTB	Bromothymol Blue
MR	Methyl Red
PT	Phenolphthalein
TT	Thymolphthalein
MB	Methylene Blue
MV	Dimethyl Viologen
CD	Cyclodextrin
CB	Cucurbituril
SDT	Sodium Dithionite
ALP	Alkaline Phosphatase
TRIS	Tris(hydroxymethyl)aminomethane
GdL	Glucono delta-lactone
GOx	Glucose Oxidase
TCA	Trichloroacetic Acid
TBA	Tribromoacetic Acid
GSH	Glutathione
CIE	Commission Internationale de l'Eclairage (International Commission on Illumination)
QR	Quick Response
CT	Charge Transfer
ESIPT	Excited-State Intramolecular Proton Transfer
PET	Photoinduced Electron Transfer
SQ	Static Quenching
DQ	Dynamic Quenching
CSB	Cyanostilbene
ATP	Adenosine Triphosphate

POCT	Point of Care Testing
OLED	Organic Light Emitting Devices
CTOLED	Colour Tunable Organic Light Emitting Devices
NIR	Near Infra-Red
ROS	Reactive Oxygen Species
LHS	Light Harvesting System
HBS	Human Blood Serum
PS	Protamine Sulphate
ACT	Activated Clotting Time
aPTT	Activated Partial Thromboplastin Time
RTP	Room Temperature Phosphorescence
BP	4-(4-bromophenyl)-pyridine
HU	Human Urine
CSB-2Bn	Benzylated Pyridinium Bromide Salts of Pyridine functionalized CSB
MA	Myristic Acid
TPEA	Butyric Acid Functionalized TPE
CAC	Critical Aggregation Concentration
τ_{av}	Average Lifetime
τ_r	Rotational Correlation Time
Φ_f	Fluorescence Quantum Yield



Chapter 1

Introduction





1.1. Colours in Nature

It is impossible to imagine a world without lights and colours. They encompass every nook and corner of all imaginable natural phenomena. They not only serve as a visual enigma but also convey essential information about the natural world's working principles. When nature's physical events are considered, observable colours provide us with fundamental knowledge concerning them. For example, distinct colours of oceans at their physical boundaries indicate the difference in their densities and mineral content that affect their miscibility. Similarly, the appearance of colours in the sky can be indicative of the air quality index and signify the presence of particulate matter. Simple variations in shades, hues and intensities clarify the periodic changes of nature. For example, one can have an idea of the changing seasons and climates by observing the changing colours of seasonal foliage. These gradual changes are often transitional and cyclic, and are directly related to the periodic nature of seasons.

Colours also play a key role in living organisms. Many organisms are apparently identical but often belong to distinct species. Where look-alike physical features often lead to confusion, colours are indicative of speciation. Evolutionary colours throw light on the path of evolution of a particular species. A particular colour might be absent, but can appear over time in the phenotype of a species that might point to the environmental changes of a habitat. For example, the colours of leaves vary with the quality and chemical constituents of the soil. Colours are pivotal in the struggle for the existence of life. Bright and fluorescent colours are often flashed as warnings to ward off predators. The neon colours of the poison dart frog designate their toxins, while the ladybird's patterns are associated with bad taste for their predators. Some animals, like the squid, generate psychedelic-coloured patterns instantaneously through upregulation of chromophores that create hypnotic trances to lure in prey. The importance of colour lies in the attraction of mates, also. Birds know how to exhibit their colourful plumage through playful courtship dances. The better the colours better the chances of the progeny to move ahead. All these examples prove that colours act as significant markers in the natural world. Nature's mastery of the utility of colours has also influenced human life in several aspects.

1.2. Importance of Colours in Daily Life

Colours have always had an impactful influence on human lives from the beginning of modern human civilization. From dawn to dusk, humans rely on the implications of colours without noticing. The most dominant utility of colours lies in their ability to be used as signals and indicators. Some colours like red are indicative of dangers, alerts or risks, and hence they find their applications in warning signals. On the contrary, humans have associated certain colours, such as green, with safety,

security and reliability. The food marketing and packaging industry very cleverly propagates ideas of healthy lifestyles, organic nature and benignity by employing green. Freshness and quality indications are also signified through colours. Quick identification and easy recognition can also be an outcome of the application of colours. For example, in a medical emergency, we look around for a red cross. Even our currency notes and denominations are discernibly coloured to make them identifiable. Every colour has its own characteristics and often plays a role in interpreting human psychology, too. Colour-coded data and encryption styles have also gained immense importance in the modern day. They find application in security systems that have anti-counterfeiting properties and aim for safe data protection.

From fashion to textiles, from marketing to advertisements, from health to finance, from security to lifestyle, from entertainment to aesthetics, every colour and every shade has its special place. Recent research has revealed that the world is losing colours at an alarming rate. Many colours are losing their utility and implementation in the desire for aesthetic representations, and these are adversely affecting human response and preference to certain colours over others. In this context, upscale research is being focused on developing smart colour-based systems that exploit the ability to convey information through chromatic modifications and transitions.

1.3. Colours in Chemistry – Molecular Origin and Energetics

Colours and chemistry have had an age-old relationship. It is undeniable that every chemist has been intrigued by the colourful observations of their subject at some point in life. Colours always pique our interest, and chemistry answers most of our curiosity with colours. A major section of chemical colour has its origin at the molecular level. Although physical factors like reflection and refraction govern the colour of visible light, chemists are more concerned with the molecular origin of colours. In general, molecules interact with electromagnetic radiation in many ways owing to their structural and electronic properties that are responsible for their observable colours. These interactions vary with the physical state of matter (solid, liquid or gas) as well, affecting the colours. When molecules are considered in a particular solution, the visible colours can mainly be due to transmitted or emitted light.

1.3.1. Colours from Transmitted Light – Absorption and Transmittance

White light is composed of electromagnetic radiation of variable wavelengths that lie in the visible spectrum. When this light passes through a molecular solution, a certain section of the wavelength is absorbed more than the other. The unabsorbed wavelength of light is transmitted through the solution, which appears as the visible or complementary colours (Figure 1.1 A). For example, a

solution of potassium permanganate appears purple because when white light passes through it absorbs mainly in the green-yellow region. The complementary components of blue and red wavelengths are transmitted, making it appear purple. The wavelength that is absorbed selectively more than the others closely corresponds to the energy gap between the electronic states of the molecules that undergo transition. These energy transitions can arise from ligand to metal charge transfers for coordination complexes or HOMO to LUMO electron transitions as seen in conjugated organic dyes. Selective stabilization of one energy level over another by solvent molecules can alter the energy gaps, leading to changes in colours also. The colours can also be produced by scattering effects in solution. Selective scattering of certain wavelengths over others affects the solution's colour. The wavelength of scattered light depends on the particle size in the colloidal solution. In general, organic dyes are coloured organic molecules that are generally soluble in a particular solvent and impart colour to a material by chemically infusing with the material matrix. The dye solutions are usually homogeneous and offer uniform colouring. On the other hand, pigments are generally insoluble in many solvents, and their dispersions generate colours through scattering. They are usually opaque and impart colours mainly to the surface of a material unless physically mixed.

1.3.2. Colours from Emitted Light – Excitation and Emission

When irradiated with light of a particular wavelength that exactly matches the energy of absorption (λ_{max}), the electronic transitions make the molecules reach their excited state. These energetically excited molecules can either participate in photochemical transformations, transfer the excess energy to another partner (FRET) after some loss through internal relaxation or release it through radiative transitions and come back to their ground state. All these outcomes may liberate energy in the form of photons, resulting in a different form of molecular colours arising from emitted light (Figure 1.1 B). Energy transfer to another molecule ultimately ends up in photonic release if not used up for reactions. The emitted light leads to molecular luminescence. The energy can be lost from the same singlet spin states, leading to fluorescent colours (fluorochromes) (Figure 1.1 C) or from triplet states after ISC, as observed in phosphorescent molecules or phosphors. Reverse ISC leads to delayed fluorescence. All these possible transitions and crossovers are represented by the Jablonski diagram (Figure 1.1 D). Most of the emitted lights are red-shifted from their respective absorption wavelengths owing to loss from internal relaxations and other non-radiative pathways. Sometimes, the ideal environment that leads to molecular emissions might be absent under regular conditions. These ideal conditions can be induced through supramolecular interactions that favour self-assembly.

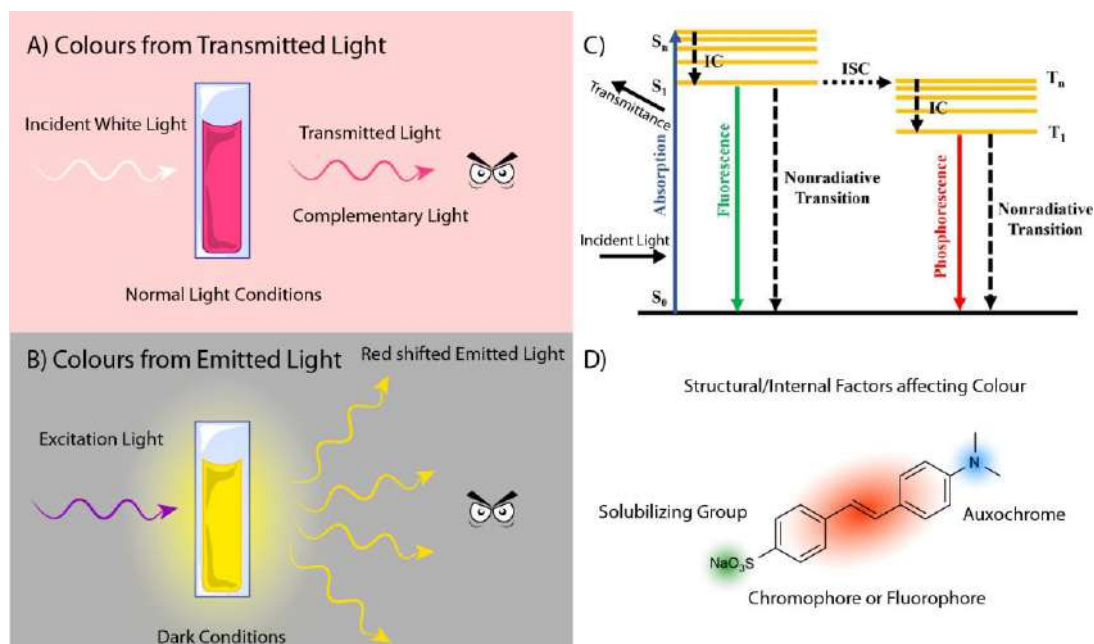


Figure 1.1. Schematic representation of colours from A) transmitted light and B) emissive light. C) General structure of a dye. D) Energy level Jablonski diagram.

1.3.3. Induced emissive colours from Supramolecular Assemblies and Host-Guest Complex

Molecules often interact with each other through non-covalent interactions that assist them in undergoing ordered structures called self-assemblies. Assemblies frequently provide the ideal conditions that induce different electronic transitions leading to different colours. For example, many hydrophobic fluorochromes like NR and ANS fail to exhibit their characteristic fluorescent colours in aqueous environments. They mainly fluoresce in aprotic or hydrophobic solvents. However, these molecules can emit if they are entrapped in isolated hydrophobic microenvironments of bulk aqueous phases. Such conditions can be provided by molecular self-assemblies. NR shows strong red fluorescence when entrapped in the vesicular hydrophobic bilayer of a liposome. Some dyes are highly specific to certain types of microdomains for their fluorescent properties. One such example is ThT, which shows its characteristic emission when it gets trapped in the β -sheet structures of proteins.

In this context, AIE is noteworthy. Certain molecules can get excited under irradiation but quickly dissipate the absorbed energy through non-radiative mechanisms like faster intermolecular rotations and vibrations. They return to their ground states without any radiative emission, and hence no colours are observed. When these molecules are aggregated, steric hindrances and molecular clustering arrest their molecular motions, preventing them from showing any non-radiative loss. RIM leads to AIE producing bright emissive colours. RIM can not only be achieved through self-aggregation but also through chemical or supramolecular crosslinking. Molecules that

show AIE effect are termed as AIEgens (TPE, TPA, stilbenes, etc.). Self-assembly confinement also protects luminescent molecules from ACQ, a major drawback in conventional organic luminescent dyes. They shield molecules from interactions with the bulk solvent or other quenching agents that would otherwise lead to the loss of emissive pathways. In this context, the host-guest complex is significantly effective. Host-guest chemistry can be effectively employed to secure emissive pathways. These complexes can direct self-assembly processes or can even directly capture fluorochrome molecules that augment emissive colours. Usually, molecules are arrested as guests in large macrocyclic cavities of host molecules due to favourable non-covalent interactions. Fulfilling proper stereo electronic demands produces thermodynamically stable host-guest complexes, which are often accompanied by chromatic changes. CDs, CBs, pillarenes and calixarenes find wide applications in host-guest-based smart luminescent materials, especially with phosphorescent molecules, which are otherwise quenched by oxygen in free solution. Hierarchical supramolecular confinements lead to secure radiative relaxations. Host-guest bindings can also promote charge transfer complexes, which result in coloured solutions. Colour changes can be achieved in 2 major ways – one is the generation or intensification of a fixed colour, as seen in AIE of TPE, or a shift in wavelength producing a transition in colours, like the cyan colour of pyrene excimers as compared to the blue emissions of monomeric pyrene. (Figure 1.2).

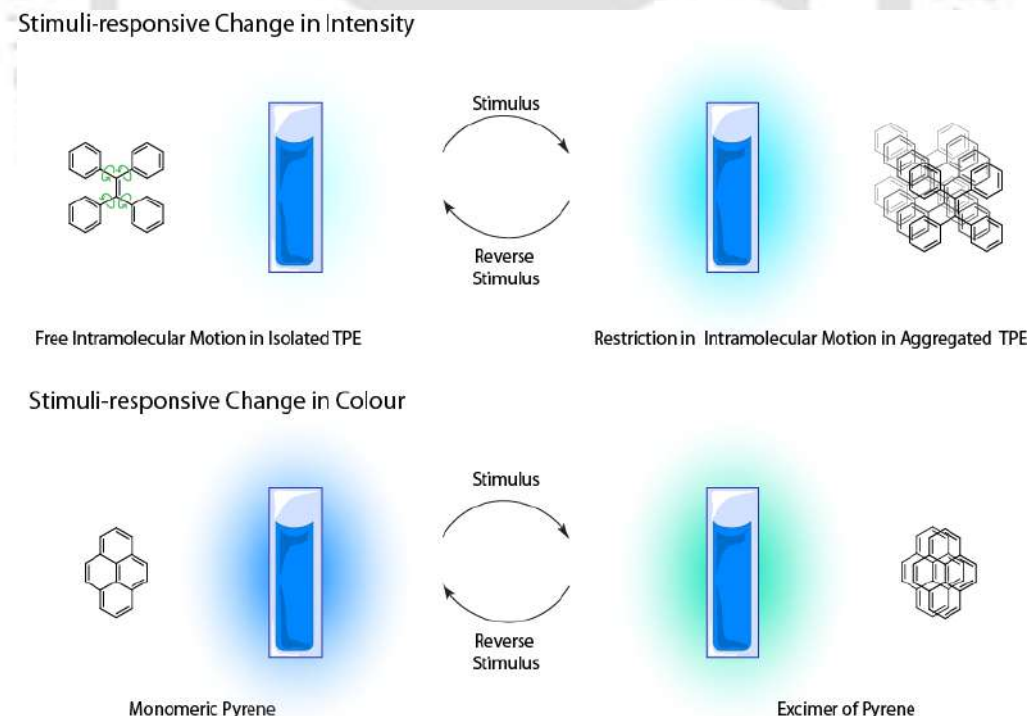


Figure 1.2. Schematic representation of A) reversible AIE by TPE and B) reversible excimer formation by pyrene.

1.4. Factors affecting Colours

The beauty of colours lies in their diversity. Molecular colours can be fine-tuned by calculated manipulation of several factors that affect them. Any element that affects the energy of molecular transitions will invariably be reflected in their transmitted and/or emitted colours. These components can broadly be categorized into internal and structural elements or external factors, which are subjected to physical and chemical parameters.

1.4.1. Internal or Structural Factors

When molecules in solution undergo transitions, their structures and electronic distributions play a key role. Extended conjugations in organic dyes reduce the HOMO-LUMO energy gap, which permits absorptions in the UV-vis spectrum. Any structural modifications or functionalizations which perturb the electronic distribution and conjugation can lead to chromatic changes. A simple example can be considered with nitrophenolic compounds. Deprotonated nitrophenols impart a yellow colour to the otherwise colourless solution due to extended conjugation through the aromatic ring to the nitro group. For any molecule, the main skeletal framework that is responsible for colour or luminescence is called a chromophore or a luminophore (fluorophore or phosphor) (Figure 1.1 D). Any functional group that augments absorptions and intensifies the colours is an auxochrome, like the nitro groups in nitrophenols.

Again, certain changes in structures and geometries can also increase conjugative properties. As mentioned earlier, in the case of AIEgens, coplanarity of aromatic rings is essential for switching on fluorescence. Non-planar structures of TPEs show almost no fluorescence when they are in free solution. Planarity is induced under aggregations, resulting in bright emissive colours. Sometimes, stereochemical factors are responsible for visual colours. For example, the *cis* and *trans* isomers of azobenzene have different absorptions and hence different colours in solution. Similarly, merocyanine and spiropyran are isomers having distinct colours in solutions. Hence, stereo electronic modifications can be carefully strategized for bringing in desirable changes in colours.

1.4.2. External Factors or Stimuli-Responsive Colours

Certain factors can be controlled externally, which force the molecules to adopt different stereo-electronic structures. These are stimuli that bring about changes in the molecular structure by affecting the physical parameters or chemical environment of the molecule. These factors drive the molecule to adopt changes that are thermodynamically or kinetically more favourable under an altered environment. For instance, changes in solvent polarities can induce changes in the emission properties of a fluorophore. An aqueous solution of DBT shows low yellow-orange fluorescence.

Increasing the percentage of THF in water reduces the overall solvent polarity, which increases the fluorescence of DBT. Proper functional groups and derivatives that respond specifically to external stimuli are an essential requirement for the molecules. The functionalization is placed according to the kind of stimuli-responsiveness and purpose needed. Sometimes, including multiple functional groups that are independent of each other can endow the properties of orthogonal stimulus-responsiveness in a single molecule. External factors are more advantageous over the internal elements on several grounds.

1.4.3. Advantages of External Stimulus over Structural Factors

Conducting structural modifications on a fluorophore is not a convenient process. These modifications require complex synthetic steps and extensive theoretical calculations. Several assumptions are needed before designing a luminophore or simple organic dye. Many of these assumptions cannot be predicted with exact accuracy, leading to falsities. Again, synthetic steps involving chromophores and fluorophores are complicated, often yielding lower conversions with unwanted side products. Additionally, they run the risk of quenching during synthetic modifications. In this regard, controlling colours and emissions with external stimuli is more advantageous. The changes induced through stimulus are instantaneous. Moreover, since the stimulus is applied externally, it does not tamper with the molecule directly and mainly brings changes in the environment and physical components. External operations are inexpensive and convenient to handle. The most important feature of stimuli-responsive colours is their reversibility. Most of the molecular changes that occur under stimulus are temporary and can easily be reversed. Since the stimulus alters the environment of a molecule, it can be expected that the original conditions can be restored by the removal of the source of stimulus or by applying a reverse stimulus. Carefully balancing the conditions between the opposite stimuli can result in intermediate molecular structures that show a range of colours. Obtaining such a library is difficult and time-consuming through chemical synthesis. Organic dyes like BTB can vary in colour with a change of pH in their chemical environment. Different combinations of colours can be produced with a fine acid and base balance.

1.5. Stimuli-Responsive Colours

1.5.1. Rationale for Stimuli-Responsive Colours

Introducing a specific stimulus to a stable solution affects the physical and chemical behaviour of the system. Since the molecules are endowed with stimuli-responsive groups, they specifically react to the changes in the solution medium. These groups can directly be a part of the conjugations,

which might be affected by chemical changes. The changes might also assist the molecule in some assembly-disassembly process that can bring about chromatic features.

1.5.2. Categories of Stimulus

Depending on the nature of the source and the kind of changes that are introduced in the system through the stimulus, it can be broadly classified into physical and chemical (Figure 1.3).

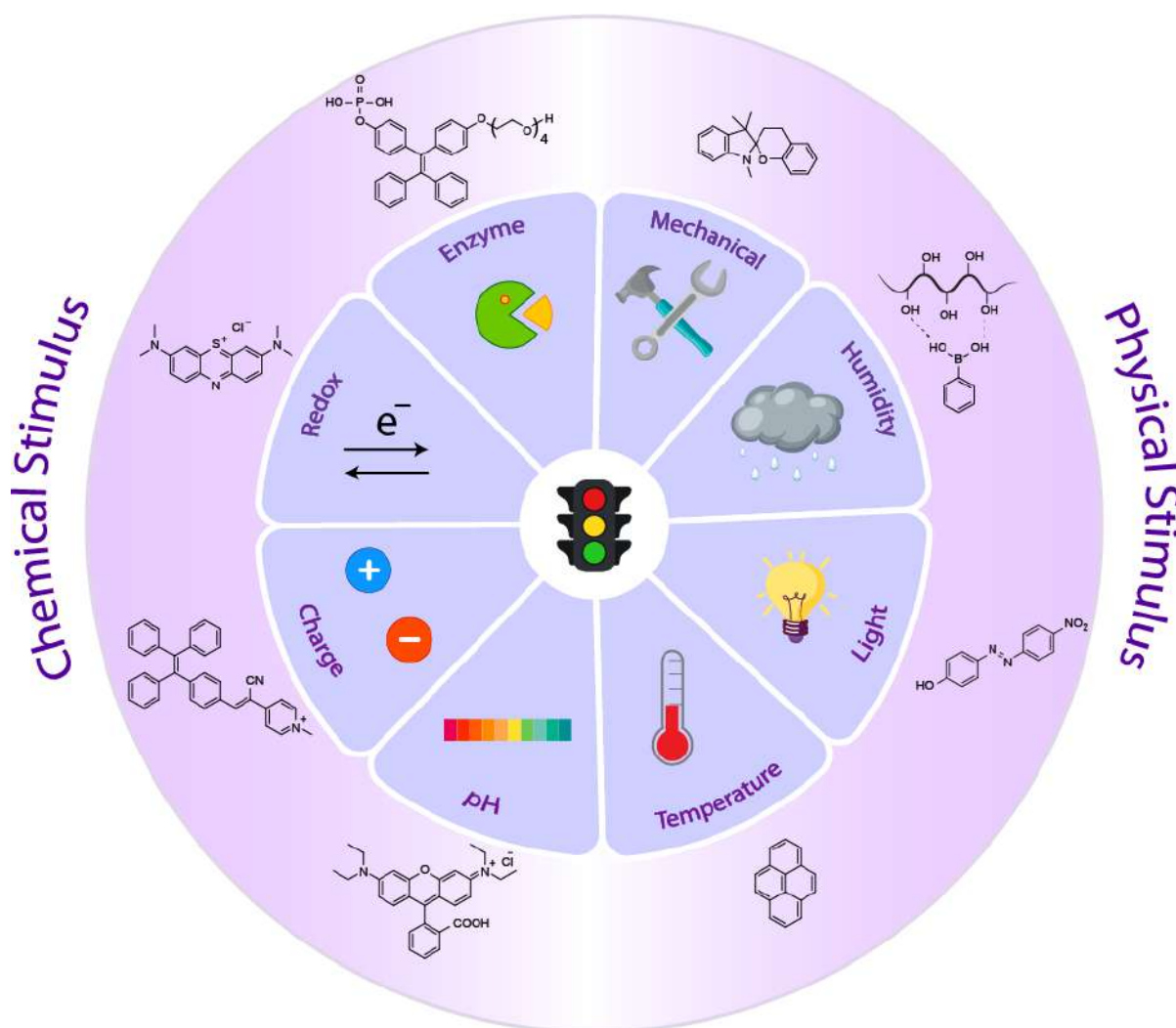


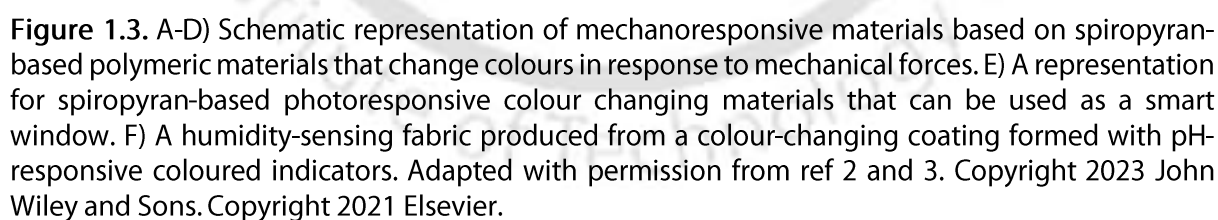
Figure 1.3. A representative depiction of categorization of stimuli-responsive colours obtained from molecules and assemblies with their representative examples.

1.5.2.1. Physical Stimulus

If a stimulus originates from an external method that affects the physical parameters of the solution, then it comes under a physical stimulus. Any changes that tamper with the physical characteristics of a system without the introduction of any new chemical agent belong to a physical stimulus. The sources of this kind of stimulus are usually physical elements or forces like heat, mechanical forces,

humidity, light, touch and sound that are applied externally. These factors mainly affect the temperature, water content and stress-strain ratios that affect stable assembly-disassembly equilibria or host-guest complexes. These changes are often brought along with chromatic transitions. Sources like light and sound might impart energy for chemical transformations and geometric rearrangements at the molecular level, showing colour changes. If the constant source of any of these physical stimuli is withdrawn, normalcy conditions are generally imposed, and the system reaches equilibrium. If reversible molecular changes have occurred, the molecules return to their initial equilibrium states with the original colours.

For example, azobenzene, when irradiated at 365 nm, shows a trans to cis isomerization accompanied by a colour change. The original colours are obtained by keeping the solution in the dark for some time. Similarly, colourful spiro compounds can be reversibly switched between differentially coloured forms through mechanical forces. For example, to investigate the effect of mechanical force on SP, a team at UIUC first prepared a linear poly(methyl acrylate) having precisely one SP unit near the centre of the chain.¹ When an acetonitrile solution of the polymer was sonicated, the colour altered from colourless to red, indicating the SP $\rightarrow$ MC reaction. The process was reversible. The solution turned transparent when exposed to visible light. No such effects were observed for the controls, indicating that the isomerisation was indeed induced by stress. It could also be incorporated into chains of bulk polymers, giving them shapes as shown in the dogbone-shaped samples of elastomeric polymer containing SP (Figure 1.3A). When the samples were subjected to tensile loading, red colour – an indication of ring opening – emerged, and its intensity increased with increasing levels of strain (Figure 1.3B). Similar behaviour was observed for SP-containing glassy PMMA. Compression gave an intense purple colour because of the tensile stress in the direction perpendicular to the loading direction, with the maximum tensile stress in the centre of the bead (Figure 1.3C, D). Importantly, intense colours, appeared well before the samples failed at high strain levels, suggesting possible uses of these materials for the detection and mapping of mechanical stress before catastrophic failure. The photochromic property of spiropyran is also well exploited in smart chromic materials. Luo et. al. have shown the application of an adhesive supramolecular film that can behave as a switchable smart window (Figure 1.3E).² They were prepared by mixing 3',3'-Dimethyl-6-nitro-spiro[2H-1-benzopyran-2,2'-indoline]-1'-ethanol (D) or 1,3,3-Trimethylindolino- β -naphthopyrpylospiran (T) into a supramolecular adhesive prepared by mixing (β -CD)/L-malic acid (L-MC). These films were capable of changing their transparencies between a coloured and a colourless state based on physical stimuli like sunlight and heat. Later on, they have been used as stimuli-responsive smart windows. These adhesives had appreciable shape-



Humidity severely reduces the emissive intensities of phosphorescent materials, and drying them restores the emissive properties. Humidity can also be sensed with the help of organic dyes that change colours in response to it. Zhang et. al. have reported humidity-sensitive discolouration materials that use the simple pH indicator dyes (Figure 1.3F).³ They have simply mixed indicators like thymol blue and cresyl red with a silica-based hydrophilic aerogel material, boric acid and silica gel to form a hydrochromic printing paste. When polyester fabrics are coated with these pastes, they

behave as humidity-sensitive materials. Multiple colours are displayed depending on the level of relative humidity exposure, and the materials show high repeatability over multiple cycles. Boric acid changes the pH of the system in response to humidity, bringing a change in the material's colours through the pH indicators. Pyrene derivatives can exist as excimers in concentrated aqueous solutions and exhibit emissions in the cyan-green region, while monomeric solutions emit blue. At higher temperatures, complexes become more dynamic, and the colour is blue-shifted. Thermal relaxation restores the original colours of emission (Figure 1.3).

1.5.2.2. Chemical Stimulus

Introducing a chemical agent to bring about transformations in colour is called a chemical stimulus. These agents alter the chemical environment of the surrounding solutions, which drives chemical transformations in the molecule. Factors that affect solvent polarity, ionic strength and viscosity of medium promote or demote self-assembly processes and bring consequent colour changes. Such changes can be brought about by mixing solvents or soluble salts in solutions.

Since a chemical trigger is added into the solution medium, a reverse stimulus is often mandatory to restore original conditions for the molecules to revert. pH-dependent and redox-sensitive groups also contribute significantly to reversible chromatic changes. Indicator dyes like methyl red can reversibly switch a solution between red and yellow by alternate triggers of acid and base, respectively. Similarly, pH-responsive emissive assemblies can also be used for obtaining wide-spectrum chemical colours. Gao and coworkers have reported a general strategy to produce pH-tunable, highly activatable multicoloured fluorescent nanoparticles (Figure 1.4A). They utilized common pH-insensitive dyes with emission wavelengths from green to near-IR range. They established a broad set of multicoloured (500-820 nm) nanoparticles with pH-induced fast micelle formation. Since the pH sensitivity was very high (fluorescence ON/OFF < 0.25 pH unit), incubation with human lung cancer cells was directly correlated to the pH transitions of endocytic compartments. Herein, the block copolymer of poly(ethylene oxide)-*b*-poly[2-(diisopropylamino)ethyl methacrylate-co-2-aminoethyl methacrylate hydrochloride], PEO-*b*-P(DPA-co-AMA) (PDPA-AMA) was used for the pH-responsive micellization. 5-Carboxytetramethylrhodamine was conjugated to yield PDPA-TMR copolymer. The fluorescence was quenched using BODIPY, coumarin and other dye derivatives that quench through various mechanisms inside the micelle, which is formed in response to pH. This pH-responsive ON/OFF mechanism allows for monitoring minute pH changes involved in cellular activities for physiological processes.⁴

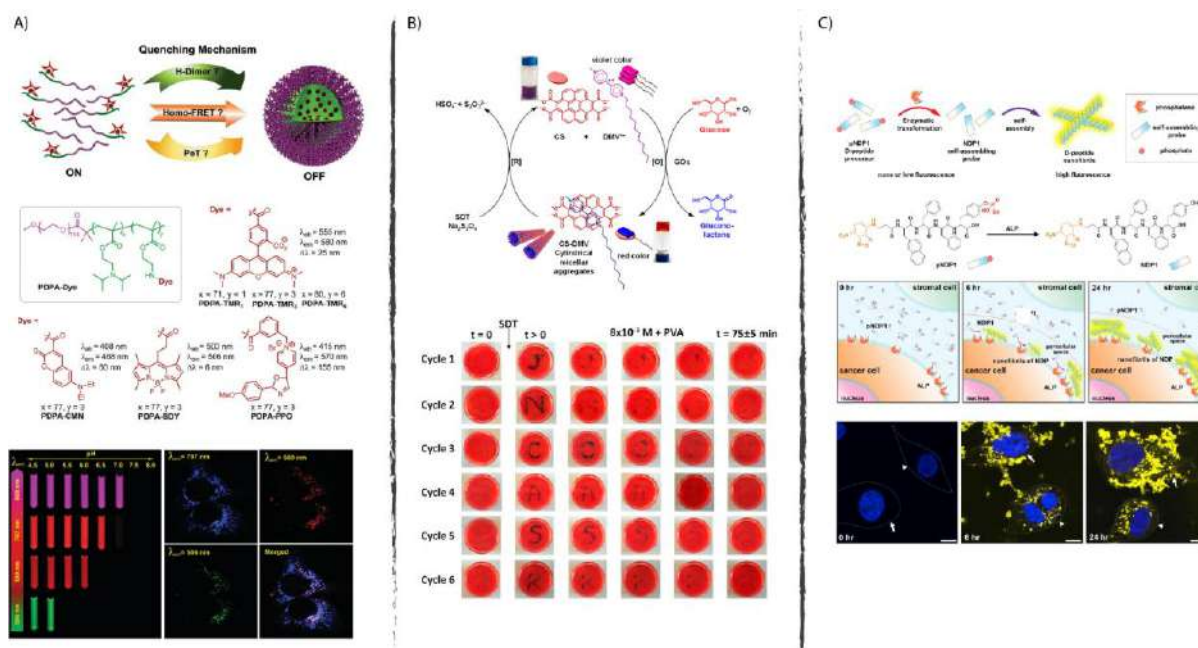


Figure 1.4. A) a pH-responsive biomimetic probe with multi-colour emissions for the diagnosis of cellular activities concerning pH through multimodal fluorescence quenching. B) A redox-responsive transient gel with variable colours from a charge transfer complex used as a self-erasable ink. C) An ALP-responsive biomimetic probe for cancer cells based on the ALP-mediated phosphate cleavage of an NBD-tagged peptide with phosphorylated tyrosine. Adapted from ref 4, 5 and 6. Copyright 2012 American Chemical Society. Copyright 2020 American Chemical Society. Copyright 2016 Elsevier.

MB imparts a blue colour to a solution. Reducing it turns the solution colourless, while the blue colour is restored on chemical oxidation. This redox-responsive property can also tune colours obtained from supramolecular charge transfer complexes. George and coworkers have reported a redox-mediated supramolecular gel with transient attributes. The gel has been used as an ink. The gel is formulated from the charge transfer complex between a polyanionic coronene derivative and an amphiphilic viologen molecule. Electrostatic interactions and a strong charge transfer complex between electronically dense and deficient species drive the gelation process. Reduction of viologen with SDT forms radical cations, which no longer form a stable gel network, and the gel disintegrates. The gel is reconstituted through an enzymatic oxidation process. The gel-sol-gel transitions are accompanied by intense colours – the gel is deep red due to charge-transfer interactions, and the sol exhibits purple from radical cations. The transient property was applied as a self-erasable ink (Figure 1.4B).⁵ The chemical transformations can also be regulated enzymatically, which can be instrumental in constructing enzyme-directed assembly-induced luminescent systems. Xu and coworkers have exploited this enzyme-instructed luminescent self-assembly for spatiotemporal profiling in live cells.⁶ They prepared an NBD tagged self-assembling peptide functionalized with a phosphate group, bringing its hydrophilicity as a precursor. Higher concentrations of ALP in cancer cells cleaved the phosphate group, driving the self-assembly

process. The nanofibrils show yellow fluorescence from NBD and are implemented in the spatiotemporal bioimaging of cancer cells. (Figure 1.4C). However, these enzyme-triggered systems often use unidirectional chemistry where the chemical transformation is irreversible (Figure 1.3).

1.5.2.3. Challenges in Chemical Stimulus-Reversible Colours

One major challenge with chemical stimuli is that most of them are permanent unless a reverse stimulus is externally added to force the original conditions back. Frequently, it is difficult to exactly determine the extent of reverse stimulus needed to negate the effect of the forward stimulus. In that case, the change of colours may not come back completely. Also, modern research aims at developing advanced and sophisticated systems where the applied stimuli are dissipative in nature. These stimuli are usually introduced through the help of a chemical trigger that initiates responsive changes in solution and maintains them as long as the trigger exists in the medium. Basically, the reverse stimulus conditions are developed in situ once the trigger is consumed and the equilibrium conditions are set back. This style of autonomous reversible colour changes has modern, life-like properties and counters the need to add any additional reverse trigger. However, the design and execution of such systems hold immense challenges and require intricately balanced chemical communications and feedback. The probabilities of interference and dampening from waste accumulations are major problems in this sector.

1.6. Autonomous Reversal of Stimulus

1.6.1. Chemically Triggered Transient Cycles under Non-equilibrium Conditions – Emergent Properties and Significance

Autonomous reversal of stimulus, implementing chemical triggers that bring about non-equilibrium conditions, is currently in focus to create biomimetic features. Usually, a chemical system is driven by the laws of equilibrium thermodynamics. When the particular chemical trigger is introduced as the stimulus, it first disrupts the equilibrium of the system. The responsive molecules (dyes, luminophores, self-assembling components, host-guest complexes) of the system experience this disturbance. They respond to the stimulus accordingly and transform. The transformations range from the formation of assemblies, self-organized structures, gelations, disassemblies, geometrical isomerizations, ionizations, etc. This pathway of chemically triggered transformation follows fast kinetics, occurs instantaneously and is often denoted as the activation routes.

The chemical trigger is formulated in such a way that it either contains the reverse stimulus in a dormant stage or slowly activates the reverse stimulus when introduced into the solution. The

reverse stimulus that can restore the original equilibrium conditions comprises a deactivation pathway where molecular transformations are reversed. The key lies in the difference in the kinetics of both these activation and deactivation routes, which makes the molecular transformations discernible. In short, the transformations appear quickly but disappear slowly because of the slower kinetics of the deactivation route. This allows the transformations to have a finite lifespan and brings transient features under non-equilibrium conditions (Figure 1.5). The deactivation cycle is always operative, but its effects are visible only when the activator molecules are consumed in the trigger, and the activation pathway is slowed down. The reverse stimulus is then more pronounced and is generated in situ without any additional intervention. These transient transformations bear dynamic qualities as they are constantly being balanced between forward and backward equilibrium. The balance can be regulated as a function of time by monitoring the ratio of the activator and deactivator. In general, higher quantities of activators favour a longer lifetime of transformations, while increasing the deactivator leads to short-lived changes. These changes are carefully coordinated through feedback mechanisms, which dictate how far and how fast the transformations should go. These mechanisms can regulate not only the time but also the spatial position of the transitions, giving the transient systems chemically triggered spatiotemporal control over dynamicity.

These features resemble biological attributes, where most chemical and morphological changes are conducted under non-equilibrium conditions, mainly on the instruction of chemical fuels. Biochemical feedbacks play a major role in regulating biological functions and structures. The study of these transient systems comes under systems chemistry, which aims at mimicking emergent properties of biological systems under abiotic conditions. These include transient features under non-equilibrium conditions, compartmentalizations, growth and division, feedback mechanisms, dynamic properties, adaptabilities, self-regulatory kinetics, etc.

If the changes occurring under out-of-equilibrium kinetics are accompanied by colour and emissive transitions, then fascinating and mesmerizing colour changes can occur temporally. These colour transitions will then have transitory features. They will be generated instantaneously, exist for a finite time and eventually return to the original state. In the process, they will produce time-gated colour programmes where multiple colours will originate, transition into different shades and return to previous conditions. These transitory colours resemble transient bioluminescence in many aspects.

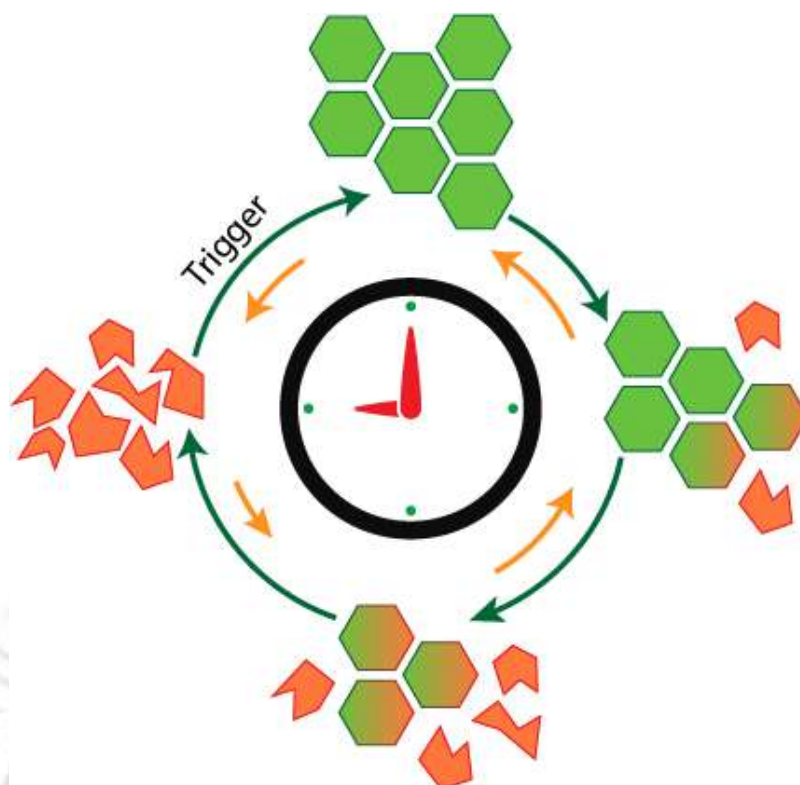


Figure 1.5. Schematic representation of a temporally regulated chemical clock guided transient assembly under non-equilibrium.

1.6.2. Time Domain in Non-equilibrium Cycles – Chemical Clocks

The time domain is a very crucial attribute of chemically fueled transient systems. The concept of temporal regulation of chemical transformations through a single trigger is not only novel but also promising in offering newer avenues in the field of self-assemblies. The temporal feature is crucial in understanding the time cycles of biology. The best quality of the time domain is that it can be modulated conveniently and implemented in several applications. Temporally operated non-equilibrium cycles are constructed through chemical clocks. Chemical clocks are time-locked chemical programmes where a single molecular trigger is used to coordinate activation-deactivation pathways of non-equilibrium transformations (Figure 1.4). The concentration ratio of activator: deactivator is tuned to control the time coordinates in solutions. When the clocks are operated with a fixed ratio, a distinct time programme is conducted through the clock. When repeated in the same solution with the reintroduction of the same composition of chemical trigger, theoretically, the chemical clock should run over the same duration. However, due to the accumulation of wastes from activation and deactivation routes, the clocks get impeded, and the effective duration is reduced with every iteration. Accumulation of chemical wastes retard the chemical cycles as they disturb the equilibria of chemical reactions. Several chemical clocks have

been constructed and widely implemented in designing temporally regulated assemblies. However, every chemical clock has its drawbacks, be it in terms of chemical dampening or applicability in solutions, etc. This makes the quest for developing a newer chemical clock with more upgraded and advanced properties that aim at the betterment of dynamic features. These chemical clocks can be classified based on the type of chemical triggers used or on the particular chemical parameter of the system they affect. In this context, the next two sections discuss two such chemical clocks in detail.

The time domain can be controlled to obtain dynamic colours under dissipative conditions or under transient conditions under non-equilibrium thermodynamics. For example, pyrene forms excimers that emit yellow to green, while the monomers show blue emissions. The colours can be regulated in a time-controlled manner. For instance, Da-Hui Qu and coworkers have shown time-dependent multicoloured fluorescence with supramolecular coordination assemblies (Figure 1.6A).⁷ A pyrene-based ligand, which generally emits blue fluorescence, coordinates to metal ions that kinetically trap it in nanofibers that show yellow emission. The fibres slowly evolve into nanosheets over time, which emit green and eventually generate the free molecules back in solution, showing blue emission. In another work, they have also achieved similar observations, but this time they controlled the time domain with an enzymatic degradation process (Figure 1.6B).⁸ A pyrene-based amphiphile was self-assembled into yellow emissive vesicles with the host-guest complexation using CD. Amylase degraded CD over time, which released the free amphiphiles in solution. The slow decomposition process was accompanied by similar colour transitions. The time coordinate of both the colour transitions could be manipulated. In the first case, it could be done simply by changing the concentrations of metal ions that kinetically trap them. In the other case, it could be regulated by changing the ratio of CD (substrate) to that of amylase.

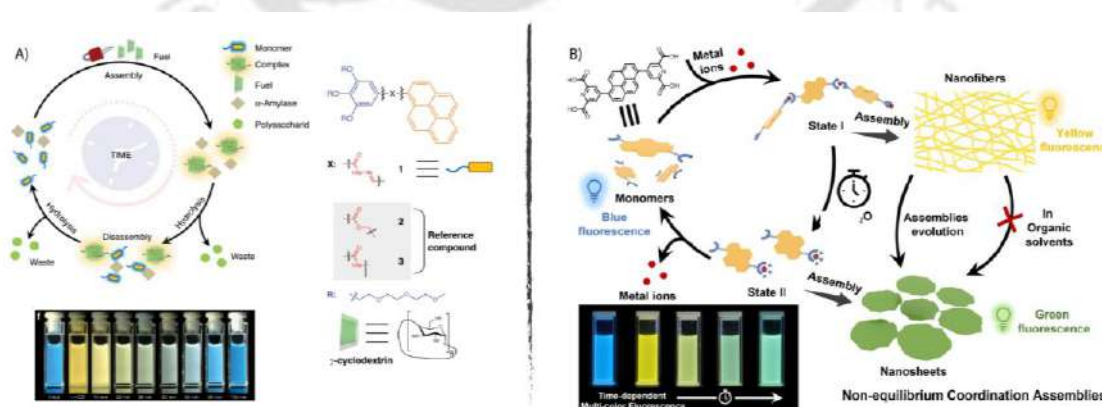


Figure 1.6. Two chemically fueled colour programmes obtained from the transient aggregation of pyrene-based amphiphiles with – A) a dissipative chemical clock obtained from the kinetically trapped fluorescent aggregates and B) a transient vesicle formation regulated by an enzymatic degradation process. Adapted from ref 7 and 8. Copyright 2024 John Wiley and Sons.

1.6.2.1. pH Clocks

A chemical programme that demonstrates the temporally cyclic variation of pH is termed a pH clock. An aqueous solution of any molecule has a particular pH. When a pH clock trigger is added chemically to the solution, a sharp and quick pH change is registered in the solution. This is the activation step for a pH cycle. The deactivation pathway counters the change in pH and brings the solution back close to its original pH. Depending on the direction of the triggered pH change, the clock can be called a forward or a reverse pH clock (Figure 1.6).

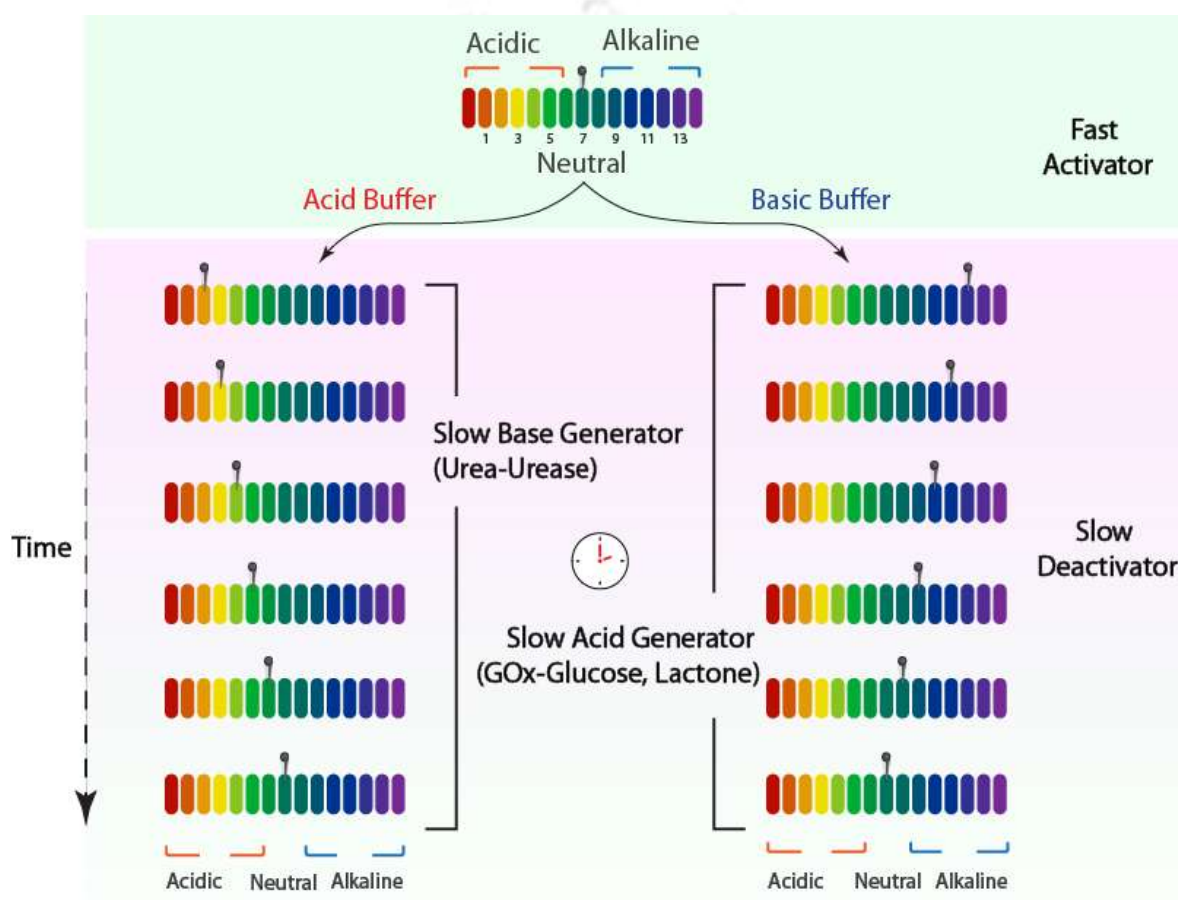


Figure 1.6. A generalized representation of a forward and reverse pH clock.

In a forward pH clock, the initial pH is usually low. When the trigger is added, the pH swiftly jumps to a high value and then slowly decreases over time. Since the first change is a quick incremental jump followed by gradual decremental steps, this pH clock is regarded as the forward clock. This clock is most commonly used for designing temporal pH functions. Very often, the fast activation is brought about by the urea/urease enzymatic couple or by a simple basic buffer like the TRIS buffer. Enzymatic reactions and buffers can reach high pH of limited values. Higher alkaline ranges can be achieved with stronger bases, such as NaOH, which has its own drawbacks. The high pH is

sometimes difficult to counter by the deactivation step, and sometimes a strong alkaline medium adversely affects molecular integrity.

The decline in the pH requires a chemical or enzymatic reaction that eventually releases acid over time. Base-mediated saponification reactions of esters release carboxylic acids through slow kinetics, which gradually reduce the pH. Several esters (esters of acetic acids, etc) and cyclic lactones (GdL, butyrolactone, etc) can be employed for this process. The rate of acid production is dependent on the hydrolysis propensity of the ester. Reactive esters that produce stronger acids show faster deactivation and can reach lower values of pH. This not only controls the lifespan of the pH clock but also the pH range that is covered by the clock. Hydrolysis of sultones liberates stronger inorganic acids, which can make solutions reach a low pH value of 2.0. However, dealing with extreme pH has its own consequences, as mentioned before. Extreme pH denatures enzymes in solution. Enzymatic couples like the glucose/GOx and long-chain esters/lipases can also be instrumental in bringing the pH of a solution down with time. Combinations of different activation and deactivation methods can give rise to different chemical, enzymatic or chemoenzymatic pH clocks depending on the goal. Enzymatic clocks are more modifiable as they provide several parameters, like enzyme and substrate concentration, that govern the overall pace, duration and range of the pH clock (Figure 1.6). Das and coworkers have shown dynamic broad-spectrum transitory emission with size-adaptive vesicles using the TRIS-GdL pH clock (Figure 1.7A).⁹ The broad-spectrum emissions have been brought by breathing vesicles that alternatively capture and release FRET pair dye in and from confinement in their hydrophobic domains. The pH clock enables the transient generation of transitory colours with different ratios of the FRET pairs, bringing temporal regulation of multicolour emission. Similarly, George and coworkers have demonstrated a living and transient supramolecular polymerization process using a chemical fuel under the influence of the urea-urease enzyme couple and the hydrolysis of lactones (Figure 1.7B).¹⁰ Basically, a red transient gel was formed by a charge transfer complex between a coronene derivative and a viologen amphiphile. The formation of the amphiphile was imine-based and hence promoted by the urea hydrolysis. Deactivation was brought by lactone hydrolysis or lipase activities that reduced the pH and disintegrated the amphiphile.

A reverse pH clock initially begins with a high pH value. The chemical trigger pulls the pH down to the acidic range, and the basic conditions are imposed slowly over time. One easy way is to utilise an acid buffer to reduce the pH, and the popular urea/urease couple to slowly basify the solution. It is imperative to mention here that autonomous pH regulation with in situ generation of base is best exemplified through pH clocks involving decarboxylation of activated carboxylic acids and photoacid triggers. When an activated carboxylic acid (TCA, TBA, α -Nitroacetic acid, 2-cyano-2-

phenyl propanoic acid) is added to a solution, it acidifies instantaneously. The time-gated decarboxylation of acids generates conjugate bases, which trap acids in solution and raise the pH. The utility of these pH programmes lies in their differential solubility range. Some acids work more efficiently in organic media, making them widely employed in designing time-regulated molecular machines which have limited water solubility. The acid-mediated molecular machine has been implemented in achieving transient fluorescent systems. Leigh and coworkers have shown the transient fluorescence output from a pulsed fuel molecular ratchet using the dissipative nature of TCA (Figure 1.7C).¹¹ Fueled motions across the molecular ratchet transiently prevented the quenching process, leading to strong fluorescence. Dissipative changes in pH change the position of the molecular ring, causing changes in fluorescence. Using a similar concept, Stefano and coworkers depicted the dissipative control over fluorescence in a pyrene-decorated calixarene (Figure 1.7D).¹² Dissipative action of TCA initially protonated the calixarene, bringing conformational changes that showed low excimer fluorescence from pyrenes. After decarboxylation, when the initial pH was restored, the equilibrium conformation was regained, and the emission was again observed. Under all these examples, the time period could be varied with different concentrations of activated acids. Some acids can be used in aqueous systems as effectively as in organic media, making them applicable for temporal control of pH-responsive aqueous assemblies. They also cover a wide range of pH depending on their ionisation strengths (Figure 1.5.). Photoacids, on the other hand, are controlled through light irradiation. Reversible photochemical transformations that can create temporary variations in pH can build pH clocks. Irradiation of light of a certain wavelength can cause photoresponsive molecules (spiropyran) to undergo cyclizations and structures that are accompanied by acid release. Removal of the light source leads to slow thermal relaxation, and equilibrium structures are retained, which consume the liberated acids. However, these photoacid pH clocks are very short-lived owing to the fast relaxation process of most photoacids. The photoacids are mainly organic molecules and chiefly produce a short window of pH changes. These carboxylic acid-based and photo-regulated pH clocks have one tremendous advantage absent in most chemical clocks. They depend on a clean and green source of fuel and generate no chemical wastes, which reduces the efficiency of cycles. Decarboxylation generates gaseous wastes that never accumulate in solutions and permits higher repeatability of the pH clock with similar efficiency in subsequent iterations. In light-mediated transitions, the same molecule acts as a source and sink for acids, leaving no waste behind.

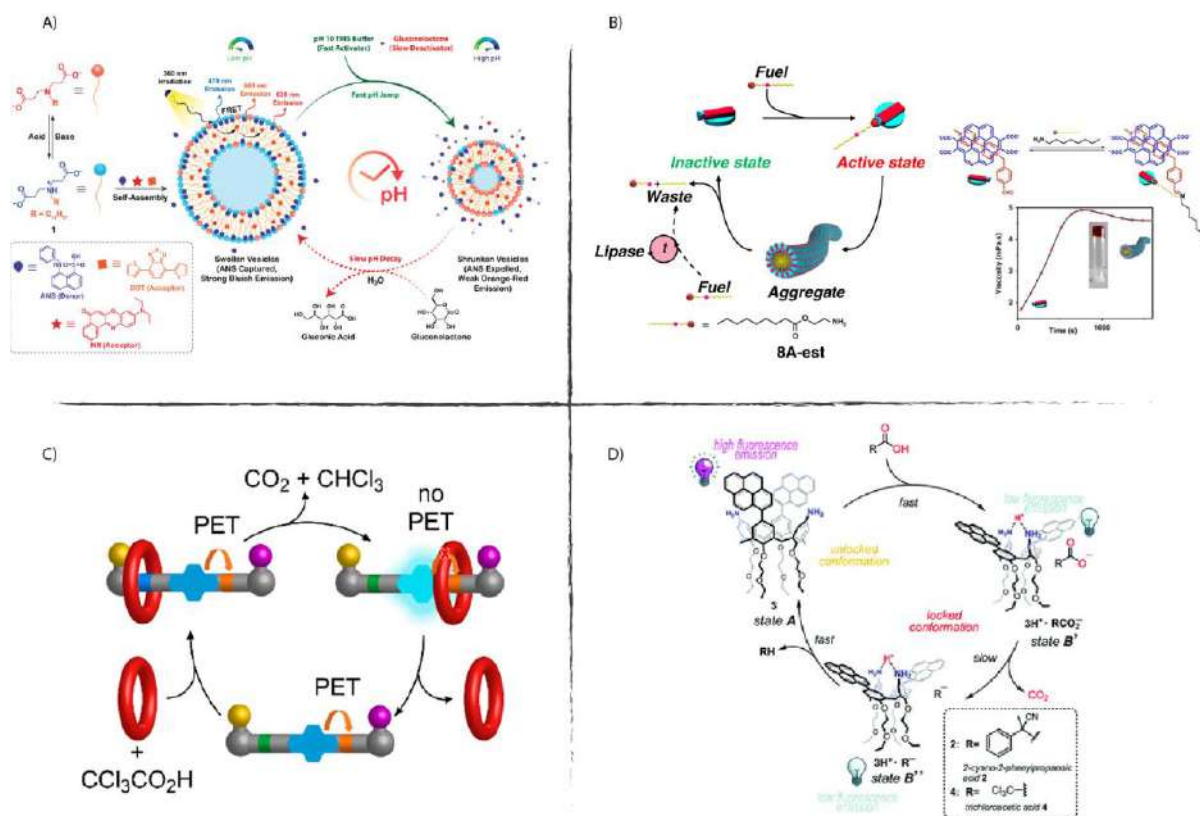


Figure 1.7. A) pH-clock guided dynamic vesicles with multicolour emission. B) Chemically fueled transient supramolecular polymerization guided by an enzymatic pH clock. Transient emission obtained from the dissipative pH clock with decarboxylation of trichloroacetic acid in C) molecular ratchet and D) pyrene modified calixarene. Adapted from ref 9, 10, 11 and 12. Copyright 2023 John Wiley and Sons. Copyright 2022 Royal Society of Chemistry.

Depending on the nature of responsiveness, molecular properties and the purpose of the goal, one can select a pH clock as per the requirement. pH-dependent molecular transformations that accompany chromatic transitions can be coupled with pH clocks to generate temporal colour programmes. These transitory colours can be used to create smart optical systems with modern state-of-the-art applications. Modulable pH clocks bring modulable time domains that can create time dimensions in transitory colours. pH clock guided transitory colours and emissions are similar to bioluminescent systems in many aspects because biological or cellular pH has a tremendous effect on the colours and nature of bioluminescence in many animals.

1.6.2.2. Redox Clocks

Similar to pH clocks, redox clocks are chemical programmes that temporally regulate the reductive and oxidative propensity of a solution in a temporally cyclic manner. Generally, a thermodynamically open system is under an oxidative environment due to the abundance of aerial O_2 . Adding a reducing agent forces the system into a reductive environment. Redox-active molecules in solution then undergo chemical reductions under such circumstances. The reduced

species remain in solution as long as the reducing agent supports reductive environments (Figure 1.8). The reductants used are generally strong reducing agents like GSH or SDT, which cause rapid reactions. The selection of reducing agents depends on the difference between the reduction potentials of the molecules and the reducing agent. The larger the difference faster the reduction kinetics. Once the reductants are consumed, the molecular species are gradually oxidized back to their equilibrium forms. Consumption of reductant fuels reestablishes the equilibrium between the aerial and dissolved O_2 . A constant dynamic redox transformation is maintained in this non-equilibrium environment, where initially the overall equilibrium is more directed towards the reduction step. The backward oxidation is always present but is less predominant. When O_2 is utilized for oxidation through slow attainment of equilibrium, more atmospheric O_2 is introduced into the solution. The lifetimes of the reduced species are longer when a higher concentration of reductants is added to the trigger. Redox clocks involving aerial deactivation are popular as they offer no chemical wastes and can be tuned easily with only the activation route. However, this is possible only in open systems.

