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Photophysical behavior of heme group: Unfolding of hemoglobin and myoglobin in the presence of Gemini surfactants of different molecular architectures

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ABSTRACT

Fluorescence studies were performed to determine the photophysical behavior of heme group in the presence of cationic Gemini surfactants of different architectures. Both hemoglobin and myoglobin were used to understand the heme group interactions with Gemini surfactants under the influence of temperature variation and were compared with homologous monomeric surfactants. The results were also supplemented from the size and zeta potential measurements of both proteins. Gemini surfactants showed marked effect on the unfolding behavior of hemoglobin that mainly contributed by the stronger hydrophobic interactions of double hydrocarbon chains as well as methylene spacer in the head group region with the hydrophobic domains of hemoglobin. Myoglobin with single polypeptide chain did not show similar unfolding behavior in the presence of Gemini surfactants rather it was readily solubilized in the surfactant solution and that too in the presence of monomeric surfactants rather than Gemini surfactants. The results highlighted the mechanistic aspects by which water soluble globular proteins interact with amphiphilic molecules of different functionalities and thus, helped to predict the interactions of both hemoglobin and myoglobin with the complex biological molecules possessing similar functionalities.

1. Introduction

Hemoglobin is a highly water soluble protein and is an important component of blood with a molar mass of 64.5 kDa. It contains four heme groups bound to four protein chains. Out of which, two are the α chains of 141 amino acids whereas two are the β chains with 146 amino acids [1,2]. Likewise, myoglobin is another heme protein that consists of a single polypeptide chain and is mainly available in the muscles [3]. Both proteins can bind many different types of amphiphilic molecules [4–6] while heme group is the most active site which can interact with different kinds of species [7]. Surfactants belong to an important category of surface active molecules which are the active ingredients of several pharmaceutical formulations. This allows surfactant molecules to freely interact with hemoglobin. Although, several studies have demonstrated the hemoglobin – surfactant interactions [8–10], this study particularly focuses on the fate of heme group when Gemini surfactants interact with hemoglobin. Since heme group is the main driving force for the electron exchange reactions involving the oxygen binding ability of hemoglobin, therefore, it is important to understand that how molecular architecture of a Gemini surfactant influences the stability of heme group. Surfactants are also known to induce unfolding in protein structure [11,12] that in turn is expected to significantly affect the heme group environment because iron in heme group is covalently bound to the globular protein via N atoms of the imidazole ring. Thus, unfolding in hemoglobin not only leads to a change in the symmetry of heme group but also induces conformation changes those are reflected in the absorbance of Soret as well as Q bands [13,14]. In this study, our focus remains on the photophysical behavior of heme group upon interacting with Gemini surfactants where we

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systematically change the dimeric head group, spacer length, and the length of double hydrocarbon chains of Gemini architecture to understand the unfolding behavior of hemoglobin.

Hemoglobin is highly water soluble protein and hence, demonstrates strong hydrophilic interactions with ionic surfactants [8–10]. The presence of Gemini surfactants with dimeric ionic head groups is expected to enhance such interactions as well as they have the ability to interact with the hydrophobic domains of protein through hydrophobic interactions. Both hydrophilic and hydrophobic interactions dramatically influence the unfolding behavior of hemoglobin that in turn should influence the photophysical properties of heme group. Heme group absorbs over an extended range of wavelength from 400 to 600 nm, therefore, the best way to study the photophysical behavior of heme group is by following the synchronized fluorescence which effectively deals with the emission from the multiple species over an extended wavelength range. We highlight some of the important characteristic properties of heme group upon interacting with Gemini surfactants of different molecular structures that may act as model for predicting the interactions of other surface active complex biologically active molecules with hemoglobin.

2. Experimental

2.1. Materials

Hemoglobin from bovine blood, product # H2625, myoglobin from equine heart, product # M1882, sodium dodecyl sulphate (SDS), dodecyltrimethylammonium bromide (DTAB), tetradecyltrimethylammonium bromide (TTAB), and hexadecyltrimethylammonium bromide (HTAB), were purchased from Aldrich. Gemini surfactants trimethylene- (16-3-16), tetramethylene- (16-4-16), pentamethylene- (16-5-16), and hexamethylenebis(hexadecyldimethyl ammonium bromide) (16-6-16), and their homologous surfactants with thio spacer i.e. 1,2-bis(2-(3-methylimidazolium-1-y)dodecylthio)ethane bromide (12-S-2-S-12), 1,2-bis(2-(3-methylimidazolium-1-y)tetradecylthio)ethane bromide (14-S-2-S-14), and 1,2-bis(2-(3-methylimidazolium-1-y) hexadecylthio)ethane bromide (16-S-2-S-16) were synthesized as reported elsewhere [15,16]. Double distilled water was used for all preparations.

2.2. Methods

Synchronized fluorescence and DLS measurements were performed by taking aqueous solution of hemoglobin (100 mg/100 ml) along with Gemini surfactant (1 mM) from 20 to 70 °C. All reactions were monitored simultaneously by spectrofluorometer (Shimadzu-Model No. RF-5301 PC) in the synchronous mode in the wavelength range of 300–800 nm to observe the progress of reactions. This instrument was equipped with a TCC 240A thermoelectrically temperature controlled Cell Holder that allowed to measure the spectrum at a constant temperature within ±1 °C. Multi-angle particle sizing and Low angle zeta potential analyses were done by DLS and ELS (Electrophoretic light scattering), respectively, using a minimum number of optical components in apparatus (NICOMP Nano Particle Size Analyzer system, model: Z3000 ZLS). It was equipped with peltier thermoelectric element which regulated the temperature of the sample cell within ±0.2 °C with lower limit of 0 °C and upper limit of 90 °C. The Particle size analysis was calibrated with nano-sphere size standards of 90 nm and 240 nm, while zeta potential was calibrated using zeta reference standards. The measurements were made using a quartz cuvette with a path length of 1 cm. The particle size analysis was recorded for both Gaussian system and NICOMP distribution.

