Development of Fluorescent Water Soluble Polyfluorene for Chemical and Biological Sensors

A thesis submitted by

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DEDICATED TO MY FATHER

LATE ***A. P. DWIVEDI*** *AND*

MOTHER, MRS. ***ASHA DWIVEDI***

Indian Institute of Technology Guwahati

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Atul Kumar Dwivedi

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CERTIFICATE

This is to certify that the work contained in the thesis entitled **'Development of Fluorescent Water Soluble Polyfluorene for Chemical and Biological Sensors'** by Atul Kumar Dwivedi, a Ph.D. student of Department of Chemistry, Indian Institute of Technology Guwahati, for the award of degree of Doctor of Philosophy has been carried out under my supervision and this work has not been submitted elsewhere for any degree.

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October, 2012

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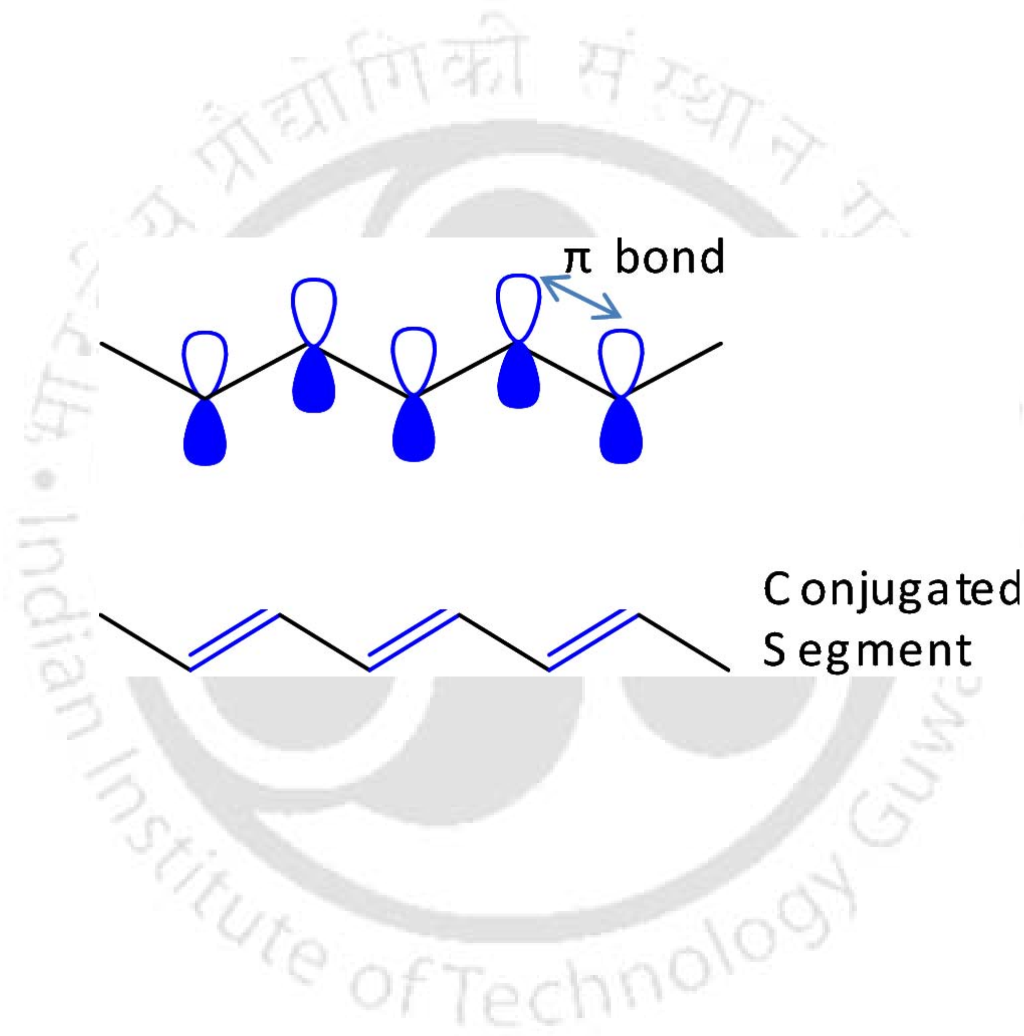
Chapter 1

Introduction



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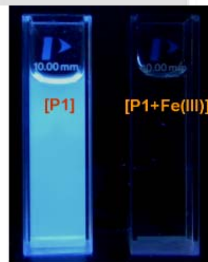
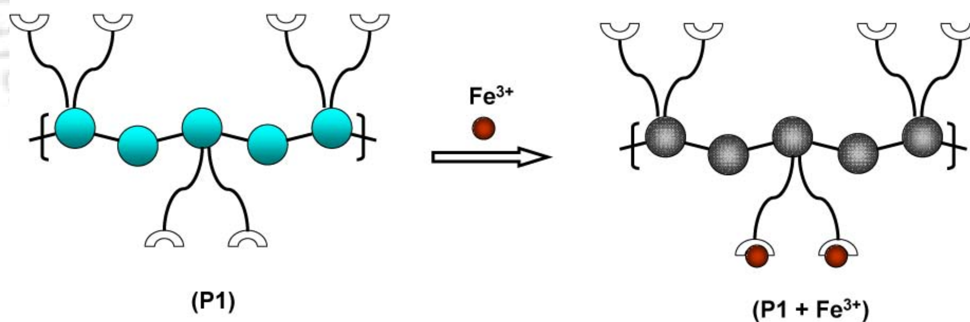
Chapter 2

A Turn off Aqueous Polyfluorene Probe for the Selective Detection of Ferric iron





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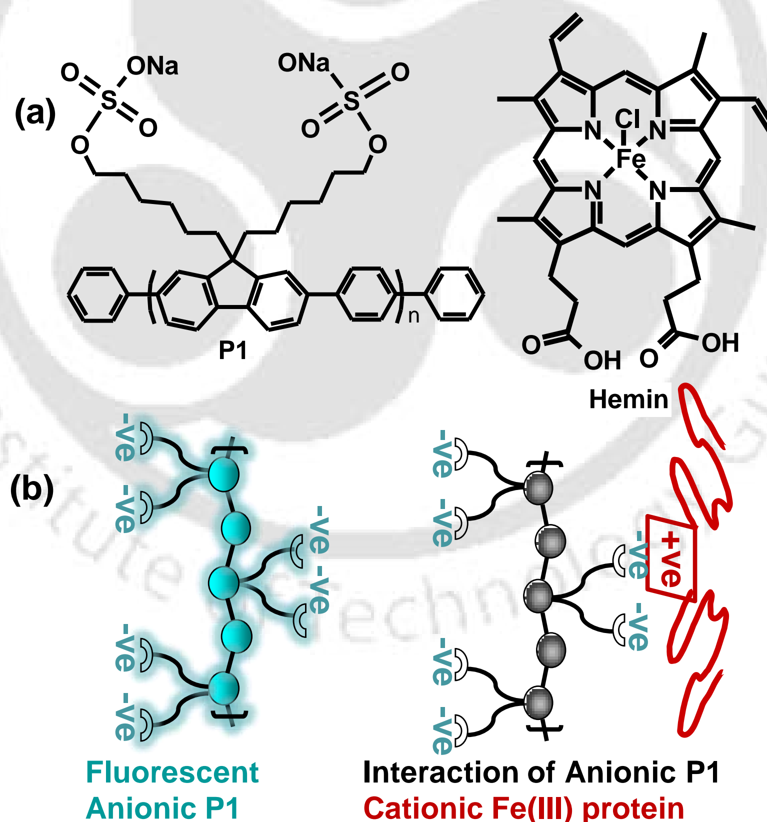
Chapter 3

Interactions of Anionic Polyfluorene with Heme Proteins: Insights into Physiological Effects, Folding Events and Inhibition Activity



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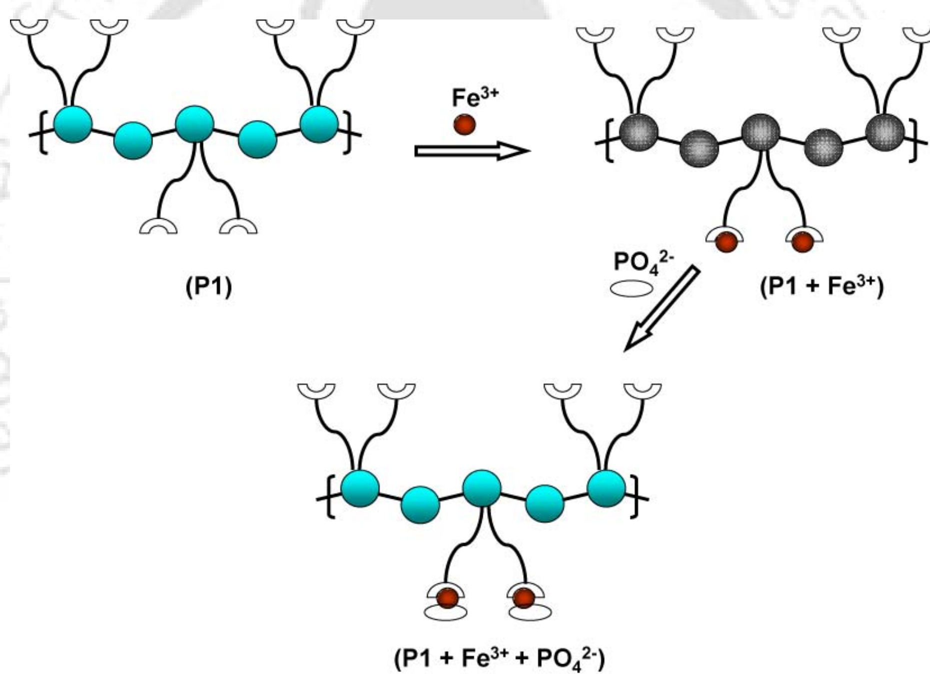

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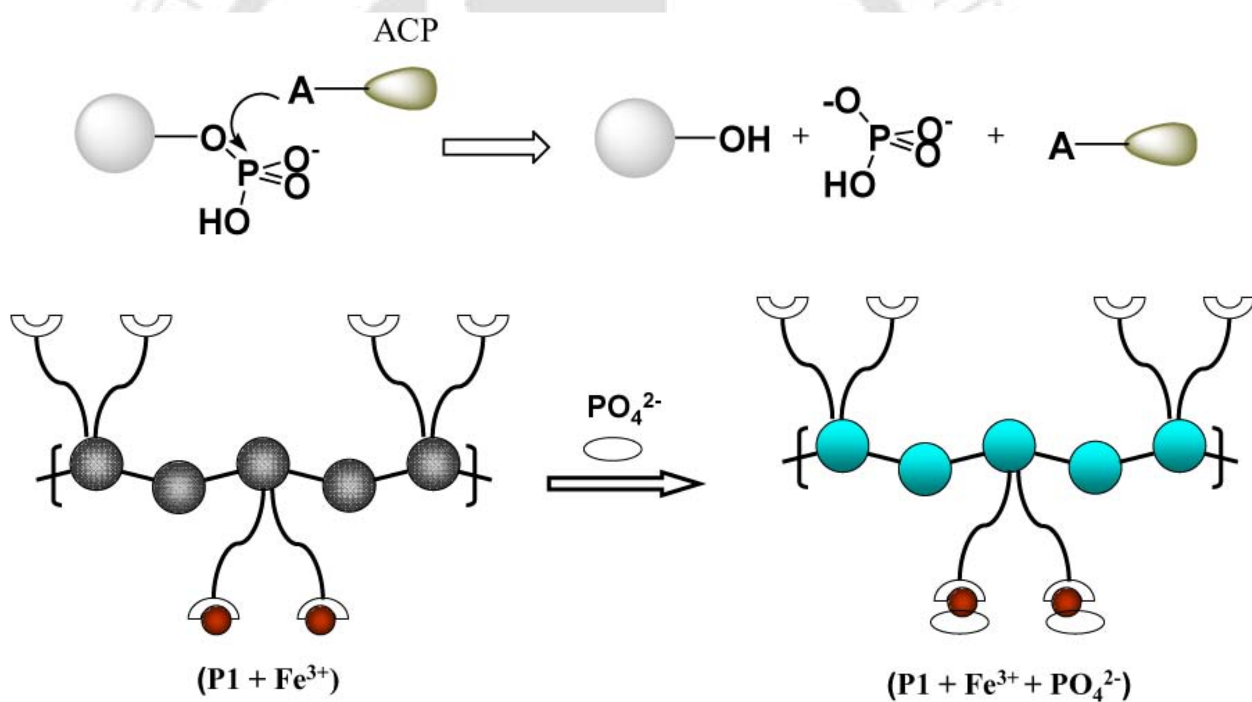
Chapter 5

A Fluorescence Turn On Acid Phosphatase Assay Based on Aqueous Polyfluorene



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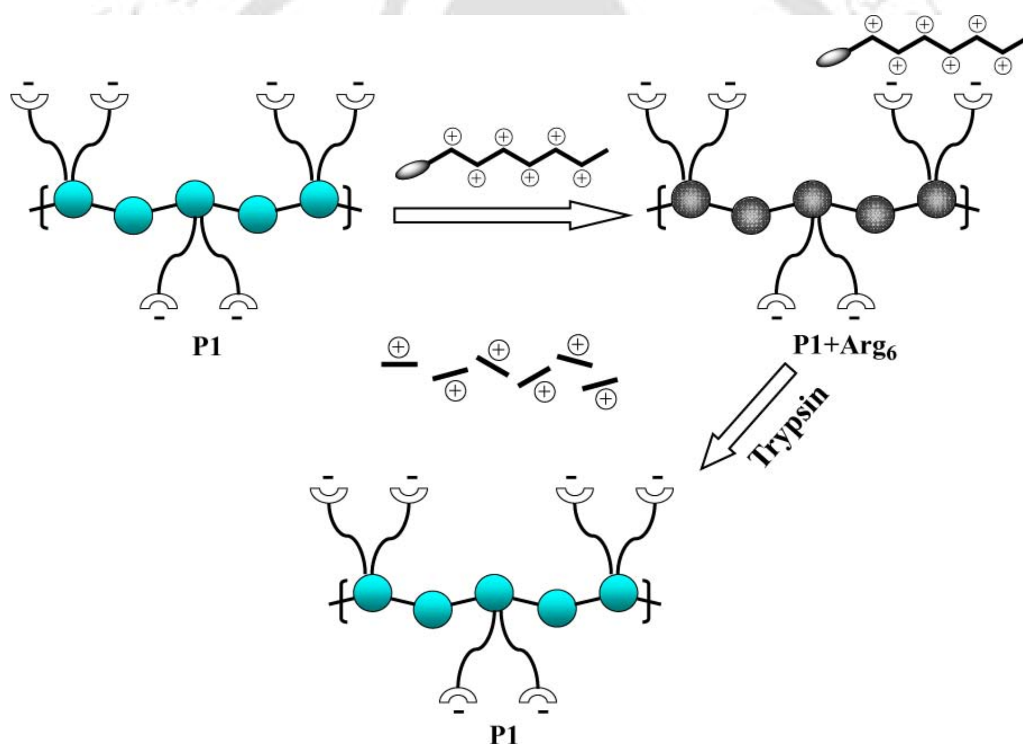

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1.1 Introduction

1.1.1 Discovery of Conjugated Polymer

Conjugated polymers (CPs) are an important class of materials which are used in a wide variety of applications. In 1977, it was first discovered that chemical doping of polyacetylene causes several order enhancement of electrical conductivity. It was believed that stable charge-transfer π complex was formed during the halogen doping. This great contribution was made by Hideki Shirakawa of the University of Tsukuba in Japan, Alan MacDiarmid of the University of Pennsylvania at Philadelphia and Alan Heeger of the University of California at Santa Barbara and this led them to get Nobel Prize in chemistry in the year 2000. CPs are unsaturated organic macromolecules having alternating saturated and unsaturated bonds along the backbone. All CPs consist of σ-bonds through the overlapping sp^2 hybrid orbitals and the remaining out-of plane p_z orbitals which overlaps with neighboring p_z orbitals and forms π-bonds. Therefore, the electrons that constitute the π-bonds are delocalized over the entire polymer backbone even though the chemical structures of CPs are presented as alternating single and double bonds. These continuous delocalized π-bonds along the backbone was the origin of the emissive property and conductive property of CPs. CPs can be obtained with a variety of backbone structures, such as poly(para-phenylenes) (PPP),[1] poly(para-phenylene vinylene) (PPV),[2] poly(para-phenylene ethynylene) (PPE),[3] polythiophene (PT),[4] polypyrrole (PPy),[5] polyaniline (PANI)[6] and polyfluorene (PF)[7] as shown in Figure 1.1.

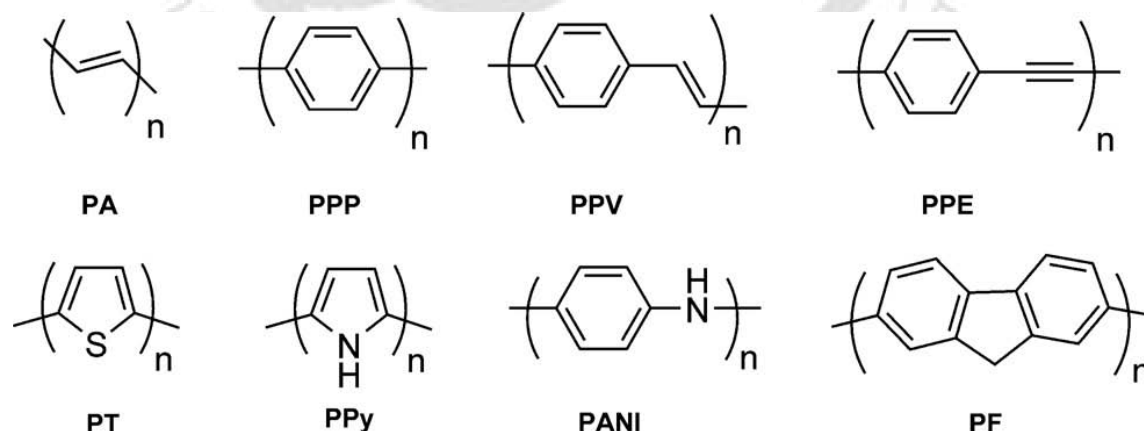

Figure 1.1 Molecular structure of some commonly used CPs.

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Because of the significant photophysical and electrochemical properties, CPs has been synthesized and investigated extensively. Therefore in past decades we have witnessed a wide reaching use of the conducting polymers that includes light emitting diodes (LEDs),[2,8] light-emitting electrochemical cells,[9] plastic lasers,[10] solar cells,[11] field effect transistors,[12,13] and as sensors.[14,15]

CPs containing hetero-donor system such as bipyridine, crown ethers, terpyridine, quinoline, phenothiazoline, benzimidazole and pyridine have also been reported as sensory materials.[16]

1.1.2 Conjugated Polyelectrolytes

Conjugated polyelectrolytes (CPEs) are conjugated polymers that contain ionic side group functionality that make the material water soluble in aqueous and other polar solvents.[17] Earlier it was realized that the optical and electronic properties of CPs are directly related to the organization of material. Several groups tried and were able to design and synthesize CPEs through a better understanding of structure-property relationship. As a result with new modifications water soluble CPEs were synthesized. Incorporation of cationic or anionic functionalities into CPs allowed them to possess beneficial properties with aqueous solubility for biological application. Generally CPEs with ionic pendant functionalities are capable of ionizing in highly dielectric media.[18] In this way it was supposed that CPs may serve as a link between electronic world and biological world. Aqueous solubility of polymer bestows potential to revolutionize bionics because most of the biological entities are present in aqueous environment. However, achieving complete water-solubility and high emissive properties remains a challenging task. To make CPs water soluble, research groups generally introduce sulphonate (SO_3^{2-}), sulphate (SO_4^{2-}), phosphonate (PO_3^{2-}), carboxylate (CO_2^-) and quaternary ammonium (NR_3^+) group in the side chain. Some representative examples of this class are given below in Figure 1.2.

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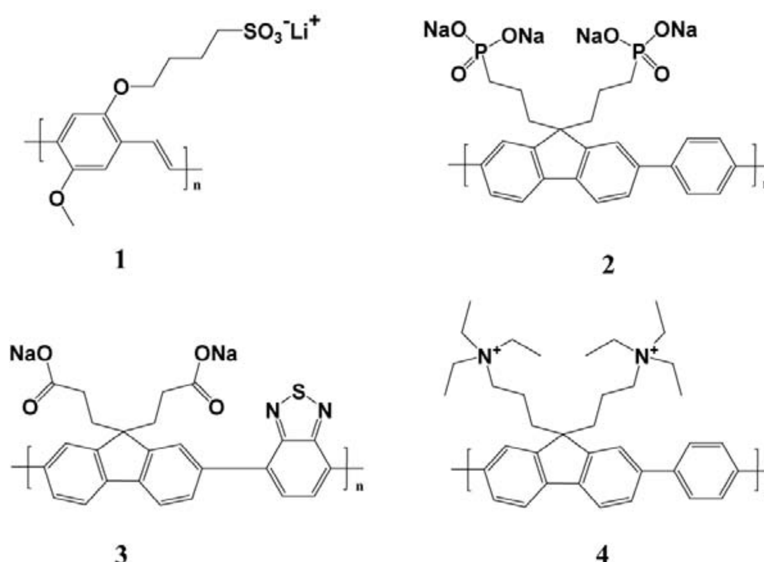

Figure 1.2 Molecular structure of some representative CPEs.

This modification opened the door to the combination of CPs with biological molecules. The first CPEs, **1**, as shown in Figure 1.2 was reported by Shi and Wudl in 1990 and others have been developed in recent years for biosensor applications.[19] CPs display tremendous optoelectronic / redox behaviour because whenever charges are in excess on the polymer chains, the charges can hop along the conjugated backbones easily. CPs in their neutral form normally are wide band gap semiconductors that exhibit efficient absorption or emission at the band edge. The strong luminescence of CPs is directly related to the delocalization and polarization of the electronic structure.[21] This inspired chemists and physicist all over the world to disclose the beneficial properties of CPs as a device and sensor. These conducting materials were thus used as a device and sensor very frequently. After the discovery of high sensitivity or superquenching concept of polyelectrolytes, researchers gave attention to use these polymers as chemical and biological sensors.[15,16]

1.1.3 Fluorescence Quenching

Fluorescence technique is widely used in sensing of analyte because of availability of the diverse transduction schemes, which are based on changes in fluorescence intensity, fluorescence lifetime and excitation or emission wavelength. Fluorescence quenching is an important phenomenon which is competing with fluorescence, in which fluorescence intensity or life time can be decreased through the interaction of quencher. There are

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many types of interaction that includes energy or electron transfer, molecular rearrangement, static complex formation and dynamic collision.[20] There are two types of fluorescence quenching mechanisms reported extensively, one is the static quenching and the other is dynamic quenching. These both require molecular contact between the fluorophore and quencher. However, static quenching is due to the formation of a non-fluorescent complex between the fluorophore and the quencher. On the other hand, dynamic quenching, which is also called collisional quenching, is due to random collisions between fluorophore and quencher molecules. Hence, it is clear for static quenching that quencher has to diffuse the fluorophore within the lifetime of the excited states.

There are several ways to distinguish between these two mechanisms. First, the lifetime in static quenching does not change, because the fluorescence occurs from the uncomplexed fluorophore, which remains the same during the quenching process. In other words in static quenching, the fluorescence lifetime is independent of the quencher concentration since complexation takes place in the ground state. However, the lifetime in dynamic quenching decreases in proportion to the intensity and in the presence of an additional deactivation pathway shortens the observed fluorescence lifetime. Second, static quenching is decreased at higher temperature due to dissociation of weakly bound complexes formed in the quenching process, while dynamic quenching is increased at higher temperature due to faster diffusion and collision.

The efficiency of both types of quenching is enumerated by Stern-Volmer constant

$$I_o/I = 1 + K_{sv}\,[Q]$$

Where I_o and I are fluorescence intensity in the absence and presence of the quencher, respectively, [Q] is quencher concentration and K_{sv} is the Stern-Volmer constant.

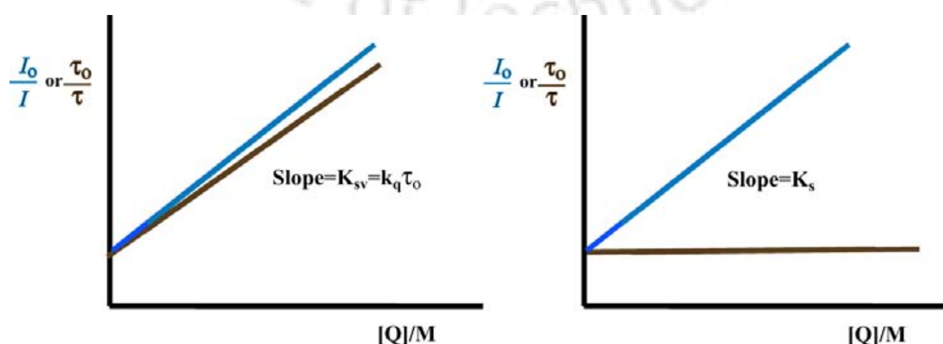

Figure 1.3 Stern-Volmer plot and changes in the life time in dynamic and static quenching.

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In case of dynamic quenching, the fluorescence intensity is quenched to the same extent as the fluorescence life time change (Figure 1.3-left). In this case equation can be written as

$$I_o/I = \tau_o/\tau = 1 + k_q \tau_o [Q] = 1 + K_D [Q]$$

Where τ_o and τ are the life times in the absence and presence of quencher. In the above equation K_D was written in place of K_{sv}, which is equal to k_q τ_o. Usually in static quenching K_{sv} is represented by K_s, which is the association constant for the formation of ground state complex. The equation for static quenching may be written as

$$I_o/I = 1 + K_s [Q]$$

Where K_s is the binding constant for the ground state association between the fluorophore and quencher. The lifetimes of the fluorophore in static quenching remain unchanged (Figure 1.3-right) by the presence of Q and the ratio of τ_o to τ is 1.