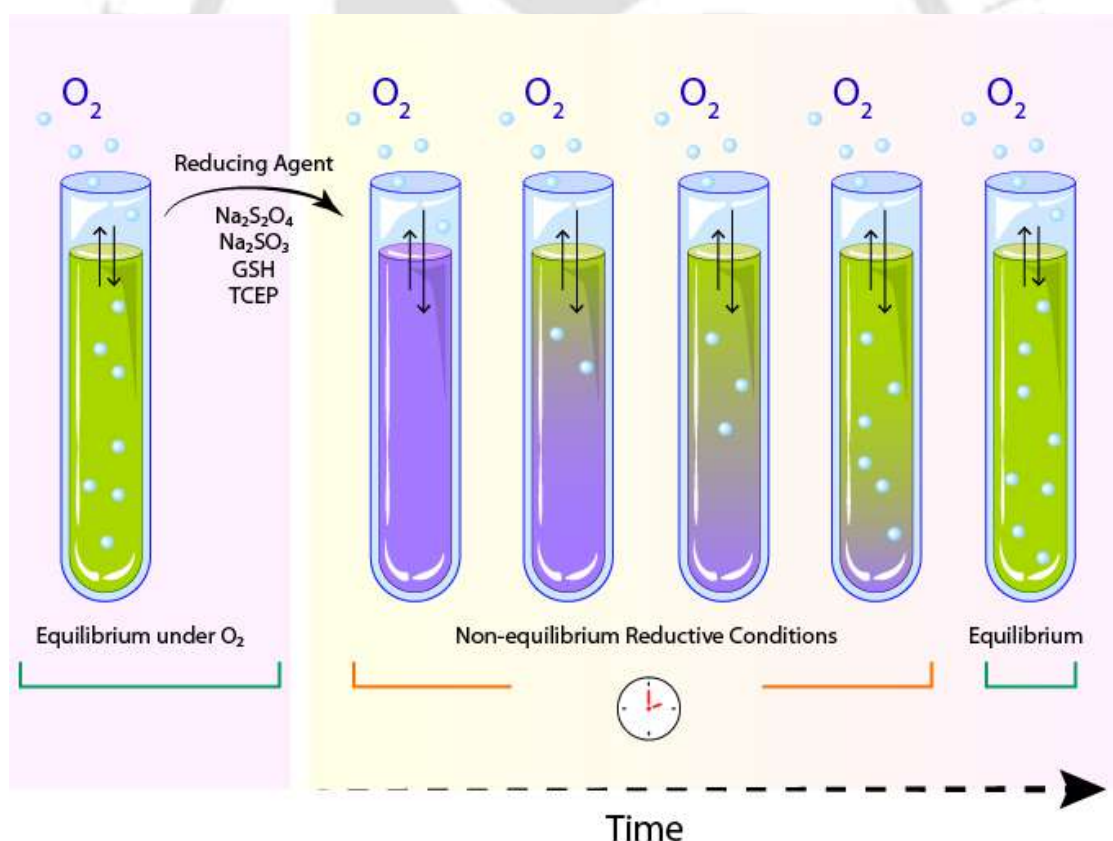


Figure 1.8. Schematic representation of a redox-clock under an open environment.

In this context, George and coworkers have demonstrated a redox-mediated transient reconfiguration using the SDT- O_2 redox clock in a redox-responsive NDI-based supramolecular

assembly (Figure 1.9A).¹³ They demonstrated that an NDI-based compound is capable of forming vesicles under oxidative conditions. When they are pushed out of equilibrium with SDT, they are reduced to their anionic states, which change their morphologies to nanosheets and emit pink-red. Aerial oxidation brings them back to the original vesicular states with green emission. This transitional emission is modulable with respect. time depending on the equivalents of SDT used. Interestingly, Kim and coworkers have shown that this SDT-O₂ system can be employed in designing coloured spatiotemporal patterns that can be controlled with audible sound (Figure 1.9B).¹⁴ These patterns are generated from differential gaseous solubility produced in aqueous surfaces from audible sound frequencies. SDT and methyl viologen have been used for this purpose, and variable patterns have been controlled efficiently through the selective diffusion of aerial oxygen in different domains of waves produced on the water surface with sound frequencies.

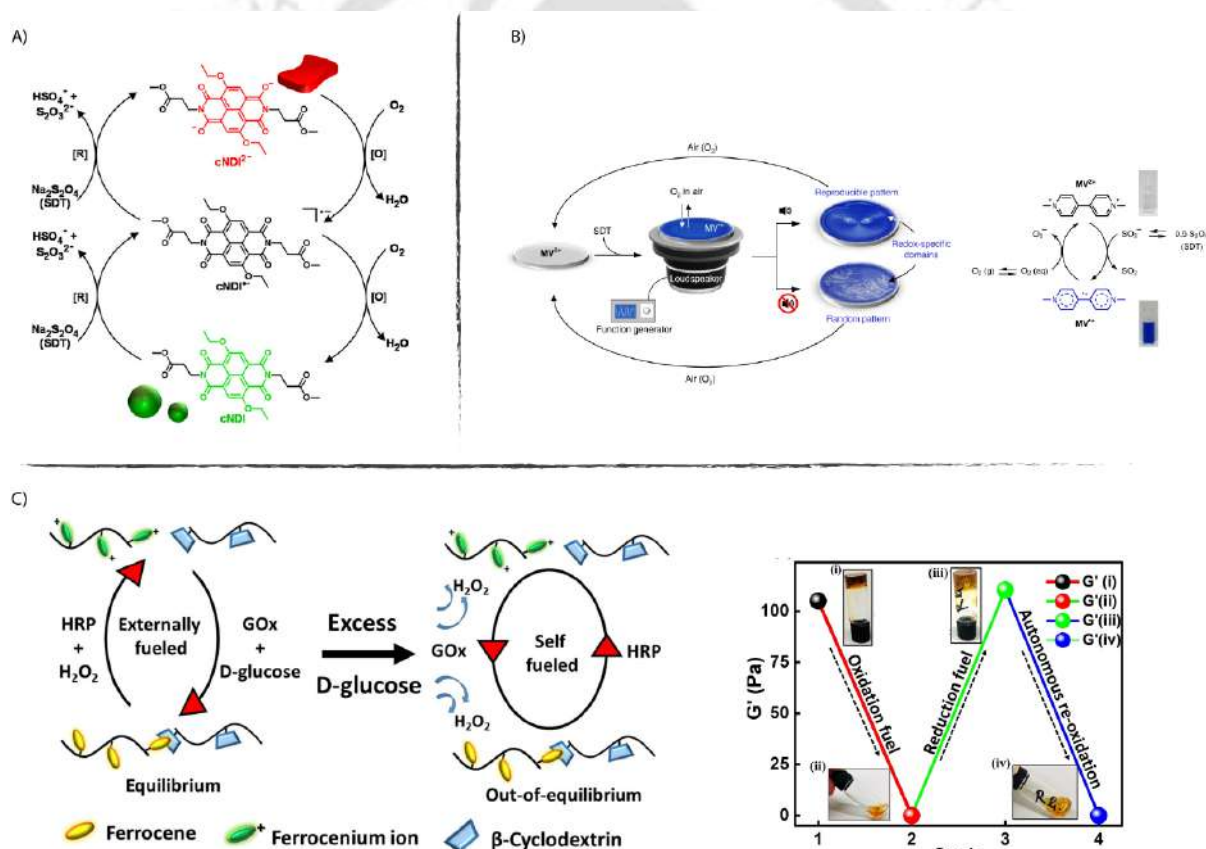


Figure 1.9. A) Redox-mediated transient reconfiguration of NDI-based supramolecular assembly. B) Spatiotemporal control of colour patterns with audible sounds with a redox-responsive viologen dye. C) Host-guest mediated gelation with redox-responsive gel-sol transition. Adapted from ref 13, 14 and 15. Copyright 2019 John Wiley and Sons. Copyright 2020 Springer Nature. Copyright 2021 John Wiley and Sons.

Under closed or isolated conditions, the reverse oxidation can be controlled by including a slow oxidant like H₂O₂ in the solution. The oxidants can also be produced sustainably in situ through

enzymatic reactions like glucose/GOx. The enzymatic kinetics can be cautiously regulated to produce H_2O_2 in a controlled manner to tune the duration of the redox clock. Peroxidases that consume H_2O_2 can be combined with these enzymes to create complex reaction networks. Balancing the kinetics of these antagonistic enzymatic routes involving multiple enzymes can also fine-tune the redox clocks. Such systems are both significant and difficult to achieve as they strategically mimic biological complexities, where multicomponent systems operate in unison through feedback processes. Ravoo and coworkers have reported the transient gelation of a redox-responsive host-guest mediated gelation process, accompanied by colour changes (Figure 1.9C).¹⁵ The redox cycle has been achieved by enzymatic activities. The crosslinking between a ferrocene-functionalized polymer and a CD-functionalized polymer from host-guest inclusion can be controlled by the reversible redox reaction on ferrocene. Gels are formed from the inclusion of ferrocene in hosts. Ferrocenium groups fail to enter the cyclodextrin cavity, and hence, sol is generated from a gel when the system is oxidised. The redox balance is achieved by an enzymatic reaction network of GOx and HRP, which generate and consume H_2O_2 , respectively. The kinetics and time of gel-sol-gel transitions are controlled by regulating the enzymes. The switchable colours appeared from different oxidation states of ferrocene that absorb in different regions of the spectrum. Light-mediated redox clocks can also prove to be cycles with no waste generation in cases where photo-redox molecules are considered. Irradiating these molecules with specific wavelengths reduces them, and they persist as long as the source of light is maintained. Under dark oxidative conditions equilibrium is reached slowly.

These examples depict how different redox triggers can be monitored and adjusted to regulate autonomous stimuli-reversal in redox clocks in a time-gated manner. These clocks can temporally make or break supramolecular emissive assemblies, chemical covalent bonds and host-guest complexes, mainly accompanied by chromatic transitions. Generally, when redox clocks are executed with organic molecules containing pyridinium groups, radical cations are produced that impart intense blue-violet colours to solutions under non-equilibrium conditions. Many redox-responsive organic dyes, when coupled to redox clocks, produce cyclic variations in colours and patterns like the blue bottle experiment and the Belousov-Zhabotinsky reaction. Basically, in these redox programmes, there are periodic variations of reductants and oxidants over time that shuttle redox equilibrium forward and backwards repeatedly over time. These are autonomous cycles in the truest sense, which can generate iterations over time through single triggers. A variety of redox clocks can thus be utilized to construct colour-tunable temporal programmes. However, one must deal with certain drawbacks concerning redox clocks. Very often, reactive radical species can be generated, which tend to interfere in emissive systems through paramagnetic quenching, leading

to the disappearance of colours. Reactive radicals can also side-step into chemical reactions, making the redox cycles redundant.

1.7. Colour Components and Representations

To understand and comprehend colours, an idea of their components and representation is necessary. Human eyes are sensitive mainly to three different regions of the full visible spectrum. The short, medium and long wavelengths appear as blue, green and red to the human eyes, respectively. Intermittent wavelengths produce intermediate colours from the combination of these primary colours. Hence, every colour is generally expressed as the extent or proportion of these three colours in them, giving them a unique red (R), green (G) and blue (B) constituent.

A colour is described from its set of components that depict the characteristic nature of a colour – hue, saturation and value (luminance). Hue refers to the basic or central component of the colour – red, blue or green. The saturation of that colour basically informs us about the intensity or the purity of the colour. If the saturation is high, the colour appears more vivid and lively, while a less saturated colour means a more diluted or muted version. Luminance reveals the brightness or darkness of the colour. Increasing whiteness in a colour gives it a characteristic ‘tint’ while increasing the blackness produces different shades. So, hue is determined by which component (R, G or B) is higher. The degree of saturation is a measure of how high that component is. Different shades and tints are obtained by mixing different proportions of black and white into that component of a specific intensity.

Colours are represented in 2D formats through coordinates in CIE plots or chromaticity diagrams. These diagrams are two-dimensional plots of differentially coloured regions in a 2D space where an infinite number of unique coordinates can represent a unique colour through endless variations in R, G and B components. The RGB values corresponding to a particular coordinate are unique to a particular colour, and that coordinate is its associated visual representation.

RGB components are real quantitative values that uphold colour properties, while CIE coordinates are representative values obtained for depiction purposes. Although other modes of representation, like the CMYK format, are also popular in colour depictions, most of these formats are updated or derived versions of the fundamental RGB style of representations.

1.8. Applications of Stimulus-Responsive and Autonomous Regulation of Colours in Chemical Systems

1.8.1. Information Encryption and Anti-Counterfeiting Strategies

We currently live in a world surrounded by data and information. This calls attention to the smart, safe and secure utility, storage, transmission and access to data. If information and data are mishandled, then it could destabilize human society. Data misuse adversely affects several aspects of social integrity, like economics, health, national security, etc. Forged data and counterfeited information are rising threats that openly challenge the well-being of society. This has focused researchers to develop more advanced methods and sophisticated techniques that aim at safe data storage with updated anti-counterfeiting features. In this regard, applying colours that respond to stimuli can prove to be effective. The correct and authentic information should be conveniently discerned directly through visual identification marks and techniques. Colours can help in this identification, and stimulus-responsiveness can help in maintaining the secrecy and discretion involved in the condition-specific identification process. This can not only prevent an important piece of information from being illicitly pirated but also help us in differentiating between real and fake information. A specific colour can act as an identification mark of authenticity when it appears, disappears, or transitions into another under different conditions. For example, specifically coloured logos or watermarks or symbols are crucial in discerning real currency notes from forged notes. Like holograms, these marks are visible only at certain angles, otherwise not.

To this end, stimuli-responsive chemical colours can be used in designing such anti-counterfeiting data storage strategies. When data is stored or encrypted through coloured cryptographic patterns, a large amount of information is stored in them smartly and shrewdly. These patterns usually comprise a specific colour combination and arrangement that encodes particular information. Cryptic features are ascribed to it when the colours appear under a stimulus, while disappearing or morphing into other colours under reverse stimulus. Colours obtained from luminescent assemblies are noteworthy in this aspect because they have an inherent cryptic feature. These colours are visible only under UV light, bringing in the concept of condition or stimulus-specific decryption. They are inconspicuous under normal light, making them secure encryption formats. Sometimes these luminescent systems emit only under the effect of a specific stimulus, like pH, small molecules, temperature, etc, making the colours and hence the information multi-stimuli responsive. Several unique colour-encoded cyphers have been reported through the development of smart multicolour emissive systems. Emissive assemblies involving fluorescent dyes, phosphorescent-based afterglow materials, light-harvesting systems, luminescent gels, and nanoparticle-based aggregates have

been extensively exploited to fabricate anti-counterfeiting systems for safe data storage. They encompass a wide variety of encryption styles like barcodes, 3D colour codes, QR codes, watermarks, holograms, etc. Da-Hui Qu have shown an assembly-encoded information encryption with multidimensional properties (Figure 1.10A).¹⁶ The multicoloured emission from different assemblies of a pyrene-based amphiphile was regulated with solvent exchange. As the colour changed with the changing stimulus a new information was generated.

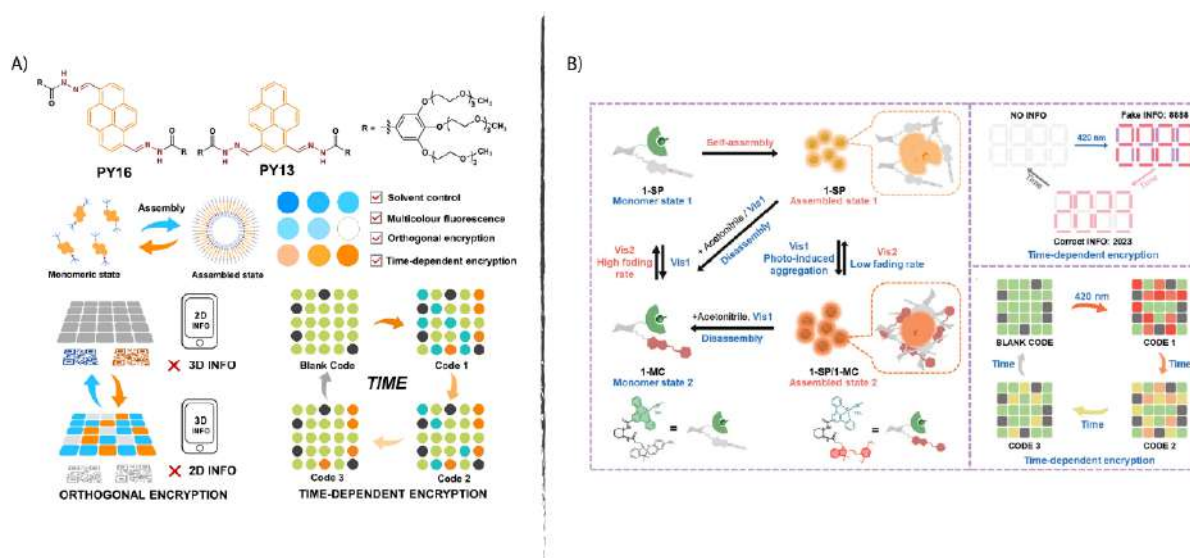


Figure 1.10 A) Time-controlled information encryption through A) assembly-encoded emission in a pyrene-based self-assembling amphiphile through solvent-induced aggregation and B) light-induced chiroptical switching of emission in a spiropyran-based Luminogen. Adapted from ref 16 and 17. Copyright 2023 John Wiley and Sons.

Although much has been done in this colour-based encryption study, most colour codes are simply stimulus-responsive and operate under UV light, which brings their own limitations. In this context, there are only a handful of reports where autonomous change of chemical colours has been exploited. Temporally regulated colours from dynamic assemblies and modifiable colours from dyes can introduce time as a new dimension that enhances the level of security for safe data representation. If the colours are chemically fueled and regulated over time, then a time window emerges that governs the colour patterns via a chemical clock. The right information is valuable if it is accessed at the right time. Time-controlled colour changes the true information with time and allows deciphering the information with constraints that enhance the security standards. For example, a report on a SP-based time-resolved optical switch has clearly demonstrated how colours both from emission and organic dyes can be used for encryption (Figure 1.10B).¹⁷ The photoresponsive behaviour of spiropyran has been used to change the assembly-induced colours in solution. These changes are all time-locked and regain their original colours once the irradiation

is stopped. In both examples, the information is linked to the colour code and can be decoded at the right time with real-life scanning of the dynamic codes.

1.8.2. Sensors

It is crucial to maintain environmental and health standards in this modern world of ever-changing surroundings. They are effectively monitored through the detection and quantification of certain markers and analytes. A feasible way to estimate them is through visual changes, implementing chemical tools. Supramolecular systems and organic dyes that change emissions and colours in response to specific chemical components can be used to detect them. The visual changes in colours can help in qualitative sensing, and the intensity of change gives a quantitative measure. The analyte can be sensed either through transitions in colours or alterations in the intensities of the same colour.

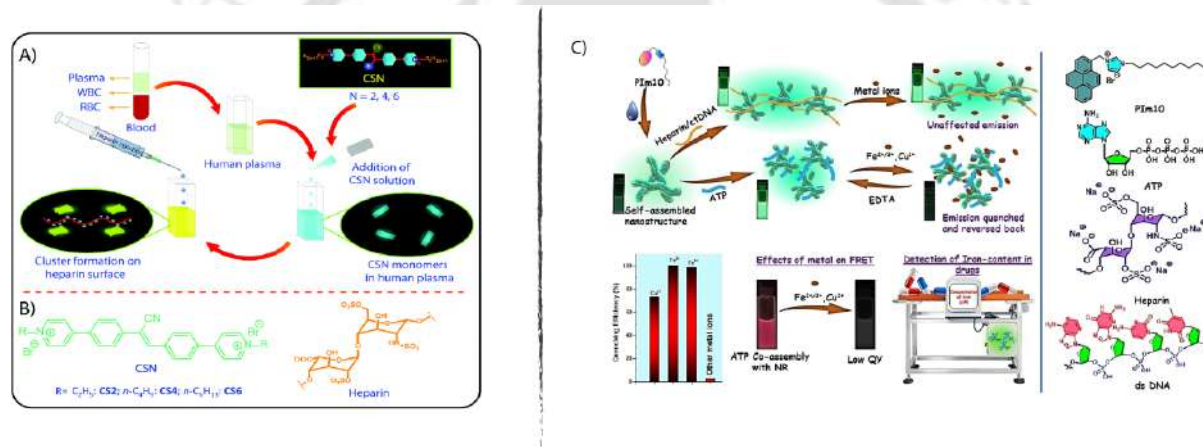


Figure 1.11. A) Sensing of heparin with a CSB-based cationic AIEgen. B) Structures of CSB-based sensor and heparin. C) Sensing of metal cations with a pyrene-based sensing probe through fluorescence quenching. Adapted from 18 and 19. Copyright 2021 Royal Society of Chemistry. Copyright 2024 John Wiley and Sons.

When an analyte interacts with a supramolecular assembly involving a luminescent dye, it changes the spectroscopic behaviour of the system by altering the energy levels of the fluorophore or phosphor. It affects the visible colours of organic dyes similarly. There are several underlying mechanisms, like CT, ESIPT, AIE, PET, FRET, SQ, DQ, etc, that can lead to changes in spectral patterns. The operative mechanism is dependent on the molecular design. A typical sensor has to be designed with a receptor moiety (the part responsible for specific recognition of the target analyte), a signal unit (the portion that produces a detectable signal triggered by interaction at the receptor site), and a signal transducing group (functional group that relays the chemical interaction into a signal through changes in electronic or conformational properties). Other units might be introduced for advanced features or specific attachments and functions. If there is an enhancement in the

colour signal on encountering the analyte, the sensor is said to be a 'turn on' sensor, while quenching behaviours and reduction in intensities of colour signals make it a 'turn off' sensor. Banerjee and coworkers have developed methods of sensing polyanionic species as analytes. In one of their methods of heparin sensing, they have utilized a CSB-based AIEgen that shows turn-on fluorescence for heparin detection. The otherwise water-soluble cationic probe shows low fluorescence in water but forms aggregates with negatively charged heparin to show strong fluorescence (Figure 1.11A).¹⁸ Utilizing a similar heparin-based fluorescent aggregate with a pyrene-based amphiphilic molecule with ATP, they demonstrated detection of metals through fluorescence quenching as well (Figure 1.11B).¹⁹

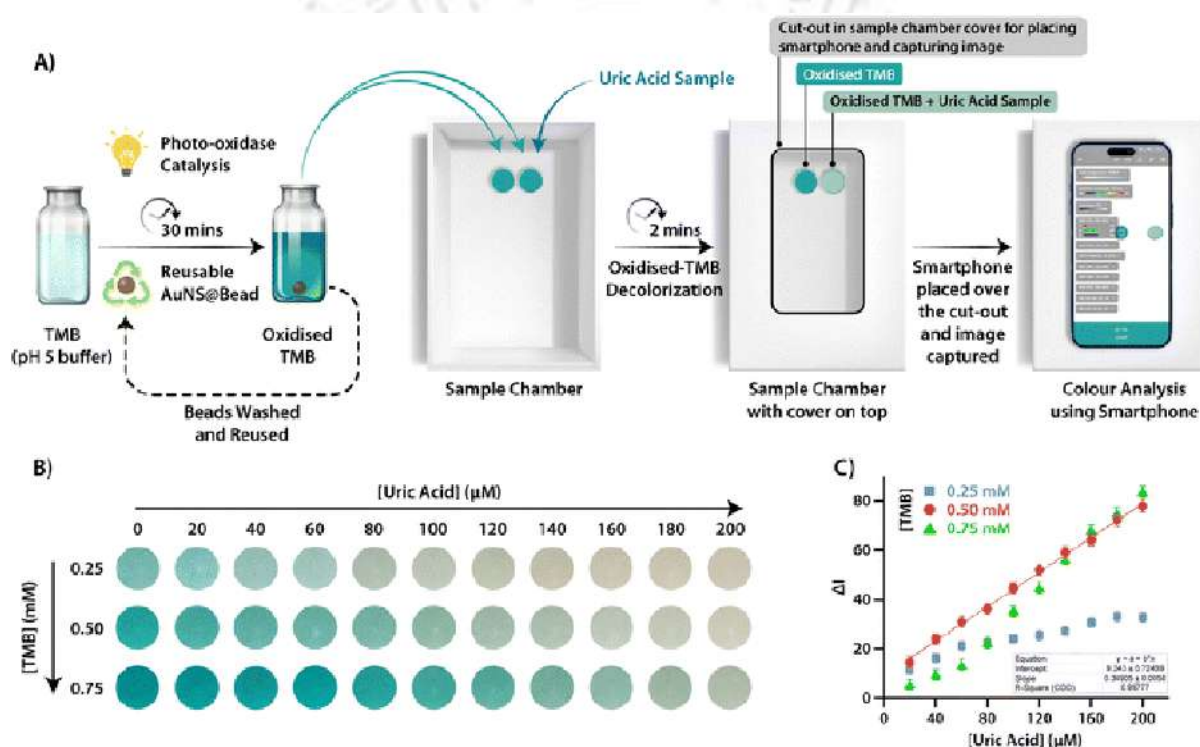


Figure 1.12. POCT device developed for uric acid detection and quantification with gold-nanostar decorated hydrogel beads. Adapted from ref 20. Copyright 2025 Royal Society of Chemistry.

Colourimetric sensing of analytes is also a crucial aspect of analyte detection, particularly in the context of point-of-care testing. Analytes can be easily detected and quantified through colourimetric changes that are visible to the naked eye, without the need for UV-visible light or sophisticated instruments. Such techniques are in demand for their user-friendly and convenient properties. Das and coworkers developed a colourimetric detection technique for uric acid, an essential biomarker for physiological activity (Figure 1.12).²⁰ In this report, they developed gold nanostars surface-functionalized hydrogel beads that show peroxidase-like activity. This was used for the colourimetric detection of uric acid in combination with TMB, which produced a blue colour

on oxidation. The colour changes could be followed to give both quantitative and qualitative measurements in POCT devices.

1.8.3. Optoelectronic devices

Introduction of stimuli-responsive colours into materials and fabrication of devices enhances the practical applicability of a system. In this regard, OLEDs are an advanced class of smart optical materials that use organic molecules to emit light from an electric current. Devices fabricated from them are colour tunable, flexible, wearable and energy efficient. They find extensive uses in wearable technology and biomedical devices. The basic colour devices have stable spectra varying with voltage and angle. This is needed for the sake of colour accuracy and reproducibility. In this context, colour-tunable organic light-emitting devices yield a wide range of colours by a single device, thereby having a minimum pixel size and maximize fill factor. There is also a growing demand for voltage-responsive coloured materials. Moreover, the incorporation of sensors and CTOLEDs can fabricate state-of-the-art optoelectronic devices which can render invisible signals like electrical, optical, magnetic, chemical and thermal into visual detections.

1.8.4. Bioimaging and Theranostics

A large section of medical treatments fails only due to poor or incorrect diagnosis. A wrong diagnosis misguides medical attention in the wrong direction. Modern researchers must focus on developing advanced methods that efficiently qualify and quantify anomalous biological microenvironments before administering the appropriate medicine. Bioimaging and theranostics face several challenges, including biological, technical and economic. Limited spatial resolution, deep tissue accessibility, need for labelling, interference from background noise, photo stability, and signal weakness are some major problems in bioimaging. On the other hand, theranostics face challenges in biocompatibility, toxicity, resistance, poor release kinetics, manufacturing, and scalability.

Molecular colours obtained from organic dyes and emissive assemblies are of critical significance in this regard. The stimuli-dependent colours from molecules are especially exploited in designing these target-specific probes. Bioimaging probes can be designed with appended functional units that specifically bind to certain organelles of the cells. The design principle is based on the specific ability of that targeting group or dye to particularly recognize and bind to certain overexpressed features in that particular organelle. For instance, mitochondria tracker dyes are used to study fusion and fission processes in cells. Janus green B and Rhodamine 123 are widely used to observe mitochondria in living cells. Some dyes that contain rhodol also provide an indication of mitochondrial activity. Even AIEgens that do not specifically bind to the mitochondria can be made

to do so by appending a target-specific peptide sequence.²¹ Sometimes, precursor dyes can also be synthesized that specifically emit colours when they encounter a specific microenvironment of the target organelle. Certain AIEgen-based precursor molecules can be designed which specifically respond to enzymes of lysosomes, like Lysosomal β -*N*-acetylhexosaminidase (Hex). These enzymes can effectively convert the precursor dye to actively emissive assemblies that aid in visualizations. Certain pH-responsive dyes that emit under acidic pH can be utilized for the same purpose. BODIPY and spirocyclic rhodamine-based dyes that are pH sensitive can show differential and contrasting images of lysosomes due to the presence of an acidic microenvironment.

Utilizing the anomalous microenvironments, one can target specific tissues as well. Cancer cells have augmented metabolic activities. This leads to a cellular environment that has low pH and high GSH concentration. pH-responsive organic dyes can be used as stains for the analysis of cancer cells. For example, hematoxylin and eosin are considered foundational stains in cancer cell diagnosis. The Papanicolaou (PAP) stain plays a role in screening out precancerous cells from cancerous cells in cervical tissues. Other strategies that aim for nuclear environments can also be used for cancer cell diagnosis. DAPI can bind to the nucleus and show strong blue fluorescence. It is used to monitor nuclear changes like condensation and fragmentation associated with cancer cells.

Sometimes, dyes that emit in the NIR region can not only help in bioimaging but also offer therapeutic solutions. The light energy emitted from these dyes controls the growth of cancer cells in many aspects, such as photodynamic and photothermal therapy. The NIR light generated from derivatives of cyanines, squarines, porphyrins and BODIPY increases cellular temperature, leading to cellular death by affecting metabolic routes. The light energy can also give rise to reactive oxygen species that participate in photodynamic therapies and treat cancer growth. Porphyrin-based dyes can behave as a photosensitizer and disrupt the redox equilibrium of the cellular environments through the generation of ROS. Studies like drug release, growth, transportation, inhibition and colocalizations can be analyzed using these tools. Orthogonal targeting and contrasting colours from different dyes with orthogonal stimuli-responsiveness can be used in the simultaneous theranostics of different organelles. Hence, stimuli-responsive properties and special targeting features of dyes and functionalized dyes can act as major tools for observing biological environments and also for the treatment of anomalies.

1.8.5. Photocatalysis

Light is a green form of energy. Using light for chemical conversions is one of the most efficient and eco-friendly ways to perform chemical transformations. Photocatalysis has thus garnered immense research attention, and more efforts are being focused on designing photocatalytic systems.

External light sources of specific wavelengths and power are required to maintain the kinetics of a particular reaction. Different reactions need light sources of different wavelengths.

Coloured lights from molecular emissions can serve the purpose in this aspect. Emissive dyes and luminescent supramolecular assemblies can play a major role in photocatalyzing reactions. The luminophores can act as a photosensitizer and trap the light energy in the UV-blue region and release it at a more suitable red-shifted wavelength, which is essential for reactions. They behave as artificial light-harvesting systems that relay energy from one end of the spectrum to another. The energy is then utilised for some chemical conversions. Rhodamines, eosin-Y, cyanines, and porphyrins are some popular emissive molecules in this regard. Supramolecular assemblies are noteworthy in this regard. The assemblies can sequester emissive dyes in them depending upon their hydrophilicities in an aqueous medium. Hydrophobic emissive dyes can get entrapped in the hydrophobic assemblies, showing increased emissions. Other acceptor dyes can also come into proximity within these hydrophobic confinements and produce higher wavelengths after energy transfer. These assemblies can serve as reaction pockets or microchambers where suitable substrates are also sequestered with upregulated local concentrations, which can efficiently use this light energy for chemical conversions. In this regard, in a report by Zhang and Feng, it has been demonstrated how an artificial light-harvesting system based on a cyanostilbene-based dendritic molecule (Figure 1.3A).²² Entrapment of FRET pair leads to efficient multicolour emission through sequential energy transfer and high antenna effect. Later, the system was applied in the photocatalytic reaction of a cross-dehydrogenative coupling between an indole and an N-aryl derivative of tetrahydroisoquinoline.

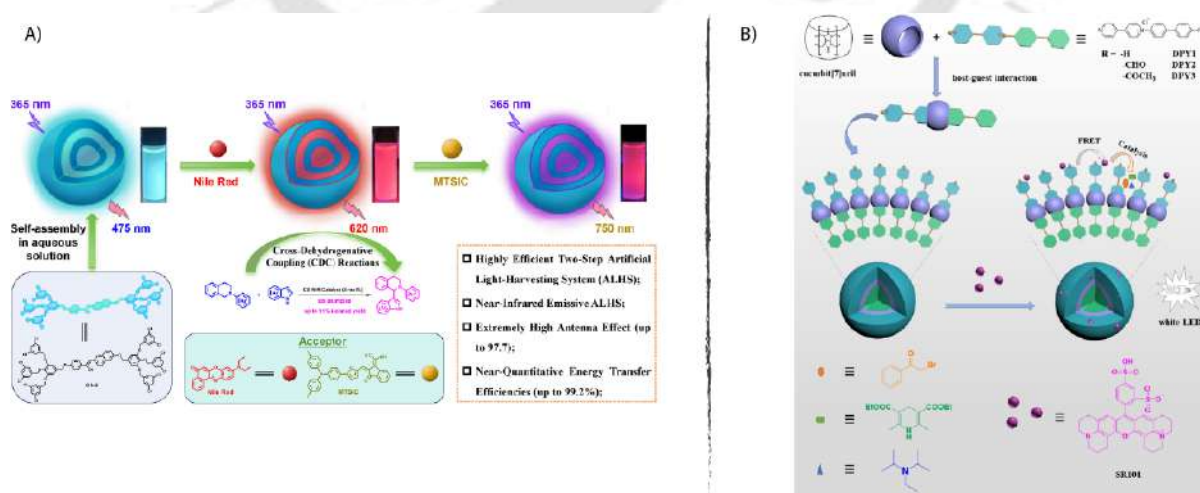


Figure 1.3. An artificial LHS for A) dehydrogenative cross-coupling reaction and B) dehalogenation of aromatic ketones. Adapted from ref 21 and 22. Copyright 2026 Elsevier. Copyright 2022 Elsevier.

The specific colour light emitted can perform a multitude of chemical transformations like dehalogenations, cross dehydrogenative couplings, photooxidations, hydrogen evolutions, and alkylations of C-H bonds. A report where a CB-[7] based supramolecular assembly could behave both as an LHS and a nanoreactor shows how host-guest reaction can act as a stimulus for photocatalytic conversion (Figure 1.3B).²³ The generated emission through energy transfer to FRET pairs was used in the dehalogenation reaction of α -haloketones aromatic ketones entrapped in the aggregates of the host-guest complex assemblies serving as nanoreactors for photocatalytic conversions. These supramolecular assemblies can be triggered systems that are generated in response to a particular stimulus. In that case, the colours that are produced from these assemblies are tunable with respect to the stimulus and are hence tunable. Tunability is one such aspect of molecular colours that increases its demands in photocatalytic reactions. They are tunable in both intensity and wavelength and thus can be used for a wide variety of reactions.

1.9. Objectives of the Present Thesis

Inspired by these stimuli-responsive molecular colours, we envisioned that colours obtained from luminescent assemblies and organic dyes can be implemented in smart ways to design modern systems that have unconventional implications and unique applications. We aimed at particularly utilizing the stimuli-responsive properties of these chemical colours in a specific role. We anticipated that if a crucial and complicated function can be achieved simply through optical signals, like change or intensity, then the system bears a convenient yet impactful outlook. We aimed at implementing colours both from fluorescent and phosphorescent assemblies, as well as transmitted colours from regular organic dyes. This is because all these colours have different origins and hence different attributes, and can be used according to the purpose. We have also attempted to create temporally regulated colour programmes that show time-controlled transitory multicolours. For this purpose, we have even used multiple chemical clocks that show time-dependent reversal of stimulus conditions and are repeatable. Autonomous change of colours was found to be useful for applications in anti-counterfeiting strategies, while the static emergence and disappearance of colour was used was sensing applications for detecting analytes and in creating logic gate representations

Chapter 2: An Aggregation-Induced Room Temperature Phosphorescence Probe for the Efficient and Selective Detection of Heparin and Protamine²⁴

This chapter demonstrates the application of a host-guest complex-based phosphorescent probe in the detection of heparin and protamine. Heparin is a vital macromolecule that regulates blood coagulation, while protamine is an essential polypeptide clinically used to counteract heparin

overdose. Detecting both heparin and its antidote protamine under physiological conditions is crucial for biological and clinical applications. This chapter introduces a cucurbituril[8] (CB[8])-based phosphorescent probe for their detection. The method employs a nanoassembly induced phosphorescence switch-on mechanism for heparin sensing and a disassembly-induced phosphorescence switch-off approach for protamine detection. An arginine-rich guest forms a supramolecular complex with heparin, enhancing phosphorescence under secondary confinement and enabling its detection. Conversely, protamine sulfate, as a stronger competitor for heparin, disrupts the probe-heparin aggregates, leading to emission quenching and protamine sensing. This sensor demonstrated high selectivity in detecting both analytes in biological samples, such as human blood serum and urine. The detection limits for heparin and protamine were determined to be 61 and 82 ng/mL in 10% HBS, respectively.

Chapter 3: Time-Encoded Information Encryption with pH Clock Guided Broad-Spectrum Emission by Dynamic Assemblies²⁵

In this chapter, we have shown the application of a transitory multicolour fluorescent assembly in time-encoded information encryption. With growing threats from counterfeiting-based security breaches, multi-level and specific stimuli-responsive anti-counterfeiting devices and message encryption methods have attracted immense research interest. To create a system with a hierarchical security level, we bring forward a pH-switchable fluorescent assembly of an AIEgen and an aliphatic acid. We later temporally direct the molecular assembly with the aid of a chemical trigger-regulated pH clock, generating a transitory multicolour emission, including transient white light generation. The pH-dependent emissions were further implemented in constructing smart multi-input fluorescent chemical AND gates. Subsequently, we integrate the time-gated emissive system to develop an advanced multidimensionally secure data encryption strategy. This novel approach enhances anti-counterfeiting measures by introducing an additional layer of security based on temporal characteristics.

Chapter 4: Bioinspired Chemoenzymatically Controlled Artificial Light Harvesting Nanoaggregates with Multicolour Transient Emissions for Time-Gated Information Encryption

Transient lights in biological systems are mainly governed by chemical reaction networks, mostly modulated by enzymes. In the quest to emulate these transient lights, we have developed bioinspired enzyme-regulated, broad-spectrum artificial light-harvesting nanoaggregates with pH-clock-guided transient emissions in this chapter. pH-adaptable AIEgen-based nanoaggregates have

been implemented in this study that can sequester a suitable FRET acceptor in their hydrophobic domain, leading to multi-colour emissions. These nanoaggregates can be temporally regulated with a chemoenzymatic pH clock, which generates time-programmed emissive colours with different FRET donor-acceptor combinations. The multi-colour transient emissions essentially mimic the fleeting lights of fireflies. These time-controlled nanoaggregates and their corresponding emissions can be implemented for information encryption strategies with anti-counterfeiting features. Since data encryption is popularly performed through cryptic codes, we have employed these emissive nanoaggregates to demonstrate time-encoded information encryption through ASCII, Morse and 4-bit codes. Herein, time plays a pivotal role in elevating the security level of code-based safe data storage. Simple molecular design, complex multi-component environment, adaptive properties, synchronized temporal control – all operate in unison to imitate the fundamental attributes of biological emission.

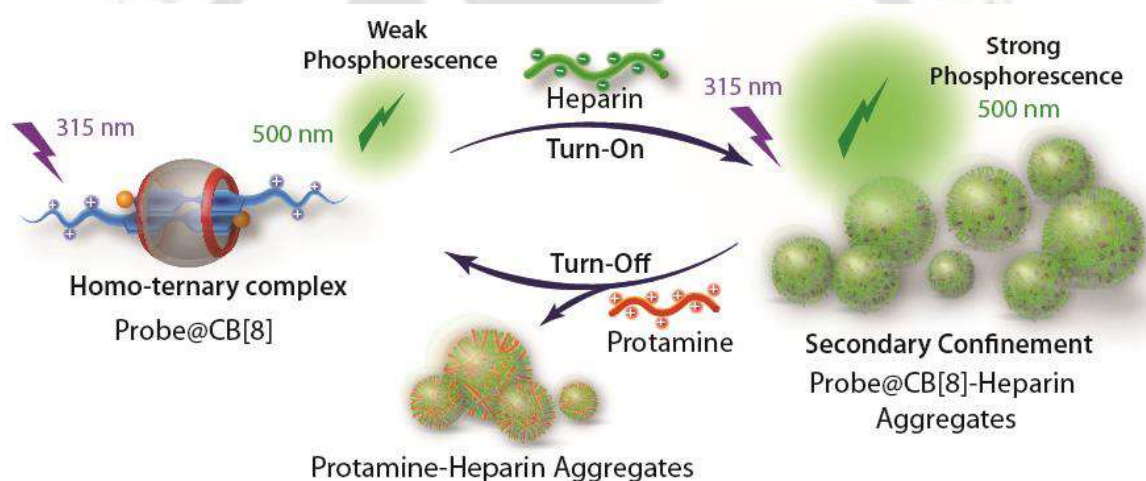
Chapter 5: Parallel Time-Scale Guided Encryption of Information with Broad-Spectrum Transitory Colours from Common Organic Dyes

Fluorescence-based materials have been widely used for encryption purposes, but they often require complex synthesis and rely on UV light for decoding, posing practical and safety challenges. To overcome these issues, this chapter presents a suite of colour-coded encryption methods using stimuli-responsive common organic dyes. These dyes offer smarter alternatives to fluorescent systems, eliminating the need for extensive synthesis or UV light while retaining all essential features of secure encryption. Selected for their specific pH- or redox-responsiveness, the dyes undergo time-dependent colour changes when driven into non-equilibrium conditions by chemical triggers. This dynamic behaviour enables the creation of time-gated, multi-dimensional colour codes. Secrecy in decryption is ensured through smart window setups – stimuli-responsive barriers that can be activated on demand, regulating access to information along independent time scales. These parallel, programmable timelines are controlled by unrelated chemical or physical triggers, enhancing security without complicating the read-out process. Our approach uniquely combines complex, multi-tiered encoding with simple, safe decryption. The resulting dual time-locked colour codes provide hierarchical protection, governed by orthogonal triggers, all within a user-friendly and synthesis-free system – a rare but highly desirable balance in secure information encryption.



Chapter 2

An Aggregation-Induced Room Temperature Phosphorescence Probe for the Efficient and Selective Detection of Heparin and Protamine





2.1. Introduction

Ions and electrolytes are crucial for life. Besides the smaller ions, numerous polymeric macroions play vital roles in physiological functions.^{26, 27} It is imperative for scientific research to explore analytical methods that probe into their selective identification, detection, and quantification, particularly under physiological conditions.^{28, 29} Amongst the various biological polymeric macro-anions, heparin (Hep) is distinct for having the highest negative charge density due to the presence of multiple sulfate and carboxylate groups.³⁰ This linear heterogenous polysaccharide belongs to the glycosaminoglycan (GAG) family and is connected by 1-4 linkages between uronic acid and glucosamine.^{31, 32} Heparin behaves as a potential antithrombin binder and plays an essential role in blood clot formation.³³ It is a widely implemented anti-coagulant drug for clinical purposes.³⁴⁻³⁶ Heparin is often administered for treating thrombotic diseases, venous pulmonary embolism, thromboembolism, and chronic consumptive coagulopathies.³⁴⁻³⁶ Different surgical procedures and post-surgical therapies demand condition-specific heparin dosage in the blood. An overdose can cause complications like hyperkalemia, osteoporosis, haemorrhages, and thrombocytopenia.^{37, 38} To prevent complications, it is essential to monitor the heparin concentration in body fluids during surgery and in post-operative therapy.³⁹ PS is routinely implemented for neutralizing this excess unfractionated heparin.⁴⁰ It is a small polycationic peptide molecule (4-6 kDa) flanked by multiple arginine residues (67% of arginine).⁴¹ It exhibits strong alkaline properties (pI 13.8).⁴² Presence of several positive charges under physiological conditions enables protamine to bind strongly to heparin and thus is a popular antidote for heparin.⁴³ Besides this, it also plays a significant role in gene expression and transmission, tissue formation, diabetic treatments, and bacteriostatic preservatives.⁴⁴⁻⁴⁷ Again, adverse doses of protamine can be lethal, causing hypotension, right ventricular failure, and other anaphylactic reactions.^{43, 48} So, in addition to heparin, it is also critical to estimate the concentration of its antidote, protamine, in biological fluids for clinical purposes

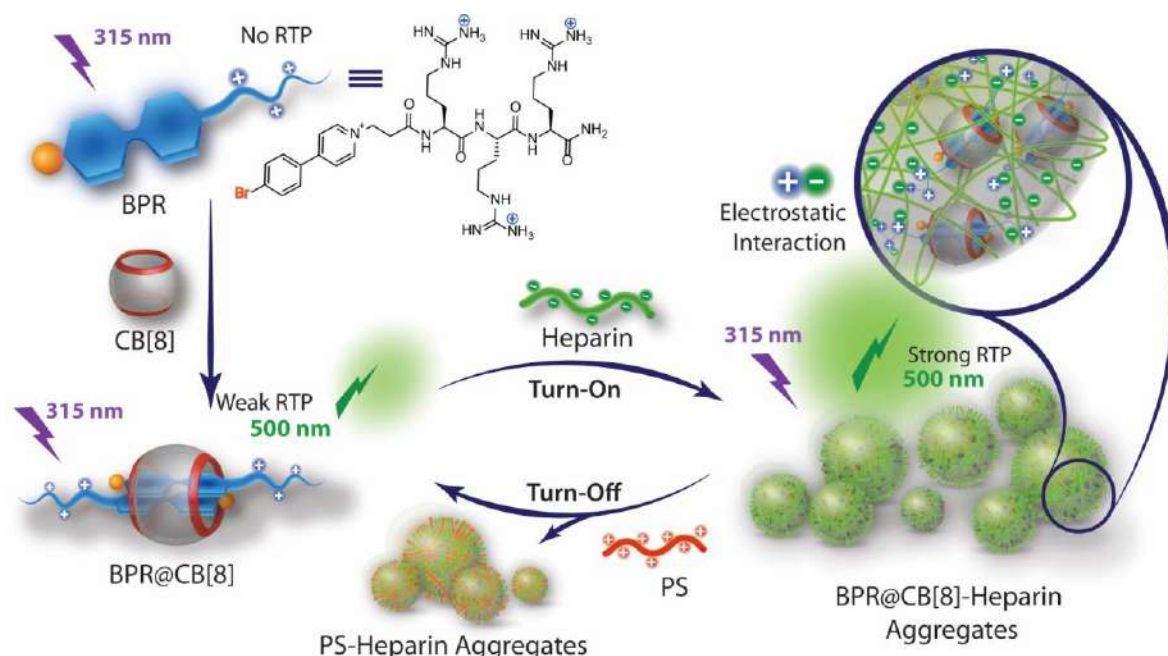
Generally, heparin and protamine are quantified by monitoring the ACT or an activated partial thromboplastin time (aPTT).⁴⁹⁻⁵¹ These expensive and indirect methods are unreliable as they lack specificity, sensitivity, and fast response time. Substantial progress has been made in recent years to develop detection methods based on colourimetric assays, capillary electrophoresis, electrochemical assays, high-performance liquid chromatography, surface-enhanced Raman spectroscopy, etc.⁵²⁻⁵⁹ These methods involve drawbacks like complex extraction methods, high cost, long operation time and possibilities of interferences. Despite these efforts, direct detection assays based on turn-on luminescence probes are advantageous owing to their quick response time, high selectivity, better reliability, and visual perceptibility.⁶⁰⁻⁶⁶ Several strategies based on fluorometric detection have been reported.^{61, 63, 67-72} However, sensing probes involving RTP are

seldom found in literature⁷³⁻⁷⁷, especially in the solution phase, that can detect an analyte and its antidote.

To this end, supramolecular phosphorescent sensing has an added advantage over fluorescence sensing. It offers longer lifetimes (milliseconds), larger Stokes shift from the excitation wavelength, and lower background fluorescence interferences.⁷⁸⁻⁸⁰ Despite these advantages, the RTP-based probes remained less explored as sensors because quenching prevails in the solution medium due to faster deactivating pathways.⁷⁹ In our current attempt to develop a phosphorescence-based sensor, we circumvent the complication of quenching by implementing a new Cucurbit[8]uril (CB[8])⁸¹⁻⁸⁵-based phosphorescent probe. Inclusion complexes of organic dyes with macrocyclic hosts like cyclodextrins, cucurbiturils, pillararenes, etc., screen the host from interactions with solvent molecules and other quenchers, securing the radiative pathways.⁸⁶⁻⁹⁴ Additional confinement through assemblies over the inclusion complex increases phosphorescence emission intensity.⁹⁵⁻⁹⁹ These secondary confinements, be they macromolecular or nano-assemblies, arrest the otherwise faster deactivating pathways and facilitate the phosphorescent emission.⁹⁰ Employing this principle of hierarchical supramolecular confinement, we attempted to generate a supramolecular phosphorescent complex that can be used to detect heparin.

BP is a well-established molecule that produces green phosphorescence when entrapped in macrocyclic hosts or assemblies.^{87, 95, 97} However, poor water solubility limits its application. To deal with it, we appended a hydrophilic tri-Arginine (Arg) residue to a carboxylic acid functionalized BP derivative (BPR, Scheme 2.1, 2.2). The molecule was designed to facilitate multi-step confinement-assisted turn-on phosphorescence.¹⁰⁰ The presence of the pyridinium group introduced a permanent positive charge that ensures strong homoternary complexation^{101, 102} with CB[8] (BPR@CB[8]) and also brings the phosphorescent emission into the picture through macrocyclic confinement.¹⁰³ The presence of the tri-Arg residue on BPR not only enhanced the hydrophilicity of the molecule but also secured the larger goal of binding with heparin. Triple Arg residues were premeditated as the BPR@CB[8] would then be flanked terminally by multiple pendant arginine residues (Scheme 2.1). Thus, the BPR@CB[8], having multiple positive charges, should easily interact electrostatically with heparin. To meet our anticipation, there was successful nano-assembly entrapment of the complex with heparin, which augmented the phosphorescence even further. This turn-on emission proved to be the key to sensing heparin. Since PS contains more arginine residues than BPR@CB[8], the introduction of PS to the BPR@CB[8] and heparin mixture leads to the detachment of heparin. The disassembly process was accompanied by a phosphorescence

“turn-off” phenomenon. This helped in the detection of PS utilising the same probe. Further, the heparin sensing behaviour was also performed with commercially available clinical heparin, and the effectiveness of our method was compared by performing the standard aPPT assays with HBS as well.



Scheme 2.1. Chemical structures of the molecules and a schematic presentation of the sensing mechanism.

2.2. Results and Discussions

2.2.1. Host-Guest and Induced Phosphorescence

To begin with, the UV-Visible absorption and fluorescence emission spectra of BPR were measured to study the self-assembly behaviour of the molecule. BPR showed characteristic absorption at 315 nm (Figure 2.1A) and emission maxima of 385 nm ($\lambda_{\text{ex}} = 315$ nm) (Figure 2.1B).^{94, 100} The concentration-dependent studies showed the linear dependency of both absorption and emission, indicating no possible molecular aggregation even up to 70 μM concentration (Figure 2.1A-B inset). Additionally, the FESEM images recorded for the 2 μM BPR solution showed no clear morphology. However, the gradual addition of CB[8] to a fixed concentration (2 μM) of BPR brought prominent changes in the absorption (Figure 2.1C) and emission spectra (Figure 2.1D).

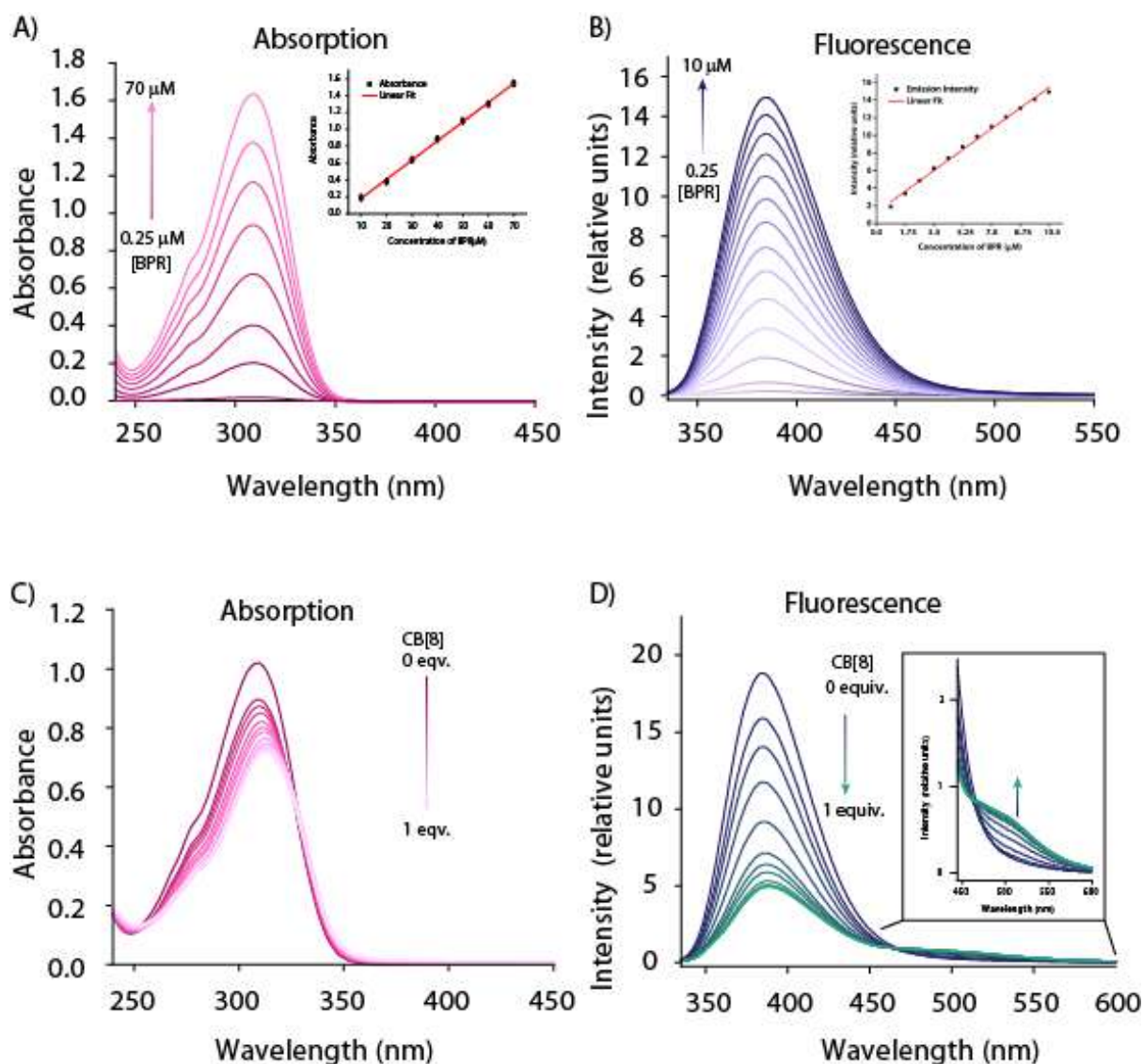


Figure 2.1. A) UV-Visible absorption and B) fluorescence emission spectra of BPR with varying concentrations and their linear variations in insets. C) UV-Visible absorption and D) fluorescence emission spectra of BPR (2 μM) with increasing concentrations of CB[8].

With increasing concentrations of CB[8], the absorption at 315 nm dropped continuously and reached a constant value after 0.5 equivalents of CB[8] (Figure 2.2A). Similarly, in the emission spectra, the peak intensity at 385 nm decreased with increasing concentrations of CB[8] up to 0.5 equivalent and flattened thereafter (Figure 2.2B). A concomitant rise in the intensity of a new broad peak at 485 – 500 nm range was also visible (Figure 2.1D inset, 2.2C). These results clearly indicate a 2:1 complexation of BPR and CB[8]. To further confirm the formation of the inclusion complex, ^1H NMR studies were performed. As can be seen from (Figure 2.2D), upon addition of CB[8] to a BPR solution, the aromatic peaks showed a significant upfield shift, confirming the inclusion complex formation.

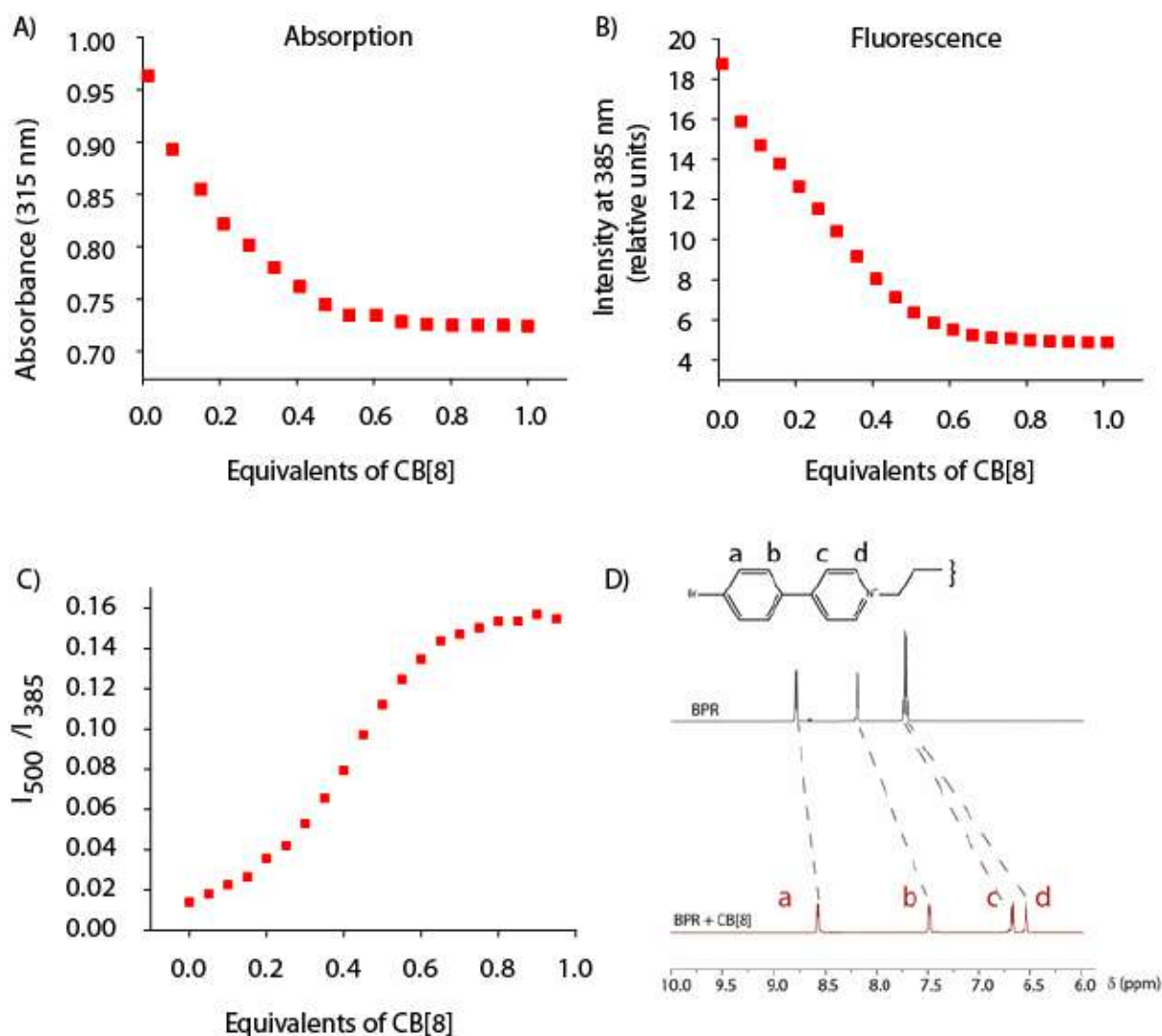


Figure 2.2. Dependence of A) absorbance and B) emission of BPR on the equivalence of CB[8] added. For fluorescence, $\lambda_{\text{ex}} = 315$ nm. C) Ratio of intensities at 500 nm and 385 nm of BPR against CB concentration. D) Partial (aromatic region) ^1H NMR spectra of BPR in the absence and presence of CB[8] measured at room temperature.

