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

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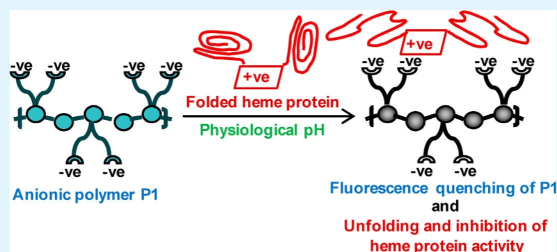
Supporting Information



ABSTRACT: Because of the toxicity caused by the heme redox-active iron proteins, their elevated levels, localization, and accumulation in the brain, many forms of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, occur as a result of which the brain becomes vulnerable to oxidative stress, ultimately resulting in neuronal death. An anionic water-soluble conjugated polyfluorene derivative poly(9,9-bis(6-sulfate hexyl) fluorene-*alt*-1,4-phenylene) sodium salt (P1) that binds Fe^{3+} proteins with very high selectivity and sensitivity is reported here. The photophysical properties of P1 were modified by the interaction with ferric heme-containing proteins cytochrome *c* (C*c*), methemoglobin (MetHb), and hemin. P1 was found to be highly sensitive toward Fe^{3+} heme proteins as compared to nonmetalloproteins. We observed that the respective activities of ferric heme proteins were inhibited and proteins were unfolded, due to modification in their heme microenvironment in the presence of the polymer P1. The observations reported in this article provide the first example for the use of a water-soluble conjugated polymer in applications, such as (1) to detect small quantities of iron proteins in aqueous medium/physiological condition with the highest K_{sv} values of 2.27×10^8 M^{-1} for C*c*, 3.81×10^7 M^{-1} for MetHb, and 5.31×10^7 M^{-1} for hemin; (2) to study the physiological effects of heme metalloproteins; (3) to visualize the folding events in real time; and (4) the inhibition activity of metalloproteins can be selectively studied using a conjugated polymer based assay system rapidly without interference from nonmetalloproteins at biological pH. 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.

KEYWORDS: *heme proteins, protein unfolding, biosensor, iron(III), neurodegenerative diseases, anionic polyfluorene*

INTRODUCTION

The selective detection of proteins and the study of its aggregation, folding/unfolding behavior, and fibril formation are vital in biochemical studies, proteomics, and 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.[1,2] 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. 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.[3,4] 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.[5,6] Through various reports, it is recognized that, in the cells, C*c* is also involved in apoptosis, signaling the cells to program cell death due to generation of reactive oxygen species by mitochondrial enzyme oxidation.[7−9] 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.[10−12] MetHb is the naturally occurring oxidized metabolite of hemoglobin. Methemoglobinemia occurs when red blood cells (RBCs) contain greater than 1% MetHb.[13,14] This occurs either from 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 their structural variation into various undesired configurations are of particular significance. Since proteins are much more complex and sensitive, designing artificial assay systems with an appropriate receptor for the target protein is still a crucial challenge in protein biosensors.[15] In this regard, polymers possessing a π-conjugated backbone system present a tremendous platform for designing protein biosensors because this backbone can facilitate electron delocalization and exciton migration through energy and electron transfer, resulting in amplified signals.[16−28] Therefore, interaction with a small fraction of analyte alters the photophysical properties of conjugated polymers, which is exploited for the protein