However, in many cases Stern-Volmer plot displays a nonlinear upward curvature, indicating the quenching by both static as well as dynamic. In such a case the Stern-Volmer equation can be written as

$$I_o/I = (1 + K_D [Q])(1 + K_s [Q])$$

This equation combines dynamic and static quenching effect on the fluorescence intensity and this behaves as a second order with respect to Q.

1.1.4 Amplified Quenching of Conjugated Polyelectrolytes

One inherent feature associated with CPEs was that, only fractional binding of analyte can cause an amplified signal gain due to delocalization of exciton along the conjugated chain. This amplified signal gain was termed as the molecular wire effect and this concept was first explored by Swager group in 1995.[21,22] According to this concept amplification was due to the delocalization of electronic structure which facilitates efficient energy migration over a large distance. To prove this concept Swager group incorporated a well known electron transfer quenching agent bis(p-phenylene)-34-crown-10 (BPP) into neutral PPEs[23a] as well as corresponding monomer. This particular group BPP was earlier shown by Stoddart to be exceptional receptor for paraquat (PQ^{2+})[23b] (Figure 1.4).

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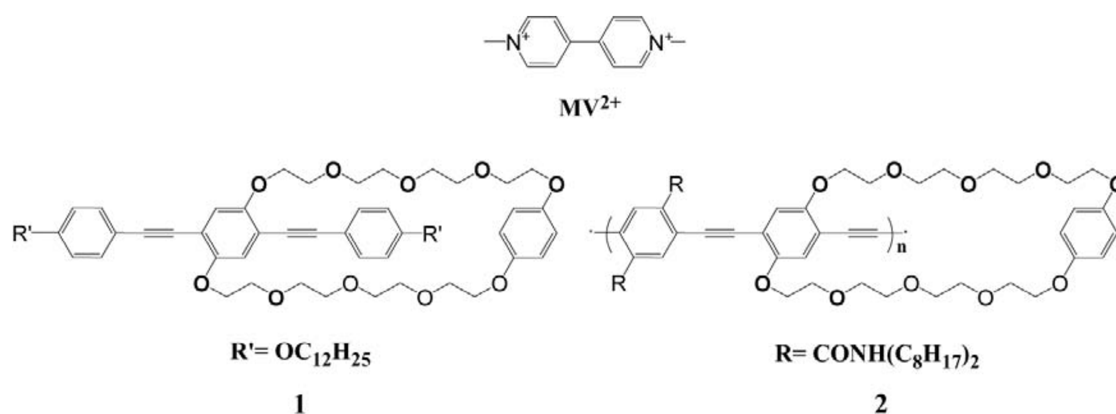

Figure 1.4 Molecular structures of monomer and polymer utilized by Swager group.

It was found that polymer as well as monomer both resulted in quenching after the interaction with paraquat but sensitivity was found to be 67 fold higher for polymer than its corresponding monomer. The K_{sv} value for this enhanced sensitivity was reported to be about 10^5 M^{-1}. In fact during excitation a combination of electron-hole pair (exciton) is generated that migrate along the polymer backbone. During migration each exciton passes through multiple receptor sites and when exciton encounters a quencher bound receptor site, excitation is quenched. Therefore a fractional binding event results in complete quenching (Figure 1.5), whereas in case of monomer every receptor must be occupied for complete quenching. Therefore CPs were found to exhibit collective transport properties and were very sensitive to minor perturbations as it contains a large number of optically active absorbing units with delocalized electronic state.[23]

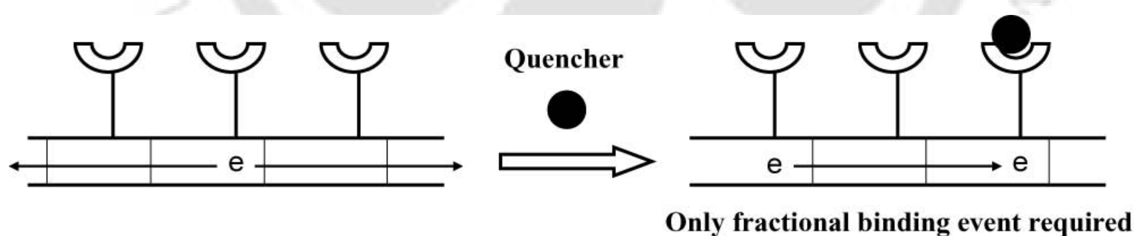

Figure 1.5 A systematic diagram showing molecular wire effect.

Emission wavelength is directly dependant on chain length of polymer but after "effective conjugation length" it is independent. Swager also explored the fluorescence efficiency of polymer delocalization length and thus influenced the amplified quenching effect.[22] It was found that quenching efficiency increases with increasing molecular weight of polymer, but if molecular weight is above 65000, there is no effect on quenching efficiency. Polymers were found to exhibit collective transport properties and were very sensitive to minor perturbations. CPs contain a large number of optically active absorbing units with

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delocalized electronic state, called as exciton, that facilitates efficient intra and interchain energy transfer.[19] In other words in CPs based sensors, only fractional binding of analyte can cause an amplified signal due to delocalization of exciton along the conjugated chain. The amplified quenching of water soluble CPEs was first discovered by Chen and Whitten in 1999[24] and this amplified quenching was called as superquenching. Chen and Whitten used an anionic derivative of poly(phenylene vinylene), PPV-SO_3^- in their work. They reported that MV^{2+} was an highly efficient quencher of anionic polymer via photoinduced electron transfer with a K_{sv} value of 1.7×10^7 M^{-1} and this K_{sv} value was million fold amplified than the isolated stilbene in dilute solution. After the work of Chen and Whitten the concept of amplified quenching was applied on other CPEs with various quenchers and as a result it was found that amplified quenching was a general property of CPEs. However, Schanze et al reported the amplified quenching with an anionic derivative of PPE (Figure 1.6), showing amplified quenching by MV^{2+} in water as well as methanol with a high K_{sv} value of 10^7 M^{-1}.[25-27] This was four orders of magnitude greater than that of the small molecule.

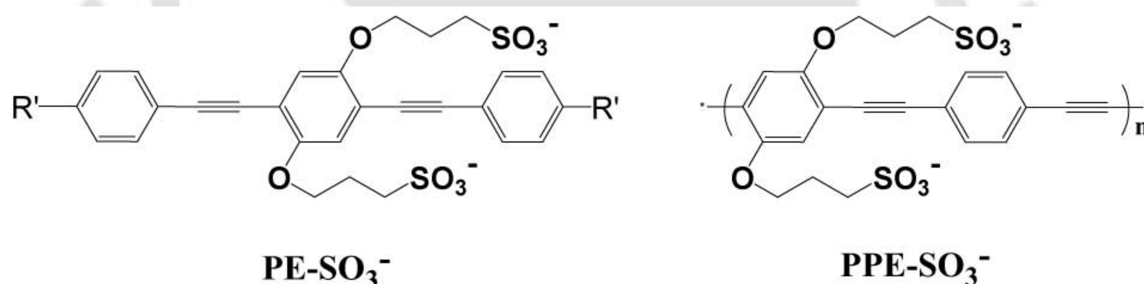

Figure 1.6 Molecular structures of monomer and polymer used by Schanze group.

Hence, in comparison to neutral CPs, charged CPEs exhibits more amplified quenching efficiency due to the formation of ion pair complex between CPEs and quencher. Therefore due to the formation of ion pair complex and molecular wire effect, CPs exhibit amplified quenching while in the contact with a quencher. From the above result it was concluded that any factor affecting these two effects can influence the amplified quenching of CPEs and thus Stern-Volmer constant. Some of these factors are polymer chain length, polymer aggregation, quencher properties, solution conditions and additives.

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1.1.5 Sensor application of Conjugated Polyelectrolytes

Over the past decade CPEs have been widely used as a sensor for chemicals[28,15] and biological analytes[29,30] because these were highly sensitive towards the minor perturbation of the environment and also there exists amplification action by a collective response with typical detection limit in nanomolar[31] or even in zeptomolar range[32] and thus establish the macromolecule superiority over small molecule. Coulombic attraction between the water soluble CPEs and oppositely charged quenchers make them selective for charged analytes. Multiple charges on CPEs offer the high solubility in aqueous medium which push the limit of material to assay the biological analytes because most of the biological entities are in aqueous environment. Homogeneous approach in CPEs is less labor intensive and less time consuming compared with heterogeneous assay such as enzyme-linked immunosorbant assay (ELISA). Therefore CPEs based assay are simple, faster and readily adapted to a fluorescence-based high-through put screening (HTS) format. Moreover the unique structural and optical properties of CPEs provide advantages over routine sensors.

CPE based optical sensors can be categorized into two types of detection scheme, colorimetric and fluorometric. Colorometric detection is mainly based on the change in absorption wavelength of CPE whereas fluorometric detection scheme is based on the change in intensity, wavelength and lifetime. However in most cases, fluorescence can be either turn off or turn on upon direct or indirect interaction with analytes. But both turn off and turn on approach are taking place by superquenching process, light harvesting via Förster resonance energy transfer (FRET) and conformational changes.

1.1.6 Small ion sensing

Charged CPEs are well known to interact with oppositely charged small ions such as metal ions, metal complexes and inorganic anions by coulombic interaction as well as collective response in a very dilute condition. As a result, interaction with small ions influences their optical properties via superquenching,[33] energy transfer[34] and conformational changes.[35] In recent years several reports related to small ion sensing has been published. In 2008 Wang and co-workers reported a sensitive Fe^{3+} sensor which was based on Fe^{3+} induced fluorescence quenching of a phosphonated PF[18] as shown in Figure 1.2. They attributed the origin of high sensitivity because of strong association interaction between Fe^{3+} ions and phosphonate group and trapping of the excitons along the CPEs

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was considered. In 2009 Wang and co-workers reported Hg^{2+} sensor based on amino acid functionalized water soluble PF (Figure 1.7.1) with a high photoluminescence quantum yield.[36] They reported fluorescence quenching of CPE selectively in presence of Hg^{2+} and also this spectral sensing was reversible. With EDTA the fluorescence was nearly recovered to original intensity of CPE.

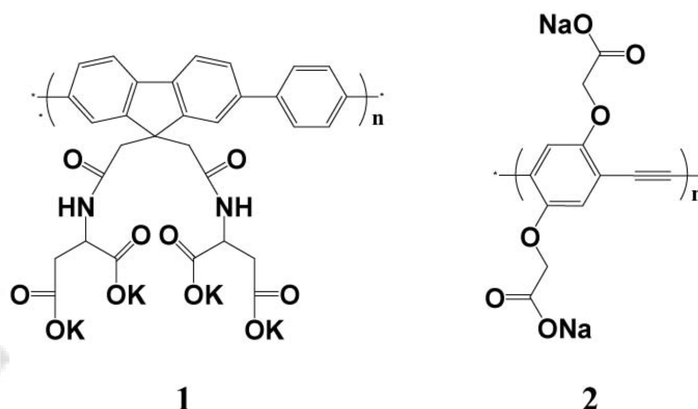

Figure 1.7 CPEs 1 and 2 were reported by Wang and Bunz group for Hg^{2+} and Pb^{2+} sensor.

In 2005, Bunz and co-workers reported a sensitive Pb^{2+} sensor which was based on fluorescence quenching of a carboxylated PPE (Figure 1.7.2) in presence of Pb^{2+} and this was attributed to the combination of multivalent effect between CPE and Pb^{2+} as well as the exciton migration along the polymer backbone. Same group also reported the Hg^{2+} sensor by using the same polymer. This method was based on the formation of an electrostatic complex between CPE and papain. In 2006 Chen et al reported a nickel sensor based on nickel induced fluorescence quenching of a polyfluorene, 2,2’-bipyridyl PF.[37] The spectral response was polymer to metal ions and was believed to be based on the chelation between 2,2’-bipyridyl units and the metal ions. It was proposed that chelation with metal ions forces the bipyridyl group, which had 20 dihedral angles between two pyridine planes to become more planar and may result in an increased effective conjugation length on the entire polymer.

1.1.7 Detection of Biomolecules with CPEs

The water solubility of CPs bestows potential to revolutionize bionics because most of the biological entities are present in aqueous environment. In this way it was supposed that aqueous solubility of CPs may serve as a link between electronic world and biological

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world. After the pioneering invention of avidin assay by David Whitten in 1999, these water soluble CPs were frequently applied in the detection of biological entities including small biomolecules,[38,39] proteins, DNA [40,41] and enzymes.[42]

Schanze group in 2002 reported a sensitive turn on sensor for saccharides by utilizing amplified quenching of a PPE-SO_3^- CPE as shown in Figure 1.6, with a boronic acid functionalized benzyl viologen (p-BV^{2+}).[43] Upon addition of sugar, carbohydrate bound p-BV^{2+} was unable to complex with PPE-SO_3^- and as a result fluorescence was turned on. In 2002 Leclerc group reported a CPE based DNA sensor by using polythiophene derivative (PT)[44] as shown in Figure 1.8.1.

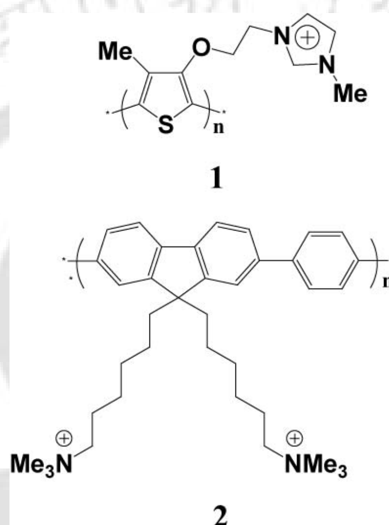

Figure 1.8 Polymers 1 and 2 were reported by Leclerc group and Bazan group for DNA detection.

In further development in the field of biosensor, Bazan group pioneered the growth of DNA sensor by incorporating fluorescence resonance energy transfer (FRET) technique in his method that was based on cationic PF structure shown in Figure 1.8.2. This FRET technique was initially applied to probe RNA-RNA[45] assemblies and RNA-protein interactions.[46] Again a modified method was proposed by Bazan group in 2006 in which it was reported that this method was able to determine the accurate DNA concentrations.[47]

1.1.7.1 Detection of Proteins with CPEs

Proteins are considered to be highly important for the life processes but at the same time because of variation in protein expression they are also responsible for several fatal diseases and even death.[1] Therefore protein detection by using some facile assay is of particular interest in the research field. In this regard quencher-tether-ligand (QTL) proposed by Whitten group[48] was one of the earliest technique for the protein detection.

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This technique was based on the change of fluorescence intensity of an anionic CPE, MS-PPV because this polyelectrolyte was considered to be a sensitive probe for biotin-avidin binding event. They linked a cationic quencher methyl viologen to biotin to prepare QTL and on addition of a very dilute solution of biotin linked methyl viologen, fluorescence of MS-PPV was significantly quenched while polymer fluorescence was significantly recovered by the addition of avidin. In fact direct detection of protein refers to monitoring the optical signal changes observed by the direct interaction between protein and CPE, there is no need for the involvement of other molecules such as ligands and quencher and thus generating the turn off signal via superquenching or conformational perturbation. Over the past few years there are several reports on the direct interactions between CPEs and proteins.

In 2002 Heeger and co-workers reported superquenching of an anionic CPE, MBL-PPV structure shown in Figure1.1, by a heme containing protein C*c* with a K_{sv} value of 3.2 x 10^8 M^{-1}.[49] The author attributed the superquenching effect to the coulombic interaction and electron transfer from photoexcited polymer to Fe^{3+} centre of protein and thus reducing Fe^{3+} heme to Fe^{2+}. Modulation of surface charge on C*c* by adjusting the pH was found to alter the K_{sv} value, thus for efficient electron transfer electrostatic complex formation was necessary. However a nonspecific quenching was also observed by the author with positively charged Lysozyme protein and it was reported that fluorescence self quenching of anionic CPE was occurring due to protein induced aggregation.

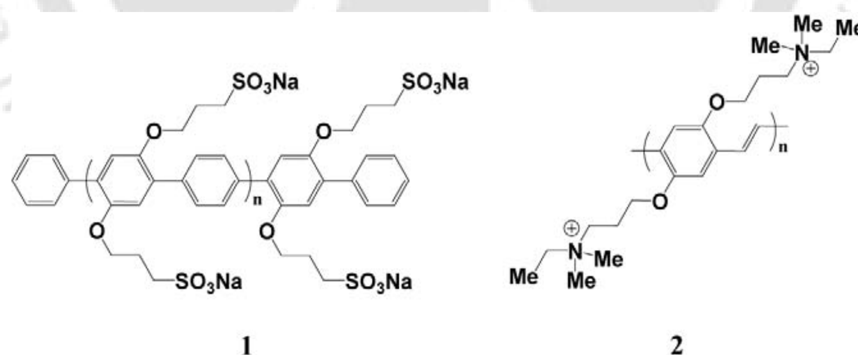

Figure 1.9 Polymer 1 and 2 were reported by Waldeck and Haung group for protein sensing.

In 2005 Waldeck and co-workers reported fluorescence quenching of PPP based anionic CPE as shown in the structure (Figure 1.9.1) by ferric as well as ferrous form of Cytochrome *C* (C*c)* and myoglobin with K_{sv} values 0.52 x 10^7, 0.36 x 10^7 and 0.28 x $10^7 M^{-1}$ respectively.[50] Stern-Volmer constant K_{sv} values for both the form of C*c* were

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closed, therefore they ruled out the possibility of electron-transfer induced quenching and a less K_{sv} value for myoglobin was attributed to smaller surface charge than C*c*. In 2005 Bunz's group published the fluorescence quenching of a sugar functionalized CPE based on PPE upon complex formation with ConA and also it was suggested that by increasing the linker length and introducing more complicated sugar units, the assay sensitivity may be increased.[51] In 2006 Haung and co-workers reported the interaction of cationic CPE as shown in the structure (Figure 1.9.2) with an anionic iron containing protein, rubredoxin via coulombic interaction as well as electron transfer from CPE to iron centre.[52] They also performed controlled experiments showing that quenching of CPE with insulin and C*c* was remarkably less than that of rubredoxin indicating the good selectivity.

Generally the existence of nonspecific interaction restricts the application of CPE in direct protein detection. However an important concept taking advantage of nonspecific interaction between CPE and protein was array based protein sensor. Bunz and coworkers proposed an array based protein sensor containing six functionalized PPE polymer to identify and quantify 17 proteins.[53] These polymers interaction with each protein was based on different mechanisms and distinct fluorescence responses in presence of a particular protein. Linear discriminate analysis was performed to differentiate 17 proteins at nanomolar to micromolar concentrations and identification accuracy was reported 97%.

1.1.7.2 Indirect detection of enzyme

Detection of protease,[54-56] kinase and phosphatases[57] by using CPEs revolutionized the research interest in the drug discovery based on the enzymes, as a result in past several years there are several reports of the enzyme detection based on CPEs and they have adopted the indirect detection of enzymes strategy that is very common. Generally in this strategy a substrate that is very prone for enzymatic activity is labeled with either the quencher or fluorophore for that particular polymer and after enzymatic activity signal is gained in the form of either fluorescence turned off or turned on.

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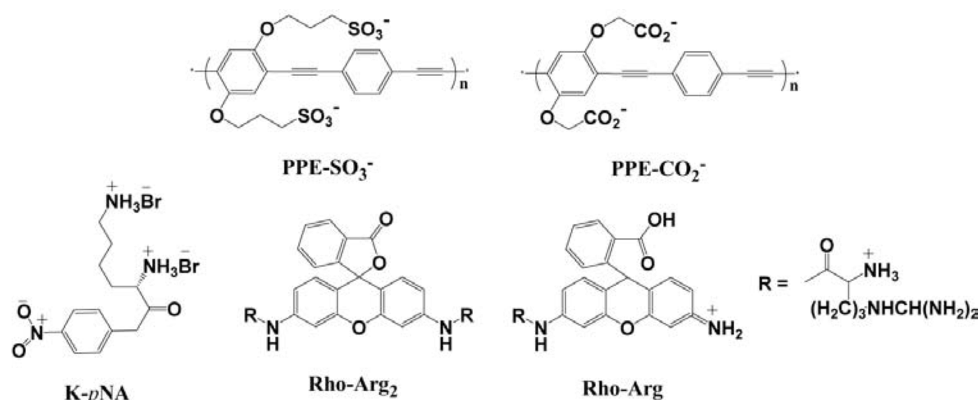

Figure 1.10 Substrate for enzymatic hydrolysis and polymers PPE-SO_3^- and PPE-CO_2^- utilized by Pinto and Schanze group are presented.

In 2004 Pinto and Schanze reported a fluorescence turn on and turn off approach for protease activity by utilizing two anionic CPEs, PPE-SO_3^- and PPE-CO_2^- respectively (Figure 1.10). A cationic substrate peptide labeled with a quencher p-nitroanilide (*p*NA) was used to quench the fluorescence of PPE-SO_3^- polymer. While the addition of protease to the solution of quencher-substrate and polymer induces hydrolysis of *p*NA-substrate and thus leading to the formation of a charge neutral *p*NA moiety, which then losses the ability to quench the polymer fluorescence. As a result fluorescence intensity from the polymer increases with progress of enzymatic hydrolysis time. Whereas in turned off approach, a colourless, nonemissive bis-arginine derivative of Rhodamine-110, Rho-Arg_2 was used as the cationic substrate for enzymatic hydrolysis. This cationic substrate was not able to quench the polymer fluorescence but it was supposed that this may electrostatically bind the polymer. Enzymatic hydrolysis generated mono arginine derivative that could efficiently quench the polymer fluorescence by FRET due to a good spectral overlap.

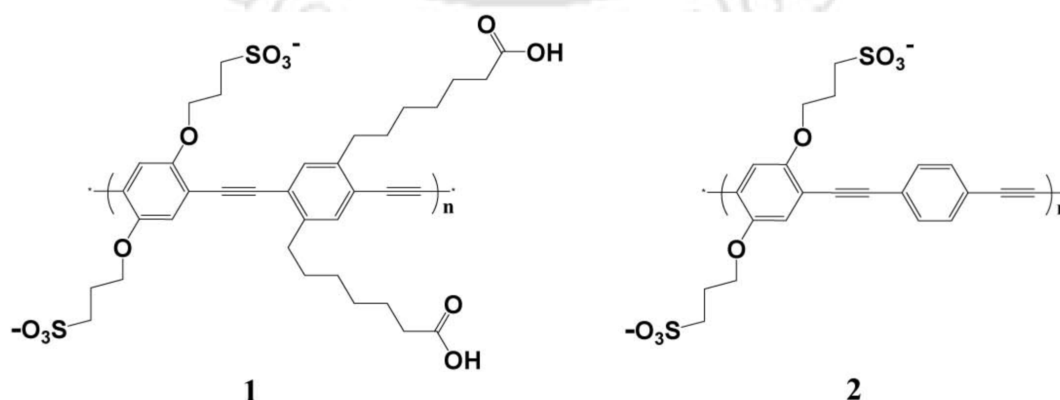

Figure 1.11 Polymers reported by Whitten group for kinase and phosphatase activities are presented.

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Simultaneously in 2004, Whitten group reported a turn on fluorescent assay for protease activity by utilizing biotin functionalized CPE-coated microsphere as well as biotin labeled polypeptide at one end and quencher on the other end. In 2004 Whitten group reported a kinase and phosphatase turned off and turned on assay based on an anionic PPE derivative containing both sulphonate and carboxylate groups as shown in Figure 1.11. Similar to their work in protease detection microspheres were coated with PPE derivative. The carboxylate group was complexed with Ga^{3+} without any quenching and Ga^{3+} are well known to have affinity towards phosphorylated peptides. Following QTL approach rhodamine labeled peptide was designed and used for the substrate of kinase activity. After kinase acivity, due to phosphorylation of peptide the polymer fluorescence was turned off whereas in turn on assay dephosphorylation by phosphatase was assayed by the same strategy. In a series of development of CPEs based enzymatic assay, Zhu et al in 2007 developed a fluorometric assay for Alzheimer disease (AD) related enzyme Acetylcholinesterase (AChE) activity by using anionic CPEs, PFP-SO_3^-.[58] In the present strategy authors employed a substrate labeled with dabcyl quencher and this labeled substrate was able to efficiently quench the polymer fluorescence through FRET and after enzymatic hydrolysis fluorescence was turned on due to the repulsion of same charges of polymer and generated species as a result of enzyme activity.

In recent years several reports on the development of phosphatase and protease assay based on CPEs have appeared. Phosphatases are present almost in all living organism ranging from bacteria to man. The main function of phosphatase is to catalyze the hydrolysis of phosphate esters with the release of inorganic phosphate and this released phosphate is then implicated in phosphate transport across biological membrane. Trypsin is one of the most important digestive enzymes present in mammalians and the assay development for trypsin activity may be a great potential for protease detection in future.

