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Solvation dynamics of LDS 750 in micelles, reverse micelles and proteins

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Abstract

In the present study, steady state, picosecond resolved fluorescence and polarization-gated anisotropy have been used to characterize the binding of LDS 750 (LDS) in biomimetics and in the transporter protein bovine serum albumin (BSA). Competitive binding studies with well-known BSA binding drugs warfarin and diflunisal show that LDS binds in domain III of the protein. Time resolved emission spectra (TRES) have been used to determine the dynamics of environmental relaxation in restricted media. Our studies open the possibility of using the dye as a potential fluorescence and solvation reporter in other proteins and biomimetics of interest.

1. Introduction

The dynamics of biological macromolecules play pivotal role in the expression of their functionality [1–3]. A wide variety of biological processes like recognition of drugs and substrates, transcription and damage recognition by DNA and proteins require considerable flexibility of the macromolecule. The macromolecular flexibility arises from collective motions of amino acid residues/base pairs (protein/DNA) and the hydration waters associated with the macromolecules. The close correlation of the dynamics of macromolecules with important biological functions has given a boost to research on macromolecular dynamics [4–11], DNA and proteins being popular choices of biomacromolecules. To avoid the complicacies of a biomolecule, environmental dynamics have been studied in simpler systems like micelles/reverse micelles [12–15]. Many of these studies [4,5,7] employ time resolved stokes shift (TRSS) to estimate the environmental relaxation of biomolecules. The TRSS technique employs a fluorescent molecule to probe the dynamics of the environment and is based on the principle that when a fluorophore is excited, its properties, e.g., polarity or polarizability, change significantly, causing the local solvent structure to reorganize. This reorganization reduces the energy of the system and causes the fluorescence to shift to longer wavelengths, i.e., a Stokes shift develops. The rate of shifting of the fluorescence spectrum directly reflects the rate of reorganization of the fluorophore's environment. However, the choice of fluorophore is crucial to the technique. The presence of internal dynamics of the fluorophore having comparable time scales with that of the environmental relaxation makes the application of fluorophores in TRSS less straightforward. This leads to the search for standard solvation fluorophores like Coumarin 480 and LDS 750 (LDS). Moreover, the efficacy of fluorophores to report environmental dynamics in simple liquids does not ensure that it is a good fluorescence reporter of restricted environments. This is because of the fact that excited state relaxation dynamics including proton transfer and electron transfer are retarded by orders of magnitude in restricted environments [16], increasing the possibility of the interference of these processes to the TRSS. Also, in TRSS, the location of the probe in the restricted environment becomes important. For example, a probe residing in the interface of a reverse micelle cannot successfully explore the dynamics/energetics of the water core.

In this regard, it is worthwhile to mention that the solvation probe LDS has been to study the ultrafast dynamics

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of simple liquids like aniline [17] and other solvents [18]. The use of LDS as a solvation probe in solvents has sparked controversy regarding the involvement of internal dynamics of the probe in the observed solvation relaxation process [19]. In the present study, we attempt to investigate the interaction of LDS with micelles, reverse micelles and proteins and thereby explore the possibility of using the dye as a reporter of the slow dynamics associated with the environments, using steady state and time resolved spectroscopic techniques. To explore the specific site of interaction of the dye LDS with the protein, we have also used competitive binding studies with other well-known BSA-binding drugs. Time resolved area normalized spectra (TRANES) has been used to investigate the possibility of dye occupying multiple binding sites in the protein.

2. Materials and methods

Bis(2-ethylhexyl) sulphosuccinate (AOT), Bovine Serum Albumin (BSA), and phosphate buffer are obtained from Sigma. The fluorescent probe LDS 750 (LDS) is from Exciton. Cetyltrimethylammonium bromide (CTAB) and Sodium dodecyl sulphate (SDS) are from Fluka. TX-100 and isooctane are from Romil and Spectrachem, respectively. The sample solutions are prepared in phosphate buffer (pH 7) using water from Millipore system. The procedures for sample preparations are detailed in our previous works [20].

The steady state absorption and emission are measured with Shimadzu UV-2450 spectrophotometer and Jobin Yvon Fluoromax-3 fluorimeter, respectively. Fluorescence transients are measured in a commercially available spectrophotometer (LifeSpec-ps) from Edinburgh Instrument, UK (excitation wavelength 633 nm, 80 ps instrument response function, (IRF)). The observed fluorescence transients are fitted by using a nonlinear least square fitting procedure to a function comprising of $(X(t) = \int_0^t E(t')R(t-t')\mathrm{d}t')$ convolution of the IRF $(E(t))$ with a sum of exponentials $(R(t) = A + \sum_{i=1}^{N} B_i\, \mathrm{e}^{-t/\tau_i})$ with pre-exponential factors (B_i), characteristic lifetimes (τ_i) and a background (A). Relative concentration in a multi-exponential decay is finally expressed as; $c_n = \frac{B_n}{\sum_{i=1}^{N} B_i} \times 100$. The quality of the curve fitting is evaluated from reduced chi-square and residual data. The calculated lifetimes, thus obtained are within 5% error range. To construct time resolved emission spectra (TRES) and time resolved area normalized spectra (TRANES), we follow the technique described elsewhere [20–23]. The solvation correlation function, $C(t)$ is constructed following the equation, $C(t) = \frac{\nu(t)-\nu(\infty)}{\nu(0)-\nu(\infty)}$ where $\upsilon(0)$, $\upsilon(t)$ and $\upsilon(\infty)$ stand for the wavenumber in cm^{-1} at the emission maxima at time zero, t and infinity, respectively. For anisotropy $(r(t))$ measurements, emission polarization is adjusted to be parallel or perpendicular to that of the excitation and anisotropy, is defined as, $r(t) = \frac{[I_{\mathrm{para}} - G \times I_{\mathrm{perp}}]}{[I_{\mathrm{para}} + 2 \times G \times I_{\mathrm{perp}}]}$. G, the grating factor, is determined following longtime tail matching technique [24].