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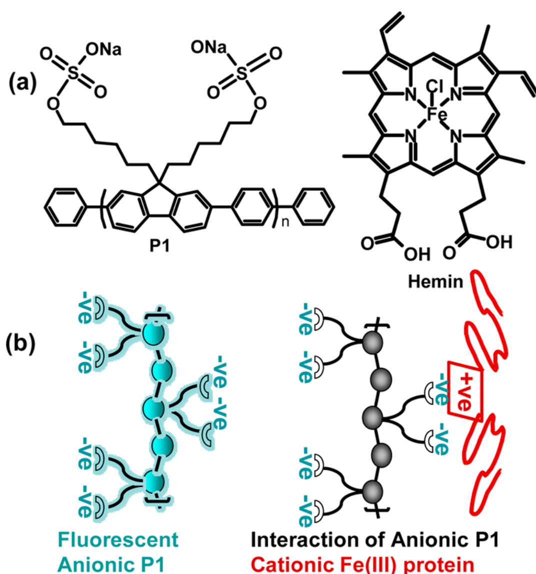
detection here. In the presence of protein, the photophysical properties of the conjugated polymer P1 (Scheme 1a) are significantly modified. In addition, the presence of P1 in small quantities also affects the structure of the protein, resulting in unfolding and alteration in its activity. Similarly, intrinsic fluorescence properties of proteins due to the presence of tyrosine and tryptophan residues have also been used to follow protein unfolding because their fluorescence properties are very sensitive to their environment, which changes when proteins fold or unfold.[29,30]

Scheme 1. (a) Structure of P1 and Hemin. (b) Representative Interaction of Fluorescent Anionic P1 and Cationic Fe(III) Protein



■ RESULTS AND DISCUSSION

Herein, heme group-containing proteins C*c* and MetHb as well as the hemin molecule (Scheme 1a) have been carefully examined to understand their activity, folding dynamics, effect of protein redox state, and ligand binding to the heme using several control examples using anionic water-soluble polyfluorene derivative poly(9,9-bis(6-sulfate hexyl)fluorene-*alt*-1,4-phenylene) sodium salt (P1).[31] A combination of spectroscopic and gel electrophoresis techniques revealed complexation of the proteins with P1. These studies also revealed that P1 was able to induce loss of activity and unfolding of ferric heme proteins by modifying the heme microenvironment and decreasing the fluorescence intensity with a shift in wavelength of the tryptophan residue.[32,33]

Evaluating the Interactions of Proteins with P1 by UV–visible and Fluorescence Spectroscopy. Since P1 shows distinct photophysical properties in the presence of ferric iron,[31] we studied its interaction with a few ferric proteins (Figure 1). The metalloproteins used in this study include C*c*, MetHb, and ferritin, and nonmetalloproteins, such as lysozyme, BSA, and casien. P1 has an absorption maximum at 334 nm and emission at 411 nm in aqueous conditions. P1 is highly fluorescent at pH 7 in 25 mM tris-HCl buffer solution with an excitation wavelength at 335 nm. The fluorescence intensity of P1 (0.4 μM) significantly decreased with increasing concentration of C*c* (0.033 μM) (Figure 1a). We also investigated whether this decrease in intensity was due to cationic and anionic interaction between the cationic protein and anionic P1 (Scheme 1b), as reported earlier for anionic polymer and MV^{2+} interactions.[34] Therefore, the interaction with the high p*I* value protein lysozyme, at the same concentration as C*c*, was investigated at physiological pH, and the results showed that lysozyme was not able to reduce the fluorescence intensity of P1 (Figure 1d). This was because the lysozyme lacks the heme unit with the ferric state as in C*c*, which is able to quench the excited state of P1 mainly by photoelectron transfer.[16,35−38] To