2.2.2. Nano-assembly dependent Phosphorescence with Heparin and Protamine

The free BPR molecule exhibited negligible phosphorescence in the free state. The phosphorescence of a fixed concentration of BPR ($2\ \mu\text{M}$) was recorded with varying amounts of CB[8] (Figure 2.3A). A clear phosphorescence emission peak at 500 nm appeared, and the intensity of the peak steadily increased with increasing CB[8] up to 0.5 equiv. (Figure 2.3A inset). Further addition of the host brought no noticeable changes. The results further confirmed the 2:1 homoternary complexation and the complex's consequent room-temperature phosphorescence. For simplicity, BPR@CB[8] will represent a 2:1 complex of BPR and CB[8] for the rest of the document,

and the concentration that may be mentioned will be of BPR. The complexation was also proven by adding 1-adamantylamine (ADA) to a solution of BPR@CB[8]. ADA, being a better guest for CB[8], easily substitutes BPR in the CB[8] cavity.⁸² This intensified the 385 nm peak again and diminished the 485-500 nm band as free BPR is released in solution from the macrocyclic confinement (Figure 2.3B). Next, we checked the effect of secondary confinement of the phosphorescent probe (BPR@CB[8]) by heparin. The gradual addition of heparin to a solution of BPR@CB[8] attenuated the intensity of the 385 nm peak and intensified the 485-500 nm band in the fluorescence emission (Figure 2.3C). The emissions at the respective wavelengths varied linearly with the addition of heparin and thereafter remained constant beyond a certain point (Figure 2.3D).

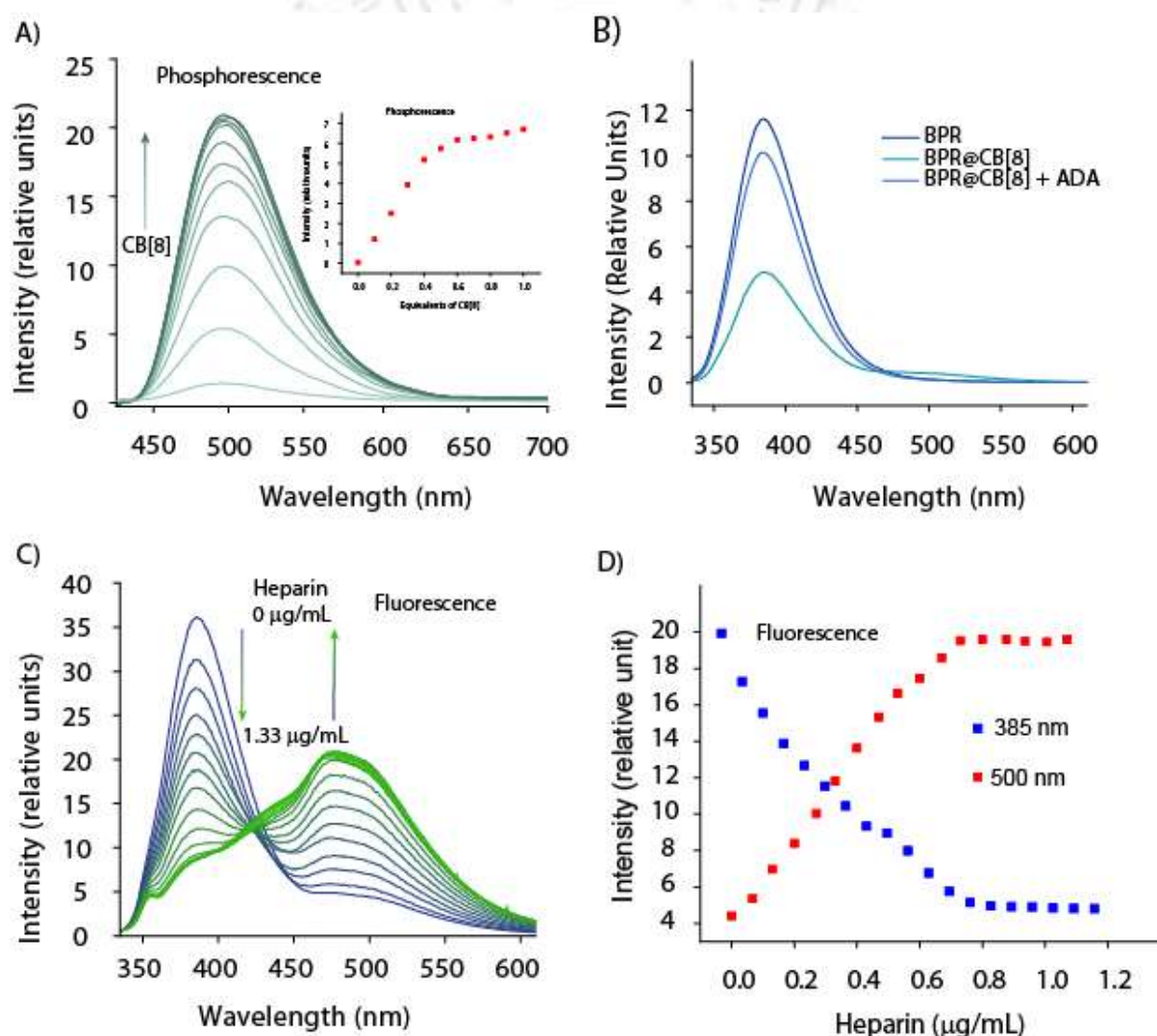


Figure 2.3. A) Phosphorescence of BPR with increasing concentration of CB[8], with dependence of the phosphorescence emission intensity on the CB[8] equivalence in the inset. B) Fluorescence as exhibited by BPR (2 μ M) with CB[8] in the absence and presence of ADA. C) Fluorescence emission of BPR@CB[8] (2 μ M) with an increasing amount of heparin. D) Change in fluorescence intensities of BPR@CB[8] (2 μ M) as a function of heparin concentration.

When heparin was introduced to the solution of BPR@CB[8], the phosphorescence emission, which had reached constancy after the addition of CB[8], showed further increment. The peak at 500 nm was enhanced with increasing additions of heparin and registered a similar linear trend, finally reaching saturation (Figure 2.4A). The increase in phosphorescence in the presence of heparin is attributed to the strong electrostatic interaction between the negatively charged heparin and the guanidinium groups of pendant Arg residues. Moreover, arginine residues on either side of the BPR@CB[8] probe allow it to behave as an electrostatic crosslinker and enable better interaction with heparin. The 2:1 binding was also established with fluorescence measurements of BPR with CB[7]. Since CB[7] is incapable of accommodating two hosts in its smaller cavity, only a 1:1 binding is observed between CB[7] and BPR. As a result, a stoichiometric amount of CB[7] is needed to produce the same spectral changes as was done by 0.5 equiv. of CB[8] (Figure 2.4B). More importantly, the resultant BPR@CB[7] probe is a tri-Arg species only like BPR itself. Unlike the BPR@CB[8], the probe with CB[7] lacks a polycationic and electrostatic bridging nature and thus fails to produce a significant spectral change even with a much higher concentration of Hep. These observations highlight the importance of a polyarginine species and the significance of a 2:1 binding fashion in the designed probe. To demonstrate the electrostatic nature of the interaction between BPR@CB[8] and Hep, control experiments were performed. Adding a saturated sodium chloride solution to a Hep – BPR@CB[8] mixture resulted in the disappearance of the increment in phosphorescence. The excess salt screens the charges and proves the electrostatic interaction between Hep and the BPR@CB[8] complex. With the increasing salt concentration of the medium, the intensity of the broad band of BPR@CB[8] with Hep at 485-500 nm is significantly hampered (Figure 2.4C). The effect of salt is more pronounced in samples of 10% HBS because the total salt concentration in solution is a cumulative effect coming from both the salt added externally and the one already present in HBS. However, the trend for both remains identical. Additionally, to highlight the binding propensity of Arg residues, a tri-Glycine derivative of BP (BPG) was also synthesized (Scheme 2.2). Although CB[8] successfully accommodated the molecule in the homoternary fashion, the BPG@CB[8] molecule produced no interaction with Hep. The emission profile with Hep showed no noticeable spectral changes, which indirectly proves the importance of Arg residues, electrostatic interactions, and hierarchical secondary confinement essential for the detection purpose (Figure 2.4H).

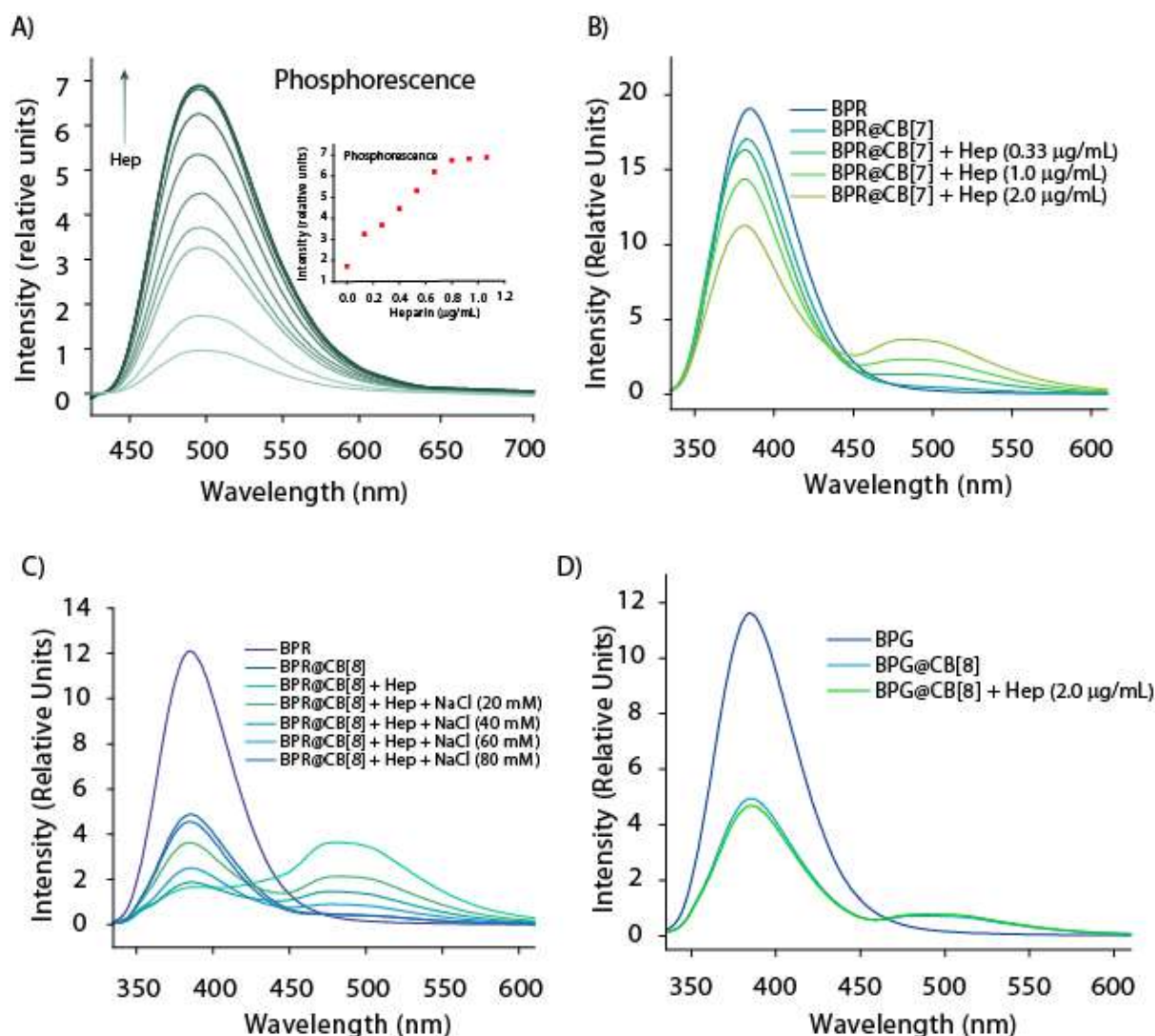


Figure 2.4. A) Phosphorescence of BPR@CB[8] (2 µM) with an increasing amount of Hep, with dependence of the phosphorescence emission intensity of BPR@CB[8] as a function of Hep concentration. ($\lambda_{\text{ex}} = 315$ nm) in the inset. B) Fluorescence emission of BPR@CB[7] (2 µM, 1:1) with increasing concentrations of heparin. C) Fluorescence emission of BPR@CB[8] (2 µM) with heparin (2.0 µg/mL) in a medium of different NaCl concentrations, pH 7.0 buffer. D) Fluorescence emission of BPG@CB[8] (2 µM) with heparin (2.0 µg/mL).

Next, the aggregation due to Hep addition to the BPR@CB[8] complex was studied. Hep, CB[8], BPR, and BPR@CB[8] individually showed particles of very negligible dimensions (Figure 2.5A). However, the solutions of BPR@CB–Hep showed particles of 220 nm average diameter. This confirmed the ability of Hep to form assembled structures with BPR@CB[8] effectively confining them. The observations were reinforced by the FESEM and FETEM images, where spherical aggregates were seen (Figure 2.5B, C). Control experiments were performed with free BPR, BPR@CB[8], CB[8], and Hep. In all cases, no noticeable aggregate morphology was observed. The energy-dispersive X-ray analysis (EDX) and elemental mapping shows the presence of sulfur and bromine in the aggregates of BPR@CB[8]–Hep (Figure 2.5D), confirming the involvement of both Hep and BPR to form the

aggregates. The decay profile measurements also complemented the increment in phosphorescence by BPR@CB[8] due to Hep addition (Figure 2.6A, Table 2.1). The unconfined BPR (2 μ M) showed a very low phosphorescence lifetime of 0.014 ms. The lifetime increased to 0.331 ms after inclusion in the CB[8] cavity. With the introduction of Hep, the lifetime increased even further. In the presence of 3.99 μ g/mL Hep, the same solution showed a lifetime of 2.22 ms. The increasing phosphorescence lifetime also supports the idea that secondary confinement is responsible for the switch-on mechanism.

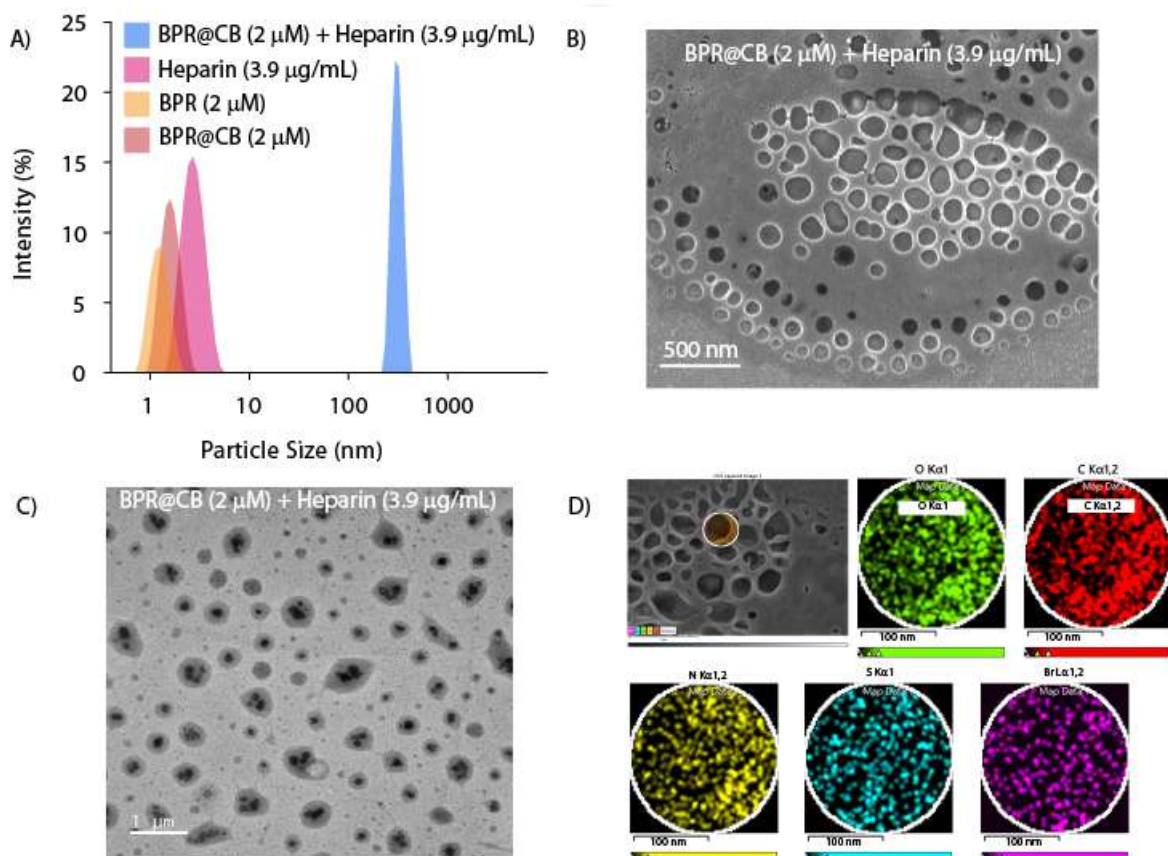


Figure 2.5. A) DLS profile of different compositions of BPR, CB[8] and Heparin. B) FESEM and C) FETEM of BPR@CB[8](2 μ M)-Hep (3.9 μ g/mL). D) Elemental mapping of nano-assemblies of Heparin with BPR@CB.

Table 2.1. The phosphorescence lifetime of BPR@CB[8] with different compositions of heparin was measured at room temperature.

Sl. No.	Entry	Lifetime (τ) (ms)	χ^2
1	BPR	0.0144	0.8417
2	PBR@CB[8]	0.3312	0.9837
3	PBR@CB[8] + Hep (1.33 μ g/ml)	1.6143	0.9536
4	PBR@CB[8] + Hep (2.66 μ g/ml)	2.0852	0.9770
5	PBR@CB[8] + Hep (3.99 μ g/ml)	2.2222	0.9873

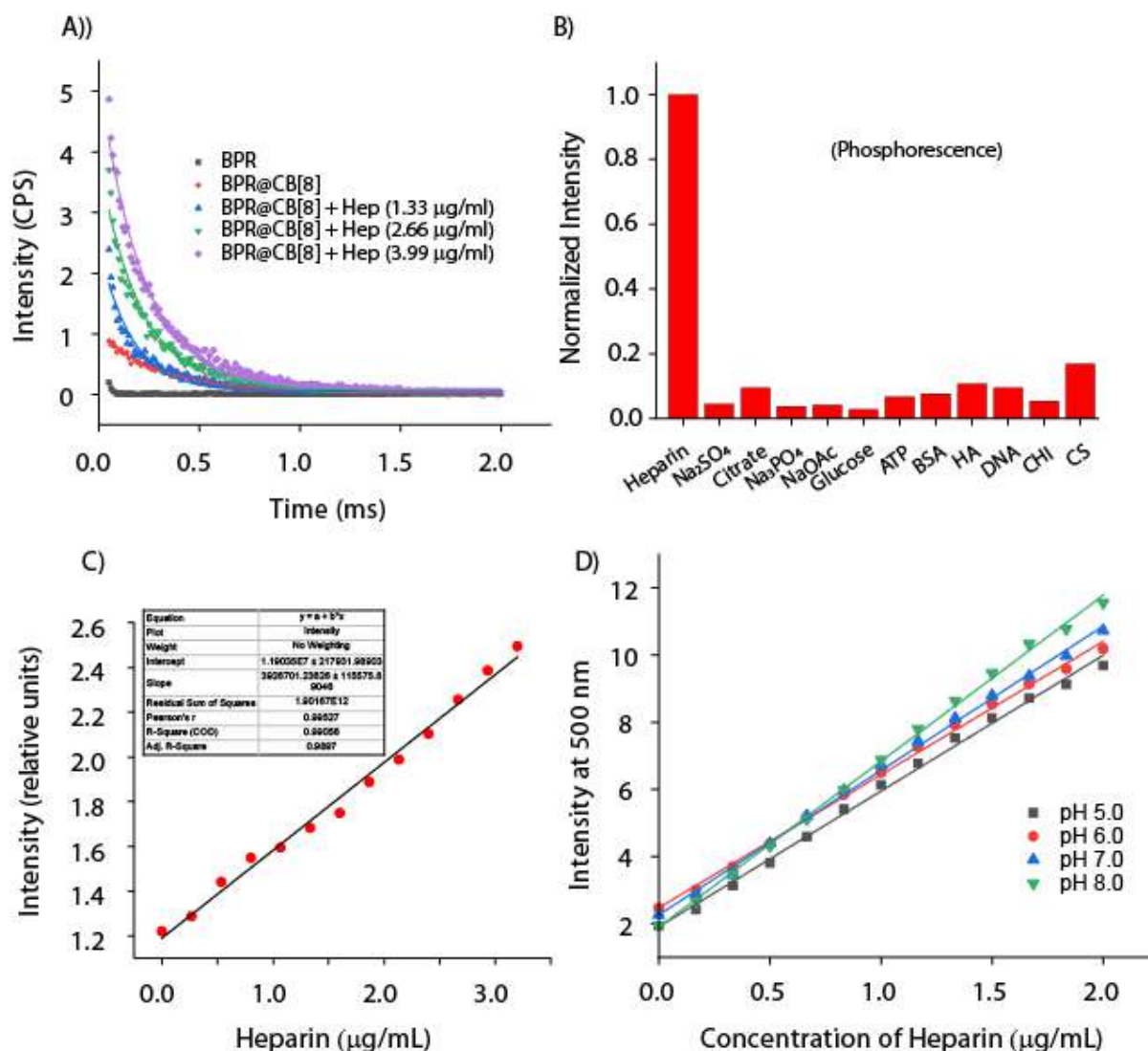


Figure 2.6. A) Phosphorescent decay traces of different compositions of BPR, CB[8] and Hep. B) Increase in phosphorescence @500 nm for BPR@CB[8](2 µM) in the presence of different analytes. C) Calibration curve for the increase in phosphorescence @500 nm for BPR@CB[8](2 µM) with increasing concentration of Hep. All studies were performed at RT. D) Linear phosphorescence calibration plot for detection of Hep with BPR@CB[8] (2 µM) in buffers of different pH.

2.2.3. Detection of Heparin and Protamine

The switch-on emission property of the BPR@CB[8]–Hep assemblies formed the basis of our sensing activity. Before proceeding into the sensing application, it was essential to ensure the selectivity of BPR@CB[8] toward Hep. Biological fluids contain multiple polyanions that often interfere during the detection of a desired analyte. To eliminate the possibility of interfering analytes restricting the Hep selective detection, phosphorescence (Figure 2.6B) emission of BPR@CB[8] (2 µM) was recorded in the presence of multiple possible interfering analytes. For most of the small anions, no noticeable change in the intensity was recorded. Small enhancements in the intensity were noticed for polyanions like hyaluronic acid (HA), DNA, and chitosan (CHI). The increment was larger for

Chondroitin Sulfate (CS) than for all other interfering anions. This might be because of greater negative charge density in CS compared to other possible interfering analytes. However, compared to the change in the presence of Hep, these enhancements were found to be insignificant. These results verified the selectivity and specificity of BPR@CB[8] towards Hep. It is noteworthy to mention that the response with heparin over CS is much more prominent (over 4 times), a feature that is absent in many sensors interacting with Hep. This might be attributed to the highest negative charge density on heparin amongst all other analytes studied and to the stronger affinity of Arg residues for sulfate groups of Hep. A linear calibration curve was obtained to detect Hep in an aqueous solution with the phosphorescence changes in BPR@CB[8] (Figure 2.6C). A pH-dependent study was also performed to note the pH sensitivity of the probe in the detection process at relevant biological pH levels. We obtained calibration curves for the addition of Hep to BPR@CB[8] in the pH range of 5.0 to 8.0. (Figure 2.6D). For all cases, we observed that in the same linear range studied, the magnitude of increment and sensitivity (LOD and LOQ values, Table 2.2) did not differ significantly. This can be attributed to the fact that the probe and analyte interaction is independent of the pH range monitored. The ionic interactions that are responsible for the binding are mainly because of positively charged side chain arginine residues and negatively charged sulphate and carboxylate groups from hep. These groups hold their respective ionic charges in the pH range studied and hence show no distinct pH-dependent behaviour in the detection process.

Table 2.2. LOD and LOQ values for Hep and PS detection in buffers of different pH.

Analyte Detected (0.0-2.0 μg/mL)	pH of medium	Method	LOD (ng/mL)	LOQ (ng/mL)
Heparin	5.0	Phosphorescence Turn-on	17.27	51.81
Heparin	6.0	Phosphorescence Turn-on	22.76	68.28
Heparin	7.0	Phosphorescence Turn-on	17.02	51.06
Heparin	8.0	Phosphorescence Turn-on	24.29	72.87
Protamine	5.0	Phosphorescence Turn-off	23.25	69.75
Protamine	6.0	Phosphorescence Turn-off	26.21	78.63
Protamine	7.0	Phosphorescence Turn-off	31.47	94.41
Protamine	8.0	Phosphorescence Turn-off	37.21	111.63

To apply the sensor for a practical use, it should be able to detect Hep in blood serum and urine samples. However, sensing in blood serum is extremely challenging as it contains a high concentration of electrolytes and charged proteins. We obtained calibration curves of phosphorescence changes in BPR@CB[8] within the concentration range of 0.0 – 2.0 μg/mL of Hep

studied in samples containing 10% HBS (Figure 2.7A) and HU (Figure 2.7B). All the curves obeyed a linear dependency with Hep in the concentration range inspected. The coefficient of linear fitting for 10% HBS and HU samples was observed to be 0.9985 and 0.9986, respectively. The limit of detection (LOD), limit of quantification (LOQ), and values for other detection parameters were estimated from the linear variation in the phosphorescence plots for both HBS and HU samples and have been summarised (Table 2.3). Hence, the detection method proved to be appreciably sensitive to Hep and could accurately detect the analyte in low concentrations with precision in the ng scale. A comparison table with other reported sensors for Hep is provided in the supporting information (Table 2.4).

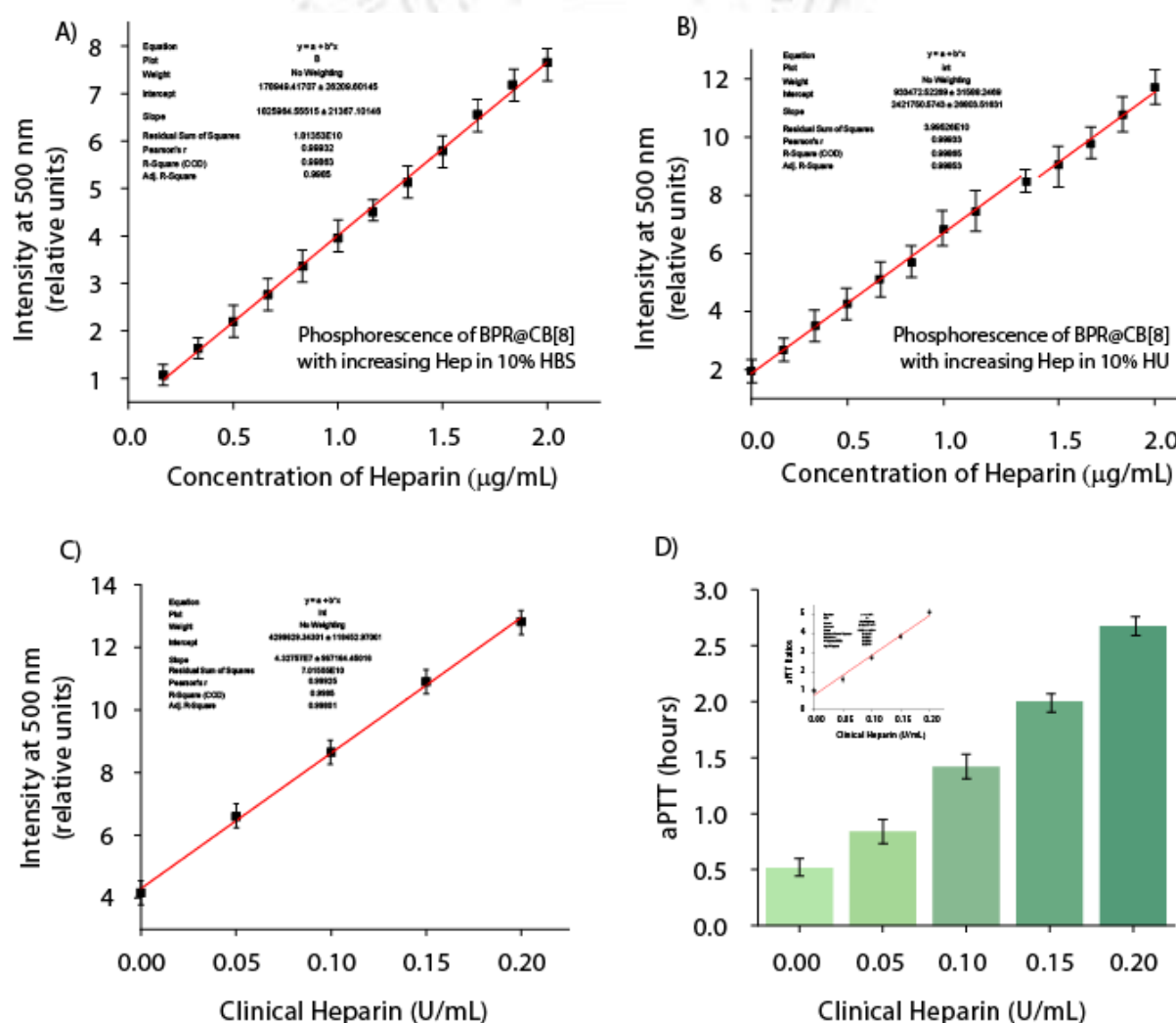


Figure 2.7. A) Linear phosphorescence calibration curve with BPR@CB[8] (2 μM) for detecting heparin in samples containing A) 10% HBS and B) 10% HU. C) Calibration curve for clinical heparin based on change in phosphorescence. D) Activated clot formation time against the concentration of clinical heparin in 40% HBS obtained using aPTT assay. Inset - Calibration curve for clinical heparin based on change in aPTT ratios.

Table 2.3. Detection parameters of phosphorescence-based sensing of heparin and PS.

Analyte Detected (0.0-2.0 µg/mL)	Medium in pH 7.0 Buffer	Method	LOD (ng/mL)	LOQ (ng/mL)	Added (µg/mL)	Found (µg/mL)	Recovery %	RSD % (n = 3)
Heparin	1% HS	Phosphorescence Turn-on	17.27	51.81	-	0.133± 0.008	-	6
					0.267	0.390± 0.014	97.5	3.6
					0.4	0.793± 0.035	99.1	4.4
					0.8	1.592± 0.030	99.5	1.9
Heparin	1% HU	Phosphorescence Turn-on	1.89	5.67	-	0.133± 0.007	-	5.3
					0.267	0.410± 0.014	102.6	3.4
					0.4	0.779± 0.025	97.3	3.2
					0.8	1.606± 0.032	100.4	2.0
Heparin	10% HS	Phosphorescence Turn-on	60.87	182.61	-	0.133± 0.009	-	6.7
					0.267	0.387± 0.024	96.8	6.2
					0.4	0.785± 0.039	98.1	4.9
					0.8	1.574± 0.042	98.4	2.7
Heparin	10% HU	Phosphorescence Turn-on	52.19	156.57	-	0.133± 0.005	-	3.8
					0.267	0.412± 0.020	103.0	4.9
					0.4	0.825± 0.043	103.1	5.2
					0.8	1.639± 0.091	102.4	5.5
Protamine	1% HS	Phosphorescence Turn-off	24.58	73.74	-	0.200± 0.009	-	4.5
					0.4	0.585 ± 0.016	96.7	2.7
					0.6	1.235± 0.017	102.5	1.4
					0.8	1.984± 0.025	99.2	1.2

Our detection method was also applied to estimate clinical Hep in the 10% HBS medium following a similar protocol of phosphorescence studies (Figure 2.7C). Clinical heparins used in injections are marketed and quantified in terms of their activities (U/mL). The clinical heparin was also utilized to perform aPTT assays to compare and determine the efficacy of our method. Increasing Hep concentrations (0.0 – 2.0 U/mL) in 40% HBS enhanced clot formation times (Figure 2.7D). A calibration curve was obtained for aPTT ratios (time ratios) against Hep concentration, from which the detection limit could reach 3.61 U/mL (Figure 2.7D inset). In the same concentration range, utilizing the BPR@CB[8] phosphorescent probe, a lower concentration of clinical Hep could be detected. From the phosphorescence calibration plot for clinical Hep, a detection of 0.0075 U/mL could be achieved. Considering the dilution factor, this value corresponds to 0.3 U/mL in 40% HBS, which is approximately 12 times lower than the detection limit reached by the aPTT assay. The LOQ for 100% HBS then reaches 2.25 U/mL, which lies in the range of the therapeutic dose level required for cardiovascular surgery (2-8 U/mL).¹⁰⁴ This study confirms not only the efficacy of our detection method in real clinical samples of Hep but also projects a comparison with the aPTT assay.

Table 2.4. Comparative data for the current work and reported luminescence-based heparin sensors.

Material	Method	Linear Range	Test Media	LOD (ng/mL)	Reference
Mn-doped ZnS quantum dots/polybrene self-assemblies ¹⁰⁵	Phosphorescence turn-on	0.38-10.76 $\mu\text{g/mL}$	Aqueous buffer	160	1
Pyrene based cationic sensor ¹⁰⁶	Ratiometric Luminescence	0-0.7 μM	Aqueous buffer	153	2
DNA templated gold nanoclusters ¹⁰⁷	Fluorescence turn-on	0.5-12.0 $\mu\text{g/mL}$	Aqueous buffer	150	3
Polyadenosine-coralayne complex ¹⁰⁸	Fluorescence turn-off	0.4-4 μM	2.5% HBS	100	4
Polycarboxyl derivative of anthracene based AIEgen ¹⁰⁹	Fluorescence turn-off	0.08-8 $\mu\text{g/mL}$	Aqueous buffer	37	5
Tetraphenylethylene-Peptide Conjugate ¹¹⁰	Fluorescence turn-on	0.32-5.5 $\mu\text{g/mL}$	Aqueous buffer	35	6
Cationic Cyanostilbene Assemblies ¹⁸	Ratiometric Luminescence	0-20 μM	2% HBS	33	7
CB[8] based polyarginine probe	Phosphorescence turn-on	0-2.0 $\mu\text{g/mL}$	1% HBS	17.27	This work
Cationic naphthalimide derivative ¹¹¹	Fluorescence turn-on	0-15 $\mu\text{g/mL}$	Aqueous buffer	7.56	8
Gold nanoclusters ¹¹²	Phosphorescence turn-on	0.006-25 $\mu\text{g/mL}$	Aqueous buffer	6	9
Host-guest based pyrene aggregate ¹¹³	Fluorescence turn-off	0-49 ng/mL	Aqueous buffer	2.4	10
Cationic Pyrene-Peptide Conjugate ¹¹⁴	Ratiometric Luminescence	0.1-1.0 $\mu\text{g/mL}$	Aqueous buffer	2.12	11

As we established the efficient phosphorescence turn-on sensing of Hep, we realized that the same sensor could detect another important analyte, PS, a well-known antidote of Heparin and often administered in treating Hep overdose. The high positive charge on PS could be a good candidate to detach Hep from the BPR@CB[8] complex and thereby turn off the phosphorescence produced in the presence of Hep. Considering this mechanism, a 2 μM solution of BPR@CB[8] containing 2.0 $\mu\text{g/mL}$ of Hep was titrated with PS solution while the fluorescence and phosphorescence of the system were monitored (Figure 2.8A-B). As expected, the emission intensity of the broad fluorescence band at 500 nm declined, and the intensity of the sharp peak at 385 nm increased (Figure 2.8A, C). The phosphorescence intensity at 500 nm also dropped significantly upon adding

PS (Figure 2.8B, D). Both the changes in fluorescence (385 nm) and phosphorescence (500 nm) followed a linear path (Figure 2.8C, D).

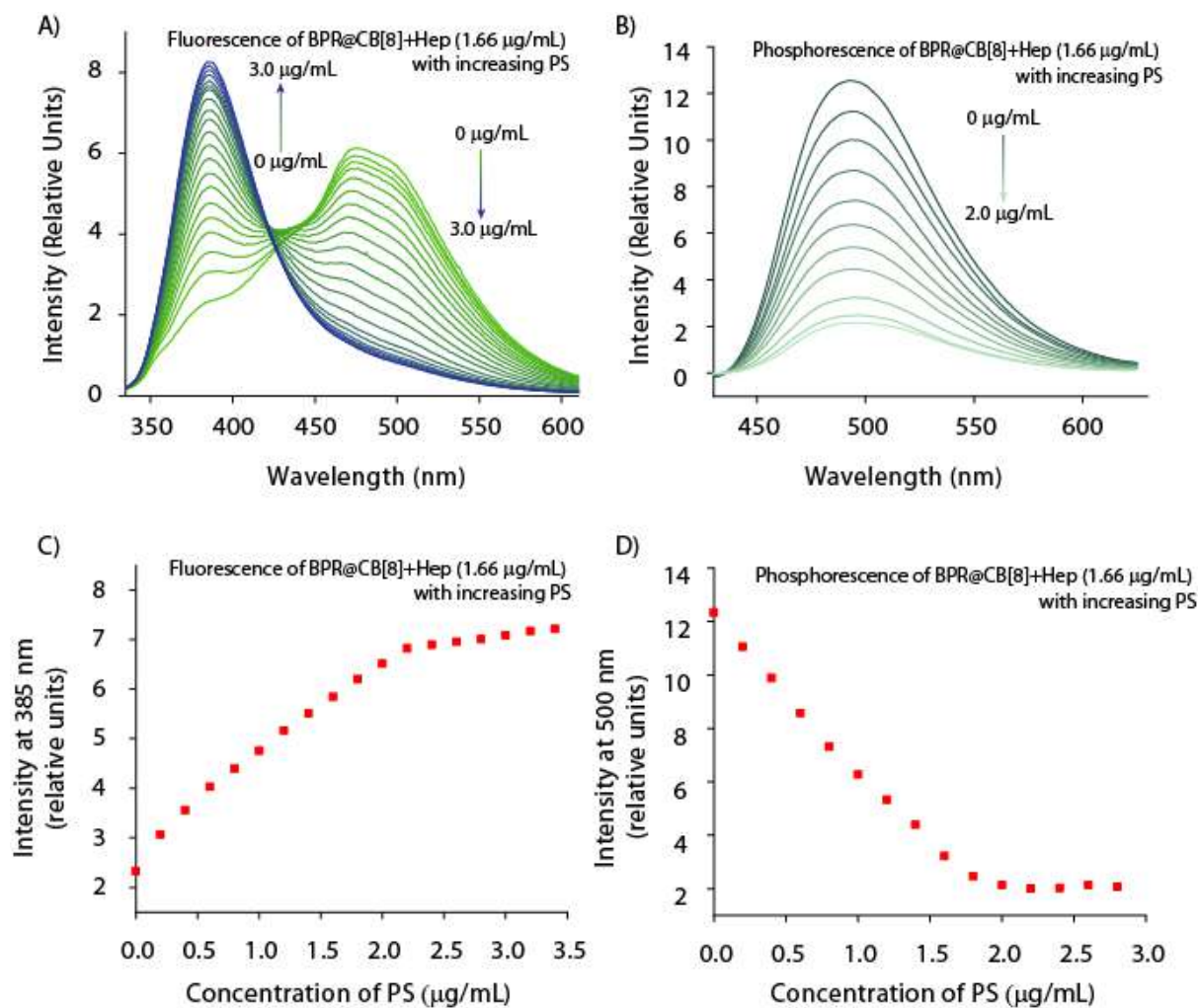


Figure 2.8. Change in A) fluorescence and B) phosphorescence of BPR@CB[8]-Hep (2.0 $\mu\text{g/mL}$) with an increase in the concentration of PS. Changes in C) fluorescence and D) phosphorescence intensities with an increase in the concentration of PS as obtained from A and B, respectively.

The gradual introduction of PS resulted in the disassembly of the BPR@CB[8]-Hep nano-assemblies and the formation of newer aggregates between Hep and PS. A stronger affinity of heparin toward PS resulted in the release of free BPR@CB[8] in solution, leading to the loss of phosphorescence emission from nano-assembly confinement. The disassembly process and the concomitant formation of new assemblies between heparin and PS were studied with DLS (Figure 2.9A). PS in the presence of BPR@CB[8] showed particles of very negligible dimensions. The solutions of BPR@CB[8]-Hep showed particles of 220 nm average diameter. The addition of PS to a solution of heparin with BPR@CB[8] resulted in the formation of larger particles with an average diameter of 300 – 400 nm (Figure 2.9A). This confirmed the ability

of PS to break the aggregates of Hep with BPR@CB[8] and itself form newer aggregates with heparin, which effectively releases BPR@CB[8] from confinement.

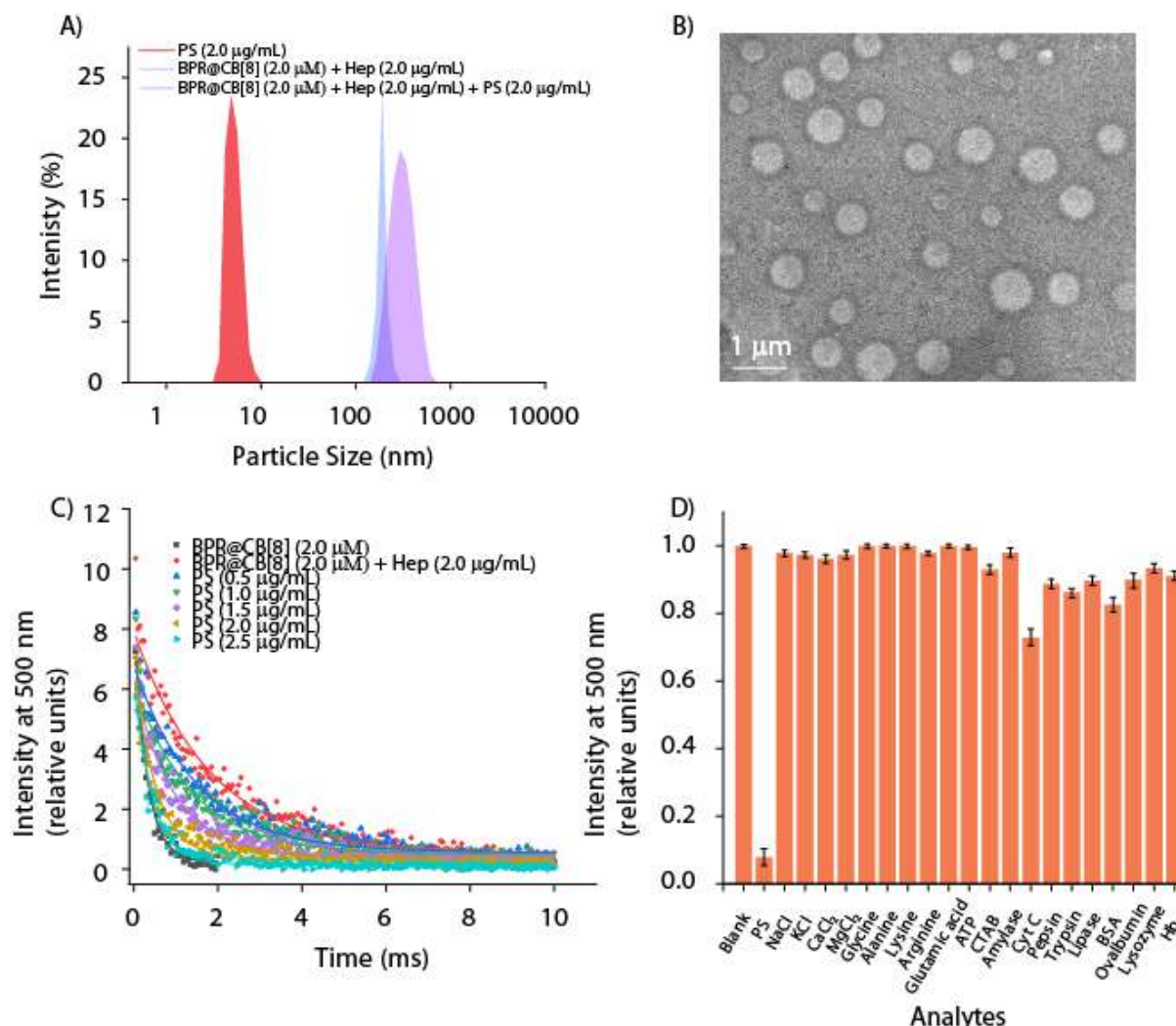


Figure 2.9. A) DLS profile of different compositions of PS, Hep, and BPR@CB[8]. B) FESEM image of a mixture of Hep and PS. C) Phosphorescence decay profiles of a solution of BPR@CB[8]-Hep with different concentrations of PS (0.5 – 2.5 µg/mL). D) Quenching of phosphorescence @500 nm for BPR@CB[8](2 µM) - Hep (2 µg/mL) in the presence of different analytes.

The observations were reinforced by the FESEM images, where larger and more regular spherical aggregates of heparin and PS were seen (Figure 2.9B). The release of BPR@CB[8] from nano-assembly confinement was also supported by the phosphorescence decay profiles of a solution of BPR@CB[8]-Hep with different concentrations of PS (0.5 – 2.5 µg/mL) (Figure 2.9C). The aggregates of BPR@CB[8] (2 µM)-Hep (2.0 µg/mL) showed a lifetime of 1.81 ms (Table 2.5). As PS is gradually added, BPR@CB[8] is released free into solution. The advantage in the phosphorescence radiative pathway obtained from nanoconfinement is lost with increasing concentrations of PS. This is reflected in the decrease of phosphorescence lifetimes from 1.81 ms to 0.44 ms with increasing

concentration of PS. The value reaches very close to that of free BPR@CB[8] (2 μ M) (0.33 ms) in solution. This trend in decay profiles proved the phosphorescence turn-off process in the presence of PS. Before proceeding into the sensing application, it was essential to ensure the selectivity of this turn-off process towards PS. To eliminate the possibility of interfering analytes restricting the PS selective detection, the quenching of phosphorescence (Figure 2.9D) emission at 500 nm of BPR@CB[8] (2 μ M)-Hep (2.0 μ g/mL) aggregates was recorded in the presence of multiple possible interfering analytes. For most of the salts (2 mM), amino acids (2 mM), and smaller analytes (1 mM), no noticeable change in the intensity was recorded. A small reduction in the intensity was noticed for larger analytes like proteins (0.1 mg/mL). The decrement was larger for Cytochrome C (Cyt-C) than for other analytes. This might be because of greater positive charge density in Cyt-C compared to the other possible interfering analytes studied. However, compared to the quenching in the presence of PS, most of these reductions were found to be insignificant. These results verified the selectivity and specificity of the turn-off mechanism towards PS. It can be said that PS contains way more arginine residues and much higher positive charge density compared to all other analytes studied. This enables PS to successfully bind to Hep and lead to the phosphorescence quenching, unlike other analytes with lower positive charge densities.

Table 2.5. Phosphorescence lifetimes of BPR@CB[8] in heparin (2.0 μ g/ml) with different concentrations of PS measured at room temperature.

Sl. No	Entry	Lifetime (τ) (ms)	χ^2
1	BPR@CB[8]	0.3118	0.9741
2	BPR@CB[8] + Hep (2.0 μ g/ml)	1.8116	0.9569
3	BPR@CB[8] + Hep (2.0 μ g/ml) + PS (0.5 μ g/ml)	1.4850	0.9489
4	BPR@CB[8] + Hep (2.0 μ g/ml) + PS (1.0 μ g/ml)	1.3682	0.9312
5	BPR@CB[8] + Hep (2.0 μ g/ml) + PS (1.5 μ g/ml)	0.9475	0.9319
6	BPR@CB[8] + Hep (2.0 μ g/ml) + PS (2.0 μ g/ml)	0.5239	0.9254
7	BPR@CB[8] + Hep (2.0 μ g/ml) + PS (2.5 μ g/ml)	0.4439	0.9579

Utilizing this principle of phosphorescence turn-off, the detection of PS was also performed in water and in samples of serum and urine (10%) (Figure 2.10 E-G). An inverse linear phosphorescence calibration curve was obtained within the concentration range of 0.0-2.0 μ g/mL of PS. The linear coefficient values for fitting were 0.9985 and 0.9933 for samples of 1% HBS and HU, respectively. The linear coefficient values for fitting were 0.9940 and 0.9964 for samples of 10% HBS and HU, respectively. From these phosphorescence calibration plots, the detection parameter values were estimated and summarised in Table 2.3. A pH-dependent study was also performed to note the pH sensitivity of the probe in the detection process for PS as well. We obtained calibration curves for

the addition of PS to BPR@CB[8]-Hep in the pH range of 5.0 to 8.0. (Figure 2.10F). For all cases, we observed that in the same linear range, the magnitude of increment and sensitivity (LOD and LOQ values, Table 2.2) did not differ significantly. Hence, from our studies, we have proved that by implementing the BPR@CB[8] probe, we could detect PS precisely in biological samples reaching low values. We have compared the LOD and LOQ parameters we obtained in HBS for PS samples (Table 2.6) with certain recent reports. Our CB[8]-based system involving the phosphorescence method of detection is new, precise, accurate, and can detect low concentrations of analyte with high sensitivity.

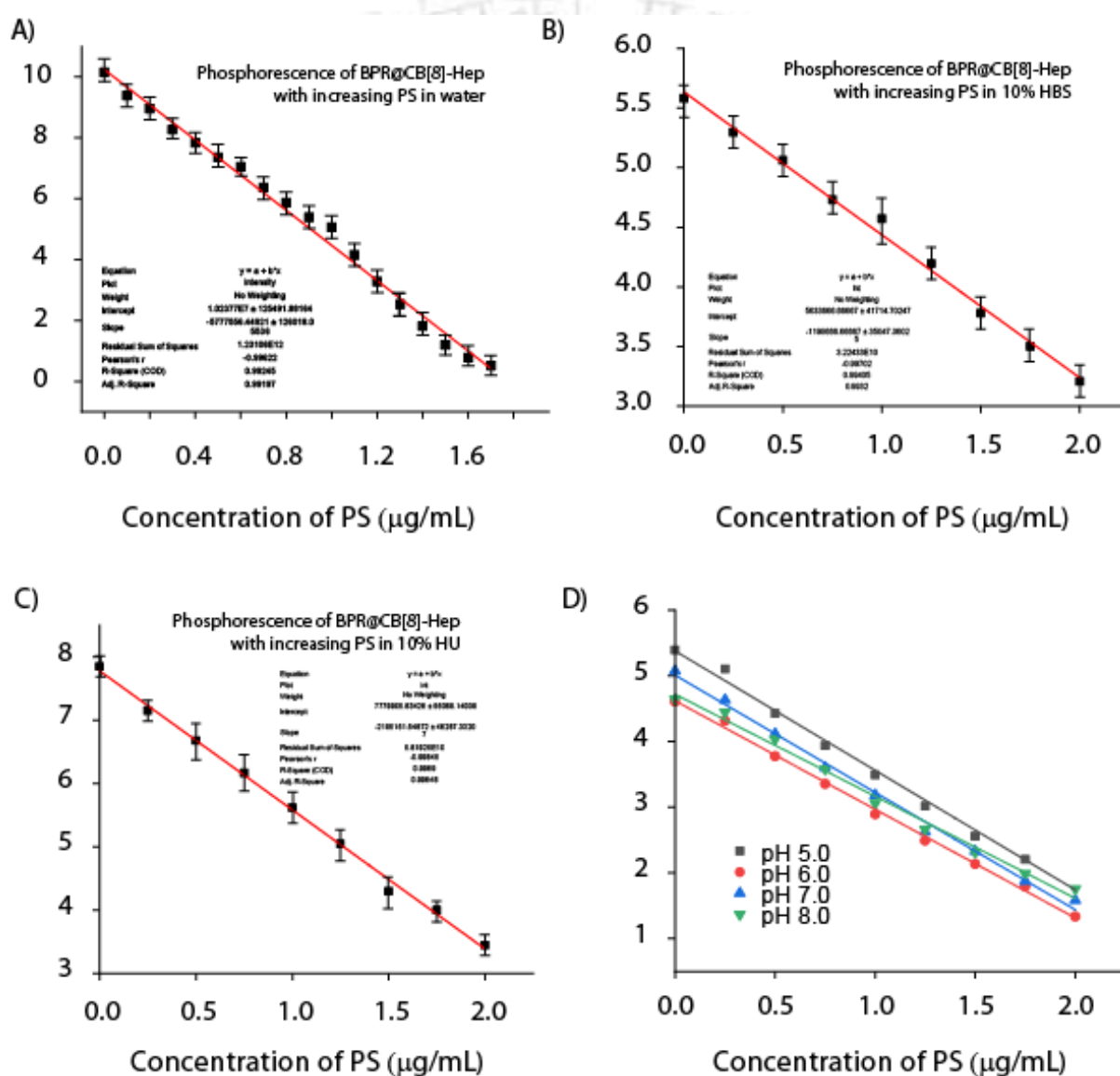


Figure 2.10. A) Calibration curves obtained for quenching phosphorescence of BPR@CB[8] (2 µM) - Hep (2 µg/mL) with increasing concentration of PS in A) aqueous medium, B) 10 % HBS and C) 10% HU. D) Linear phosphorescence calibration plot for detection of PS with BPR@CB[8] (2 µM)-Heparin (2.0 µg/mL) in buffers of different pH.

2.3. Conclusion

To summarize, a phosphorescence sensing method is established employing a host-guest complex that can detect heparin by a turn-on process and its antidote, protamine, by a turn-off process. Both sensing was successfully achieved in chemical and biological samples with high sensitivity and accuracy. A CB[8] based host-guest complex with an Arginine-rich molecule (BPR@CB[8]) is employed to aid the phosphorescence pathway. Heparin, because of its multiple negative charges, binds with highly positively charged complex, BPR@CB[8], leading to a secondary confinement that enhances the phosphorescence of the probe. The enhanced phosphorescence allowed quantitative and selective detection of heparin not only in aqueous medium but in biofluids like HBS and urine. The strong affinity of protamine toward heparin allowed us to use the same BPR@CB[8]-heparin aggregate as a probe to detect protamine. The addition of protamine to this aggregate detaches heparin due to stronger binding and thereby releases the BPR@CB[8] complex, resulting in a decrease in phosphorescence. The detection limits for heparin and protamine were estimated as 17 and 24 ng/mL in 1% HBS, respectively. Most importantly, the sensor showed high selectivity for both the analytes. This report brings forward dual heparin and protamine sensing with high sensitivity and selectivity, and it is promising in its application as a conventional method of analyte detection.

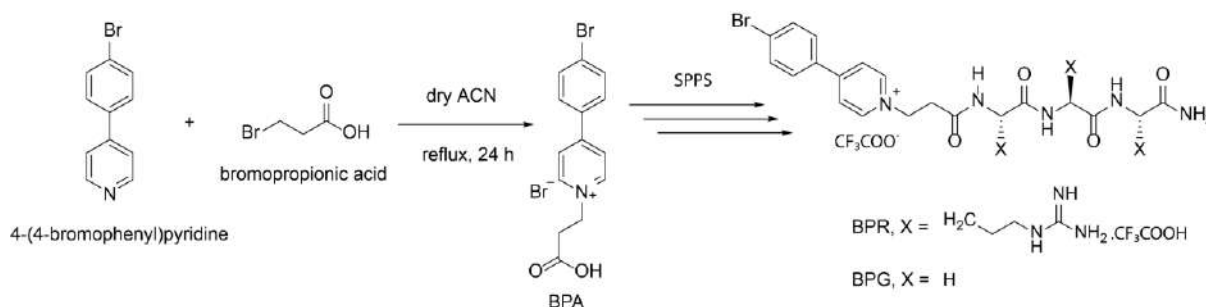
2.4. Experimental Section

2.4.1. General Information and Materials

BP, 3-bromopropionic acid, trisodium salt of citric acid and ATP were purchased from Sigma-Aldrich (USA). Heparin sodium salt ex. bovine intestinal mucosa (150 IU/mg), protamine sulfate for molecular biology (90-110%), all enzymes, all proteins and salts were procured from SRL (India). Dodecyl dihydrogen phosphate was purchased from TCI Chemicals. CB[8] was synthesized following the previously published protocol and characterized accordingly.¹¹⁵⁻¹¹⁷ Rink amide MBHA resin, protected amino acids, and coupling reagents were purchased from Novabiochem. HPLC-grade DMF, DCM, ACN, diethyl ether, diisopropylethylamine (DIPEA), silica gel (100-200 mesh) for column chromatography and calcium chloride were procured from Spectrochem (India) and Fisher Scientific (India). Clinical heparin for injection (5000 IU/mL) derived from porcine intestinal mucosa was purchased from Gland Pharma Limited, India. Solvents were dried whenever required according to the reported procedures. Milli-Q water with a conductivity of less than $2\ \mu\text{S cm}^{-1}$ was used for all sample preparations. ^1H NMR and ^{13}C NMR spectra were recorded using a Bruker Ascend 600 MHz (Bruker, Coventry, UK) spectrometer and referenced to deuterated solvents. ESIMS analyses were performed with a Q-ToF-Micro Quadrupole mass spectrometer (Micromass). MALDI analyses were performed with Bruker Daltonics - autoflex™ speed MALDI-TOF instrument.

2.4.2. Synthesis

BPR was synthesized following Scheme S2.2.



Scheme 2.2. Synthetic route for BPR and BPG.

Synthesis of 4-(4-bromophenyl)-pyridinium propionic acid (BPA)

500 mg (2.14 mmol, 1 equiv.) of 4-(4-bromophenyl)-pyridine and 390 mg (2.56 mmol, 1.2 equiv.) of 3-bromopropionic acid were measured and transferred into a 10 mL sealed tube equipped with a magnetic bar. To it, 4 mL of dry ACN was added, quickly sealed, and stirred at 90 °C in an oil bath for 24 hours. The reaction mixture was cooled, and a white precipitate was obtained. The precipitate was filtered and washed once with ACN and thrice with diethyl ether. The precipitate was dried under reduced pressure to obtain the pure product. Yield = 74%. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 9.13 (d, J = 6.0 Hz, 2H), 8.54 (d, J = 6.0 Hz, 2H), 8.04 (d, J = 8.1 Hz, 2H), 7.87 (d, J = 8.1 Hz, 2H), 4.78 (t, J = 6.7 Hz, 2H), 3.11 (t, J = 6.8 Hz, 2H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ (ppm) = 171.57, 153.73, 145.43, 132.73, 132.67, 130.17, 126.29, 124.17, 55.71, 34.33. Mass (ESI-MS): m/z calcd. for $\text{C}_{14}\text{H}_{13}\text{NO}_2^+ [\text{M}]^+$: 306.0124, found 306.0119, 308.0109.

General Synthesis of the Peptide by SPPS (BPR and BPG)

The peptide was synthesized on Rink amide MBHA resin using standard Fmoc (9-fluorenylmethoxycarbonyl) - solid phase peptide synthesis (SPPS) protocol. In a typical coupling, 3 equiv. of a protected amino acid (with respect to the loading of the resin), 3 equiv. of HBTU, and 6 equiv. of DIPEA were taken in 5 mL of DMF (for 0.1 mmol scale with respect to the resin loading) and stirred for 5 minutes before the addition of the mixture to the swelled deprotected resin. The reaction mixture was shaken for 60 min, and the resin was washed several times with DMF. The Fmoc-deprotection was achieved by treatment of the resin thrice with 5 mL of 20% piperidine in DMF for 5 minutes, followed by thorough washing of the resin with DMF and DCM. The Fmoc-deprotection and coupling steps were repeated until the designed peptide sequence was obtained. The resin with the loaded peptide was washed several times with DMF and DCM and dried. The dried resin was then treated with a mixture of freshly prepared mixture of 8.5:1:0.5 (TFA/TES/ H_2O) and stirred for 1 h. The resin was finally washed with DCM several times. The cleavage cocktail and the

washings combined were concentrated to a minimum volume on a rotary evaporator. The cleaved peptide was then precipitated from the cold, dry ether, centrifuged, and lyophilized to get the crude peptide. Purification was done in a semi-preparative HPLC using a Luna 5 μm (C18) column (Phenomenex) and an eluent of acetonitrile and water starting at 5% ACN in H_2O , reaching 100% ACN in 15 min to complete the chromatogram in 17 min.

BPR: ^1H NMR (500 MHz, D_2O) δ 8.71 (d, $J = 6.7$ Hz, 2H), 8.20 (d, $J = 6.8$ Hz, 2H), 7.75 – 7.68 (m, 4H), 7.32 – 7.28 (m, 1H), 7.21 – 7.14 (m, 2H), 6.87 – 6.76 (m, 1H), 4.83 – 4.74 (m, 5H), 4.22 – 4.06 (m, 4H), 3.04 (d, $J = 7.0$ Hz, 2H), 3.03 – 2.97 (m, 3H), 2.94 (dt, $J = 7.1, 3.4$ Hz, 3H), 1.65 – 1.35 (m, 12H). Mass (ESI-MS): m/z calcd. for $\text{C}_{32}\text{H}_{53}\text{BrN}_{14}\text{O}_4^{4+}$ $[\text{MH}_4]^{4+}$: 194.0884, found 194.0894. for $\text{C}_{32}\text{H}_{50}\text{BrN}_{14}\text{O}_4^+$ $[\text{M}]^+$: 773.3317 found 773.3577.