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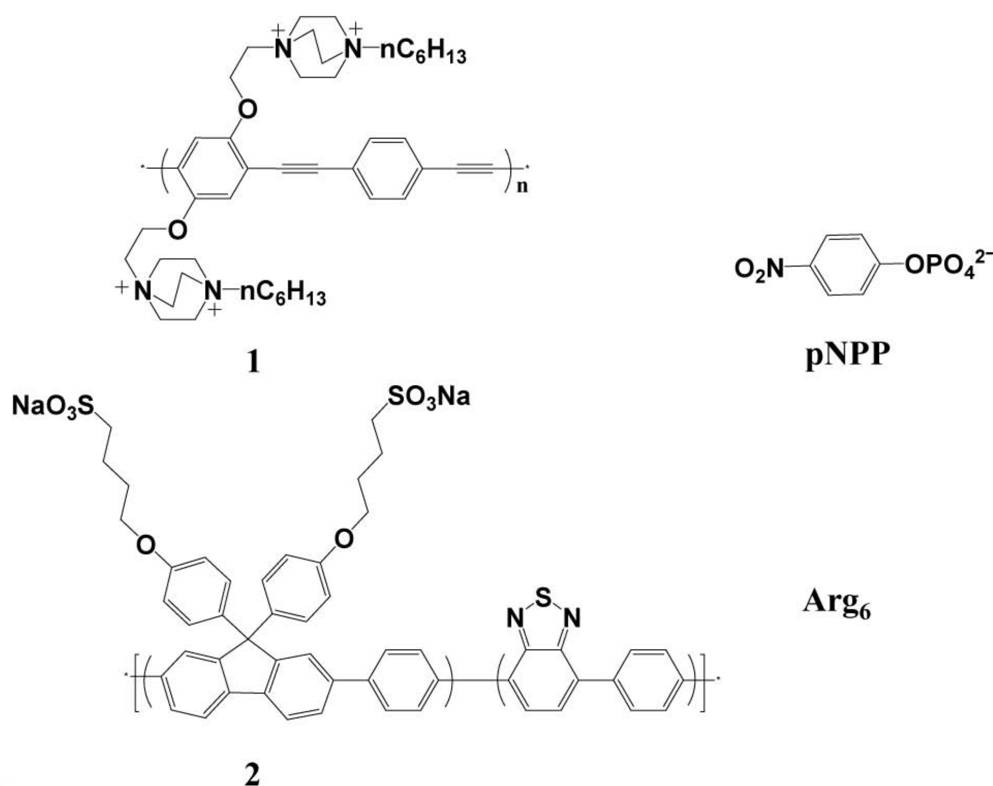

Figure 1.12 Polymers and substrates for enzymatic activity of Acid Phosphatase and Trypsine, reported by Jiang and and Wand group respectively are listed in figure.

In 2008 a fluorometric turn off assay for alkaline phosphatase activity was reported by Schanze group that was based on the anionic CPE, having carboxylate group.[59] They utilized Cu^{2+} metal to quench the polymer fluorescence and found that the fluorescence was efficiently recovered by Pyrophosphate and after enzymatic activity of alkaline phosphatase this fluorescence was turned off due to the enzymatic cleavage of Pyrophosphate into Pi at physiological pH. Recently acid phosphatase turn on assay based on the cationic CPE (Figure 1.12.1) was reported by Jiang group and it was also shown that this assay can screen the inhibitors for phosphatase activity.[60] Cationic polymer fluorescence in acidic condition was significantly quenched by negatively charged substrate p-nitrophenyl phosphate (pNPP) and due to enzymatic hydrolysis of pNPP into a neutral moiety, fluorescence from polymer was turned on and in presence of inhibitor the increase in fluorescence intensity was retarded. Trypsine turn on detection based on CPE was reported in 2007 by Wang group as shown in Figure 1.12.2. They used the multiple cationic charged polypeptides Arg_6 to quench polymer fluorescence and after fragmentation of polypeptide into single positively charged fragmented products, polymer fluorescence was turned on.

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1.2 Objective of the present work

To investigate the sensor applications of a newly synthesized sulphate functionalized water soluble polyfluorene in presence of chemical as well as biological analytes and to establish it as an fluorescent assay for examining activity of various enzymes.

1) Biologically important metals play an essential role in various life processes. Therefore selective detection of biologically important metals has been in constant attention in recent years. CPE based on PF with very high aqueous solubility can facilitate the fractional binding event with metal by coulombic interaction and facile exciton migration through its conjugated backbone resulting in an amplified fluorescence turn off signal. The work in chapter 2 is focused on the design and synthesis of a novel anionic conjugated polyfluorene with sulphate group as a receptor capable of recognizing the ferric iron metal ion at physiological pH, utilizing the rapid change in optical property.

2) Protein detection is considered to be highly important in biochemical studies, proteomics as well as in medical diagnostics. Fractional binding event of protein changes the optical properties of CPE due to signal amplification and also protein itself may alter their conformation and lose their activities. In the chapter 3, we demonstrate the interaction of ferric heme protein with anionic polyfluorene at physiological pH and also the alteration in the protein conformation and inhibition of their respective activities due to change in their heme microenvironment.

3) The interaction of CPE with specific metal ion and biologically important anions in the presence of competitive biological environment pushes their limit for the potential application in chemical and biological sensing has been demonstrated. We present in chapter 4, a novel anionic conjugated polyfluorene with sulphate group receptor initially binds ferric iron metal as well as phosphate anion selectively by turn off and turn on signals respectively in aqueous and biological environment at physiological pH.

4) ACPs in human are normally found in low concentration but an abnormal elevated level of ACP is the indication of several diseases. The strategy to detect ACPs and screen the inhibitors of its activity is of significant interest. As in several reports CPEs offer detection of biological analyte in a very less concentration due to the amplified signals. Therefore the work presented in chapter 5 is focused on an indirect detection strategy of ACP which was employed by utilizing the fluorescence property of anionic polyfluorene.

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5) It is well known that trypsin play an essential role in controlling pancreatic exocrine function, but an increased level is also associated with a few types of pancreatic diseases. Therefore efficient method to detect the trypsin and screen the inhibitors of its activity has been in constant attention in recent years. In the chapter 6 trypsin activity was assayed by using anionic polyfluorene and one inhibitor for trypsin activity was employed to check the feasibility of assay.

1.3 Conclusion

A wide variety of water soluble CPEs have been extensively studied for their unique properties. Coulombic interaction ability and amplification action by a collective response make them an excellent material for detection of chemical as well as biological analytes with typical detection limit in nanomolar or even in zeptomolar range. Utilizing these properties, CPEs have been employed as a chemical and biosensor. Small ion such as proton, metal ions, metal complexes and inorganic anions interacts with oppositely charged CPE and alter their photophysical properties via superquenching, energy transfer and conformation changes. In CPE based sensor fluorescence turn off and turn on approach due to some analyte binding event either by electron transfer, coulombic interaction or energy transfer has been followed from earlier and we have also utilized this approach in our present work. The direct detection of proteins via turn off and indirect detection of enzyme via turn off or turn on have been extensively utilized and it is very common recently.

However nonspecific interaction associated with optical sensor application of CPEs has been regarded as a significant challenge. There are a limited number of sensors those are free from unwanted nonspecific interaction. Generally a particular type of receptor is employed to provide the specificity towards a particular analyte. Therefore investigation of their synthesis, structural and property relationship studies not only intriguing but also will prove to be relevant to the understanding of their biochemical activities.

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Abstract

A novel anionic sulphate functionalized polyfluorene derivative, poly(9,9-bis(6'-sulfate)hexyl) fluorene-*alt*-1,4-phenylene sodium salt (P1) was synthesized. P1 showed blue photoluminescence under UV light in aqueous solution and it's solubility in water is 100mg/mL which is very high as compared to other reported water soluble polymers. P1 exhibits exemplary activity towards the selective and sensitive detection for Fe^{3+} in aqueous solution under physiological conditions. On binding Fe^{3+}, exceptional fluorescence quenching of P1 occurred, demonstrated by >97 % reduction in the fluorescence intensity. This remarkable ability of P1 to accomplish in-situ monitoring of indispensable biological targets like Fe^{3+} in real-time and label-free conditions, corroborate the extension of this assay system for clinical validation.

2.1 Introduction

CPs are attractive materials for modern sensor technologies. Sensor design is one of the attractive applications of the CPs for their advantages, such as intrinsic sensitivity, ease of detection, low cost and rapid implementation.[1,2] Moreover collective system response produces a high signal amplification on changing the environment even on a single site.[3-6] CPs with pendant charged (Cationic or anionic) functionality, capable of ionizing in highly dielectric media,[7] render the polymer soluble in aqueous media. The past decade has witnessed the emergence of water-soluble CPs for diverse sensor applications.[8,9] Polyphenylene vinylene (PPV) based anionic CPs used as dual sensors were able to achieve up to million-fold fluorescence amplification.[10] Several such outstanding reports on the sensitivity of CPs that are quenched by oppositely charged or neutral quenchers in aqueous medium led to the development of very versatile systems for the detection of a plethora of chemical and biological analytes.[11-13] The strong association of ions with aqueous soluble CP is attributed to columbic and hydrophobic interaction, in many respects the association between polymer and quencher in aqueous solution is similar to the association of same ions with charged micelles or vesicles.[14] Most of the biological analytes are soluble in water, so inevitably a biological sensor should manifest a good solubility in aqueous medium. But most of the reported water soluble CPs are poorly soluble in water due to the low charge density and aromatic hydrophobic backbone.[15] Hence critical requirements for a superior sensor system, in addition to aqueous solubility and high sensitivity, is to have appropriate binding

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sites, showing selectivity in label-free conditions and the ability to perform detection at physiological pH, particularly for the development of advanced biosensors. It is highly dubious that such a system exists. We report here the extraordinary sensing ability of poly(9,9-bis(6’-sulfate)hexyl)fluorene-*alt*-1,4-phenylene sodium salt, (P1), a novel anionic polyfluorene derivative, to accomplish selective recognition of Fe^{3+}, exhibiting very high solubility in aqueous medium compared to other reported polymers. Most of the groups have used trialkyl phosphite and sultone to introduce phosphonate and sulphonate groups in the side chains of conjugated polymer. We report a new anionic sulphate group containing polyfluorene derivative with high solubility in aqueous media, prepared by using conc. sulphuric acid deprotection reaction, replacing sultone or triethylphosphite. Polyfluorene based polymers are widely used in biological and chemical sensing applications because of their facile substitution at C9 position, good chemical and thermal stability.

Iron(III) is the most abundant metal in life forms having indispensable importance in metabolism, oxygen transport and oxidative phophorylation. However, excess Fe^{3+} accumulation leads to kidney, liver and DNA damage, while, its deficiency causes anemia and breathing problems. Thus a chemical sensor, that is sensitive and selective towards Fe^{3+} at physiological pH, was developed. Nonetheless, meager effort have been directed towards the design and development of sensitive and selective Fe^{3+} detection systems.[15-19] Although label-free anionic CPs that are known for their high sensitivity to small perturbations have been developed, most of them have some drawbacks like, delayed response to Fe^{3+}, low aqueous solubility and interference with other metal ions. Herein, we demonstrate that P1 with pendant sulphate group fulfils the requirements of detecting indispensable biological targets like Fe^{3+} at physiological pH, corroborating this system for clinical and diagnostic validation.

2.2 Result and discussion

To synthesize anionic water soluble polymer P1 we effected the post polymerization deprotection reaction of P0 in concentrated H_2SO_4 at room temperature. The method gives pendant sulphate group containing polyfluorene with very high solubility in aqueous media. This method was very simple, it requires only room temperature conditions and expensive inert gases or expensive catalysts are not required. Sulphated monomer may also be prepared in the same way followed by polymerization, but because of purification problems, post-

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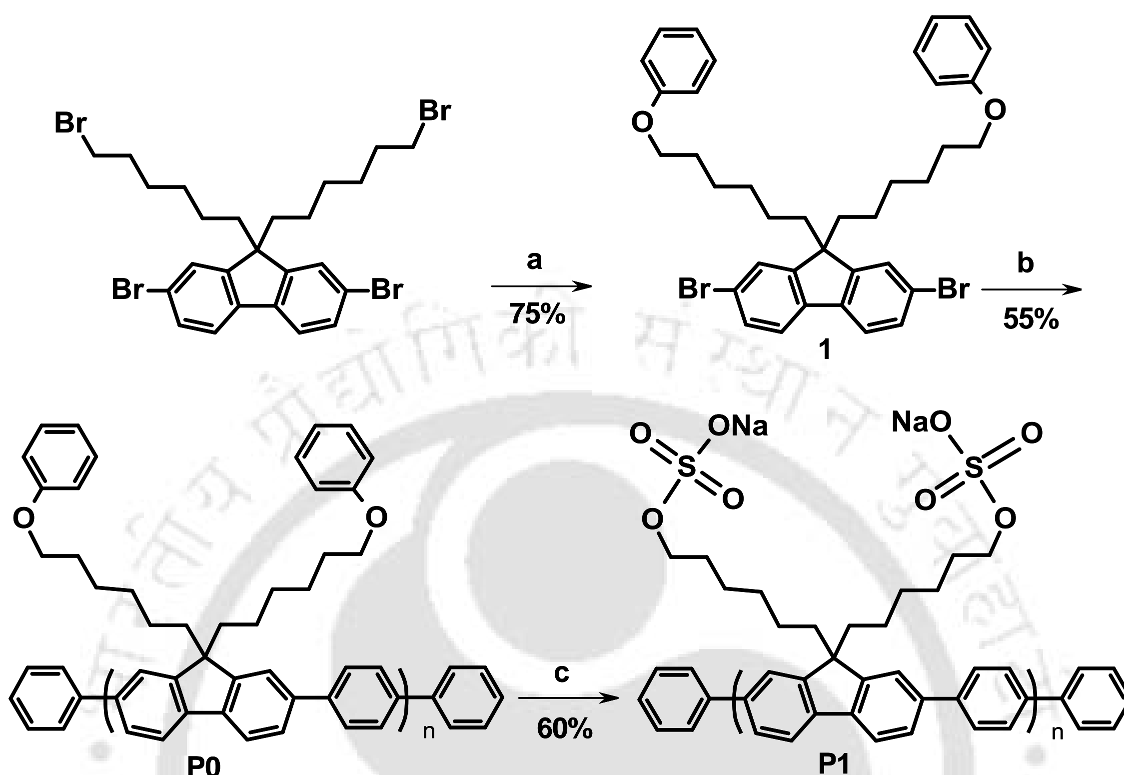
sulphation was affected in the present scheme. The synthetic entry of the polymer **P1** is mentioned in scheme 2.1.



Scheme 2.1 Synthesis of the polymer poly(9,9-bis(6'-sulfate)hexyl) fluorene-*alt*-1,4-phenylene sodium salt, (P1). **a**-2,7-dibromo-9,9-bis(6-bromohexyl)-*9H*-fluorene, phenol, K_2CO_3, dry acetone, reflux, 24 hours. **b**-Monomer 1, tetrakistriphenylphosphine palladium(0), benzene-1,4-diboronic acid, 2M aqueous K_2CO_3 and THF, reflux, 18 hours. **c**-Polymer P0, conc. H_2SO_4, room temperature, 8 hours.

The first step involves the synthesis of monomer (1), 2,7-dibromo-9,9-bis(6-phenoxyhexyl)-9H-fluorene in which phenol was O-alkylated with 2,7-dibromo-9,9-bis(6-bromohexyl)-9H-fluorene in presence of base K_2CO_3 in dry acetone, to give monomer 1 in 75% yield. Suzuki coupling of monomer 1 with benzene-1,4-diboronic acid gives the polymer P0, as pale yellow solid in 55% yield. Post polymerization of P0 results in desired polymer P1 as yellow solid in 60% yield. P1 was purified by dissolving in methanol and precipitation in acetone and for removal of small inorganic impurities dialysis was done with membrane of 5 kDa cutoff. P0 was well characterized by ^{1}H and ^{13}C NMR and was found to have MW of 30982, PDI-1.81 (GPC in THF-PS as internal standard). Since P1 was highly soluble in water (and in methanol) with solubility of 100 mg/mL, the detection of biologically important analytes

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could be carried out easily in the same phase. This remarkably high solubility of P1 in aqueous medium may be attributed to the sulphate group in the side chain of P1 that remains ionized within a large pH range.

Absorption and emission spectra of P1 were measured in deionized water. Absorption and emission maximum were found at 334 nm and 411 nm with a prominent blue color under UV light illumination as shown in Figure 2.1.

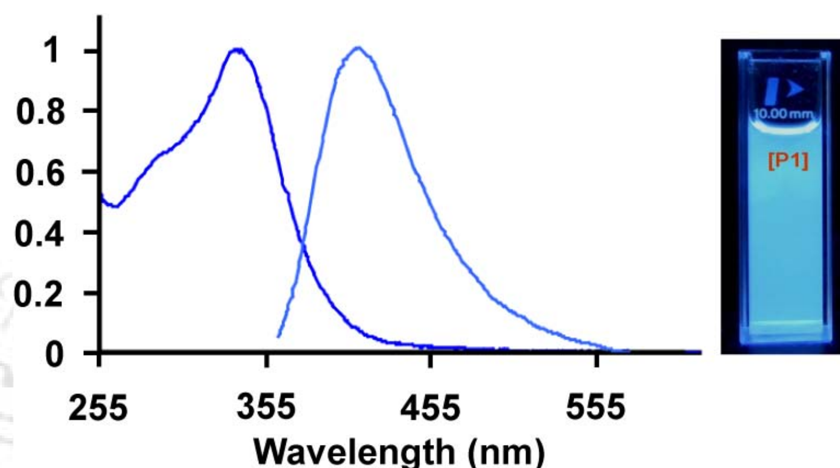

Figure 2.1 Absorption and emission spectra of P1 in aqueous solution.

Metal ions alter the optical properties of CPs by associating with their binding sites, resulting in optical events that can be visualized either by the shift in wavelength or quenching of the fluorescence intensity or both.[20] P1 was chosen to study the viability of metal binding since the novel sulfate terminated side chains would facilitate its association with oppositely charged molecules by quenching the excited state of the CPs.[10] The resulting optical changes can be conveniently studied by fluorescence spectroscopy. Salts of Mg^{2+}, Ca^{2+}, Mn^{2+}, Mn^{3+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} having concentrations 2 μM aqueous solution were titrated with P1 (0.4 μM aqueous solution) and monitored by fluorescence spectroscopy. No significant changes were observed with these metal ions even after 8 μM addition. Metal salts like Fe^{2+}, Co^{2+}, Al^{3+} and Cu^{2+} caused fluorescence quenching of P1 at very high concentrations. All other metals studied here caused no quenching or very minor quenching of the polymer P1.

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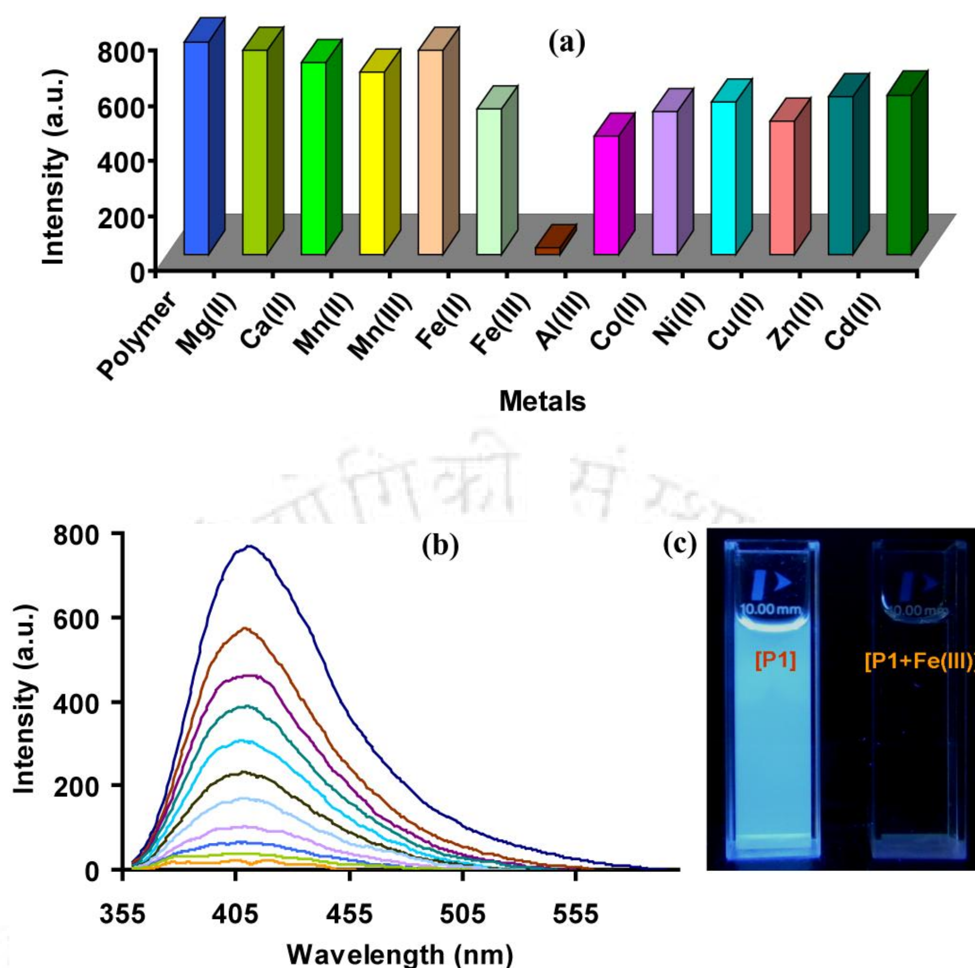

Figure 2.2 (a) Bar diagram depicting effect of various metals on the fluorescence intensity of P1 in 25 mM Tris-HCl buffer (pH 7.4). (b) PL spectra of P1 (4.0 X 10^{-7} M) with increasing concentration of Fe^{3+} shows >97% quenching in 25 mM Tris-HCl buffer (pH 7.4). (c) Blue color of P1 under UV light (left) and its quenched form (right).

But upon addition of Fe^{3+} (chloride and perchlorate) a large quenching in the fluorescence (97%) of P1 (Figure 2.2b) was observed. Such robust quenching of P1 by Fe^{3+} is possibly due to a combination of factors like competent energy migration and exciton delocalization along the polymer backbone. Quenching of P1 is observed to be maximum at lowest concentration (2.0 X 10^{-7} M) of Fe^{3+} and >50% quenching occurred at concentrations as low as 6.0 X 10^{-7} M. The fluorescence of P1 is further quenched on increasing the concentration of Fe^{3+} but was less dramatic.

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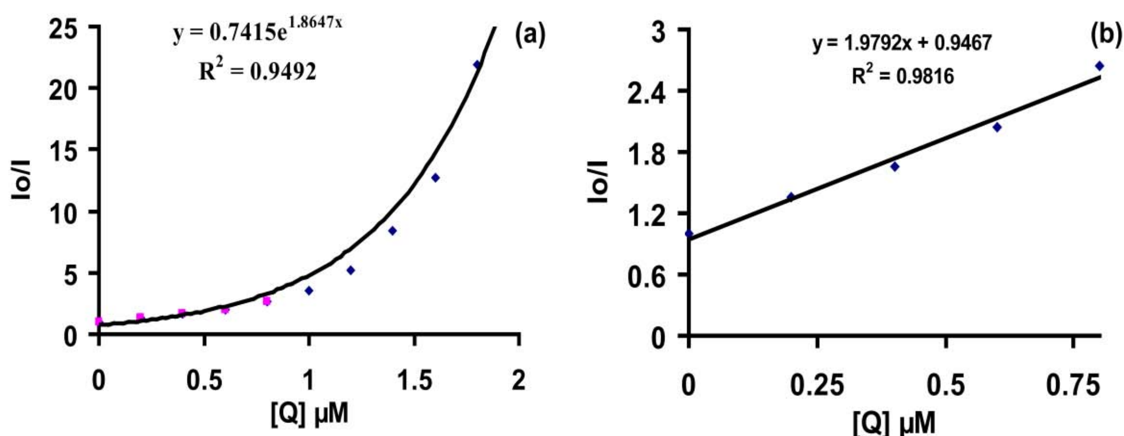

Figure 2.3 (a) Stern-Volmer plot of P1 (0.4 μM) with higher quencher (Fe^{3+}) concentration. (b) and linear static fitting with quencher (Fe^{3+}) concentration.

The efficiency of fluorescence quenching was concluded in terms of Stern-Volmer constant. Figure 2.3 shows Stern-Volmer plot of P1 upon addition of Fe^{3+} in aqueous solution. (I_o/I vs. [Q]) The Stern-Volmer equation may be written as

$$I_o/I = 1 + K_{sv}\,[Q]$$

where I_o is the fluorescence intensity of P1, and I is the fluorescence intensity of P1 after addition of a given concentration of quencher [Q] where [Q] = Fe^{3+}. K_{sv} value concluded from the plot was 1.98×10^6 M^{-1}. From the figure it was seen that as the quencher concentration increases the plot becomes superlinear, indicating quencher induced aggregation of conjugated polymer.

However in absorption spectra no significant change was observed with above metal ions but on addition of Fe^{3+} absorption intensity increased simultaneously accompanied by a blue shift of about 8 nm, (Figure 2.4) indicating fluorescence quenching behavior to be static (associated complex) type.[15] Further, lifetime values of P1 were not modified on titration with Fe^{3+}, signifying the static quenching mechanism.

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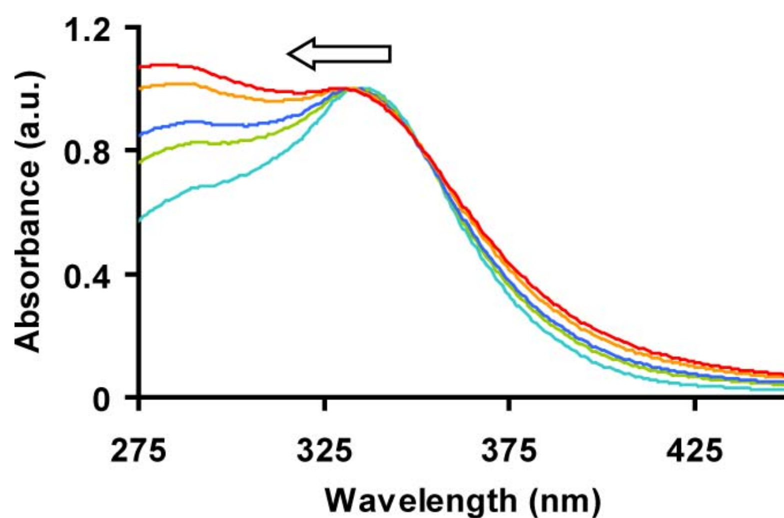

Figure 2.4 Changes in the absorption spectrum of P1 on addition aqueous solution of Fe^{3+} metal salt in 25 mM Tris-HCl buffer (pH 7.4).

This remarkable ability of P1 as a highly selective and sensitive probe for Fe^{3+} in aqueous medium irrespective of Fe^{3+} salts opens avenues for its utility in probing Fe^{3+} in biological systems with a broad range of likely applications that include iron metabolism, anemia and redox reactions. P1 sensitivity towards biological analytes at physiological pH has been described in the next chapters.