3. Results and discussion

3.1. Unfolding temperature (Td)

Fig. 1a shows the synchronized fluorescence spectra of hemoglobin in pure water at 20–70 °C. A sharp peak close to 310 nm belongs to the tryptophan emission whereas prominent peaks in the range of 400 to 700 nm are due to the emission of the heme group. Similar synchronized spectrum is obtained for myoglobin (supporting information, Fig. S1) which is a monomeric protein while hemoglobin is a tetrameric hemeprotein. Our focus in this study is not on the tryptophan emission but on the fate of the heme group. In order to understand the temperature influence on fluorescence emission of heme group, we have plotted intensity of 580 nm peak with temperature in Fig. 1b for both hemoglobin as well as myoglobin. The variation in the emission intensity of both proteins is almost identical and it is largely unaffected within the temperature range of 20–50 °C. But it starts increasing thereafter with the increase in the temperature up to 70 °C. An increase in the intensity after 50 °C is due to the unfolding of hemoglobin and myoglobin that breaks the covalent linkages of heme group with protein structure and hence, aqueous exposes the heme group [17–19]. A break in the respective curve indicates the unfolding temperature (Td) of myoglobin and hemoglobin which is 52 and 56 °C, respectively (Table S1). A low unfolding temperature can be attributed to the single polypeptide chain of myoglobin in comparison to tetrametic polypeptide structure of hemoglobin.

3.2. Monomeric surfactants

When the same measurements are carried out in the presence of DTAB, TTAB, and HTAB, a dramatic change in the heme behavior is observed. The representative fluorescence spectra for hemoglobin are shown in Fig. S2. A variation in the fluorescence intensity of heme group of hemoglobin in the presence of HTAB (Fig. S2c) is opposite to that of Fig. 1a, where it decreases with the increase in temperature. The emission intensity versus temperature plots (Fig. 1c) show that Td of hemoglobin reduces from 56 to 51 °C in the presence of DTAB whereas it decreases significantly to 34 °C when TTAB is used. HTAB on the other hand, completely changes this behavior where intensity shows an instant fall with temperature and then becomes almost constant around 26 °C. This trend is clearly governed by the hydrophobicity from C12 (DTAB) to C16 (HTAB). The presence of DTAB increases the solubility of hemoglobin and hence, Td reduces from 56 to 51 °C. But further increase in the hydrophobicity (as of TTAB) facilitates the unfolding of hydrophobic domains that reduces Td dramatically from 51 to 34 °C [20]. However, Td is not observed in the presence of HTAB because it produces micellar solution with five times higher concentration (i.e. [HTAB] = 5 mM) than its cmc_{HTAB} = 1 mM [21–23] which favorably solubilizes protein in the micellar state. Since, heme is highly hydrophobic, therefore, it prefers to associate with a more hydrophobic surfactant. This was not the case with 5 mM of DTAB as well as TTAB with cmc values of 15.3 mM and 3.43 mM, respectively [21–23], because both surfactants were in their pre-micellar range. Thus, increase in the temperature dehydrates the HTAB micelles that entrap heme group and hence, reduces heme fluorescence emission due to the temperature induced fluorescence quenching which converts the radiative decay into non-radiative decay [24].

The above results are further supplemented from the DLS analysis [25,26]. Some of the representative size distribution histograms are shown in Fig. S3. Fig. 2a shows the size variation of aqueous solubilized hemoglobin with respect to temperature. A 100 mg/100 ml aqueous solution of hemoglobin produces a size around 600 nm of its self-aggregated state that reduces significantly with the increase in temperature due to the temperature induced dehydration of globular protein. Around 56 °C, the size reduces to 100 nm and thereafter, it tends to constant with the further increase in temperature. Thus, 56 °C fully