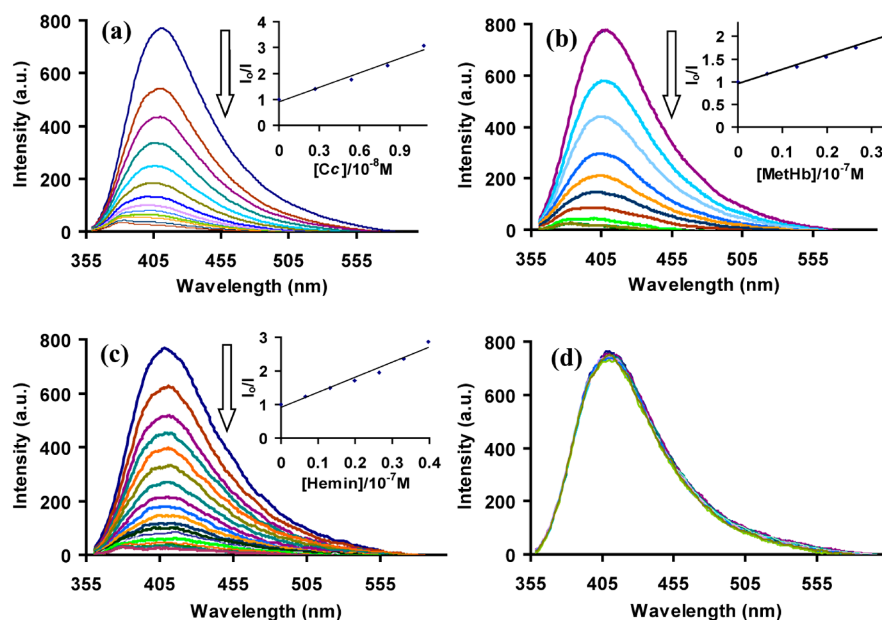


Figure 1. Fluorescence response of P1 (0.4 μM) toward proteins and hemin was checked in 25 mM tris-HCl solution. (a) C*c* was added up to a concentration of 0.033 μM. Inset is the Stern−Volmer plot. (b) Methemoglobin was added up to a concentration of 0.1 μM. Inset is the Stern−Volmer plot. (c) Hemin was added up to 0.16 μM. Inset is the Stern−Volmer plot. (d) Lysozyme was added up to 0.8 μM.

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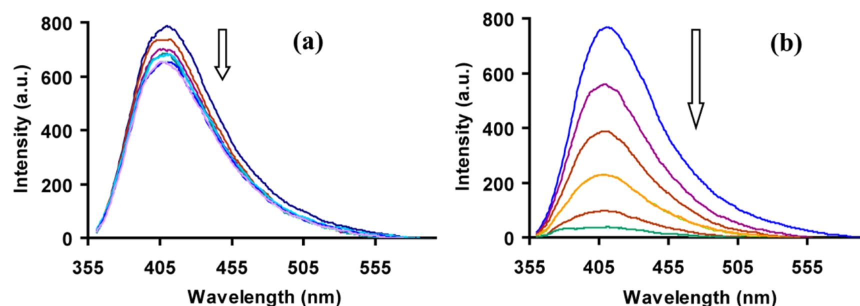

Figure 2. (a) Changes in the fluorescence intensity of P1 (0.4 μM) in the presence of the apoenzyme part ranging from 0 to 0.2 μM. (b) Changes in fluorescence intensity of P1 (0.4 μM) in the presence of the hemin part ranging from 0 to 0.17 μM.

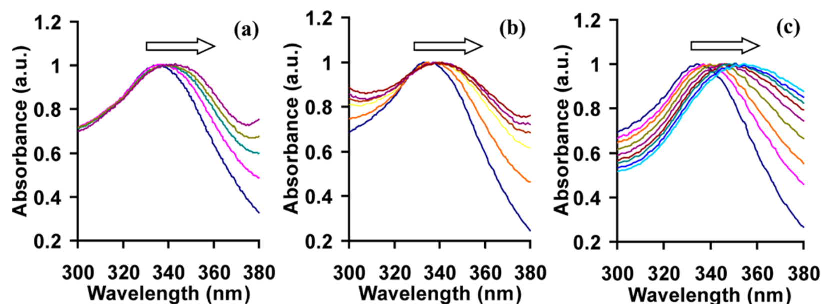

Figure 3. Normalized absorption spectra of P1 with metalloproteins and hemin recorded in 25 mM tris-HCl buffer solution. (a) P1 with C*c* up to 1 μM shows a 7 nm shift. (b) P1 with MetHb up to 4 μM shows a 7 nm shift. (c) P1 with hemin up to 5 μM shows an 18 nm shift.

