J Fluoresc (2011) 21:365–374
DOI 10.1007/s10895-010-0725-y

ORIGINAL PAPER

# Fluorescent Probing of Protein Bovine Serum Albumin Stability and Denaturation Using Polarity Sensitive Spectral Response of a Charge Transfer Probe

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Received: 26 April 2010 / Accepted: 8 September 2010 / Published online: 5 October 2010


**Abstract** The polarity sensitive photo-induced intramolecular charge transfer (ICT) fluorescence probe (E)-3-(4-methylamino-phenyl)-acrylic acid ethyl ester (MAPAEE) has been used to study the model protein Bovine Serum Albumin (BSA) in its native and thermal and urea induced denatured states. The interaction between BSA and the regular surfactant Sodium Dodecyl Sulphate (SDS) as well as the biologically relevant steroid-based amphiphile Sodium Deoxycholate (NaDC) has also been very keenly followed using this ICT probe. The variation of micellar properties of both SDS and NaDC with increasing ionic strengths and in presence of the chaotrope urea has also been well documemted by the same probe. Steady-state spectroscopy, FRET, and fluorescence anisotropy measurements have been used to gain better insight into these processes and the molecule MAPAEE to be a full-bodied fluorescent probe for studying such intricate biological systems, their properties and interactions.

**Keywords** Fluorescence probe · (E)-3-(4-methylamino-phenyl)-acrylic acid ethyl ester · Sodium Dodecyl Sulphate · Sodium Deoxycholate · FRET · Anisotropy

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## Introduction

Serum albumins are major transport proteins found in plasma and have been extensively studied for years [1–5]. These are involved in binding and carriage of an array of biologically important compounds like fatty acids, bilirubin, bile salts, and lecithin. However major interest in these albumins is a result of their strong drug-binding capacities and a large volume of literature is available on this. Bovine serum albumin (BSA) has 583 amino acid residues arranged in a single polypeptide chain and 17 disulphide bridges leading to a series of nine loops. This gives the protein a roughly heart-shaped structure. The protein has three domains I, II and III each with two subdomains IA, IB, IIA, IIB, IIIA and IIIB. BSA has two tryptophan residues Trp-134 and Trp-212. While Trp-212 is more exposed, Trp-134 resides deep inside a hydrophobic pocket. Serum albumins have served as model proteins for a large variety of bio-chemical and bio-physical studies and continue to garner interest owing to their easy handling, drug-binding capacities and other standard model properties.

Surfactants bind strongly to proteins leading to major conformational changes in the protein and these surfactant induced uncoiling of proteins are widely studied owing to the structural information that can amassed from them. Interactions of the model protein BSA with various cationic, anionic and neutral surfactants have been reported and a multitude of models proposed to explain some of these binding processes [6–11]. The binding isotherms for these surfactants-protein systems were constructed from the various studies carried out and show distinct regions of binding.

Surfactants and their micellar systems also find diverse applications owing mainly to their biological and membrane-mimetic nature and study of the micellisation

phenomenon of diverse amphiphiles form a vast area of research today. Serum albumins as mentioned before bind to an array of biologically active substances and among them bile salts have amphiphillic properties very similar to surfactants. Their interaction with serum albumins hence has immense biological significance and their binding isotherms show regions similar to those of the other reported surfactant-BSA systems [10, 12]. Bile salts [13–15] are biological amphiphiles biosynthesized in the liver and form mixed micelles with fats, lipids and monoglycerides thereby playing one of the most crucial roles in fat digestion. They are also majorly involved in the absorption of fat soluble substances like vitamins and certain drugs and in cholesterol solubilzation. The peculiar facial amphiphilicity of the bile salt molecules gives rise to micelles very different in shape, structure, rigidity and properties from the conventional surfactants with polar head groups and hydrophobic tails. Primary aggregates are formed at low bile salt concentrations and at higher concentrations secondary aggregates form by hydrogen-bonding of these primary aggregates.

Fluorescence probe techniques to study biologically important systems are rapidly gaining popularity in the study of biological microheterogenous systems due to their non-invasive nature and the large extent of information gleaned thereby [16, 17]. Probes that show massive spectral changes on being tagged to such systems are being designed and developed and their spectral responses are then exploited to gain information about the structure and dynamics of the system under study. In this context probes exhibiting proton transfer, charge or electron transfer are much in demand. Probes like ANS [18–22] show polarity dependant spectral characteristics and are being used widely as fluorescent labels for studying proteins, vesicles, micelles, mixed micelles and similar biological or biomimetic micro-heterogeneous systems.

We have earlier reported on the dual fluorescence exhibited by (E)-3-(4-methylamino-phenyl)-acrylic acid ethyl ester (MAPAEE) (Scheme 1) in polar solvents due to an excited state intra-molecular charge transfer from the donor secondary amine group to the acceptor ester group [23]. The red-shifted ICT emission band was sensitive to the polarity of the solvents owing to the polar nature of the corresponding excited charge transfer state. Greater stabilization of this CT state in more polar solvents produced the observed red shift of this ICT band maximum in more polar solvents and this sensitivity has been employed to study the model protein BSA and its chemical denaturation with urea. Two amphiphillic micellar systems Sodium Dodecyl Sulphate and the biologically relevant bile salt Sodium Deoxycholate were studied and the effect of increased ionic strengths and the presence of chaotrope urea on the Critical Micellar Concentration (CMC) of these systems were determined fluorimetrically again using this ICT probe MAPAEE. Their interactions with BSA were also studied employing the same probe and correlated with the already established binding isotherms. The results were found to be in excellent agreement with the established binding curves of BSA to SDS and BSA to NaDC respectively. This study, in the end points to the possible large spectrum of applicability of the polarity sensitive ICT exhibiting molecule MAPAEE as sensitive probe for studying micro-heterogeneous systems of relevance like proteins, micelles and bile salt aggregates.