2.3 Conclusion

In summary, a novel anionic polyfluorene derivative (P1) having sulfate as terminal groups was designed and synthesized using Suzuki coupling reaction. P1 was utilized for rapid, label-free detection and estimation of indispensable biological targets like Fe^{3+} in aqueous medium. These significant and unique properties of anionic P1 will certainly inspire fundamental development of sulfate terminated conjugated fluorescent polymers that have shown outstanding ability as amplifying fluorescent polymers in clinical applications. Additionally, their ability to transport electronic excited states and inherent film forming properties on desired substrates promises a bright future for interdisciplinary applications like healthcare, security, environmental monitoring and optoelectronic devices.

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2.4 Experimental

2.4.1 Reagents and materials

All the reagents and solvents were purchased from Aldrich Chemicals (India), Merck (India) or Ranbaxy (India) and were used as received. Milli-Q water was used in all the experiments.

2.4.2 Instrumentation

FT-IR spectra were recorded on a Perkin Elmer spectrometer with samples prepared as KBr pellets. ^{1}H NMR (400 MHz) and ^{13}CNMR (100 MHz) spectra were obtained with a NMR spectrometer Varian-AS400. Electro spray ionization mass (ESI-MS) spectra were recorded on a Waters (Micro mass MS-Technologies) Q-Tof Premier mass spectrometer. GPC was recorded with Waters-2414 instrument (Polystyrene calibration). UV/Visible and PL spectra were recorded on a Perkin Elmer Lambda-25 spectrophotometer and Varian Cary Eclipse Spectrophotometer respectively. Fluorescence lifetimes were measured with the use of the Jobin Yvon Spex Fluorocube instrument. IBH 375 nm LED was used for excitation and emission intensity was recorded using 380 nm Scotch cut-off filters. The Fluorocube instrument employs a TBX-04 detector with jitter timing of 250 ps FWHM or better. The fluorescence decays were analyzed with the reconvolution method using software provided by IBH.

2.4.3 Synthesis of monomer 1

A mixture of 2,7-dibromo-9,9-bis(6-bromohexyl)-9H-fluorene (2.0 g, 3.08 mmol), phenol (1.16 g, 12.32 mmol) and K_2CO_3 (2.12 g, 30.8 mmol) was refluxed in dry acetone for 24 hours. Reaction progress was monitored by TLC. (1:9 Ethyl acetate: Hexane) The mixture was cooled, filtered over celite pad and the filtrate dried under vacuum. Repeated washing with methanol gave the desired product **1**. (1.56 g, 75 %). ^{1}H NMR (400MHz, $CDCl_3$) δ (ppm)**:** 7.53 (d, *J* = 8.8Hz, 2H), 7.47 (s, 2H), 7.27 (t, *J* = 8.4 Hz, 4H), 6.93 (t, *J* = 7.2 Hz, 2H), 6.85 (d, *J* = 8.0 Hz, 4H), 3.85 (t, *J* = 6.4Hz, 4H), 1.95 (t, *J* = 8.0 Hz, 4H), 1.62 (q, *J* = 6.8Hz, 4H), 1.25 (q, *J* = 7.2Hz, 4H), 1.15 (q, *J* = 7.2Hz, 4H), 0.64 (q, *J* = 4.0Hz, 4H). ^{13}C NMR (100MHz, $CDCl_3$) δ (ppm)**:** 159.27, 152.59, 139.33, 130.53, 129.64, 126.38, 121.81, 121.48, 120.707, 114.71, 67.90, 55.87, 40.37, 29.85, 29.40, 25.9, 23.89. HR-MS(ESI) calculated for $C_{37}H_{40}Br_2O_2$: 676.5381, found, 679.6098.

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2.4.4 Synthesis of P0

A mixture of **1** (0.5g, 0.74mmol), tetrakistriphenylphosphine palladium(0) (0.042g, 0.03mmol), benzene-1,4-diboronic acid (0.123g, 0.74mmol), 5 mL 2 M aqueous (Milli-Q water) K_2CO_3 and THF (10 mL) were taken in a flask fitted with a reflux condenser. The reaction mixture was degassed thrice by freeze thaw pump cycles followed by refluxing for 18 hours under inert atmosphere. Iodobenzene (0.03g, 0.147mmol) was added to the reaction and refluxed for 3 hours. This was followed by addition of phenyl boronic acid (0.018g 0.147mmol) dissolved in 1 mL THF and refluxing for additional 3 hours. The reaction mixture was cooled, poured into methanol and stirred for 30 minutes. The precipitates were collected by filtration, followed by washing with methanol. Soxhlet extraction of the above precipitates by acetone for 24 hours gave the desired polymer **P0** (0.24 g, 55%). ^{1}H NMR (400MHz, $CDCl_3$) δ (ppm)**:** 7.75 (b), 7.64 (b), 7.58 (b), 7.47 (b), 7.22 (b), 6.88 (b), 6.82 (b), 3.81 (b), 2.04 (b), 1.59 (b), 1.22 (b), 0.76 (b). ^{13}C NMR (100MHz, $CDCl_3$) δ (ppm)**:** 159.27, 153.21, 151.77, 151.1, 140.43, 130.36, 129.56, 127.70, 126.30, 121.55, 120.62, 114.67, 67.90, 55.67, 40.56, 29.87, 29.32, 25.85, 23.88.

2.4.5 Synthesis of P1

Polymer **P0** (0.2 g) was stirred with 10 mL conc. H_2SO_4 for 8 hours at room temperature. The reaction mixture was cooled to 0 °C and treated with 20 % aqueous NaOH solution carefully until neutralization. The precipitates obtained were filtered, washed with cold water and dried to get desired **P1.** P1 was further purified by dissolving in methanol and precipitation in acetone and for removal of small inorganic impurities dialysis was done with membrane of 5 kDa cutoff. (0.13 g, 60 %). ^{1}H NMR (400MHz, CD_3OD) δ (ppm): 8.54 (b), 8.35 (b), 7.85 (b), 7.06 (b), 4.02 (b), 2.17 (b), 1.29 (b), 1.09 (b), 0.95 (b). FTIR (ν_{max}, KBr pellet): 3460, 2956, 1645, 1558, 1448, 1415, 1195, 1037, 848, 605 cm^{-1}.

2.4.6 UV titration of P1 with Fe^{3+}

To a solution of **P1** (2×10^{-5} M) was carefully titrated a solution of Fe(III) metal salts up to 2×10^{-4} M concentrations. The changes in the absorption were monitored and recorded carefully.

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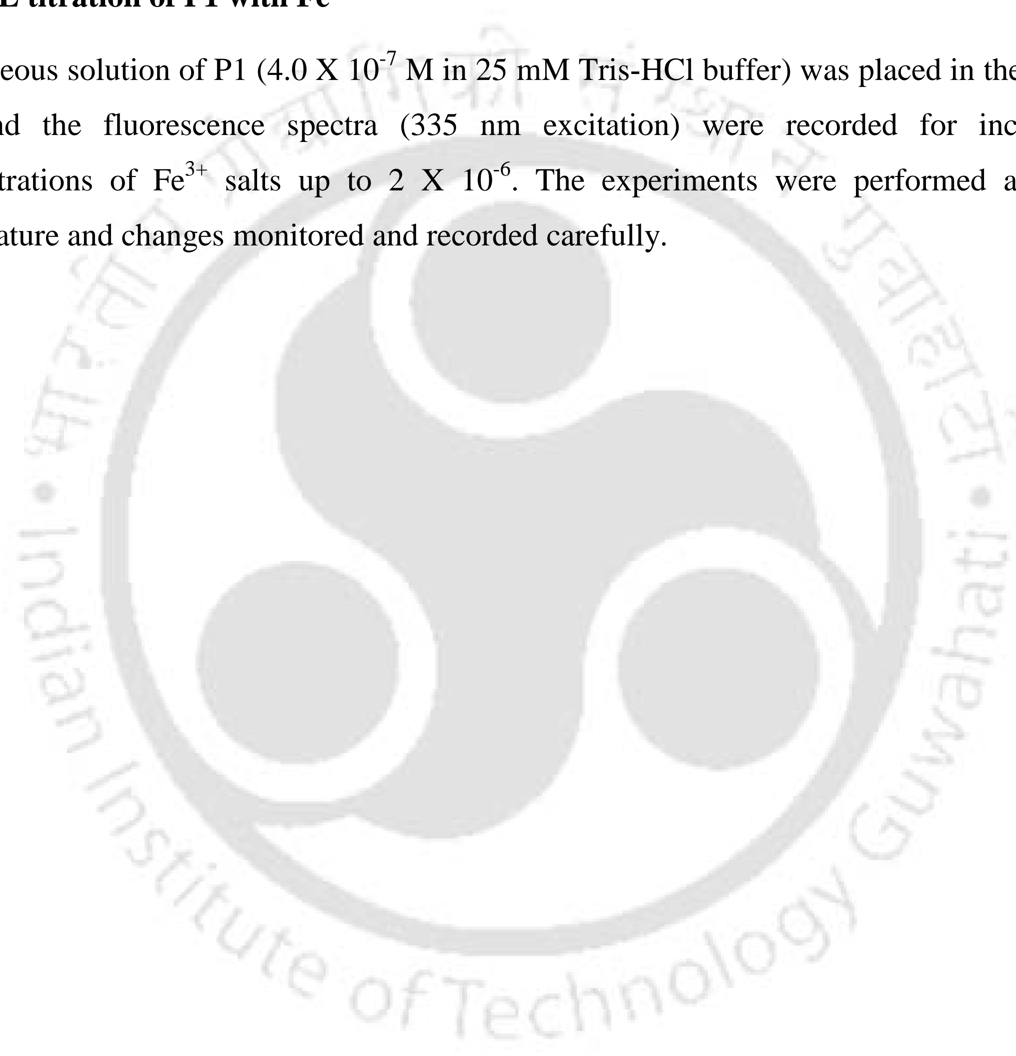
2.4.7 PL titration of P1 with metals

An aqueous solution of P1 (4.0×10^{-7} M in 25 mM Tris-HCl buffer) was placed in the quartz cell and the fluorescence spectra (335 nm excitation) were recorded for increasing portions of metal salts up to 8 μM. The experiments were performed at room temperature and changes monitored and recorded carefully.

2.4.8 PL titration of P1 with Fe^{3+}

An aqueous solution of P1 (4.0×10^{-7} M in 25 mM Tris-HCl buffer) was placed in the quartz cell and the fluorescence spectra (335 nm excitation) were recorded for increasing concentrations of Fe^{3+} salts up to 2×10^{-6}. The experiments were performed at room temperature and changes monitored and recorded carefully.



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Abstract

Anionic water-soluble conjugated polyfluorene derivative poly(9,9-bis(6-sulphate hexyl) fluorene-*alt*-1,4-phenylene) sodium salt (P1) that binds Fe(III) proteins is reported. The photophysical properties of P1 were modified by the interaction with ferric heme containing proteins Cytochrome *c* (C*c*) and Methemoglobin (MetHb). P1 was found to be highly sensitive towards Fe(III) heme proteins and since the absorption spectra of hemin and heme containing proteins (~410 nm) had major overlap of >95% with the 411 nm emission of anionic polymer P1, accompanying resonance energy transfer from P1 (donor) to hemin unit (acceptor) of metalloproteins and the fast photo induced electron transfer (ET) from anionic P1 to proteins is observed. We also observed that the respective activities of ferric heme proteins were inhibited and proteins were unfolded, due to modification in their heme micro environment. Due to the toxicity caused by these heme redox active iron proteins, their elevated levels, localization and accumulation in the brain many forms of disease occur. The observations reported in the present work, provide the first example for the use of water soluble conjugated polymer in applications such as (1) to detect small quantities of iron-proteins in aqueous medium / physiological condition with highest K_{sv} value of 2.27 X 10^8 M^{-1} for C*c* and 3.81 X 10^7 M^{-1} for MetHb, (2) to visualize structural modifications in real time and (3) effect of a conjugated polymer on the inhibition activity of metalloprotein. All this is achieved by generating optical events, taking advantage of the bright fluorescence of anionic polyfluorene P1 in this case, that can be observed and monitored by modification in the absorption and emission color in real-time. Since the removal of iron containing proteins reduces the redox imbalance and diminishes oxidative stress, efficient strategies for early as well as sensitive detection and binding of these heme containing proteins could influence the understanding of key pathological processes in neurodegenerative diseases.

3.1 Introduction

The selective detection of proteins and the study of its aggregation, folding / unfolding behavior and fibril formation are vital in biochemical studies, proteomics as well as in medical diagnostics. Proteins are highly important for various life processes but at the same time because of variation in protein expression they are also responsible for several fatal diseases and death.[1] It is well known that iron is the most abundant metal in most life processes and it plays a major role in the progression of a number of metabolic activities.

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The cell iron is bound with proteins like cytochrome c (C*c*), ferritin, hemoglobin etc. in almost all biological systems, whereas only a small fraction is found to be in labile state.[2] C*c* is one of the most extensively studied mitochondrial, small heme protein that plays an essential role in life process, but it is also known to be a key opening for the door to death.[3]

Through various reports, it is recognized that in the cells, C*c* is also involved in apoptosis, signaling the cells to programmed cell death due to generation of reactive oxygen species by mitochondrial enzyme oxidation.[4] Hemoglobin that consists 70% of total body iron continuously converts to methemoglobin (MetHb) by oxidative process, but if it does not convert back to hemoglobin, the MetHb level increases substantially, raising the possibility of methemoglobinemia. MetHb is the ferric form of Hb with water or hydroxide as a sixth coordination ligand to the iron.[5] MetHb is the naturally occurring oxidized metabolite of hemoglobin. Methemoglobinemia occurs when red blood cells (RBCs) contain greater than 1% MetHb.[6,7] This occurs from either congenital changes or from exposure to toxins that acutely affect redox reactions involving MetHb. When MetHb does not bind oxygen, it leads to a functional anemia. Therefore protein detection and study of variation into these configurations are of particular significance. Since proteins are much more complex and sensitive, designing artificial assay systems with appropriate receptor for the target protein is still a crucial challenge in protein biosensor.[8] In this regard polymers possessing π–conjugated backbone system present a tremendous platform for designing the protein biosensors because this backbone can facilitate electron delocalization and exciton migration through energy and electron transfer, resulting in amplified signals.[9] Therefore, interaction of small fraction of analyte alters the photophysical property of conjugated polymer which is exploited for the protein detection here. In the presence of protein, the photophysical properties of the conjugated polymer P1 are modified that can be easily visualized. In addition, the presence of P1 in small quantities also affects the structure of the protein resulting in unfolding and modification in its activity. External agents such as guanidine hydrochloride are well-known to denature the proteins which have been characterized by performing systematic optical spectroscopic studies. Similarly, intrinsic fluorescence property of proteins due to the presence of tyrosine and tryptophan residues have also been used to follow protein

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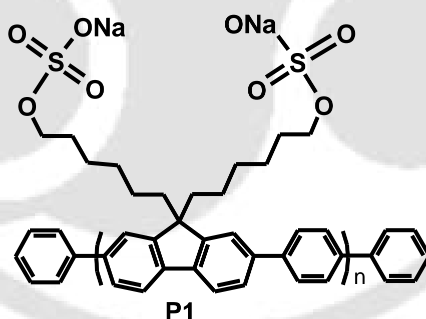
unfolding because their fluorescence properties are very sensitive to their environment which changes when protein fold or unfold.[10,11]

Herein, we report an anionic water soluble polyfluorene derivative poly(9,9-bis(6-sulphate hexyl) fluorene-*alt*-1,4-phenylene) sodium salt (P1) (Scheme 3.1), as an efficient fluorescent probe for the detection of ferric heme proteins C*c* and MetHb as well as hemin molecule. We describe here the utilization of intrinsic optical properties of P1, followed by examining their fluorescence turn off in aqueous solution in the presence of metalloproteins at physiological pH. Stern-Volmer quenching pattern represents the fluorescence quenching efficiency of the P1 polymeric system and confirms to be the highest among artificial assays. Ferric heme proteins C*c*, MetHb and active site ferric heme were found to be highly efficient quenchers of P1 fluorescence than non-metalloproteins. Absorption spectroscopy and gel technique revealed complexation of the proteins with P1. Further systematic spectroscopic studies revealed that P1 was also able to induce loss of activity and unfolding of ferric heme proteins by modifying the heme micro environment and decreasing the fluorescence intensity with a shift in wavelength of tryptophan residue. [12,13]

3.2 Result and Discussion



Scheme 3.1 Chemical structure of P1

Since P1 shows distinct photophysical properties in the presence of ferric iron[14] we studied its interaction with few ferric proteins. The metalloproteins used in this study include the C*c*, MetHb, Ferritin and non metalloproteins such as Lysozyme, BSA, and Casien. P1 has absorption maximum at 334 nm and emission at 411 nm in aqueous conditions as shown in chapter 2. P1 was highly fluorescent at pH 7 in 25 mM Tris-HCl buffer solutions with excitation wavelength at 335 nm (Figure 3.1a). In these studies, we found that P1 (0.4μM) fluorescence intensity was significantly decreased with increasing

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concentration of C*c* (0.05μM) (Figure 3.1b). We also investigated whether this decrease in intensity was due to cationic and anionic interaction between cationic protein and anionic P1 as reported earlier for anionic polymer and MV^{2+} interactions.[15] Therefore interaction with high p*I* value protein lysozyme at the same concentration as in C*c* was investigated at physiological pH and the results showed that it was not able to reduce the fluorescence intensity of P1 at any noticeable significance. The reason behind this was supposed to be more positive character of C*c* than Lysozyme at physiological pH leading to the electrostatic interaction[16-18] along with the heme integrity of protein which may be a dominant factor towards high sensitivity. To test this hypothesis, the interaction of hemin was investigated with P1 fluorescence and it was observed that 0.16 μM hemin could efficiently quench the fluorescence of P1. (Figure 3.1a)

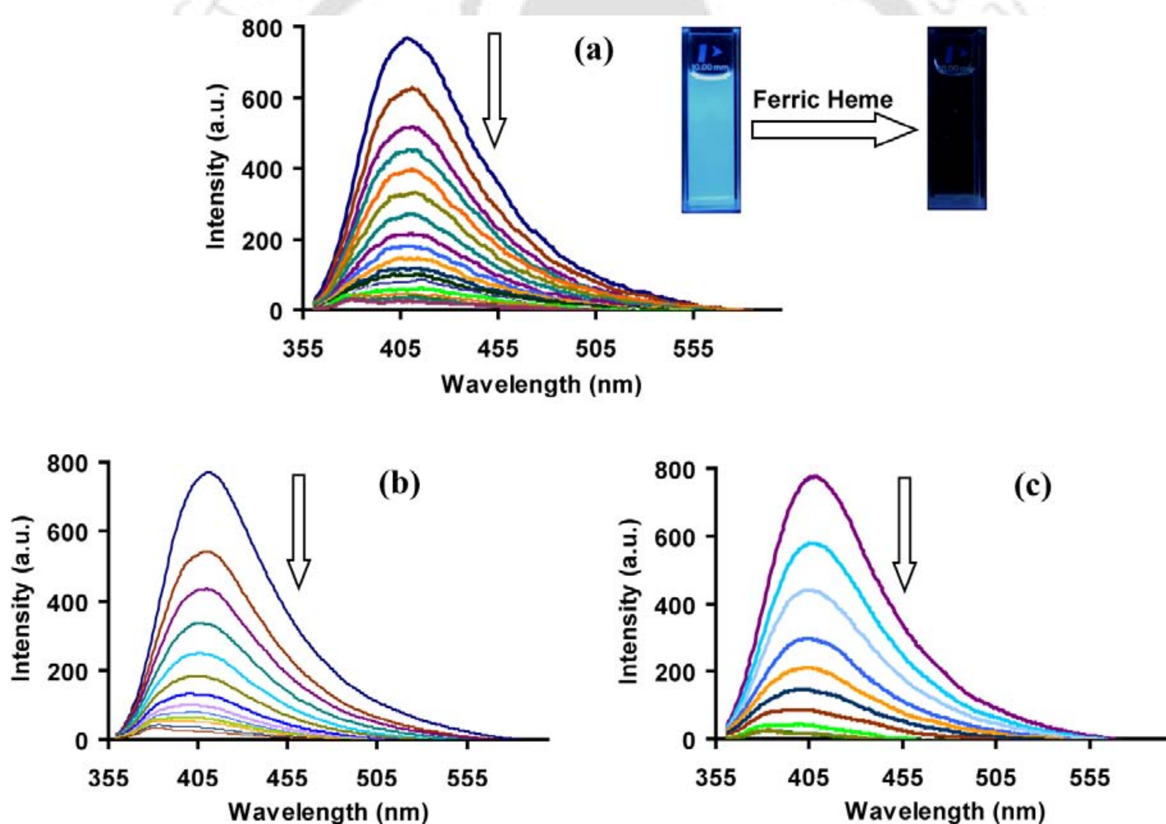

Figure 3.1 Fluorescence response of P1 (0.4 μM) towards proteins and hemin was checked in 25 mM Tris−HCl solution. (a) Hemin was added upto a concentration of 0.16 μM. (b) C*c* was added upto 0.033 μM. (c) Methemoglobin was added upto 0.16 μM with P1.

Despite MetHb being negative at physiological pH, the fluorescence quenching studies showed that the ferric heme unit of MetHb was also an efficient quencher of P1 proving

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the dominant effect of heme proteins towards high sensitivity (Figure 3.1c). However, non-metalloproteins BSA and casein, that lack ferric heme unit, were unable to quench the fluorescence of P1 and hence is not shown here. Non heme protein ferritin also quenched the fluorescence intensity of P1 but the quenching was less efficient as compared to ferric heme proteins. Hence, the interaction of ferritin with P1 is not presented along with these results. The efficiency of fluorescence quenching of P1 was concluded by generating a Stern–Volmer plot and compared.

$$I_o/I = 1 + K_{sv}\,[Q]$$

where I_o and I are fluorescence intensity in the absence and presence of the quencher, respectively and [Q] is quencher concentration.

Protein	Stern-Volmer values (M^{-1})
C*c*	2.27×10^{8}
MetHb	3.81×10^{7}
Hemin	5.31×10^{7}

Table 3.1: K_{sv} values for C*c*, MetHb and hemin.

High K_{sv} value of an artificial assay indicates the probe to have high sensitivity in the protein biosensor application.[19] Since C*c* quenches the fluorescence of P1 at a lower concentration than other metalloproteins reported above, we got a higher K_{sv} value in the order of 10^8 whereas the values of MetHb and hemin were 10^7 orders respectively (Table 3.1). It can be inferred from the above table that C*c* is a highly efficient quencher of P1 than heme and MetHb with all three values being highest in literature. But non-metalloproteins showed insignificant fluorescence quenching in comparison to metalloproteins indicating that the fluorescence of P1 decreases due to porphyrin functionality with ferric iron.

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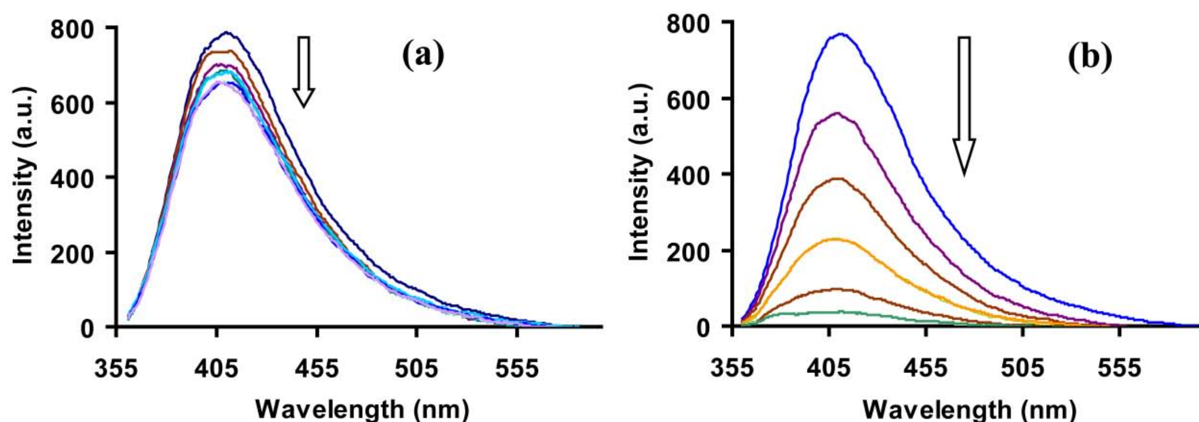

Figure 3.2 (a) Changes in the fluorescence intensity of P1 (0.4 μM) was checked with apoenzyme part (0–0.2 μM) (b) Changes in the fluorescence intensity of P1 (0.4 μM) was checked with hemin part (0–0.17 μM).