3. Results and discussion

Fig. 1a shows the absorption spectra of the LDS in buffer and in different restricted environments. The absorption spectra of the dye in micelles and BSA shows a red shift compared to the absorption of the dye in buffer (absorption maximum at 514 nm) indicating the ground state stabilization of the dye in the restricted environments. The shift is maximum for micelles (TX-100/CTAB/SDS) indicating maximum ground state stability of the probe in micelles. The proximity of the absorption maxima of LDS in RM $(w_0 = 2.5)$ and BSA suggest comparable ground state stabilization of the dye in both the environments. Fig. 1b shows the emission spectra of LDS in the restricted environments. The emission spectrum of the dye in buffer is also shown for comparison. It is interesting to observe that the emission spectrum of the dye in micelles and reverse

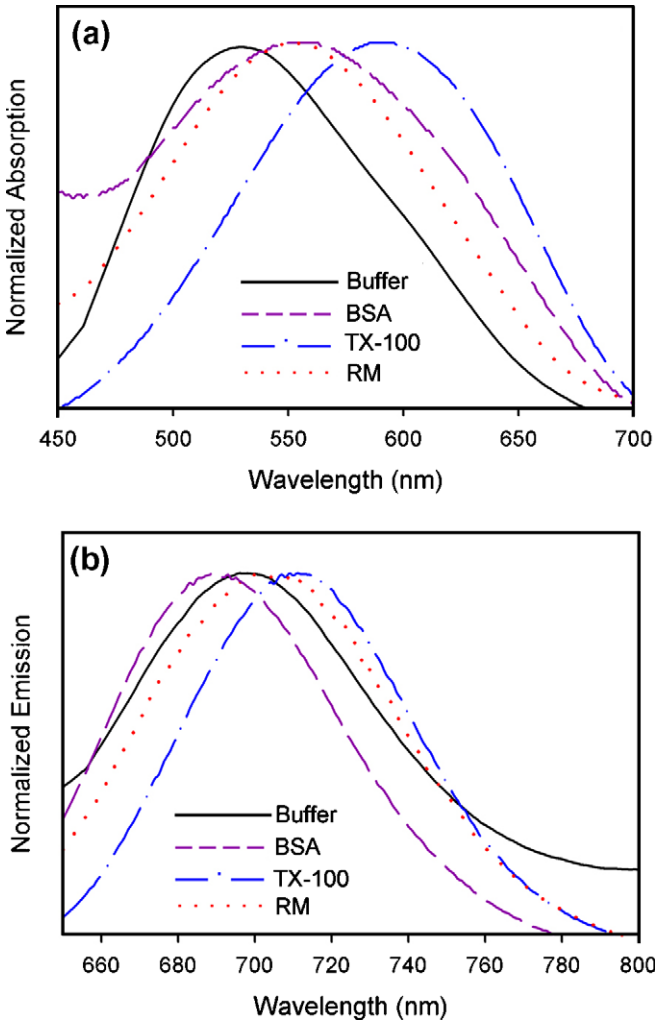

Fig. 1. The absorption (a) and emission spectra (b) of LDS in restricted environments. For the sake of clarity only representative micelles (TX-100) and RM $(w_0 = 2.5)$ are shown.

micelles are red shifted compared to those of the buffer. The red shift in the emission spectra can be rationalized considering internal Stark effect [25]. The static electric field created due to the presence of charged/polar surfactant head groups affects the excited state of the probe and that is indicated by the red shift in the emission spectra. This shift is expected to be different from that of the polarity dependent solvatochromic shift as reported by Maroncelli et al. [26]. Only, the emission of LDS in BSA is blue shifted indicating the destabilization of the excited state in the binding pocket of BSA.