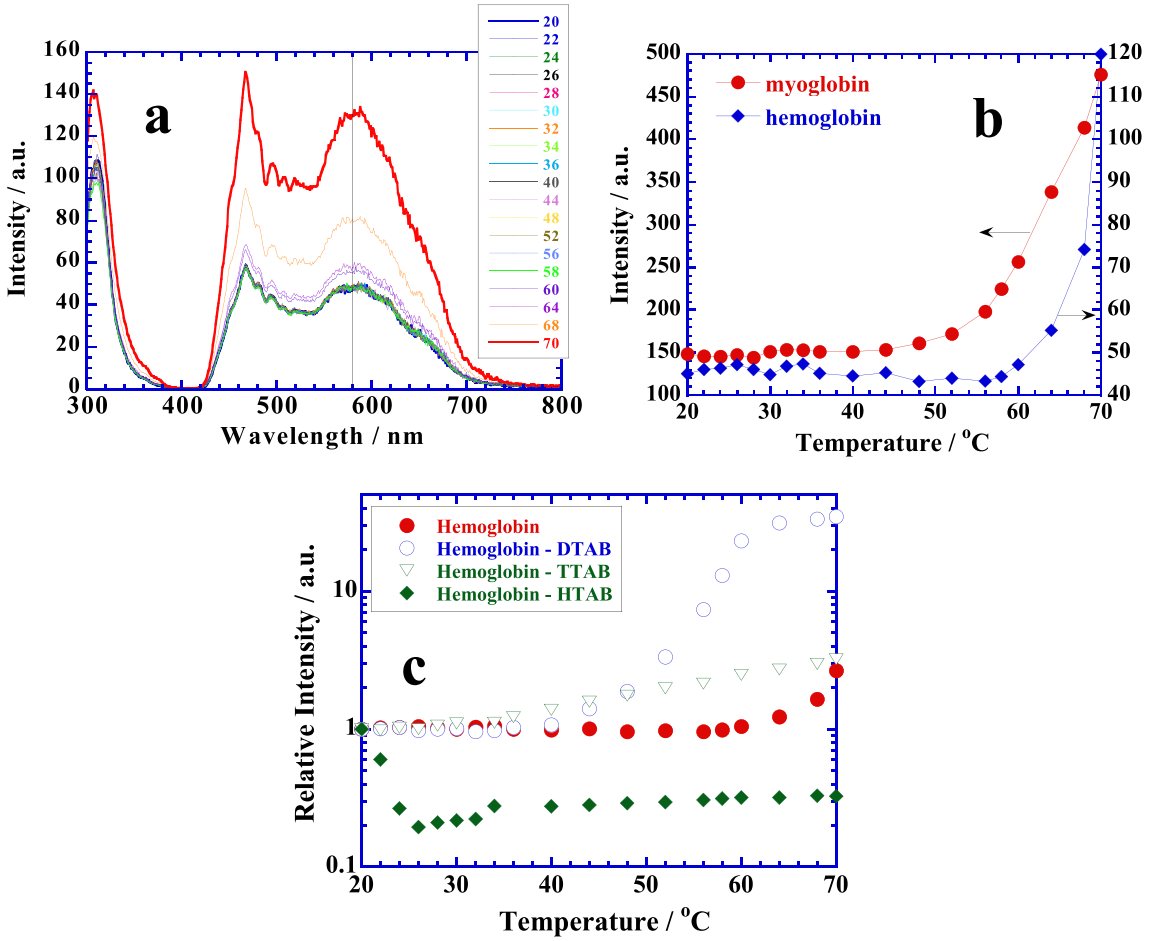

Fig. 1. (a) Fluorescence emission spectra of 100 mg/100 ml aqueous hemoglobin from 20 to 70 °C. (b) Plots of fluorescence intensity of aqueous solutions of hemoglobin and myoglobin with temperature. (c) Plots of relative intensity of hemoglobin in the absence and presence of 5 mM DTAB, TTAB, and HTAB with temperature.

dehydrates hemoglobin aggregates and induces unfolding by exposing heme group to the aqueous phase that results in an instantaneous increase in the fluorescence intensity as depicted in Fig. 1b. However, the unfolding behavior of hemoglobin in the presence of DTAB, TTAB, and HTAB is not clear from the size variation (Fig. 2a). There is an instant fall in the size at Td for DTAB (51 °C) and TTAB (34 °C) (indicated by black arrows) contrary to a constant size variation in their absence. This demonstrates that the hemoglobin – DTAB/TTAB complex is not fully dehydrated at Td which is obviously expected due to the presence of ionic surfactant molecules in the complex [27,28]. Ionic head group in aqueous phase retains its primary hydration sphere that is little influenced by temperature within a range of 20–70 °C. This is also evident from the overall greater size of protein – surfactant complex than that in the absence of surfactant especially after 56 °C (Td of pure protein). All hemoglobin – DTAB/TTAB/HTAB complexes show a bigger size after 56 °C than that of pure hemoglobin and it further depends on the hydrocarbon chain length as discussed in the next section.

3.3. Effect of monomeric head group and chain length on size

In the presence of DTAB, the overall size of hemoglobin remains higher than the control because of the protein – DTAB complexation and its hydration. Ionic surfactant molecules are known to interact with protein through different modes of interactions which include both hydrophilic mainly due to the oppositely charged electrostatic interactions between the polar surfactant head groups and amino acid residues of opposite polarity; as well as due to the hydrophobic interactions between the non-polar hydrocarbon chains and non-polar hydrophobic domains of protein. Hemoglobin interactions with ionic surfactants are predominantly hydrophilic rather than hydrophobic because hemoglobin is predominantly hydrophilic protein. Thus, hydrophilic interactions are expected to promote greater hydration that increases the size of protein – surfactant complex. Since all DTAB/TTAB/HTAB are made up of identical tetraalkylammonium head group, therefore, they are expected to induce equal amount of hydration. However, increase in the hydrocarbon chain length in the order of DTAB < TTAB < HTAB increases the hydrophobicity that in turn promotes the hydrophobic interactions which reduce the hydration [29–31]. That is why the overall size of hemoglobin – surfactant complex decreases in the order of DTAB > TTAB > HTAB (Fig. 2a).

Zeta potential (Fig. 2b) is another important parameter that helps in understanding the surface charge of hemoglobin – surfactant complex especially when ionic surfactant is the part of the complex. Zeta potential of pure hemoglobin remains close to zero mV (isoelectric point ~7) with predominantly more low positive values over the temperature range studied [32]. In the presence of DTAB, although the overall value of zeta potential still remains close to zero, it produces more low negative values. For TTAB, the average negative zeta potential lies between 10 and 20 mV, whereas it becomes significantly negative for HTAB. This trend highlights the predominance of the micellar phase from DTAB to HTAB that solubilizes hemoglobin. Since, 5 mM concentration of HTAB produces sufficient amount of micelles ($cmc_{HTAB} = 1$ mM) [21–23] to solubilize hemoglobin, therefore, zeta potential acquires a negative value because cationic micelles (solubilizing protein) are electrostatically neutralized by the negatively charged Br^- counterions in the form of electrical double layer. Furthermore, it is not possible to