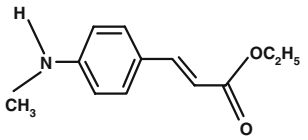
## Experimental Section

The synthesis of the charge transfer probe MAPAEE was described elsewhere [17]. BSA from SRL, India and Sodium Deoxycholate (NaDC) from Merck, India were used as received. All NaDC, protein and protein-surfactant solutions were prepared in 0.01 m Tris-HCl buffer maintained to pH 7.5 by using 1:1 HCl. Triply distilled water was used for preparing all solutions. For the studies on the interactions with the micellar system all solution were prepared in triply distilled, de-ionised water to rule out any effect of ionic strength on the micellization process. Once prepared, probe–protein, probe–protein–urea and probe–protein–SDS solutions were kept undisturbed overnight to allow complete equilibration. Probe-NaDC and probe-protein-NaDC solutions were however equilibrated for 3 hours and measurements were taken immediately to avoid the problem of aging.

All steady state absorption spectra were recorded on a Hitachi UV/VIS U-3501 spectrophotometer and emission spectra were recorded on a Perkin Elmer LS50B fluorimeter. Steady state anisotropies were measured on Perkin Elmer LS55 fluorimeter. Concentration of the CT fluorescence probe was kept at $<10^{-7}$M for all measurements to ensure no occurrence of self-aggregation and/or quenching and also to maintain probe concentrations at a much lower value than the protein concentrations in all studies.

Steady state anisotropy (r) was defined by the following relation [24]

$$r = (I_{VV} - G.I_{VH})/(I_{VV} + 2.G.I_{VH}) \quad (1)$$




**Scheme 1** Structure of fluorescence probe (E)-3-(4-Metylamino-phenyl)-acrylic acid ethyl ester

where $I_{VV}$ is the fluorescence intensity when both the excitation and emission polarizers are oriented vertically and $I_{VH}$ is the fluorescence intensity when the excitation polarizer is vertically oriented and the emission polarizer is horizontally oriented. The factor G is defined as

$$G = I_{HV}/I_{HH} \quad (2)$$

$I_{HV}$ is the fluorescence intensity with the excitation polarizer horizontally and the emission polarizer vertically oriented and $I_{HH}$ is the fluorescence intensity with both the excitation and emission polarizers oriented horizontally.

The time resolved fluorescence measurements have been done with a time correlated single photon counting (TCSPC) set up [25]. Excitation at 350 nm was achieved by using a picosecond diode laser (IBH Nanoled-07) in an IBH Fluorocube apparatus. A Hamamatsu MCP photomultiplier tube (5000U-09) collected the emission at a magic angle polarisation. The TCSPC set up consists of an Ortec 9327 CFD and a Tennelec TC 863TAC. Data collection was done with a PCA3 card (Oxford). An IBM DAS6 software was used to deconvolute the fluorescence decays. The relative contribution of each component was obtained from a triexponential fitting finally was expressed by the following equation [26]:

$$a_n = \frac{B_n}{\sum_{i=1}^{N} B_i} \quad (3)$$

$B_i$ being the pre-exponential factor. The mean fluorescence lifetimes for the decay curves were calculated from the decay times and the relative contribution of the components using the following equation

$$\langle \tau_f \rangle = \frac{\sum a_i \tau_i^2}{\sum a_i \tau_i} \quad (4)$$

## Results and Discussions

### Steady-State Spectral Characteristics of MAPAEE

The detailed spectral properties of probe MAPAEE have been described elsewhere [23]. The absorption spectra of MAPAEE in different solvents (Fig. 1a) comprised of a strong band at ~350 nm with a less intense shoulder at ~318 nm. The strong band was assigned to the $\pi \rightarrow \pi^*$ transition of the benzene chromophore of MAPAEE. This band was almost independent of solvent polarity and all throughout this study the probe has been excited at this band maximum, i.e. 350 nm.

The emission spectral characteristics of MAPAEE are however very sensitive to the solvent polarities. As seen in Fig. 1b, in polar solvents, dual fluorescence is observed due to photoinduced charge transfer (CT) process. A solvent polarity independent band of low intensity is observed at lower wavelengths with a maximum at ~385 nm in all solvents and this is assigned to the emission from the locally excited (LE) state. The red-shifted band has much higher intensity and in all probability arises from the polar CT state. This CT maximum is at 435 nm in acetonitrile, but in water it is red-shifted to 467 nm. In fact, the emission band maxima of MAPAEE are found to be more red-shifted in higher polarity solvents which has been explained on the lines of the generation of a polar emissive species, i.e. the CT state and its' greater stabilization in more polar environments.