To support this result we separated out hemin part from MetHb by deproteinising the protein by reported method[20] and then repeated the PL experiment with both hemin and the apoenzyme part. According to Figure 3.2(a), the apoenzyme part shows an insignificant fluorescence quenching providing evidence that this fluorescence of polymer system P1 is unaffected by non-metalloproteins. However, more than 97% fluorescence quenching of P1 occurs on addition of separated hemin part as seen from the Figure 3.2(b). The UV/Vis spectra of P1 (6 μM) at physiological pH in 25 mM Tris–HCl buffer solution, with C*c* (2 μM), MetHb (4 μM) and hemin (5 μM) are shown in Figure 3.3. One inherent feature we observed was that the absorption spectra of polymer P1 (λ_{max}-334 nm) was seen shifting towards longer wavelength, 7 nm in case of C*c*, 7 nm in case of MetHb and 18 nm in case of hemin. These results suggest that P1 associates with the metalloproteins resulting in the observable shift in the absorption spectra of P1.[21]

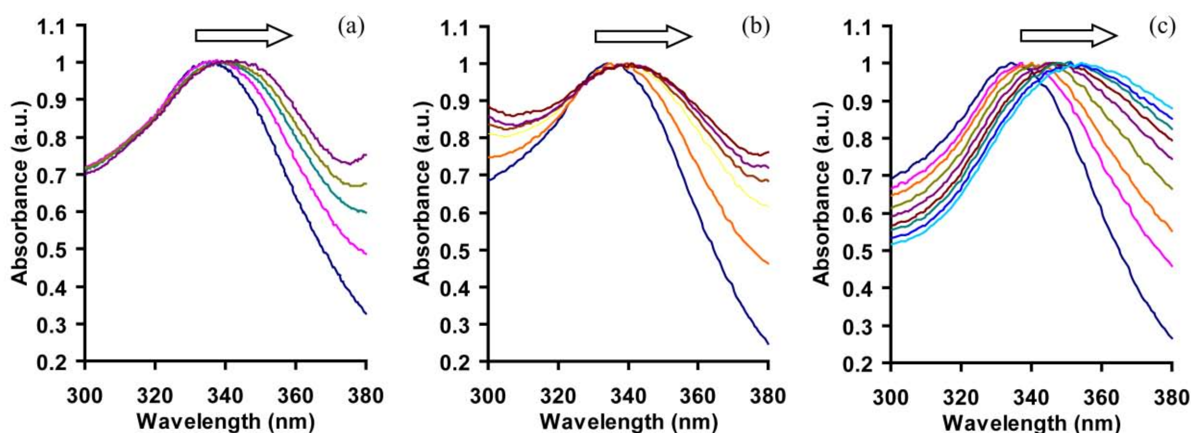

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Figure 3.3 Absorption spectra of P1 with metalloproteins and hemin were recorded in 25 mM Tris−HCl buffer solution. (a) Normalized absorption spectra of C*c* upto 1 µM show 7 nm shift. (b) Normalized absorption spectra of MetHb upto 4 µM shows 7 nm shift. (c) Hemin upto 5 µM show a drastic shifting pattern of 18 nm in normalized absorption spectrum.

The interaction of metalloproteins and P1 was also monitored by native gel electrophoresis experiments (Figure 3.4). For all the metalloproteins 1% agrose gel was prepared in 1X Tris-glycine buffer. For C*c* and lysozyme, the pH of buffer solution was maintained at 8.6 whereas for MetHb, the buffer pH was maintained at 6.0.

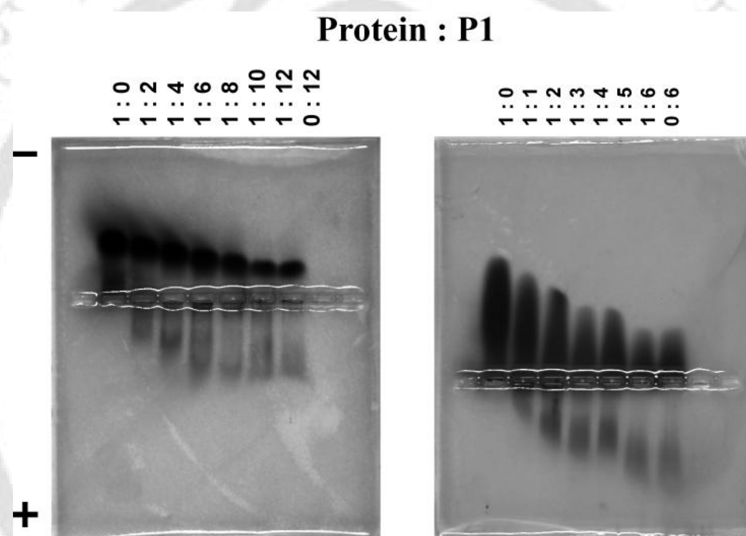

Figure 3.4: Native gel electrophoresis of metalloproteins and polymer P1 in Tris−glycine buffer with 1% agrose was performed keeping metalloprotein concentration constant in each lane and varying P1 concentration. (a) C*c*, 50µM was the concentration in each lane and the first extreme left lane contained only C*c* but P1 was varied according to ratio 0, 2, 4, 6, 8, 10, 12 from left to right lanes and in the last extreme right lane contained only P1 (0.6 mM). (b) MetHb 50 µM concentration in each lane and first extreme left lane contained only MetHb but P1 was varied according to ratio 1, 2, 3, 4, 5, 6 and last extreme right lane contained only P1 (0.030 mM).

The movement of C*c* and MetHb towards cathode shows the cationic nature of the proteins. For P1 no bands were observed even after staining. The controls of metalloproteins C*c* (50 µM) and MetHb (50 µM) without P1 showed high intensity of

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band but when P1 concentration ratio along the well was increased, intensity of protein band decreased and these were found in close proximity to the well. In case of C*c* band (Figure 3.4a), the intensity was seen decreasing from protein control to increasing concentration ratio of P1 but an additional increasing band intensity pattern appeared towards the cathodic side. Additional bands near the well was the almost neutral complex of protein and P1 but as the polymer P1 concentration ratio was increased, additional bands were observed shifting towards anodic side well to well, conforming that the protein bound complex is negative in nature. In a similar manner, MetHb also showed (Figure 3.4b) protein band intensity decreasing pattern on the cathodic side and additional complex band intensity increasing pattern on the anodic side from the concentration ratio of protein to polymer P1, 1:0 to 1:6.

Further, to understand the nature of interaction between P1 and protein, systematic spectroscopic studies were performed. In the native state of C*c*, heme iron is ligated to His^{18} and Met^{80} which are the axial ligands under physiological conditions that play a central role in the folding and unfolding mechanism.[22] Coordination of these ligands produce a low spin complex with a soret absorption maximum at 410 nm. When P1 (35 μM) was added into C*c* (6 μM) at pH 7.0 in Tris−HCl buffer, soret band at 410 nm was blue shifted to 402 nm (Figure 3.5a) with increase in absorption intensity, indicating the replacement of axial ligand Met^{80} by the solvent water, since the Met^{80} ligand is more labile than His^{18} ligand.[23] Whereas, in the case of MetHb this soret band intensity decreased with increasing concentration of P1 (Figure 3.5b).

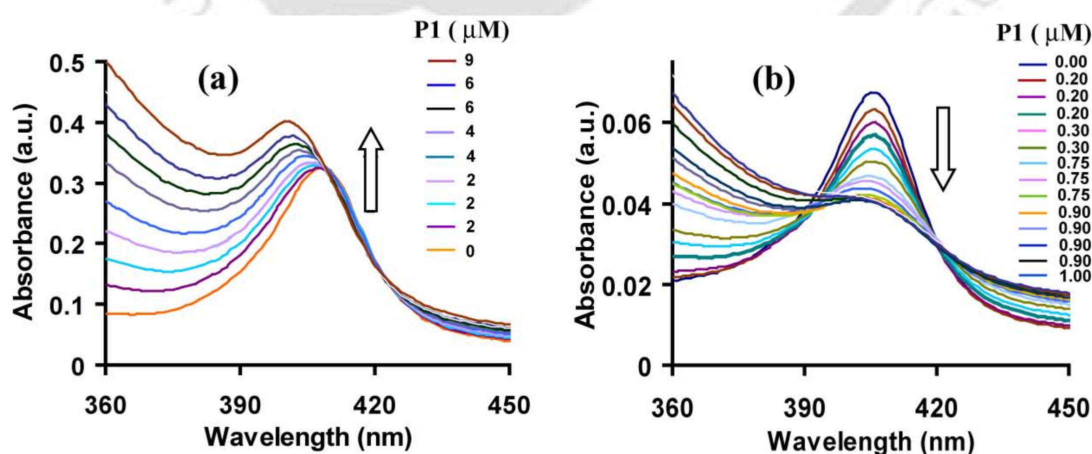

Figure 3.5 Absorption spectra (Soret region) of (a) Native C*c* (6 μM) and (b) MetHb (1 μM) at pH 7.4 in Tris−HCl buffer with increasing concentration of P1.

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In case of both these proteins, the P1 is responsible for changes in the heme microenvironment and this change leads to structural modification (folding/unfolding) of the proteins, which was confirmed by fluorescence emission of tryptophan residue. In proteins, typically, fluorescence parameters give direct interpretation of the degree of exposure of the fluorophore to the solvent.[24] For tryptophan emission, excitation wavelength was kept at 290 nm and the maximum emission wavelength was observed between the range 310 - 350 nm.

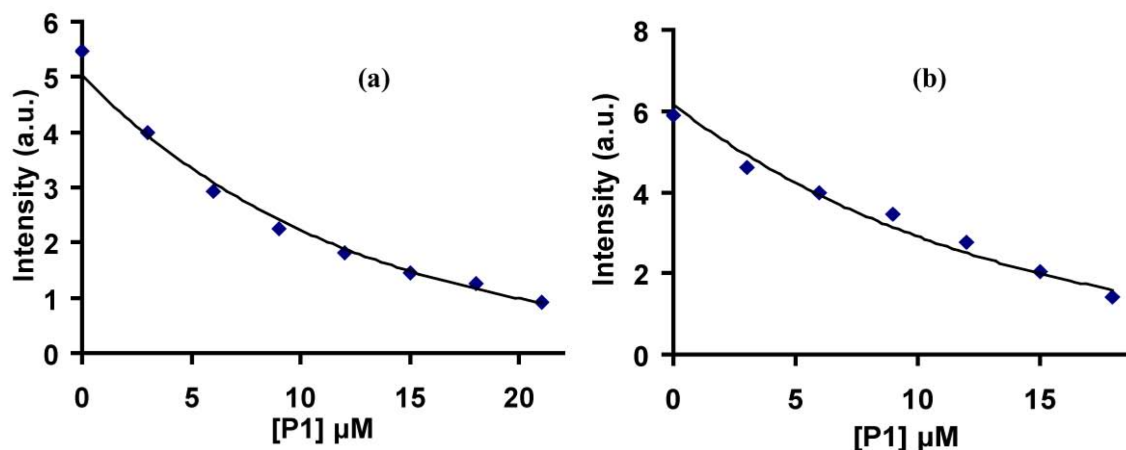

Figure 3.6 Changes in tryptophan fluorescence intensity of (a) C*c* (8 μM) on addition of P1 (0–30 μM) (b) MetHb (10 μM) on addition of P1 (0–20 μM) in Tris–HCl buffer.

On adding P1, into the solution of C*c*, (Figure 3.6a) the fluorescence emission of tryptophan residue decreased accompanied by a red shift, providing strong evidence that heme was exposed to solvent, resulting in unfolded and chemically modified C*c*.[25] However, in the case of MetHb (Figure 3.6b), the tryptophan fluorescence was decreased with a blue shift, indicating that protein conformational structure rearranges in such a way that tryptophan got buried in the relatively hydrophobic interior or interfaces in the protein. In both the cases, this led to unfolding of the proteins. Almost in all the proteins, amino acids tryptophan, tyrosine and phenylalanine are intrinsically fluorescent but tryptophan is the most useful among the three due to less extinction coefficient and high quantum yields. In addition, tryptophan and phenylalanine relatively lack environmental sensitivity of their emission profile and make them less useful than tryptophan.[24] The fluorescence properties of tryptophan are very sensitive to their environment which changes when protein conformation changes.[26,27] This blue shift and red shift of tryptophan accompanying with fluorescence quenching in MetHb and C*c* indicates that

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P1 is able to induce conformation modification and unfold both the proteins. From the above experiment we were able to get strong evidence that protein undergoes unfolding.

Hence, circular dichroism (CD) spectroscopy was also used to monitor structural transformations in the proteins. As already reported, far UV region ranging from 180 to 250 nm reflects information about the peptide bond asymmetric environment and reflects the secondary structure content of the designated protein.[28] Far UV−CD spectra can predict the conformational changes in proteins arising due to interaction with some host species. C*c* (50 μM) in 25mM Tris-HCl buffer gave a characteristic peak at 222 nm, which indicates α-helical content of the protein and this molar ellipticity at 222 nm, which was used to calculate the α-helix fraction. After increasing the concentration of P1 (0−150 μM) we observed that helicity of C*c* was decreasing (Figure 3.7a). Similarly, MetHb (40 μM) in 25 mM Tris-HCl buffer also gave a peak at 222 nm and after increasing the concentration of P1 (0−100 μM) the helicity of MetHb was decreasing (Figure 3.7b).

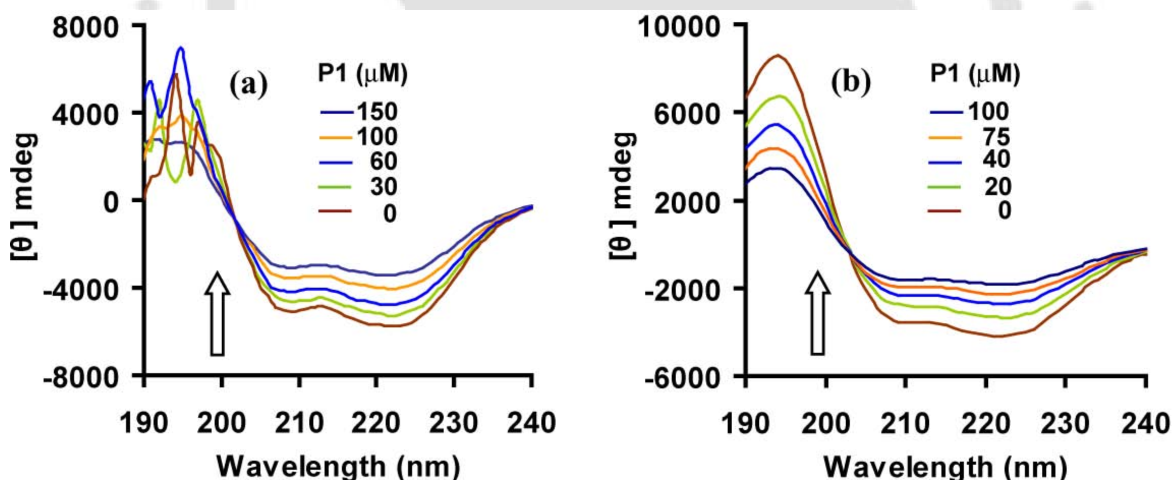

Figure 3.7 CD spectra in 25 mM Tris−HCl buffer solution.(a) C*c* (40 μM) show changes in α-helical content on interaction with P1 (0−150 μM) in 25mM Tris−HCl buffer solution. (b) MetHb (40 μM) show changes in α-helical content on interaction with P1 (0−100 μM) in 25 mM Tris−HCl buffer solution.

CD software was used to examine the changes in secondary structures of proteins. Finally, it was concluded that P1 was able to change and bring modifications in the

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secondary structure of proteins examined here which was also earlier proved by spectroscopic studies. Hence the anionic polymer P1 can induce a significant conformational change in secondary structure of metalloproteins.

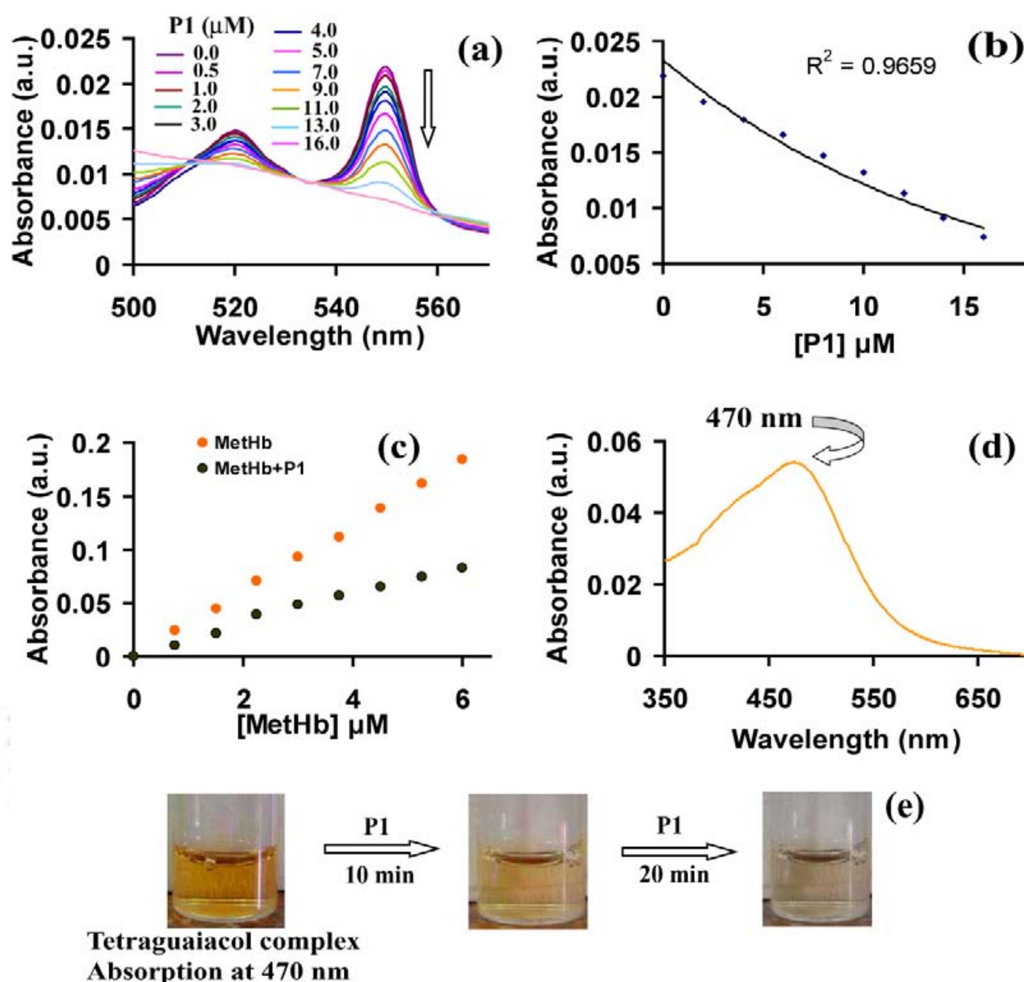

Figure 3.8 (a) and (b) Fe^{3+} in C*c* was rapidly reduced to Fe^{2+} by ascorbate (2.8 mM) with increase in the absorbance at 550 nm in the absence of P1. On increasing the concentration of P1 to 16 μM, this 550 nm band was decreased in presence of ascorbate. (c) MetHb peroxidase activity was assayed by following increase in absorption of tetraguaiacol complex at 470 nm. In presence of P1, absorption at 470 nm was less intense and according to Figure (d) color pattern was changed from intense to almost colorless. (e) visual color change of tetraguaiacol complex in presence of P1.

From the above UV−Visible and CD spectroscopy results it is confirmed that P1 was able to change the heme microenvironment of proteins, which means that the activity of proteins may enhance or decrease after interaction with P1. To check this hypothesis, the activities of both the proteins were investigated in absence and presence of P1 (Figure

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3.8). Fe^{3+}-C*c* reactivity was tested towards ascorbate in presence of P1. To perform this experiment, Fe^{3+}-C*c* (2.5 μM) was preincubated with P1 at different concentrations (0-16μM) followed by the addition of ascorbate (2.8 mM) solution to initiate the reaction (Figure 3.8a and Figure 3.8b). Fe^{3+}-C*c* was rapidly reduced by ascorbate (2.8 mM) in absence of P1 which was monitored by increase in absorbance at 550 nm. After addition of P1, we observed that absorbance at 550 nm was significantly decreased with increasing concentration of P1 (0−16 μM) which indicates that in presence of P1, C*c* reactivity towards ascorbate was decreased and P1 bounded C*c* was mainly in oxidized form.

MetHb peroxidase activity was tested with guaiacol in presence of hydrogen peroxide (Figure 3.8c). To perform this experiment, guaiacol 20 mM and H_2O_2 0.27 mM were taken in 50mM Tris buffer (pH 7.4) solution and MetHb with increasing concentration (0-5 μM) was incubated for 15 min. in absence of P1 in a final volume of 1mL guaiacol buffered solutions. All the components were added directly to the 1mL quartz cuvett and mixed by inverting atleast six times. MetHb peroxidase activity was assayed by following increase in the absorption of tetraguaiacol complex at 470 nm (Figure 3.8d). In the same way P1 (15 μM) was preincubated in guaiacol solution and then increasing concentration (0−5 M) of MetHb was added in the solution and absorption at 470 nm were recorded. We found that in the presence of P1, at the same concentrations of MetHb, absorption at 470 nm decreased. In the Figure 3.8(e), orange color of tetraguaiacol complex faded rapidly in presence of P1, indicating decreased peroxidase activity of MetHb. The increased presence and accumulation of the redox active heme iron proteins causes many forms disoders. Alzheimer's disease, Parkinson's disease, Huntington's disease etc. are related to release of C*c* from mitochondria into cytoplasm[29] and the presence of C*c* in the cytoplasm results the acute and chronic neurodegeneration after a broad range of insult to CNS system.[30,31] Whereas with elevated level of MetHb in blood causes the tissue hypoxia. In all these cases the occurrence of altered heme metabolism and the bound iron is responsible for oxidative stress, leading disorders and thus responsible for diseases. Despite extensive efforts, the pathogenic mechanism of disease is yet to be exploited because of which no treatments or cure exist,[32,33] instead preventing this form of neurodegenerative disorder,[34,35] can be an underlying factor to reduce oxidative stress by controlling the free radical generation linked with redox active metals.[36] In addition to the ability of P1 to selectively bind heme iron the above experiments also confirmed that the heme microenvironment of proteins could be modified, which means that the activity of proteins can be controlled on interaction with P1. Since P1 is also able to induce

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conformation modification and unfold the proteins it can have potential applications to study structurally transformed proteins intermediates, that may be helpful for clinical therapeutic purpose such as the amyloidogenic protein intermediates that induce aggregate forming amyloid fibrils studied by Heegaard et al.[37,38]

3.3 Conclusion

In summary, anionic polyfluorene derivative (P1) binds ferric heme proteins with very high sensitivity by complexation, altering structural conformation and inhibiting their respective activities. Due to complexation between P1 and C*c*, heme was exposed to solvent by the replacement of labile Met^{80} ligand and quenching tryptophan fluorescence accompanying blue shift, results unfolding but MetHb structural conformation was rearranged in a different way or unfolded such that tryptophan residues got deeply buried in the relatively hydrophobic interiors, but not solvent exposed after interaction with P1. Structural transformation of ferric heme proteins by P1, would help the use of polymeric system P1 in protein conformational research. These properties of anionic P1 will certainly inspire the fundamental development of new synthetic molecules such as the sulfate terminated conjugated fluorescent polymers reported here in clinical applications and healthcare.

3.4 Experimental

3.4.1 Reagents and materials

All the reagents were purchased from Aldrich Chemicals (India), Merck (India) or Ranbaxy (India) and were used as received. Milli-Q water was used in all the experiments.

3.4.2 Instrumentation

UV-Vis absorption spectra were recorded on a Perkin Elmer Lambda-25 spectrometer. Fuorescence spectra were carried out on a Varian Cary Eclipse Spectrometer. A 10 mm X 10 mm quartz cuvett was used for solution spectra and emission was collected at 90° relative to the excitation beam. Deionized water obtained by Milli-Q system (Millipore) was used. CD spectra were recorded on a JASCO, J – 815 CD Spectrometer, model no. J – 815 – 150S. Changes in the secondary structure of metalloproteins were recorded in the far UV region (190 – 270 nm) using 0.1 cm path length cells and 20 nm min^{-1} scan speed. Native Gel Electrophoresis (PAGE) technique was used to observe the interaction of

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proteins with polymer. Gels were stained with Coomassie Briliant Blue and fluorescent polymer and protein bands were visualized in a GEL LOGIC 1500 IMAGING SYSTEM.

3.4.3 PL titration of P1 with C*c*

An aqueous solution of P1 (4.0×10^{-7} M in 25 mM Tris−HCl buffer) was placed in the quartz cell and the fluorescence spectra (334 nm excitation) were recorded for increasing portions of C*c* up to 0.033 µM. The experiments were performed at room temperature and at physiological pH, changes monitored and recorded carefully.

3.4.4 UV titration of P1 with C*c*

To a solution of **P1** (2×10^{-5} M in 25 mM Tris−HCl buffer) was carefully titrated to a solution of C*c* upto 1 µM concentrations. The changes in the absorption were recorded at room temperature and physiological pH.

3.4.5 PL titration of P1 with MetHb

P1 (4.0×10^{-7} M in 25 mM Tris−HCl buffer) was placed in the quartz cell and the fluorescence spectra (334 nm excitation) were recorded for increasing portions of MetHb up to 0.1 µM. PL titration was performed at room temperature and at physiological pH, changes monitored and recorded carefully

3.4.6 UV titration of P1 with MetHb

A solution of **P1** (2×10^{-5} M in 25 mM Tris−HCl buffer) was carefully titrated to a solution of MetHb solution upto 4 µM concentrations. The changes in the absorption are shown in Figure 3.3b.

3.4.7 PL titration of P1 with Heme

Heme was dissolved in basic aqueous solution and then stock solution in Tris−HCl buffer was prepared by dilution and aliquot was added to the solution of P1 (4.0×10^{-7} M in 25 mM Tris−HCl buffer) in the quartz cell and the fluorescence spectra (334 nm excitation) were recorded for increasing portions of heme up to 0.16 µM. The experiments was performed at room temperature and at physiological pH, changes monitored and recorded carefully

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3.4.8 UV titration of P1 with Heme

Solution of **P1** (2 X 10^{-5} M) was carefully titrated to a solution of ferric heme upto 5 μM concentrations. The changes in the absorption are shown in Figure 3.3c.

3.4.9 PL titration of P1 with apoenzyme part of MetHb

MetHb solution was carefully deprotenised with acetone by known procedures, separated apoenzyme part was diluted with Tris−HCl buffer and titrated with P1 (4 X 10^{-7} M) solution. The concentration of apoenzyme part was kept 0−0.2 μM in cuvett and in the same way hemin part (0−0.17 μM) was titrated with P1 (0.4 μM).

3.4.10 Gel experiment

In gel experiment appropriately sized wells (40 μL) were prepared by placing a comb in the centre of the gel. A stock solution of metalloproteins and P1 in Tris−HCl buffer was used to prepare 20 μL samples at the varying concentration of polymer keeping metalloproteins concentration fixed. Samples were incubated for 60 min. at room temperature before running gel. 2 μL 80% glycerol was added in each samples for proper loadind inside the wells and a constant voltage (100 V) was applied for 60 min. for sufficient movement. After 60 min gels were placed in staining solution (0.5 Coomassie blue, 40% methanol and 10% acetic acid solution) for one hour. After proper staining, the gels were destained (40% methanol and 10% acetic acid solution) for 8 hours until bands were cleared. After proper destaining protein bands were visualized in a GEL LOGIC 1500 IMAGING SYSTEM.