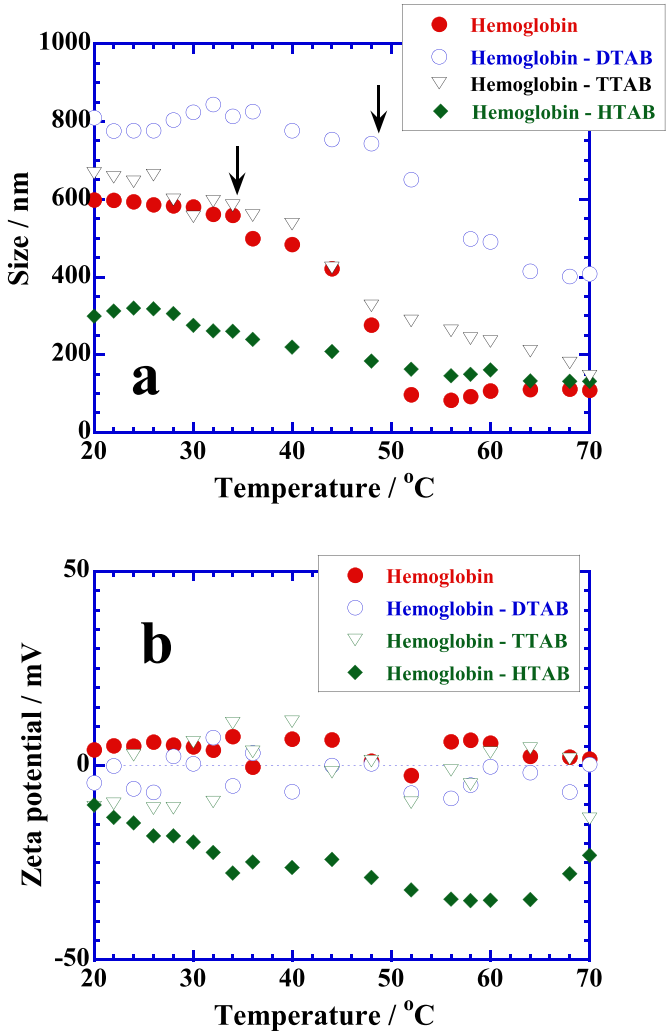

Fig. 2. Plots of size (a) and zeta potential (b) of hemoglobin in the absence and presence of 5 mM DTAB, TTAB, and HTAB with temperature. See details in text.

determine the Td from the variation of zeta potential (Fig. 2b) because we do not observe any significant change in the zeta potential close to Td due to the highly hydrophilic nature of hemoglobin – surfactant complex before and after the unfolding. Thus, both size and zeta potential profiles for hemoglobin in the presence of DTAB/TTAB/HTAB are less sensitive to the protein unfolding but they are quite helpful in determining the solubilization behavior of hemoglobin in aqueous surfactant solution.

3.4. Comparison with myoglobin

The unfolding and solubilization behavior of myoglobin in the presence and absence of DTAB/TTAB/HTAB is depicted from the variation of fluorescence intensity of heme group of myoglobin with temperature in Fig. S4. Because of the presence of single polypeptide chain in myoglobin, its fluorescence behavior is quite different from that of hemoglobin in the presence of surfactants [33,34]. In the presence of DTAB, the unfolding of myoglobin is almost similar to that in the absence of DTAB which is in contrast to that of hemoglobin (Fig. 1c). Single polypeptide chain of myoglobin makes it more hydrophilic in comparison to hemoglobin because of the reduced amount of hydrophobic domains. However, solubilization significantly increases as hydrophobicity increases in the presence of TTAB and hence, Td significantly reduces to 26 °C. On the other hand, no unfolding process is observed in the presence of HTAB because of much enhanced solubilization of myoglobin that causes a continuous increase in the fluorescence intensity with temperature due to a regular exposure of heme group to the aqueous phase with temperature.

3.5. Gemini surfactants

3.5.1. Methylene spacer

Some representative fluorescence spectra of hemoglobin in the presence of different Gemini surfactants with spacer effect are shown in Fig. S5, and the plots of normalized fluorescence intensity of heme group versus temperature are presented in Fig. 3a. In each case, the fluorescence intensity either slightly decreases or remains constant initially with the temperature and then shows an instant increase where unfolding of hemoglobin aqueous exposes the heme group. This temperature has been taken as Td and it clearly depends on the number of methylene groups in the Gemini spacer. Td decreases with the increase in the spacer length from 3 to 6 (Table S1). A Gemini surfactant is expected to have much stronger interactions with hemoglobin because of its dimeric head group as well as double hydrocarbon tails. A dimeric cationic head group interacts much more strongly through hydrophilic interactions in comparison to the monomeric cationic head group (discussed in the previous section) [35,36]. Similarly, double hydrocarbon chains can effectively penetrate into the hydrophobic domains of hemoglobin in comparison to single hydrocarbon chain of a monomeric surfactant. However, despite the presence of stronger interactions of Gemini surfactants (i.e. 16-3-16 to 16-6-16) with hemoglobin, none shows any similarity with the fluorescence behavior shown by hemoglobin in the presence of HTAB (C16) with identical hydrocarbon chain length (Fig. 1b). This is all because of the presence of spacer group in Gemini surfactant in comparison to HTAB. The presence of a spacer group produces less compact Gemini micelles with low aggregation number in comparison to those produced by HTAB [37,38]. The solubilization capacity of the micellar phase depends on the hydrophobicity in the interior of the micelle. The presence of a spacer group reduces the compactness of the micelle due to the steric strains caused by the spacer [39,40] and hence, the micelles produced by the Gemini surfactants usually of low aggregation number with low solubilization capacity. However, hydrophobicity of a Gemini surfactant increases with the increase in the spacer length and that in turn facilitates the solubilization of hemoglobin bringing Td to a lower temperature as depicted in Fig. 3a (Table S1).

As observed previously, both size (Fig. 3b) and zeta potential (Fig. 3c) variations are not sensitive to the unfolding of hemoglobin in the presence of Gemini surfactants. Although the size of hemoglobin – Gemini complex decreases with the increase in the spacer, it is more clear at low temperature rather than at high temperature. This variation is in accordance with the increased hydrophobicity with spacer length that facilitates the solubilization of hemoglobin in Gemini micelles thereby reducing the size [41]. However, high temperature reduces this effect due to the dehydration of micelles and hence, it is not clear in the higher temperature range. On the other hand, zeta potential variation is more prominent and spacer dependent in the higher temperature range. As elevated temperature induces the micelle dehydration, more Br^- counterions prefer to adsorb on the micelle – solution interface created by cationic Gemini surfactant head groups. The micelle dehydration is obviously much more rapid and significant in the case of more hydrophobic micelles produced by 16-6-16 rather than 16-3-16, and that in turn allows more Br^- counterions to adsorb at the interface making larger negative zeta potential (Fig. 3c).