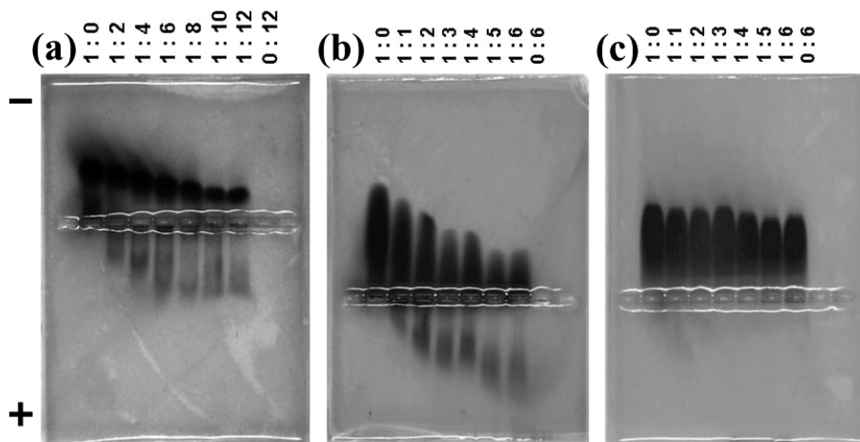

Figure 4. Native gel electrophoresis of proteins and P1 in tris-glycine buffer with 1% agarose. Metalloprotein concentration is constant in each well, and P1 concentration varied. (a) C*c* (50 μM) in each well; the left well contained only C*c*; P1 varied in the ratio 0–12 from left to right wells; the right well contained only P1 (0.6 mM). (b) MetHb (50 μM) in each well; left well contained only MetHb; P1 varied in the ratio 0–6; right well contained only P1 (0.030 mM). (c) Lysozyme (50 μM) in each well; left well contained only lysozyme; P1 varied in the ratio 1–6; right well contained only P1 (0.030 mM).

test this hypothesis, the interaction of hemin was investigated with P1 and changes were monitored by fluorescence spectroscopy.

It was observed that 0.16 μM hemin quenched the fluorescence of P1 completely (Figure 1c). Despite MetHb being negative at physiological pH, the quenching studies showed that the ferric heme unit of MetHb was also an efficient quencher of P1 (Figure 1b). However, nonmetalloproteins lysozyme, BSA, and casein, which lack ferric heme units, were unable to quench the fluorescence of P1. Nonheme protein ferritin also quenched the fluorescence of P1 but was less efficient as compared to ferric heme proteins. Hence, 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 comparing

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

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

A high K_{sv} value of an artificial assay indicates that the probe has high sensitivity in the protein biosensor application.[36] Because C*c* quenches the fluorescence of P1 at a lower

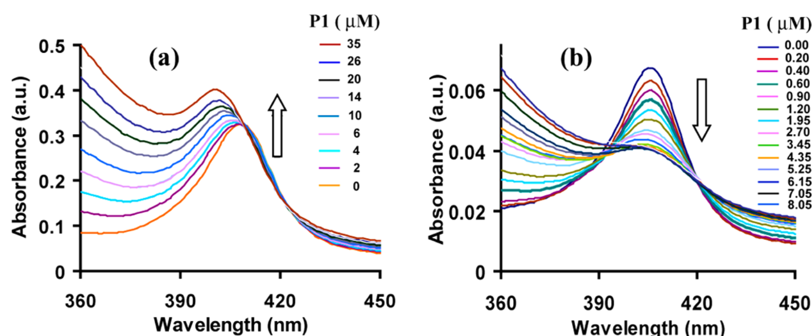

Figure 5. Absorption spectra (Soret region) of (a) native Fe(III) C*c* (6 μM) and (b) MetHb (1 μM) at pH 7.4 in tris-HCl buffer with increasing concentration of P1.

concentration than other metalloproteins, we observed a higher K_{sv} value of 2.27×10^8 M^{-1}, whereas the values of MetHb and hemin were 3.81×10^7 and 5.31×10^7 M^{-1}, respectively (inset, Figure 1a−c). Although it can be inferred that C*c* is a highly efficient quencher of P1 compared with heme and MetHb, all the K_{sv} values obtained here are among the highest for artificial assays.[36−38] Nonmetalloproteins showed insignificant fluorescence quenching in comparison to metalloproteins, indicating that the fluorescence of P1 decreases due to porphyrin functionality with ferric iron.