3.4.11 UV titration of C*c* with P1 to monitor the change in Soret band

C*c* concentration was kept 6 μM in 3mL solution cuvett at 7.4 pH in Tris−HCl buffer and P1 was added with increasing concentration (0−35 μM) in C*c* solution and the changes were recorded carefully.

3.4.12 UV titration of MetHb with P1 to monitor the change in Soret band

P1 with increasing concentration (0−8 μM) was added into the 1 μM MetHb 3 mL cuvett solution at pH 7.4 in 25 mM Tris−HCl buffer and changes were recorded carefully.

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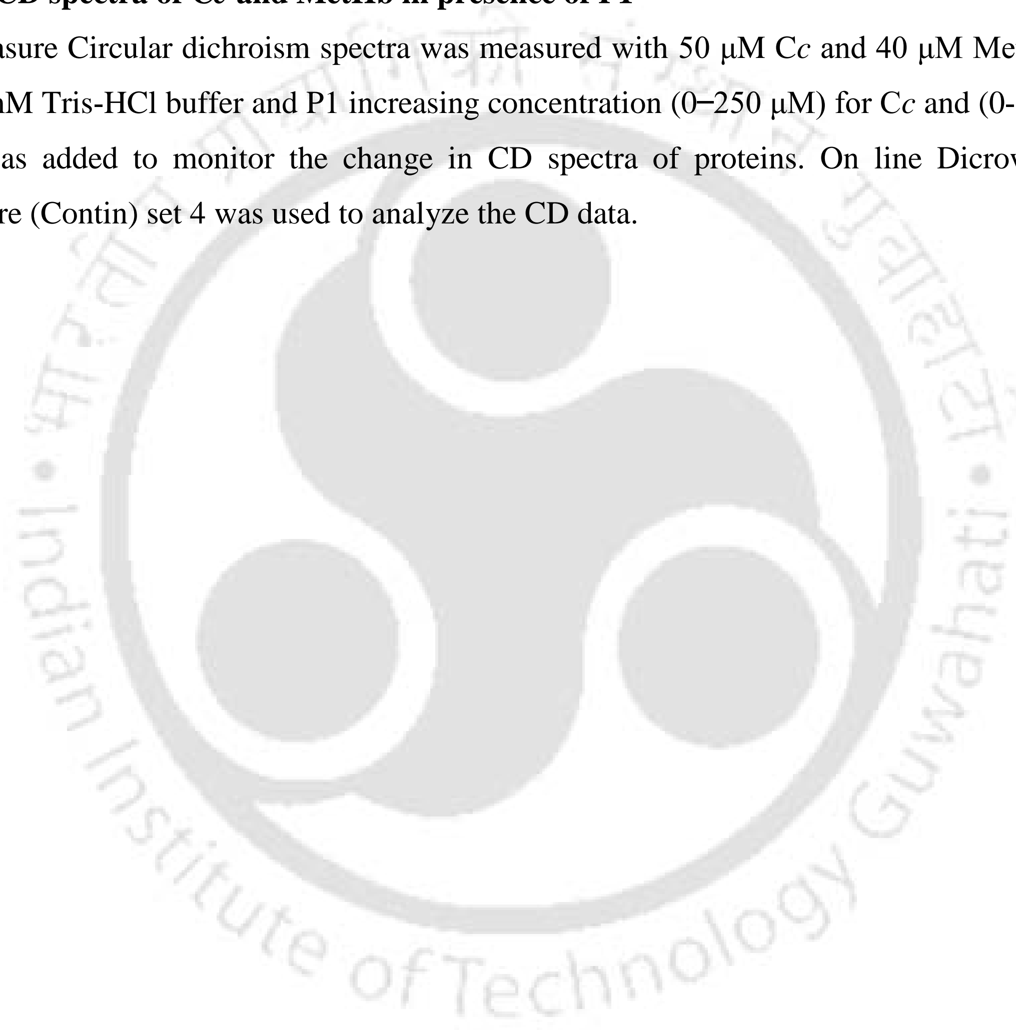
3.4.13 Emission spectra of Tryptophan in presence of P1

C*c*(8 μM) and MetHb (10 μM) concentration was maintained in Tris−HCl buffer in a 1 mL quartz cuvett at pH 7.4 and emission intensity of Trp unit was recorded keeping excitation wavelength at 290 nm. P1 with increasing concentration (0−30 μM) was added into cuvette having C*c* and (0−20 μM) for MetHb (0−10 μM) and changes in emission intensity were recorded carefully.

3.4.14 CD spectra of C*c* and MetHb in presence of P1

To measure Circular dichroism spectra was measured with 50 μM C*c* and 40 μM MetHb in 25 mM Tris-HCl buffer and P1 increasing concentration (0–250 μM) for C*c* and (0-100 μM) was added to monitor the change in CD spectra of proteins. On line Dicroweb Software (Contin) set 4 was used to analyze the CD data.



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Abstract

Anionic water-soluble conjugated polyfluorene derivative poly(9,9-bis(6'-sulphate hexyl) fluorene-*alt*-1,4-phenylene) sodium salt (P1) in combination with Fe^{3+} (P1-Fe^{3+}) assay is highly selective for inorganic phosphate (P_i) anions at biological pH values, observed by complete fluorescence dequenching confirmed through >95 % fluorescence enhancement. In order to validate its diagnostic potential, this assay was employed to monitor the P_i levels in a competing biological environment like blood serum and saliva. At pH 7.4, this assay showed high specific activity to detect P_i in the bioassay environment, observed by the unique enhancements in fluorescence intensities for varying and low P_i concentrations. Since this assay performed P_i detection at very low concentrations we utilized it successfully for the fluorometric detection of inorganic phosphates in blood serum and saliva within short duration. This remarkable ability of P1 to accomplish in-situ monitoring and detection of indispensable biological targets like Fe^{3+} and P_i in real-time and label-free conditions, corroborate the extension of this assay system for clinical validation.

4.1 Introduction

Phosphorus is the second most common mineral in the body after calcium and when it combines with oxygen, it becomes phosphate. Vital role of phosphate in a number of physiological body systems, that includes bone development, cellular membrane integrity and energy transfer (mitochondrial metabolism) are well recognized and well documented and normal phosphate homeostasis make a balance of phosphate in the blood serum ranging from 0.8 to 1.45 mmol/L concentration.[1] However serum phosphate level show a circadian rhythm which is not parallel.[2] Meals now a days contain high phosphate load but this phosphate load is managed by the phosphatonin or by adaptation.[3,4] Extra phosphorus which is two third of the dietary phosphate load, is removed from the body through urine made by healthy kidneys and even with deteriorating kidney function in CKD, this excretion rate is relatively same.[3] This may result in the elevated phosphate level in the blood causing hyperphosphatemia. According to specialists serum phosphate levels above 3.5 mg/mL were already significantly associated with mortality. Nonetheless, meager efforts have been directed towards the design and development of sensitive and selective phosphate detection systems. In recent years, Fe^{3+} compounds

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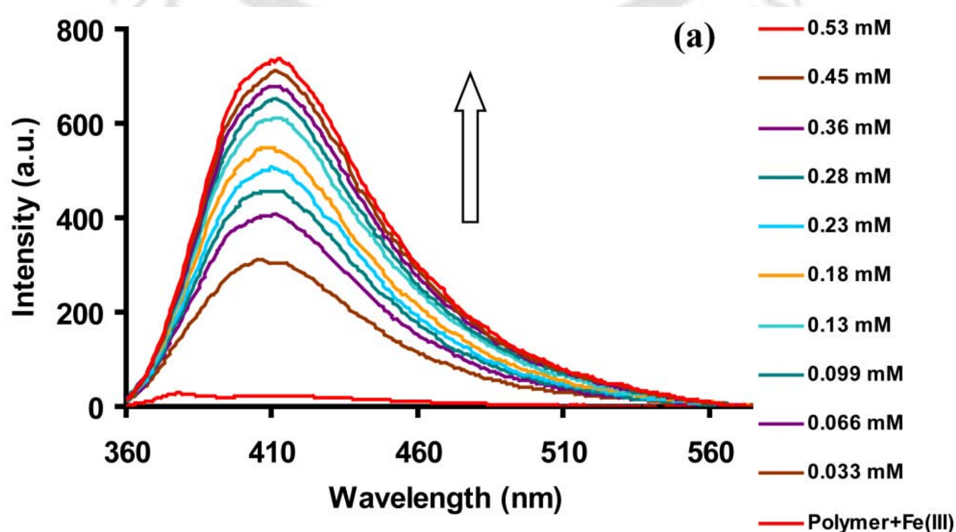
have been applied as binders of phosphate[5] in serum, whole blood and dietary phosphates, replacing conventional binders like aluminum,[6] lanthanum[7] and calcium salts, that have numerous drawbacks like toxicity, intolerance to patients, expensive and unknown side effects.[8,9] Yet, development of Fe^{3+} based phosphate binders, which prevails over all the above drawbacks, lies dormant and merits investigation. Additionally, detection of inorganic phosphate (P_i) anions in aqueous medium is compounded by the competing solvation effect[10,11] and as a consequence, reports of P_i detections are scarce.[12-18] A recent method to detect phosphate in blood serum at physiological pH highlighted the use of a tripodal ligand embedded onto a polymer matrix.[19] Yet, label-free anionic CPs that are known for their high sensitivity to small perturbations have not yet been developed for phosphate detection[20] and estimation in competitive biological environment. Herein, we demonstrate rapid detection of inorganic phosphate anions (PO_4^{2-}) in comparison to other inorganic and organic phosphates anions such as pyrophosphates, polyphosphates, ATP, ADP, AMP, G6P, o-phospho-L-serine, o-phospho-L-tyrosine, o-phospho-L-threonine, giving delayed response with less sensitivity. Hence P1 fulfills the requirements of detecting indispensable biological targets like phosphate in blood serum and saliva at physiological pH, corroborating this system for clinical and diagnostic validation.

4.2 Result and discussion

Employing the P1-Fe^{3+} assay system, we examined the binding of P_i at pH-7.4 in Tris−HCl buffer by fluorescence spectroscopy. Since HPO_4^{2-}: $H_2PO_4^{1-}$ exists in 61:39 ratio at pH 7.4, we have used the term “phosphates” in this manuscript.



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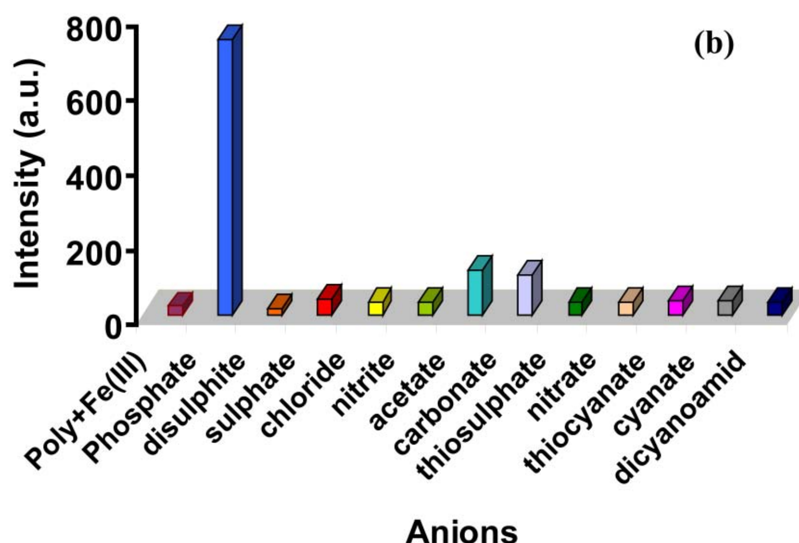

Figure 4.1 (a) PL spectra of P1-Fe^{3+} in 25 mM Tris−HCl (pH 7.4) with increasing concentration of P_i shows >95% superdequenching. **(b)** Bar diagram depicting effect of anions on fluorescence intensity of P1-Fe^{3+} at pH 7.4.

Figure 4.1(a) depicts the rapid fluorescence dequenching of >95 % on titrating aqueous P_i with P1-Fe^{3+} assay. The largest spectral enhancement is observed at the lowest P_i concentration (3.3 X 10^{-5} M), which gradually leveled off at a concentration of 5.3 X 10^{-4} M. The dequenching of P1-Fe^{3+} assay on adding aqueous P_i to the quenched P1-Fe^{3+} assay results in high amplification of the fluorescence. To determine the extent to which this P1-Fe^{3+} assay would react to other anions, similar titrations were performed with several anions. As observed in Figure 4.1(b), the fluorescence of P1-Fe^{3+} assay is barely perturbed on addition of sulfide, sulfate, chloride, acetate, carbonate, thiosulfate, nitrate, thiocyanate, cyanate and dicyanamide anions. Similarly, chelating ligands such as citrate, EDTA and picolinate caused only 21%, 13% and 5% dequenching. This observation of dequenching on adding P_i to the quenched P1-Fe^{3+} solution presents a unique, highly sensitive, label-free homogenous assay for P_i detection. As in the previous chapter P1 spectra was modified resulting in the enhancement in absorbance accompanied with blue shift on adding Fe(III) metal. Further the mechanism responsible for high fluorescence dequenching was confirmed by the change in UV/Vis spectra of P1-Fe^{3+} occurring in the presence of phosphate anions. Here, it was clearly observed that Fe^{3+} as well as phosphate are both associating with the polymer indicating the association in place of displacement as shown in Figure 4.2.

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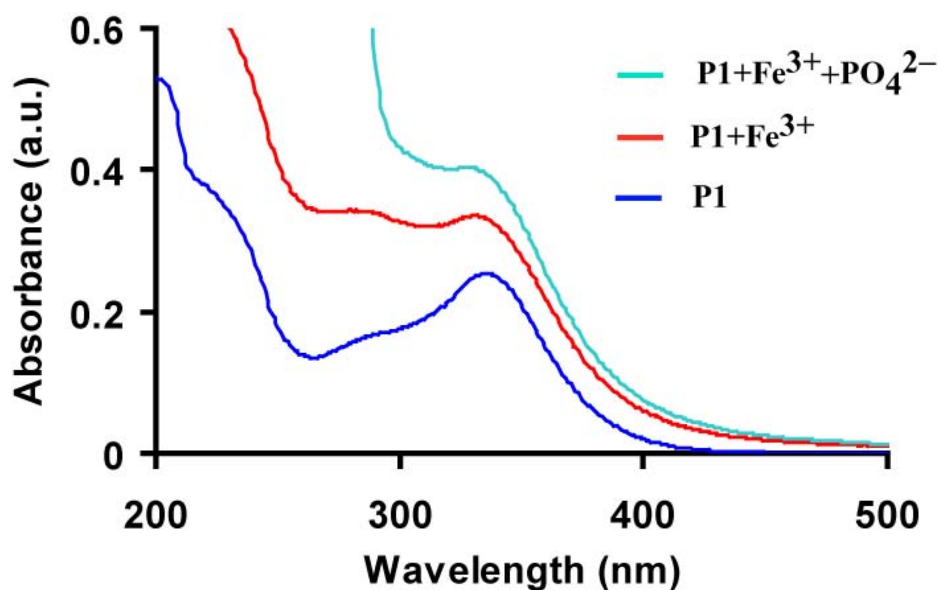

Figure 4.2 Changes in the absorption of P1-Fe^{3+} in 25 mM Tris−HCl (pH 7.4) on addition aqueous solution of phosphate anion.

The interaction of P1-Fe^{3+} with other phosphates (Figure 4.3) showed that fluorescence dequenching was very slow (2-8h incubation), incomplete and incomparable with the rapid fluorescence dequenching with P_i observed above. e.g. pyrophosphate (87%, 2h), polyphosphate (80%, 4h), ATP (71%, 8h), ADP (52%, 8h), AMP (37%, 8h), G6P (66%, 8h), o-phospho-L-serine (45%, 8h), o-phospho-L-tyrosine (48%, 8h), o-phospho-L-threonine (49%, 8h) and triethyl phosphate (0%, 8h).

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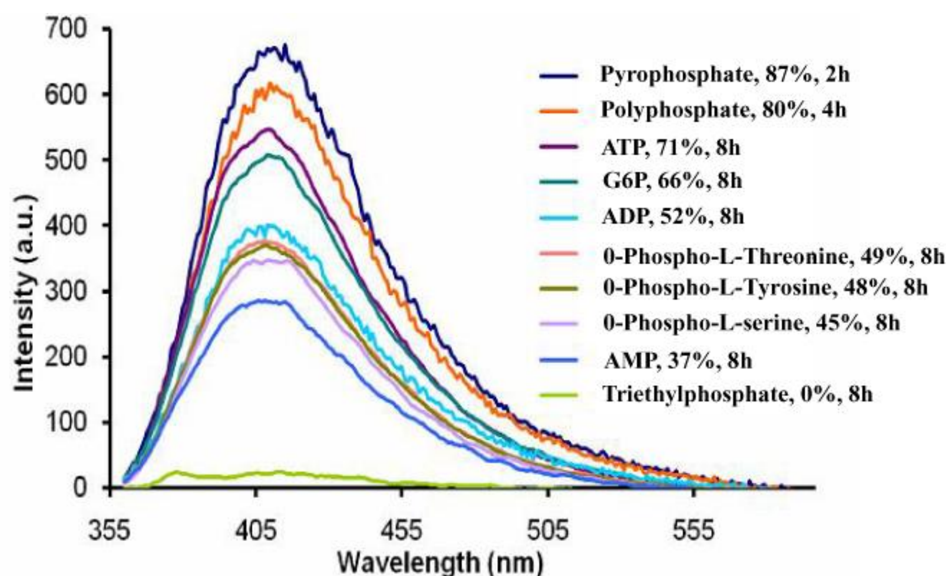

Figure 4.3 Fluorescence spectra changes of P1-Fe^{3+} in 25 mM Tris−HCl (pH 7.4) on addition of various phosphates.

These results encouraged us to study the detection of P_i in blood serum to ascertain the practical utility of the P1-Fe^{3+} assay in a competitive environment. Three samples of untreated blood serum, doped with 0.009, 0.018 and 0.03 mg/dL(0.0029, 0.0058 and 0.0097 mM/L) concentrations of P_i were prepared and added carefully to P1-Fe^{3+} assay at physiological pH. The fluorescence enhancement response (Figure 4.4) observed on adding these three blood serum samples were unique and exceptionally distinguishable even at extremely low concentrations, authenticating this assay for systematic detection and quantification of P_i in blood serum environment that consist competing proteins, globulins, biophosphates, carboxylate ions and electrolytes. Therefore, even at very low P_i concentrations the amplification of fluorescence is clearly distinguished, indicating that the homogenous fluorometric detection of P_i in competitive bioassay can be practically preformed. Acute renal failure patients have P_i levels in blood exceeding 3.5−5.5 mg/dL, (1.13−1.77 mM/L) resulting in hyperphosphatemia and imbalance of other electrolytes.[21]

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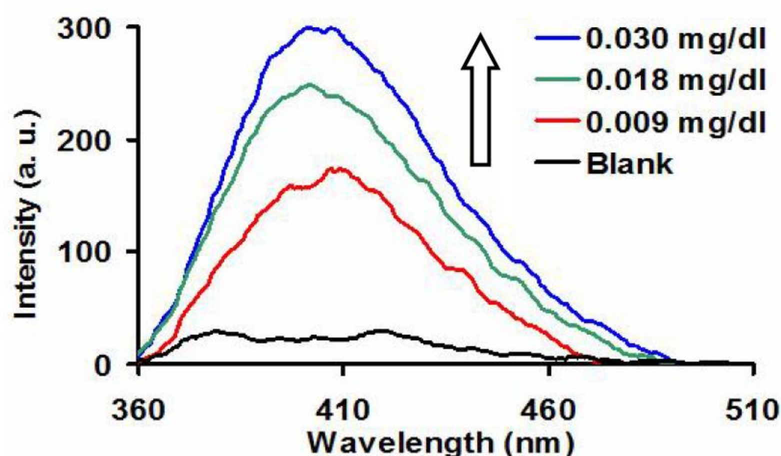

Figure 4.4 Variation in the PL spectra of P1-Fe^{3+} in 25 mM Tris−HCl buffer (pH 7.4) on adding varying concentration of P_i doped blood serum samples.

Importantly, this assay performs P_i detection without being encountered by competing solvent effect and other ions[3,22] that has hampered development of viable assays for the detection of P_i. P1-Fe^{3+} is an exceptional displacement assay that performs P_i determination in blood serum with much higher order of sensitivity as compared with clinically permitted procedures for phosphate.[23] Additionally, since the absorbance (279 nm) and the fluorescence (330 nm) peaks of serum[24] do not overlap or interfere with this assay system, P1 can be efficiently used for the detection of serum components.

Since the P1-Fe^{3+} assay can detect P_i at such low concentrations, we utilized this platform for fluorometric detection of P_i in blood serum and saliva. Since serum contains phosphate in the range of 2.5−4.5 mg/dL but saliva contains four times higher than this range and in saliva interferrence in detection is very less than serum. Therefore fresh serum sample was deproteinized to remove the metalloproteins and the supernatant was added (4 µL aliquot) to P1-Fe^{3+} assay (pH-7.4). The fluorescence enhancement observed was found to be significant. Similarly fresh saliva was collected after cleaning the mouth with water. To follow a typical collection procedure, saliva was allowed to accumulate in the mouth for 3 minutes and collected in sample vial and this was used immediately for analysis (Figure 4.5).

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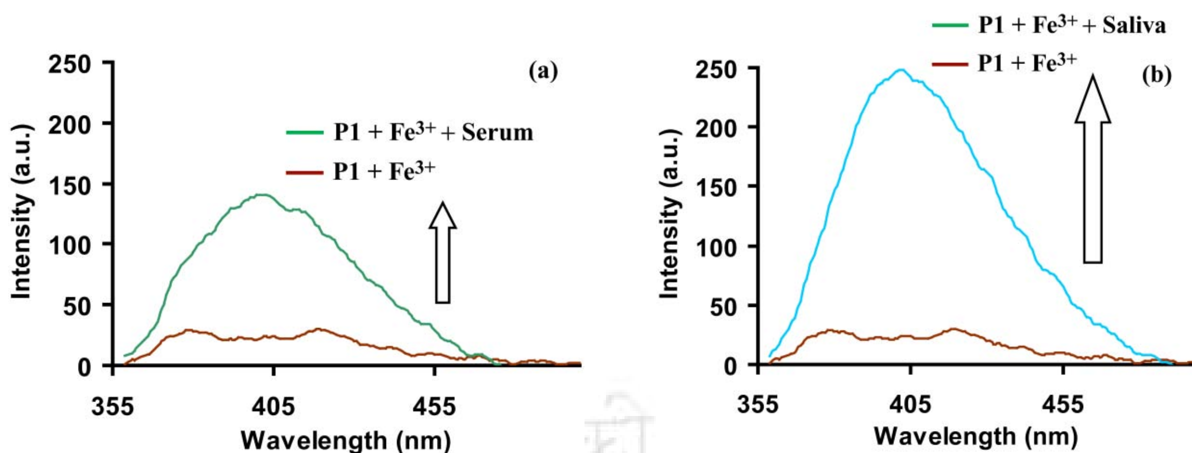

Figure 4.5 (a) Dequenching of P1-Fe^{3+} in 25 mM Tris−HCl Buffer (pH 7.4) after addition of 4μL aliquot of deprotenized serum. **(b)** Dequenching of P1-Fe^{3+} in 25 mM Tris−HCl Buffer (pH 7.4) after addition of 2 μL aliquot of freshly collected saliva.

Overall, this unique behavior of P1-Fe^{3+} displacement assay as a fluorescence amplification probe for P_i detection under biological conditions in addition to the very high selectivity and sensitivity of P1 towards Fe^{3+}, validates this homogenous assay as a useful platform for understanding the interaction and assembly in several chemical and biological entities. P1 demonstrates largest quenching and dequenching in a label-free homogenous environment while performing these detection tasks. Since P1 can be utilized both as a stand-alone probe as well as in combination with quenchers, preserving its individual photo physical characteristics, a diverse range of sensory platforms potentially exploiting these collective properties can be developed.

4.3 Conclusions

In summary, tripodal ligand embedded onto a polymer matrix of anionic polyfluorene derivative (P1) was utilized for rapid, label-free detection of P_i in aqueous medium as well as in competitive biological environment such as blood serum and saliva. These significant and unique properties of anionic P1 will certainly inspire fundamental development of sulfate terminated conjugated fluorescent polymers that have shown outstanding ability as amplifying fluorescent polymers in clinical applications. Additionally, their ability to transport electronic excited states and inherent film forming

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properties on desired substrates promises a bright future for interdisciplinary applications like healthcare, security, environmental monitoring and optoelectronic devices.

4.4 Experimental

4.4.1 Reagents and materials

All the reagents and solvents were purchased from Aldrich Chemicals (India), Merck (India) or Ranbaxy (India) and were used as received. Milli-Q water was used in all the experiments.

4.4.2 Instrumentation

Blood serum samples from healthy subjects were obtained as a gift from the host institute medical department. UV/Visible and PL spectra were recorded on a Perkin Elmer Lambda-25 spectrophotometer and Varian Cary Eclipse Spectrophotometer respectively.

4.4.3 PL titration of P1+Fe^{3+} assay with anions

A solution of P1 (1×10^{-5} M) and metal (2×10^{-4} M) solution was prepared in 25 mM Tris-HCl buffer. A solution of P1 was placed in a quartz cell and the fluorescence spectrum was recorded. First Fe^{3+} was added to quench the fluorescence and titration was performed with various anions up to 6×10^{-4} M to dequench the fluorescence. The fluorescence intensity changes were recorded at room temperature each time with excitation wavelength 334 nm.