3.5.2. Thio spacer

The purpose of choosing thio spacer is to differentiate between an entirely non-polar methylene spacer ($-CH_2-CH_2-$) and partially polar thio spacer ($-S-CH_2-CH_2-S-$). The presence of thio spacer is expected to involve in the electron exchange with heme group, thereby

affecting the fluorescence behavior of heme group due to the quenching effect. The fluorescence spectra of hemoglobin in the presence of 12-S-2-S-12/14-S-2-S-14/16-S-2-S-16 are shown in Fig. S6. But they are not much different from that in the presence of Gemini surfactants with only methylene spacer (Fig. S5). It indicates that thio spacer is not directly interacting with the heme group and the overall change in the fluorescence is almost similar to the one shown by the methylene spacer. Here, the Td decreases (Table S1) with the increase in the length of double hydrocarbon chains from 12-S-2-S-12 to 16-S-2-S-16 due to the increase in the hydrophobicity of Gemini surfactant that facilitates the hemoglobin solubilization (Fig. S7).

Fig. S8 demonstrates the variation of size of hemoglobin – surfactant complex containing thio spacer with temperature. The overall size increases with the increase in double hydrocarbon chain length. This is in contrast to the variation depicted in Fig. 2a where the overall size decreases with the increase in the length of hydrocarbon tail of monomeric surfactant. The increase in the size with the increase in the length of double hydrocarbon chain is obviously understood from the bulky molecular structure of Gemini surfactant in comparison to that of the monomeric surfactant. A dimeric Gemini head group is instrumental in accommodating higher number of water molecules [42,43] than the head group of monomeric surfactant and hence, contributes towards the increase in the size. Likewise, bulkiness of the longer and double hydrocarbon chains adds to an increase in the size. Thus, both dimeric head groups as well as double hydrocarbon chains are responsible for an overall increase in the size of hemoglobin – Gemini surfactant complex with thio spacer. However, temperature effect decreases the size due to the onset of dehydration as observed previously in Fig. 2a. The variation in the zeta potential (Fig. S8) is similar to that of Fig. 2b where increase in the length of hydrocarbon tails produces more negative zeta potential.

3.5.3. Comparison with myoglobin

Because of the single polypeptide chain in myoglobin, it demonstrates greater solubility in the micellar phase of Gemini surfactants, hence, it eludes the unfolding behavior the way it is observed in Fig. 3a. Fig. 4a shows the variation in the myoglobin fluorescence emission in the presence of different Gemini surfactants of methylene and thio spacers. In all cases, the fluorescence intensity falls which indicates the fact that heme group of myoglobin is already aqueous exposed in Gemini surfactant solution, or in other words, it is already in the unfolded state contrary to the fact depicted in Fig. 3a for hemoglobin. Fig. 4 depicts the concentration effect of 14-S-2-S-14 on the unfolding behavior of myoglobin. At low concentration of 14-S-2-S-14 = 0.25 mM, there is not much difference from that of pure myoglobin, but at high concentration (0.75 mM), no unfolding behavior is observed because of the presence of micellar phase that facilitates the solubilization of myoglobin and aqueous exposes the heme group.

Because of the greater aqueous solubilization, myoglobin – Gemini surfactant complex acquires predominantly a greater size in comparison to pure myoglobin (Fig. S9). However, the temperature induces dehydration and reduces size as observed for hemoglobin in Fig. 3b. Zeta potential of myoglobin is positive (Fig. S9) but its solubilized state in aqueous Gemini surfactant produces low negative zeta potential that depends on the hydrophobicity as observed in Fig. 3c and Fig. S8 for hemoglobin. Longer spacer and longer hydrocarbon double chains induce greater hydrophobicity that in turn produces compact micelles with