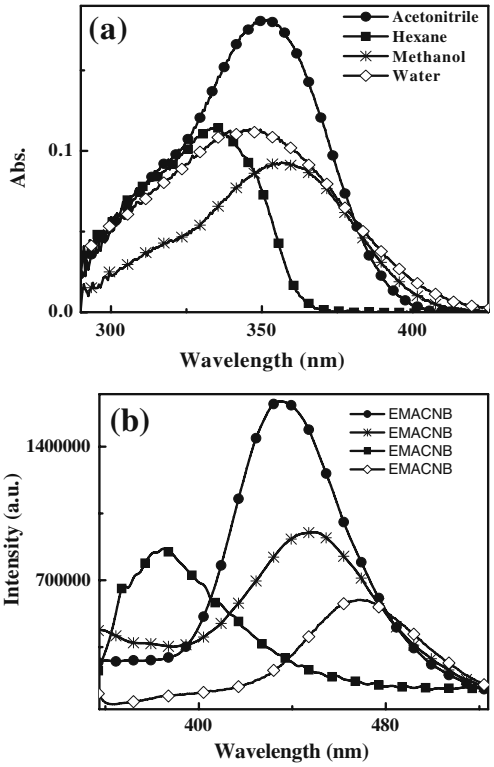



**Fig. 1** **a** Absorption spectra and **b** emission spectra ($\lambda_{ext}$=350 nm) of MAPAEE in different solvents

Introduction of MAPAEE into higher concentrations of BSA in aqueous buffer solutions is found to hardly influence the absorption characteristics of the probe. However, a pronounced blue-shift of the CT emission maximum with a simultaneous increase in emission intensity is observed on introducing MAPAEE into progressively higher concentrations of BSA (Fig. 2a). The movement of MAPAEE from the polar aqueous buffer environment to the much less polar, hydrophobic protein environment on binding to BSA may be the reason behind this sudden lowering of micropolarity leading to the observed blue-shift. It was earlier seen that non-radiative

decay channels were operative in strong hydrogen bonding solvents like water which depleted the quantum yield of MAPAEE in protic solvents. Movement and subsequent residence of the probe in the hydrophobic protein environment deactivates these channels and hence fluorescence intensity of the probe increases on binding to BSA.

The well known Benesi Hildebrand (BH) plot [27] for 1:1 complexation reaction between the probe and BSA can be expressed in terms of fluorescence intensity by the following equation.

$$\frac{1}{(I-I_o)} = \frac{1}{(I_1-I_0)} + \frac{1}{K(I_1-I_0)[BSA]} \quad (3)$$

where $I_0$, I and $I_1$ are the emission intensities of MAPAME in the absence, at intermediate and at infinite concentration of protein BSA, respectively, The term *K* is the binding constant of the complex. The BH plot for Eq. 3 is given in Fig. 2b. This plot was found to be linear through the entire concentration range of BSA and it is clear from this linearity that only 1:1 association has taken place between the probe MAPAEE and the protein BSA. The binding constant *K* and the associated free energy change, ΔG, for this binding process were then calculated to be $0.818\times 10^{-4}$mol$^{-1}$ and −22.32 kJ mol$^{-1}$ respectively. This indicates strong binding between the two partners and the process is spontaneous.

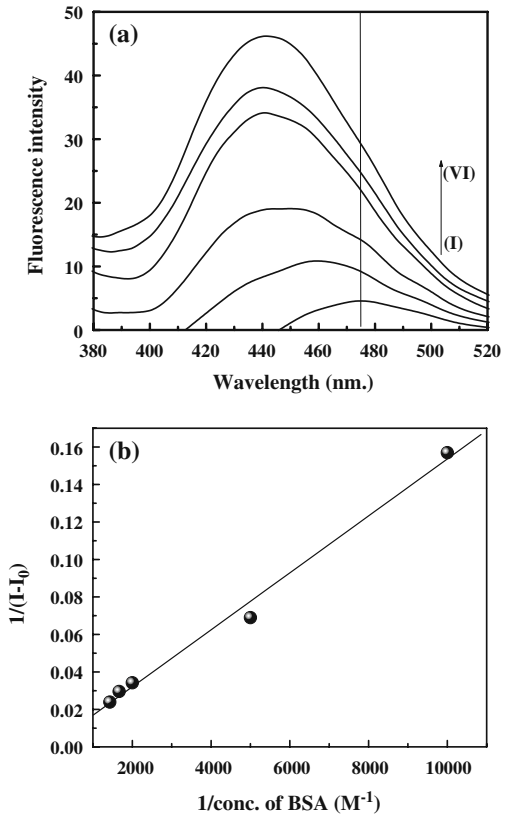



**Fig. 2** **a** Effect of binding with BSA on the emission spectrum ($\lambda_{ext}$= 350 nm) of MAPAEE {(I): 0 μM,(II):10 μM, (III): 20 μM , (IV):50 μM, (V): 60 μM and (VI):70 μM BSA in tris-HCL buffer (pH=7.0)}; **b** Benesi-Hildebrand plot for 1:1 complexation between MAPAEE and BSA following equation 3

Comparison of the spectral properties of polarity sensitive probes residing in a micro heterogeneous system to those in media of known polarities helps to determine the polarity of the immediate microenvironment of the probe in the medium under study. We used the Reichardt $E_T(30)$ scale in this context to construct the calibration curve for micropolarity determination [28]. The ICT emission position of MAPAEE in various dioxane-water mixtures was plotted against their corresponding known $E_T(30)$ values [29–31]. Then position of the ICT emission of MAPAEE bound to BSA was interpolated onto this curve. As seen in Fig. 3a, the microplarity of the probe's environment in the protein bound state was calculated to be 46.7. This value lies in between the $E_T(30)$ values of pure acetonitrile (45.6) and pure isopropanol (48.4) and is not as low as the alkanes.