4.4.4 PL titration of P1+Fe^{3+} assay with phosphate

A solution of P1 (1×10^{-5} M) and metal (2×10^{-4} M) solution was prepared in 25 mM Tris −HCl buffer. A solution of P1 was placed in a quartz cell and the fluorescence spectrum was recorded. First Fe^{3+} was added to quench the fluorescence of P1. The solution of phosphate was added in portion and the fluorescence intensity changes were recorded at room temperature each time with excitation wavelength of 334 nm.

4.4.5 PL titration of P1+Fe^{3+} assay with different type of phosphates

Same procedure as described above.

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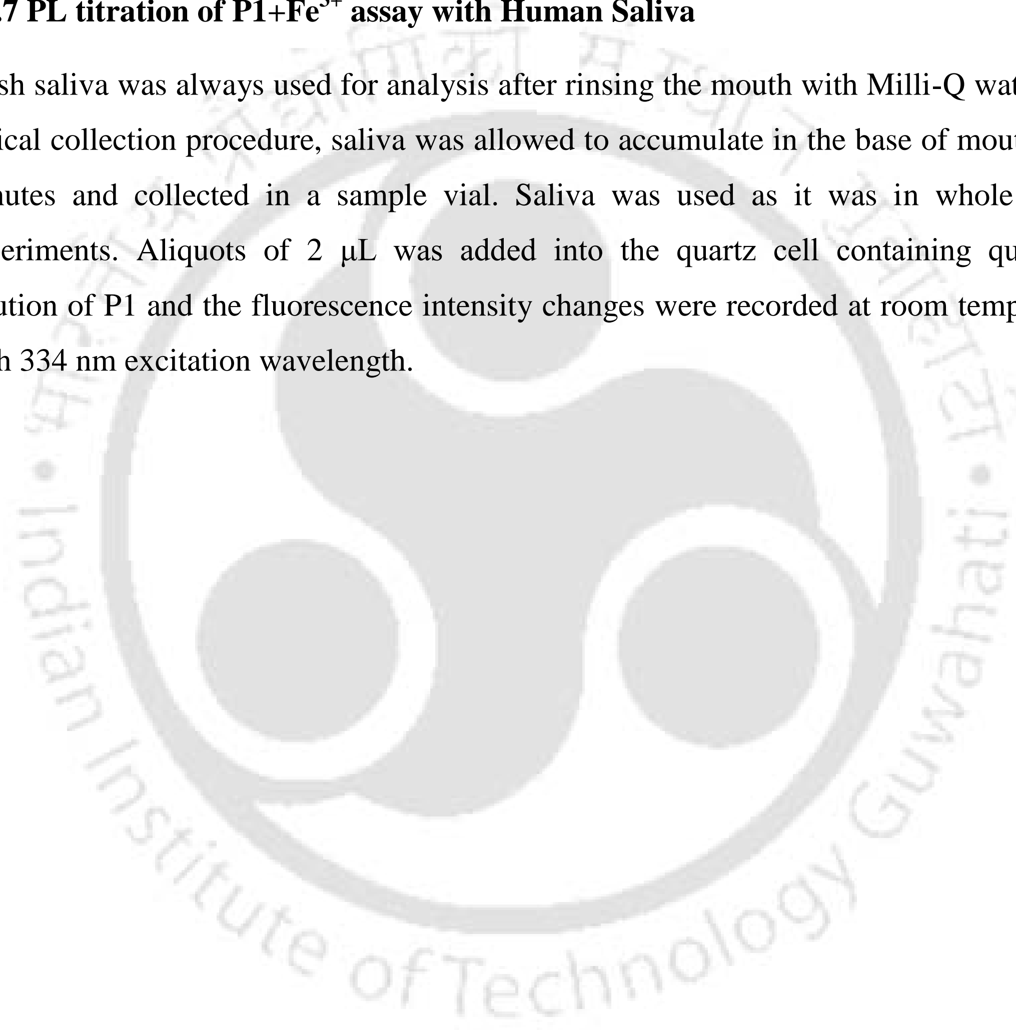
4.4.6 PL titration of P1+Fe^{3+} assay with Human Serum

Fresh serum sample was first deprotenised by adding $(NH_4)_2SO_4$ following a standard protocol to remove metalloproteins from the serum. An aliquot of 4 μL of deprotenised serum sample was added into the quartz cell containing quenched solution of P1 and the fluorescence intensity changes were recorded at room temperature with 334 nm excitation wavelength.

4.4.7 PL titration of P1+Fe^{3+} assay with Human Saliva

Fresh saliva was always used for analysis after rinsing the mouth with Milli-Q water. In a typical collection procedure, saliva was allowed to accumulate in the base of mouth for 3 minutes and collected in a sample vial. Saliva was used as it was in whole of the experiments. Aliquots of 2 μL was added into the quartz cell containing quenched solution of P1 and the fluorescence intensity changes were recorded at room temperature with 334 nm excitation wavelength.


Indian Institute of Technology Guwahati

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Abstract

A method developed that includes a ferric iron bound to a novel anionic water soluble polymer (P1) matrix for a continuous and real time turn on assay for the enzymatic activity of acid phosphatase under acidic conditions is reported in this chapter. This assay was also able to screen the inhibitors of enzyme acid phosphatase that may render the usefulness of the method in drug discovery. The method offers a sensitive rapid turn on assay for Pi, whereas organic phosphates exhibited a low activity towards the dequenching of P1-Fe^{3+} fluorescence. Commonly used phosphatase substrate p-Nitrophenyl phosphate (pNPP) had been used in our study as a model compound for the enzymatic hydrolysis and a very small concentration of enzyme in nM regime was used and the changes were monitored during enzymatic hydrolysis.

5.1 Introduction

Acid phosphatase (ACP) an abundant enzyme plays a major role in phosphatae scavenging and considered as a hydrolase enzyme. These are widely spread in nature and can be found in mammalian body fluids and tissues. ACPs in human are normally found in low concentration but an abnormal elevated level of ACP is the indication of several diseases such as prostate cancer, Gaucher disease, Paget's disease, thrombophlebitis, hyperparathyroidism, kidney disease, multiple myeloma.[1] Thus ACPs could be diagnostically useful as serological and histological marker of disease and could be useful in the pathophysiology of the associated disease, such as prostatic acid phosphatase which are considered as an important serum marker for prostate cancer[2] and in recent times, these prostatic acid phosphatase are regarded as prognostic indicator.[3] Some other type of ACPs for example phosphatidic acid phosphatase type 2C[4] and tartarate-resistant acid phosphatase (TRAP)[5] have been regarded as an emerging class of drug targets. Therefore, a sensitive and continuous assay of ACP is of interest among the various enzymes present in mammalian systems. A number of phosphatase assays that use different substrate such as pyrophosphate which operates at a physiological pH,[6] pNPP as a colorometric assay,[7] 4-methylumbellylferyl phosphate (4-MUP) as a fluorometric assay operating at a high pH,[8,9] have been developed. But most of the ACPs require a lower pH, therefore, as an improvement, FDP and DiFMUP have been used in continuous ACP assays over a pH range 5–9.[10,11]

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In recent years conjugated polyelectrolytes (CPEs) with their unique optical properties have been extensively used in the exploration of sensing chemical and biological materials such as metal ions,[12,13] anions,[14,15] small biomolecules,[16,17] proteins, DNA[18,19] and enzymes.[20] The intrinsic fluorescence signal amplification of conjugated polyelectrolytes leads to the high sensitivity towards biological analytes in very less concentrations. Because enzymes play an essential role in almost all cellular processes including signaling pathway, metabolism and gene expression and also several disease are related with enzymes. Therefore enzyme detection and activity study is highly important for the pathological screening and theraupetic development. In recent years much attention has been given on the CPEs based fluorescent assays of disease related enzymes that includes thrombin, papain, enterokinase, protein kinase, caspase-3, 7 and 8, and β-secretase.[21,22] Hence, recent years have seen several examples, that include complexes of conjugated polymer or some ligands with metal ions as useful probes for continuous, sensitive and facile fluorescence assay for enzymes.[23,24] Moreover different types of iron complexes have also been used to mimic the enzymatic hydrolysis of acid phosphatases and also iron complex substrates containing organo phosphates in the presence of acid phosphatase, towards scavenging the different types of organic phosphates and thus releasing the inorganic phosphates.[25−28]

Herein, we report a novel anionic water soluble polyfluorene derivative poly(9,9-bis(6-sulphate hexyl) fluorene-*alt*-1,4-phenylene) sodium salt (P1) in combination with ferric iron as an efficient continuous and sensitive fluorescence turn-on assay for the detection of ACPs and we have included the commonly used phosphatase substrate pNPP for the enzymatic hydrolysis. In the present method ferric iron was embedded onto polymer matrix P1 via the sulphate group as discussed in previous chapters and this combined system was sensitive towards P_i than other inorganic as well as organic phosphates giving a turn on signal as reported in chapter-4. Therefore after enzymatic hydrolysis, liberated P_i was taken by iron embedded polymer P1 resulting in the enhancement of fluorescence intensity. Overall, in this system enzyme-catalyzed pNPP hydrolysis was signaled by an increase in the fluorescence from the conjugated polyelectrolyte P1, in other words fluorescence of P1 was turned on when the concentrations of the enzyme was in the nanomolar regime.

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5.2 Result and Discussion

We already reported in previous chapter that the P1-Fe^{3+} assay system shows rapid fluorescence dequenching of >95 % on titrating aqueous P_i and a large spectral enhancement was observed at the lowest P_i concentration of 33 μM. The interaction of P1-Fe^{3+} with other phosphates showed fluorescence dequenching to be very slow (2-8 h incubation), that was incomplete and incomparable with the rapid fluorescence dequenching with P_i. Therefore P1-Fe^{3+} assay system works efficiently as turn on assay for P_i.

The sensitivity of quenched P1-Fe^{3+} assay towards P_i provided us the opportunity to establish P1-Fe^{3+} assay system as a probe for the enzymatic hydrolysis of organic phosphates such as pNPP in the presence of acid phosphatase through real time turn on assay. P1 was also found to be highly fluorescent in acidic conditions of pH with excitation wavelength at 335 nm as shown in Fig. 5.1. However a decrease in fluorescence intensity was observed on lowering the pH of medium. But still in acidic media, polymer fluorescence was strong enough and this may be attributed to the sulphate group in the side chain of P1 that remains ionized even in strongly acidic conditions. It has been described in chapter 2 that photophysical properties of P1were sensitive towards modification on addition of ferric iron. Therefore to utilize P1-Fe^{3+} system as the fluorometric assay for phosphatase enzymes, we examined the sensitivity of P1 towards ferric iron in acidic condition. We found that the addition of 3 μM of Fe^{3+} was able to quench the fluorescence of P1 (0.6 μM) solution (Figure 5.1).

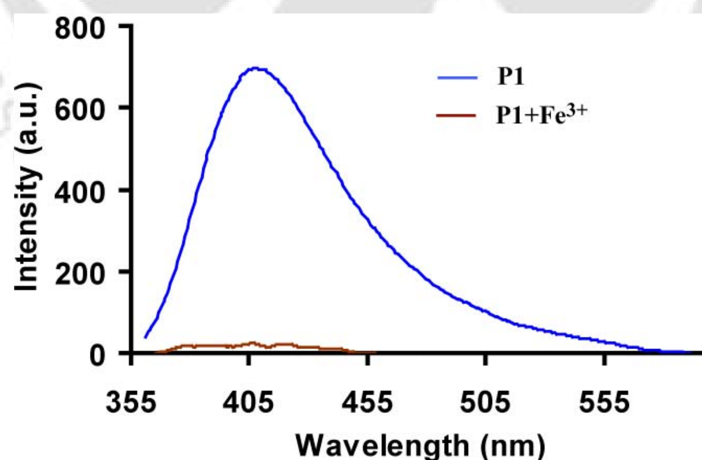

Figure 5.1 P1 (0.6 μM) fluorescence in acidic medium of pH 6.0 and it was turned off by the addition of $FeCl_3$ (3 μM).

Here acidic condition of pH 6.0 in 15 mM Tris – HCl buffer was applied in all the experimental conditions and less substrate quantity of pNPP (say 40 μM) was used in our method to achieve obvious and rapid fluorescence intensity change mainly due to the

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dequenching of P1-Fe^{3+} quenched system by enzymatic hydrolysis. First, we examined the amount of change in fluorescence intensity of P1-Fe^{3+} assay on addition 40 μM of P_i and pNPP. To perform this experiment two samples containing 40 μM of P_i and 40 μM of pNPP were prepared and incubated. Fluorescence enhancements of P1 for both samples were recorded immediately and after 8 hour incubation as shown in Figure 5.2. As reported in chapter 4, 3.3 X 10^{-4} M concentration of phosphate was able to dequench the P1-Fe^{3+} fluorescence by upto 95% immediately after 8h incubation but in the same incubation time 40 μM of pNPP dequenching was insignificant as shown in the Figure 5.1.

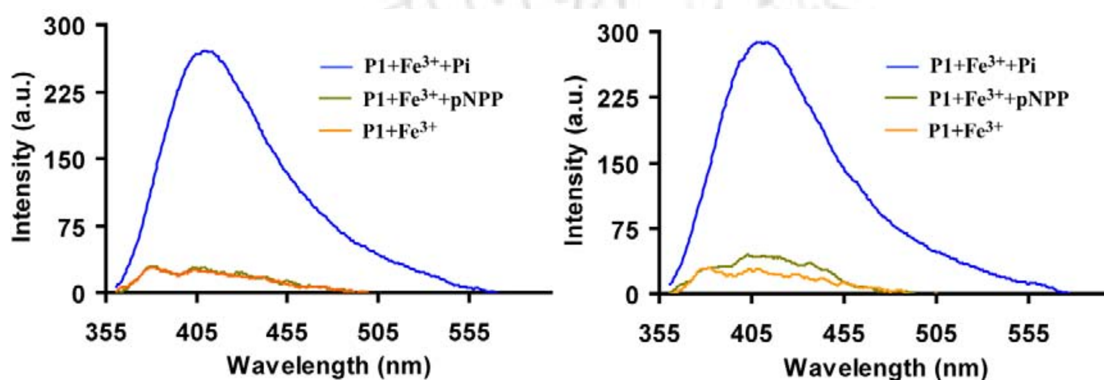

Figure 5.2 Fluorescence intensity changes of P1-Fe^{3+} after addition of Pi and pNPP (a) immediately (b) after 8 hours incubation time.

According to the Figure 5.2, P_i was able to dequench the fluorescence immediately, but pNPP was unable to dequench the fluorescence of P1-Fe^{3+} even after 8 hour incubation. Only a slight change in fluorescence intensity after 8 hours was observed which was negligible. Therefore obvious fluorescence intensity change with pNPP was observed mainly due to the dequenching of P1-Fe^{3+} quenched system by enzymatic hydrolysis.

We also examined the interference of enzyme in our method as it contains non heme ferric iron in its active site as already reported earlier that P1 fluorescence was sensitive with ferric iron in micro levels. At the same time we have also reported that P1 fluorescence was very sensitive with ferric heme proteins but non heme ferric proteins such as ferritin sensitivity was comparable to simple ferric iron salt and also the enzyme concentration used in our method is lower than ferric iron salt concentration. We now examined the change in fluorescence intensity of P1 by adding the same concentration of enzyme as we used for enzymatic assay, into the P1 solution. A slight change in the fluorescence intensity of about 2 − 3 % was observed and the quenched fluorescence intensity was almost unaffected on addition of same concentration of enzyme used in the method. However, we were more

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interested and concerned in the fluorescence intensity enhancement of P1 with negligible interference. Hence, due to the release of P_i by enzymatic action on the substrate pNPP and this enhanced intensity of P1 by enzymatic action was considered as a standard to screen inhibitor experiment and to evaluate inhibition efficiency by our method.

We monitored the enzymatic hydrolysis of pNPP in presence of P1-Fe^{3+} assay. To perform the experiment 40 μM of pNPP in 15 mM Tris−HCl buffer solution of pH 6.0 was prepared followed by the addition of 30 nM of ACP from potato and incubated for different times. The fluorescence intensity changes at 411 nm were recorded with different incubation time as shown in Figure 5.3.

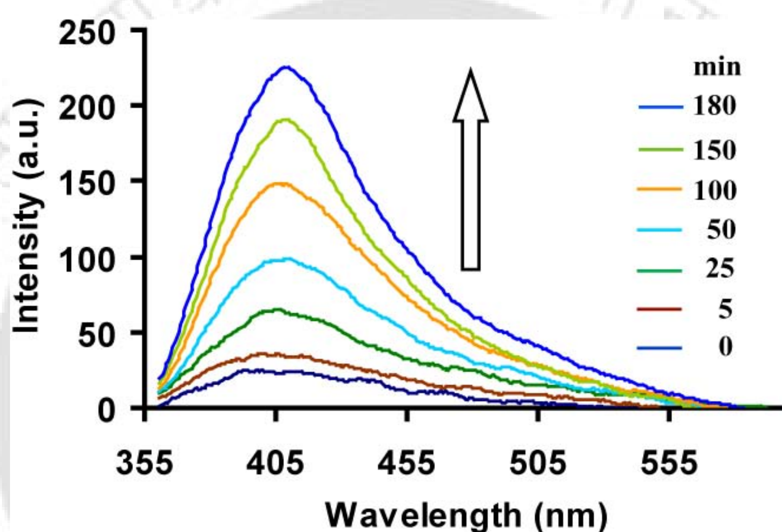

Figure 5.3 Changes in the fluorescence intensity of P1-Fe^{3+} on addition pNPP (40 μM) followed by the addition of ACP (30 nM) with respect to increasing time.

According to Figure 5.3, it was observed that after addition of ACP, the fluorescence intensity of P1-Fe^{3+} quenched system was unaffected giving an evidence that enzyme itself did not affect the polymer fluorescence at this much low concentration, as the quenched state needs some higher concentration of iron. After some time as the enzymatic hydrolysis proceeds, the quenched emission of P1-Fe^{3+} gradually increased and then reached a plateau and this fluorescence increase was leveled off at 32% as was expected for this less concentration of pNPP.

Further, to check the feasibility of P1-Fe^{3+} assay for a continuous real time ACP activity study, we investigated enzyme catalyzed hydrolysis of pNPP as a function of time and with varying concentration of enzyme because we were more interested here that whether the

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enzymatic hydrolysis of pNPP was dependant on the enzyme concentration or not and also how much less concentration of enzyme was needed for a significant change in the fluorescence intensity. Therefore we performed the controlled experiment in the absence and presence of enzyme. Six samples containing P1-Fe^{3+} and 40 μM pNPP in 15 mM Tis−HCl buffer solution at pH 6.0 were prepared followed by the addition of different concentration of enzyme, say 0 nM, 4 nM, 8 nM, 15 nM, 22 nM and 30 nM and fluorescence intensity changes were monitored at the emission intensity 411 nm with increasing concentration of ACP as a function of time (Figure 5.4).

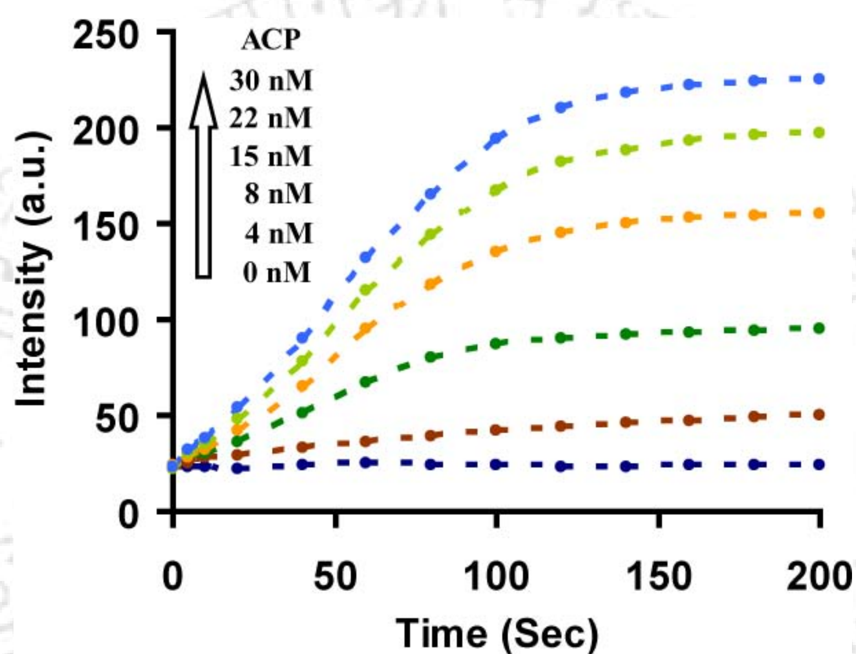

Figure 5.4 Fluorescence intensity enhancement of P1-Fe^{3+} with pNPP (40 μM) in the presence of varying concentration of ACP as function of time.

The Figure 5.4 demonstrates the increase in the fluorescence intensity of P1-Fe^{3+} system with respect to the incubation time and it was clear indications that increase in fluorescence intensity was totally dependent on the enzyme concentration. At lower enzyme concentration the fluorescence recovery was slow but as the concentration of enzyme was increased, fluorescence recovery was very high.

Therefore, we can say that iron on a polymer P1 can serve as the fluorometric assay for enzymatic hydrolysis. There are several enzymes in the human body with different functions, but an abnormal elevated level of enzymes is the indication of several type of diseases and hence in recent times drug discovery has been focussing on the enzymes. Therefore, a

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sensitive and continuous assay of ACP that is able to screen the enzyme inhibitors is of general interest. Hence, we applied our method to screen the inhibition of ACP enzyme. We also examined whether the P1-Fe^{3+}/pNPP/ACP system can be used to screen inhibitors based on the inhibition of the ACP activity. We further studied the inhibition of enzyme activity with vanadate (Na_3VO_4) ion which is a well known inhibitor of phosphatases. To perform this experiment, six samples with same concentration of P1-Fe^{3+} / pNPP and ACP were prepared followed by the addition of different concentration of inhibitor from 0 to 500 nM in each of the six samples. One sample without inhibitor (0 nM) and the other samples with inhibitor were considered and the fluorescence intensity changes were monitored at the emission intensity 411 nm as a function of time. Fluorescence pattern observed was depicted in Figure 5.5.

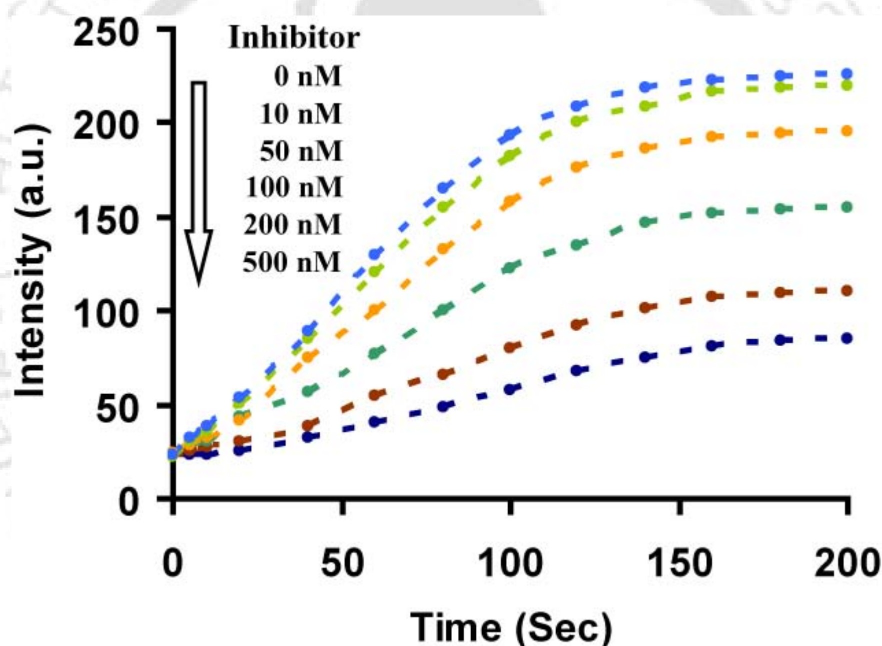

Figure 5.5 The retardation of fluorescence intensity enhancement of P1-Fe^{3+} /pNPP (40 μM)/ACP (30 nM)/ in the presence of inhibitor Na_3VO_4 (0 to 500 nM) as function of time.

Figure 5.5 demonstrates the changes in fluorescence intensity pattern as a function of time. It was very clear from the figure 5.5 that in the absence of inhibitor fluorescence intensity of P1-Fe^{3+}/pNPP/[ACP] was increasing gradually as hydrolysis time was increasing but in presence of inhibitor, this fluorescence enhancement was retarded upto a significant level. Therefore it was clear from the figure 5.5 that higher the concentration of inhibitor in the solution, slower the increase in fluorescence intensity from P1. The inhibition efficiency of

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the inhibitor was calculated using equation (1-*I*/*Io*) × 100%, where *Io* and *I* are the restored fluorescence intensities at 411 nm (Figure 5.6).

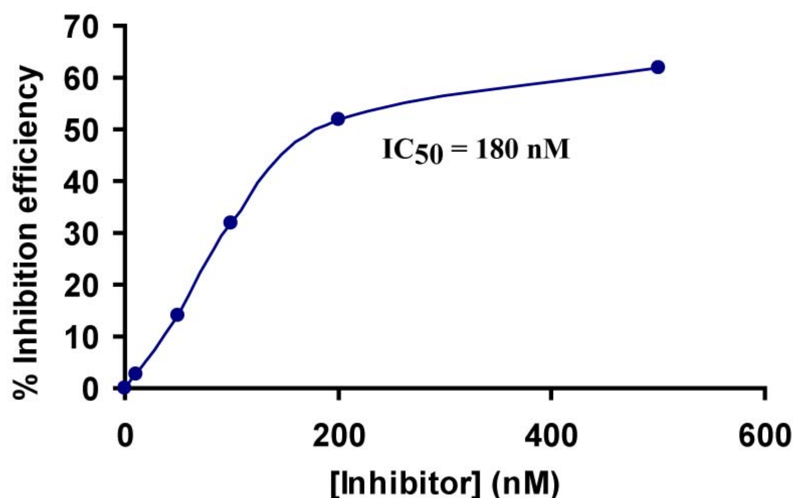

Figure 5.6 Inhibition efficiency was plotted against inhibitor concentration to evaluate the IC_{50} value.