BPG: ^1H NMR (500 MHz, D_2O) δ 8.71 (d, $J = 6.7$ Hz, 2H), 8.20 (d, $J = 6.8$ Hz, 2H), 7.75 – 7.68 (m, 4H), 4.61 (s, 2H), 4.03 (s, 2H), 3.94 (s, 2H), 3.88 (s, 2H), 3.33 (s, 2H). Mass (ESI-MS): m/z calcd. for $\text{C}_{20}\text{H}_{23}\text{BrN}_5\text{O}_4^+$ $[\text{M}]^+$: 476.0928, found 476.3063.

2.4.3. Methods and Instrumentations

2.4.3.1. UV-Vis, Fluorescence, and Phosphorescence Spectroscopy

UV-vis spectra were recorded on a Lambda 750 spectrometer (Perkin Elmer), while fluorescence measurements were performed on a Fluoromax 4 Plus spectrophotometer. Phosphorescence emission and time-resolved delayed photoluminescence decay spectra measurements were performed in a Horiba Jobin Yvon Fluoromax 4P instrument.

All experiments were recorded in 1 mL quartz cuvettes (path length 1 cm) at 298 K. All fluorescence and phosphorescence experiments were performed using a slit width of (Ex./Em.) = 5/5 nm under $\lambda_{\text{ex}} = 315$ nm at 298 K. The parameters for acquiring delayed photoluminescence emission and decay spectra were set as follows: time per flash 61 ms, sample window 200 μs , delay after flash 50 μs , flash count 100. All spectra were recorded in 10 mM phosphate buffer (pH 7.0).

2.4.3.2. Dynamic Light Scattering (DLS)

The particle sizes of the assemblies were obtained at 298 K using a 632.8 nm He–Ne laser using Zetasizer Nano-ZS90 (Malvern). The stock solutions of assemblies were prepared in water and filtered through appropriate filters to remove dust particles, if present, before the measurement. All the measurements were performed in triplicate.

2.4.3.3. Field Emission Scanning Electron Microscopy (FESEM)

5 μ L of appropriate sample solution was cast on a silicon wafer and immediately freeze-dried. FESEM imaging and elemental analysis were then performed on a Gemini SEM 300 (Sigma Zeiss) instrument.

2.4.3.4. Field Emission Transmission Electron Microscopy (FETEM)

5 μ L of the sample solution was cast on the carbon-coated copper grid (300 mesh Cu grid 8 with thick carbon film from Pacific Grid Tech, USA) and allowed to air dry for 2 minutes, and then the excess sample was blotted with a tissue paper. The grid was then air-dried, and the FETEM images were taken in JEOL JEM 2100F microscopes.

2.4.3.5. Extraction of HBS and HU Samples

Blood (5 mL) and urine samples were collected from a group of healthy men in the age group of 25-30. The blood samples were treated with 3.8% sodium citrate solution (9:1 blood: citrate), then centrifuged at 4000 rpm for 10 minutes at 5 °C. The clear transparent supernatant solution was collected as the serum and incubated at 4 °C. The urine samples required no pre-treatment or extraction procedures and were incubated as collected at 4 °C.

2.4.3.6. aPTT assays

50 mg of silica (100-200 mesh) and 100 μ L of dodecyl dihydrogen phosphate solution (incubated at 37 °C for 2 minutes) were taken in a 2 mL glass vial. 100 μ L of plasma or heparin-containing plasma (incubated at 37 °C for 2 minutes) was added to it, and after a gentle stirring, the vial was kept at 37 °C for exactly 5 minutes. Then aqueous CaCl_2 solution (incubated at 37 °C for at least 2 minutes) was added to it, and the vial was kept at 37 °C for 20 seconds. Then in every minute, clot formation was checked by gently tilting the vial. The clot formation time was noted down. The aforementioned procedure was employed for the clinical heparin injection solution. Subsequently, aPTT ratios were plotted against the concentration or activity of the injected heparin. The phosphorescence and the aPTT ratio calibration curve of the clinical heparin were compared.

2.4.3.7. Titration Procedures for Detection Parameter

The spectroscopic titrations for the detection of heparin and PS were performed in human serum and urine samples, each diluted to 10% in 10 mM pH 7.0 phosphate buffers. The calibration curve for heparin detection was obtained by gradually adding the analyte in a linear concentration range to the aforementioned solutions containing BPR@CB[8] and recording the spectra. The calibration

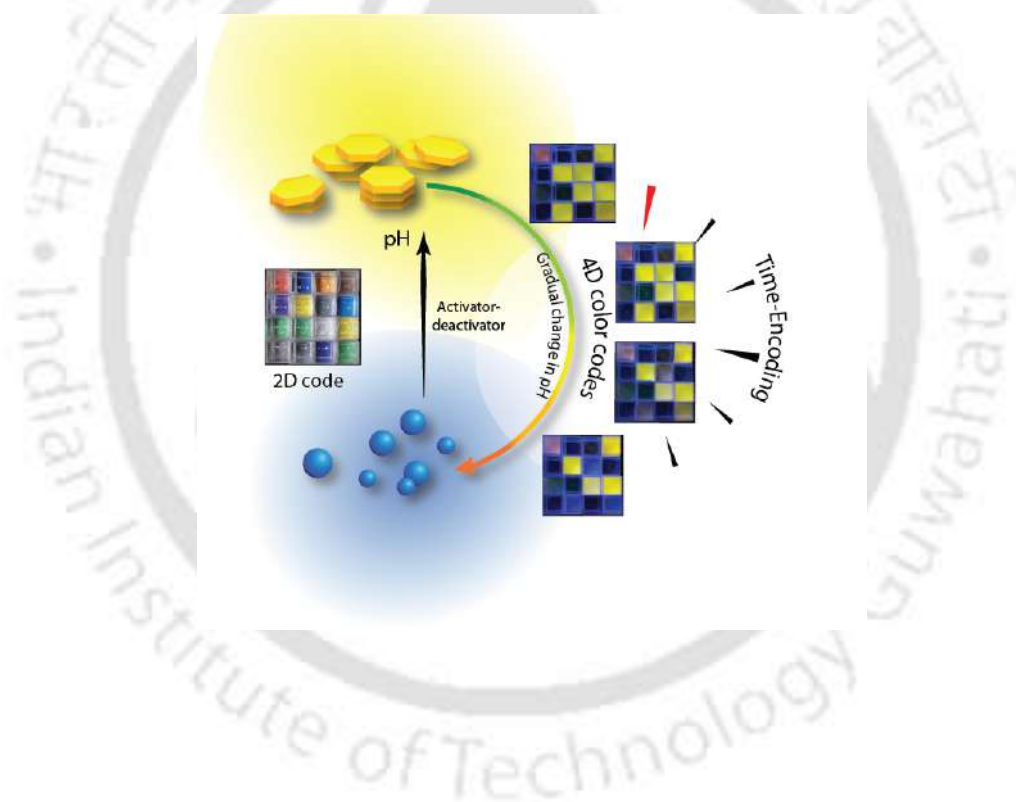
curve for PS detection was obtained by gradually adding the analyte in a linear concentration range to the aforementioned solutions containing BPR@CB[8] and heparin and recording the spectra. The LOD and LOQ were measured from the calibration curves for the analytes obeying the $3.3\sigma/m$ and $10\sigma/m$ formulas, respectively, where ' σ ' denotes the standard deviation in the measurements and ' m ' denotes the slopes of the linear fitting. Recovery (found/added) and relative standard deviation percentage were also calculated from their standard formulas.





Chapter 3

Time-Encoded Information Encryption with pH Clock Guided Broad-Spectrum Emission by Dynamic Assemblies





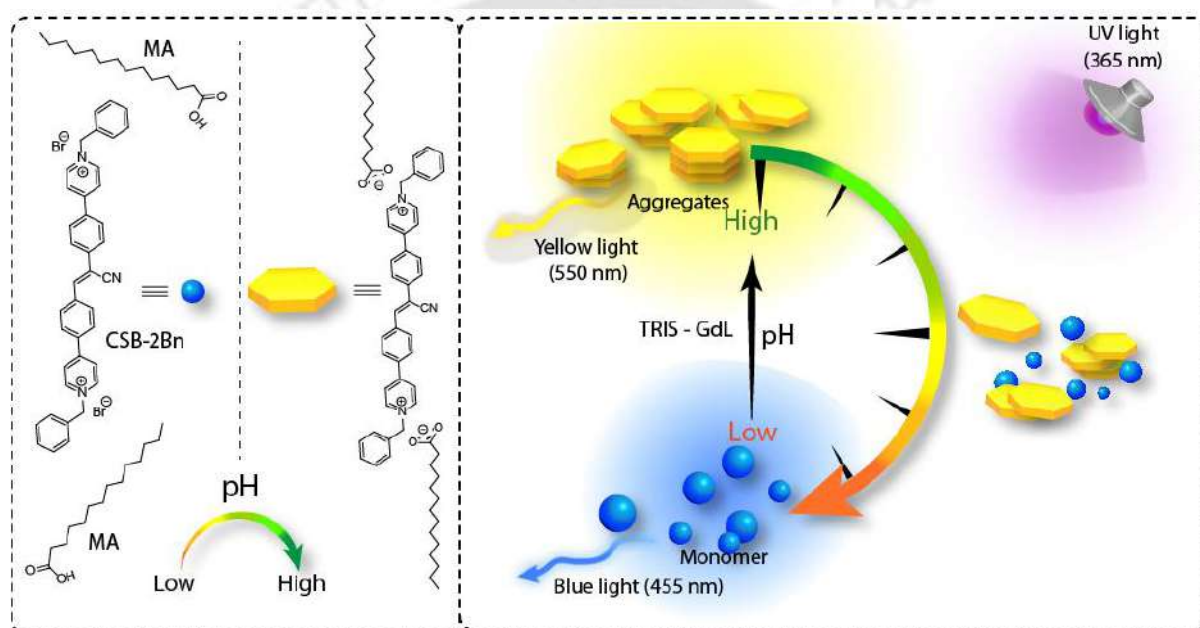
3.1.Introduction

In today's fast-paced digital age, we are constantly surrounded by vast amounts of information and data. The key lies in discerning genuine information amidst a sea of falsities. Additionally, counterfeiters have added to the alarming rise in security threats, information mishandling, and data manipulation.^{118, 119} Counterfeiting poses a significant global risk to economic stability, public health, social cohesion, and national security over time.^{120, 121} This has prompted a focused effort towards developing logic-based information processors and secure data encryption systems. These systems aim to facilitate safe and straightforward storage, access, transmission, and utilization of data. However, creating encrypted systems that securely contain data while meeting the rigorous requirements of multi-level security, adaptability, programmability, and practicality presents considerable challenges.¹²²⁻¹²⁴

To address this global concern, substantial efforts have been made with the development of anti-counterfeiting devices and materials, such as holograms, barcodes, watermarks, QR codes, and fluorescent patterns.¹²⁵⁻¹³⁵ Amongst them, scannable codes such as QR codes and barcodes, in particular, stand out due to their hassle-free decryption and quick read-out process, making them indispensable but also problematic due to their straightforward and rapid interpretation.¹³⁶⁻¹³⁸ Hence, advanced and more sophisticated 3D fluorescent scanning codes, which feature enhanced capabilities such as responsiveness to specific stimuli, multi-dimensional encryption, fluorescence switching ability, and time-encoded glows, are currently in focus.^{8, 139-146}

Adding extra layers of protection to anti-counterfeiting devices is crucial for enhancing security. We identified time as a potential new dimension to significantly improve the security of 3D fluorescent scanning codes, making them more difficult to decode. This can only be achieved by implementing time-gated encryption and decryption through temporal control of the system and its emissions. In recent years, activator-deactivator-controlled temporal assemblies have gained tremendous attention.¹⁴⁷⁻¹⁶¹ However, only a limited effort has been made to modulate the emission behaviour of the dynamic aggregates temporally.¹⁶²⁻¹⁶⁶ We reasoned that establishing a dynamic multi-colour fluorescence system, which temporally emits different colours, could provide an additional layer of protection in anti-counterfeiting systems. Time-dependent data encryption has been achieved previously.^{140, 167, 168} However, the reported systems used solid-state molecular motion and solvent-assisted colour tuning. None of these systems used fuel-driven transient self-assembly-based multi-colour emissive systems. With the help of a newly designed dynamic multi-colour fluorescence system, the fourth dimension in the form of time can be achieved. The continuous change in emission colour will allow an additional protection layer, as the encrypted data can only be decoded at a specified time.

To this end, enhanced emissions from aggregation-induced emitters (AIEgens) have proven superior to traditional fluorophores because of their high fluorescence quantum yield, colour tunability, signal responsiveness, and low quenching behaviour.¹⁶⁹⁻¹⁷⁵ Appropriate functionalization of hydrophobic AIEgens can endow them with appropriate amphiphilicity, making their aggregation behaviour susceptible to fluctuations in ambient factors like pH, ionic strength, redox potential, temperature, etc.^{164, 176-178} Such stimuli-responsive AIEgens are ideal candidates for developing activator/deactivator-controlled temporal multi-colour emitting systems. The present work aims to temporally modulate the aggregation and, consequently, the emission of such AIEgens and apply the time-gated system to introduce the time coordinate in the anti-counterfeiting mechanism. For this purpose, we relied on a well-established AIEgen, CSB.



Scheme 3.1. Schematic illustration of pH clock-instructed transient assembly of CSB-2Bn and MA and the corresponding broad-range multi-colour emission profile.

In what follows, we have attempted to strategize a pH-clock-instructed dynamic assembly of a CSB derivative in the presence of an aliphatic long-chain carboxylic acid. CSBs typically exhibit weak blue-cyan fluorescence in their free state and display intense yellow emission when aggregated or in a solid state.¹⁷⁹⁻¹⁸³ We have prepared a dicationic cyanostilbene derivative (CSB-2Bn, Scheme 1) and manipulated its assembly and fluorescence in the presence of MA through pH switchable protonation and deprotonation of MA. When subjected to our well-established Tris buffer – GdL pH clock,^{117,184,185} the system produces transitory emission profiles spanning a broad range of the visible spectrum (blue, yellow, white, and finally back to blue). The transient emissive cycles can be reproduced multiple times by re-initiating the pH cycle. In the present system, to the best of our knowledge, there is no report on the temporal variation of fluorescence quantum yield and lifetime

of dynamic assemblies. Furthermore, we have implemented the static pH fluorescent emissions to express binary and ternary chemical logic (AND) gates. The dynamic pH-adaptive emissions were successfully employed in our larger goal of introducing the time coordinate to the external signal-triggered, multi-dimensional colour-coded message encryption, thereby enhancing the security of the anticounterfeiting system.

3.2. Results and Discussions

3.2.1. pH-Responsive Assembly and Emission

CSBs normally display different emissive properties in concentrated and dilute media.¹⁷⁹ The presence of polar or electrostatically charged groups is necessary for imparting hydrophilicity to this otherwise hydrophobic AIEgen to construct multi-fluorescent materials in aqueous medium.¹⁸⁶ To meet our objective, we modified a dipyridine functionalized CSB by appending two pendant benzyl groups at its two pyridine terminals (CSB-2Bn, Scheme 3.1). CSB-2Bn expectedly showed a higher solubility in water relative to its parent CSB counterpart.

To study the self-assembling behaviour of CSB-2Bn, the absorption and emission spectra were recorded as a function of its concentration and pH of the medium. An absorption maximum (λ_{max}) centred at 365 nm (Figure 3.1A) was observed at pH 7. When excited at the λ_{max} , an emission peak in the blue region at 455 nm (λ_{em} Figure 3.1B), characteristic of the CSB monomeric species, was observed.^{179, 186} The absorption at λ_{max} and the emission intensity at λ_{em} increased linearly with increasing concentration of CSB-2Bn, indicating no probable aggregation at this concentration range (Figure 3.1A, B inset). The overlapping of the excitation and absorption spectra of 5 μM CSB-2Bn also confirms the existence of the CSB monomer (Figure 3.1C). Also, the absorption and emission behaviour of 5 μM CSB-2Bn remained unchanged when the pH of the medium varied from 4 to 9, signifying no possible assembly across this pH range (Figure 3.1D, E).

It was anticipated that charge shielding by counter-electrostatic interactions at the cationic terminals of the molecule might assist the aggregation process.^{147, 187, 188} An amphiphilic anionic molecule might assist in the assembly by balancing the positive charges on the pyridinium groups, thereby reducing the electrostatic repulsive forces amongst them and enhancing the overall hydrophobic character. Furthermore, if the charge on the selected anion depends on the pH of the medium, then a pH-directed aggregation might be obtained. We realized that a long-chain carboxylic acid could be an appropriate choice. The carboxylic acid will show a pH-dependent charge on the anion, and the hydrocarbon chain will provide the required hydrophilic-lipophilic balance (HLB).¹⁸⁹ Thus, fatty acids with varying long chains (C6 to C14) were studied.

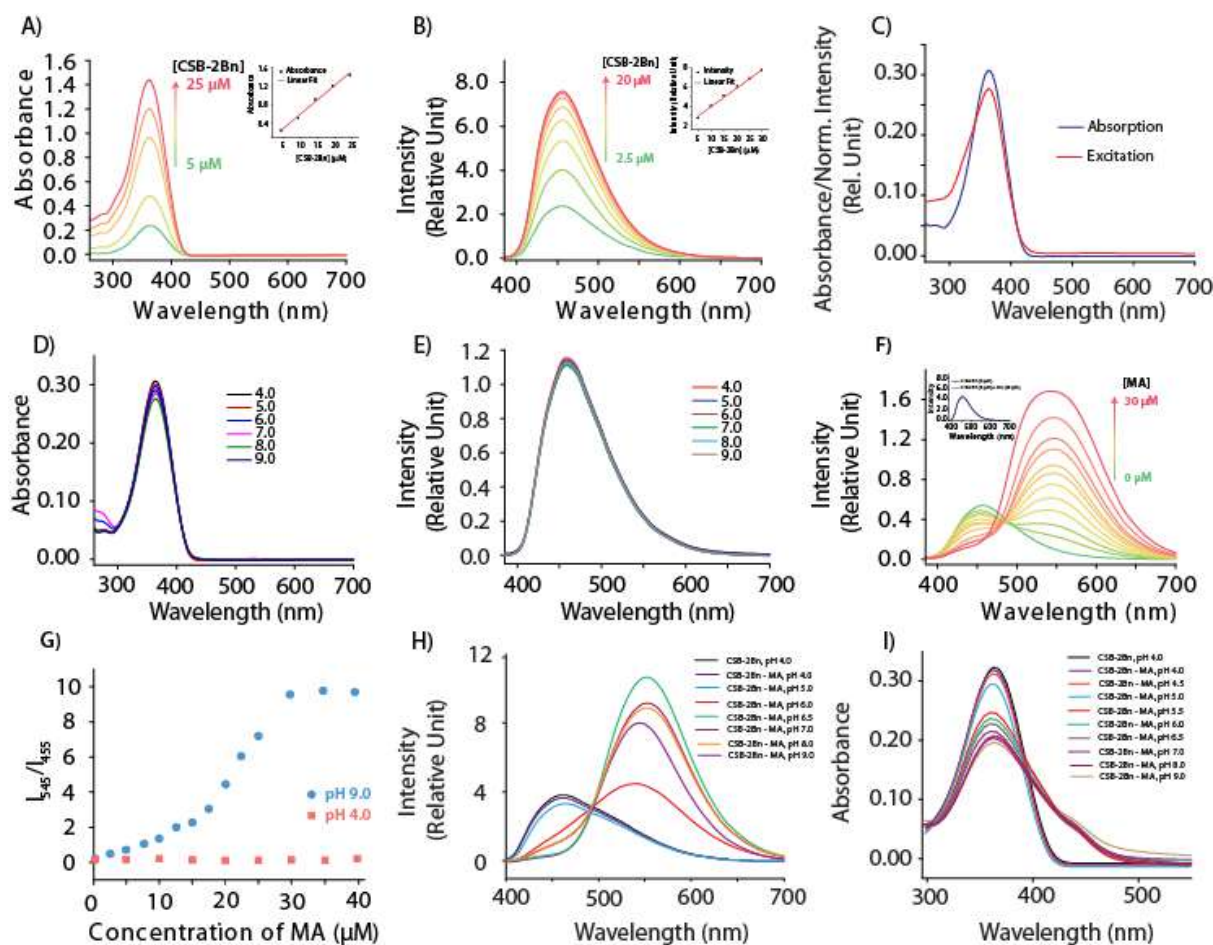


Figure 3.1. A) Concentration-dependent absorption (A) and emission (B) spectra of CSB-2Bn in phosphate buffer pH 7.0. C) Absorption (red line) and Excitation (blue line) spectra of CSB-2Bn (5 mM) ($\lambda_{em} = 455$ nm). pH-dependent D) absorption and E) emission spectra of 5 μ M CSB-2Bn. F) Emission spectra of CSB-2Bn (5 μ M) with increasing concentrations of MA in Tris buffer pH 9.0, and at pH 4.0 (inset). G) I_{545}/I_{455} ratio of CSB-2Bn (5 μ M) against increasing concentrations of MA at pH 9.0 and pH 4.0. H) Emission spectra of CSB-2Bn (5 μ M) – MA (25 μ M) in buffers of different pH. I) UV-Visible absorption spectra of CSB-2Bn (5 μ M) – MA (25 μ M) in buffers of different pH. $\lambda_{ex} = 365$ nm for all emission experiments.

CSB, being an AIEgen, the aggregation of CSB-2Bn is expected to impart a significant change in the emission behaviour.^{179-181, 186, 190} Thus, we studied the emission behaviour of CSB-2Bn in the presence of long-chain fatty acids at basic pH (>9, as the acids will remain deprotonated). While the shorter analogues ($C_6 - C_{10}$) could not impart noticeable changes, a significant enhancement of the emission is observed for longer chain acids (C_{12} and C_{14}), indicating possible aggregation (visual detection). Based on these results, MA, having a $pK_a \sim 4.9$, was selected for our purpose. When MA was added to an alkaline (pH 9) solution of CSB-2Bn, a stark change was observed in the emission spectra (Figure 3.1F). A substantial red shift in the fluorescence maximum (from 455 nm to 545 nm), accompanied by a significant overall enhancement in the emission intensity, was observed. Under acidic conditions (pH 4, < pK_a (MA), the carboxylic acid remains protonated. The addition of MA

brought no changes (Figure 3.1F inset) in the emission intensity and pattern. When (I_{545}/I_{455}) is plotted against MA concentration for both pH values, while a steady increase is shown in the basic pH, no apparent change is observed for pH 4 (Figure 3.1G). However, the increase in the intensity at pH 9 flattened after 30 μM concentration of MA, showing the upper limit. Keeping the CSB-2Bn – MA ratio fixed at 1:5 when the emission spectra were recorded at different pH values (4 – 9), the red-shifted emission band at 545 nm was observed only above pH 5.5 (Figure 3.1H), signifying aggregation above this pH value. The appearance of a red-shifted shoulder band at ~ 450 nm in the absorption spectra is characteristic of CSB-aggregation.¹⁷⁹ To verify the same, we checked the absorption profile for the CSB-2Bn – MA (1:5) mixture at various pH values (Figure 3.1I). It showed the appearance of the band at 450 nm above pH 5.5. All the observed spectral changes signify the formation of ordered aggregates of CSB-2Bn – MA at and above pH 5.5.

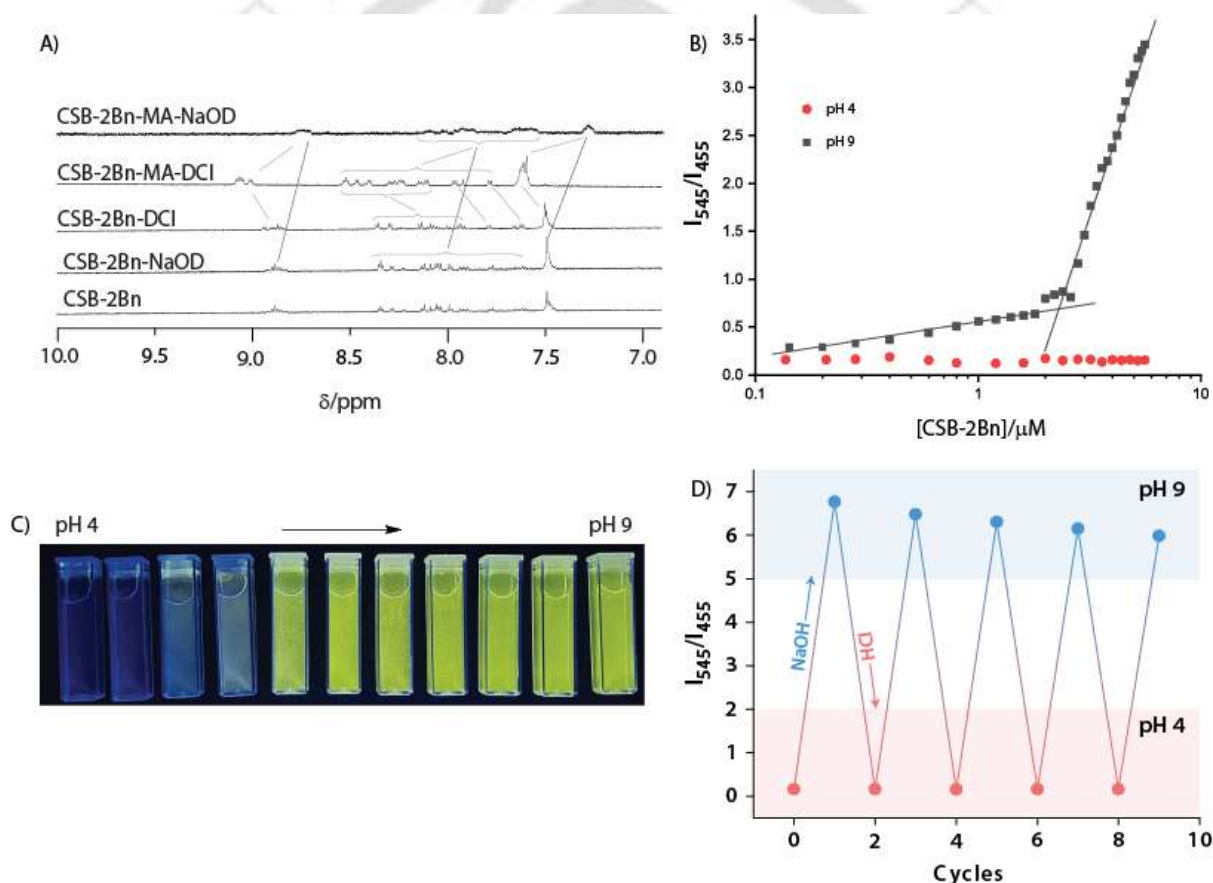


Figure 3.2. A) ^1H NMR spectra of CSB-2Bn (5 μM , D_2O) under various conditions. B) Determination of CAC of CSB-2Bn – MA (1:2) at pH 4 and 9. C) Photograph of cuvettes containing CSB-2Bn (5 μM) – MA (25 μM) in buffers of different pH under UV-light (365 nm). D) Reversible emissive behaviour of CSB-2Bn – MA (5:25 μM) across multiple cycles induced by alternate addition of NaOH and HCl.

Moreover, the ^1H NMR study (Figure 3.2A) also suggests aggregation of the CSB-2Bn – MA system under basic conditions. Additionally, we checked the CAC of the CSB-2Bn – MA (1:2) system at pH 4

and 9 using the concentration-dependent emission study (Figure 3.2B). Under acidic conditions, the I_{545}/I_{455} ratio remained unaltered, indicating no aggregation. On the other hand, a clear inflexion point at 2.3 μM (CSB-2Bn suggests the critical aggregation concentration of the CSB-2Bn – MA (1:2) system at pH 9. The ^1H NMR spectra of CSB-2Bn (5 μM , D_2O) were recorded under various conditions and the aromatic region was compared. As can be seen from the figure, acidification or basification could not impart much change in the peak position of the CSB group. However, the presence of MA (2 equiv.) in the acidic condition resulted in an overall downfield shift of the aromatic protons. On the other hand, under basic conditions, the presence of MA (2 equiv.) showed a significant upfield shift of the signals. The upfield shift is a typical sign of π – π stacking. It is to be noted that at this low concentration, it is difficult to obtain a good signal, especially in the aggregated state (basic medium, in the presence of 2 equiv. of MA). However, the overall upfield shift was clearly visible.

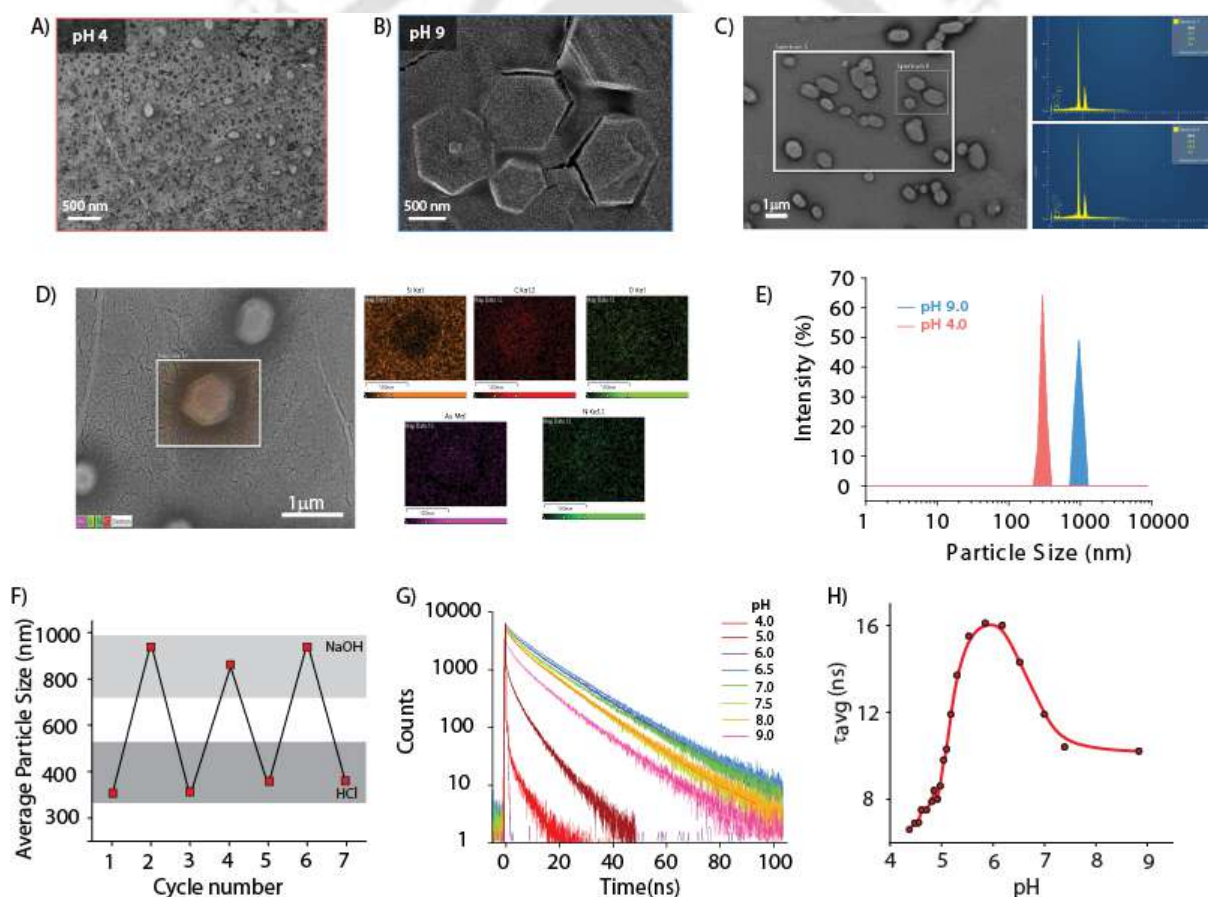


Figure 3.3. FESEM image of CSB-2Bn – MA (5:25 μM) at A) pH 4.0, and B) 9.0. FESEM image of the CSB-2Bn (5 μM) – MA (25 μM) at pH9 and the corresponding C) EDX and D) elemental mapping. E) Average particle diameters as measured by DLS for CSB-2Bn – MA (5:25 μM) at pH 4.0 and 9.0. F) pH reversible particle size of CSB-2Bn - MA assembly over multiple cycles conducted by alternate addition of NaOH and HCl. G) pH-dependent fluorescence decay profile for CSB-2Bn – MA (1:5) (λ_{ex} = 374 nm, λ_{em} = 550 nm). H) Change in average lifetime for the emission at 550 nm of CSB-2Bn - MA at different pH.

When kept under UV light of 365 nm, the sample solutions exhibited a blue colour at pH 4, while at pH 9, the colour changed to yellow-green (Figure 3.2C). To check whether changing the pH of the solution can change the emission colour and if that can be repeated, a CSB-2Bn – MA (1:5) solution at pH 4 was treated with NaOH solution to attain a pH value of 9, and the emission was recorded. To this NaOH-treated sample, HCl was added to decrease the pH to 4. This process was repeated for several cycles (Figure 3.2D). The I_{545}/I_{455} can be reversibly switched between high and low values in a repetitive cyclic way, suggesting the pH-reversible emissive properties.

Next, the morphology of the aggregates formed was analyzed using the FESEM technique. The FESEM images of 5 μ M CSB-2Bn at pH 4 and 9 showed no noticeable morphology. A 25 μ M solution of MA at pH 4 resulted in particles of 200 – 300 nm diameter, but the same at pH 9 showed no proper morphology. A mixture of CSB-2Bn – MA (5:25 μ M) at pH 4 showed irregular particles of 200 – 300 nm size (Figure 3.3A). On the other hand, at pH 9, a regular hexagonal disk-like (~1000 nm) morphology was observed for the same composition (Figure 3.3B). The elemental mapping of the hexagonal structures is provided (Figure 3.3C, D). The regular hexagonal pattern is a result of the ordered aggregation of the CSB-2Bn – MA. However, the irregular, smaller aggregates under acidic conditions are presumably the agglomeration of MA molecules that lost solubility due to protonation. These observations are in agreement with the DLS results (Figure 3.3E), where particles of average hydrodynamic sizes of 950 and 295 nm were observed at pH 9 and 4, respectively. The formation of hexagonal aggregates is an interesting observation and can be investigated in detail. However, that is beyond the scope of this work. The particle size also showed pH reversibility with alternate addition of NaOH and HCl (Figure 3.3F).

Next, the decay traces of CSB-2Bn-MA mixtures at different pH conditions were recorded and analyzed (Figure 3.3G, Table 3.1). At pH 4, the decay trace recorded at 550 nm fitted well with a tri-exponential function and provided three lifetime values, i.e. a short component of ~50 ps (fixed) with ~90 % contribution, 1.3 ns with ~3 % contribution and 5.6 ns with ~7 % contribution and the τ_{av} is estimated as 0.5 ns. This result clearly indicates that three types of species exist in the excited state. The short-lived component (τ_1) with the highest contribution, which might be ascribed to the monomeric species from CSB-2Bn that tend to remain free and stable in the acidic medium in the mixture of CSB-2Bn and MA and two kinds of smaller aggregates with smaller contributions. This free fluorophore emits mainly around 455 nm and decays faster in the observed region. With increasing pH (especially when the pH crosses the pK_a value of MA), the fluorescence decreases at 455 nm, and a fluorescence band for the aggregated form appears at 550 nm. On pH increment, the decay trace also followed a tri-exponential function with an increasing lifetime of moderate and

longer components as the conditions facilitate assembly; the free CSB-2Bn monomers form the MA salt and subsequent ordered assemblies. The contribution of the short lifetime component decreased significantly (from 90% at pH 4.0 to 1.5% at pH 6.5), whereas the long component lifetime increased from 5.6 ns to 15.4 ns along with the increase in the contribution (7% to 90%) at pH 6.5 and beyond this pH, the lifetime and its contribution gradually decrease to 12.7 ns and 77%, respectively. The moderate component lifetime increased from 1.3 ns to 4 ns at pH 6.5. The τ_{av} also increased significantly and reached the highest value of 14.1 ns at pH 6.5 and then decreased further to 10.3 ns with increasing pH to 9 (Figure 3.3H). As mentioned earlier, when the pH value goes beyond the pK_a value of MA, the aggregation of CSB-2Bn to ordered structures in the presence of MA gets facilitated.

Table 3.1. pH-dependent fluorescence lifetimes of CSB-2Bn - MA (1:5) ($\lambda_{em} = 550$ nm)

pH	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_{av} (ns)	χ^2
4.0	0.05 (89.8%)	1.3 (2.9%)	5.6 (7.3%)	0.5	1.1
5.0	0.05 (22.5%)	1.8 (23.7%)	7.1 (53.8%)	4.3	1.3
6.0	0.05 (1.3%)	3.7 (8.7%)	15.1 (90%)	13.9	1.1
6.5	0.05 (1.5%)	4 (8.9%)	15.4 (89.6%)	14.1	1.13
7.0	0.18 (1.6%)	3.8 (10.9%)	15.1 (87.5%)	13.6	1.1
7.5	0.05 (2.4%)	3.7 (13.7%)	12.6 (83.9%)	11.1	1.17
8.0	0.05 (2.6%)	3.9 (15.9%)	13.9 (81.5%)	11.9	1.17
9.0	0.05 (6.2%)	3.1 (17%)	12.7 (76.8%)	10.3	1.04

Table 3.2. pH-dependent anisotropy measurements of CSB-2Bn - MA (1:5).

pH	τ_{r1}	τ_{r2}	χ^2
5.0	0.15 (2.6%)	2.4 (97.4%)	1.11
6.5	0.40 (3.6%)	6.0 (96.4%)	1.04
7.5	---	13.8 (100%)	1.12
8.0	---	24.3 (100%)	1.16
9.0	---	23.8 (100%)	1.07

The two longer lifetime components may presumably be attributed to higher-order aggregates of different sizes, as evidenced by the fluorescence anisotropy measurements, which give insight into the dimension of the fluorophore.¹⁹¹ The τ_r obtained from the anisotropy decay trace is directly proportional to the size of the molecule. At pH 5, the anisotropy decay trace showed a biexponential fit with τ_{r1} value of 150 ps corresponding to the free monomeric species and τ_{r2} value of 2.4 ns corresponding to smaller aggregates. With increasing pH, the decay trace became mono-exponential, and the short component disappeared, and the long component τ_{r2} value increased significantly (Table 3.2). This result clearly indicates that larger restricted assemblies of higher

dimensions are present at higher pH but are absent in the low pH zone and corroborates the results obtained from DLS and FESEM measurements. Fluorescence quantum yield (Φ_f) values were also estimated using coumarin 1 and coumarin 153 in ethanol as standards (Table 3.3). In line with the fluorescence lifetime results, the fluorescence quantum yield value for CSB-2Bn monomer at pH 4 is the lowest ($\Phi_f = 0.01$), which reached the maximum value ($\Phi_f = 0.2$) at pH 6.5 and again decreased ($\Phi_f = 0.04$) by increasing pH to 9. Higher fluorescence quantum yield and a significant increase in fluorescence lifetime at pH 6.5 suggest that the smaller aggregates are more highly fluorescent than free monomeric species at pH 4, as well as higher-order aggregates at pH 9.

Table 3.3. Quantum yields from CSB-2Bn and MA co-assembly in different pH zones

pH	Reference dyes	Φ_f (ref)	Φ_f (CSB-2Bn)
4.0	C-1	0.73 (in EtOH)	0.01
6.5	C-153	0.53 (in EtOH)	0.20
9.0	C-153	0.53 (in EtOH)	0.04

3.2.2. pH-Clock Directed Transient Assembly and Multi-Colour Emission

As the pH dependence of the assembly and consequent changes in the emissive property were established, we checked the broad range of emission colours that the system generates at different pH levels (Figure 3.2C). These visual chromatic transitions are well corroborated with the emission spectra of CSB-2Bn - MA as measured along the pH range. The emission is intense in the basic pH, mainly at the greenish-yellow region at 545 nm to the existence of assembled species. As the pH drops, CSB-2Bn monomers are released freely in the solution. This is reflected in the gradual drop in the 550 nm peak and the simultaneous appearance of the 455 nm peak. The coexistence of the monomer and the assemblies in different proportions at different pH levels results in a broad spectrum of light.

We realized that the pH-dependent assembly and the consequent emission property can be temporally programmed if the system is subjected to a pH clock.^{153, 163} Such a pH-programmed assembly behaviour should also be marked by a dynamic display of multicolour emission. The Tris buffer – GdL pH clock, as optimized by us previously, was chosen for this purpose.^{163, 192} In short, this chemically-driven pH clock is governed by a Tris buffer (activator), which promptly raises the medium pH, and GdL (deactivator), which follows slower base-catalyzed saponification kinetics to gradually lower the pH by releasing gluconic acid. The lifespan of this pH clock is tunable and reproducible as it is adaptive to the variation in the activator–deactivator ratio.¹⁵³ Typically, in a solution adjusted to pH ~ 4.0, when the TRIS buffer (pH 9.0) – GdL (2:5 mM) pH clock was operated,

the pH instantaneously registered a value of 8.75, which steadily dropped to a final value of 4.2 over a span of 90 minutes (Figure 3.4 A, B).

Table 3.4. Time-dependent fluorescence quantum yield of CSB-2Bn - MA (1:5) under the effect of pH clock.

Time (min)	pH	* Φ_f
0	8.9	0.098
3	7.6	0.091
6	7.2	0.091
9	6.9	0.105
13	6.6	0.189
16	6.4	0.213
19	6.2	0.232
22	6.0	0.241
25	5.8	0.197
29	5.5	0.134
34	5.3	0.110
39	5.1	0.081
45	5.0	0.053
55	4.8	0.032
65	4.7	0.021
75	4.6	0.017
90	4.4	0.016

*Fluorescence standards used for the determination of quantum yields

C-153 in EtOH ($\Phi_f = 0.53$) for 0-75 min

C-1 in EtOH ($\Phi_f = 0.73$) for 90 min

The Tris buffer (pH 9.0) – GdL (2:5 mM) pH cycle was operated in the mixture of CSB-2Bn and MA (5: 25 μ M) to create the pH-regulated transient multicolour emission. The solution at pH 4 showed the blue emission corresponding to the monomeric emission at 455 nm (Figure 3.4C). The addition of Tris buffer immediately raised the pH to $\sim$ 9, promoting the self-assembly and, thereby, the yellow emission at 550 nm. The yellow-colored emission persisted as long as the pH remained in the basic region. However, the autonomous acid generation by GdL gradually reduced the pH of the medium and increased the intensity at 550 nm up to pH 6 ($\sim$ 20 min). With a further fall in the pH value, the fluorescence intensity at 550 nm gradually decreased, and the 455 nm band reappeared as the pH dropped below the pK_a of MA. For the quantitative measurement, the fluorescence quantum yield Φ_f at different time points during the pH clock was evaluated using suitable fluorescence standards (Table 3.4). At the beginning of the pH clock, the Φ_f value was $\sim$ 0.1, which increased to 0.24 at pH 6 ($\sim$ 20 min) and then decreased to a value of $\sim$ 0.02 at the end of the pH clock. The time-dependent emission profile was extrapolated to the CIE diagram that shows the path of the cumulative emission

during the pH clock (Figure 3.4D). Interestingly, though not perfectly, the emission path passed near the white light coordinates (0.28, 0.36). The coinciding colour changes showing the transient white light glow have been clearly visualized.²⁵ It is worth mentioning that achieving transient white light emission in a chemically triggered autonomous system is a challenging task, and only a handful have been reported.^{124, 163}

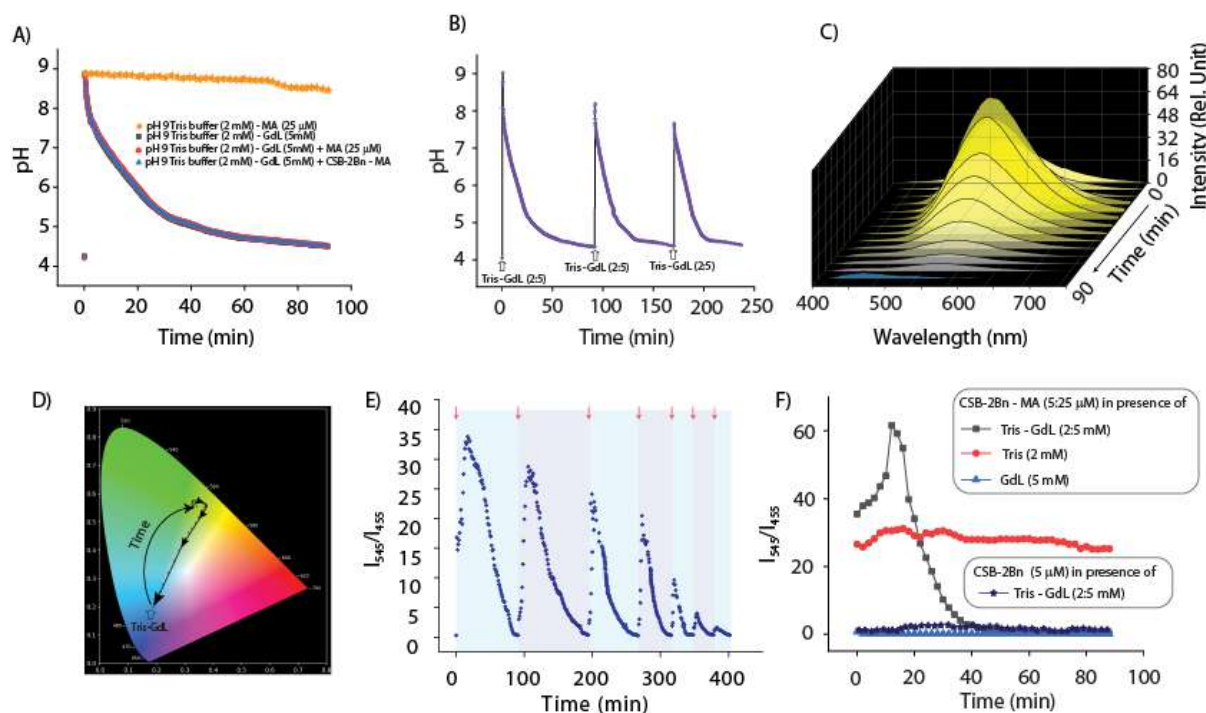


Figure 3.4. A) Change in the pH during the pH clock operation in the presence of MA (25 μ M) or CSB-2Bn (5 μ M) – MA (25 μ M). B) Multiple cycles of pH variations were generated in the pH clock by the addition of TRIS (pH 9.0)-GdL (2:5) in the same solution. C) Time-dependent emission spectra, and D) CIE diagram of CSB-2Bn – MA (5:25 μ M) under the influence of the pH clock initiated by Tris (pH 9.0) – GdL (2:5 mM). E) I_{545}/I_{455} versus time and multiple transitory emissive cycles generated by resupplying of Tris (pH 9.0) – GdL (2:5 mM). F) Time-dependent I_{545}/I_{455} values during various control experiments.

The temporal emissive change is better visualized when illustrated as I_{545}/I_{455} against time (Figure 3.4E). A quick rise in this ratio, followed by a steady, well-spread decline with time, is traced to the pH clock profile. To verify that the emission transition is an outcome of the dynamic pH clock, control experiments were run. In the absence of either the activator or the deactivator, no changes in I_{545}/I_{455} over time (Figure 3.4F) were observed. It proves that the change in fluorescence in an autonomous manner is only possible if the pH variation is programmed with both activator and deactivator and is not static. Another control experiment was performed where the pH clock was run with CSB-2Bn in the absence of MA. No prominent change in the fluorescence intensity was observed (Figure 3.4F). It proves that the system-generated gluconate anions could not interfere with the aggregation, and the presence of a hydrophobic group is essential for the aggregation. Additionally, to check any

feedback from the released MA during the pH clock operation on the system's pH, we monitored the pH of the system while operating the pH clock and also ran a control where the pH clock was operated in the presence of 25 μM MA. As can be seen from Figure 3.4A, the presence of MA (25 μM) or CSB-2BN (5 μM) – MA (25 μM) could not impart any noticeable change in the pH of the system. As the concentration of GdL (5 mM) present in the system is 200 times higher than MA, the protonation/deprotonation of MA and the corresponding change in pH remain insignificant.

Table 3.5. Time-dependent fluorescence lifetimes of CSB-2Bn - MA (1:5) under the effect of pH clock.

Time (min)	pH	τ_1 (ns) [#]	τ_2 (ns)	τ_3 (ns)	τ_{av} (ns)	χ^2
0	8.84	0.05 (2.8%)	2.9 (13.5%)	11.7 (83.7%)	10.2	1.23
3	7.39	0.05 (2.8%)	2.8 (12.2%)	11.8 (85%)	10.4	1.23
6	7.0	0.05 (1.6%)	3.7 (15%)	13.6 (83.4%)	11.9	1.28
11	6.52	0.05 (1.8%)	3.5 (10.9%)	15.9 (87.3%)	14.3	1.25
16	6.18	0.05 (2.5%)	2.8 (7.9%)	17.6 (89.6%)	16	1.21
20	5.85	0.05 (3.6%)	2.8 (7.1%)	17.8 (89.3%)	16.1	1.10
24	5.53	0.05 (4.4%)	2.4 (9.3%)	17.1 (86.3%)	15.5	1.17
28	5.3	0.05 (4.7%)	2.4 (12.0%)	16.1 (83.3%)	13.7	1.25
31	5.18	0.05 (8.5%)	2.1 (14.7%)	15.1 (70.8%)	11.9	1.36
33	5.1	0.05 (13.4%)	2.0 (16.7%)	14.2 (69.9%)	10.3	1.27
34	5.04	0.05 (13.8%)	2.0 (17.8%)	13.7 (68.4%)	9.8	1.26
37	4.97	0.05 (19.6%)	1.7 (17.4%)	13.1 (63%)	8.6	1.35
40	4.92	0.05 (24.7%)	1.6 (17%)	13.0 (58.3%)	8.0	1.39
43	4.85	0.05 (24%)	1.6 (15.2%)	13.2 (60.8%)	8.4	1.34
46	4.81	0.05 (28.2%)	1.7 (14.6%)	13.2 (57.2%)	7.9	1.21
56	4.70	0.05 (36.2%)	2.5 (12.2%)	13.6 (51.6%)	7.5	1.22
67	4.61	0.05 (39.5%)	2.7 (10.2%)	13.9 (50.3%)	7.5	1.38
77	4.54	0.05 (44.5%)	2.9 (9.2%)	13.8 (46.3%)	6.9	1.38
90	4.48	0.05 (44.2%)	3.3 (11.1%)	14.2 (44.7%)	6.9	1.53
120	4.37	0.05 (47.4%)	3.2 (9.3%)	14.1 (43.3%)	6.6	1.97

[#]For the analysis of the decay traces, the short component has been kept fixed at 50 ps (the time resolution of the present TCSPC instrument).

The τ_{av} at 550 nm at several time points during the pH clock varied according to the pH of the solution (Figure 3.5A, B, and Table 3.5). The τ_{av} is about 10 ns at the beginning, which attains a maximum value of 16.1 ns around 20 minutes of the pH clock, while towards the end, a comparatively faster decay (~6.6 ns) is observed, which is consistent with the other observation (change in fluorescence quantum yield) as well.

Reproducibility of temporal variation in properties is an essential attribute for an adaptive system like this.¹⁹³ Multiple pH cycles (Figure 3.4B) and, hence, multiple emissive cycles (Figure 3.4E) have been regenerated by reintroducing the activator-deactivator combination. Nevertheless, the dampening effects of the residual acid and dilution were observed after each run. To investigate the changes, such as the assembly modulated by the pH clock, time-dependent DLS (Figure 3.5C) and

FESEM analyses of freeze-dried samples collected at different time points during the pH clock were performed (Figure 3.5D). Before the addition of the activator, some irregular spherical aggregates arising from MA could be seen. Upon activating the pH cycle, larger, regular hexagonal coin-like arrangements were obtained. As the pH gradually decreased, the morphology slowly changed back to its original irregular spherical form. The time-dependent DLS profile, obtained during the pH cycle, showed a similar trend as the particle size increased drastically (~ 1000 nm) upon adding the Tris buffer–GdL mixture and then gradually decreased to ~ 300 nm over a time of 90 mins.

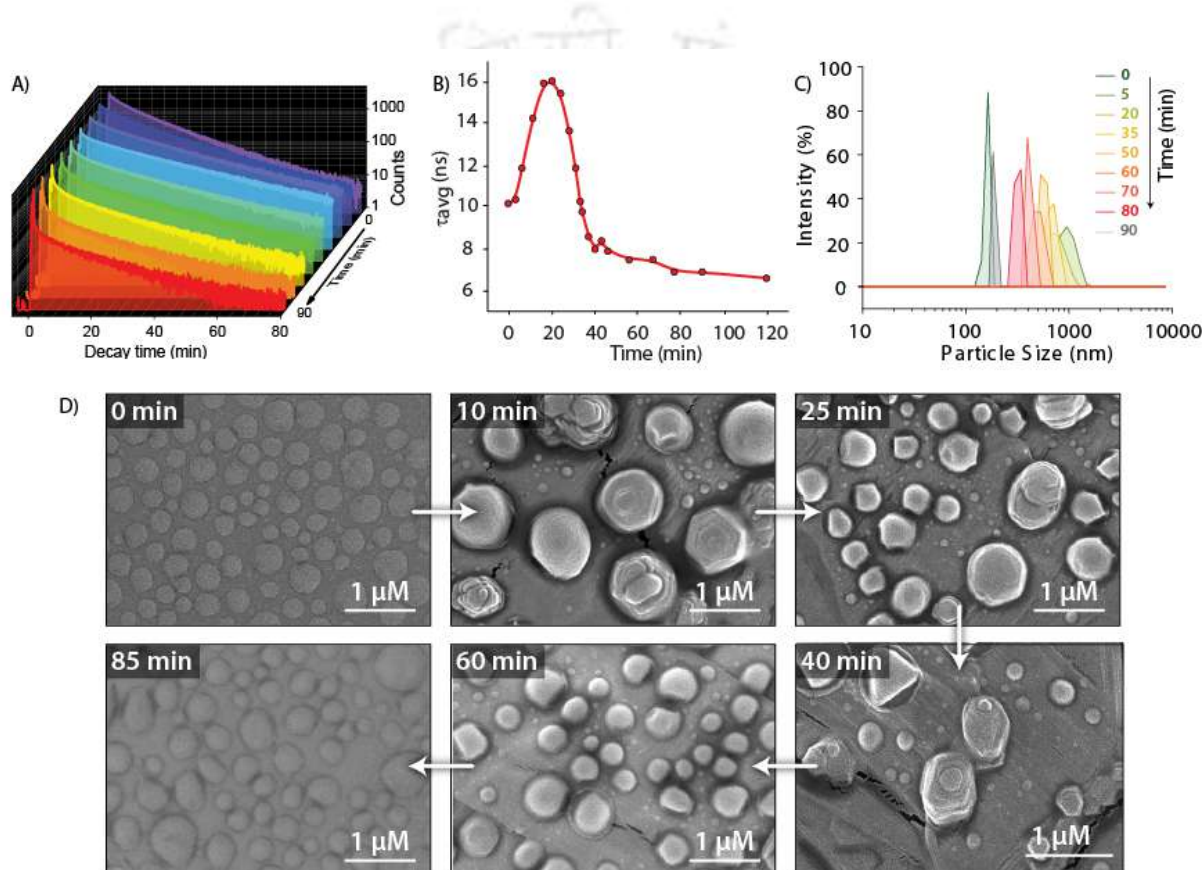


Figure 3.5. A) Change in the decay profiles for emission at 550 nm of CSB-2Bn – MA (5:25 μ M) under the influence of the pH clock. B) Change in average lifetime for the emission at 550 nm of CSB-2Bn – MA at different time points during the pH clock. C) Change in the DLS profiles of CSB-2Bn-MA (5:25 μ M) during the pH clock. D) FESEM images CSB-2Bn – MA (5:25 μ M) at different times during the pH cycle.

3.2.3. Fluorescence Chemical AND Gates

With the in-hand properties of the system, we aimed to utilize it to prepare fluorescence-based smart optical systems. Since the base-induced assembly of CSB-2Bn-MA produced a better fluorescence quantum yield compared to CSB-2Bn alone, we thought the combination of these two could be perceived in a UV-responsive binary or 2-input chemical logic (AND) gate. An AND gate

performs a logical operation and returns a logical true value (1) only if all the entries send in a true value. If any of the entries register a null (0) value, the outcome is also null.

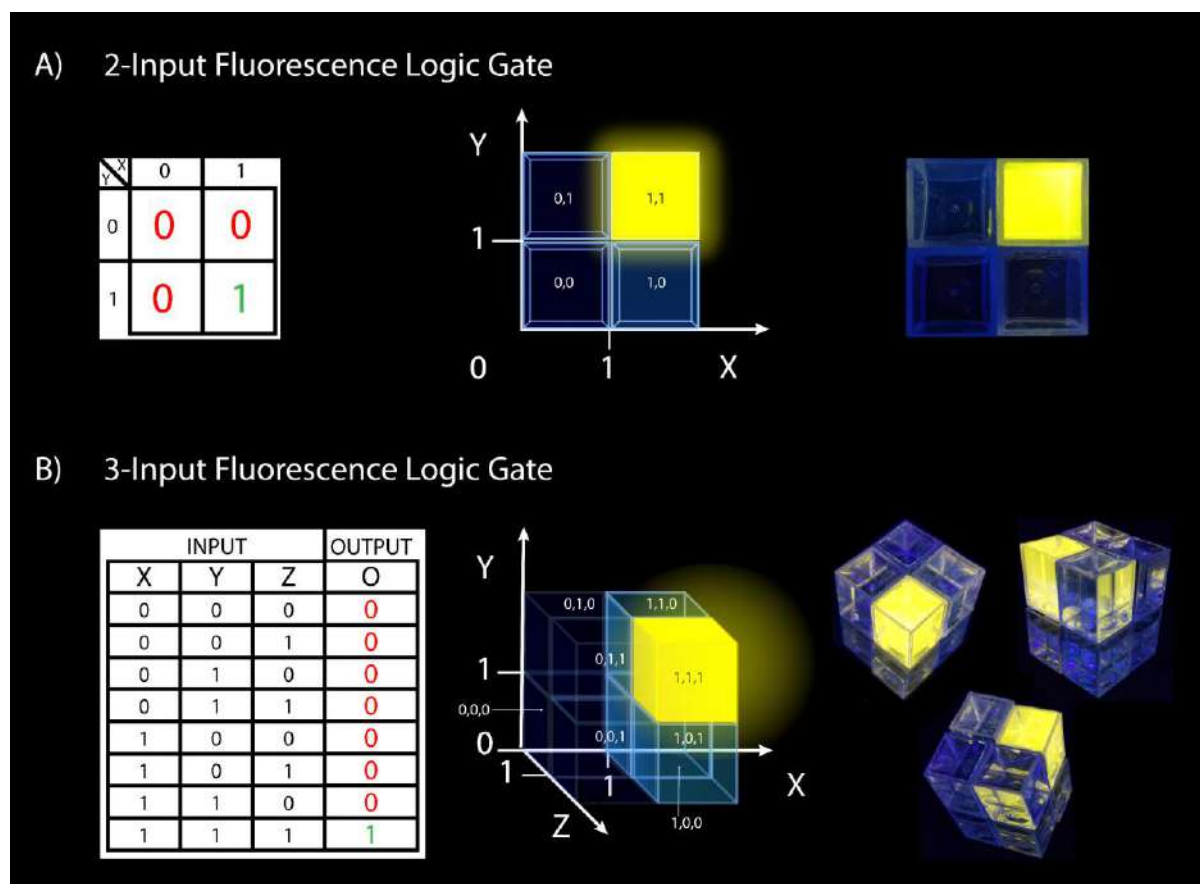


Figure 3.6. Truth table, schematic illustration, and fabricated chemical fluorescence AND gate for A) Binary inputs where X = CSB-2Bn, Y = MA, and pH 9.0 buffer is the solution medium, and, B) Ternary inputs where X = CSB-2Bn, Y = MA, Z = pH 9.0 buffer and HCl solution is the base medium.

For our fabrication, we considered that the yellow emission of high intensity ($>10^6$) from the assembly is the true value 1 for the output in the AND gate. Any other fluorescence of lower intensity ($<10^6$) or no fluorescence accounts for 0. For constructing the UV-responsive chemical AND gate, the two inputs in our case are CSB-2Bn and MA in a medium of pH 9.0 Tris buffer. Only when both the inputs are physically present (1) in the cuvette having a basic buffer solution, an intense yellow glow (1) is seen under the UV light. The absence of both or any one shows no or low fluorescence (0) under UV light (Figure 3.6A). Again, an AND gate becomes even more complex with three variables as inputs.¹⁹⁴ To realize that, we required another chemical input to go along with the two assembling molecules. A solution with a pH of less than 4 was used as the medium to fabricate such a ternary UV-light responsive chemical AND gate. Hence, now, in addition to the CSB-2Bn and MA, a basic buffer solution of pH 9.0 Tris buffer serves as the third input variable. In this situation, even if both the molecular entities are present to assemble, they would unlikely show strong yellow fluorescence,

inhibited by the acidic nature of the solution. The addition of a basic buffer that can overpower the acid content and deprotonate the MA is essential for the bright yellow glow. Only then can the chemical combination return the logical true value (Figure 3.6B). In a 3D representation, the space having coordinates (1,1,1) glows to return a logical value. Thus, by utilizing the pH-responsive property of the system, a simple and complex fluorescence-based AND gate was assembled that could be observed only under UV light.