A substantial overlap between the absorption spectrum of MAPAEE and the emission spectrum of the tryptophan residue of BSA makes Fluorescence Resonant Energy Transfer (FRET) from Trp of BSA to the bound probe possible. For FRET to take place besides this spectral overlap there should also be proper relative orientations of the donor and acceptor transition dipoles and optimum distance must be maintained between the donor and acceptor counterparts. This FRET process is shown in Fig. 3b. We find that the emission obtained on exciting the protein at the Trp absorption maximum is gradually quenched and a new band generates at the position of the BSA bound probe on addition of MAPAEE. It is worthwhile to note here that MAPAEE has absolutely no absorption at 280 nm so the emission obtained for it can generated only if FRET takes place from tryptophan residue to it. The fluorescence resonant energy transfer efficiency can be calculated by the following equation [24]

$$E = 1 - \frac{F}{F_0} = \frac{{R_0}^6}{{r_0}^6 + {R_0}^6} \quad (4)$$

where E = Efficiency of energy transfer, F = Fluorescence intensity of MAPAME when bound to BSA and $F_0$ = Fluorescence intensity of MAPAME in absence of BSA, $R_0$ is the critical distance at which the extent of energy transfer is 50%, The energy transfer efficiency (E) for FRET between donor Trp of BSA and acceptor MAPAME has been calculated to be 93.5%. The value for $R_0$ could be calculated using the following equation

$$R_0 = 8.74 \times 10^{-25} K^2 n^{-4} \Phi J \quad (5)$$

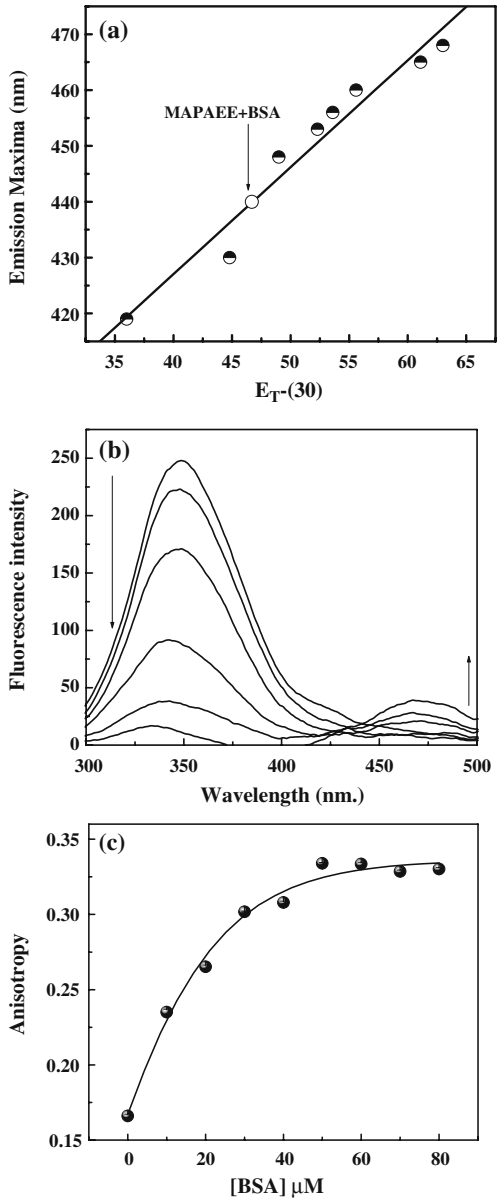



**Fig. 3** **a** Calibration curve for determination of the polarity of the microenvironment of MAPAEE in the BSA bound state; **b** Fluorescence Resonant Energy Transfer (FRET) from Tryptophan of BSA to the bound MAPAEE (*arrow* indicates increasing amounts of the probe) and **c** Change in fluorescence anisotropy of MAPAEE with increasing BSA concentrations

where $K^2$ is the spatial orientation factor, $n$ is the refractive index of the medium, and Φ is the fluorescence quantum yield of the donor (i.e. Tryptophan) and $J$ is the overlap integral between donor emission and acceptor absorption which is defined by the following equation

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}{\int_0^\infty F(\lambda)d\lambda} \quad (6)$$

Here F(λ) is the fluorescence of the donor in the wavelength range λ and λ + dλ and ε(λ) is the molar extinction coefficient of the acceptor at the wavelength λ. Using this equation the value of $J$ was calculated and then the values for $R_0$ and $r_0$ were calculated to be 2.23 nm and 1.98 nm, respectively. These values lie within the range described by many workers to be optimal for FRET, i.e. for good FRET the donor and acceptor should lie within a distance of 2 to 8 nm of each other [32] and the condition $0.5R_0<r<1.5\ R_0$ should be fulfilled [33]. Both this conditions are in fact satisfied well for BSA-bound MAPAEE.