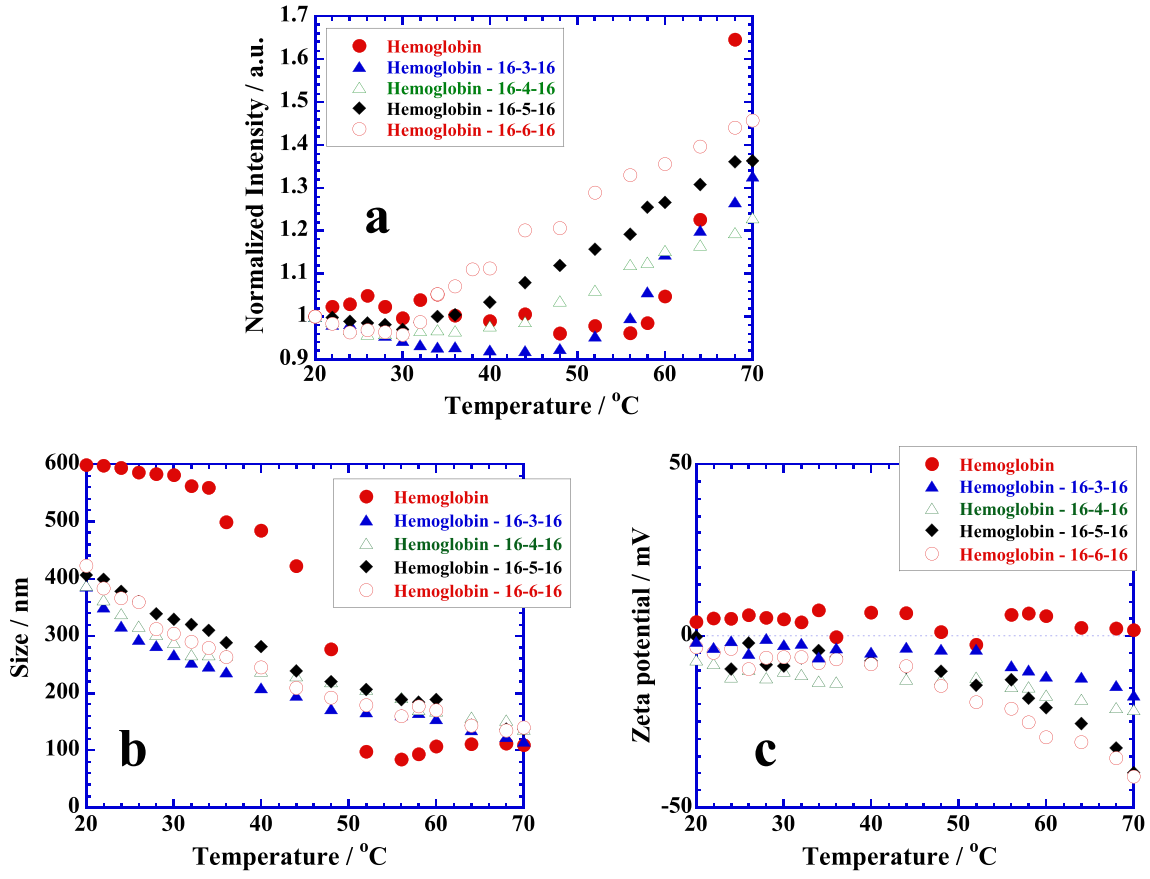

Fig. 3. Plots of relative intensity (a), size (b) and zeta potential (c) of hemoglobin in the absence and presence of 1 mM 16-3-16, 16-4-16, 16-5-16, and 16-6-16 Gemini surfactants with temperature. See details in text.

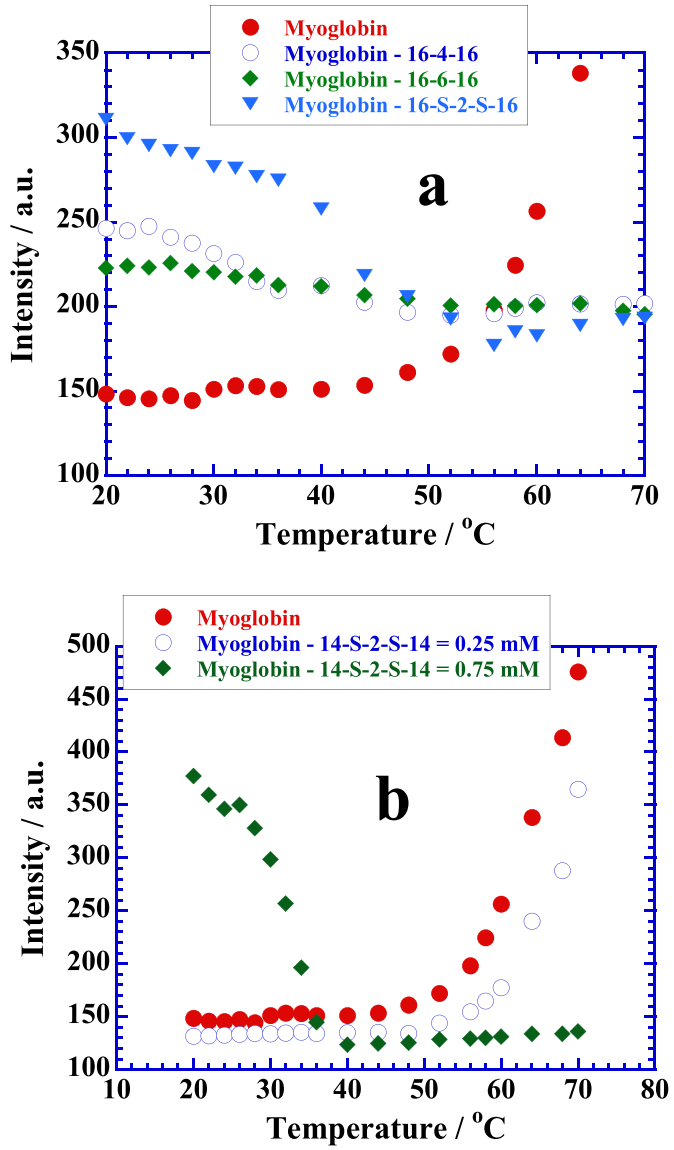

Fig. 4. (a) Plots of fluorescence intensity of myoglobin in the absence and presence of 1 mM 16-4-16, 16-6-16, and 16-S-2-S-16 Gemini surfactants with temperature. (b) Plots of fluorescence intensity of myoglobin in the absence and presence of 0.25 and 0.75 mM 14-S-2-S-14 Gemini surfactants with temperature. See details in text.

greater probability of surface adsorption of Br^- counterions producing higher negative zeta potential values.

3.6. Concentration effect

In order to understand that how Gemini surfactants influence the unfolding and solubilization of both hemoglobin and myoglobin proteins, fluorescence titrations are performed over an extended concentration range covering pre- to post micellar regions at 25 °C and 70 °C. At 25 °C, protein is considered to be in its globular form while at 70 °C, it is in its unfolded state. For this purpose, two surfactants have been chosen. One is the HTAB monomeric and other one is 16-6-16 Gemini surfactant, and the results are presented in Fig. 5. Fig. 5a shows the emission spectrum of heme at 25 °C for hemoglobin – 16-6-16 system. The tryptophan emission is stronger than the Q1-band of porphyrin functional group of heme which significantly depends on the concentration of 16-6-16. A porphyrin coordinates to metal using four nitrogens as electron-pair donors to iron in heme while the fifth position is coordinated to nitrogen of histidine residue. The sixth coordination site is occupied by O_2 in oxygenated form. As heme is highly hydrophobic, therefore, it is associated with predominantly hydrophobic domains of protein. The increase in concentration of a surfactant allows it to interact with the hydrophobic domains that partially unfold the protein and aqueous expose the porphyrin. On the other hand, when the same experiment is conducted at 70 °C (Fig. 5b), the emission intensity of whole porphyrin (Q1 – 4 bands) [44] becomes much more prominent in comparison to that of tryptophan because now heme is already aqueous exposed due to temperature induced unfolding. Thus, in the globular form (i.e. at 25 °C), heme is deep embedded in the hydrophobic domains and hence, is less aqueous exposed in comparison to tryptophan, whereas at 70 °C hemoglobin is largely in the unfolded state which allows maximum heme emission. Similar emission spectra of hemoglobin – HTAB system are shown in Fig. S10. Fig. 5c shows the variation in the emission intensity of heme group with concentration of surfactant. There is a large difference between the additive behavior of HTAB and 16-6-16 on the emission intensity. The emission intensity of heme in both globular (at 25 °C) and unfolded states (at 70 °C) continuously increases with the increase in concentration of HTAB whereas it passes through a strong maximum at low 16-6-16 concentration. This distinct difference in the fluorescence behavior of heme group is entirely related to both strong hydrophilic (head group effect) as well as hydrophobic (spacer and double hydrocarbon chain effect) interactions of 16-6-16 with hemoglobin in comparison to that of monomeric HTAB. Thus, both stronger hydrophilic and hydrophobic interactions of 16-6-16 even in the pre-micellar concentration range (cmc = 0.1 mM) instantaneously unfolds hemoglobin thereby aqueous exposes heme group which causes an instant increase in its fluorescence emission. The emission intensity falls thereafter because unfolded hydrophobic domains prefer to solubilize in the micellar phase [45] of 16-6-16 created after 0.1 mM of its concentration. That converts the radiative decay into non-radiative decay and hence, quenches the fluorescence emission of heme. Whereas this process is gradual and concentration dependent when instead of 16-6-16, HTAB is used because of its monomeric nature. In addition, the high cmc of HATB (1 mM) [22,31] in comparison to that of 16-6-16 (0.1 mM) does not allow the formation of HTAB micelles that could solubilize hydrophobic domains of hemoglobin.