3.2.4. On-Paper Message Encryption

Information storage, access, and delivery on paper is still the most trivial and primitive manner of data handling, yet it is significantly noteworthy. Though the protection of data on paper is highly unreliable, the world still runs on it because of its ease of handling and accessibility. Consequently, data encryption and on-demand stimuli-guided information decryption on paper are thoroughly sought after.¹⁷⁸ We wanted to develop an encrypted message on paper that is authentically revealed only under an externally applied condition, like the UV light. The CSB-2Bn molecule appears as a bright lemon-yellow colour solid to the naked eye under normal light. With the intention of using it as a hidden ink, we made two solutions in MeOH of a high and low concentration separately. Both were employed together as writing inks on cellulose paper (Figure 3.7). After quick evaporation of volatile MeOH, the message written with the concentrated ink was clearly visible to the naked eye as yellow letters (DO IT) from solid aggregated CSB-2Bn. It was only under the UV light that the complete true message (DON'T) was disclosed. The dilute solution lacked the necessary concentration to express the yellow-colored letters. Under normal light, it displayed an incomplete message only from the concentrated ink. Nonetheless, when held under UV light, fluorescence comes into play. Both the yellow emission from the aggregates of high concentration and the blue emission from the monomers of the dilute ink unfold the complete hidden message. Accordingly, by applying the solid-state absorbance and the concentration-dependent fluorescence nature of the fluorophore molecules, we were able to describe an on-paper message encryption method having a condition-specific (UV-light) decryptable set-up.

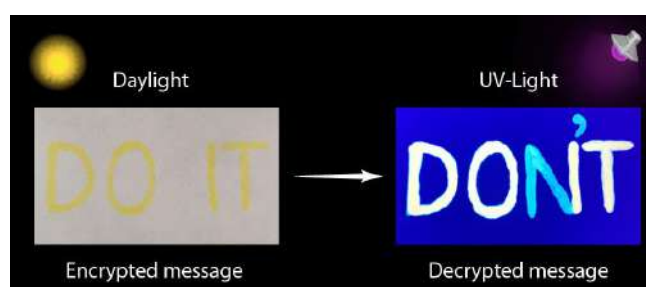


Figure 3.7. On-paper encrypted message with UV-light specific decryption.

3.2.5. Time-Encoded Multi-Dimensional Orthogonal Information Encryption

The pH-clock-regulated transient emission motivated us to develop a time-encoded message encryption system. A simple physical arrangement of seven cuvettes containing solutions of CSB-2Bn and MA in the pattern of '8' conveyed the message of the same digital number 8 under normal light. But when displayed under the stimulus of the UV light, the yellow fluorescence conveyed a separate hidden message of the digital number "3" (Figure 3.8A). The message herein appeared only under special conditions (UV light) from selected cuvettes that were pre-charged with the Tris-GdL combination. Since the message was an outcome of chemically driven assemblies under a pH clock, the transient message vanishes over time or, if attempted, cannot be read in daylight. Thus, we have successfully demonstrated a time-encoded message encryption where the true programmed message is both specifically decrypted under UV light and short-lived. The decryption here is both temporally restricted and externally condition-specific, adding to its level of security. Moreover, the time window valid for decryption can easily be modulated by altering the ratio of the activator/deactivator.

3D colour codes have drawn considerable scientific and industrial attention because of their safer information storage capacity than conventional scanning codes.^{130, 139, 143, 195} They belong to an essential class of scanning codes where different spatial three-dimensional arrangements of colours convey different messages once scanned. However, the construction of transformable codes that can be modulated as per requirement is still under scrutiny. Since our assembly-induced emission can generate transience in a broad spectrum of colours wherein yellow, blue, and white could be isolated, affixed, or dynamized, a multi-dimensional colour-coded encryption task could be performed. First, a colour pattern/QR code on the paper was laid on a black background as the 2D information. Then, 16 small cuvettes containing 1 ml of dilute HCl solution were arranged as a 4 x 4 perfect square for the 3D colour-coded information. To each one, CSB-2Bn (10 μ M) and MA (25 μ M) were added. For the ones where yellow fluorescence is desired, pH 9.0 Tris buffer was added, and for the ones where white was desired, pH 5.5 PB was added. To bring in the 4D temporal programmability, GdL was added accordingly to selected cuvettes. Filling different cuvettes with the assembling molecules in a dilute HCl medium and adding buffers of different pH as triggers can produce yellow or white light accordingly. The absence of buffers yields a blue colour in an acid medium. A particular spatial placement of different cuvettes corresponds to a particular code or message that can only be decoded when scanned under UV light. Though physical rearrangement can transform the hidden codes, we aimed to chemically program the code in a manner that shows an automated time-locked transformation with the transient pH clock.

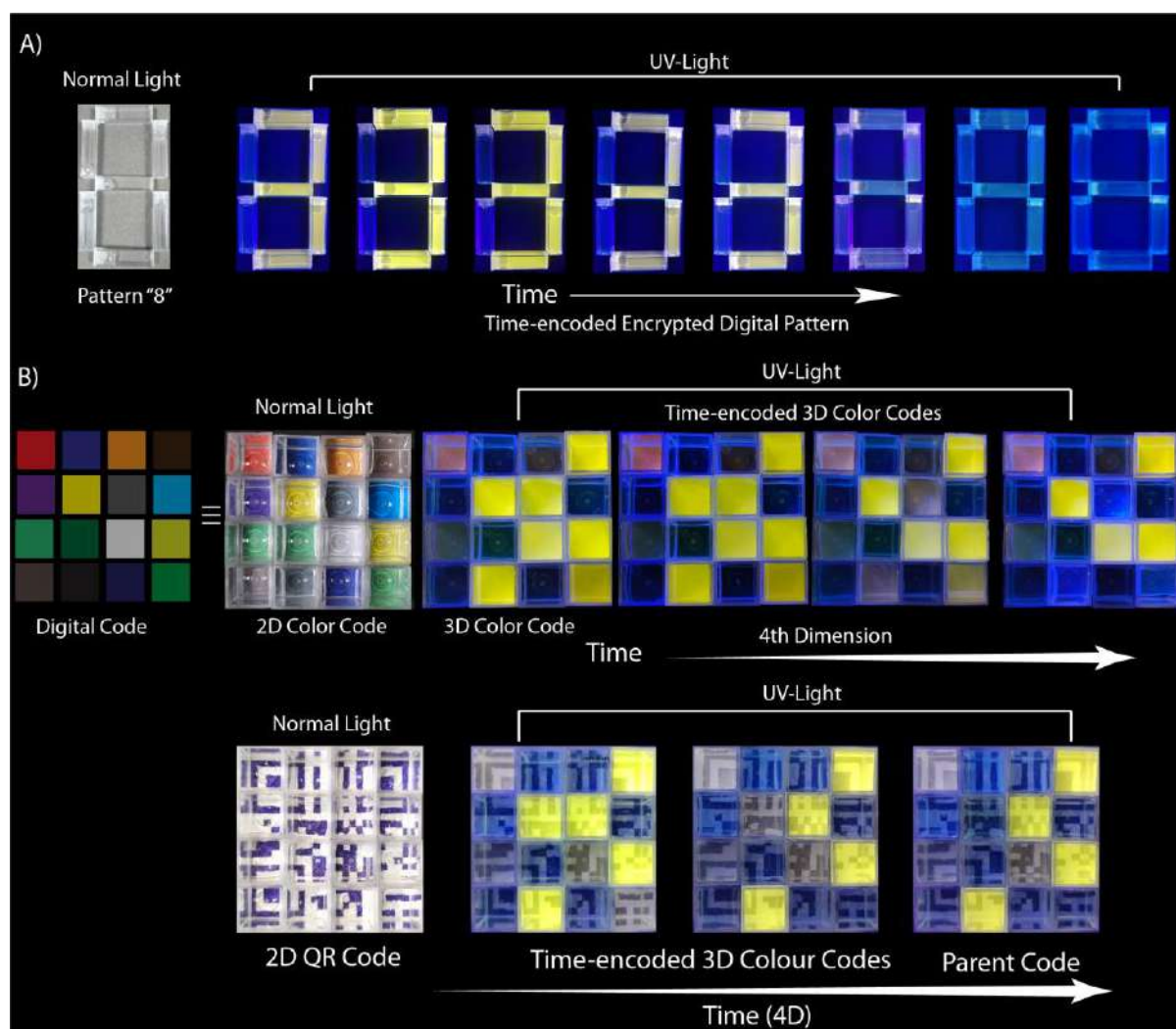


Figure 3.8. Anticounterfeiting displays generated by CSB-2Bn – MA (5:25 μM) under the influence of the pH clock. A) UV-light decryption of a chemically driven time-locked encrypted pattern. B) Time-encoded multi-dimensional colour-coded anti-counterfeiting encryption.

When the cuvettes containing the molecules were spatially organized on a colour pattern or normal QR code and observed in daylight, the solutions appeared transparent (Figure 3.8B). The 2D code present in the background could be deciphered in daylight. However, in the dark, when exposed to UV light, the original 2D code disappeared, and the new 3D code (originating from the emission) appeared. Thus, by application of a specific stimulus 2D code could be transformed into a 3D code, i.e., the dimensional level of security could be exchanged by alternating the two stimuli. Moreover, when the information from the 2D code is visible, the 3D code is not and vice versa. This demonstrates an orthogonal relation and stimulus-specific decryption, which elevates the degree of security. Furthermore, the pH clock endows the encryption system with time-locked properties when the system is under dynamic conditions. Thus, the codes that are generated are time-encoded ones and are ephemeral, building on the security levels. These codes run under non-equilibrium

conditions and transform with time automatically until they reach their final equilibrium stage (final or parent code). This, in turn, can be seen as an additional dimension. So, in this dimension, multiple codes can be inferred along the time scale, and the correct one can be identified only at the right time. Even the time dimension is also orthogonal and independent of the other two dimensions, as the required stimulus to operate it is a chemical trigger. Here, each stimulus is not only specific and orthogonal but also builds up the hierarchy of the safety levels of the designed system. Thus, to sum up, multi-dimensional colour-coded data encryption has been realized that can both be time-programmed and orthogonally switched between multiple hierarchical levels by the exchange of stimuli.

3.3. Conclusions

To summarize, by combining MA and CSB-2Bn, we have created a pH-responsive AIEgen system. Both the assemblies and fluorescence could be repetitively altered autonomously with the help of a chemically triggered pH clock. Running the pH cycle produced a multi-colour spectrum encompassing a broad range from yellow to blue and transiently emitting white light. Utilizing these dynamic properties, multi-input fluorescence AND gates were developed. The property was even extended into making message encryption systems with anti-counterfeiting attributes both on paper and in solution, which had orthogonal, time-locked, and stimuli-specific hierarchical features. The newly developed anticounterfeiting system introduces a new dimension of security in the form of time. Overall, our newly developed strategy offers hierarchical anti-counterfeiting capabilities by integrating pH-switchable fluorescence, pH clock modulation, and time-gated encryption, thereby advancing the field towards more secure and dynamic anti-counterfeiting solutions.

3.4. Experimental

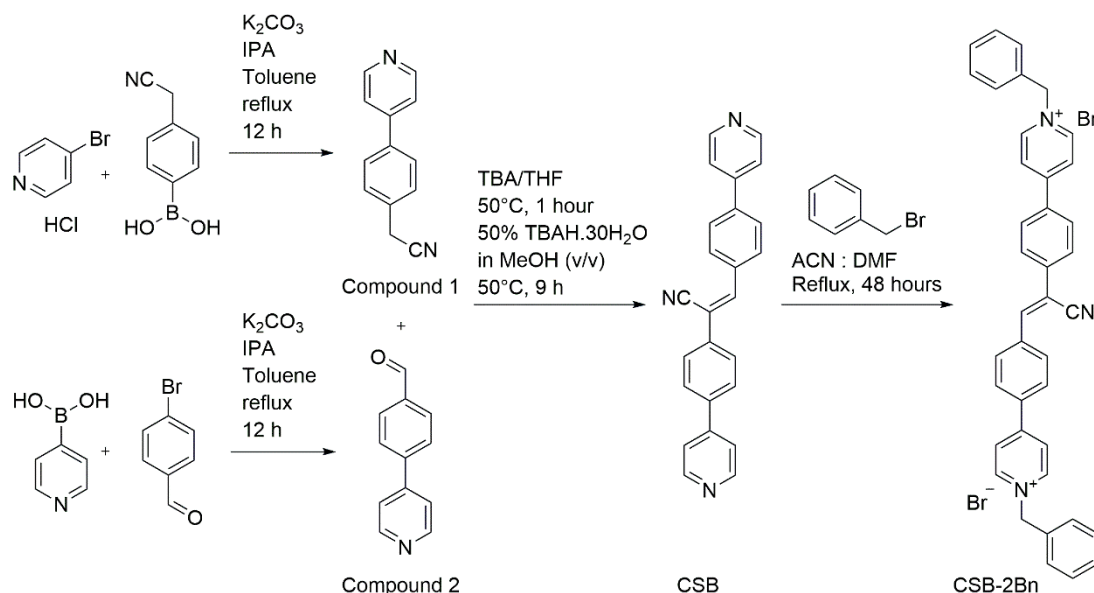
3.4.1. General Information and Materials

All fine chemicals were purchased from TCI, India, or Sigma-Aldrich, India. Solvents and other common chemicals were purchased from SRL, India, or Spectrochem, India. Milli-Q water with a conductivity of $< 2 \mu\text{S cm}^{-1}$ was used for all sample preparations and experimental measurements. ^1H NMR and ^{13}C NMR spectra were recorded using a Bruker Ascend 600 MHz (Bruker, Coventry, UK) spectrometer. UV-vis spectra were recorded on a PerkinElmer Lambda 750 spectrometer, while fluorescence measurements were performed on a Fluoromax 4 Plus spectrophotometer. pH data were recorded on a Hanna HI 2210 pH meter equipped with a HI1131 pH probe from Hanna. FESEM imaging was performed on a Gemini SEM 300 (Sigma Zeiss) instrument. The particle size analyses were performed at 298 K using Zetasizer Nano-ZS90 (Malvern). A He-Ne LASER ($\lambda=632.8$ nm) was used as an excitation source for DLS measurements. The time-resolved fluorescence measurements

were recorded using a time-correlated single photon counting (TCSPC) set-up from Horiba Scientific (UK). The chromaticity diagram and CIE coordinate points were plotted with SpectraChroma.

3.4.2. Synthesis

CSB-2Bn was synthesized following Scheme 3.2.



Scheme 3.2. Synthetic scheme for the preparation of CSB-2Bn.

Synthesis of Compound 1

4-(cyanomethyl)phenyl)boronic acid (250 mg, 1.55 mmol, 1 equiv.), 4-bromopyridine hydrochloride (330 mg, 1.7 mmol, 1.1 equiv.), K_2CO_3 (850 mg, 6.2 mmol, 4 equiv.) and tetrakis(triphenylphosphine)palladium(0) (30 mg, 0.025 mmol, 0.016 equiv) were measured in a clean, dry 100 mL two-necked round-bottom flask equipped with a magnetic bead. It was sealed, vacuumed and flushed with argon. To it, 10 mL Toluene, 5 mL isopropanol and 5 mL deionized water was added. The mixture was stirred for 30 min under an argon atmosphere and then refluxed for 12 hours. The reaction mixture was concentrated under vacuum, and the water fraction was diluted and extracted thrice with equal volumes of ethyl acetate. The combined organic layer was dried over sodium sulphate and evaporated under vacuum. The crude product was purified by column chromatography with EtOAc:Hexane (40%) to yield a white powder. Yield ~ 66%. 1H NMR (600 MHz, Chloroform-*d*): δ 8.67 (d, J = 5.4 Hz, 2H), 7.66 (d, J = 7.9 Hz, 2H), 7.49 (d, J = 5.5 Hz, 2H), 7.46 (d, J = 7.9 Hz, 2H), 3.82 (s, 2H); ^{13}C NMR (150 MHz, Chloroform-*d*) δ 150.48, 150.46, 147.50, 138.22, 130.97, 128.87, 127.89, 121.68, 117.62, 23.54; MS (ESI): m/z calculated for $C_{13}H_{10}N_2$: 194.08; found for $[C_{13}H_{10}N_2]^+$: 195.09.

Synthesis of Compound 2

Compound 2 was synthesized and purified following the same protocol and measurements as those for compound 1. Here, 4-bromobenzaldehyde (250 mg, 1 equiv.) and 4-pyridylboronic acid (1.1 equiv.) were reacted with each other. The proportions of K_2CO_3 and tetrakis(triphenylphosphine)palladium(0) were maintained accordingly. The crude product was purified by column chromatography with EtOAc:Hexane (40%) to yield a white powder. Yield ~ 61%. 1H NMR (600 MHz, Chloroform-*d*) δ 10.09 (s, 1H), 8.74 – 8.71 (m, 2H), 8.01 (d, J = 8.3 Hz, 2H), 7.80 (d, J = 8.3 Hz, 2H), 7.56 – 7.53 (m, 2H); ^{13}C NMR (150 MHz, Chloroform-*d*) δ 191.79, 150.65, 147.10, 144.13, 136.62, 130.58, 127.89, 121.92, 77.37, 77.16, 76.95; MS (ESI): m/z calculated for $C_{12}H_9NO$: 183.07; found for $[C_{12}H_{10}NO]^+$: 184.07.

Synthesis of CSB

Compound 1 (250 mg, 1.25 mmols, 1 equiv.) and compound 2 (230 mg, 1.25 mmols, 1 equiv.) were measured in a 25 mL round-bottom flask and dissolved in 4 mL tert-butanol and 0.5 mL tetrahydrofuran mixture. The reaction mixture was stirred for 1 hour at 50 °C. To it, 0.7 mL 50% TBAH.30H₂O in methanol (V/V) was then added dropwise over a period of 15 min at 50 °C until the colour of the reaction mixture reached a maroon colour after showing light yellow, orange and then red colour. The stirring was continued for 9 more hours at 50 °C. The reaction mixture was cooled to room temperature. The yellowish white precipitate formed was then filtered and washed with tert-butanol multiple times and finally dried under vacuum. Yield ~ 48%. 1H NMR (600 MHz, Chloroform-*d*) δ 8.72 – 8.69 (m, 4H), 8.05 (d, J = 8.2 Hz, 2H), 7.84 (d, J = 8.4 Hz, 2H), 7.76 (dd, J = 13.5, 8.2 Hz, 4H), 7.66 (s, 1H), 7.56 – 7.54 (m, 4H); ^{13}C NMR (150 MHz, Chloroform-*d*) δ 150.62, 150.59, 147.14, 141.67, 140.34, 139.24, 135.05, 134.32, 130.27, 127.85, 127.71, 126.91, 121.61, 121.59, 117.80, 111.90; MS (ESI): m/z calculated for $C_{25}H_{17}N_3$: 359.14; found for $[C_{25}H_{18}N_3]^+$: 360.13.

Synthesis of CSB-2Bn

CSB (50 mg, 0.139 mmol, 1 equiv.) was measured in a 10 mL sealed tube equipped with a magnetic bead. To it, benzyl bromide (330 μ L, 20 equiv.) and 4 mL of dry ACN: DMF (4:1 v/v) were added, and the solution was refluxed in the sealed tube for 48 hours at 90 °C. The reaction mixture was cooled, and a bright yellow precipitate and a turbid solution were obtained. The solution, along with the precipitate, was poured into an excess of cold, dry diethyl ether. The precipitate was collected by centrifugation and then purified by washing multiple times with Et₂O. The pure compound obtained was dried under vacuum. Yield ~ 72%. 1H NMR (600 MHz, DMSO-*d*₆) δ 9.31 (t, J = 7.3 Hz, 4H), 8.63 (d, J = 6.6 Hz, 4H), 8.44 (s, 1H), 8.28 (dd, J = 12.3, 8.4 Hz, 4H), 8.22 (d, J = 8.4 Hz, 2H), 8.08 (d, J = 8.5 Hz, 2H), 7.60 (dd, J = 7.5, 2.6 Hz, 4H), 7.46 (dt, J = 12.8, 6.9 Hz, 6H); ^{13}C NMR (150 MHz, DMSO-*d*₆) δ 153.84, 153.81, 144.96, 144.92, 143.32, 136.80, 135.30, 134.52, 134.48, 134.28, 130.34, 129.37, 129.35, 129.26,

129.15, 128.88, 128.79, 128.75, 127.07, 125.15, 125.06, 117.31, 111.29, 62.56, 62.49; MS (ESI): m/z calculated for $C_{39}H_{31}N_3^{2+}$: 270.62; found: 270.61.

3.4.3. Methods and Instrumentations

3.4.3.1. Execution of pH Clock

In a 3 mL glass vial, 2975 μ L of water was taken and acidified to pH 4.0 with 1 mM HCl solution. To it, the required volume of stock solutions of CSB-2Bn and MA was added to achieve concentrations of 5 and 25 μ M, respectively. To start the pH clock, required volumes of stock solutions of pH 9.0 Tris buffer and GdL were added simultaneously. The contents were quickly homogenized with a micropipette and analyzed by various techniques as required.

3.4.3.2. Fluorescence Measurements

All fluorescence experiments were performed in a 3 mL quartz cuvette (path length 1 cm) with a slit width of (Ex./Em.) = 2/2 nm under λ_{ex} = 365 nm at 298 K. For, any time-independent measurements, required volumes of CSB-2Bn and MA were added in the solution in the cuvette mixed thoroughly, equilibrated for 2 minutes and then recorded. In a typical time-dependent fluorescence experiment, all the contents were added following the same proportion and sequence as that in the pH clock. The contents were quickly homogenized with a micropipette, and then the emission spectra were recorded at intervals of every minute over 1.5 hours. The final concentration of CSB-2Bn and MA was 5 μ M and 25 μ M, respectively.

Fluorescence quantum yields (Φ_f) of the present systems were estimated following a comparative method where C-1 (Φ_f = 0.73) and C-153 (Φ_f = 0.53) in ethanol were used as the reference. From the experimentally measured fluorescence intensities, quantum yields at three different pH levels were determined.

$$\Phi_x = \Phi_{ref} \times \frac{A_{ref}}{A_x} \times \frac{I_x}{I_{ref}} \times \frac{\eta_x^2}{\eta_{ref}^2} \quad (3)$$

Where, Φ_x and Φ_{ref} are quantum yields of the sample and reference, A_x and A_{ref} are the absorbance of the sample and reference at the excitation wavelength, I_x and I_{ref} are the integrated fluorescence intensity (area under the curve) of the sample and reference, and η_x and η_{ref} are the refractive index of the sample and reference solution, respectively.

3.4.3.3. Time-Resolved Photoluminescence and Quantum Yield Measurements

All measurements were carried out in a 3 mL quartz cuvette with a path length of 1 cm. For all the experiments, CSB-2Bn and MA concentrations were kept at 1.67 μ M and 25 μ M, respectively. For studies under static pH buffers were used, while dynamic studies were conducted under the pH

clock as optimized previously. Herein, a 374 nm (~100 ps, 1 MHz repetition rate) diode laser was used for excitation

A reconvolution procedure was used to analyze the observed decays,^{196, 197} which could be satisfactorily fitted by tri-exponential decay functions. A deconvolution procedure was used to analyze decays using a proper instrument response function obtained by using a light scatterer (suspension of TiO₂ particles in water). The fluorescence decays $I(t)$ were analyzed in general as a sum of exponentials,^{196, 197}

$$I(t) = \sum B_i \exp\left(-\frac{t}{\tau_i}\right) \quad (1)$$

where, B_i and τ_i are the pre-exponential factor and fluorescence lifetime for the i th component, respectively. Reduced chi-square (χ^2) values and random distribution of the weighted residuals among data channels were used to judge the acceptance of the fits.

For anisotropy measurements, samples were excited with a vertically polarized excitation beam and the vertically and horizontally polarized fluorescence decays were collected with a large spectral bandwidth of ~32 nm. Using these polarized fluorescence decays, the anisotropy decay function, $r(t)$, was constructed as follows,^{196, 197}

$$r(t) = \frac{I_V(t) - GI_H(t)}{I_V(t) + 2GI_H(t)} \quad (2)$$

$I_V(t)$ and $I_H(t)$ are the vertically and horizontally polarized decays, respectively, and G is the correction factor for the polarization bias of the detection setup. The G factor was determined independently by using a horizontally polarized excitation beam and measuring the two perpendicularly polarized fluorescence decays.

3.4.3.4. FESEM Analyses

For the time-dependent FESEM experiments, 5 μ L of the sample solution under pH clock were taken out at specific times and drop-cast on a silicon wafer. The samples were immediately freeze-dried to arrest the pH clock kinetics and then analyzed. For samples under static pH conditions, 5 μ L of the sample solution prepared in buffer was cast on a silicon wafer, dried, and analyzed for morphologies.

3.4.3.5. DLS Analyses

The particle sizes were recorded in a 3 mL DLS cuvette. For measurements under static pH conditions, the buffer solution of the desired pH was taken, followed by the addition of required volumes of CSB-2Bn and MA stock solutions. The solutions were thoroughly mixed and equilibrated for 2 minutes before measurements. For a typical time-dependent dynamic experiment, all the constituents were added following the same proportion and sequence as in the pH clock. The

contents were quickly homogenized with a micropipette, and then the particle sizes were monitored at regular intervals over 1.5 hours.

3.4.3.6. Fabrication of Chemical Logic Gates

4 small cuboid-shaped cuvettes ($l = b = 1$ cm; $h = 1.5$ cm) with one side open were arranged in a square on a black chart paper for the binary logic gate. They were filled with 1 ml of pH 9.0 (Tris 10 mM) buffer. To it required volumes from stock solutions of CSB-2Bn and/or MA were added to make their final concentration 5 μ M and 25 μ M, respectively. A three-dimensional cubical arrangement was made with 8 such cuvettes for the ternary AND gate. A set of 4 in a square arrangement with another similar set of 4 on top. Each of them had 1 ml of 1 mM HCl solution. To it required volumes from stock solutions of pH 9.0 Tris buffer (1M), CSB-2Bn and/or MA were added to make their final concentration 10 mM, 5 μ M and 25 μ M, respectively. They were visualized and photographed under a 365 nm UV light.

3.4.3.7. On-Paper Encrypted Ink

1 mM and 5 mM solutions of CSB-2Bn in MeOH were used as inks on cellulose papers without any optical brighteners. MeOH was selected as the molecule is well soluble in it and because of its high volatility. They were applied with a regular brush, allowed to dry and visualized under normal light and under 365 nm UV light for decryption.

3.4.3.8. Time-Encoded Encryption

For developing the encrypted message in pattern 8, seven cuvettes (3 ml) were filled with solutions of dilute HCl. To each to them required volumes of CSB-2Bn and MA stock solutions were added to make their final solutions 5 μ M and 25 μ M, respectively. Out of them, in 5 cuvettes, Tris-GdL (2:5 mM) were added. These 5 cuvettes were arranged in the form of "3" on a black paper background. The remaining 2 were kept to complete the pattern 8. The arrangement was visualized under normal and UV light.

For the multi-dimensional colour-coded arrangement, at first, a colour pattern/QR code on the cellulose paper was laid on a black background as the 2D information. Then, 16 small cuvettes containing 1 ml of dilute HCl solution were arranged as a 4 x 4 perfect square for the 3D colour-coded information. To each one, CSB-2Bn (10 μ M) and MA (25 μ M) were added. For the ones where yellow fluorescence is desired, pH 9.0 Tris buffer was added, and for the ones where white was desired, pH 5.5 PB was added. To bring in the 4D temporal programmability, GdL was added accordingly to selected cuvettes. The arrangement was photographed in normal light to visualize the 2D information. For the 3D and 4D details, the system was photographed at regular time intervals under the UV light over time.

3.4.3.9. Preparation of Stock Solutions

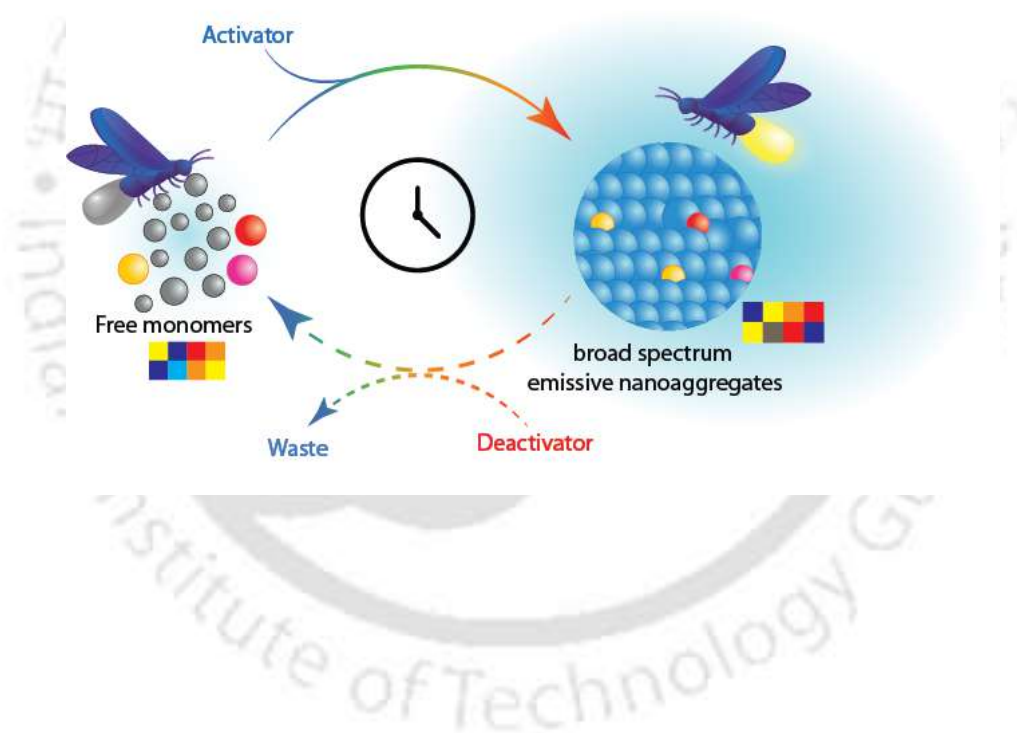
The stock solutions of different components were prepared according to the table given below and stored at 4 °C. CSB-2Bn (5 mM), MA (25 mM) and GdL (2 M) stock solutions were prepared in DMSO. 1M TRIS/HCl buffer (pH 9.0) was prepared.





Chapter 4

Bioinspired Chemoenzymatically Regulated Broad-Spectrum Transitory Emissions in Artificial Light-Harvesting Nanoaggregates





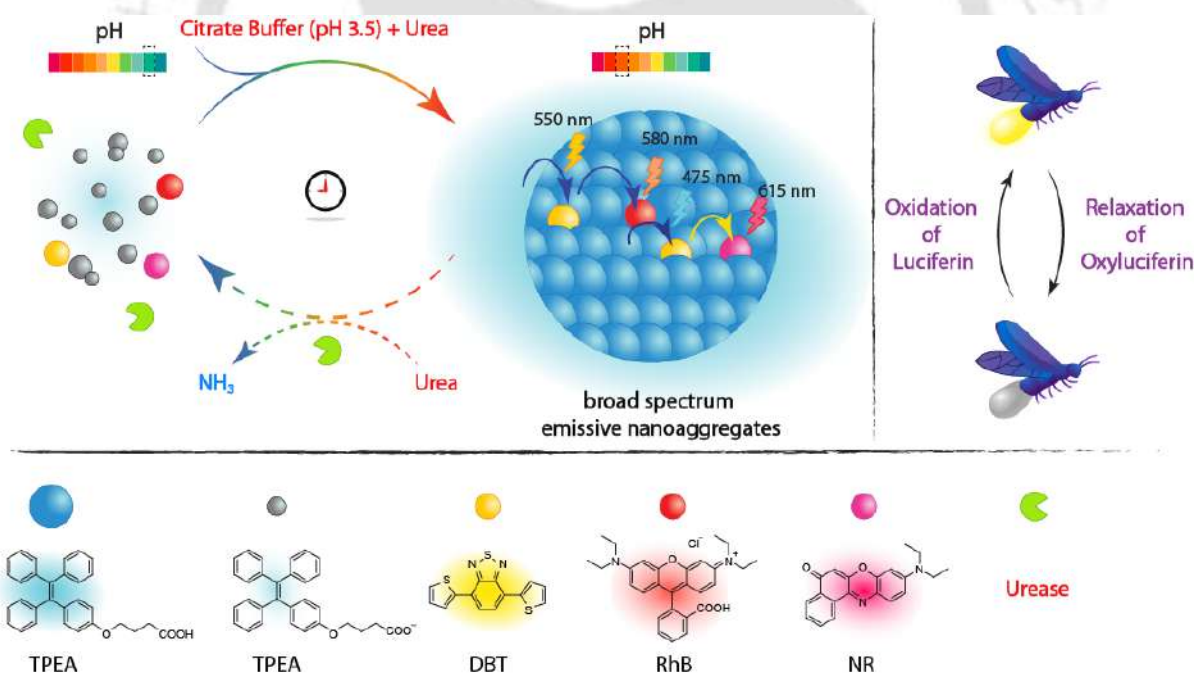
4.1. Introduction

Transient lights are ubiquitous. They encompass a wide range of natural phenomena – extending from the enormous scale of the captivating Northern Lights to the minuscule level of glowing fireflies. Physical events, such as the Northern Lights, are an outcome of interactions between charged particles from solar winds and the Earth's magnetic field.¹⁹⁸ On scaling it down, bioluminescence in organisms also illustrates transient lights at a more compact level.¹⁹⁹ The visual display is coordinated by an intricate network of chemiluminescent reactions that occur at the molecular level.²⁰⁰ Whatever the motive, transitory luminescent displays that are created instantaneously, exist momentarily and fade away lie at the heart of bioluminescence. Although these lights are momentary, they often exhibit psychedelic colours that smoothly glide from one shade to another, over a short period before completely disappearing into darkness.¹⁹⁹

In chemical processes, reaction dynamics often generate electronically excited products, releasing energy in the form of chemiluminescence. Enzymatic reactions fundamentally regulate chemiluminescence inside living organisms (bioluminescence).²⁰¹ For example, in fireflies, reactions involving luciferin-luciferase are principally responsible for the fleeting lights.²⁰² Besides the common green glow, a wide spectrum covering yellow, orange, red or even blue has been noticed.²⁰² The diversity in colours can be attributed to several factors, such as temperature, pH, or even species-specific enzymes, which affect the chemical dynamics.²⁰³⁻²⁰⁵ All these independent factors operate individually or in unison to produce multicoloured bioluminescence. In firefly, luciferase produces oxyluciferin in its excited state.²⁰⁶ The product dissipates the energy as photons. The unique wavelength emitted not only relies on the pH but also on interactions of oxyluciferin with the enzymatic microenvironment.²⁰²

A substantial amount of work has been done to imitate the luciferin-luciferase chemistry.²⁰⁷⁻²¹² Firefly-inspired smart luminescent materials have been designed through multi-stimuli-adaptive emissions in AIEgens, as well as in various systems, including hydrogels, nanoclusters, films, ultrastructures, photonic structures, and light-emitting diodes.^{139, 145, 213-226} Besides focusing on mimicking transient lights, these reports have also illustrated their extensive applications in sensing, optical materials, anti-counterfeiting, photocatalytic reactions, LHS, and device fabrication.^{223, 225, 227-233} Despite these efforts, there is a considerable lack of work that truly focuses on the enzymatic control of emission, a feature crucial to life-like systems in abiotic conditions.^{8, 234} Moreover, most of these studies rely on complex synthetic routes or an extra vessel that can trap AIEgens in proximity or restriction to show augmented fluorescence. A simplistic design of molecules that can respond to stimuli without the need for an additional vessel for fluorescence switch-on/off is desirable.²²¹

In our effort to mimic this pH- and enzyme-regulated transient emission, we have designed bioinspired, enzyme-controlled, broad-spectrum artificial light-harvesting nanoaggregates that exhibit pH-responsive, short-lived emissions. Our objective was successfully achieved by implementing a pH-responsive derivative of TPE, a well-established AIEgen.²³⁵⁻²³⁸ When TPE is aggregated, restrictions in intramolecular rotations make the molecule highly emissive.²³⁵ It is widely employed in constructing advanced emissive materials because it directly addresses the problems concerned with ACQ, as witnessed in conventional organic fluorophores.²³⁹ Highly modulable emissive properties and good quantum yields also add to the advantage of TPE-based smart luminescent materials.^{200, 229, 236-238} TPE is especially coveted while constructing artificial LHS because most of its derivatives emit in the blue-cyan range.²⁴⁰ It can act as a primary source, emitting at the end of the spectrum, so that the final energy can be transmitted to the other end through a multi-step energy transfer.^{170, 241, 242} However, one has to deal with the substantial hydrophobicity and the cumbersome multistep synthesis associated with molecular design before working with any TPE derivative in aqueous solutions. In this respect, we have employed a minimalist synthetic strategy in our simple molecular design and attempted to utilize the hydrophobicity to our advantage.



Scheme 4.1. Schematic illustration of bioinspired pH-clock guided emissive nanoaggregates with multi-colour transient emission

In what follows, we have developed a carboxylic acid functionalized TPE derivative (TPEA, Scheme 4.1). TPEA is a pH-responsive molecule that self-assembles into blue-emitting nanoaggregates under acidic pH conditions.^{221, 243-245} At higher pH, the assemblies disintegrate, resulting in

diminished emission. By trapping multiple FRET acceptors (RhB, DBT, NR, etc.) in the hydrophobic microenvironment of the pH-reversible nanoaggregates, we have established an efficient set of multistep artificial LHS that emit at longer wavelengths.²⁴⁰ Furthermore, we have pushed the nanoaggregates into non-equilibrium conditions using a chemoenzymatic pH clock.^{9, 85, 246-249} The time-programmable pH cycle produced wide-spectrum emissive assemblies that have been extended to constructing advanced information encryption strategies with anti-counterfeiting properties.²⁵⁰ Our demonstration focuses on coding techniques that are already popular, widely accepted and employed in real-life threat protections, which are often missing in several fluorescence-based encryption reports. Coding languages that are already favoured in public utility sectors need upgrades. Herein, they have been elevated in security standards using an additional protection of time-scale with temporally regulated transient colours.

4.2. Results and Discussions

4.2.1. pH-Responsive AIE

Our first step was to obtain a simple pH-responsive TPE derivative, avoiding complicated synthesis. To accomplish that, we employed a minimalistic design strategy by simply appending a single short aliphatic carboxylic acid chain to a hydroxy TPE derivative (TPEA, Scheme 4.2). The single functionalization was preferred to evade the complications often associated with multiple ionisations. We studied the aggregation pattern of TPEA at pH 3 and 9. The CAC of TPEA was close to 3.5 μ M (Figure 4.1A). Next, we recorded the pH-dependent (pH 3 – 9) UV-visible absorption and emission spectra of a fixed concentration (25 μ M) of TPEA above its CAC (Figure 4.1B, C). TPEA exhibited an absorption maximum at 350 nm in acidic solutions (pH 3). With an increase in pH, a blue shift from 350 nm to 310 nm was observed, accompanied by a concomitant decrease in absorbance (Figure 4.1D, E). In the fluorescence spectrum (λ_{ex} = 350 nm), TPEA showed an emission maximum at 475 nm throughout the entire pH range. However, to our expectation, the emission at 475 nm was significantly higher at acidic pH and gradually dropped with increasing pH (Figure 4.1F). The intensity could also be reversibly switched between lower and higher values by alternately adding NaOH and HCl to the same solution (Figure 4.1G). It could also be visually noticed that acidic solutions were more turbid, and the turbidity decreased at a higher pH (Figure 4.1H). The observations from these preliminary experiments indicated the possibility of a pH-responsive assembly (Figure 4I).

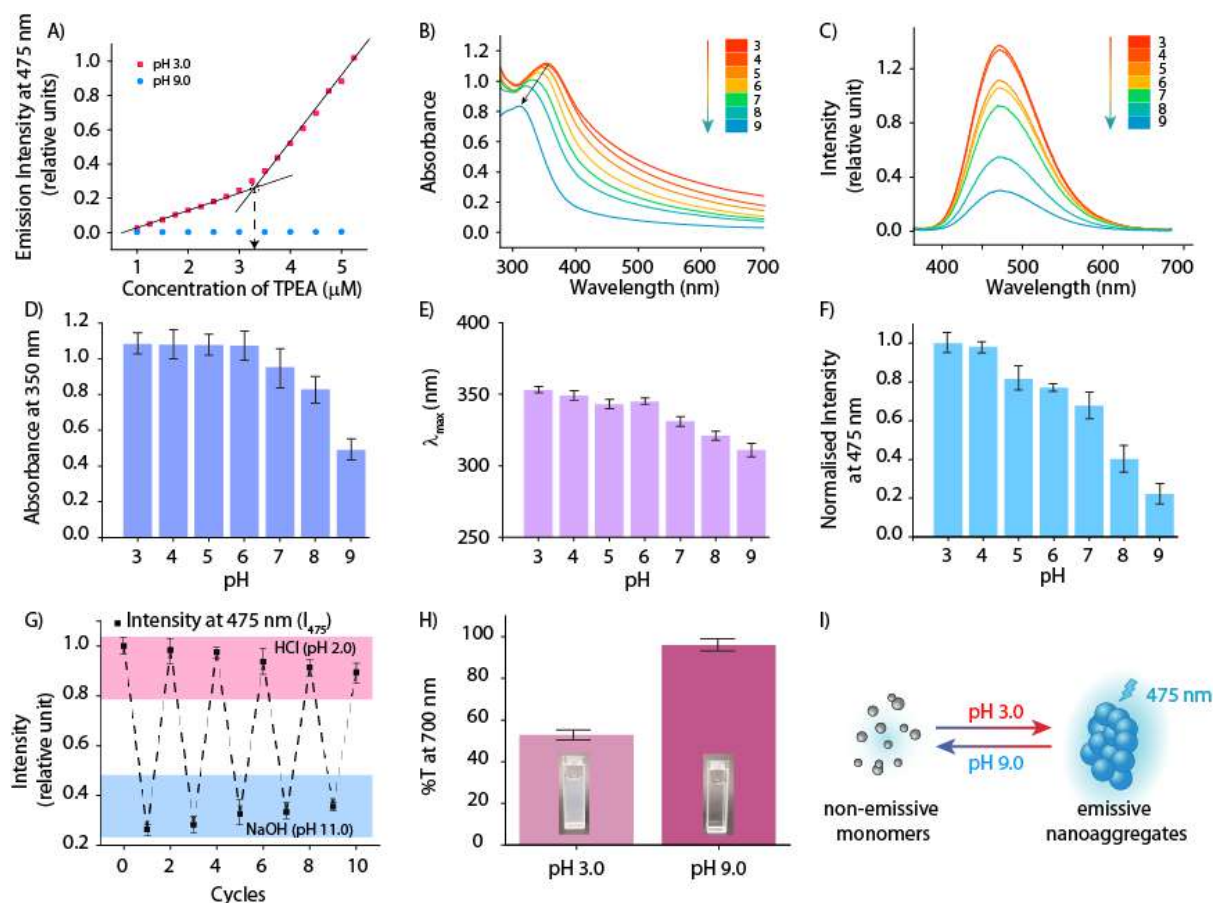


Figure 4.1. A) pH-dependent Critical aggregation concentration of TPEA. pH-dependent A) absorption spectra, B) emission spectra, C) absorption at 350 nm and D) wavelength of maximum absorption (λ_{max}) of TPEA (25 μM). F) Emission intensity of TPEA (25 μM) at 475 nm (λ_{em}) in solutions of different pH. G) Reversible emission behaviour of TPEA across multiple cycles induced by alternate additions of NaOH and HCl. H) Transmittance of solution of TPEA (25 μM) at 700 nm at pH 3.0 and pH 9.0. I) Schematic representation of pH-reversible TPEA-based emissive nanoaggregates.

To examine the aggregation process, we analyzed the pH-responsive morphologies through DLS, where particles of average hydrodynamic sizes of 150 and 850 nm were obtained at pH 9 and 3, respectively (Figure 4.2A). The pH reversibility of particle sizes was confirmed with alternate additions of NaOH and HCl (Figure 4.2A inset). The aggregates were also examined through the FESEM. Extensive clusters of polydisperse spherical nanoaggregates with an average diameter of 800-1000 nm were observed for TPEA (25 μM) in an acidic medium (Figure 4.2B). On the contrary, inconsistent nanoaggregates of lower dimensions were scattered unevenly in the basic medium (Figure 4.2C). These results were consistent with those measured by DLS. We also recorded the concentration-dependent (10 – 50 μM) absorption and emission spectra, which demonstrated that the nature of the spectra was independent of the TPEA concentration above its CAC (Figures 4.2D-G).

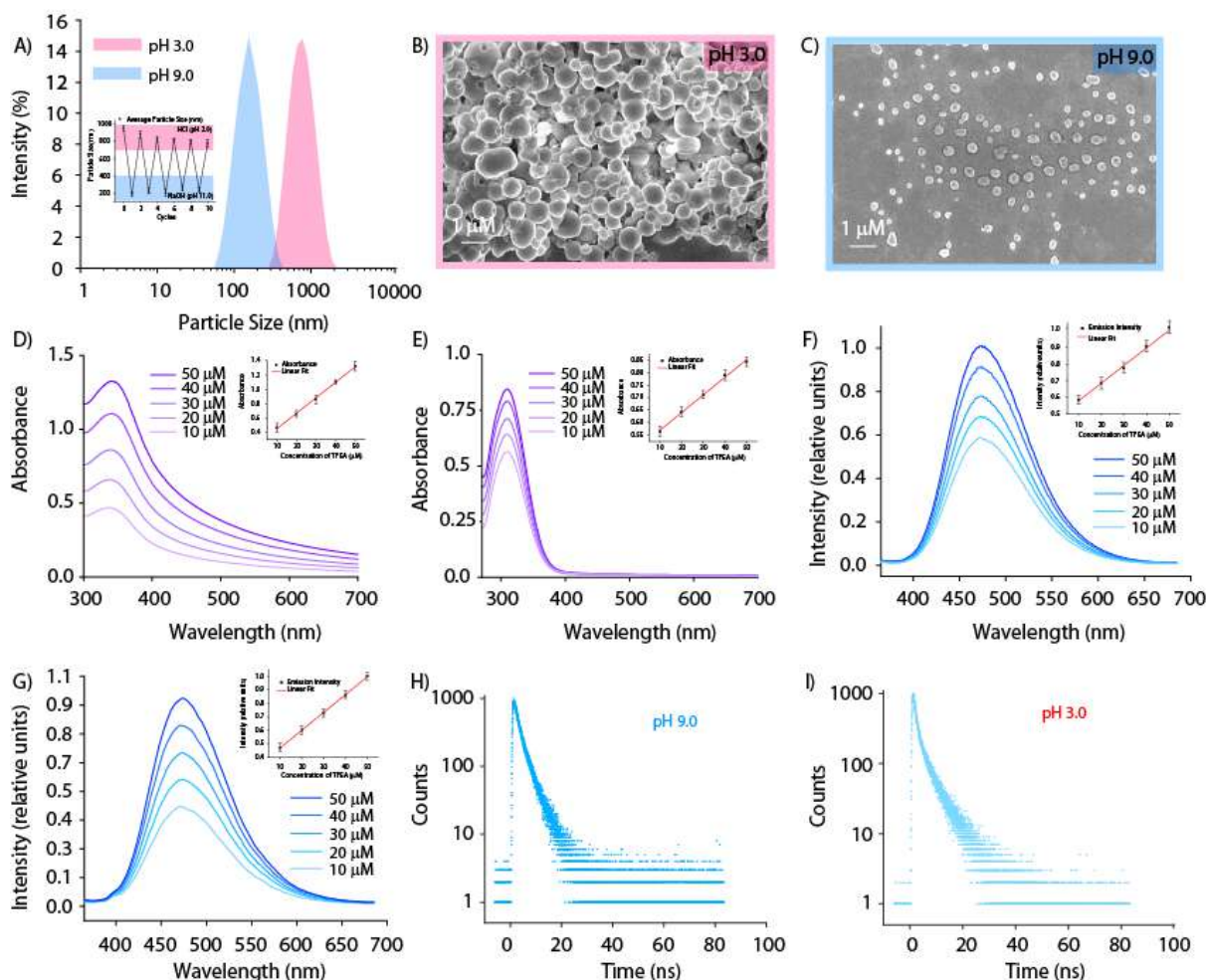


Figure 4.2. A) Average particle diameter of TPEA as measured by DLS at pH 3.0 and 9.0. **and** reversible particle size (DLS) behaviour of TPEA across multiple cycles induced by alternate additions of NaOH and HCl in the inset. FESEM image of TPEA (25 μ M) at B) pH 3.0 and C) pH 9.0. Concentration-dependent absorption spectra of TPEA at D) pH 3.0 and E) pH 9.0, with their respective linear fittings of absorbance (350 nm). Concentration-dependent emission spectra of TPEA at D) pH 3.0 and E) pH 9.0, with their respective linear fittings of emission (475 nm). Fluorescence decay profiles of TPEA (25 μ M) at H) pH 9.0 and I) pH 3.0.

The pH-dependent fluorescence decay profile and quantum yields were also recorded in acidic and basic media for the emission at 475 nm of TPEA (Figure 4.2H, I). The average lifetime (1.92 ns) and quantum yield (67%) were observed to be higher in an acidic medium compared to those in basic conditions (1.25 ns and 17% respectively, Table 4.1). Considering these observations, it can be concluded that TPEA exhibits a pH-responsive AIE. The AIE effect is observed under acidic conditions, where the carboxylic acid remains protonated. Below its pK_a , TPEA is a neutral molecule with lower water solubility. The hydrophobic effect facilitates the formation of aggregates, where, due to steric crowding and molecular clustering, free rotational intramolecular motions are substantially hindered.^{221, 235} Consequently, inside the aggregates, the emissive properties are augmented

because non-radiative pathways are significantly restricted.^{221, 223, 236, 237} As the pH is gradually increased, TPEA undergoes deprotonation when the pK_a value is exceeded. Under such circumstances, TPEA attains its carboxylate form, where the molecule is no longer neutral. It is expected that the negatively charged TPEA will be unable to aggregate as efficiently as it did in acidic conditions.^{243, 244} The comparatively higher solubility limits the charged molecule to smaller or lower-order aggregates, resulting in weaker emission. Since the protonation-deprotonation equilibrium of a carboxylic acid is a pH-reversible function, the emissive aggregates can be up- or downregulated promptly by the alternative switching of pH between low and high values, respectively.

Table 4.1. pH-dependent lifetime measurements of different combinations of dyes

System	λ (nm)	pH	τ_1 (ns)	τ_2 (ns)	τ_{av} (ns)	χ^2
TPEA	475	3	0.89 (67%)	4.04 (33%)	1.92	0.99
		9	0.59 (86%)	5.41 (14%)	1.25	1.00
TPEA + DBT	475	3	3.18 (14%)	0.45 (86%)	0.84	1.22
		9	0.47 (88%)	4.58 (12%)	0.96	1.09
	545	3	2.33 (37%)	9.54 (63%)	6.87	1.11
		9	1.55 (66%)	9.97 (34%)	4.40	1.22
TPEA + RhB	475	3	2.18 (18%)	0.43 (82%)	0.74	1.02
		9	0.33 (92%)	3.17 (08%)	0.54	0.98
	580	3	2.12 (87%)	3.90 (13%)	2.35	0.99
		9	0.35 (92%)	3.05 (8%)	0.58	0.96
TPEA + NR	475	3	0.63 (82%)	4.37 (18%)	1.30	0.99
		9	0.02 (49%)	0.02 (51%)	0.01	1.86
	615	3	0.94 (78%)	4.79 (22%)	1.77	1.04
		9	0.23 (92%)	0.99 (8%)	0.29	1.21
TPEA + DBT + NR	475	3	0.58 (83%)	4.36 (17%)	1.24	1.23
		9	0.12 (98%)	1.76 (2%)	0.15	1.37
	545	3	0.86 (76%)	4.99 (24%)	1.86	1.02
		9	0.28 (95%)	3.09 (5%)	0.41	1.38
	615	3	0.67 (84%)	4.44 (16%)	1.27	1.21
		9	0.33 (94%)	2.15 (6%)	0.45	1.26

4.2.2. pH-Responsive Multicolour Emission Through Multiple FRET Pairs

Since the acid-induced nanoaggregates were generated by hydrophobic TPEA, they could provide an ideal hydrophobic microenvironment that can be generated and dissolved with respect to pH. We anticipated that these emissive agglomerates could sequester other dyes in their hydrophobic domains, thereby separating them from the aqueous environment and preventing quenching. A careful selection of hydrophobic dyes (FRET pairs) that can absorb in the emission region of TPEA and encapsulated in the nanoaggregates should generate multicolours through FRET in an acidic medium.²⁴⁰ Fluorophores that exhibit stronger emission specifically under hydrophobic confinement or in acidic environments should prove to be suitable choices for FRET pairs.⁹

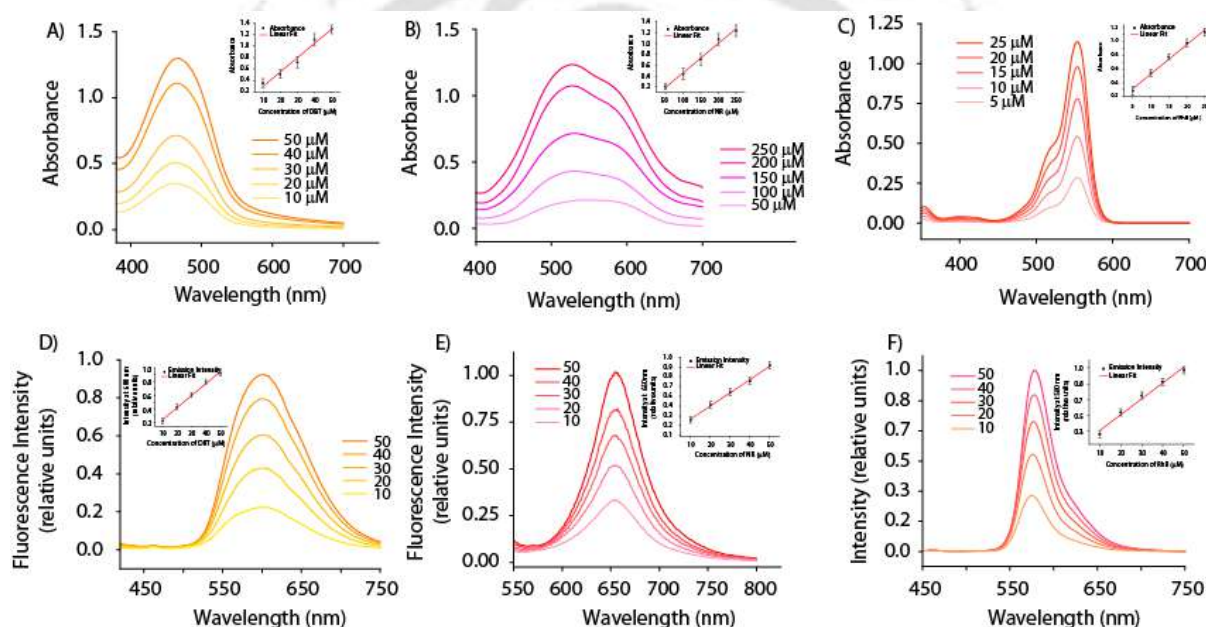


Figure 4.3. A) Concentration-dependent absorption spectra at pH 3.0 for A) DBT, B) NR and C) RhB with their respective linear variations of absorbance at λ_{max} versus the concentrations of dyes in the insets. B) Concentration-dependent fluorescence spectra at pH 3.0 for A) DBT, B) NR and C) RhB with their respective linear variations of emission at λ_{em} versus the concentrations of dyes in the insets.

Keeping all these criteria in mind, we studied the pH-dependent emission behaviour of TPEA nanoaggregates in the presence of several dyes – namely RhB, DBT and NR (Scheme 4.1). First, the concentration-dependent absorption and emission spectra for each dye were recorded (Figure 4.3A-F). For all cases, the spectral pattern was independent of the dye concentration. The λ_{max} values were 460, 525 and 550 nm, whereas the λ_{em} values were 600, 580 and 650 nm, for all concentrations of DBT, NR and RhB, respectively. The shift in the values is because of the absence of a hydrophobic domain in a completely aqueous medium. From the absorption-emission overlap spectra, it was clearly observed that there was a significant spectral overlap between the absorption of each dye

and the emission of TPEA, making them promising FRET dyes under close confinement (Figure 4.4A-D). Next, the pH dependency of the absorption and emission behaviour of a fixed concentration of these dyes was examined. For all the dyes, no pH dependency was observed.

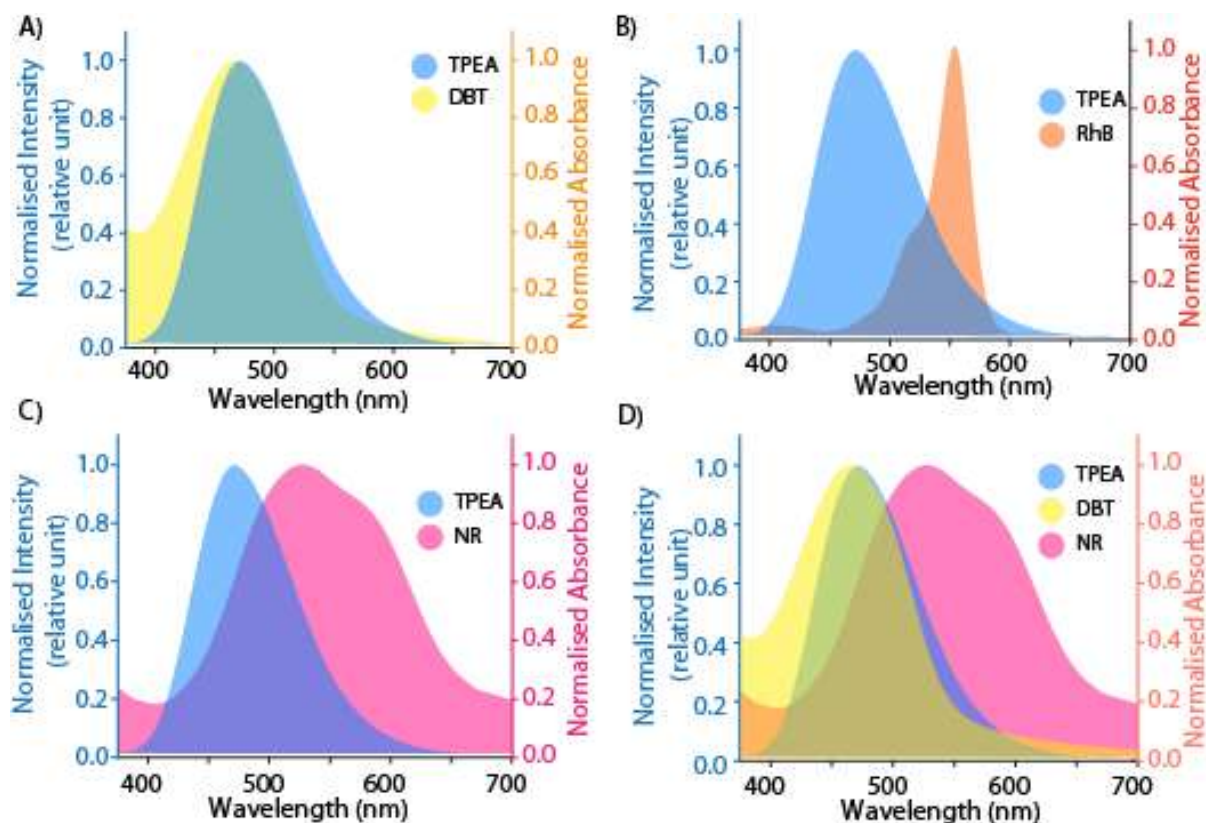


Figure 4.4. Spectral overlap of TPEA absorption with emission spectra of A) DBT, B) RhB, C) NR and D) DBT and NR at pH 3.0.