Once the motional freedom of a fluorophore is restricted, the rotational diffusion is arrested leading to a lowering of the polarization of the emitted light and hence an increase in anisotropy [24]. Interaction of fluorophores with micro-heterogeneous media like proteins can lead to both changes in polarity and rigidity of its immediate environment. Therefore monitoring the changes in fluorescence anisotropy can track the changes in rigidity of the microenvironment of a fluorophore interacting with such media. As seen in Fig. 3c we found that on introducing MAPAEE into progressively higher concentrations of BSA the fluorescence anisotropy increases from 0.164 in buffer to as high as 0.33 in 50 μM BSA after which it levels off. This high value of anisotropy is again suggestive of very strong motional restrictions imposed by the proteinous environment on the bound probe.

Average fluorescent lifetime of MAPAEE bound to 50 μM BSA was found to be around 0.727 ns when monitored at 450 nm band which is much higher than the reported average lifetime in even pure methanol, i.e. 0.012 ns [23]. This increase in average fluorescent lifetimes in the proteinous environment is in agreement with our assumption that on binding to the protein the probe moves to a hydrophobic environment from the aqueous environment. Lowering of the non-radiative decay channels in this new proteinous, hydrophobic environment is responsible for the increase in average fluorescent lifetimes. The binding of MAPAEE to BSA is hence reflected not only in the steady state spectral results but also in the time-resolved measurements.

## Effect of Urea on Denaturation of BSA

Chemical denaturation of BSA by urea is a much studied phenomenon and it has been observed that the maximum extent of denaturation is produced between 4 to 6 mM urea. We have tried to use MAPAEE as a fluorescence probe to track this denaturation process and found that the results match well with the literature data available on this process [16, 34–36]. Denaturation of probe bound BSA gradually moves and perhaps releases the bound probe back to the aqueous buffer environment. This then produces exactly

opposite changes in the spectral characteristics of MAPAEE to those involving its binding to the protein. Movement back to the more polar aqueous environment produces an expected red-shift of the polarity sensitive ICT emission band. Rejuvenation of the non-radiative decay channels operative in the hydrogen bonding aqueous environment produces the observed decrease in intensity of this band (Fig. 4a). Also the release of the probe into the buffer environment from the rigid proteinous environment on chemical denaturation relaxes the restrictions formally imposed on the motional freedom of the bound probe. This is then reflected in the decreasing anisotropies of the BSA-bound probe with increasing urea concentrations as observed in Fig. 4b. What is most noticeable in this study is that the maximum drop in anisotropy is observed from 4 to 7 mM urea which is in agreement with the widely reported data available on the urea induced denaturation of BSA. MAPAEE is hence a perfect probe to track very closely the denaturation of BSA with the chaotrope urea.

## Effect of Temperature on Denaturation of BSA

Denaturation of BSA with increasing temperature also releases the bound probe back to the aqueous environment. This is again marked by the decrease in fluorescence anisotropy of MAPAEE attached to the protein. These changes are shown in Fig. 5. We have in this case tracked the anisotropy changes of the bound probe only to ensure that the relaxation of the motional restrictions on the BSA bound probe due to the effect of denaturation of BSA is monitored and the quenching effect of higher temperature on the ICT fluorescence of the probe does not interfere in the results. The decrease in anisotropy of the bound probe again marks its journey back to the free aqueous environment from the very rigid proteinous environment on denaturation of the protein with increasing temperature. The anisotropy of the probe at 80 °C matches with that of the free probe in water and from this we can conclude that the denaturation of BSA is almost complete at this high temperature.

## Interaction with SDS Micelles

Micelles are bio-mimetic micro-heterogeneous media and as the difference in polarity between the exterior and interiors of a micelle is fairly large, the interaction of a polarity sensitive fluorophore with these systems shows pronounced spectral changes. Introduction of MAPAEE into progressively higher concentrations of the conventional surfactant Sodium Dodecyl Sulphate (SDS) produces a pronounced blue-shift of the ICT emission maximum and an increase in intensity of the same. This signals a lowering in immediate polarity and movement into more hydrophobic

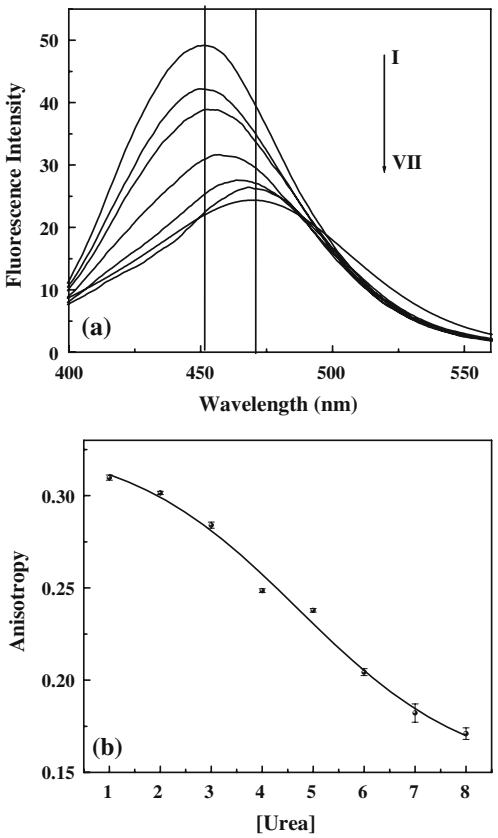



**Fig. 4** Effect of urea on the (**a**) Emission spectra ($\lambda_{ext}$=350 nm) and (**b**) Fluorescence anisotropy of MAPAEE in 50 μM BSA. (I- 0 M, II-2M, III-3M,IV- 4M, V-5M, VI-7M and VII-8M urea)

environment. Probable movement of the MAPAEE molecule from the polar water environment to less polar micellar environment explains the observed spectral changes. A plot of the emission intensities of the ICT maxima of MAPAEE in various SDS concentrations against the corresponding surfactant concentration marks an easy and well followed way to determine the CMC of this micellar system. This plot produces two separate lines corresponding to two sets of