To support these results, we separated the hemin part from MetHb by deproteinizing the protein and then repeated the PL experiment with both hemin and the apoenzyme part[39] (Supporting Information). In Figure 2a, the apoenzyme part shows an insignificant fluorescence quenching, providing evidence that P1 is unaffected by nonmetalloproteins. However, more than 97% of fluorescence quenching of P1 occurs on addition of up to 0.17 μM hemin part obtained from the deproteinization experiment. 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(a−c). An inherent feature observed was that the absorption of P1 (λ_{max} = 334 nm) was seen shifting toward longer wavelength, 7 nm in the case of C*c*, 7 nm for MetHb, and 18 nm for hemin, suggesting that P1 associates with the metalloproteins, resulting in this shift.[40]

Evaluating the Interactions of Proteins with P1 by Native Gel Electrophoresis. The interaction of metalloproteins and P1 was also monitored by native gel electrophoresis experiments. For C*c* and lysozyme, the pH of the buffer solution was maintained at 8.6, whereas for MetHb, the buffer pH was maintained at 6.0 (Figure 4). The movement of C*c* and MetHb toward the 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 a high intensity band, but when the P1 concentration ratio along the well was increased, the intensity of the protein band decreased and these were found in close proximity to the well. In the case of the C*c* band (Figure 4a), the intensity was seen decreasing from the protein control to increasing concentration ratio of P1, but an additional increasing band intensity pattern appeared toward the cathodic side. Additional bands near the well were the almost neutral complex of protein with P1, but as the P1 concentration ratio was increased, additional bands were observed shifting toward the anodic side well to well, conforming that the protein bound complex is negative in nature. In a similar manner, MetHb also showed (Figure 4b) a protein band intensity decreasing pattern on the cathodic side, and an additional complex band intensity increasing pattern on the anodic side from the concentration ratio of protein to P1 of 1:0 to 1:6. The interaction of P1 with lysozyme (50 μM) (Figure 4c) was also checked by gel electrophoresis, but the decreasing pattern of protein band and development of additional bands, as observed for metalloproteins, were absent, providing strong evidence of no interaction of lysozyme and P1.

Evaluating the Structural Modifications Occurring in Proteins in the Presence of P1. Further, to understand the nature of the 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.[41] Coordination of these ligands produces a low-spin complex with Soret absorption maxima at 410 nm. When P1 (35 μM) was added into C*c* (6 μM) at pH 7.0 in tris-HCl buffer, the Soret band at 410 nm was blue shifted to 402 nm (Figure 5) with an increase in absorption intensity, indicating the replacement of the axial ligand Met^{80} by the solvent water, since the Met^{80} ligand is more labile than the His^{18} ligand,[42] whereas in the case of MetHb, this Soret band intensity decreased with increasing concentration of P1. In the case of both of these proteins, P1 is responsible for changes in the heme microenvironment, and this change leads to unfolding of proteins, confirmed by fluorescence emission of the tryptophan residue.

In proteins, typically, the fluorescence parameters give a direct interpretation of the degree of exposure of the fluorophore to the solvent.[43] For tryptophan emission, the excitation wavelength was kept at 290 nm and the maximum emission wavelength was observed between the range of 310−350 nm. On adding P1, to the solution of C*c* in tris-HCl buffer, the fluorescence emission of the 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*.[44] However, in the case of MetHb in tris-HCl buffer, the tryptophan fluorescence decreased with a blue shift, indicating that the protein conformational structure rearranges in such a way that tryptophan got buried in the relatively hydrophobic interior or interfaces in the protein (Figure 6). In both cases, this led to unfolding of the proteins. In almost all the proteins, amino acids tryptophan, tyrosine, and phenylalanine are intrinsically fluorescent, but tryptophan is the most useful among the three due to a lower extinction coefficient and high quantum yields. In addition, tyrosine and phenylalanine relatively lack environmental sensitivity of their emission profile, which makes them less useful than