Next, the fluorescence patterns ($\lambda_{\text{ex}} = 350 \text{ nm}$) were recorded by gradually increasing the concentration of each dye in TPEA solutions ($25 \mu\text{M}$, pH 3; Figure 4.5A-D). In each case, the intensity at 475 nm for TPEA decreased with increasing concentration of the added dye. Concomitantly, a new peak eventually appeared at the respective λ_{em} values for the individual dyes that stabilized after attaining a certain ratio (TPEA: DBT – 250:1, TPEA: RhB – 50:1, TPEA: NR – 33:1). The spectral shifts can also be visualized under a 365 nm lamp (Figure 4.6A-D). Before introducing any dye, a solution of TPEA nanoaggregates appeared blue-cyan. On adding DBT, RhB and NR, the colour shifted from blue to yellow, orange and pink, respectively, for each dye. The CIE coordinates were also plotted for each dye combination (Figure 4.6E-H). It is clear from the CIE coordinates that the generated colours were unique not only for a particular dye but also for a specific concentration ratio of FRET donor-acceptor pair. It can be noted that for the RhB–TPEA pair, the transition passes through pure white light with coordinates of 0.33,0.33. As expected, these dyes were entrapped

within the nanoaggregates, bringing them into proximity to the highly emissive TPEA. Variations in spectral overlaps and different extents of energy transfer, achieved through a single-step FRET, have produced distinct wavelengths of emission that serve as a signature for a particular combination. This is analogous to the species-specific multicoloured bioluminescence. Thus, one-step energy transfer LHS with wide-spectrum emissions could be formulated.

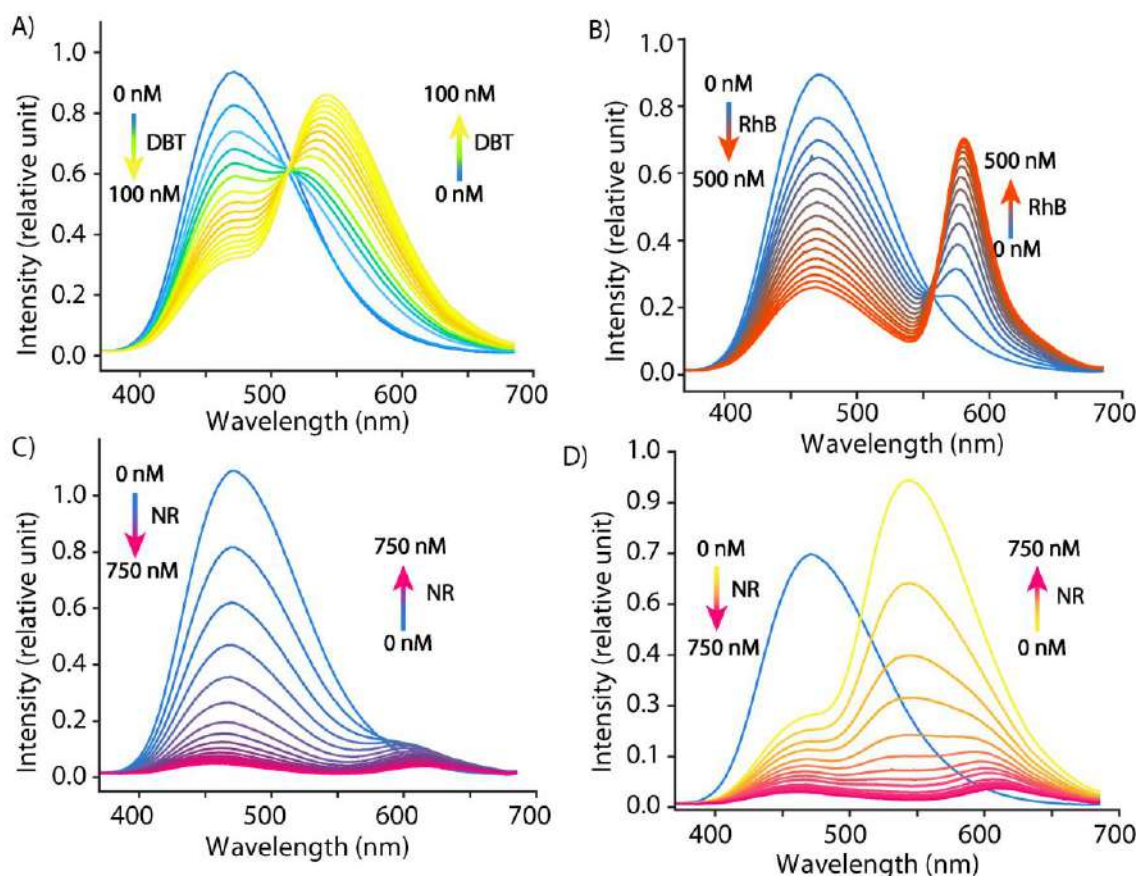


Figure 4.5. Emission spectra of TPEA (25 μM) at pH 3.0 with varying concentrations of FRET pair dyes – A) DBT, B) RhB, C) NR. D) Emission spectra of TPEA (25 μM) with DBT (0.1 μM) at pH 3.0 with varying concentrations of NR.

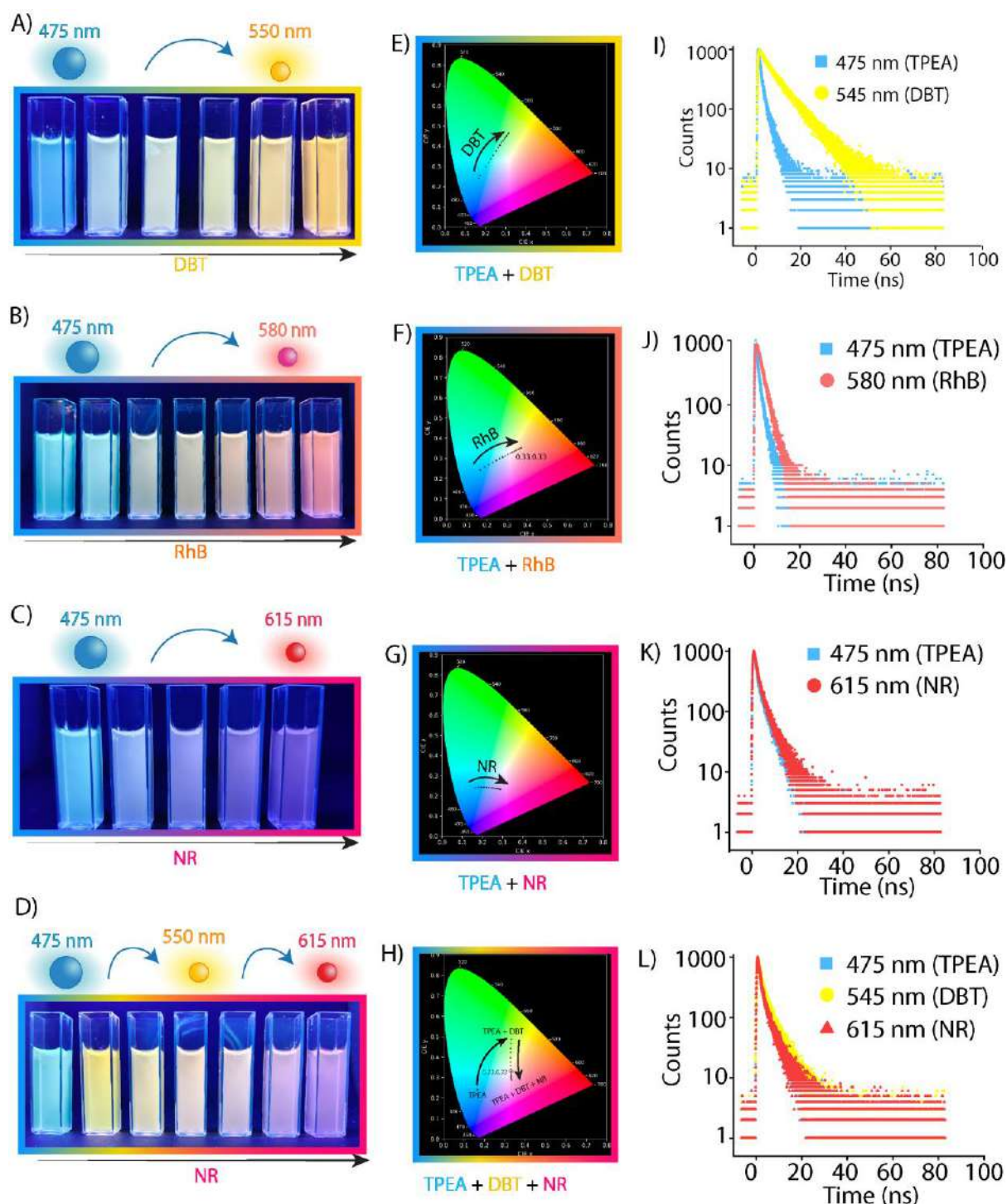


Figure 4.6. Images of broad-spectrum fluorescent colours of TPEA in combination with FRET pairs A) DBT, B) RhB, C) NR and D) DBT and NR. CIE plots of TPEA with FRET pairs - E) DBT, F) RhB, G) NR and H) DBT and NR. Fluorescence decay profiles of dye combinations of TPEA with I) DBT, J) RhB, K) NR and L) DBT and NR. All measurements have been performed at pH 3.0.

We realized that since TPEA-DBT and TPEA-NR could produce yellow and pink light, respectively, combining the three could lead to an even more diverse fluorescence emission, encompassing white light. Moreover, the absorption spectra of NR overlapped with the emissions of both TPEA and

DBT (Figure 4.4D). The combination of the three dyes, which would presumably produce a two-step FRET, was also analyzed through emission spectra (Figures 4.5D and 4.6D). Several combinations were attempted to develop broad-spectrum light-harvesting nanoaggregates, where the TPEA:DBT:NR (25:0.1:0.075) combination was found to be the best for producing a white light (0.33, 0.33) (Figure 4.7H). For all subsequent measurements, we maintained a fixed donor: acceptor ratio and colour (TPEA – 25 μM - blue, TPEA:DBT - 25:0.1 μM - yellow, TPEA:DBT - 25:0.05 μM - green, TPEA:RhB – 25:0.5 μM – orange, TPEA:NR - 25:0.75 μM - pink, TPEA:DBT:NR - 25:0.1:0.08 μM – white or TPEA:RhB – 25:0.25 μM - white) unless otherwise mentioned.

Table 4.2. pH-dependent quantum yields of dye combinations.

System	$\lambda(\text{nm})$	pH	$\Phi\%$
TPEA	475	3	67
		9	17
TPEA + DBT	475	3	26
		9	4
	545	3	77
		9	62
TPEA + RhB	475	3	42
		9	10
	580	3	19
		9	9
TPEA + NR	475	3	31
		9	4
	615	3	6
		9	1

The decay traces, lifetimes, and quantum yield for all the combinations were measured. It was observed that the quantum yield and lifetime for TPEA emission at 475 nm were higher under acidic conditions (Figure 4.1I). When any FRET pair was introduced, the lifetime and the quantum yield for the emission of the FRET dye at its corresponding wavelength increased, while that for TPEA decreased (Figure 4.6I-L, Table 4.1-4.2). Similar trends were also observed for these combinations in a basic medium, however, with much lower values than in the acidic medium (Table 4.1-4.2). All the emissions were highly pH dependent. For all cases, considerably higher emission intensities were observed in acidic medium, indicating that the presence of nanoaggregates is crucial for strong emission (Figure 4.7A-C).

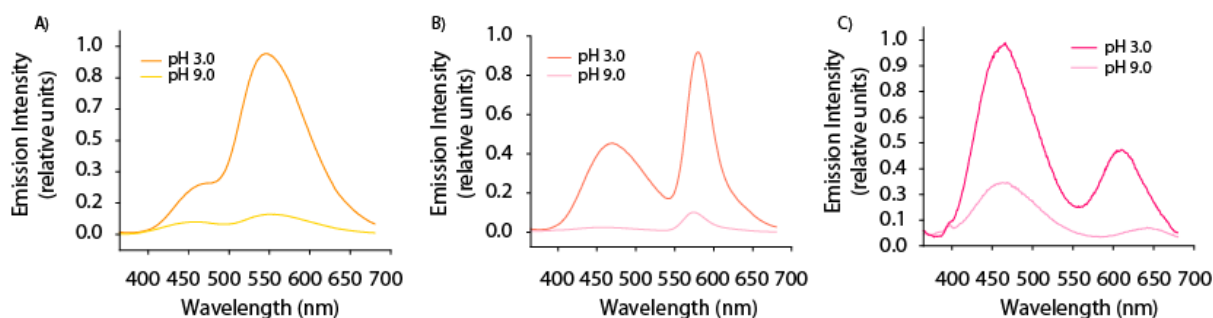


Figure 4.7. pH-dependent emission spectra of TPEA with A) DBT, B) RhB and C) NR.

4.2.3. pH-Clock Guided Broad-Spectrum Transitory Emission

We achieved a command over the emissive patterns with pH fluctuations; however, such a responsive behaviour is still quite far from original bioluminescence. If the nanoaggregates are subjected to a temporal pH program, then a time domain can be introduced in the pH-regulatory emissive behaviour, bringing it closer to real biomimetic on-off luminescence. In this context, we relied on a chemoenzymatic pH clock because enzymatic regulations sit at the heart of biological systems. The citrate buffer-urea/urease chemoenzymatic reaction cycle, which introduces autonomous pH variations, can influence the formation and deformation of emissive nanoaggregates.²⁵¹ The addition of an acidic (citrate buffer) urea solution to a urease solution triggers the pH clock. The buffer action of the citrate buffer swiftly pulls the pH down. The enzymatic hydrolysis of urea by urease was initiated, simultaneously generating ammonia in the solution, which eventually increases the pH. However, since enzymatic kinetics are themselves highly pH-dependent and require a proper pH for optimum function, a lag phase is observed before a high alkaline pH is registered. This is because the pH clock was slow at the beginning due to the low activity of urease outside its optimum pH range, as well as the buffer action of citrate on the generated ammonia, which causes it to spend more time in the acidic zone. The slow generation of ammonia increases the pH steadily, generating positive feedback. After this lag phase, urease soon entered its optimum pH zone, close to the near-neutral region where the rate was accelerated. This is followed by a faster pH rise, where the pH clock quickly entered the basic zone. The production of excess ammonia creates a negative feedback, which again retards the clock once it reaches a pH level outside its optimum range. Hence, a time-controlled chemoenzymatic pH profile can be obtained, featuring a rapid pH drop followed by a steady pH rise reaching the starting point. Then overall time and duration of the lag phase can be manipulated by altering any constituent of this multicomponent pH clock. We analyzed the possible modulations in the time scale by varying the individual components of the pH clock (Figure 4.8). Following the optimization study, all further studies were conducted using a pH clock, initiated by adding 5 mM citrate buffer (pH 3.5) and 60

mM urea to a urease solution (5 U/mL) at pH 9.0. The initial pH dropped quickly from 9.0 to 3.8, then gradually returned to the original pH over 15 minutes.

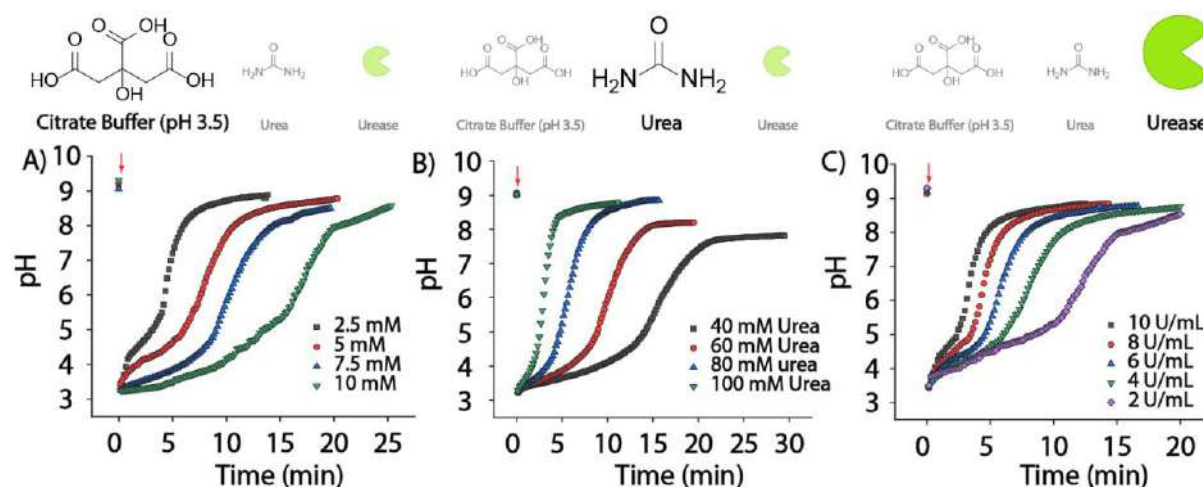


Figure 4.8. Optimization of citrate buffer-urea/urease pH clock. pH vs time profile with varying concentrations of A) citrate buffer under fixed concentrations of urea (100 mM) and urease (10 U/mL), B) urea under fixed concentrations of citrate buffer (10 mM) and urease (10 U/mL) and C) urease under fixed concentrations of citrate buffer (10 mM) and urea (100 mM). Additions of trigger have been indicated by the red arrows.

Next, the changing dimensions of the nanoaggregates under the effect of the pH clock were analyzed. A time-dependent DLS profile reveals that upon initiation of the pH clock, the nanoaggregates attain a larger size (900 nm). As the clock progresses, the distribution of particle size gradually moves towards lower dimensions (Figure 4.9A). The results were complemented by the FESEM analyses showing similar changes in the particle size during the pH clock (Figure 4.9B). The emission intensity of the TPEA (25 μ M) solution at pH 9.0 was significantly low. When the pH clock was triggered in the solution, the intensity of the peak at 475 nm rapidly increased (Figure 4.9C). The peak remained stable over time as long as the pH clock resided in the acidic range. Over time, as the pH increased, the peak's intensity reduced, almost returning to its original value after the end of the pH cycle. When the pH clock was executed in the solutions of the mixed dyes, similar observations were recorded. For all cases, the intensities of the respective emission peaks increased rapidly and decreased slowly over time. The emission profiles were also extrapolated to time-dependent CIE coordinates (Figure 4.10A-F). These temporal changes were recorded under UV light, and transient on-off fluorescence of different colours was visualized, resembling real-life bioluminescence. These observations established that chemoenzymatic pH clock-guided size-regulatory TPEA-based nanoaggregates with FRET acceptors could behave as artificial LHS, exhibiting temporal control over multicolour emission similar to the enzymatically controlled switching of bioluminescence. Since TPEA solutions form aggregates under acidic conditions, the

solutions appear turbid. The turbidity was visibly reduced at higher pH. The time-dependent transmittance measurement at 700 nm under the pH clock revealed that turbidity appeared instantaneously at the onset of the pH clock, and with an increase in pH, the solutions were rendered transparent due to the disassembly of TPEA (Figure 4.10G, H).

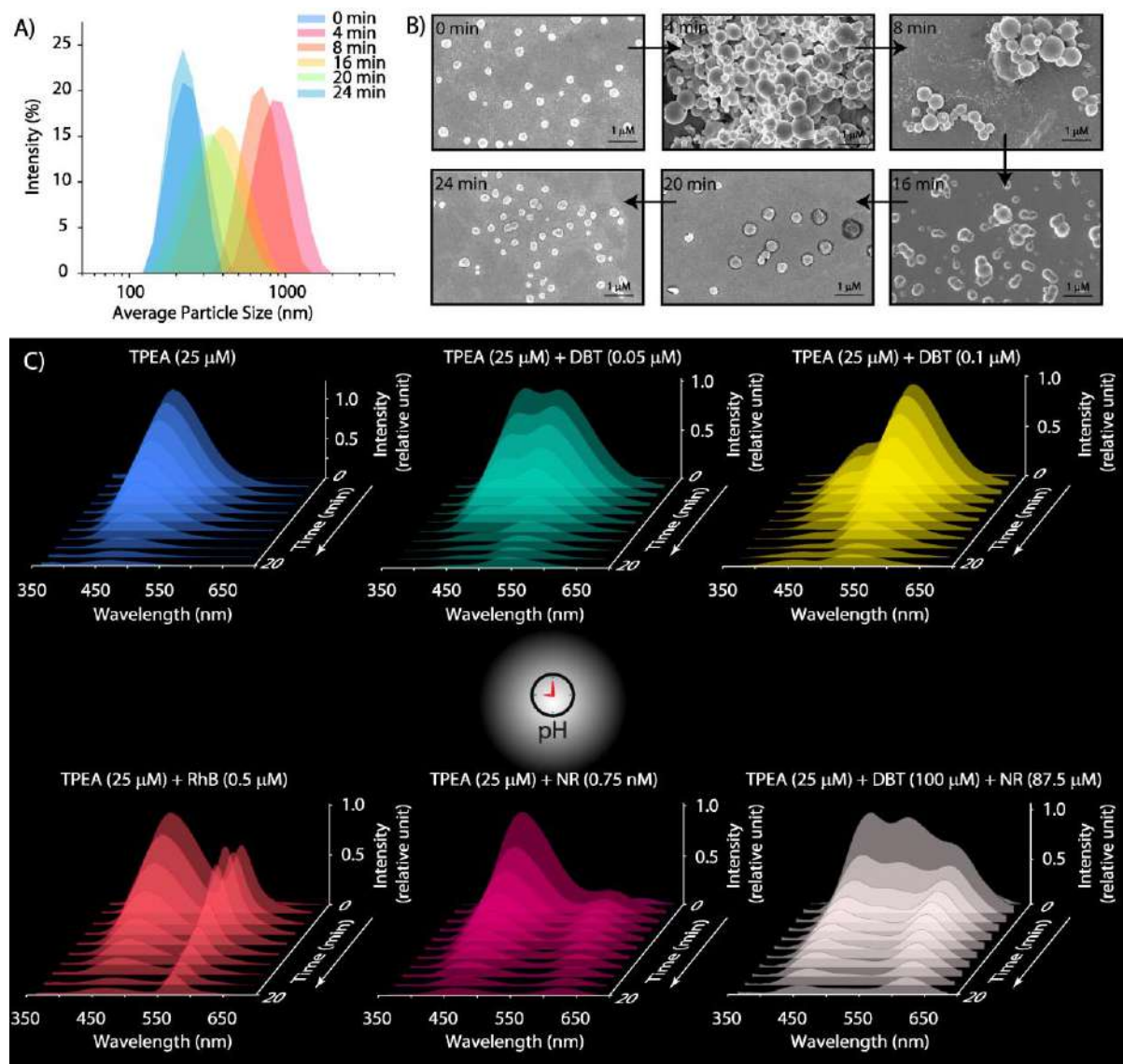


Figure 4.9. A) Time-dependent A) particle size analysis through DLS and B) FESEM images of TPEA (25 μ M) nanoaggregates under the effect of the pH clock. C) Time-dependent multicolour emission spectra of TPEA emissive nanoaggregates with different FRET dyes.

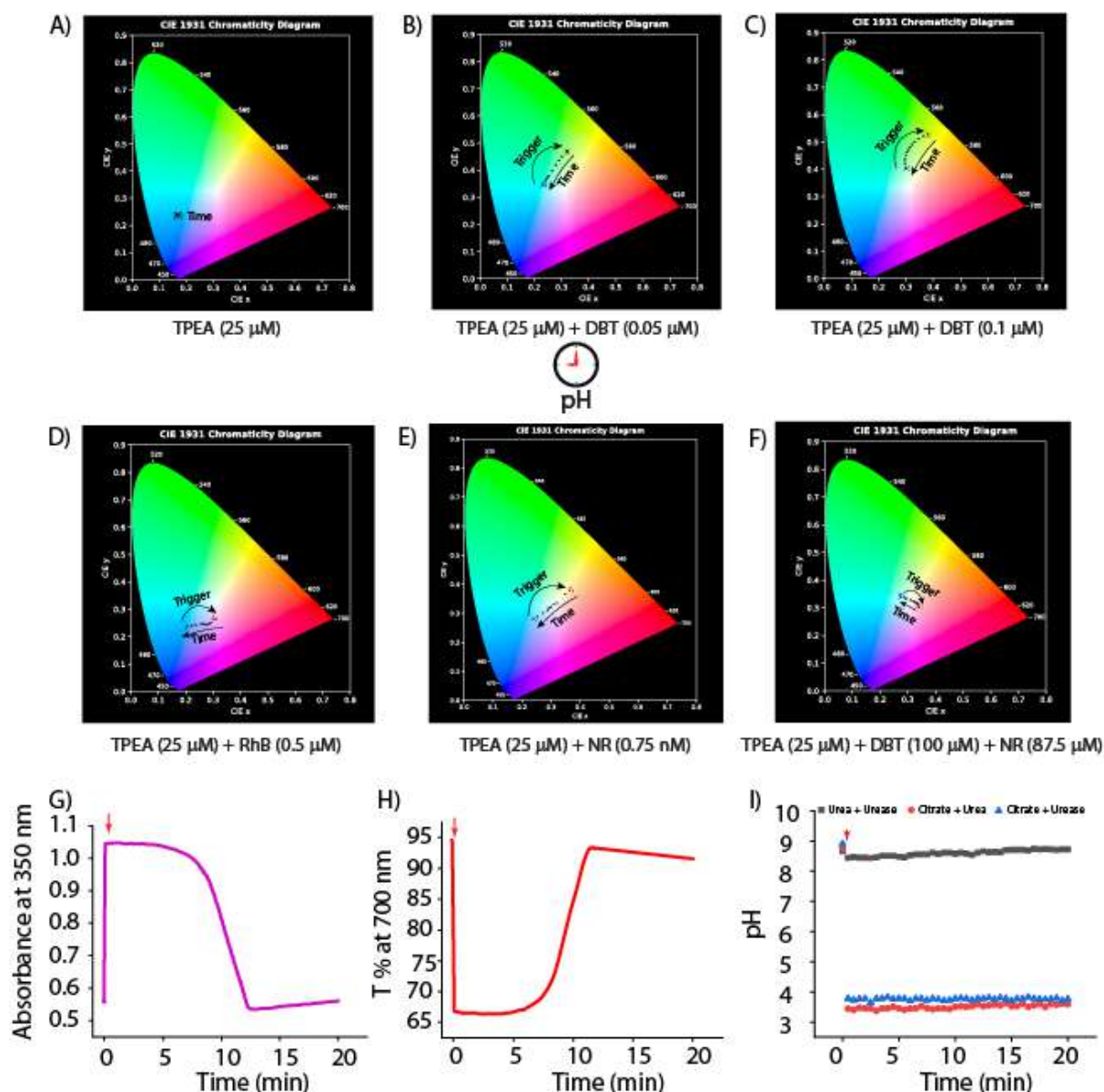


Figure 4.10. Time-dependent CIE coordinates for the combination of dyes under the effect of the pH clock (A-F). The red and blue circled dots indicate the coordinates before and after the addition of the pH clock trigger. Time-dependent G) absorption at 350 nm and H) transmittance (T%) at 700 nm for TPEA (25 μM) under pH clock. Additions of trigger have been indicated by the red arrows. I) pH clock control experiments. The addition of the pH-clock trigger is indicated by the red arrow.

Cellular systems are essentially multicomponent systems in which multiple constituents work together to achieve a significant functional outcome. Although each component has its unique role, the function and operation of one element are invariably influenced by the others. It is often observed that failure or intracellular miscommunication in a single step can disrupt the entire biological network, resulting in a cascading effect. What we developed also illustrates a similar, intricate, and advanced multicomponent coordination. Each participant in the pH clock influences the transient, broad-spectrum emissive nanoaggregates, plays its own part, and orchestrates its

signature time-controlled, colour display. To demonstrate the significance of each component, we performed time-dependent control experiments in the absence of individual components (Figure 4.10I). For all cases, the pH remained constant where the pH clock ceased to operate. Under such conditions, the transient glow would be absent. Either there will be no emission at all, or there will be no disappearance in emission once generated. Hence, we proved that to achieve unique transient emission, we not only need a particular composition of mixed dyes but also a smoothly coordinated temporal pH profile, all in sync.

4.2.4. Application of the Transient Emission for Advanced Time-encoded Anti-counterfeiting

To this end, transmission of messages maintaining secrecy is an integral part of information encryption and data protection. Securing valuable data, facts, figures, and statistics from mishandling is a crucial aspect of an anti-counterfeiting-based encryption strategy. In this context, ASCII codes and Morse code have garnered immense attention in the field of message encoding. ASCII codes encode alphanumeric characters with numeric digits, while Morse codes employ binary symbols of dots and dashes, where a unique combination of dots and dashes is designated as a discrete signature corresponding to each character. Besides being a fundamental platform for encrypting letters, it offers a secure and covert method for transmitting confidential information. We envisioned that the developed time-gated multicolour emission system could efficiently be used to create advanced-level information encryption strategies.

The ASCII numbers corresponding to each character can also be represented in their binary forms. For example, the ASCII code for 'H' is 72, which is 01001000 in the binary representation. We assigned the binary digit '0' to a transparent solution of TPEA under basic conditions and '1' to a turbid acidic solution of TPEA. Before the pH clock is initiated, a linear arrangement of 8 clear (basic) solutions of TPEA depicts 00000000. Selective initiation of the pH clock in a specifically placed solution produces different binary combinations that encode for a particular character that turns clear over time (Figure 4.11A). We have performed encryption of the message "HIDE" using the pH clock-controlled turbidity of TPEA nanoaggregates, employing the binary code versions of the ASCII codes for each character. This encryption strategy simply aims to achieve data storage under daylight.

Being a pH-reversible fluorescent assembly, encoding data in binary patterns can also be accomplished with the emissive nanoaggregates. When fluorescence is involved, decryption is performed under UV light. We considered that intense emissions under an acidic medium can be used to convey a hidden message, and weaker emissions in basic solutions will lead to the deletion of this secret message under UV light. We executed time-controlled Morse code with pH clock-

Figure 4.11. Time-controlled A) ASCII and B) Morse codes with pH clock guided emissive nanoaggregates.



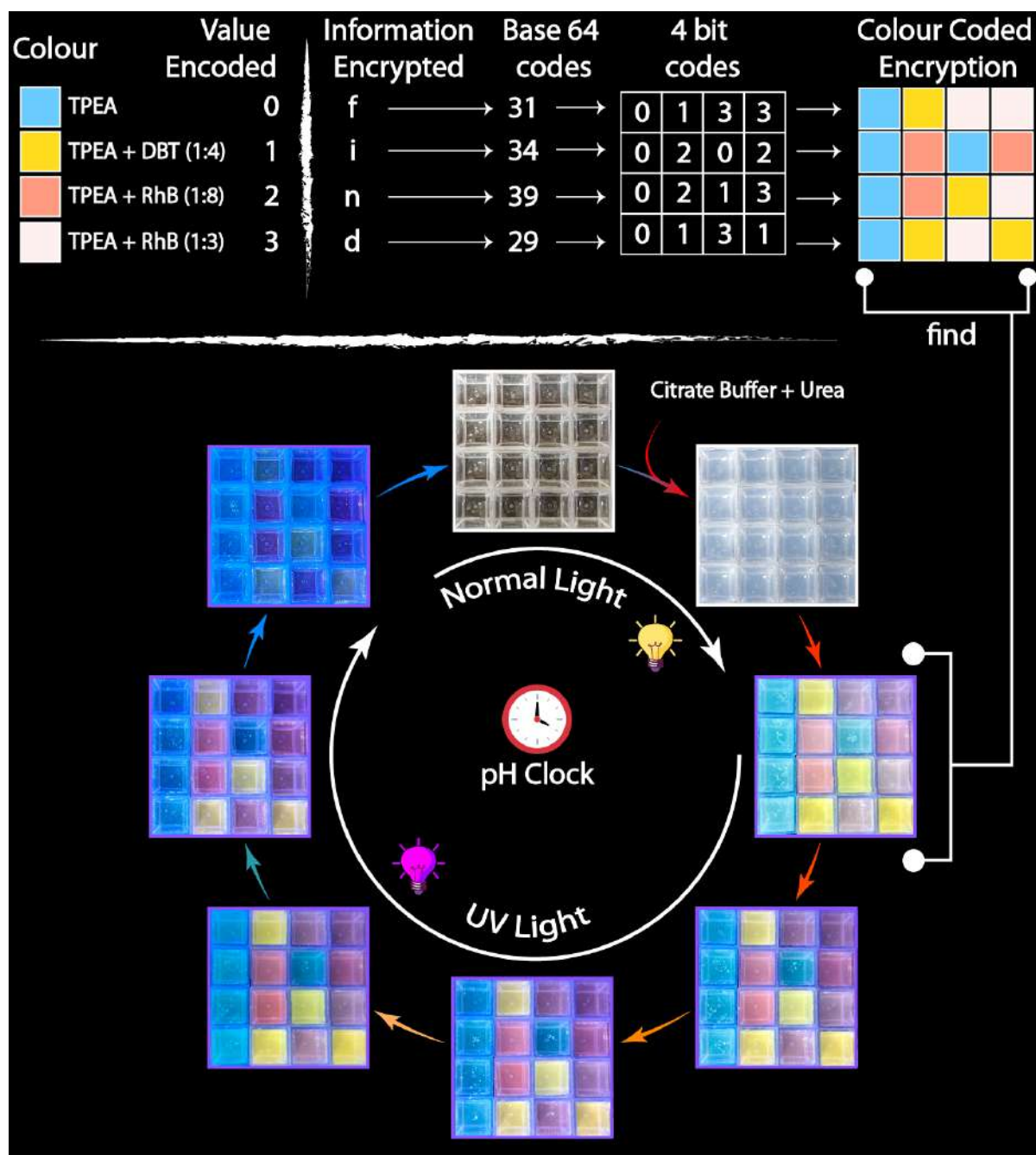


Figure 4.11. Time-controlled colour codes from pH clock guided multicolour emission from TPEA nanoaggregates with different FRET pairs.

Colour codes have gained immense importance in the field of information encryption with anti-counterfeiting properties. We anticipated that since our nanoaggregates could emit multiple colours, we could utilise these variable colours in an unconventional approach. Mostly in colour-coded encryption, the entire colour arrangement is assigned to a specific piece of information. We proceeded by assigning individual colours to specific information that can be encrypted for a logical message when combined in a specific arrangement. We assigned 0 to blue, 1 to yellow, 2 to orange

and 3 to white for encryption purposes. The 64 base codes of alphanumeric characters can be expressed in their 4-bit codes, which mainly comprise a specific sequence of 0,1,2,3. For our strategy, we chose to encrypt the message “find” using a 4×4 colour-coded encryption (Figure 4.11). Under daylight, the multicoloured nanoaggregate simply appeared turbid. Under UV light, the colour code appeared, and the interpretation of the code revealed the message through the 4-bit codes. Since the pH clock was executed in each solution, the colours gradually faded, and the true message was secured through time-locked disappearance. The codes can be read through their colours, appearing in sequence from left to right. Each colour represents a digit (0,1, 2, 3). When read from left to right in a single row, it generates a 4-bit code from that row. This code, when converted to its 64-base code, gives a number that encrypts for an alphanumeric character. In this way, if the entire setup is decrypted row by row at the right time along the time scale, it reveals a hidden message that dissipates over time. All our strategies involve the demonstration of popular encryption codes that inherently bear the quality of secrecy. When additionally controlled temporally through the pH clock, an extra layer of security is introduced through the time dimension. The time regulatory features create a narrow window through which true information can be accessed, thereby elevating the level of security in every aspect.

4.3. Conclusions

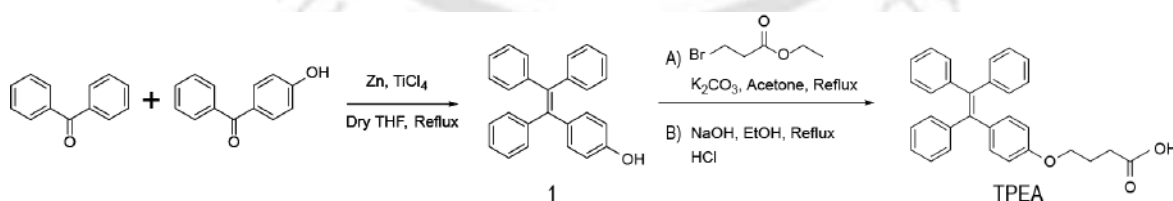
To summarize, in this work, we have successfully mimicked the fascinating phenomenon of transient lights, as seen in fireflies, using the pH-dependent aggregation of a TPE derivative. Minimalistic molecular design, followed by stimuli-responsive properties, has been utilized to achieve multicolour-fluorescence on-off behaviours. FRET pairs have been employed to channelize a single wavelength of light energy into multiple colours through ordered energy transmission. Further to closely stick to biological systems, where enzymatic regulation creates autonomously manipulated switch-on/off mechanisms, we also followed a chemoenzymatic route for temporal control of stimulus. We demonstrated how biotic systems can be mimicked through complex yet coordinated chemistry under abiotic conditions when cautiously synchronized. On successfully generating multicolour time programmes through a chemoenzymatic pH clock-guided modulation of broad-spectrum emissive light-harvesting nanoaggregates, we have been able to display an aspect of nature’s works. Extrapolating these concepts further, we have extended these time-programmed multicolour displays into information encryption strategies with anticounterfeiting properties. Time-locked versions of popular cryptographic codes have been demonstrated where the time coordinate has been presented as an additional parameter that enhances the level of protection in these security code.

4.4. Experimental

4.4.1. General Information and Materials:

Benzophenone, 4-hydroxybenzophenone, 4,7-dithien-2-yl-2,1,3-benzothiadiazole (DBT), Rhodamine B (RhB) and Nile red (NR) were purchased from TCI Chemicals (India) were purchased from Tokyo Chemical Industry (TCI) Chemicals (India). Ethyl 4-bromobutyrate was purchased from Sigma-Aldrich (USA). Urease ex. Jack Beans (Activity: 394 U/mg) and urea were purchased from SRL. Tris(hydroxymethyl)aminomethane (TRIS), NaOH, tri-Sodium citrate dihydrate and Citric acid monohydrate were purchased from Merck. Titanium tetrachloride was purchased from Spectrochem. Milli-Q water with a conductivity of $<2\mu\text{Scm}^{-1}$ was used for all sample preparations and experimental measurements. 60-120 mesh silica gel (SRL) was used for column chromatography. ^1H NMR and ^{13}C NMR spectra were recorded using a Bruker Ascend 600 MHz (Bruker, Coventry, UK) spectrometer. Coupling constants (J values) are reported in hertz, and chemical shifts are reported in parts per million (ppm). FESEM imaging was performed on a Gemini SEM 300 (Sigma Zeiss) instrument. The particle size analyses were performed at 298 K using Zetasizer Nano-ZS90 (Malvern). A He-Ne LASER ($\lambda=632.8\text{ nm}$) was used as an excitation source for dynamic light scattering (DLS) measurements. UV-vis spectra (absorbance and transmittance) were recorded on a PerkinElmer Lambda 365+ spectrometer. Fluorescence measurements were performed on a Fluoromax 4 Plus spectrophotometer. pH data were recorded on a Hanna HI 2210 pH meter equipped with a HI1131 pH probe from Hanna. The PL decay measurements were performed using a time-correlated single photon counting (TCSPC) setup with MCP detection (Horiba Jobin, Model: Ultrafast-01-DD). An LED of 340 nm was used for the excitation of the sample. The absolute quantum yield measurements were performed using an Edinburgh Life Spec II instrument. The chromaticity diagram and CIE coordinate points were plotted with SpectraChroma.

4.4.2. Synthesis



Scheme 4.2. Synthetic scheme for the preparation of TPEA

Synthesis of Compound 1

Zinc powder (1.65 g, 25 mmol, 10 equiv.) was measured in a double-necked 100 mL round-bottom flask equipped with a magnetic stirrer and purged with Argon. Dry THF (20 mL) was added to it. The mixture was stirred at 0°C in an ice bath for 30 minutes. TiCl_4 (1.4 mL, 12.5 mmol, 5 equiv.) was added

slowly to it with a syringe in the argon medium at 0 °C. The suspension was gradually allowed to reach room temperature, after which it was refluxed for 3 hours. The suspension was again cooled to 0 °C in an ice bath and stirred. A solution of benzophenone (250 mg, 1.37 mmol, 1 equiv.) and 4-hydroxybenzophenone (270 mg, 1.37 mmol, 1 equiv.) in THF (10 mL) was added dropwise to this suspension under cold conditions and stirred in the ice bath for 30 minutes after complete addition. The reaction mixture was then refluxed for 6 hours. The reaction mixture was cooled and concentrated, and then diluted with EtOAc. The EtOAc fraction was washed thrice with saturated NaHCO₃ solution and concentrated to obtain the crude product. The crude product was purified over a column with 10% EtOAc in Hexane to obtain the pure product as a white powder (Yield ~ 51 %). ¹H NMR (600 MHz, Chloroform-*d*): δ 7.12 – 7.06 (m, 9H), 7.05 – 6.99 (m, 6H), 6.89 (d, *J* = 8.6 Hz, 2H), 6.56 (d, *J* = 8.6 Hz, 2H), 4.80 (s, 1H); ¹³C NMR (150 MHz, Chloroform-*d*) δ 154.41, 144.35, 144.25, 144.23, 140.79, 140.53, 136.69, 133.08, 131.71, 131.69, 131.67, 128.05, 127.95, 126.72, 126.60, 114.93; MS (ESI): *m/z* calculated for C₂₆H₂₀O: 348.15; found for [C₂₆H₂₀O]: 348.19.

Synthesis of Compound TPEA

Compound 1 (350 mg, 1 mol, 1 equiv.), K₂CO₃ (165 mg, 1.2 mol, 1.2 equiv.) and ethyl 4-bromobutyrate (186 μL, 1.3 mol, 1.3 equiv.) were measured in a 100 mL double-necked round-bottom flask equipped with a magnetic stirrer and purged with argon and sealed. To it, 20 mL of dry acetone was added, and the reaction mixture was refluxed for 12 hours. The reaction mixture was cooled to room temperature, concentrated, and then diluted with EtOAc. The EtOAc fraction was washed with dil. HCl and the water. The organic fraction was concentrated to obtain the crude product, which was purified over a column with 5% EtOAc in hexane to give a white powder as the pure product. The product was taken in a round-bottom flask and refluxed with NaOH (xx mg, xx mol, xx equiv.) in EtOH (15 mL) for 18 hours. The reaction mixture was concentrated under vacuum and diluted with a minimum amount of water. The solution was acidified dropwise with 6M HCl to a very low pH – 1.0. until a white turbid solution appeared. The precipitate was isolated and dried under vacuum to obtain the pure product as a white powder (Yield ~ 58 %). ¹H NMR (600 MHz, Chloroform-*d*) δ 7.13 – 7.06 (m, 9H), 7.05 – 6.99 (m, 6H), 6.92 (d, *J* = 8.7 Hz, 2H), 6.61 (d, *J* = 8.7 Hz, 2H), 3.94 (t, *J* = 6.0 Hz, 2H), 2.55 (t, *J* = 7.3 Hz, 2H), 2.07 (p, *J* = 6.7 Hz, 2H); ¹³C NMR (150 MHz, Chloroform-*d*) δ 177.97, 157.61, 144.36, 144.31, 140.83, 140.46, 136.62, 132.89, 131.73, 131.70, 131.67, 128.07, 127.94, 126.70, 126.60, 126.57, 113.90, 66.65, 30.65, 24.75; MS (ESI): *m/z* calculated for C₃₀H₂₆O₃: 434.19; found for [C₃₀H₂₆O₃]: 434.20.

4.4.3. Methods and Instrumentations

4.4.3.1. Preparation of Stock Solutions of Dyes

The stock solutions of all the fluorophore dyes were prepared in ethanol and stored at 4 °C.

4.4.3.2. Execution of Citrate-Urea/Urease pH clock

In a glass vial, 2.8 mL of water was taken, and the required volume of urease solution was added from its stock (1000 U/mL). The solution was basified to pH 9.0 with 5 μ L of 1 mM NaOH solution. This was immediately followed by the simultaneous addition of required volumes of sodium citrate/citric acid buffer (pH 3.5, 100 mM stock) and urea (2 M stock) solutions. The solution was quickly homogenised with a micropipette, and then the pH change was monitored from an initial pH of 3.7 to a final pH of 8.6 over time.

4.4.3.3. Absorption measurements

All absorption experiments were recorded in a 3 mL quartz cuvette (path length 1 cm) with a slit width of 1 nm at 298 K. To 3 mL of water or buffer of desired pH, the required volume of dye was added from its respective stock solution to obtain the desired concentrations. It was then evenly mixed with a micropipette and equilibrated for a minute before data acquisition. For a typical time-dependent experiment, all the required contents were added to a dye solution following the sequence as in the pH clock. The contents were uniformly homogenised, and the spectra were recorded at regular time intervals or at a particular wavelength over a time span as needed.

4.4.3.4. Fluorescence Measurements

All fluorescence experiments were performed in a 3 mL quartz cuvette (path length 1 cm) with a slit width of (Ex./Em.) = 2/2 nm at 298 K under $\lambda_{\text{ex}} = 350$ nm unless otherwise mentioned. For any time-independent measurements, required volumes of dyes were added to the solution in the cuvette from their respective stock solutions, mixed thoroughly, equilibrated for 1 minute and then recorded. In a typical time-dependent fluorescence experiment, all the contents were added following the same proportion and sequence as that in the pH clock. The contents were quickly homogenized with a micropipette, and then the emission spectra were recorded at intervals of every minute over 30 minutes. The final concentration of TPEA was 25 μ M and was variable for variable dyes.

4.4.3.5. Time-Resolved Photoluminescence and Quantum Yield Measurements

All measurements were performed in a 3 mL quartz cuvette with a 1 cm path length. For all the experiments, the required concentration of the dyes and their combinations was maintained, respectively. For studies conducted under static pH conditions, buffers were used. For time-resolved

photoluminescence lifetime measurements, a 340 nm (~100 ps, 1 MHz repetition rate) diode laser was used for excitation. A deconvolution procedure was used to analyze the observed decays, which could be satisfactorily fitted by bi-exponential decay functions. A deconvolution procedure was used to analyze decays using a proper instrument response function obtained by using a light scatterer (suspension of TiO₂ particles in water). The fluorescence decays $I(t)$ were analyzed in general as a sum of exponentials. The fluorescence decays $I(t)$ were analyzed in general as a sum of exponentials,^{196, 197}

$$I(t) = \sum B_i \exp\left(-\frac{t}{\tau_i}\right) \quad (1)$$

where, B_i and τ_i are the pre-exponential factor and fluorescence lifetime for the i th component, respectively. Reduced chi-square (χ^2) values and random distribution of the weighted residuals among data channels were used to judge the acceptance of the fits.

4.4.3.6. FESEM and FETEM Analyses

For the time-dependent FESEM experiments, 5 μ L of the sample solution under pH clock were taken out at specific times and drop-cast on a silicon wafer. The samples were immediately freeze-dried to arrest the pH clock kinetics and then analyzed. For samples under static pH conditions, 5 μ L of the sample solution prepared in buffer was cast on a silicon wafer, dried, and analyzed for morphologies.

4.4.3.7. DLS Analyses

The particle sizes were recorded in a 3 mL DLS cuvette. For measurements under static pH conditions, the buffer solution of the desired pH was taken, followed by the addition of required volumes of dyes from their stock solutions. The solutions were thoroughly mixed and equilibrated for 1 minute before measurements. For a typical time-dependent dynamic experiment, all the constituents were added following the same proportion and sequence as in the pH clock. The contents were quickly homogenized with a micropipette, and then the particle sizes were monitored at regular intervals over 30 minutes.

4.4.3.8. Time-encoded encryption in chemically triggered colour codes

For the time-regulated ASCII codes, 8 small glass cuvettes (1 cm * 1 cm * 1 cm) were arranged side by side. Each cuvette contained clear solutions having urease and TPEA (25 μ M) at pH 9.0 adjusted with dilute NaOH. pH clock was initiated in the desired cells by the addition of the citrate buffer/urea trigger, which generated turbidity in them. The time-dependent changes in the turbidity were visually observed.

For the time-regulated Morse codes, solutions of TPEA (blue) and TPEA: DBT (yellow) were placed in well plates at specific positions. In basic conditions, under UV light (365 nm), no emission was visible.

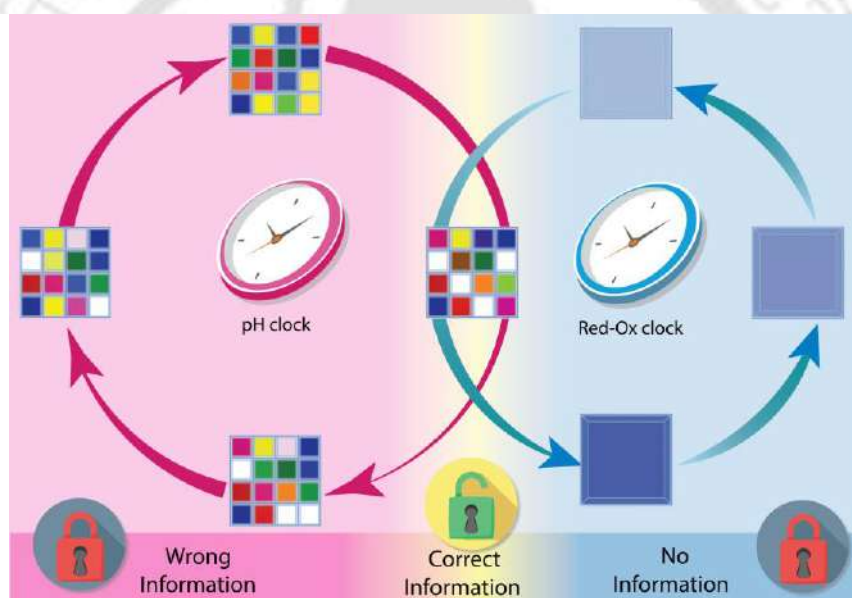
When the pH clock was initiated, the blue and yellow fluorescence appeared that depicted the Morse code representations, which were time-locked and vanished temporally.

For the time-regulated transitory colour code, 16 small glass cuvettes (1 cm * 1cm * 1cm) were arranged in a 4 x 4 perfect square. Each of them was filled with 1 mL of water. Adequate volumes of desired dyes were added to them from their respective stock solutions to produce the desired colours. Under basic pH, no turbidity nor any emission was seen. Initiation of the pH clock produced turbid solutions, which appeared colour under UV light of 365 nm. The colours were time-encoded and thus vanished after the end of the cycle. The codes can be read through their colours, appearing in sequence from left to right. Each colour represents a digit (0,1, 2, 3). When read from left to right in a single row, it generates a 4-bit code from a single row. This code, when converted to its 64-base code, gives a number that encrypts for an alphanumeric character. In this way, if the entire setup is decrypted at the right time along the time-scale, it reveals a hidden message that dissipates over time. Once initiated, the entire setup was monitored over time and photographed at regular time intervals to capture the necessary change and different codes.



Chapter 5

Parallel Time-Scale Guided Encryption of Information with Broad-Spectrum Transitory Colours from Common Organic Dyes





5.1.Introduction

Potentially accurate information becomes lethal when misused²⁵². The right information, even if handled properly, can be adverse or unproductive if handled at the wrong time. Mishandling or manipulation by counterfeiters poses serious threats to global safety, affecting key sectors such as economics, public health, social integrity, and national security.^{118, 253, 254} In response, extensive research has focused on developing secure data encryption methods to address these risks.^{122, 255-257} Effective strategies enable safe storage, controlled access, and reliable data transmission. An ideal encryption system should ensure secure containment while offering multi-level protection, programmability, adaptability, and practical usability.^{8, 122, 258-261} Substantial efforts have focused on developing advanced anti-counterfeiting 3D colour codes to tackle these challenges.^{8, 16, 20, 139, 262} Similar to quick response (QR) codes and barcodes, these scannable systems offer easy decryption and fast read-out.²⁶³ To avoid overt and immediate interpretation, many designs incorporate stimuli-responsive luminescent molecules/materials, enabling controlled decryption only under specific conditions.^{139, 264-268} Compared to conventional methods, these systems offer notable advantages, including multi-level encryption, condition-specific decoding, fluorescence switching, and hierarchical structural complexity.^{8, 16, 20, 139}

In our previous work, we introduced, for the first time, time as an additional and orthogonal dimension (4D) to significantly advance the security features of three-dimensional (3D) fluorescent scanning codes.²⁰ Conventionally, such systems employ emissive molecular components to impart a cryptic and covert character to the encryption process.^{262, 264-266, 268} The encoded information is typically revealed only under ultraviolet (UV) light exposure, which acts as an activation trigger.^{129, 269, 270} In these systems, data is embedded within 3D colour codes, where the emissive response is perceptible solely under dark conditions upon UV irradiation.²⁷¹⁻²⁷³ Remarkably, the encrypted message resides in plain sight yet remains concealed, as the fluorescent glow becomes visible only under specific spatial and temporal lighting conditions – highlighting the dual camouflage afforded by both the environment and stimulus.^{274, 275}

Despite their success, these systems face several limitations. A) They often rely on fluorescent components – organic fluorophores, polymers, nanoparticles, or quantum dots – typically requiring complex, multi-step syntheses with low yields.^{255, 265, 276-279} Most are hydrophobic, making aqueous processing difficult and necessitating further functionalisation to enhance solubility, adding to synthetic complexity.^{280, 281} B) The emissive behaviour of the fluorophores is influenced by multiple factors apart from the intended external stimulus. This further complicates the design process. As a

result, numerous variables must be carefully considered early on to achieve reliable, stimulus-specific encryption at the application stage.²⁸²⁻²⁸⁵ C) A single fluorogenic molecule generally cannot emit multiple distinct colours without dopants, limiting the variety of possible code combinations.²⁸⁶⁻²⁸⁸ D) Most of these methods rely on UV light for decryption.^{129, 273, 278} Though effective, UV exposure poses significant health risks, requiring stringent safety protocols and making the approach less user-friendly.²⁸⁹

To this end, nature excels at using colour for communication. Many animals change their colours in response to stimuli – squids and octopuses display vivid patterns to hunt, toxic frogs and snails use bright hues as warnings, birds flaunt colourful feathers to attract mates, and chameleons shift shades based on mood or environment.²⁹⁰⁻²⁹² These changes result from precise spatiotemporal regulation of skin pigments in chromatophores, lasting only as long as the stimulus persists.^{170, 291} Though purposes vary, the core intent is to convey information. Inspired by nature, we see coloured organic dyes as a promising alternative to fluorescence-based anti-counterfeiting systems. Traditionally used as indicators in acid-base and redox titrations, these dyes respond quickly and sharply to changes in pH or redox conditions.^{293, 294} By skilfully controlling these fluctuations over time, it's possible to achieve temporal control over colour changes, allowing a broader palette of stable, tunable colours beyond simple, instantaneous shifts.

When governed by pH or redox clocks, these dyes can enable time-encoded information encryption. Unlike fluorescence systems, their colour transitions are visible under normal light, offering safer and more user-friendly decryption. These dyes are also cost-effective, readily available, and avoid complex synthesis routes. Their colour depends on the relative population of two forms with different visible absorption profiles, and balancing the stimulus can yield intermediate hues. Thus, a single dye can produce multiple colours, and combinations of dyes can yield even more, minimising material use while maximising coding diversity. Notably, these systems avoid complications from molecular assembly or aggregation.

In this work, we screened several well-known pH- and redox-responsive dyes commonly used in graduate laboratories and fabricated time-gated, 3D colour codes for anti-counterfeiting encryption.²⁹⁵ These time-encoded systems are regulated by established pH and redox clocks.^{20, 85, 247, 296-300} The transient colour changes across the time scale enabled diverse code combinations and introduced additional layers of data security. A limitation of our method is that the visible colour changes under ambient light may make decryption too obvious. To address this, we introduced solution- and gel-based stimuli-responsive smart windows that concealed the dynamic codes. These

covers revealed the hidden information only at specific time points when triggered, and revert quickly after stimulus withdrawal, restricting information access to a narrow time window.

For the first time, we present a dual time-encoded encryption system where multiple orthogonal time scales operate simultaneously to enhance security. Our approach is entirely synthesis-free, highly adaptable, and modular. The proposed strategies offer a comprehensive framework for data storage, combining sophisticated encryption with simple, cost-effective, safe, and non-invasive decryption.

5.2. Results and Discussions

5.2.1. Stimuli-Responsive Colours in Organic Dyes

Amongst several chemical stimuli, pH and redox changes are the most popular as they can be easily executed, repeated without significant dampening effects, involve inexpensive chemical compounds and are conveniently safe. Moreover, in our case, the stimulus-responsivenesses should have been orthogonal and unrelated to each other. This would direct our larger goal of achieving independently parallel time coordinates that would be operated by separate chemical triggers, not influencing one another. Consequently, we affixed pH and redox triggers for our purpose. The first step towards our goal involved the selection of organic dyes that exhibited pH and redox-responsive colours. In this context, we realised that there is a great deal of common organic dyes used as indicators for acid-base titrations in graduate-level chemistry laboratories. The selection criterion was based on many factors like water solubility, pK_a of colour transition (pK_{in}), response time and number of discernible colours during transition. Some dyes, although water-soluble, exhibited colour changes only under extreme pH conditions that cannot be achieved unless strong acids or bases are used. Again, some dyes displayed only two transitional colours that were not visually distinctive. We looked for dyes that displayed more than two transitional colours in different pH windows of the entire pH region that was encompassed by the TRIS-GdL pH clock that we intended to use (pH 9.0 – 4.0).²⁰

Amongst all the tested dyes, we screened out bromothymol blue (BTB, Fig. 1) and methyl red (MR, Figure 5.1). We recorded their respective UV-Visible absorption spectra in solutions of varying pH (4.0 to 9.0). BTB imparted a blue colour to a basic solution, which transitioned into yellow when the solution was acidified (Fig. 5.1A-B), passing through an intermediate green colour near its pK_{in} (7.0).³⁰¹ On the other hand, MR displayed a yellow, red, and intermediate orange colour above, below and around its pK_{in} (5.1) respectively (Fig. 5.1D-E).³⁰² The absorption spectra of BTB and MR showed

intense peaks at 615 nm and 430 nm above their respective pK_{in} values (Fig. 5.1C and 5.1F). On acidification to a pH below the pK_{in} , the absorption maxima changed to 430 nm (BTB) and 520 nm (MR) with isosbestic points at 500 nm and 440 nm, respectively.

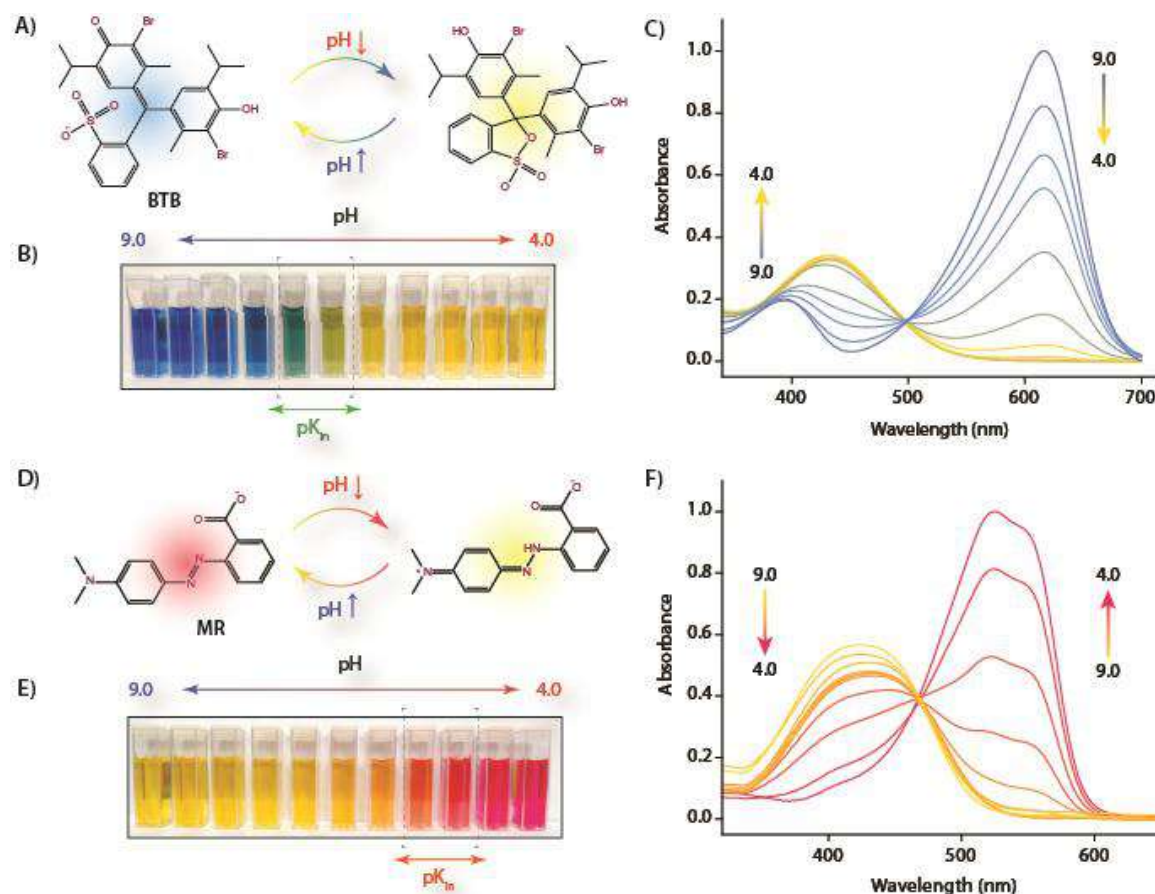


Figure 5.1. pH-dependent structures and colours of halochromic dyes. Acidic and basic forms of A) BTB and D) MR. pH-dependent colours of B) BTB and E) MR. pH-dependent absorption spectra of C) BTB and F) MR. The concentrations of BTB and MR are 10 and 25 μ M, respectively.