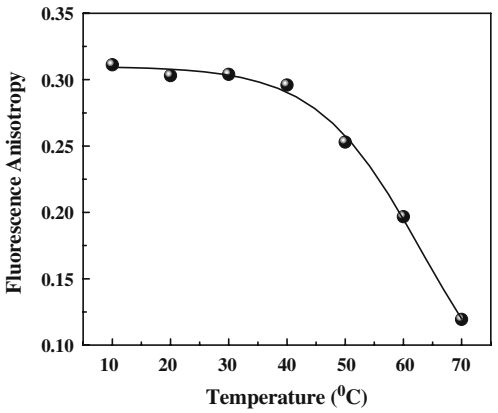



**Fig. 5** Effect of increasing temperatures on the fluorescence anisotropy of MAPAEE bound to 50 μM BSA

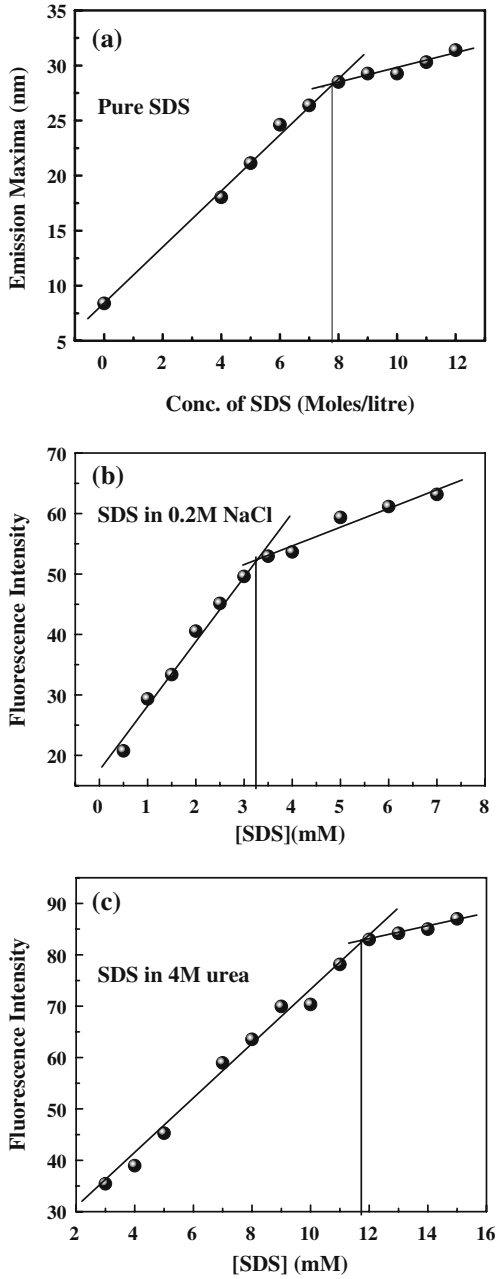



**Fig. 6** Plot of emission maxima intensity of MAPAEE against corresponding concentration of SDS for (**a**) pure SDS, (**b**) SDS in 0.2 M NaCl and (**c**) SDS in 4 M urea

points one before and one after the CMC of SDS. The point of interaction of these two lines marks the CMC of the system [37, 38]. We have determined the CMC of SDS in this way in different environments and find the values to be in very good agreement with reported literature [30]. These plots for pure SDS, SDS in 0.2 M NaCl and in the presence of chaotrope urea (4 M) are shown in Fig. 6a, b and c. Greater ionic strength produces an expected decrease in CMC as is observed from the probe fluorescence. Again in the presence of urea the hydrophobic

**Table 1** Critical Micellar Concentration determined from the study of MAPAEE emission changes

| Detergent | Critical Micellar Concentration | | |
|---|---|---|---|
| | Pure | In 0.2 M NaCl | In 4 M urea |
| SDS | 7.85 mM | 3.22 mM | 11.8 mM |
| NaDC | 3 mM | 0.9 mM | 6.33 mM |