From the above plot between inhibition efficiency and inhibitor concentration IC_{50} value of the inhibitor was calculated and this was found as 180 nM. This indicates the very high sensitivity of the method towards screening the inhibition of enzyme. Therefore, it was concluded that P1-Fe^{3+} quenched system may serve as a fluorescent probe for enzymatic hydrolysis and can also be used to screen the inhibitors for their enzyme activity.

5.3 Conclusion

From the above results we found that ferric iron on a novel anionic water soluble polymer P1 can serve as the fluorometric assay and was able to screen the inhibition of enzyme acid phosphatase very efficiently. The organic phosphate pNPP was less active towards dequenching of P1-Fe^{3+} fluorescence in acidic as well as at physiological pH, but with the release of inorganic phosphate P_i during enzymatic hydrolysis, quenched fluorescence of P1-Fe^{3+} was dequenched, indicating the eligibility of method to monitor the enzymatic action in vitro. Therefore anionic polyfluorenne P1 in combination with ferric iron demonstrate the ability as a turn on assay for the enzymatic activity of acid phosphatase under acidic condition. Thus complexes of conjugated polymer with some metal ions could be useful as probes for continuous, sensitive and facile fluorescence assay for acid phosphatase enzymes. This system offers a convenient mixing and detect strategy which represents a continuous

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approach for rapid assay for enzymatic activity as well as the use of water soluble conjugated polymer imparts the sensor high sensitivity.

5.4 Experimental

5.4.1 Reagents and materials

All the reagents and solvents were purchased from Aldrich Chemicals (India), Merck (India) or Ranbaxy (India) and were used as received. Milli-Q water was used in all the experiments. Acid phosphatase (ACP) from potato lyophilized (3.0 − 10 units/mg solid) powder was obtained from sigma.

5.4.2 Instrumentation

Fluorescence spectra were carried out on a Varian Cary Eclipse Spectrometer. A 10 mm X 10 mm quartz cuvette was used for solution spectra and emission was collected at 90° relative to the excitation beam. Deionized water obtained by Milli-Q system (Millipore) was used.

5.4.3 Fluorescence quenching and dequenching experiments

The experiment was carried out in 15 mM Tris−HCl buffer solution of acidic pH 6.0. The addition of 3 μM Fe^{3+} solution was able to quench the fluorescence of P1 (0.6 μM) solution. For the dequenching experiment two samples containing 40 μM of P_i and 40 μM of pNPP were prepared and incubated with P1/Fe^{3+} solution and fluorescence intensity changes were observed immediately after 8 hours.

5.4.4 Real Time acid phosphatase assay in the absence and presence of inhibitor

The experiment was carried out in 15 mM Tris−HCl buffer solution of acidic pH 6.0. Fluorescence quenching experiment was performed as described above. Six samples containing P1-Fe^{3+} and 40 μM pNPP in 15 mM Tris−HCl buffer solution at pH 6.0 were prepared followed by the addition of different concentration of enzyme, say 0 nM, 4 nM, 8 nM, 15 nM, 22 nM and 30 nM and fluorescence intensity changes were monitored at the

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emission intensity 411 nm with increasing concentration of ACP as a function of time and the same experiment was performed in presence of inhibitor.

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5.5 References

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Abstract

A new method based on the electrostatic interaction of a novel anionic water soluble polymer P1 with a positively charged polypeptide Arg_6 was developed for a continuous and real time turn on assay for the enzymatic activity of trypsin under alkaline condition and this method was also able to screen the inhibitors of trypsin. P1 fluorescence intensity was significantly decreased by the positively charged Arg_6 due to the electrostatic interaction, whereas the enzymatic action recovered P1 fluorescence due to the fragmentation of Arg_6 into small positively charged fragments and these were unable to quench the P1 fluorescence. Therefore, by triggering the fluorescence intensity change, it was possible to assay the enzymatic activity. Use of water soluble conjugated polymer P1 and no labeling on the substrate may enhance the utility of method.

6.1 Introduction

Trypsin, one of the most important digestive enzyme produced in pancreases as the inactive proenzyme trypsinogen,[1] plays a key role in cleaving the proteins into smaller peptides on the c-terminal side of arginine and lysine residues[2] and this protein cleaving into the components of amino acids facilitates the digestion of proteins. Moreover, the generated proenzyme trypsinogen self-cleaves to produce the more active form as needed[3] and this active form then functions to activate additional trypsinogens within intestine and this may be called as autolysis. Only a small amount of enteropeptidase is necessary to initiate the reaction for autodigestion. Therefore trypsin is involved in major role in controlling pancreatic exocrine function. Trypsin becomes more active under alkaline conditions and in presence of metals Ca^{2+}, Mg^{2+} and Mn^{2+}.[4] This proteolytic enzyme is a medium sized globular protein with application in mass spectroscopy based proteomics,[5-7] wound healing components, in washing agents and in biotechnology, mainly to perform enzymatic reactions.[8-12] Hence, it is confirmed that trypsin plays an essential role in controlling pancreatic exocrine function, but an increased level is also associated with some type of pancreatic disease.[13-15] Therefore, a new continuous assay for trypsin and inhibitor screening may open a door for the development of new diagnostic methods and therapeutic strategies for the implication in pancreatic diseases.

Several methods for trypsin assay have been reported. Among them traditional methods involve multiple clinical tests including radioimmuniassay[16] and gelatin based film techniques[17] whereas recently amperometric,[18] colourometric[19] and fluorometric assay

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methods based on, labeled substrate peptide,[20-23] were reported. However most of the reported methods were found to respond slowly towards enzyme modified electrodes and labeling was necessary to respond. Label free fluorescent assay for trypsin based on water soluble conjugated polymers[24-26] were also reported. Past few years witnessed that conjugated polyelectrolytes (CPEs) with their unique optical properties and intrinsic fluorescence signal amplification have been extensively used for sensing the biological materials, such as small biomolecules,[27,28] proteins, DNA [29,30] and enzymes.[31] However, a convenient and continuous label free water soluble conjugated polymer based fluorometric assay for trypsin and inhibitors screening are still very limited.

In the present work, we report that a novel anionic water soluble polyfluorene derivative poly(9,9-bis(6-sulphate hexyl) fluorene-*alt*-1,4-phenylene) sodium salt (P1) interacts with a cationic peptide Arg_6 by electrostatic interaction and serve as an efficient continuous and sensitive fluorescence turn-on assay for the detection of trypsin. Enzymatic hydrolysis completion was signaled by turn on response from the conjugated polyelectrolyte P1 as a result of the products, that were fragmented as single positively charged Arg and free chain conjugated polyelectrolyte.

6.2 Result and discussion

We reported in chapter-3 that P1 fluorescence intensity was highly sensitive towards C*c* and the reason was supposed to be highly positive nature at physiological pH and heme integrity of protein. Charged CPEs are well known to bind oppositely charged analytes via electrostatic interaction. This property of CPEs has been utilized in the present work. Positively charged polyarginine peptide (Arg_6) was selected as a substrate for enzymatic activity of trypsin. It is already reported that Arg_6 peptide have 6 positive charges at pH 8.5, whereas arginine has only one positive charge at the same pH.[32] High positive charged species may interact more efficiently with anionic P1. Therefore pH was kept at 8.5 in the present work and P1 was highly fluorescent at this basic pH of 8.5 in 2.0 mM phosphate buffer solutions with excitation wavelength 334 nm and emission at 411 nm. First, we investigated the amount of change in fluorescence intensity of P1 on addition of positively charged Arg and peptide Arg_6. To perform the experiment P1 (0.4 μM), Arg and Arg_6 peptide solutions were prepared in 2.0 mM PBS buffer solution. Arg and Arg_6 peptide solution (0−5 μM) were added into two separate solutions having P1 with same concentration of 0.4 μM and the fluorescence intensity change were monitored as shown in Figure 6.1.

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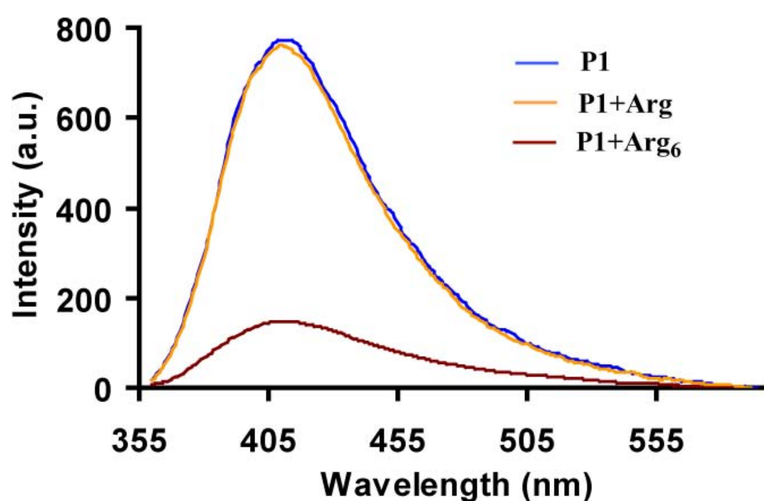

Figure 6.1 Changes in fluorescence intensity of P1 (0.4 µM) on addition of 5 µM of Arg and Arg_6 at pH 8.5 in 2 mM PBS solution containing $[Ca^{2+}]$ = 10 µM.

For instance, P1 solution in the absence of peptide shows a strong fluorescence. However, after addition of peptide solution the P1 fluorescence intensity was decreased by more than 80% due to opposite charge interaction. However, Arg with a single positive charge was unable to change the fluorescence intensity of P1. Therefore, we expect that Arg with one positive charge that will be the fragmented product after enzymatic hydrolysis will not interfere in the turn on signal from P1 and this will be solely from the enzymatic hydrolysis. Before the investigation of enzymatic hydrolysis we also investigated whether the quenched fluorescence of P1 as a result of association with Arg_6 was changing with time or this will remain intact during hydrolysis. We observed that this association with P1 was quiet stable to perform the enzymatic hydrolysis. Also the controlled experiments demonstrate that fluorescence intensity of P1/Arg_6 was unchanged during addition of trypsin, indicating that trypsin itself has no effect on intensity of P1/Arg_6 and the fluorescence intensity change will be due to the enzymatic hydrolysis. We further examined the enzymatic hydrolysis of P1/Arg_6 in presence of trypsin. To perform the enzymatic hydrolysis of Arg_6, trypsin (300 nM) was added into P1/Arg_6 solution in PBS pH 8.5 containing 10 µM of $CaCl_2$. The main purpose to add $[Ca^{2+}]$ was to activate trypsin and increase its stability against autolysis, as this was already reported in literatures.[33] After addition of trypsin, it was allowed for incubation. The gradual increase in fluorescence intensity at 411 nm was recorded for different incubation time from 0 to 20 min (Figure 6.2).

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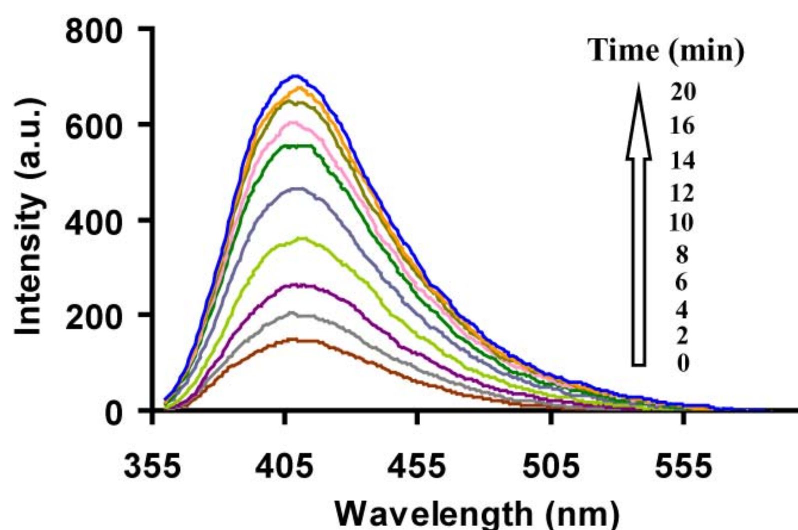

Figure 6.2 Changes in fluorescence intensity of P1 (0.4 μM)/Arg_6 (0−5 μM) on addition of 350 nM of trypsin at pH 8.5 in 2 mM PBS solution containing [Ca^{2+}] = 10μM.

According to Figure 6.2 it was observed that after addition of trypsin, the fluorescence intensity of P1/Arg_6 was unaffected giving an evidence that enzyme itself have no effect on the fluorescence intensity and after some time as the enzymatic hydrolysis proceeds, the quenched emission of P1/Arg_6 gradually increased and then reached a plateau and this fluorescence increase was leveled off at 48%. Therefore these results give evidence that trypsin catalyzes the hydrolytic cleavage of peptide Arg_6 into small positively charged fragments which are incapable to quench the P1 fluorescence and thus leading to an emission turn on response of P1/Arg_6.

Further increase in fluorescence intensity of P1/Arg_6 assay for a continuous real time was also investigated with different concentration of enzyme as a function of time to check whether the enzymatic hydrolysis of Arg_6 was dependant on the trypsin concentration and also how much less concentration of enzyme was needed for a significant change in the fluorescence intensity. Therefore, we performed the controlled experiment in the absence and presence of trypsin. Six samples containing P1/Arg_6 and 2.0 mM PBS buffer solution at pH 8.5 were prepared followed by the addition of different concentration of enzyme, say 0 nM, 40 nM, 80 nM, 120 nM, 220 nM and 350 nM and fluorescence intensity changes were monitored at the emission intensity 411 nm with increasing concentration of trypsin as a function of time as reported in Figure 6.3.

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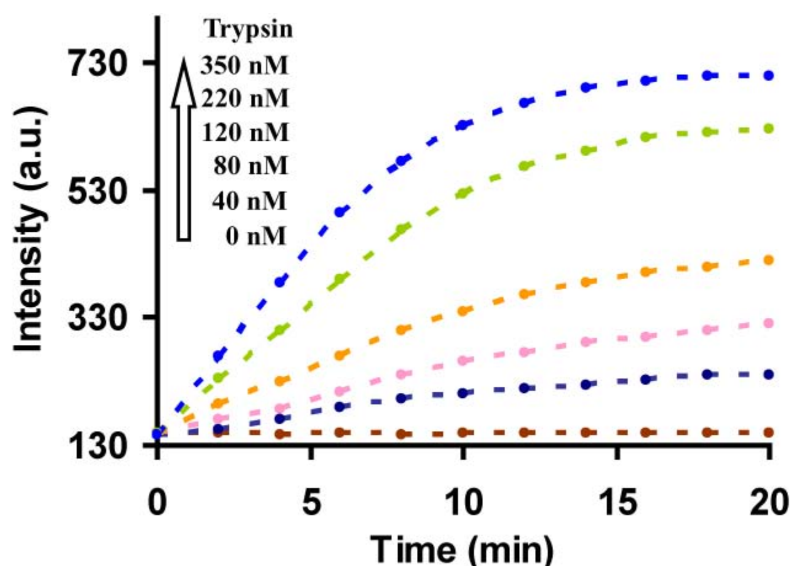

Figure 6.3 Fluorescence intensity enhancement of P1 (0.4 μM)/Arg_6 (0−5 μM) in presence of different concentration of trypsin as a function of time in 2 mM PBS solution containing $[Ca^{2+}]$ = 10μM.

The Figure 6.3 demonstrates the increase in the fluorescence intensity of P1/Arg_6 with respect to the incubation time and it was evident from the fluorescence increasing pattern that increase in fluorescence intensity was dependent on the enzyme concentration. At the lower concentration of trypsin the fluorescence recovery was slow whereas at higher concentration, fluorescence recovery was found to be high. At the same time a linear relationship between the maximum emission intensity and trypsin concentration was observed over the enzyme concentration from 0 to 220 nM.

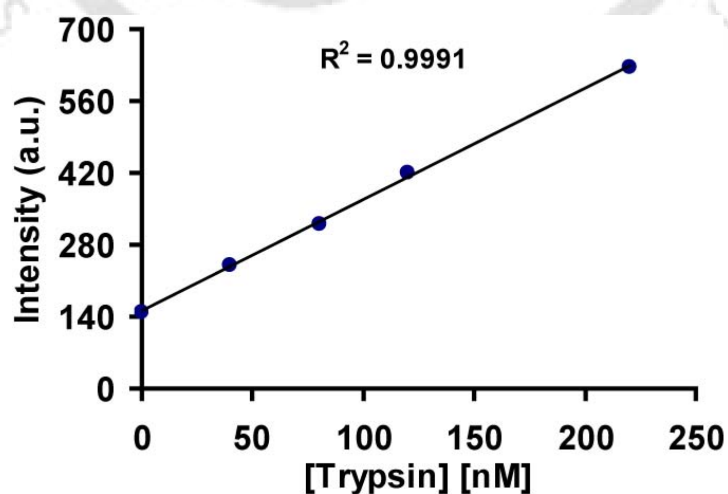

Figure 6.4 A linear relationship between maximum intensity from P/Arg_6 and enzyme concentration.

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Therefore it can be expected that due to this linear relationship, P1/Arg_6 association can assay the trypsin quantitatively (Figure 6.4). Therefore from these results it was evident that anionic polymer P1 in association with Arg_6 can serve as the fluorometric assay for hydrolytic cleavage by trypsin. Trypsin is the most important digestive enzyme present in the human body but an abnormal elevated level of enzymes is the indication of several types of diseases. These days, several drugs have been discovered and this drug discovery is based on the enzymes and their activity. Therefore a sensitive and continuous assay of enzyme that will be able to screen the enzyme inhibitors is of interest. We assumed that the hydrolysis of Arg_6 peptide catalyzed by trypsin will be retarded in presence of corresponding inhibitor into the solution. Therefore we carefully examined the inhibition of trypsin activity with Bowman-Birk protease inhibitor (BBI) which is a commonly used inhibitor of trypsin. To perform this experiment, six samples with same concentration of P1/Arg_6 and trypsin (350 nM) were prepared followed by the addition of different concentration of inhibitor from 0 to 2 µg/mL in each of the six samples. Therefore one sample without inhibitor (0 nM) and other samples with different concentration of inhibitor, and then fluorescence intensity changes were recorded at the emission intensity 411 nm as a function of time. Fluorescence pattern observed was depicted in Figure 6.5.

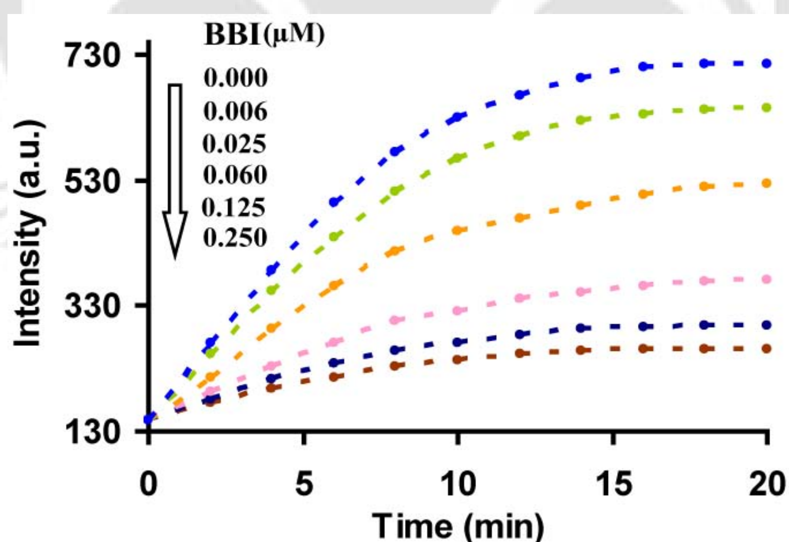

Figure 6.5 The retardation of fluorescence intensity enhancement of P1 (0.4 µM)/Arg_6 (0−5 µM) in the presence of inhibitor BBI (0 to 0.250 µM) as function of time in 2 mM PBS solution containing [Ca^{2+}] = 10µM.

Figure 6.5 demonstrates the changes in fluorescence intensity pattern as a function of time. As expected it was well evident from the figure that in the absence of inhibitor fluorescence

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intensity of P1/Arg_6/ [trypsin] was increasing gradually as hydrolysis time was increasing but in the presence of inhibitor, this fluorescence enhancement was retarded. Moreover, higher the concentration of inhibitor into the solution, slower was the increase in fluorescence intensity from P1. Inhibition efficiency of the inhibitor was calculated using the equation $(1-I/Io) \times 100\%$, where *Io* and *I* are the restored fluorescence intensities at 411 nm (Figure 6.6).

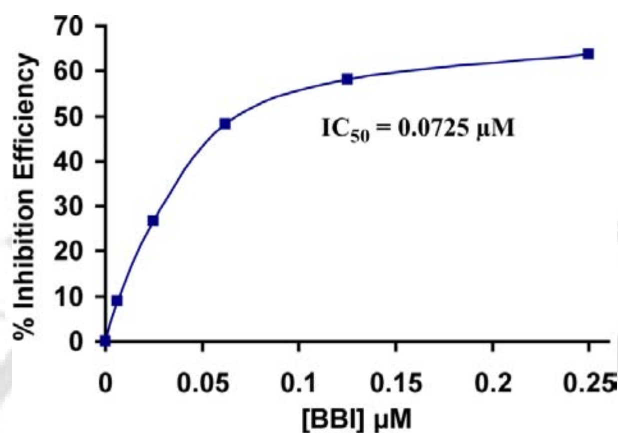

Figure 6.6 Inhibition efficiency was plotted against inhibitor concentration to evaluate the IC_{50} value.

From the above plot between inhibition efficiency and inhibitor concentration IC_{50} value of the inhibitor was calculated and this was found as 0.0725 μM. This indicates the high sensitivity of the reported method towards screening the inhibitors of trypsin. Therefore, it was concluded that anionic P1 may serves as a fluorescent probe for enzymatic hydrolysis of trypsin and can also be used to screen the inhibition of the enzyme activity.

6.3 Conclusion

From the method established above, it was concluded that the interaction of a novel anionic water soluble polymer P1 and Arg_6 under alkaline pH, can serve as the fluorometric assay as well as to screen the inhibitors of enzyme trypsin, on the basis of light harvesting properties of the conjugated polymers. The method does not require any labeling on the substrate, thus reducing the cost of method and during enzymatic hydrolysis turned on signal was visible, indicating the eligibility of this method to monitor the enzymatic action in vitro. Therefore anionic polyfluorene P1 in association with Arg_6 demonstrates the turn on assay for the enzymatic activity of trypsin under alkaline condition could be established. This system also offers a convenient mixing and detect strategy, similar to the previous chapter, which

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represents a continuous approach for rapid assay for enzymatic activity as well as the use of water soluble conjugated polymer imparts the sensor with high sensitivity.

6.4 Experimental

6.4.1 Reagents and materials

All the reagents and solvents were purchased from Aldrich Chemicals (India), Merck (India) or Ranbaxy (India) and were used as received. Milli-Q water was used in all the experiments. Trypsin (porcine pancreas) and BBI were obtained from sigma Aldrich.

6.4.2 Instrumentation

Fluorescence spectra were carried out on a Varian Cary Eclipse Spectrometer. A 10 mm X 10 mm quartz cuvette was used for solution spectra and emission was collected at 90° relative to the excitation beam. Deionized water obtained by Milli-Q system (Millipore) was used.

6.4.3 Fluorescence quenching and dequenching experiments

The experiment was carried out in 2 mM phosphate buffer solution at basic pH of 8.5. P1 (0.4 μM), Arg_6 and Arg_6 peptide solutions were prepared in 2.0 mM PBS buffer solution Arg and Arg_6 peptide solution (0-5 μM) were added into two separate solutions having P1 with same concentration 0.4 μM and the fluorescence intensity changes were monitored at 411 nm.

6.4.4 Real Time trypsin assay in the absence and presence of inhibitor

Six samples containing P1/Arg_6 and 2.0 mM PBS buffer solution at pH 8.5 containing 10 μM of $CaCl_2$ were prepared followed by the addition of different concentration of enzyme, such as 0 nM, 40 nM, 80 nM, 120 nM, 220 nM and 350 nM and fluorescence intensity changes were monitored at the emission intensity 411 nm with increasing concentration of trypsin as a function of time.

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6.5 References

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LIST OF PUBLICATIONS

1. **Dwivedi, A. K.**; Saikia, G.; Iyer, P. K. Aqueous polyfluorene probe for the detection and estimation of Fe^{3+} and inorganic phosphate in blood serum. *J. Mater. Chem.*, **2011**, *21*, 2502-2507. (**Appeared as Journal Inside Cover Picture**)
2. **Dwivedi, A. K.**; Saikia, G.; Iyer, P. K. Neutral Conjugated Polymer Demonstrates Remarkable Activity as Non-invasive Sensor For Inorganic Phosphate Detection. *Polymer Perprint* (American Chemical Society, Division of Polymer Chemistry) **2011**, *52*, 963-964.
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7. **Dwivedi, A. K.**; Iyer, P. K. Continuous and real time turn on assay for monitoring the enzymatic activity of trypsin under alkaline conditions. (Manuscript under preparation)
8. **Dwivedi, A. K.**; Iyer, P. K. Anionic polyfluorene based assay system to screen the inhibitors of acid phosphatase enzyme. (Communicated)

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STATEMENT

I do hereby declare that the work contained in the thesis entitled '**Development of Fluorescent Water Soluble Polyfluorene for Chemical and Biological Sensors**' is the result of investigations carried out by me in the Department of Chemistry, Indian Institute of Technology Guwahati, Guwahati, Assam India under the supervision of Dr. Parameswar Krishnan Iyer, Associate Professor, Department of Chemistry, Indian Institute of Technology Guwahati, Guwahati, Assam, India. This work has not been submitted elsewhere for the award of any degree.

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October, 2012

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