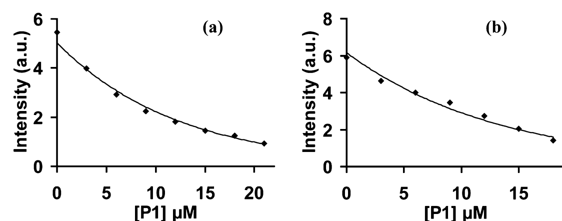

Figure 6. (a) Changes in tryptophan fluorescence intensity of C*c* (8 μM) (tris-HCl buffer pH 7.4) in the presence of P1 (0−30 μM). (b) Changes in tryptophan fluorescence intensity of MetHb (10 μM) (tris-HCl buffer pH 7.4) in the presence of P1 (0−20 μM). Both the solutions, C*c* and MetHb, were excited at 290 nm.

tryptophan.[43] The fluorescence properties of tryptophan are very sensitive to its environment, which changes when the protein conformation changes.[30,45,46] These blue and red shifts of tryptophan, accompanying the fluorescence quenching in MetHb and C*c*, indicate that P1 is able to induce conformational modification and unfold the proteins. From the above experiment, we were able to obtain strong evidence that the protein undergoes unfolding. Hence, circular dichroism (CD) spectroscopy was also used to monitor structural transformations in the proteins (Figure 7).

The far-UV region ranging from 180 to 250 nm reveals information about the peptide bond asymmetric environment, reflects the secondary structure, and can predict the conformational changes in proteins arising due to interaction with some host species.[47] C*c* (50 μM) in 25 mM tris-HCl buffer gave a characteristic peak at 222 nm, assigned to the α-helical content of this protein. After addition of P1 (0−150 μM), the helicity of C*c* significantly changed (Figure 7a). Similarly, MetHb (40 μM) in 25 mM tris-HCl buffer also gave a peak at 222 nm, and in presence of P1 (0−100 μM), the helicity of MetHb was also altered (Figure 7b). From the obtained CD data, we can conclude that P1 is capable of causing modification in the α-helix content of proteins, thereby bringing conformational changes in the metalloprotein structure, which was earlier proved by spectroscopic studies.[48] Hence, P1 induces conformational changes in the secondary structure of metalloproteins.

These results confirm that P1 changes the heme microenvironment of proteins, which suggests that the activity of proteins is enhanced or decreased after interaction with P1. To check this hypothesis, the activities of both the proteins were investigated in the absence and presence of P1. Fe^{3+}−C*c* reactivity was tested toward ascorbate in the presence of P1. Here, Fe^{3+}−C*c* (2.5 μM) was preincubated with P1 at 0−16 μM concentrations, followed by the addition of ascorbate (2.8 mM) solution to initiate the reaction. Fe^{3+}−C*c* was rapidly reduced by ascorbate (2.8 mM) in the absence of P1, monitored by an increase in absorbance at 550 nm (Figure 8a). After addition of P1, we observed that the absorbance at 550 nm decreased significantly with increasing concentration of P1 (0−16 μM), indicating that, in the presence of P1, C*c* reactivity toward ascorbate decreased and P1 bound C*c* was mainly in the oxidized form (Figure 8b). The MetHb peroxidase activity was tested with guaiacol in the presence of H_2O_2. Here, guaiacol (20 mM) and H_2O_2 (0.27 mM) were taken in 50 mM tris-HCl buffer (pH 7.4) solution and MetHb with increasing concentration (0−5 μM), which was incubated for 15 min in the absence and presence of P1 in a final volume of 1 mL of guaiacol buffered solutions (Figure 8c). MetHb peroxidase activity was assayed by following the increase in the absorption of tetraguaiacol complex at 470 nm (Figure 8d). In the same way, P1 (15 μM) was preincubated in guaiacol solution, and then increasing concentrations (0−5 μM) of MetHb were added in the solution. The absorption at 470 nm was recorded. In presence of P1, at the same concentrations of MetHb, absorption at 470 nm decreased. The color of the tetraguaiacol complex faded rapidly in the presence of P1 (Figure 8e), indicating a decreased peroxidase activity of MetHb.