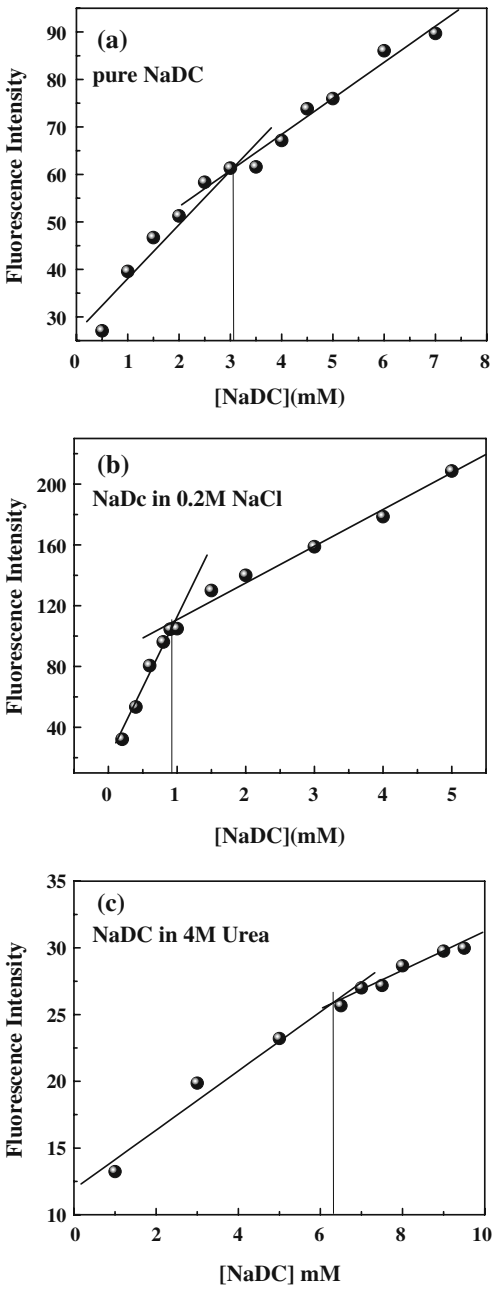



**Fig. 7** Plot of emission maxima intensity of MAPAEE against corresponding concentration of NaDC for (**a**) pure NaDC, (**b**) NaDC in 0.2 M NaCl and (**c**) NaDC in 4 M urea (All solutions were made in 0.01 M Tris-HCl buffer, pH=7.5 to avoid gel formation)

interactions required for production of stable micelles is much disturbed which leads to a pronounced increase in the CMC of any surfactants [39, 40]. This increase is also observed for SDS in 4 M urea as seen in Fig. 6c. The observed CMCs are presented in Table 1 and are in good agreement with reported. Therefore, MAPAEE has a quite good applicability to study micro heterogeneous systems.

## Interaction with NaDC Micelles

Sodium deoxycholate is a dihydroxy bile salt and one of the three major bile salts found in humans. Here we report the primary CMC's of NaDC in tris-HCl buffer (0.01 M, ph= 7.5, to avoid any chance of gel formation) in pure state as well as in presence of 0.2 M NaCl and 4 M urea using MAPAEE as a probe as described before. The results are shown in Fig. 7a, b and c and Table 1. As expected, increase ionic strength decreases the CMC and presence of chaotrope urea increases the CMC of NaDC like standard surfactants. These studies are important considering the importance of bile salts and formation of their micelles in animals for digestion of fats and fatty acids. In this context we find again that MAPAEE with its special photo physical properties can serve as a very sensitive probe for not only bio-mimetic micellar systems but also biologically important amphiphillic bile salts.

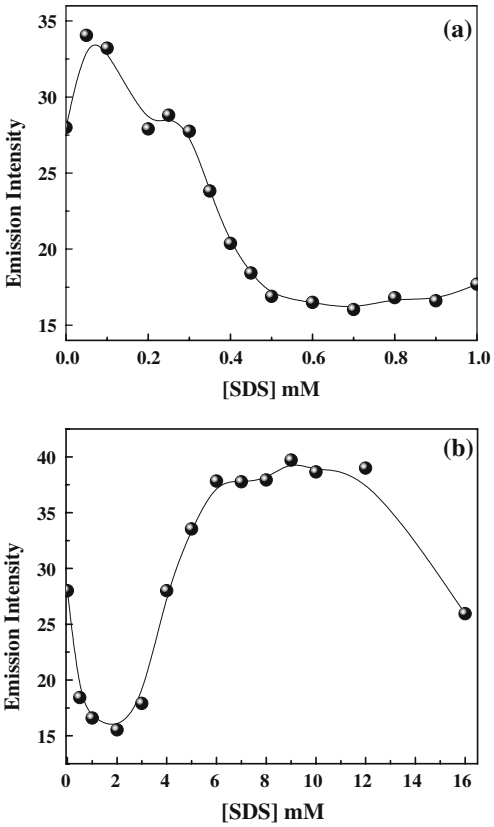



**Fig. 8** (**a**) and (**b**): Variation of intensity of ICT emission maxima of MAPAME bound to BSA with increasing SDS concentration (traversing the binding curve between BSA and SDS along increasing SDS concentration)