Our next pair of dyes was phenolphthalein (PT) and thymolphthalein (TT). Both these dyes showed their respective colours above their pK_{in} but were colourless below it. These were leuco dyes where one of the species was coloured and the other colourless. Although these dyes had pK_{in} values very high in the basic pH, they seemed promising for similar applications with varying properties and were also screened out. PT displayed a bright pink colour above its pK_{in} (8.3) (Figure 5.2A-B) with an absorption maximum at 555 nm (Figure 5.2C).³⁰³ TT exhibited a blue colour (λ_{max} = 595 nm) when the pH was above its pK_{in} (9.5) (Figure 5.2D-F). The absorbances of the respective peaks were reduced as the pH of the solutions reached near the pK_{in} . No peak was observed in the respective colourless solutions having a pH below the pK_{in} .

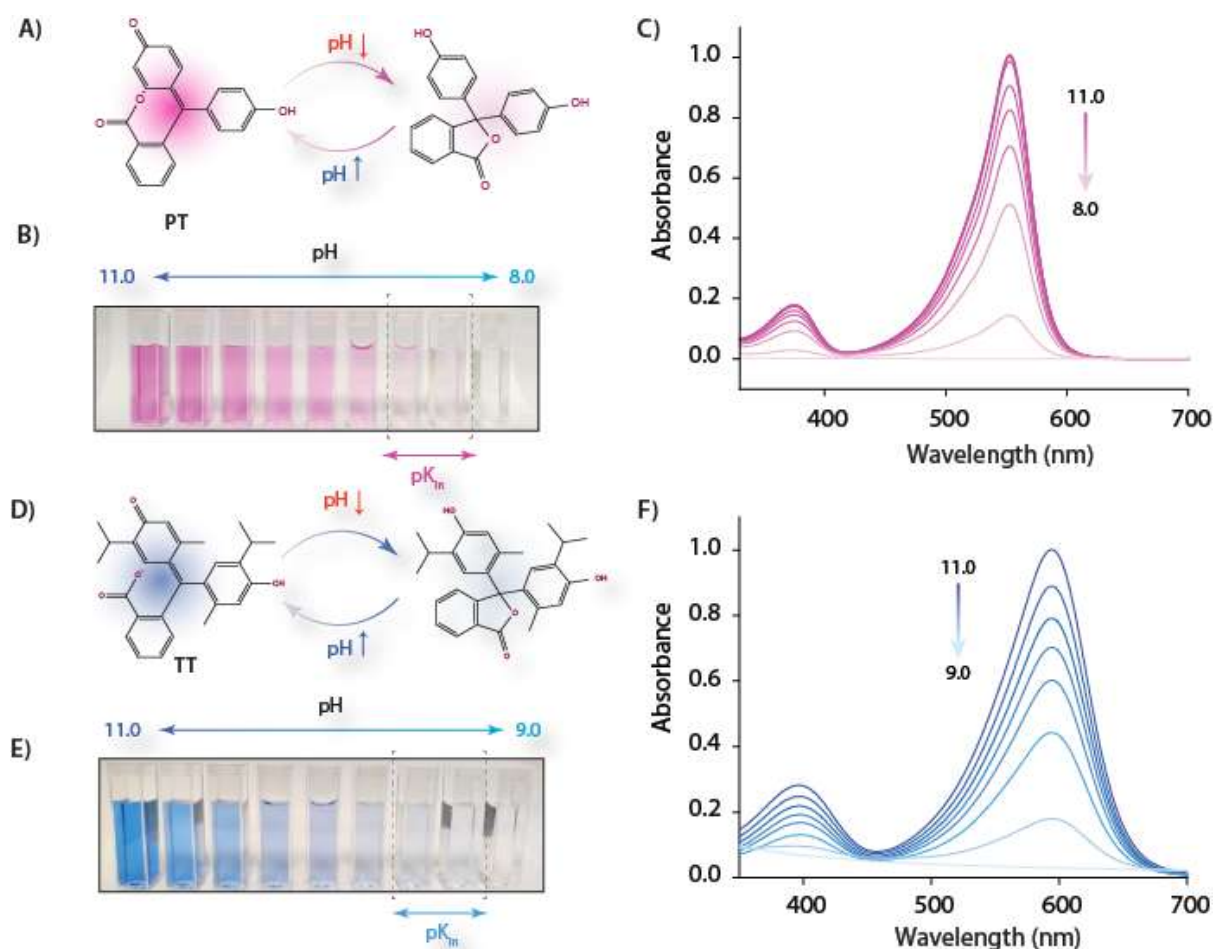


Figure 5.2. pH-dependent structures and colours of halochromic dyes. Acidic and basic forms of A) PT and D) TT. pH-dependent colours of B) PT and E) TT. pH-dependent absorption spectra of C) PT and F) TT. The concentrations of PT and TT are 20 μM , respectively.

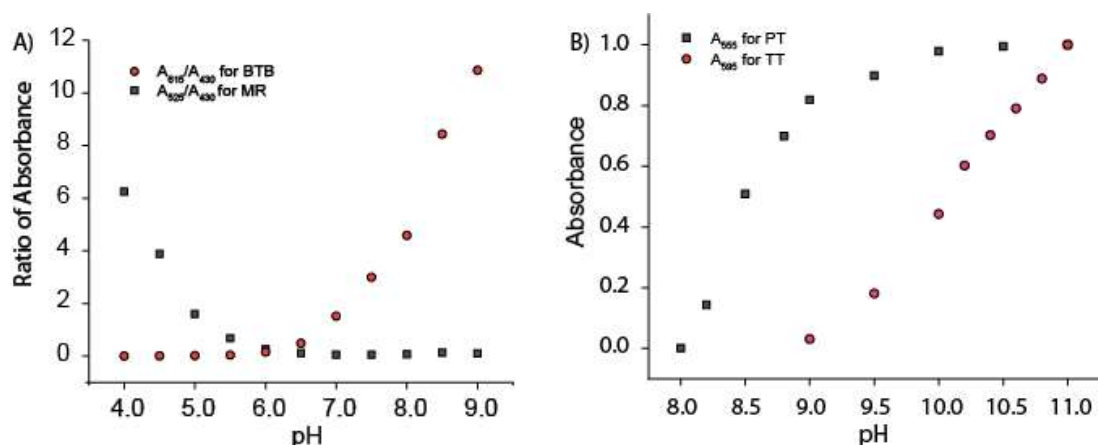


Figure 5.3. A) Ratio of Absorbances for BTB (A_{615}/A_{430}) and MR (A_{525}/A_{430}) in solutions of different pH. B) Absorbances for PT (A_{555}) and TT (A_{595}) in solutions of different pH.

The pH dependency of the colours was better understood when the ratio of absorbances at different wavelengths (A_{615}/A_{430} for BTB and A_{525}/A_{430} for MR) was plotted as a function of pH (Figure 5.3A). For

BTB, it was observed that the change in the ratio was prominent towards the basic or near-neutral region, but the value showed a negligible change in acidic pH. For MR, a reverse observation was noted. Depending on the pH, these dyes existed either in their protonated or deprotonated form, which had different spectroscopic properties and hence showed different colours (Fig. 5.1A, D). The observed colour depended on the population ratio of these species at a particular pH.³⁰⁴ A gradual variation of pH distributed the population abundance of the two species, generating a palette of transitional colours and, in particular, around the pK_{in} . In an ideal case where the pH of the solution exactly matches the pK_{in} of the dye, a perfect intermediate colour should be displayed from an equal share in the abundance of both species, for example, green for BTB.³⁰⁵ For both the leuco dyes, their absorbance values as a function of pH (A_{555} for PT and A_{595} for TT) were altered in the basic region only (Figure 5.3B). TT showed a change at a relatively higher pH (>9.5) than PT (>8.3) because of its higher pK_{in} value. Below pK_{in} , the molecular conjugation was disrupted, leading to a spectral shift that lay outside the visible range, making it colourless.

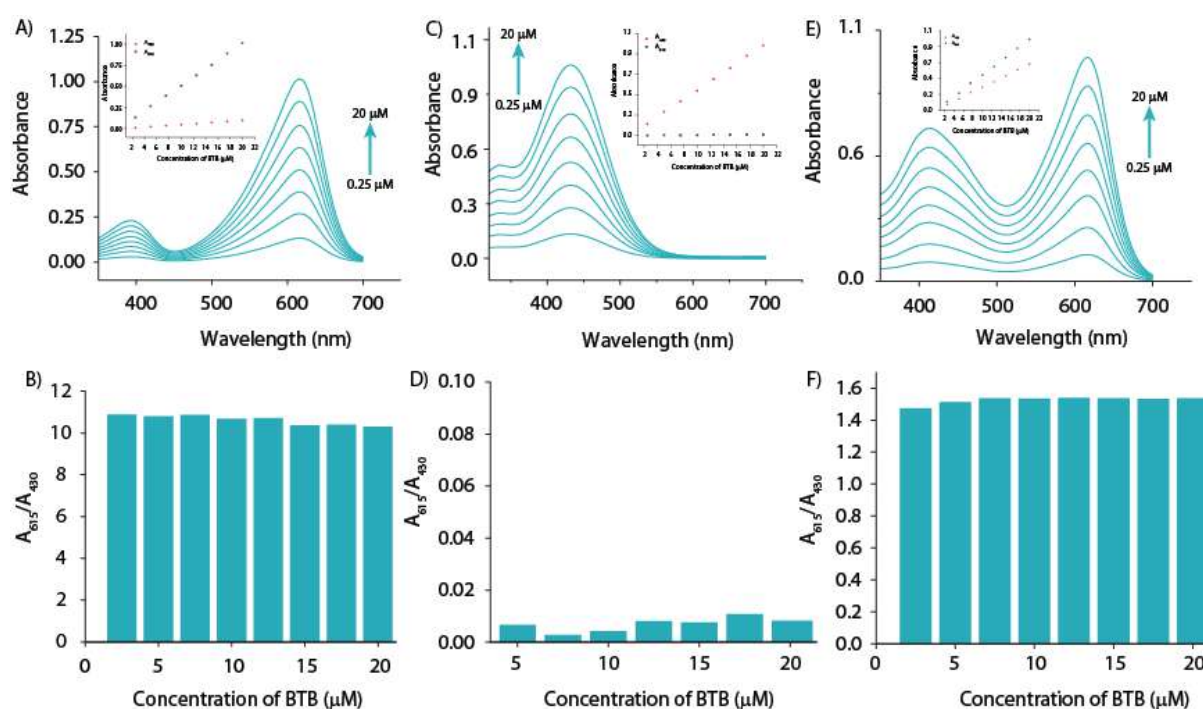


Figure 5.4. The absorbance of different concentrations of BTB A) above, C) below and E) around pK_{in} , with their respective linear fitting for absorbance (A_{615} and A_{430}) versus concentration of BTB in their insets. A_{615}/A_{430} for different concentrations of BTB B) above, F) below and D) around pK_{in} .

To ensure that all four dyes imparted colours depending mainly on the pH of the solution and not on their concentration, we recorded the concentration-dependent absorbance of the dyes (Figure 5.4A-F, 5.5A-H) at different pH levels (above, around and below pK_{in}). For each case, it was observed that the absorbance at different wavelengths increased linearly with concentration at a particular

pH. So, under a fixed pH, if the concentration was increased, the colour of the solution only intensified but remained unaffected as there was no change in the position of the peak (the ratio of absorbance at two different wavelengths did not change). This disapproved the possibility of a concentration-aided assembly that might alter the spectral properties, leading to a change in colour as often witnessed in the case of fluorescence-based molecules.

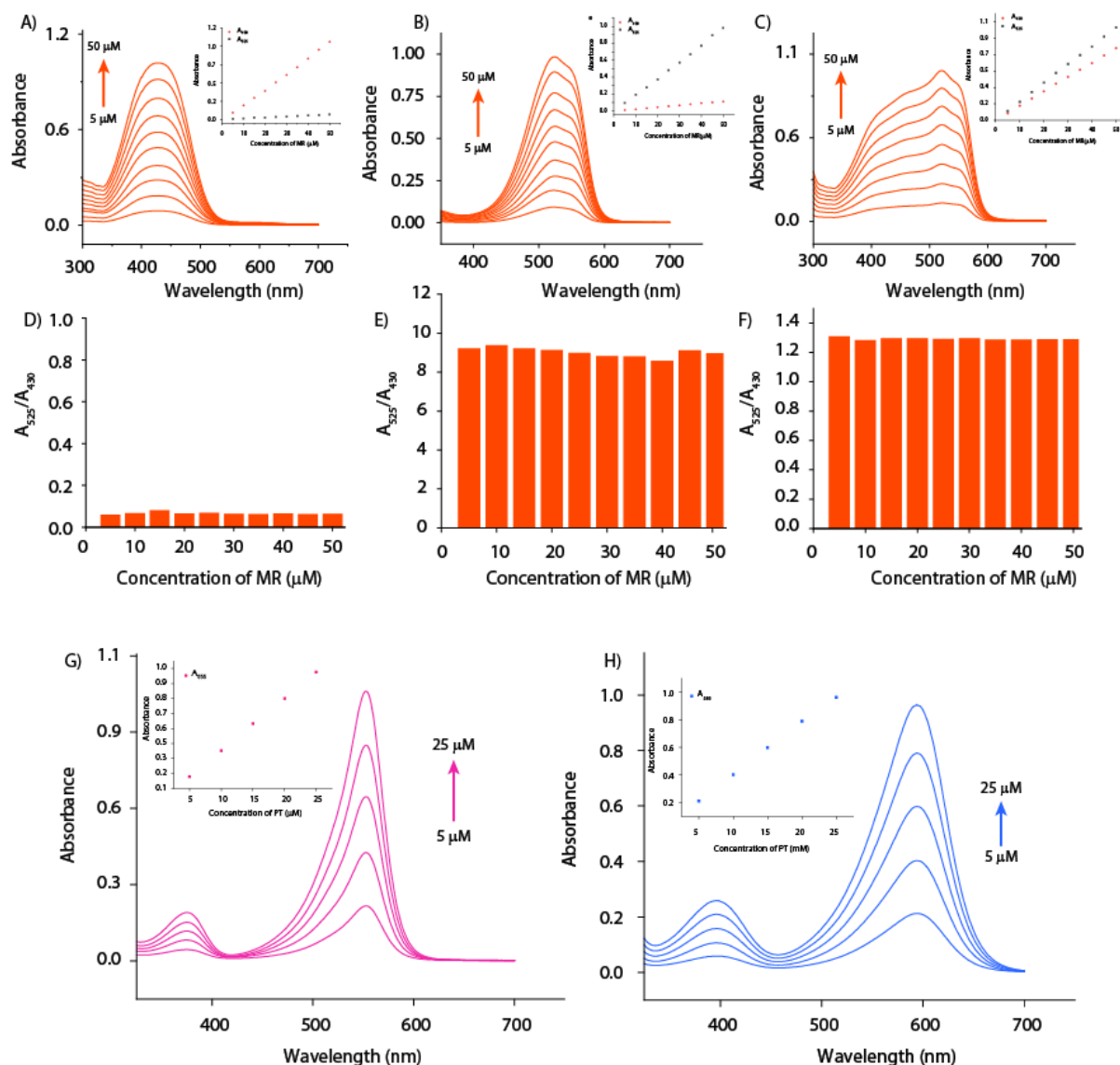


Figure 5.5. The absorbance of different concentrations of MR A) above, C) below and E) around pK_{in} , with their respective linear fitting for absorbance (A_{525} and A_{430}) versus concentration of BTB in their insets. A_{525}/A_{430} for different concentrations of BTB B) above, F) below and I) around pK_{in} . G) Absorbance of different concentrations of G) PT and H) TT above pK_{in} and their respective linear fitting for absorbance versus concentration of PT and TT above pK_{in} in their insets.

Next, we identified two well-established redox-responsive leuco dyes, methylene blue (MB) and dimethyl viologen (MV). MB formed a blue colour in water, whereas MV showed an almost colourless solution in water. Upon the addition of a reducing agent like sodium dithionite (SDT), MB turned

colourless, while MV generated an intense blue colour (Figure 5.6).^{306, 307}. At a fixed concentration of MB, the absorbance at λ_{max} (665 nm, Figure 5.6C, inset) linearly decreased with increasing concentration of SDT due to the reduction of MB. With increasing concentrations of MB, higher amounts of SDT were required to completely decolourise the solution. The reduction of MB by SDT is always accompanied by aerial oxidation of the reduced species. Thus, to keep the solution completely colourless ($A < 0.1$) for a substantial period of time, a huge excess of SDT was required. For example, 50 equivalents of SDT were required to keep MB solutions colourless for more than 5 min (Figure 5.6D). We also ascertained that the peak position for MB under a fixed redox condition did not vary with dye concentration (Figure 5.6E-F), confirming no effect of self-assembly of the molecules.

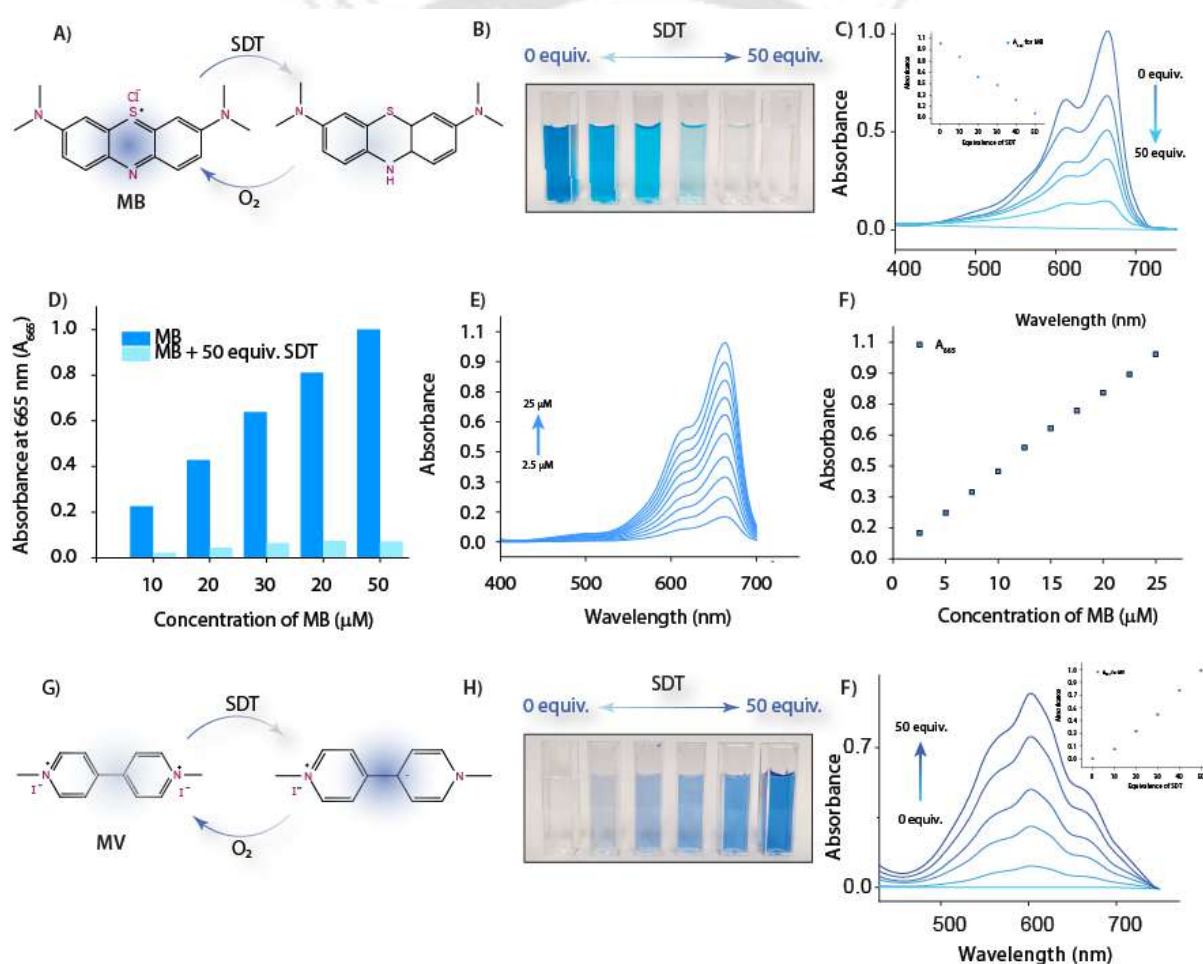


Figure 5.6. A) Structures and colours of redox-responsive dyes MB. Reduced and oxidised forms of A) MB (10 μM) and G) MV (25 μM). Visual colours of B) MB (10 μM) and H) MV (25 μM) under different concentrations of SDT. Absorbance of C) MB and I) MV under different concentrations of SDT with their respective linear fittings in the inset. D) Absorbance (A_{665}) of different concentrations of MB (10 - 50 μM) in the absence and presence of SDT (50 equiv. w.r.t. MB). E) Absorbance of different concentrations of MB in water. F) Linear fitting for absorbance (A_{665}) versus concentration of MB in water.

In contrast to the case of MB, the absorption at λ_{max} (600 nm, Figure 5.6G-I) for MV increases linearly with an increase in SDT concentration.³⁰⁶ The one-electron reduction of MV generated the radical cation, leading to the intense blue colour.³⁰⁶ Like in the case of MB, ~50 equivalents of SDT were required to keep the solution equally intense for 5 min (Figure 5.7A). It is noteworthy to mention that for a sustained change in these redox-active systems, several measures need to be taken care of, like excess addition of SDT, degassing of water and maintaining a closed set-up where the solution is inaccessible to aerial O_2 .

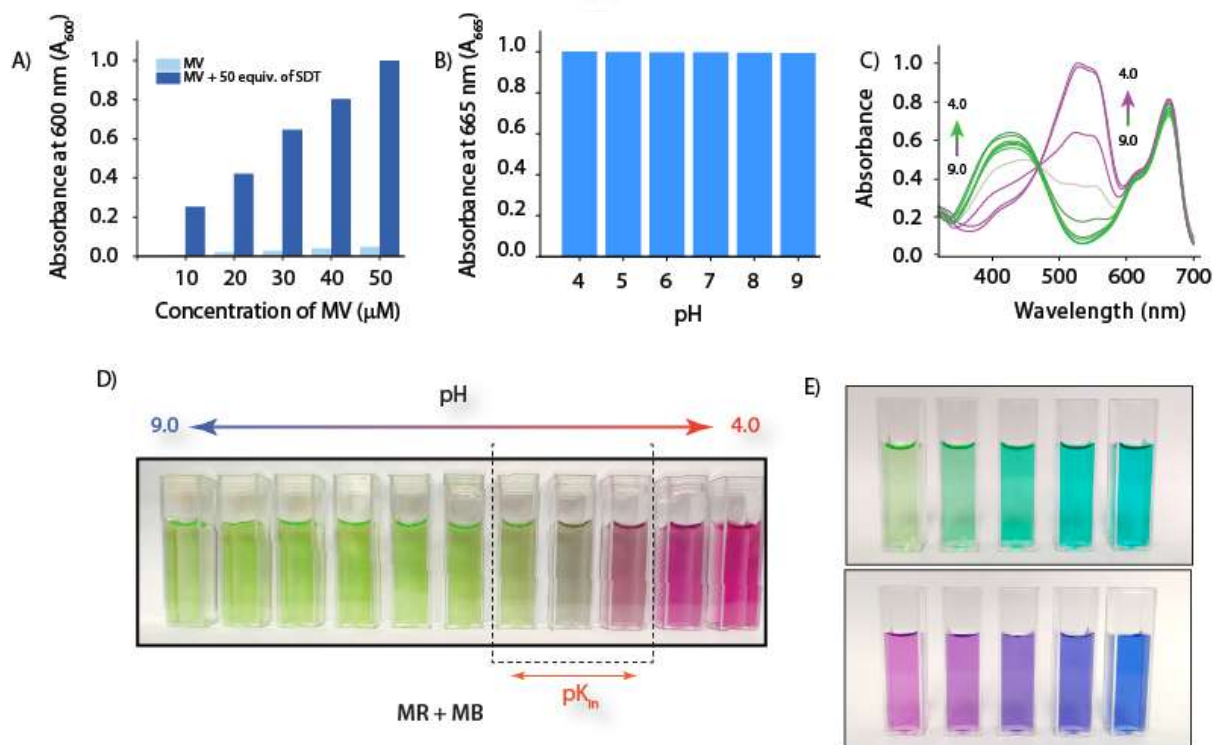


Figure 5.7. A) Absorbance (A_{600}) of different concentrations of MB (10 - 50 μM) in the absence and presence of SDT (50 equiv. w.r.t. MV). B) Absorbance (A_{665}) of MB (50 μM) in solutions of different pH. C) pH-dependent absorption spectra of MR (25 μM): MB (5 μM). D) Solutions MR (25 μM): MB (5 μM) in solutions of different pH. E) Different shades of green and purple generated by varying ratios of MR: MB in the solutions of basic (above) and acidic (below) pH. MR is 25 μM in all solutions. MB varies from 5 to 9 μM from left to right in each case.

Next, we confirmed that the absorption profile of a fixed concentration of MB was independent of the solution pH (Figure 5.7B). We presumed that if MB could be combined with a pH-responsive dye that did not show much spectral overlap with MB, then a separate set of recombinant colours could be produced in a solution that still had not been achieved in our colour palette. In this regard, MR could turn out to be a good candidate because it did not show any significant peak under any pH in the absorption region of MB. On adding MB (5 μM) to a solution of MR (25 μM), different colours were obtained in the pH range of 4.0 to 9.0 (Figure 5.7C-D). Above the pK_{in} of MR, the solutions

appeared green instead of yellow because of the presence of blue MB. Below the pK_{in} of MR, the solutions displayed a purple colour instead of red. When the pH of the medium was varied for this mixture (Figure 5.7D), as expected, no change in the absorption at 665 nm (arising from MB) was observed. However, the absorptions of MR peaks varied depending on the pH. This combination of dyes generated a new set of colours in solution without the introduction of any new dyes to the list. Though the colours could be varied by varying the ratio between the concentrations of MR and MB (Figure 5.7E), we stuck to the ratio of MR (25 μ M): MB (5 μ M) for further studies. It is worth mentioning that for all these dyes, the formation of different species due to the change in pH or redox reactions was confirmed either by 1H NMR and the data and related discussion are provided (Figure 5.8).

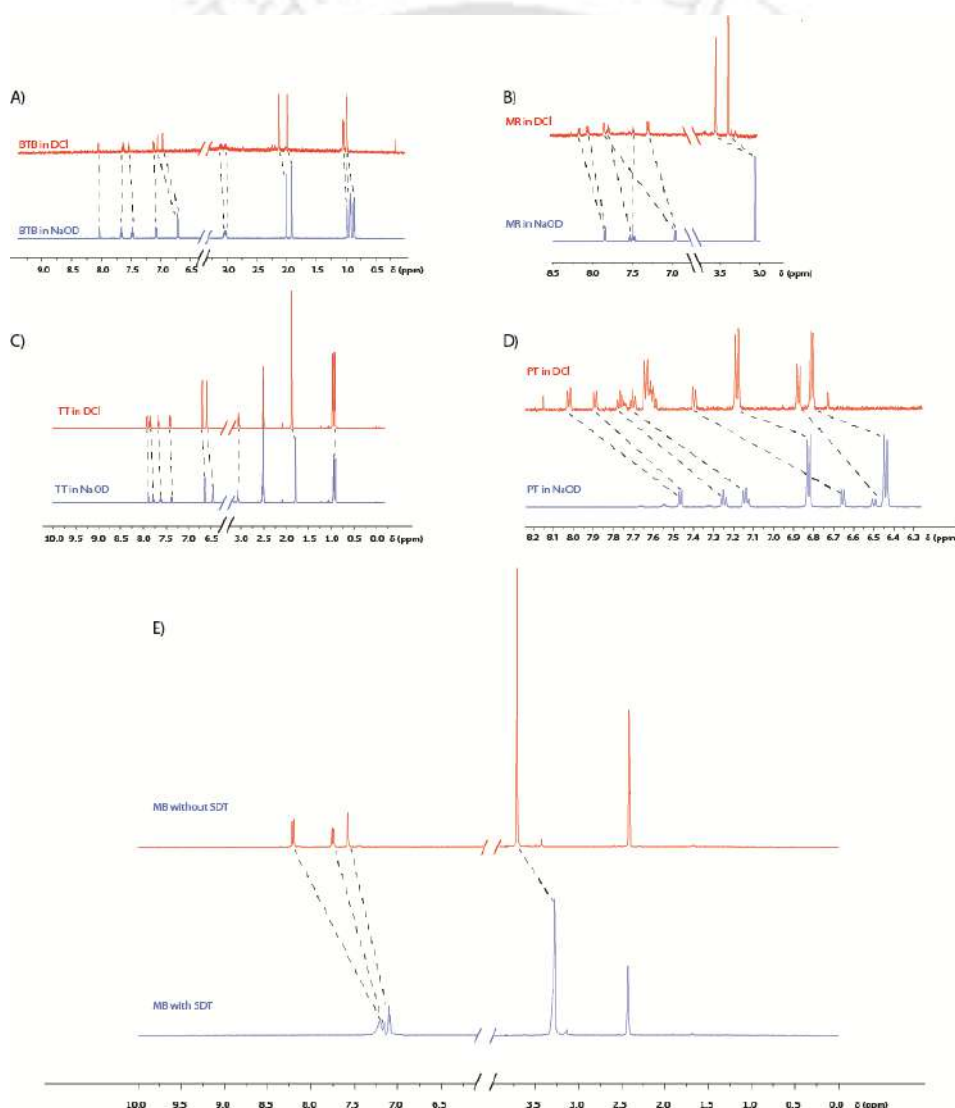


Figure 5.8. 1H NMR Spectra of A) BTB in D_2O , B) MR in D_2O , C) TT in $DMSO-d_6$, D) PT in D_2O in acidic and basic pH. E) 1H -NMR Spectra of MB in the absence and presence of SDT in 30% $ACN-d_3$ in D_2O . The shift in the signals indicates the pH and redox-dependent structural changes in the molecules. MV generated a radical cation on reduction that gave no signals in the aromatic region.

5.2.2. pH Clock Guided Transitory Colours

As we screened out our dyes, we focused on developing transitory colours. For this purpose, we chose our already established TRIS-GdL pH clock.²⁰ This pH clock comprised a fast activator (TRIS buffer pH 9.0) and a slow deactivator (GdL). When both components were added as chemical triggers in the solution, a time-regulated pH variation (pH 4.0 to 9.0 to 4.0) was generated. TRIS buffer (2 mM) quickly raised the pH of the solution while GdL (5 mM) followed a slow saponification kinetics and brought the pH down gradually over time (90 min) (Figure 3.4A, B). The pH clock could be repeated several times, and by varying the TRIS: GdL ratio, the duration of the cycle can also be modulated.

From the pH-dependent experiments (Figure 5.1, 5.2), it was clear that for both BTB and MR, the desired colour changes occurred within the pH range (4.0 – 9.0) that was covered by the TRIS-GdL pH clock (Figure 3.4B). The time-controlled transition in colours (from blue to yellow, passing through the green for BTB; from yellow to red via orange for MR) was clearly observed. The time-dependent absorption spectra were recorded for BTB, MR and MR: MB solutions under the influence of the TRIS: GdL pH clock. For BTB, as the pH clock started (~pH 9.0), the peak at 615 nm appeared, and the absorbance of the peak gradually decreased over time. After a certain point, the peak at 430 nm started appearing, which intensified till the end of the pH cycle (~pH 4.0) (Figure 5.9A). A similar effect was observed for MR, where the 430 nm peak decreased while the 525 nm peak increased with the progress of the pH clock (Figure 5.9B). For both dyes, the changes in the absorbances of the peaks showed sigmoidal curves as a function of time with crossover points of ~ 6 min and ~19 min for BTB and MR, respectively (Figure 5.9C-F). These crossover points indicated the attainment of the pK_{in} of the individual dyes in the pH clock. The appearance of the crossover points at different time zones was mainly because of the different pK_{in} of these two dyes and the differential hydrolysis kinetics of GdL at different pH.³⁰⁸ The hydrolysis followed a slower rate in the acidic zone, while it was much faster in the basic pH. Thus, under the pH clock, BTB reached its pK_{in} (7.2) much faster compared to MR (5.1). So, it can be concluded that using the same pH clock, we have produced two transitory colour programs that followed the same temporal route, but exhibited colour changes at different time regions of the pH clock and also covered the entire visible range. In the context of our final goal of information encryption, it is important to note from the that though the pH clocks were separately initiated at the same time, the blue-to-yellow (BTB) colour transition was completely over before the yellow-to-red (MR) transition started. Next, we moved to the mixture of dyes with the hope of getting the missing green-to-purple transition during the temporal colour program. To achieve that, we mixed MB and MR and applied the pH clock. Since MB was independent of pH, the

system only showed a change in absorbance of the peak belonging to MR (Figure 5.10A-B). The solution appeared green as the pH clock was initiated ($\sim$ pH 9.0) and ended as purple ($\sim$ pH 4.0) towards the completion of the clock. The transition time scale followed that of MR.

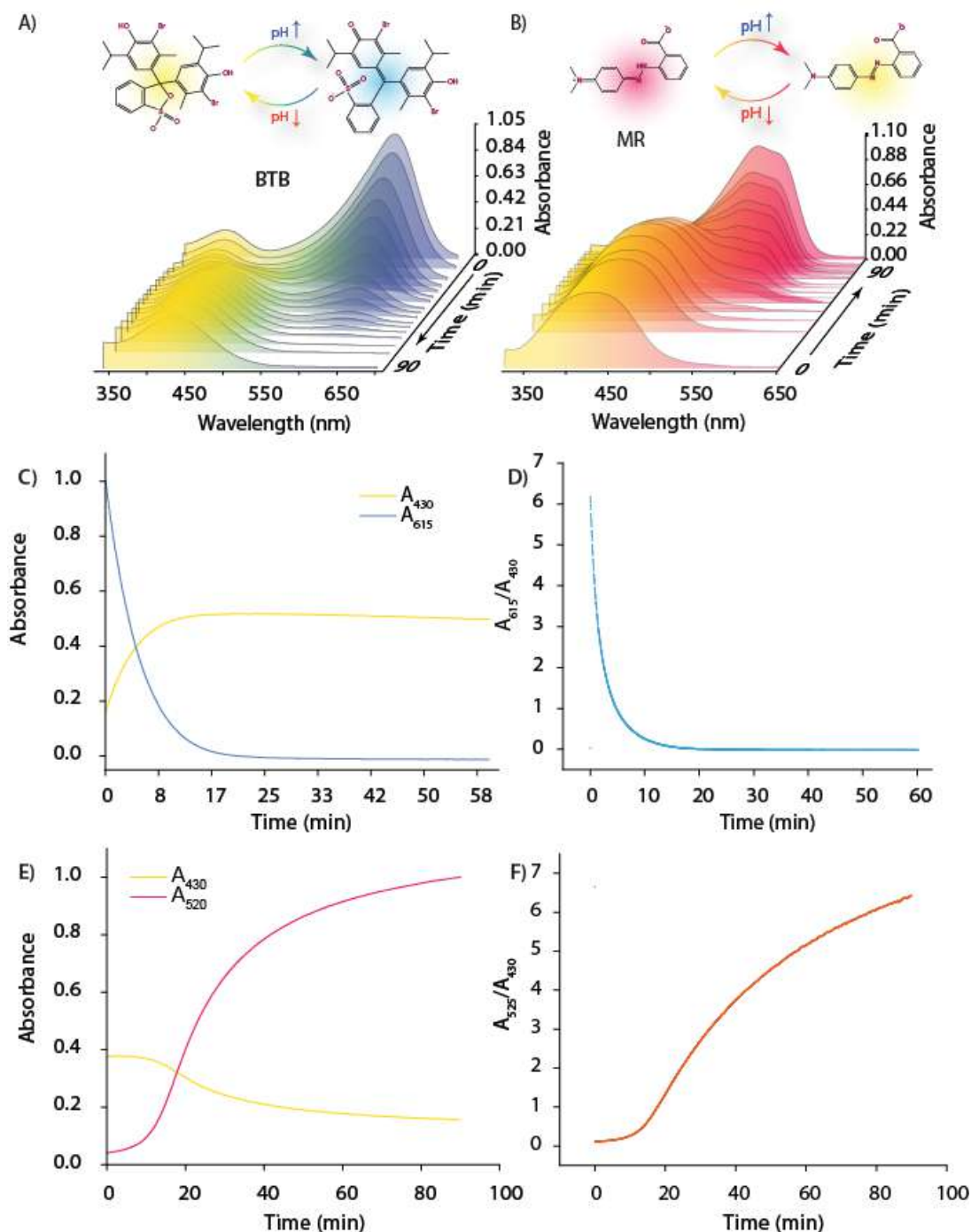


Figure 5.9. Time-dependent absorption spectra of A) BTB (10 μ M) and B) MR (25 μ M) under the TRIS-GdL pH clock. C) Absorbance at 615 nm (A_{615}) and at 430 nm (A_{430}), and D) Ratio of absorbance (A_{615}/A_{430}) as a function of time for a BTB solution under the TRIS-GdL pH clock. E) Absorbance at 525 nm (A_{525}) and at 430 nm (A_{430}), and F) Ratio of absorbance (A_{525}/A_{430}) as a function of time for an MR solution under the TRIS-GdL pH clock.

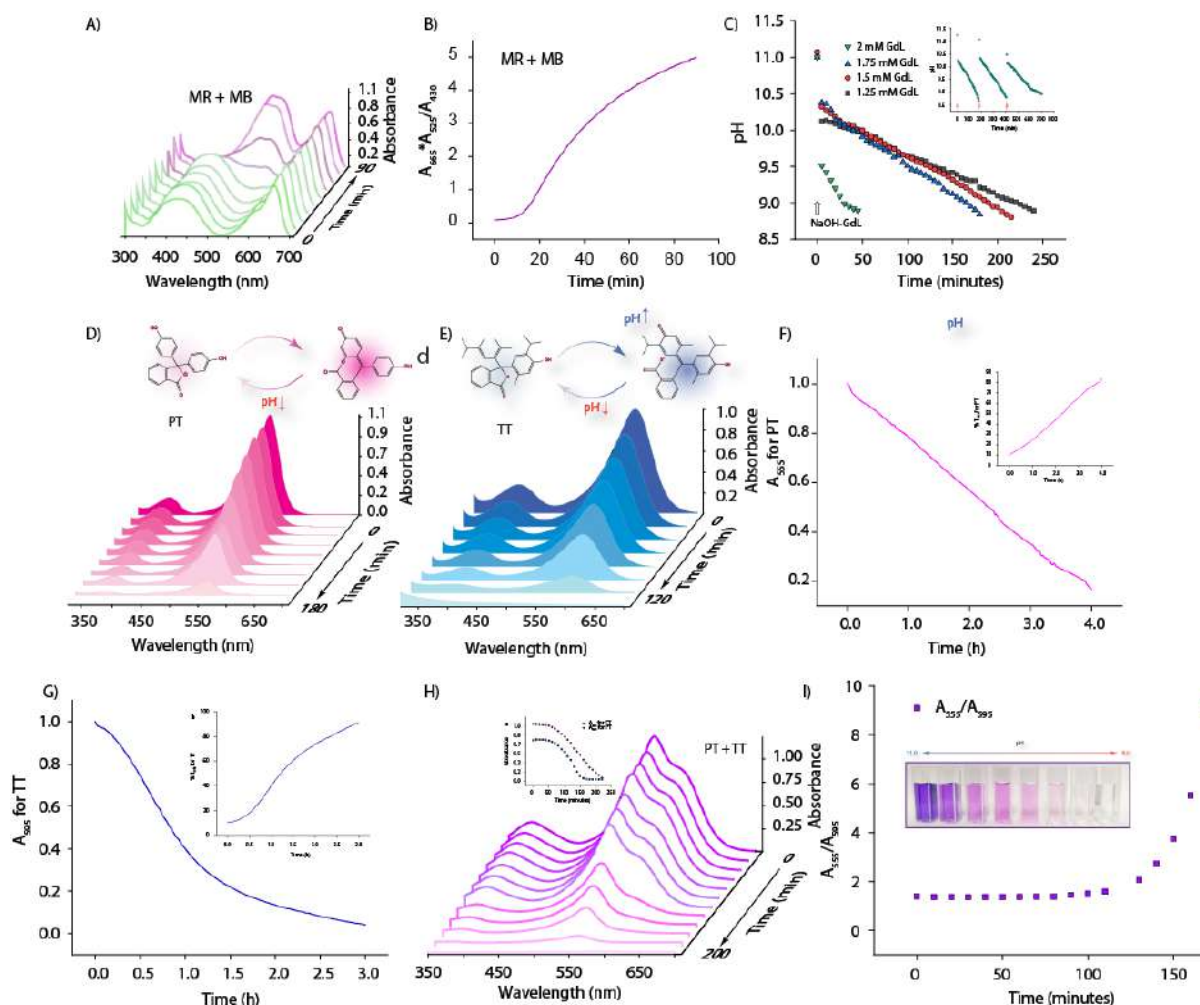


Figure 5.10. A) Time-dependent absorbance and B) Variation of function $A_{665} \cdot A_{525} / A_{430}$ with time for MR: MB in a solution under the TRIS-GdL pH clock. C) pH vs time profile for NaOH-GdL clock with different concentrations of GdL. 2 mM NaOH was used for each cycle. Inset - Repeatable pH vs time profile for NaOH-GdL pH clock with addition of fresh NaOH-GdL trigger after every cycle, indicated by the arrows. Time-dependent absorption spectra under the pH and redox clocks for D) PT (20 μ M) and E) TT (20 μ M) under the NaOH-GdL pH clock. F) Absorbance at 555 (A_{555}) for PT and G) Absorbance at 595 nm (A_{595}) for TT against time under the NaOH-GdL pH clock. H) Time-dependent absorption spectra of an equimolar mixture of PT and TT in solution under the NaOH-GdL pH clock. Inset - Time-dependent absorbance at 555 nm (A_{555}) and at 595 nm (A_{595}) for PT and TT mixture under the NaOH-GdL pH clock.

Moving to the leuco dyes, PT and TT, we realised that although their colour transitions occurred at basic pH, the TRIS-GdL pH clock was not suitable for these systems, as the pK_{in} values lay beyond this pH clock range. We felt the most convenient way to achieve a high pH was to add a strong base, such as NaOH. Adding NaOH (2 mM) to water swiftly raised its pH to a very high value of 11.0 (Figure 5.10C). In the same solution, when GdL is added, there is a gradual drop in the pH, similar to the TRIS-GdL clock. The NaOH-GdL pH clock operated at a higher pH window than the TRIS-GdL clock,

as NaOH, being a strong base, acted as a sharper and more far-reaching activator that pushed the pH up to extreme values. Again, since a strong base was directly used, a buffer action was not possible. This allowed us to use only a low concentration of GdL to pull the pH down. If a high concentration of GdL was added, due to the absence of buffer action, there was a sudden decrease in the pH. The immediate dip in the pH was attributed to the burst kinetics in the hydrolysis of GdL, which was assisted by a very high pH in the presence of NaOH.³⁰⁹ Hence, to manage a controlled pH decrement, a low concentration of GdL was permissible as the deactivator (1-2 mM GdL). Based on our optimization, for our subsequent studies, we selected NaOH-GdL (2:1.75 mM) combination that registered a pH of 8.8 from 11.0 over 3 hours and was repeatable for multiple cycles (Figure 5.10C inset).

Next, PT, TT and their mixtures were subjected to this newly developed NaOH-GdL clock. For PT, the addition of NaOH-GdL quickly generated a pink colour (555 nm), which very slowly diminished over time with the decrease in pH (Figure 5.10D).³⁰³ For TT, a similar peak was generated at 595 nm, resulting in the blue colouration that dissipated with time to become colourless. However, the decolouration was much quicker in the case of TT owing to its higher pK_{in} (9.5) (Figure 5.10E). To further prove the complete decolourization, the changes were monitored through transmittance that provided similar results (Figure 5.10F-G inset). It was expected that in the case of the mixed dyes, TT would lose its colour before the complete loss of colour from PT. This was exactly what happened when a solution containing both dyes was studied under the pH clock (Figure 5.10H). A violet colour was displayed, arising from the combination of blue (TT, λ_{max} = 595 nm) and pink (PT, λ_{max} = 555 nm). With time, though both the absorption peaks reduced in intensity, the 595 nm peak disappeared faster, leading to a situation where the system showed a transition from violet to pink to colourless (Figure 5.10I inset). The visual changes in the pH clock-guided transitory colours of these leuco dyes were recorded.

5.2.3. Redox-Clock Guided Transitory Colours

For the redox-responsive dyes, we utilised the well-established SDT-O₂ cycle.^{13, 297, 299} When performed in an open environment, in this cycle, SDT reduces the species having a suitable reduction potential, and with time, the influx of aerial and soluble O₂ balances the conditions back to an oxidative environment.^{13, 297, 299} The lifetime of the reduced species and, hence, the overall cycle depends on the amount of SDT added. When SDT was added to a solution of MB, the absorption maxima at 665 nm vanished instantly (Figure 5.11A). The peak and the blue colour reappeared after a lag phase. However, the reappearance was almost instant instead of a gradual increase in

absorbance and colour. This indicated that the redox transformation was very quick and sharp. This was also supported by the time-dependent absorbance and transmittance studies (Figure 5.11B). The redox cycle was repeatable by subsequent additions of SDT, and the duration (lag phase) of colourlessness was directly proportional to the concentration of SDT added (Figure 5.11C-D).

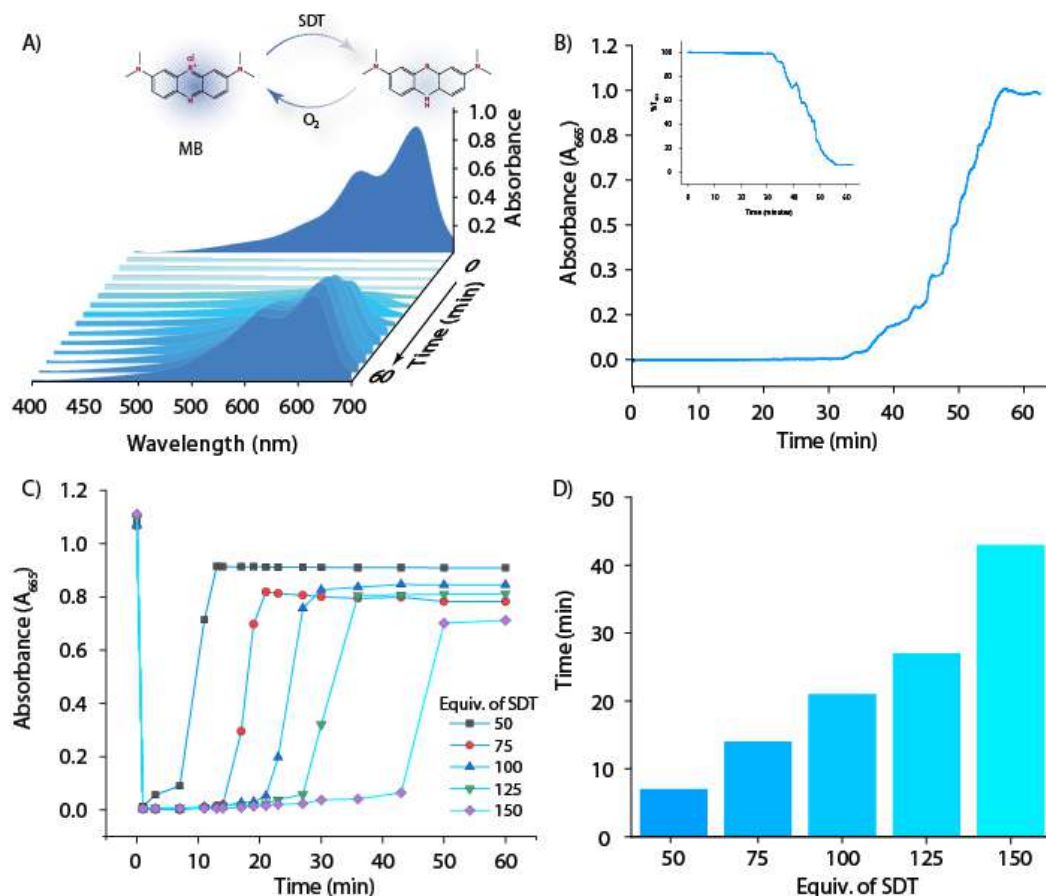


Figure 5.11. Time-dependent A) absorption spectra of MB (10 μ M), B) absorbance at 665 nm (A_{665}). Inset - transmittance at 665 nm (%T₆₆₅) for MB under SDT- O_2 redox clock. C) Time-dependent absorbance at 665 nm (A_{665}) for MB under SDT- O_2 redox clock with varying concentrations of SDT. D) Lifetime of decoloured MB (lag phase, $A < 0.1$) with varying concentrations of SDT.

On the contrary, in the case of MV, a peak at 600 nm and the blue colour appeared instantly upon addition of SDT (Figure 5.12A). After a while, the peak and the colour disappeared quickly once the SDT was consumed. This was also reflected in the time-dependent absorbance and transmittance at 600 nm (Figure 5.12B). The transient colour could be reproduced over multiple cycles, and the lifetime of the coloured species could be related to the concentration of SDT (Figure 5.12C-D). Hence, with a common SDT- O_2 redox clock, we could establish a time-regulated colour transition of two dyes, but in an opposite sense of appearance and disappearance of colours.

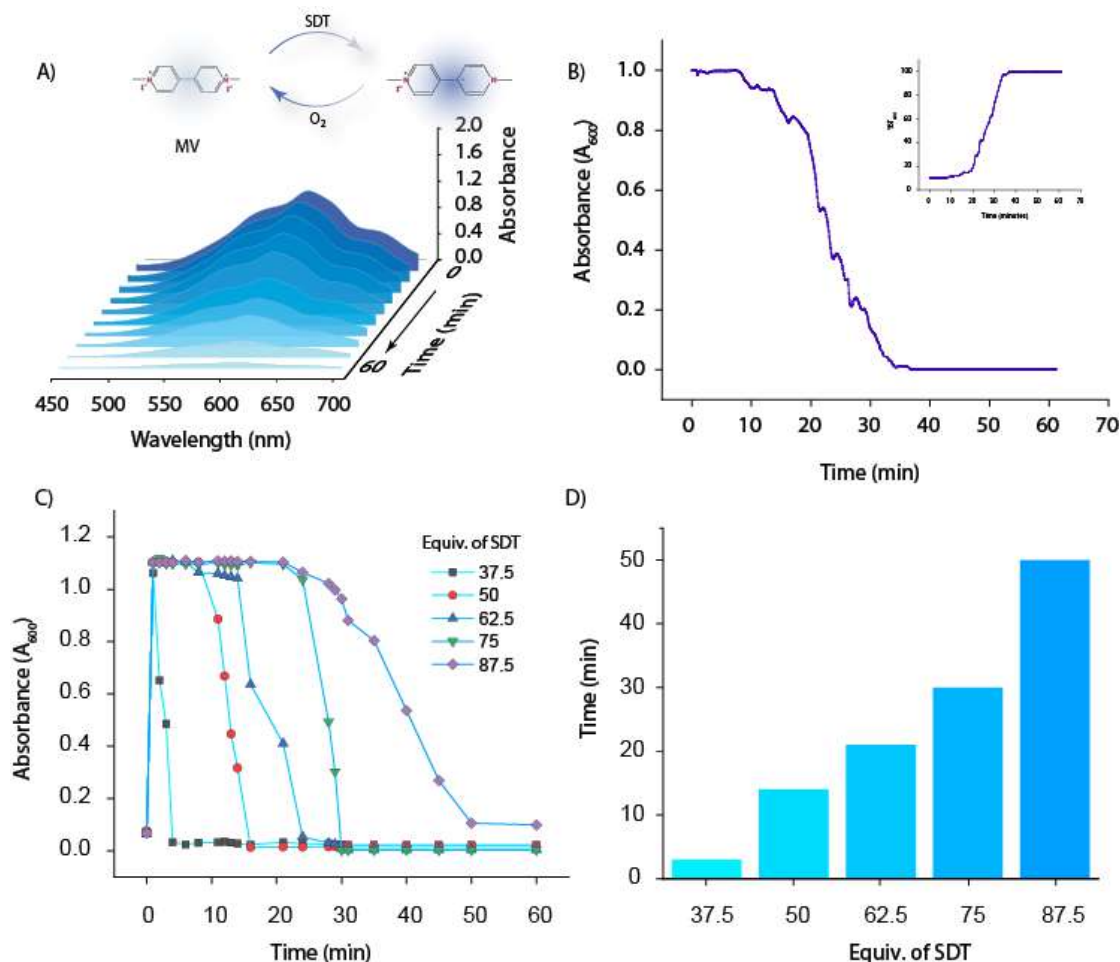


Figure 5.12. Time-dependent A) absorption spectra of MV (10 μ M), B) absorbance at 600 nm (A_{600}). Inset - transmittance at 600 nm (%T₆₀₀) for MV under SDT- O_2 redox clock. C) Time-dependent absorbance at 600 nm (A_{600}) for MV under SDT- O_2 redox clock with varying concentrations of SDT. D) Lifetime of decoloured MV (lag phase, $A > 0.1$) with varying concentrations of SDT.

5.2.4. Time-Gated 2D to 3D Information Transformation with pH Clock-Instructed Transitory Colours from the Leuco Dyes

On successfully identifying dyes whose colours can be manipulated in a time-regulated fashion under the operation of a pH or redox clock, our final task was to implement these features in designing time-encoded information encryption strategies in colour codes. Our first attempt was to construct an encryption method similar to our previous work²⁰ but without the implementation of UV light for decoding purposes. To demonstrate a multi-dimensional information encryption system, we relied on the pH clock-guided colours from the leuco dyes (Figure 5.13). Herein, we arranged a QR code at the background of the colour code formed by the spatial arrangements of cuvettes containing the pH-responsive leuco dyes. Under normal scenarios, the QR code was clearly

visible and served as a source of direct two-dimensional (2D) information (State A). When the pH clock was not initiated, the aqueous solutions of the dyes were colourless and transparent and thus the information conveyed in the QR code at the backdrop of the arrangement was distinctly apparent (State A).

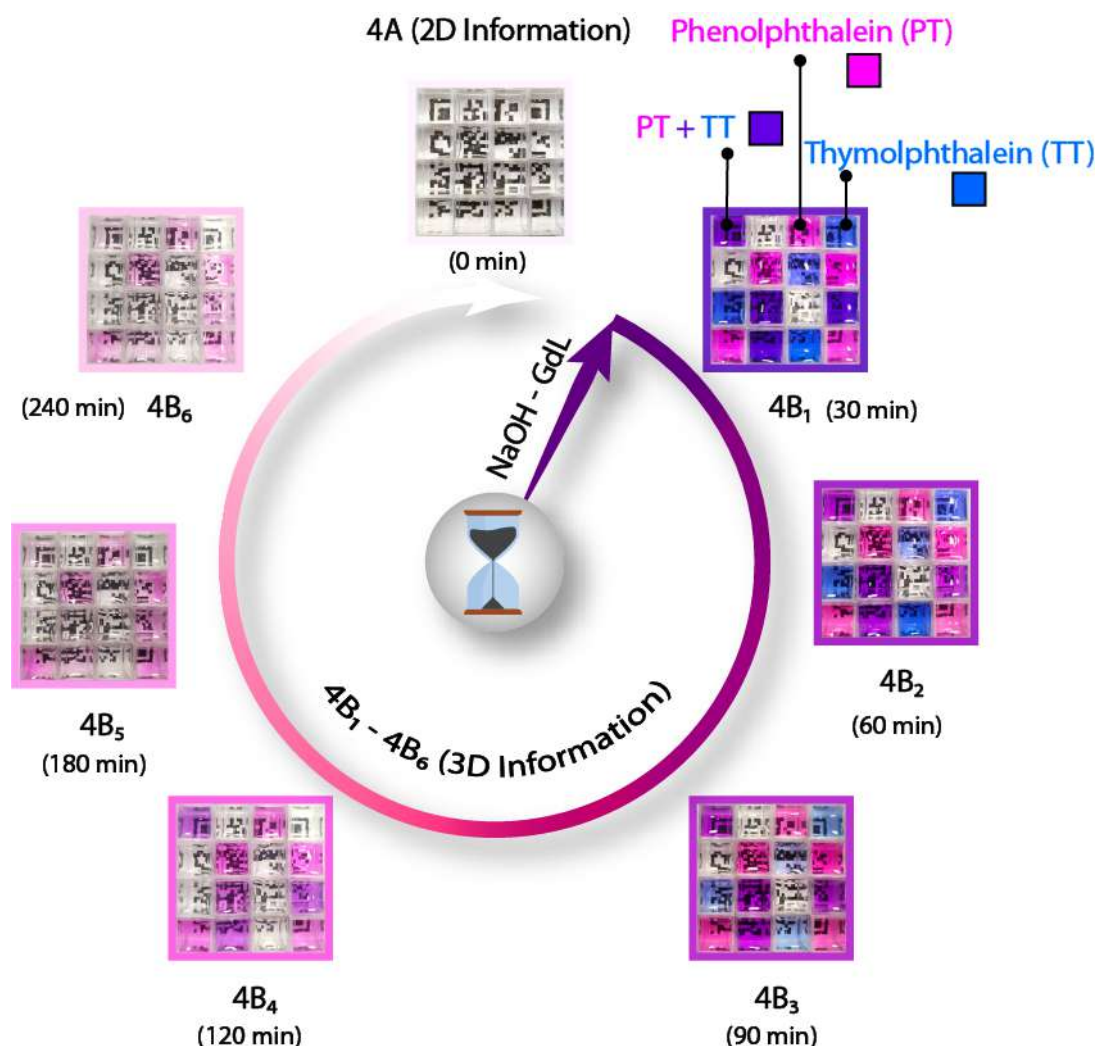


Figure 5.13. Time-dependent images of different 3D transitory colour codes generated from leuco dyes at different time points of the NaOH-GdL pH clock. On-set of the trigger is indicated with the purple arrow. Addition of the trigger to State A converts 2D information to 3D information (States B1 to B6). The dyes and their combination used for each colour is indicated against the box of that respective colours.

The addition of the NaOH-GdL trigger started off the pH clock at once. A three-dimensional (3D) colour code was generated instantaneously, constituting a spatial arrangement of pink, blue, purple and white cubes that originated from PT, TT, a mixture of PT - TT, and a solution with no dye, respectively (State B1). A piece of new information was now available from the 3D colour code in addition to the 2D QR code that was always present behind it. Since the 3D colour code was an

outcome of the pH clock, it obeyed a time-locked property which introduced time as an added curtain of protection. Time operated as a fourth dimension (4D) that directed the lifespan of the 3D information (States B1 to B6). Along the timescale, the information encoded was not stagnant; rather, it morphed and altered its pattern continuously. If observed, one can notice that the parent code did not vanish at once but went through many intermediate codes (States B2-B5) to reach the final, distinctly different pattern (State B6). All these colour changes were due to the different lifetimes of colours generated from the dyes under the same pH clock as discussed before (refer to section, pH Clock Guided Transitory Colours). TT transited through its higher pK_{in} faster than PT. Thus, even when the blue cuvettes (B1 - B3) turned colourless (B4 - B6), the pink ones still held their colours (B1 - B6). The purple cuvette's transition into pink generated a new code in combination with the already pink present (B3 to B4). As the pH clock reached its completion, the transitory codes also disappeared, leaving behind the original state (State A). All the solutions in the entire arrangement were again colourless with the 2D code perceived in clear vision, and the entire system was ready for another run of the pH clock (State A). In this system, we achieved a simple multi-dimensional message encryption method having multiple levels of hierarchical security, including a time-gated behaviour and a decryption route that did not demand UV light.