In contrast, no distinct behavior between the additive effect of HTAB and 16-6-16 is observed on the emission intensity of myoglobin that obviously stems from the presence of much weaker hydrophobic domains in myoglobin in comparison to that of hemoglobin. Thus, the hydrophobic effects which drive the difference between the additive effects of HTAB and 16-6-16 are not observed as depicted by Fig. 5c. Rather, the additive effect is much more pronounced for HTAB than 16-6-16 (Fig. 5d) because it is now predominantly driven by the hydrophilic interactions as observed previously in Fig. S4. Although 16-6-16 contains dimeric head group which is expected to have much stronger hydrophilic interactions with myoglobin, the presence of a long spacer of six methylene group substantially reduces this effect and hence, the emission intensity of heme in myoglobin becomes much stronger than that in the presence of 16-6-16. It instantaneously increases in aqueous HTAB due to stronger hydrophilic interactions that aqueous expose the heme group to a greater extent than induced by 16-6-16. Thereafter, intermolecular self-quenching among the heme groups or between the heme and surfactant molecules reduces the emission. Thus, it is clear that Gemini surfactants have stronger interactions with bigger globular protein like hemoglobin rather than a smaller one like myoglobin. The origin of such interactions stems from the bigger hydrophobic domains which preferentially interact with the hydrophobic functional groups such as double hydrocarbon chains and longer spacer group. Smaller protein like myoglobin lacks appropriate hydrophobic domains that in turn allow the monomeric surfactant like HTAB to have stronger hydrophilic interactions in comparison to homologous Gemini surfactant (i.e. 16-6-16).

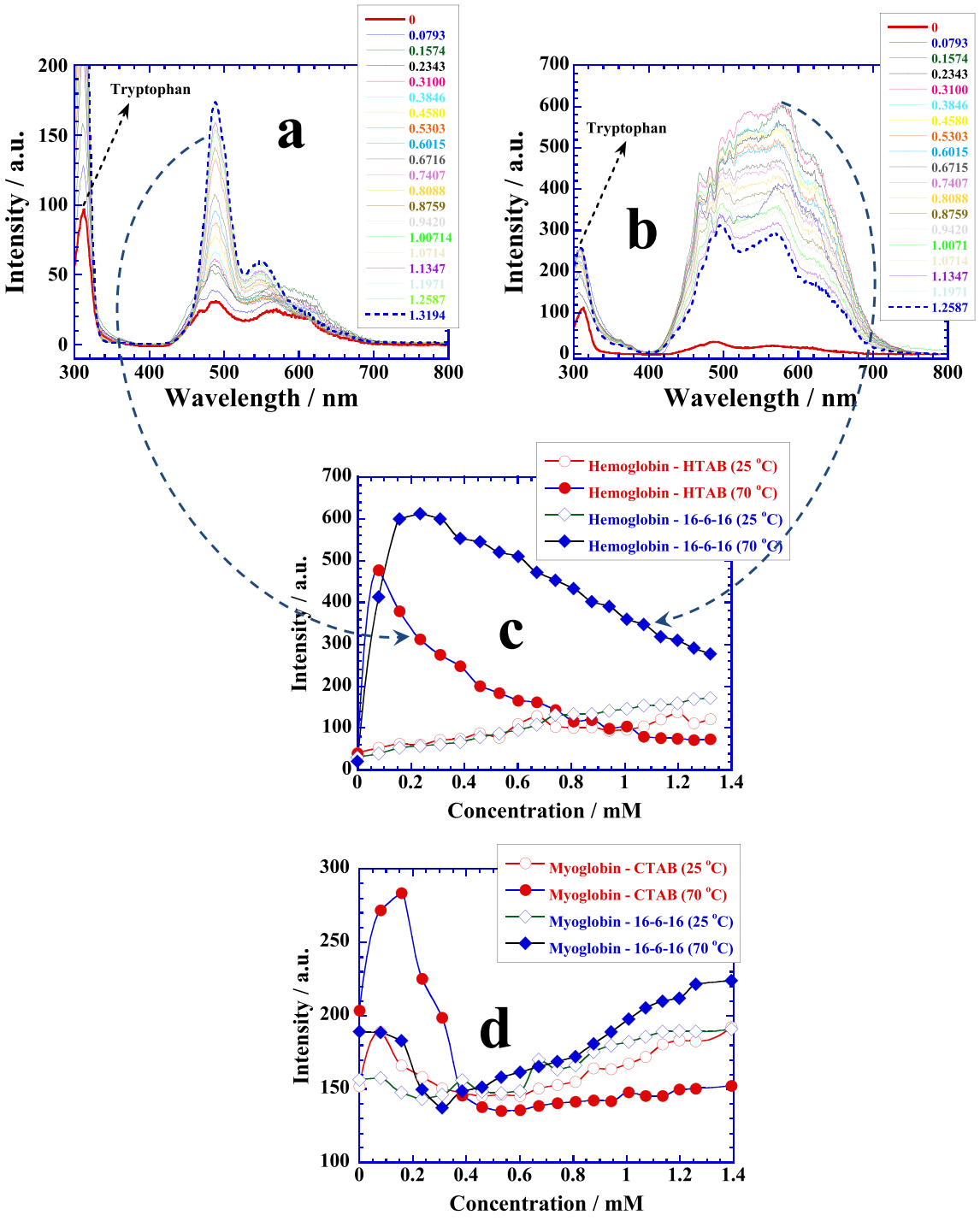

Fig. 5. Fluorescence spectra of hemoglobin at varying concentrations of 16-6-16 at 25 °C (a) and at 70 °C (b). Plots of intensity versus concentration of HTAB and 16-6-16 for hemoglobin (c) and myoglobin (d). See details in text.