Because of the increased presence and accumulation of the redox-active heme iron proteins, such as C*c* and MetHb, the toxicity in the brain increases, which is responsible for many forms of old age neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD). Despite extensive efforts, the pathogenic mechanism of AD is yet to be exploited, because of which no treatments or cure exist,[49,50] instead preventing this form of neurodegenerative disorder,[51,52] can be an underlying factor to reduce oxidative stress by controlling the free radical generation linked with redox-active metals.[53] P1 selectively interacts with ferric heme proteins, inducing a change in their structural conformation, which is highly sensitive in comparison to *N*-methyl-D-aspartate (NMDA) receptor antagonists (memantine) or acetyl cholinesterase inhibitors donepezil that are very expensive, but not specific, yet being used currently for relieving cognitive symptoms.[54,55] In addition to the ability of P1 to selectively bind heme iron, the heme microenvironment of proteins could be modified, which indicates that the activity of proteins can be controlled on interaction with P1. Since P1 is also able to induce conformation modification and unfold the proteins, it can have potential applications to study structurally

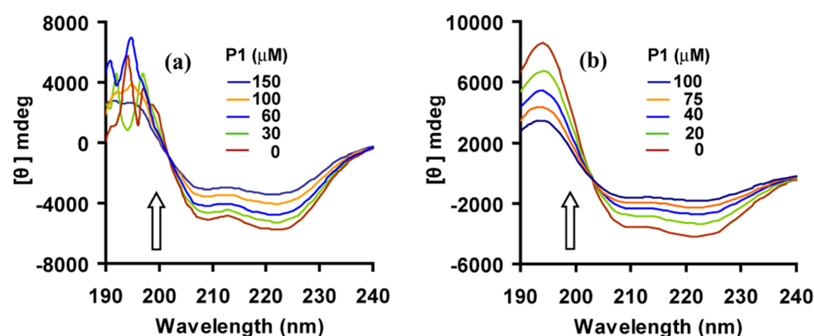

Figure 7. CD spectra in 25 mM tris-HCl buffer solution. (a) C*c* (40 μM) shows changes in α-helical content on interaction with P1 (0−150 μM). (b) MetHb (40 μM) shows changes in α-helical content on interaction with P1 (0−100 μM).