5.2.5. Time-Locked Switching of Colour Codes of Opposing Senses with Redox Clock

We aimed to design a single system that can store two different pieces of information with opposite senses. The idea was to convert the initial message to the opposite one in a time-gated way. To achieve this, it was essential to switch the position of a pair of colours at the beginning and at the end of a cycle. If that can be executed, then a code at the beginning of a cycle will flip over to another code that would be the opposite colour. To understand this, let us treat blue and white hypothetically as a pair of opposing colours. Therefore, if a code 'b' having blue and white regions switches over into a code 'd' (Fig. 5 Inset) where the blue and white regions have exchanged their places, then codes **b** and **d** can be considered opposing codes. So, if the code **b** encrypts for 'YES' and it transforms it into **d** that encrypts for 'NO' after the addition of a trigger, then it can be considered that two conflicting messages have been accessed on the time scale with a single arrangement. At the end of the timeline, the code 'b' is regenerated. We utilised the redox clock to achieve this. Here, we have considered two pairs of opposing colours – blue and white as one opposing pair while green and yellow as the other (Figure 5.14). The blue colour was generated either from MB or the reduced MV, while the reduced MB and oxidised MV provided the white colour. Similarly, the yellow was obtained from MR (basic form) and its opposing colour, green, was generated from the mixture of MR with MB or reduced MV. In this approach, the initial arrangement

(State A, Fig. 5) comprised blue, white, green, and yellow colours. All the dyes were in an equilibrium oxidative environment. When the redox clock was initiated in each cuvette (State B1) by the introduction of SDT, the blue cuvettes switched colours to white while the green ones switched to yellow. This happened because MB was decolourised, MV was reduced to a blue colour, and MR remained unaffected. The respective changes remained intact over time as long as SDT was present in the solution and a non-equilibrium reductive environment was maintained (State B1 – B7). As the SDT was consumed by oxidation, the codes gradually transitioned into their original pattern (State B8, which resembled State A). The chemically triggered fast colour exchange between the colour pairs got sorted back into their original positions. We obtained a time-locked encryption strategy where information of conflicting sense (code) could be reached in the same timeline.

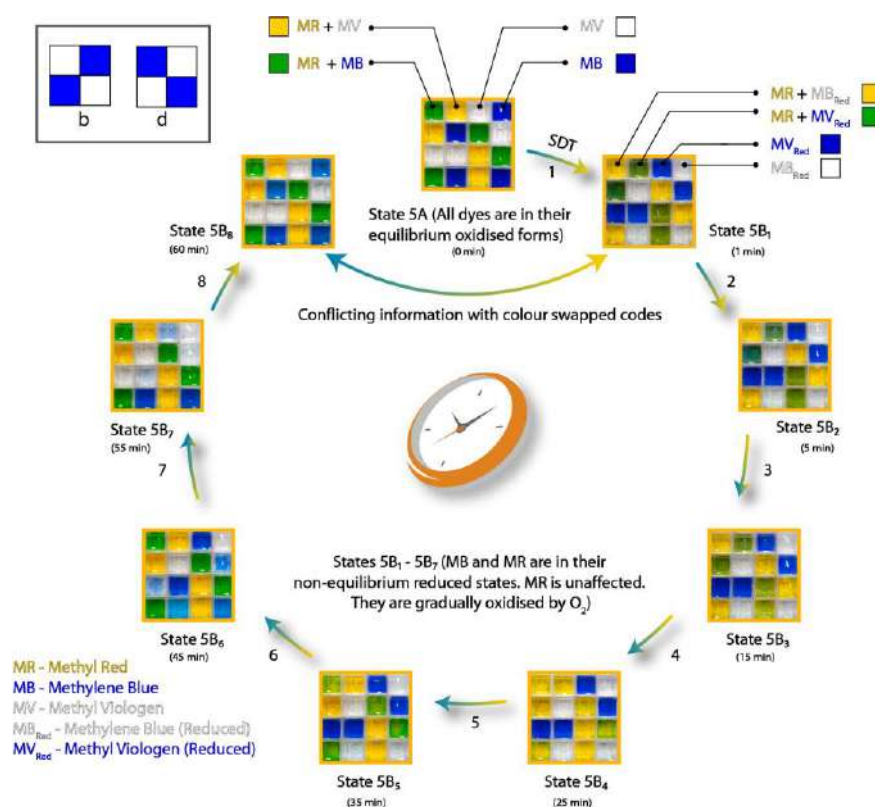


Figure. 5.14. Time-dependent images of different 3D transitory colour codes encoding conflicting messages generated from dyes at different time points of the SDT-O₂ redox clock. The inset shows schematic representations of opposing codes 'b' and 'd' with the reversed position of colours. Arrow 1 indicates the addition of the SDT trigger to initiate the redox clock. State A represents the initial equilibrium condition under an oxidative environment. States B1-B7 represent the non-equilibrium redox states under reductive conditions.

5.2.6. Dual Timeline Guided Message Encryption Regulated by Orthogonal pH and Redox Clocks

We envisioned that we could create novel and more advanced avenues in anti-counterfeiting strategies if we focused on manipulating the time coordinate. We anticipated that if a common arrangement can be modulated back and forth between two colour codes over time, or if a single code is partially omitted and regenerated at different time points of the long timescale, then a more progressive system can be constructed with advanced features. The NaOH-GdL pH clock constituted a longer time span, but the SDT-O₂ was more temporally flexible and adaptable. As a result, our motive could be realised by implementing both the pH and redox clocks in the same array (Figure 5.15). We organised the cuvettes in the usual 4 × 4 pattern. In some, we added MB or PT, and the rest were left as they are (only water). This generated a pattern with only blue and white (State A1). Under neutral conditions, PT remained colourless in water, while MB generated a blue colour. In this state, all the solutions were in their stable equilibrium condition. When NaOH-GdL was added as a trigger in the white (colourless) cuvettes (only water or PT), a pink colour was immediately produced in those that had PT (State B1). Now, a complete parent colour code originated from the spatial position of blue, pink, and white cuvettes. To introduce the concept of a secondary or dual time scale, a calculated amount of SDT was added to the blue cuvettes (with MB), decolourising them (State C1). This deleted the parent colour code partially (State C1–C2). As the SDT was exhausted in a short while, the blue colours reappeared, and the parent code was again re-established (State B2). The SDT-O₂ cycle ran over a substantially shorter time scale (State C1 – C2; State C3 – C5) than the pH clock that was continuously running in the pink cuvettes (State B1 – C5). The entire code could be partially omitted and reproduced multiple times throughout the longer pH cycle by repeating the redox clocks. Herein, the SDT-O₂ clock introduced 'shorter time loops' (State C1 – C2; State C3 – C5) of imperceptible information amidst the longer central pH clock (State B1 – C5). The redox clocks ran as shorter parallel time coordinates that pushed the complete codes away into an incomplete state where the true information could not be accessed. The original (similar to state B1) information could only be visualised when these smaller loops merged back into the main timestream. These smaller zones of inaccessibility on top of the overall time scale added an extra layer of protection. When the central pH clock ended (State A2), the parent code (State B1) was eliminated, as there were only blue and white cuvettes with no pink (similar to State A1). In this approach, we obtained an information encryption method which was more advanced than the previous setups. In addition to the absence of UV light, the entire system was more secure because, in an overall time-locked encryption, there are mini time zones of data inaccessibility. Further, even more complex arrangements could be made if redox cycles of different timescales are initiated for different blue

cuvettes at the same time. These blue colours would reappear after different durations in the 'shorter time loops', creating different codes step-wise as they emerge back into the main code. To the best of our knowledge, there are no reports where dual time scales have been implemented from two independent and non-interfering temporal functions in executing a single physical outcome.

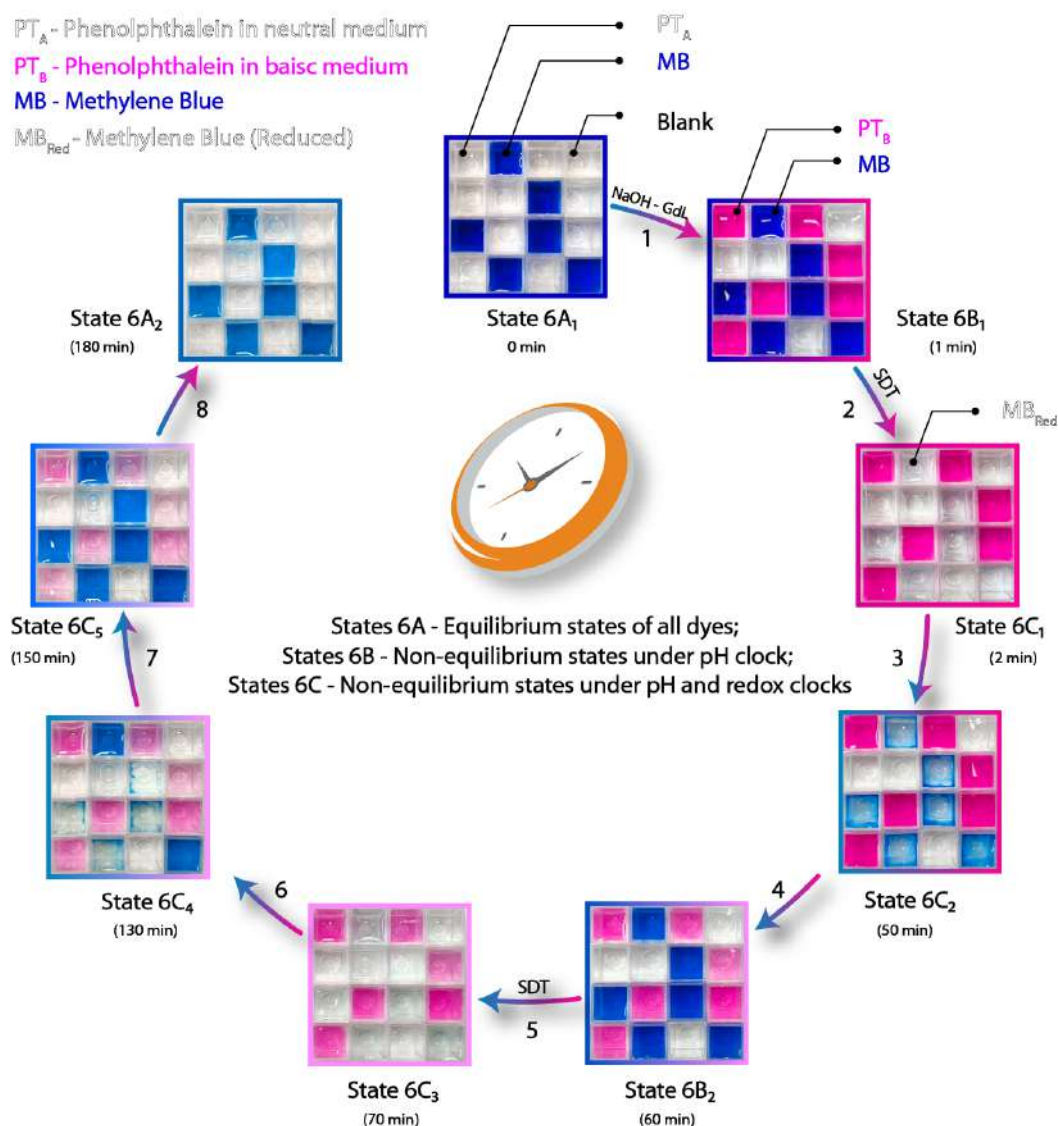


Figure 5.15. Time-dependent images of different 3D transitory colour codes encoding messages generated from dyes at different time points under the NaOH-GdL pH and SDT-O₂ redox clocks. All states A are under normal equilibrium conditions. All states B indicate conditions where only pH clock is operational. All states C indicate conditions where both pH and redox clocks are running simultaneously.

5.2.7. Parallel Time-Scale Encryption via pH-Clocked Transient Colours Shielded by a Smart Window

We have already addressed message encryption techniques that have the attributes of multi-level, hierarchical and time-encoded security. However, in our achievement, we have made a small compromise in the covert and cryptic nature of the encryption technique. When UV light is used for decryption, usually the codes are invisible in normal light. We felt the need to introduce an added layer that would be an alternative to the UV light. The decryption route could then be modified into an upgraded version. We envisioned that an alternative could be a chemical or physical smart window in the path of vision that needs to be triggered with an orthogonal stimulus to open it, so that the underlying code could be seen. We have designed a smart window-protected information encryption method with multi-level security, having a dual parallel time-encoded behaviour. First, we ran a simple TRIS-GdL pH clock-guided colour code in a time-gated manner (Figure 5.16). We used BTB, MR and a combination of MR and MB to generate a colour code that had an ample number of colours so that we could produce innumerable combinations from only three dyes. When the pH clock was triggered from the initial state I all the dyes and their combinations showed their respective colour in their basic pH (State B1). As the GdL was hydrolysed, the dyes showed their respective colour transitions (State B1 to A4). Since BTB exhibited a faster colour change than MR in the TRIS-GdL pH clock regime, it was observed that the blue cuvettes containing BTB only showed the transitions, while the yellow and green cuvettes containing MR showed no observable colour changes at the early stages (States B1 – B3, basic to near neutral range). By the time blue cuvettes turned completely yellow, the other cuvettes started showing their respective changes in the middle phase of the pH clock (States C1 – C4, neutral to slightly acidic zone). Finally, in the end, the remaining cuvettes also showed their colour changes from yellow to red and from green to purple (States A1 to A4, acidic region). This constructed a pH clock-regulated information encryption that displayed multiple colour codes along the time scale. It could be noticed that no two adjacent codes visually appear different (for example, B1 and B2 or C1 and C2), but any two codes that were well spaced in the time scale exhibited significantly different colours (for example, B1 and C2).

Thus, multiple visually non-identical codes could be generated along the time scale of the pH clock that could securely store information and varied messages. Importantly, this time-gated process generated a vast array of CIE coordinates or RGB combinations, thereby enabling the creation of an extensive number of unique codes. We developed a method to decrypt these codes by scanning with an indigenous program. After applying the appropriate triggers to the system, the camera took a picture at a particular moment. Following their extraction from this image, the RGB combinations

were mapped to the URL www.iitg.ac.in. Then, another cycle started, and the scanner continuously scanned the system from the beginning of the decryption process. The scanner immediately connected to the specified output link after capturing the correct image at the exact moment that corresponded to the pre-assigned code. This example shows a straightforward and efficient method for decoding.

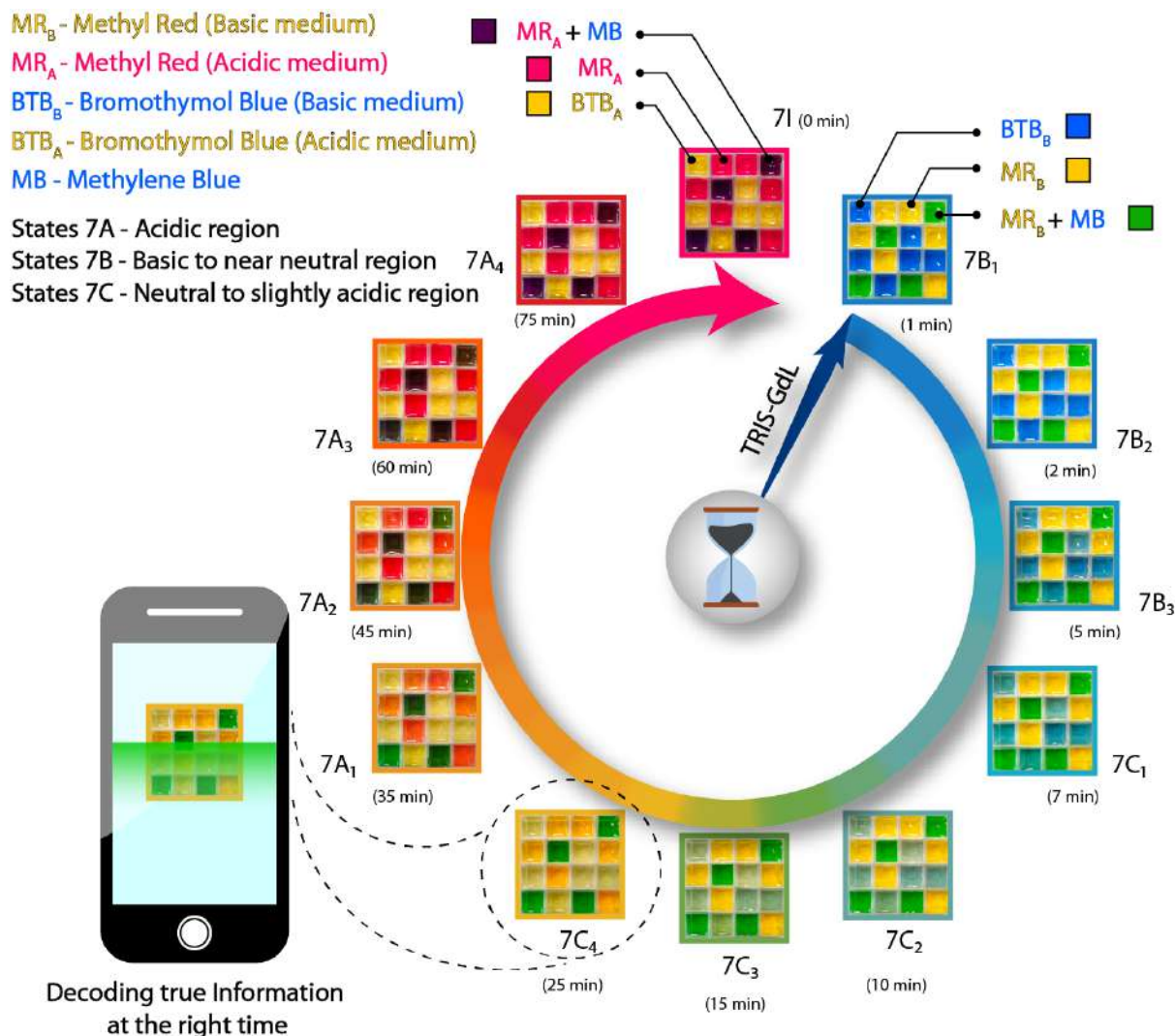


Figure 5.16. Time-dependent images of different 3D transitory colour codes encrypting information generated from dyes at different time points under the TRIS-GdL pH clock. The blue arrow indicates the addition of the trigger for the onset of the pH clock.

To uplift the security standard, we introduced a very simple design for a chemical screen as an addition to our previous setup of Figure 5.16 (Figure 5.17). Firstly, we had the entire arrangement under a slightly acidic medium (Figure 5.17, State I). The TRIS-GdL trigger was added (Arrow 1) to initiate the pH clock, and a petri dish containing water was placed on the top of the colour code arrangement (State A1). The pH clock triggered a new colour code that was clearly visible through

MR_B - Methyl Red (Basic medium)
 MR_A - Methyl Red (Acidic medium)
 BTB_B - Bromothymol Blue (Basic medium)
 BTB_A - Bromothymol Blue (Acidic medium)
 MB - Methylene Blue
 MB_{red} - Methylene Blue (Reduced)

8I

TRIS-GdL 1

8A₁

8B₁

8C₁

8D₁

8E₁

8F₁

8B₂

8B₃

8C₂

8C₃

8C₄

8D₂

8D₃

8D₄

8E₂

8E₃

8E₄

21

16

15

14

13

12

11

10

9

8

7

6

5

4

3

2

1

SDT

TRIS-GdL

MB

MB_{red}

BTB_B

MR_B

MR_A + MB

BTB_A

When repeated (Arrow 7) after some time (State C1), the blue screen again vanished (State C2). A new colour code (State C3) was visible underneath, which was quite different from the previously observed code (State B3). This, too, was short-lived because the redox clock very quickly shut the screen (State C4 to C1). The colour codes beneath the screen continuously kept morphing into another (similar as seen in Figure 7) because of the running pH clock (from state A1 to E4 or from arrow 1 to 20). Hence, each time (State B1, C1, D1 and E1) the shorter redox clock (B1 to B3, C1 to C4, D1 to D4 or E1 to E4) was operated, a new code was seen behind the shield. The entire route could be retraced by re-initiating the pH clock in the cuvette arrangement underneath and replacing the MB screen (Arrow 21).

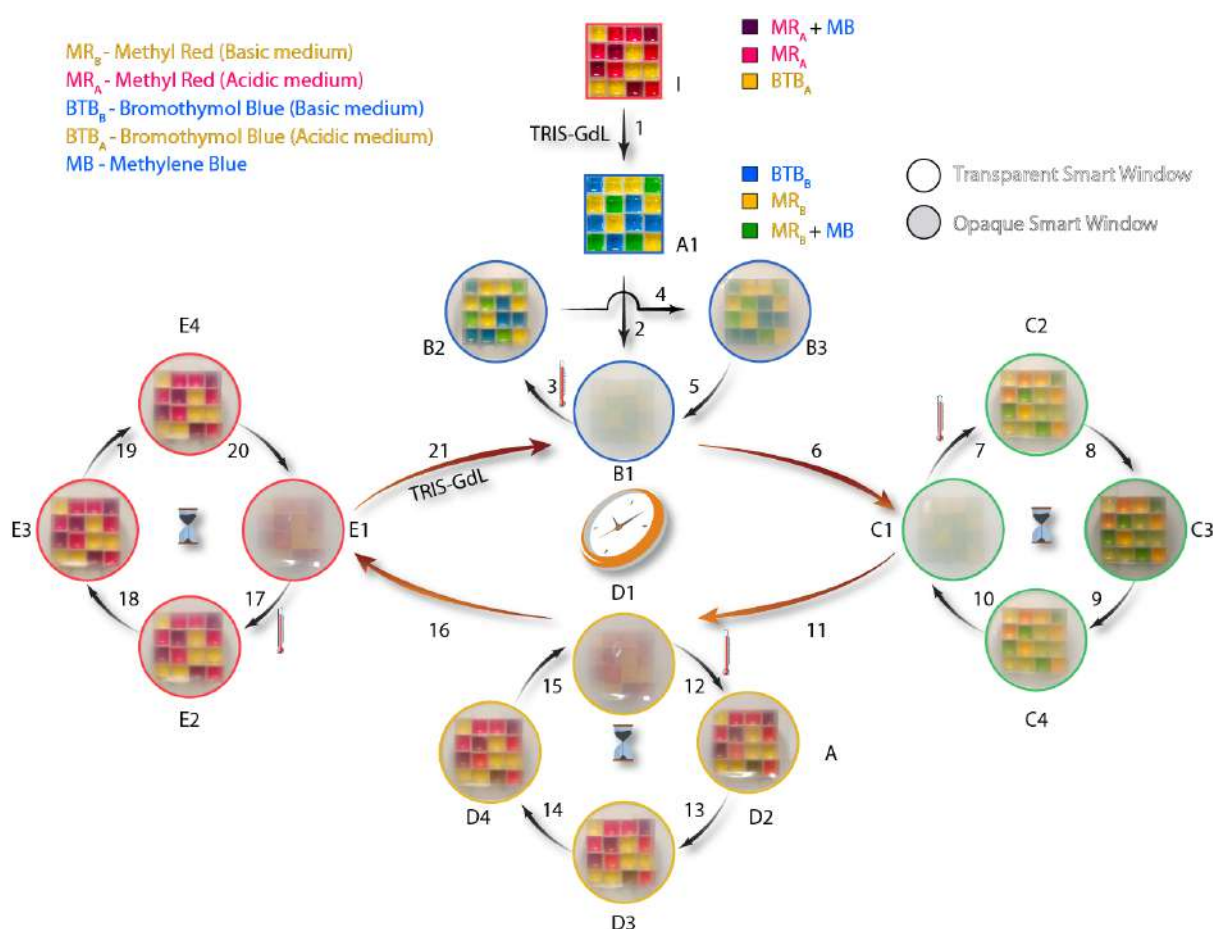


Figure 5.18. Parallel time-scale encryption via pH-clocked transient colours shielded by thermo-responsive smart window. Time-dependent images of different 3D transitory colour codes encoding messages generated from dyes at different time points under the parallel time scales of a primary TRIS-GdL pH clock and a secondary thermo-responsive smart window hydrogel.

The same demonstration was also executed using a smart window that was responsive to a physical stimulus (temperature) instead of a chemical stimulus (Figure 5.18). For this purpose, we fabricated an already reported polyacrylic acid/acrylamide and hydroxypropyl cellulose-based temperature-

responsive hydrogel.³¹⁰ The hydrogel remains opaque at room temperature and upon heating becomes transparent. This thermo-responsive hydrogel could act as a smart window similar to the MB solution in the previous case. However, in this case, heat (increase in temperature) acted as the trigger. As can be seen in Figure 5.18, a similar arrangement was made as the previous setup, but herein the protection was removed by heating the hydrogel whenever required. Heating at different time points revealed the different underlying codes at that time, similar to the case of Figure 5.16. The absorbed heat was soon dissipated, and the temperature dropped, making the material regain opacity and hiding the codes.

The reason for choosing such a thermo-responsive material for the smart window application is because of its popularity in the biomedical field, robotics and optics.^{311,312} Amongst various types of external stimuli, physical parameters like temperature/heat are highly coveted because they do not involve additional substances, introduce no extra unwanted wastes, are easy to handle and dissipate over time into the surroundings without adversely affecting them. This dissipation of heat occurs over time once the source of the stimulus is removed and can be reapplied multiple times without much fatigue or hazard. Thus, if the extra layer of protection can be controlled by manipulating a factor like temperature, then it will form a more advanced and intricate method of decryption.

The strategies (Figure 5.16, 5.17 and 5.18) were endowed with salient features that made them unique and advanced. A) Keeping in parity with our previous strategies, this, too, had attributes of multi-dimensional, hierarchical, time-encoded levels of security. B) Without compromising all these features, we could eliminate the use of harmful UV light. C) The decryption style preserved the cryptic, secretive, and covert nature of the process. D) For the decryption process, different orthogonal stimuli were used. E) In the course of designing a discrete decryption technique, we have split the time dimension (4D) into two independent and orthogonal time components, which can be considered as the fifth dimension (5D).

5.3. Conclusions

In conclusion, we have developed a multi-dimensional data encryption strategy using simple acid-base and redox chemistry of common organic dyes. Carefully selected based on their pH- and redox-responsive colour-changing properties, these dyes were studied under stimuli-responsive and chemically triggered non-equilibrium conditions. This enabled the generation of dynamic, time-programmed transitory colour changes – either from single dyes or their combinations – producing a rich palette of visible colours not typically observed in conventional fluorophore-based systems.

Importantly, these colours remained unaffected by concentration-dependent aggregation or quenching effects often seen in conventional fluorophores. Leveraging these time-controlled chromatic transitions, we designed a series of anti-counterfeiting encryption techniques, each unique in its design and functionality. With each successive method, we enhanced the complexity of encryption without compromising operational simplicity. Despite the intricate encoding-decoding processes for heightened security, the methods remained user-friendly and easily executable. All techniques were time-encoded, introducing time as the fourth and fifth dimensions to fortify data protection. We avoided UV-dependent decryption, maintaining cryptic integrity through stimuli-responsive smart windows. These smart windows also enabled the introduction of orthogonal, dual time scales for enhanced security. Looking ahead, we believe these organic dye-based strategies can be extended to practical, safer applications for time-regulated secure data repositories. The next challenge lies in translating these approaches into functional materials. Nevertheless, this work lays the foundation for innovative, secure, and adaptable data storage systems with transformative potential.

5.4. Experimental Section

5.4.1. General Information and Materials

All dyes used in the work and N,N'-methylene bis-acrylamide were purchased from Tokyo Chemical Industry (TCI) Chemicals (India). GdL, acrylic acid (AAc), hydroxypropyl cellulose (HPC), hydrochloric acid and all deuteriated solvents used for NMR were purchased from Sigma-Aldrich (USA). Acrylamide (AAm), and ammonium persulfate (APS) were purchased from SRL. TRIS and NaOH were purchased from Merck. Milli-Q water with a conductivity of $< 2 \mu\text{Scm}^{-1}$ was used for all sample preparations and experimental measurements. UV-vis spectra (absorbance and transmittance) were recorded on a PerkinElmer Lambda 365+ spectrometer. pH data were recorded on a Hanna HI 2210 pH meter equipped with a HI1131 pH probe from Hanna. ^1H NMR spectra were recorded using a Bruker Ascend 600 MHz (Bruker, Coventry, UK) spectrometer.

5.4.2. Methods and Instrumentations

5.4.2.1. Preparation of Stock Solutions of Dyes

The stock solutions (10 mM) of all the dyes were prepared in ethanol and stored at 4 °C.

5.4.2.2. Execution of TRIS-GdL pH clock

In a glass vial, 3 mL of water was taken and acidified to pH 4.0 with 1 mM HCl solution. This was immediately followed by the simultaneous addition of 6 μ L and 7.5 μ L each from the stock solutions of pH 9.0 TRIS buffer (1M) and GdL (2 M), respectively. The contents were quickly homogenized with a micropipette, and then the pH change was monitored over 1.5 hours.

5.4.2.3. Execution of NaOH-GdL pH clock

In a glass vial, 3 mL of water was taken. To it required volume of desired dye was added from their respective stock solutions. This was immediately followed by the simultaneous addition of 6 μ L and 2.25 μ L each from stock solutions of freshly prepared NaOH (1 M) and GdL (2 M), respectively. The contents were quickly homogenized with a micropipette, and then the pH change was monitored over 3-4 hours.

5.4.2.4. Execution of the SDT-O₂ clock

In a glass vial, 3 mL of 0.25 M NaOH was taken. To it required volume of desired dye was added from their respective stock solutions. This was immediately followed by the addition of required volumes (3 – 7.5 μ L) of SDT from its freshly prepared stock solution (1M). The contents were quickly homogenized with a micropipette, and then the pH change was monitored over 3-4 hours.

5.4.2.5. Absorption measurements

All absorption experiments were recorded in a 3 mL quartz cuvette (path length 1 cm) with a slit width of 1 nm at 298 K. To 3 mL of water or buffer of desired pH, the required volume of dye was added from its respective stock solutions to obtain the desired concentrations. It was then evenly mixed with a micropipette and equilibrated for a minute before data acquisition. For a typical time-dependent experiment, all the required contents were added following the same proportion and sequence as that in the pH or redox clock. The contents were uniformly homogenized, and the spectra were recorded at regular time intervals as needed. In all experiments, 10 μ M each of BTB and MB, 25 μ M each of MR and MV, and 20 M each of PT and TT were used for the single dye measurements. For the mixed dye experiments, the combinations of MR: MB (25 μ M: 5 μ M) and PT: TT (10 μ M: 10 μ M) were used.

5.4.2.6. NMR studies

All NMR spectra were recorded in D₂O unless specified. DCl and NaOD were used to maintain acidic and basic conditions, respectively.

5.4.2.7. Preparation of PACA-HPC (Polyacrylic acid/acrylamide – hydroxypropyl cellulose) Hydrogels

The PACA-HPC hydrogels were prepared via radical polymerization following a previously reported procedure.³¹⁰ Firstly, 665 mg of hydroxypropyl cellulose (HPC) was weighed and slowly dissolved in 12 mL of deionized water to afford a hydroxypropyl cellulose solution (5 wt%). A few drops of dilute hydrochloric acid were added to make the system acidic. Next, 2.850 g of acrylamide and 1.312 g of acrylic acid were weighed and dissolved in the above solution, and 5 mg radical initiator APS (0.1 wt% of monomer) and 6 mg (0.2 wt% of monomer) crosslinker methylene bis-acrylamide were added. The above pre-polymerization solution was stirred well for 60 min to properly homogenize it. It was further centrifuged for 5 min to remove air bubbles. The mixed solution was then transferred to a glass mould and polymerized at 90 °C for 2h.

5.4.2.8. Time-encoded encryption in chemically triggered colour codes

For the time-regulated transitory colour code, 16 cuvettes (1 cm * 1cm * 1cm) were arranged in a 4 x 4 perfect square. Each of them was filled with 1 mL of water. Adequate volumes of desired dyes were added to them from their respective stock solutions to produce the desired colour. The specific activator-deactivator triggers were added to initiate the pH or redox clock. Once initiated, the entire setup was monitored over time and photographed at regular intervals to capture the necessary changes and different codes. For the parallel time setup, which involved the additional smart windows as the extra layer of protection, the clocks were initiated, and the smart window was placed on top to conceal the arrangement of codes beneath it. The smart window was first operated on by their respective and specific stimulus (redox stimulus for MB and temperature for the PACA-HPC hydrogel), and then the changes were photographed.

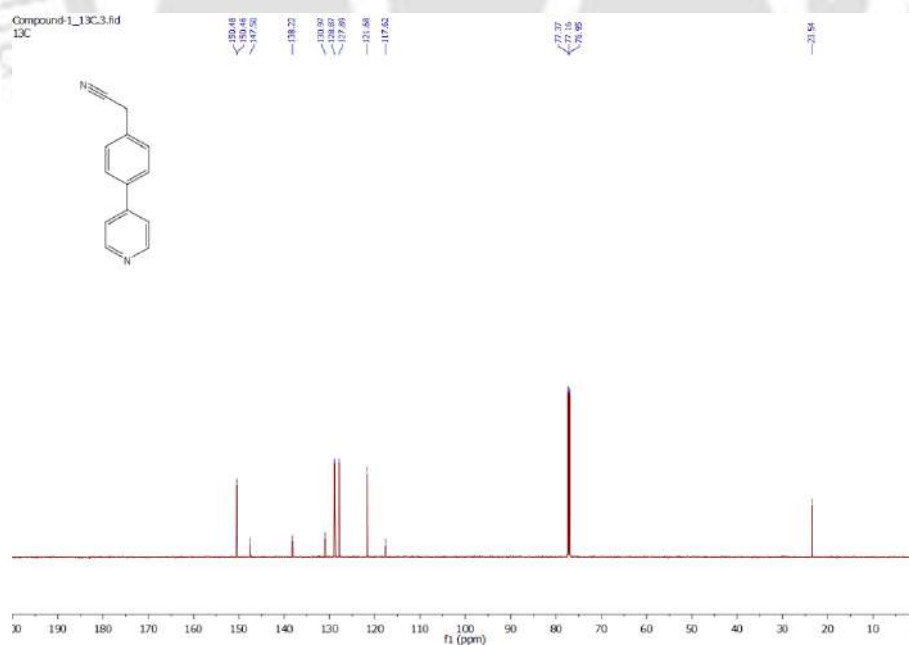
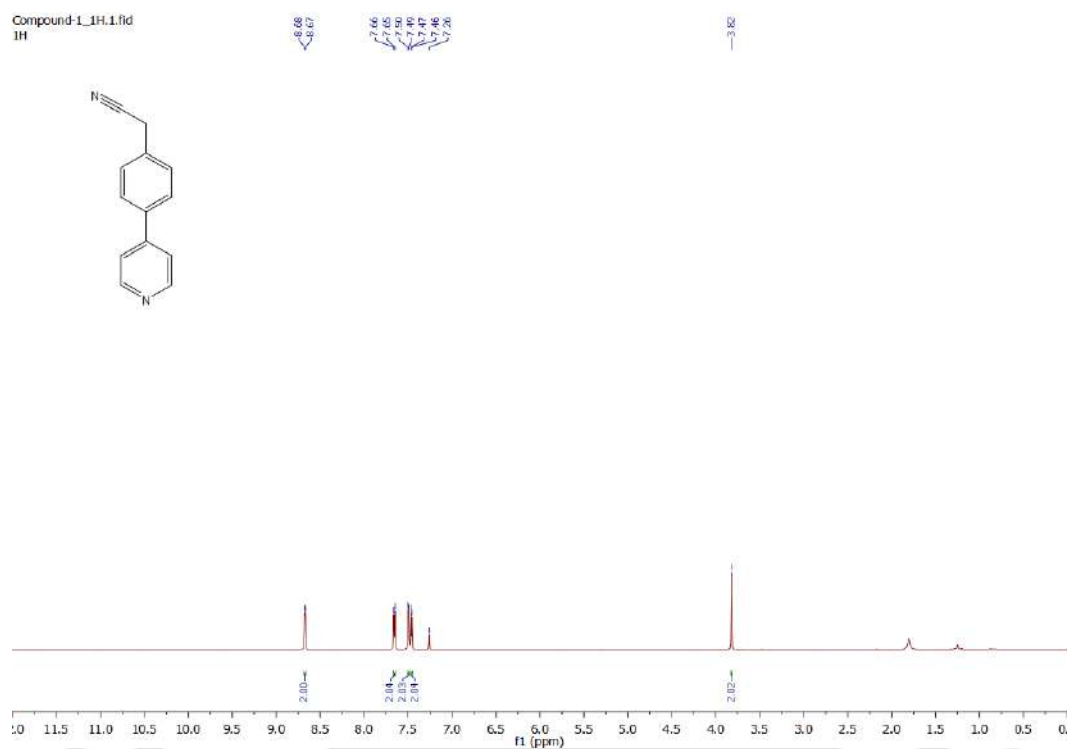


6. Outlook

This thesis has a comprehensive outlook that shows how conventional molecules can be used in unconventional ways. The underlying works in the thesis not only aim at developing systems that show stimulus-responsive changes in chromatic properties but also at explaining how those features can be implemented in modern and advanced applications that rely on colour transitions. The stimulus studied here encompasses a wide variety of independent sources that affect the molecules in different ways – namely, pH, redox and small molecules. The stimuli have been autonomously regulated through chemical clocks, which introduce time-gated features into the stimuli-responsive colours. The time-locked features have allowed us to explore novel avenues of smart applications – be it information encryption with advanced anti-counterfeiting techniques or mimicking biological features in abiotic conditions. These works highlight how modern applications can be realized through an intrinsic property of chemistry – colour transitions. This set of works demonstrates that colours obtained from chemical systems hold much more potential than visual enigma and are immensely capable of promising futuristic applications.



7. Characterization Data of the Synthesized Compounds



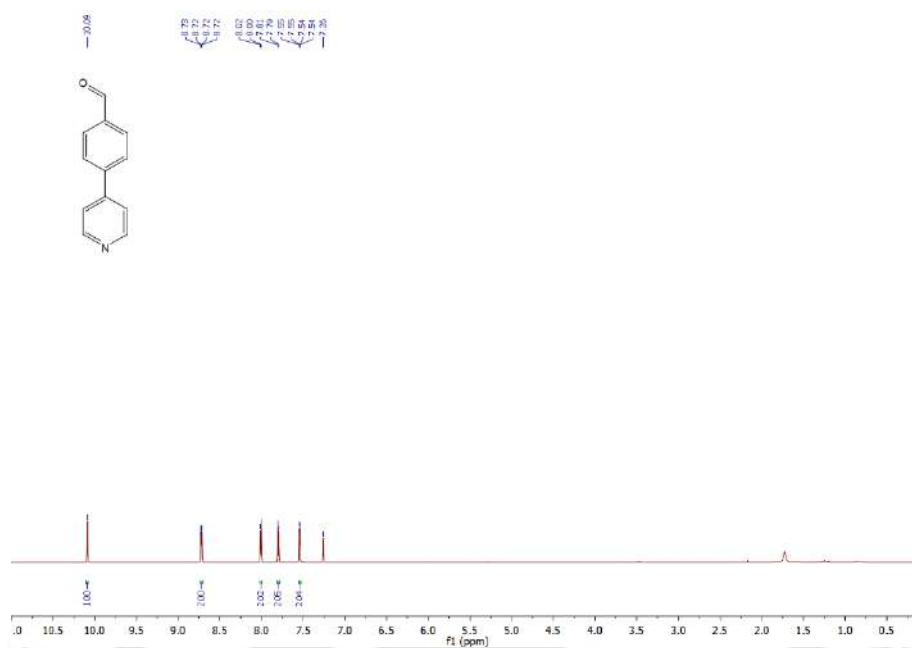


Figure 6.3. ¹H NMR spectra of 2 in CDCl₃.

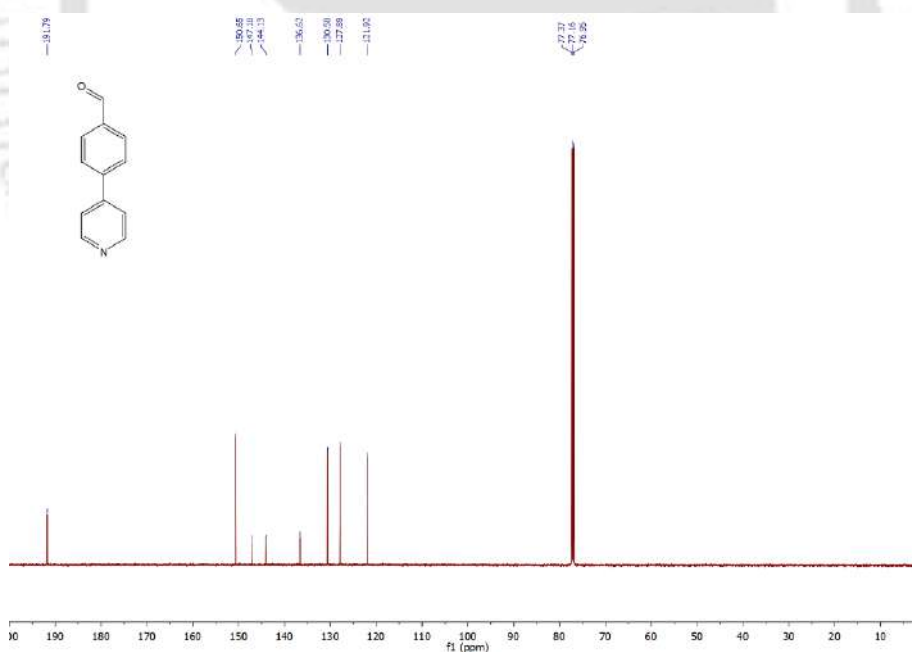


Figure 6.4. ¹³C NMR spectra of 2 in CDCl₃.

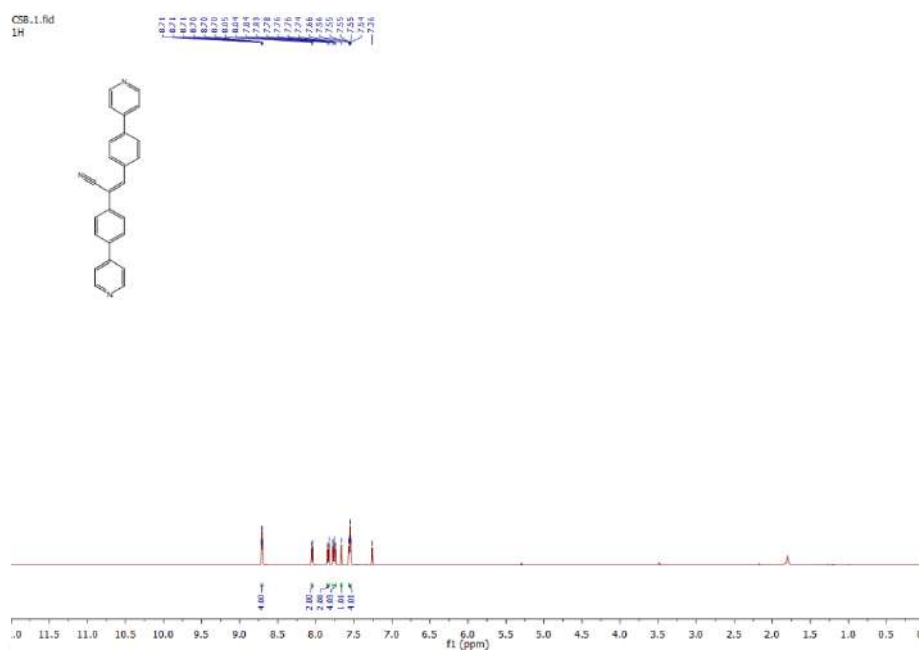


Figure 6.5. ^1H NMR spectra of CSB in CDCl_3 .

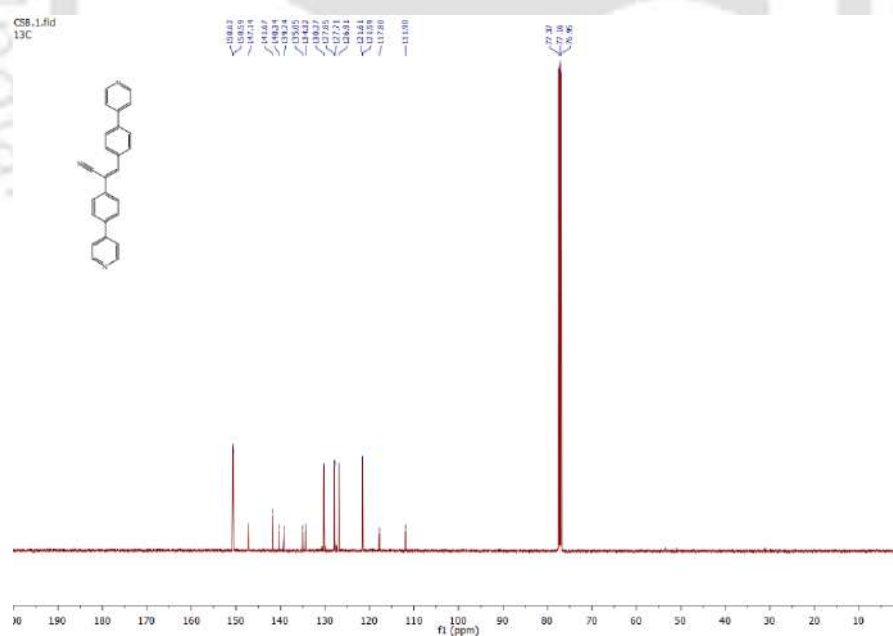


Figure 6.6. ^{13}C NMR spectra of CSB in CDCl_3 .

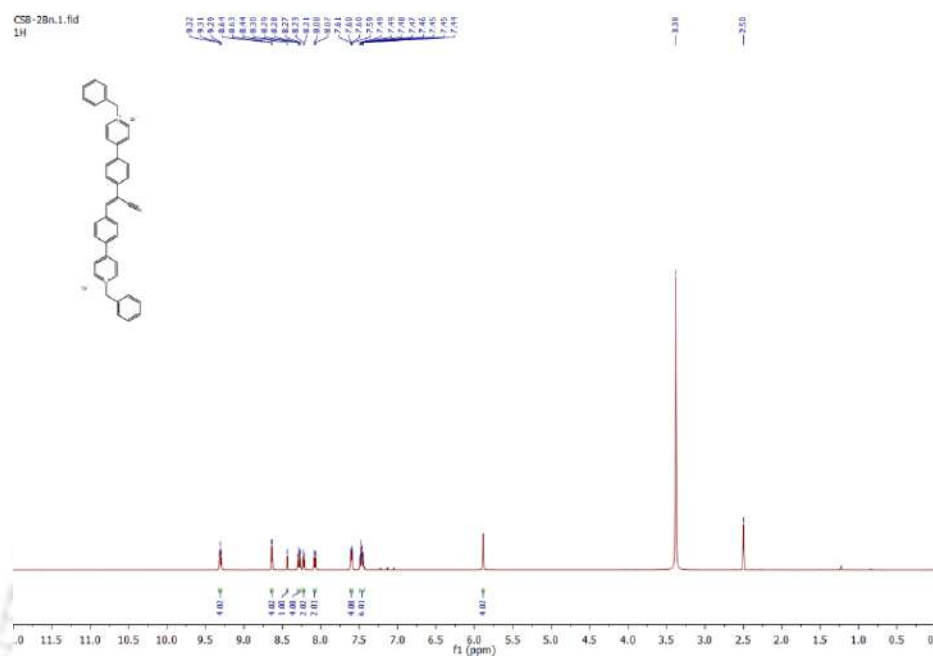


Figure 6.7. ^1H NMR spectra of CSB-2Bn in DMSO-d_6 .

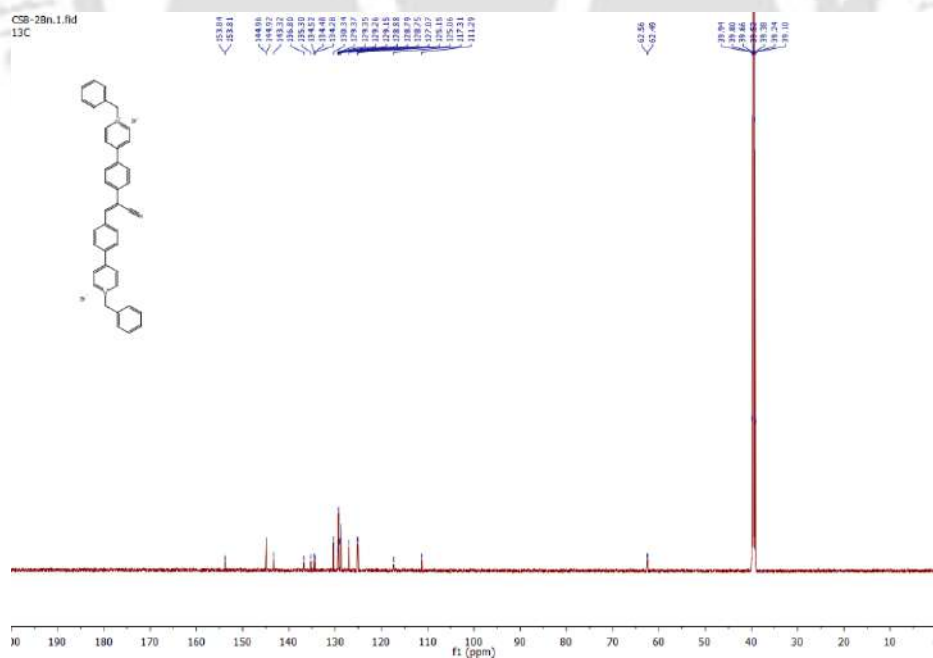


Figure 6.8. ^{13}C NMR spectra of CSB-2Bn in DMSO-d_6 .

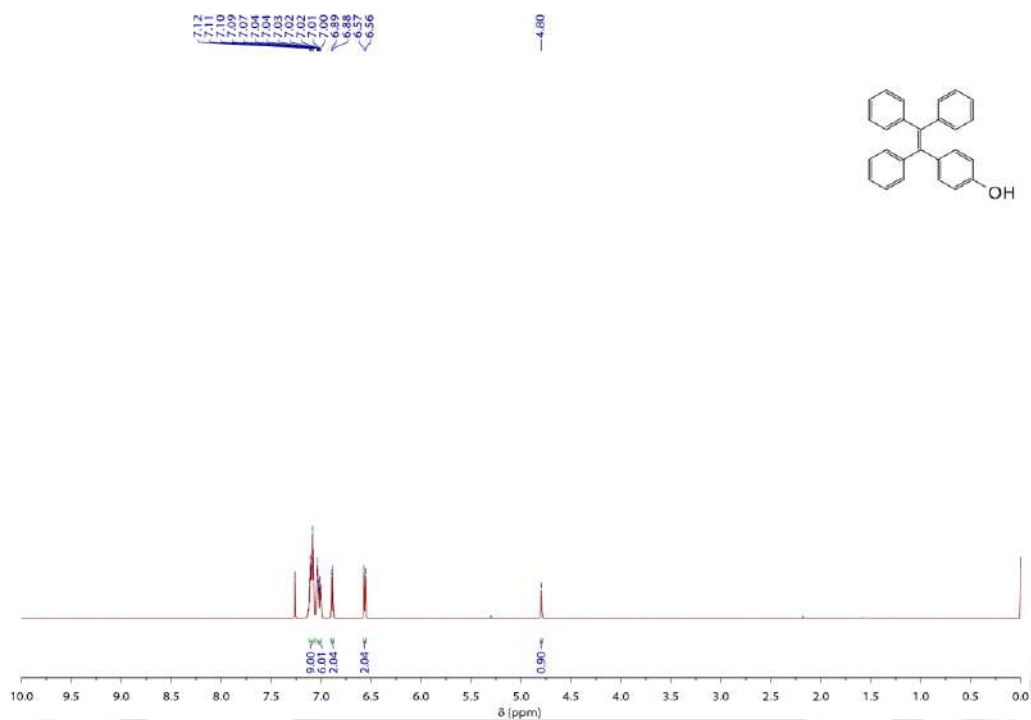


Figure 6.9. ¹H NMR spectra of TPE-OH in CDCl₃.

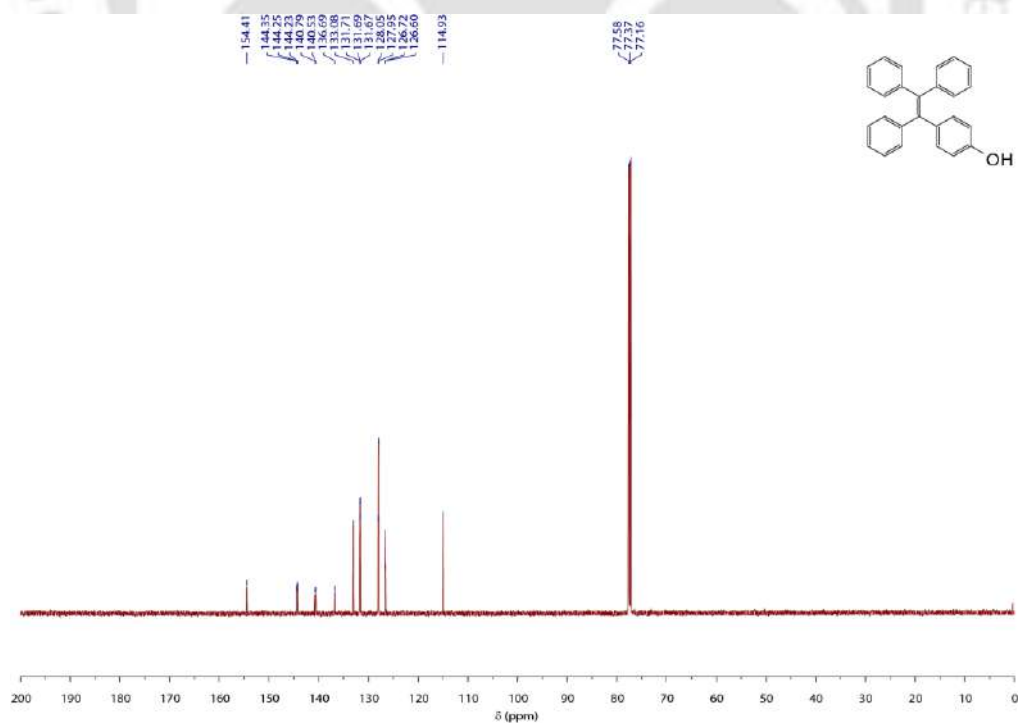


Figure 6.10. ¹³C NMR spectra of TPE-OH in CDCl₃.

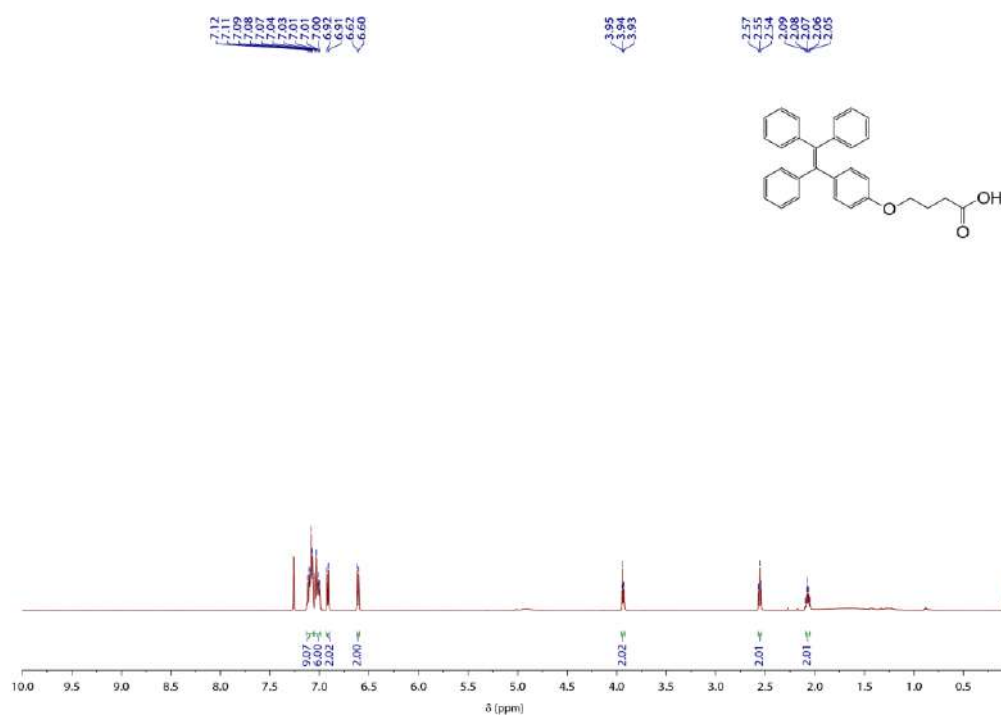


Figure 6.11. ¹H NMR spectra of TPEA in CDCl₃.

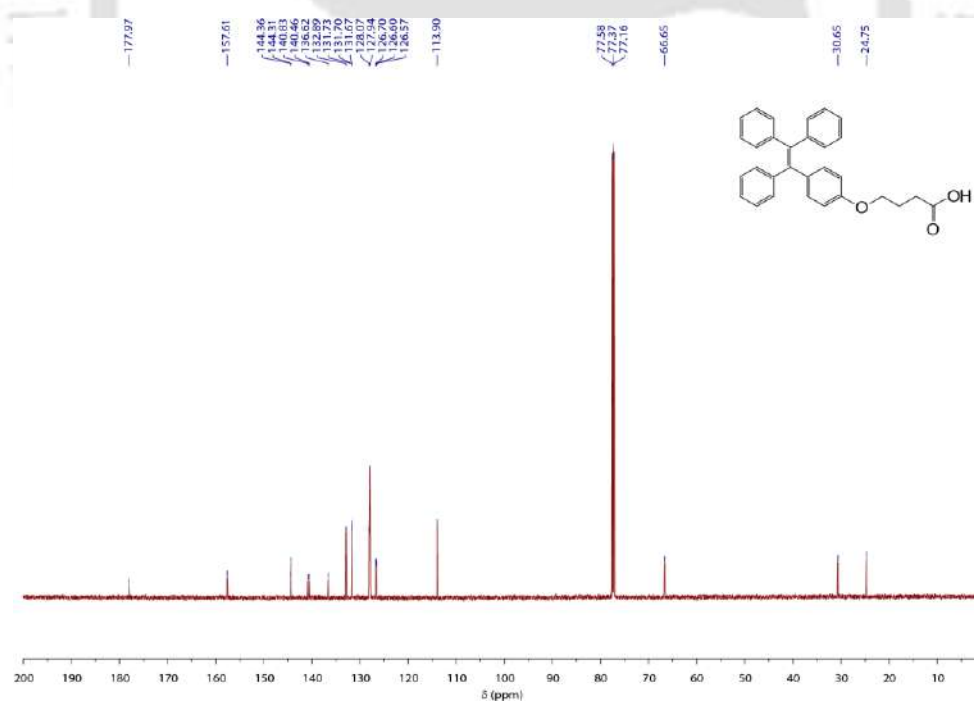


Figure 6.12. ¹³C NMR spectra of TPEA in CDCl₃.

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9. List of Publications

1. Advanced Information Encryption via Parallel Time-Scale Modulation Using Broad-Spectrum Transient Colors From Organic Dyes. P. Das, M. K. Baroi, S. Routray and D. Das* *ChemSystemsChem* 8, no. 2 (2026): e00067.
2. The journey of short peptides: from molecules to materials. S. Routray, P. Das, M. K. Baroi and D. Das*. *Chem. Commun.*, 2026, Advance Article
3. Time-encoded information encryption with pH clock-guided broad-spectrum emission by dynamic assemblies. P. Das, T. Das, S. Koley, M. K. Baroi, S. Das, J. Mohanty and D. Das* *Angew. Chem. Int. Ed.*, 2025, 64, e202414239.
4. An Aggregation-Induced Room Temperature Phosphorescence Probe for the Efficient and Selective Detection of Heparin and Protamine. P. Das, S. Routray, M. K. Baroi, T. Das and D. Das* *Anal. Chem.*, 2025, 97, 20, 10628–10637.
5. Book Chapter: Peptide Amphiphile Nanomaterials. P. Das and D. Das. (2023). In: Elsayy, M.A. (eds) Peptide Bionanomaterials. Springer, Cham.
6. Controlling the lifetime of cucurbit[8]uril based self-abolishing nanozymes. S. Das, T. Das, P. Das and D. Das* *Chem. Sci.*, 2022, 13, 4050-4057.
7. Bi-directional feedback-controlled transience in Cucurbituril-based tandem nanozyme. S. Das, P. Das, P. Dowari, B. K. Das and D. Das* *J. Colloid Interface Sci.*, 2022, 614, 172–180.
8. Bioinspired chemoenzymatically regulated broad-spectrum artificial light-harvesting nanoaggregates with pH-sensitive transitory emissions and information encryption. P. Das, S. Routray, R. Kushwaha and D. Das* (Communicated).
9. A Bioinspired Monolayer Hydrogel with Efficient Omnidirectional Moisture-Driven Actuation for Humidity Sensing. S. Routray, M. K. Baroi, R. Kushwaha, P. Das and D. Das* (Communicated).