4. Concluding remarks

The above results conclude that the fluorescence emission of the heme group is significantly affected by the molecular architecture of Gemini surfactants which in turn unfold the water soluble globular protein and aqueous expose the heme group (Fig. 6). Since surfactant molecules exist in both monomeric and micellar phases, therefore, critical micelle concentration also plays a significant role in the protein – surfactant interactions where most of the surfactant molecules exist in the micellar phase [46,47]. The 1 mM concentration of Gemini surfactants allows them to exist in the micellar phase. The unfolding of protein depends on both hydrophilic as well as hydrophobic interactions with surfactants but specifically depends on the magnitude of the hydrophobic domains when protein interacts with Gemini surfactant molecules. A bigger hydrophobic domain as of hemoglobin (Fig. 6a) invites Gemini surfactant molecules that can undergo favourable hydrophobic interactions while the hydrophobic interactions of smaller hydrophobic domain as of myoglobin are screened by the predominantly hydrophilic interactions (Fig. 6b). This happens in a much distinct manner between hemoglobin and myoglobin due to four and single polypeptide units, respectively. Thus, the unfolding of hemoglobin depends on both the length of double hydrocarbon chains as well as the spacer length of

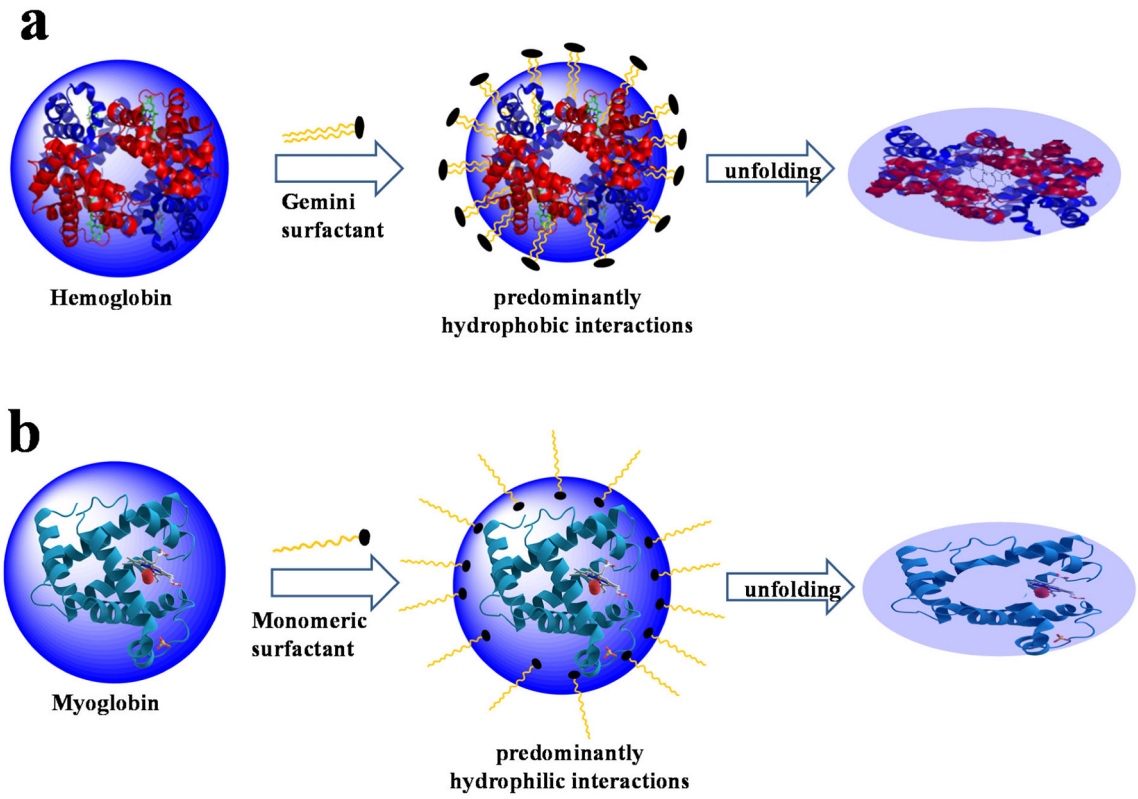

Fig. 6. (a) Schematic representation of the predominant hydrophobic interactions between hemoglobin and Gemini surfactants inducing unfolding in hemoglobin. (b) Schematic representation of the predominant hydrophilic interactions between myoglobin and monomeric surfactants inducing unfolding in myoglobin.

Gemini surfactants. Both functionalities prove to be instrumental in facilitating the unfolding and hence, aqueous expose the heme group that enhances the fluorescence emission of heme group.

Author statement

Gurinder Kaur Ahluwalia: Methodology, Investigation. Lavanya Tandon: Validation, Formal analysis. Pankaj Thakur: Visualization, Investigation. Poonam Khullar: Supervision. Kultar Singh: Software, Investigation. Mandeep Singh Bakshi: Conceptualization, Writing-Reviewing and Editing,

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Appendix A. Supplementary data

Fluorescence spectra, DLS size histograms, and zeta potential profiles. This information is available free of charge via the internet. https://doi.org/10.1016/j.ijbiomac.2020.04.008.

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