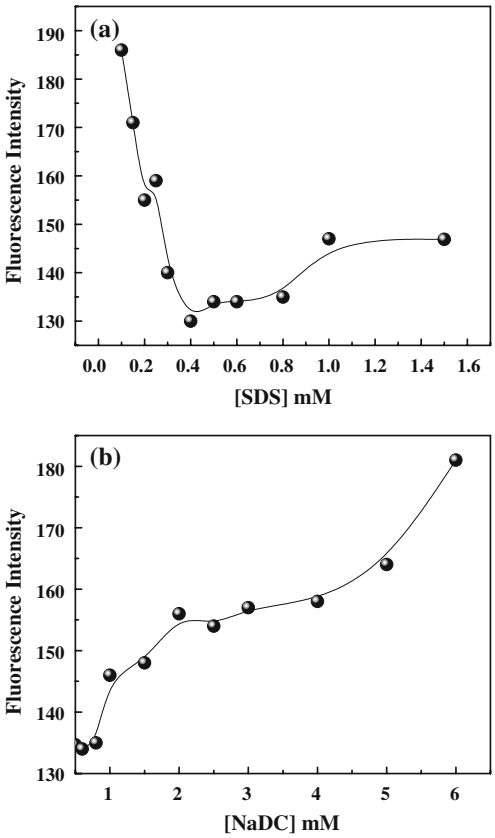



**Fig. 9** (**a**) and (**b**): Variation of intensity of ICT emission maxima of MAPAME bound to BSA with increasing NaDC concentrations (traversing the binding curve between BSA and NaDC along increasing NaDC concentration)

## SDS Binding with BSA

The surfactant induced uncoiling of BSA has been studied widely and a lot of models were proposed to explain this phenomenon [36, 41]. An attempt to traverse the established binding isotherm for the SDS-BSA interaction has been made here and we follow the uncoiling of BSA along this isotherm [41] by using the protein bound probe MAPAEE's spectral response to increasing SDS concentrations. The results are shown in Fig. 8. The uncoiling of BSA by SDS follows three distinct regions. Initially at very low SDS concentrations specific binding takes place as SDS binds to some specific high energy binding sites on BSA. This is followed by a non-cooperative binding region followed by the region for cooperative binding where a very large number of SDS molecules bind to BSA. Figure 8 outlines the changes in the intensity of fluorescence maxima of BSA-bound MAPAME in various concentra-

tions of SDS. Initially (Fig. 8a) a smart rise in ICT emission intensity is observed followed by a decrease till about 0.7 mM SDS corresponding to the initial specific binding regions of the SDS-BSA binding isotherm. After this, a very gradual rise in intensity is observed but from around 2mM SDS concentration a sharp rise till about 7 mM SDS is observed which corresponds with the cooperative binding region of the binding isotherm. This is then followed by a plateau signifying the region of saturation of binding (Fig. 8b). The observed spectral changes very well correlate with the binding isotherms reported earlier by many workers including us for this system and make way for use of MAPAEE as a sensitive probe. Therefore, it is found to be able enough to monitor SDS induced uncoiling of BSA which is not a very smooth change and is marked by various regions of different behaviors.

NaDC Binding with BSA

The success with which MAPAEE could monitor the SDS induced uncoiling of BSA led us to try and use it for probing a more biologically relevant interaction. We chose to apply it for studying the interaction of BSA with the bile salt NaDC which is a steroidal biologically active detergent. The binding isotherm for the NaDC-BSA system [12] is already established and found to show three distinct regions: a specific binding region from 0 to ~0.6 mM NaDC followed by a non-cooperative binding region ranging between ~0.6 mM to ~3 mM and finally at higher concentrations of NaDC co-operative binding sets in leading to massive uncoiling of the protein. BSA attached to MAPAEE was made to interact with increasing concentrations of NaDC following the established NaDC-BSA binding isotherm and the ICT emission intensity of the probe is monitored at each step. Initially, as shown in Fig. 9a, we find that the emission intensity decreases steadily with increasing NaDC concentrations till about 0.5 mM NaDC corresponding very well to the specific binding regions of the binding isotherm. After 0.6 mM NaDC, further increase in NaDC concentrations leads to a slow increase in MAPAEE emission intensity till around 3 mM NaDC marking the non-co-operative binding region of the isotherm. After 3 mM NaDC larger bile salt concentrations lead to a massive increase in the fluorescence intensity of the bound MAPAEE marking the cooperative binding region (Fig. 9b). The changes in intensity of the probe MAPAEE are in very good agreement with the established binding isotherms for this system. Also noteworthy is the difference in the spectral response between that for the binding of SDS to BSA and for NaDC to BSA, although the binding isotherms for both have similar regions. This might be due to the intrinsic difference in the structures and hence properties of the micelles of conventional SDS and the steroidal NaDC molecule which makes the association of these two amphiphiles with BSA different in nature.

## Conclusion

The special polarity responsive photo-physical properties of the ICT probe MAPAEE has been used with ease as a very sensitive yet robust probe for studying the model protein Bovine Serum Albumin and its thermal and chemical denaturation by urea. The ICT emission band of MAPAEE shows definite changes in position, intensity and anisotropy on binding to BSA and these changes are reversed when the bound protein is made to denature chemically and thermally. Tracking these spectral changes makes it possible to monitor these biologically relevant processes making way for use of MAPAEE as a good fluorescent probe for monitoring these biological micro-heterogeneous systems. A successful attempt was also made to use the spectral responses of MAPAEE to changes in polarity and viscosity to study the uncoiling of BSA by SDS and the bile salt NaDC and excellent correlation with the binding isotherms for both the systems were obtained. This study opens up a multitude of possibilities for using the probe MAPAEE as a good fluorescent probe for spectroscopic probing of biological and bio-mimetic micro heterogeneous systems.

**Acknowledgement** This work is supported by CSIR, India (Project no. 01(2161)07/EMR-II) and DST, India (Project no. SR/S1/PC/26/2008). The authors would like to thank Prof. T. Ganguly of IACS, Kolkata for allowing them the fluorescence lifetime measurements

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