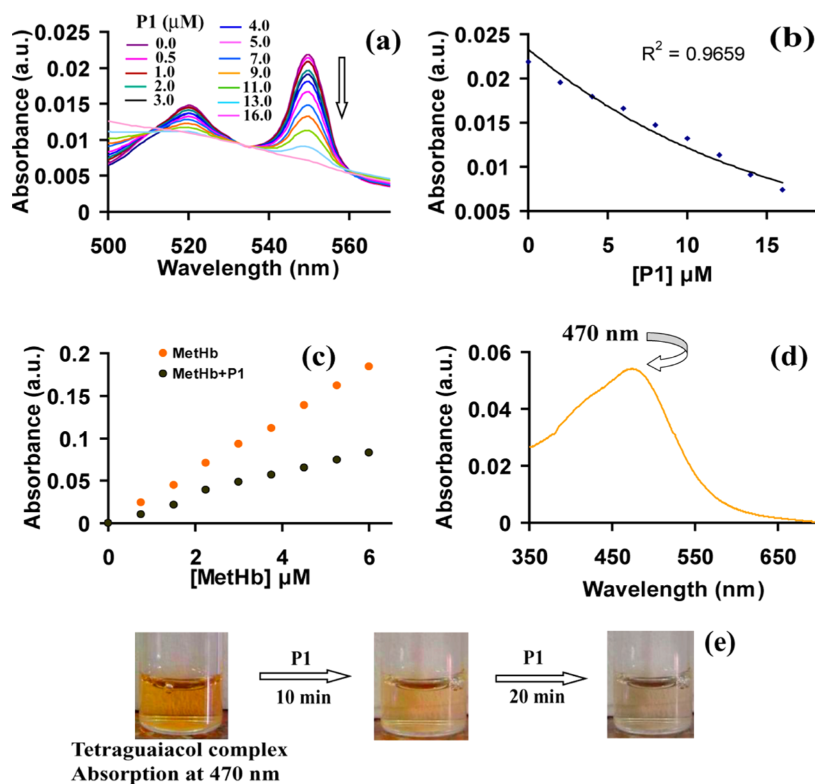

Figure 8. (a) Increase in the 550 nm peak, Fe^{3+} in C*c* (2.5 μM) reduced to Fe^{2+} by ascorbate (2.8 mM) in the absence of P1. (b) Decrease in 550 nm peak in the presence of P1 (16 μM) and ascorbate. (c) Guaiacol (20 mM) and H_2O_2 (0.27 mM) in 50 mM tris-HCl buffer (pH 7.4) solution with increasing concentration of MetHb (0−5 μM) in the absence and presence of P1. (d) MetHb peroxidase activity was assayed by following the increase in the 470 nm peak of tetraguaiacol complex. (e) Visual color change of tetraguaiacol complex in the presence of P1.

transformed protein intermediates that may be helpful for clinical therapeutic purposes, such as the amyloidogenic protein intermediates that induce aggregate forming amyloid fibrils.[56,57]

■ CONCLUSIONS

The increased level and accumulation of heme redox-active iron proteins in the brain are confirmed to be responsible for metabolic perturbations in chronic neurodegenerative diseases, such as AD, PD, HD, etc., as a result of which the brain is exposed to oxidative stress, causing irreversible transformation in the brain of patients suffering from these neurodegenerative diseases. Although the exact pathological consequences of heme binding to the amyloid fibrils in AD are not known yet, it provides a new avenue of research into studying the mechanism of AD, PD, and HD. P1 binds ferric heme proteins C*c*, MetHb, and hemin with very high sensitivity, altering the structural conformation and inhibiting their respective activities. However, nonmetalloproteins, such as lysozyme, BSA, and casein, which lack a ferric heme unit, were unable to quench the fluorescence of P1, confirming the selective nature of P1 toward ferric heme. Because of the complexation between P1 and C*c*, heme was exposed to solvent by the replacement of the labile Met^{80} ligand, and the quenching tryptophan fluorescence accompanying blue shift results in the unfolding. The MetHb structure unfolded in a different way as compared to C*c* such that the tryptophan residues got deeply buried in the relatively hydrophobic interiors, but are not solvent exposed after interaction with P1. Structural transformation of ferric heme proteins by P1 provided valuable insights to elucidate the role of the proteins involved in iron metabolism. The application of P1 to study physiological effects of heme metalloproteins sheds important light on mechanistic strategies involved in heme protein conformational research and provides vital clues to rationally design artificial assay systems. P1 is the first example for the use of a water-soluble conjugated polymer to detect small quantities of iron proteins in aqueous medium/physiological condition with K_{sv} values of 2.27×10^8 M^{-1} for C*c*, 3.81×10^7 M^{-1} for MetHb, and 5.31×10^7 M^{-1} for hemin, which are the highest in the literature. P1 also facilitates examining the physiological effects of heme metalloproteins, the folding events, and the inhibition activity of metalloproteins without interference from nonmetalloproteins. These findings open the possibility to investigate and rationally design artificial assay systems to examine physiological effects of heme metalloproteins in neurodegenerative disorders and to study mechanistic strategies involved in heme protein conformational research and the role of the proteins involved in iron metabolism.

■ ASSOCIATED CONTENT

Supporting Information

It consists of the details of instruments used in performing all the experiments reported here. Details of polymer synthesis and characterization and metalloprotein and nonmetalloprotein interactions studied by UV−visible spectroscopy, fluorescence spectroscopy, CD spectra, gel electrophoresis, and protein activity studies are mentioned in the Supporting Information file. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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