To explore the possibility of using LDS as a reporter of the environmental dynamics, fluorescence transients are taken across the emission spectrum in all the restricted environments. Fig. 2a shows the fluorescence transients of LDS in AOT/isooctane reverse micelles (RM) having $w_0 = 2.5$. It is to be noted that the observed lifetimes (~nanoseconds) associated with LDS in RM are much longer than that of the probe in buffer (<80 ps), indicating the residence of the probe in hydrophobic environments. The transients show fast decay in the blue end and rise in the red end indicative of solvation. The constructed TRES (Fig. 2b) gives a shift of 700 cm^{-1} in a 3 ns window. To confirm that the observed dynamics represents environmental stabilization, time resolved area normalized emission spectra (TRANES) are constructed. The absence of iso emissive points in TRANES excludes the possibility of interference of any internal dynamics of the probe to the observed relaxation dynamics [23]. The temporal decay of the solvation correlation function (Fig. 2c) is fitted with time constants of 110 ps (50%) and 700 ps (50%). The slower time constant agrees well with the value of 660 ps observed by Zhang and Bright [27], and is associated with the dynamics of bulk like water present in the RM. The absence of additional nanosecond solvation time scales, characteristic of the relaxation of bound water molecules in close vicinity of the interface [27] suggests that LDS is located inside the water pool of the RM. The temporal decay of the fluorescence anisotropy (Fig. 2c, inset) is fitted with a nanosecond component (Table 1) (in contrast to the fast (<80 ps) re-orientational motion of the probe in buffer), reflecting restricted rotation of the probe in RM. The overall tumbling motion of the RM remains as an offset in our experimental window. The observance of two separate motions corresponding to that of the probe and the overall system (RM) is in accordance with the model suggested by Lipari and Szabo [28]. To explore the effect of increase in the pool size of the RM, the dynamics of environmental relaxation are monitored in RM having different w_0 values. The time constants (Table 1), associated with the (Fig. 3) $C(t)$ decay in RM with different w_0s become progressively faster with increase in the size of the RM. The result is consistent with enhanced water mobility [13] consequent to the increase of free type water molecules [29] possessing greater orientational flexibility [12] in the RM. The increased water mobility in the bigger sized RM is also responsible for the faster rotational

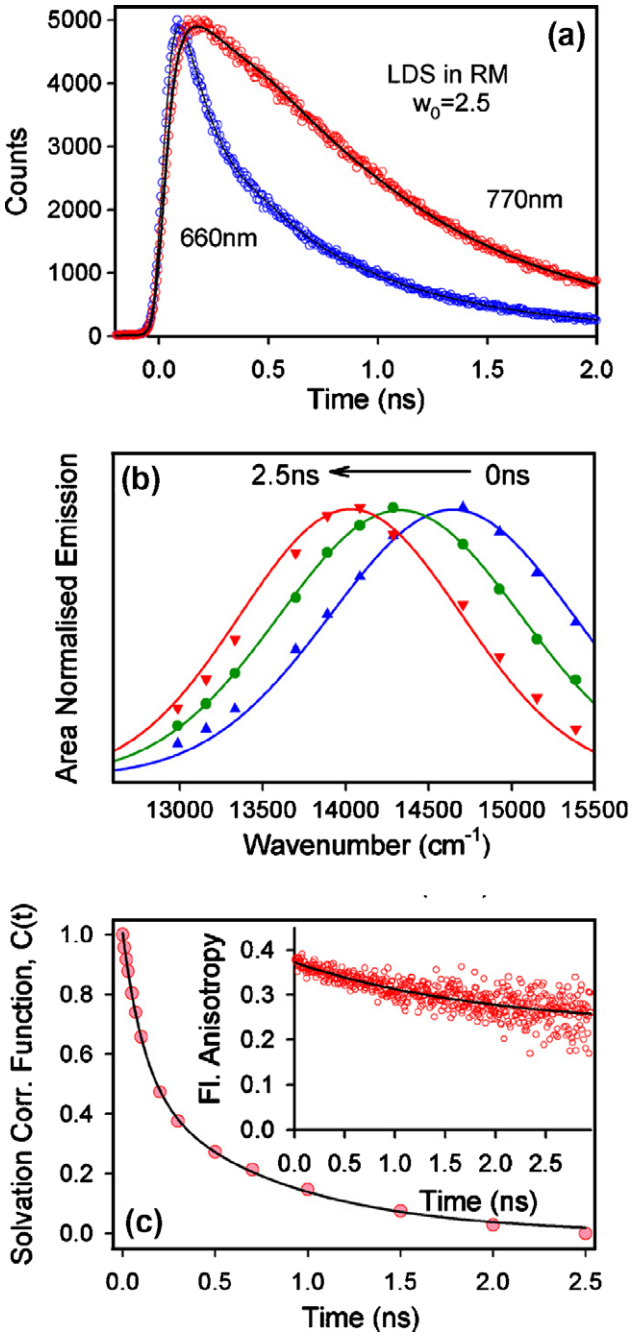

Fig. 2. The fluorescence transients (a) TRES, (b) temporal decay of solvation correlation function (c) and fluorescence anisotropy (c, inset) of the LDS in RM ($w_0 = 2.5$).

motion of the probes, borne out by a decrease in time constants (Table 1) associated with the temporal decay of fluorescence anisotropies (Fig. 3, insets) in RM of different sizes, the observation being consistent with a recent study [14].

To study the effect of the surface charge on the recognition of biomimetics by LDS we studied the dynamics of the probe in neutral (TX-100), cationic (CTAB) and anionic (SDS) micelles. As discussed above, the probe shows ground state stability in all the micelles, whereas, the ionic environment created by the charge/polarity of micellar

Table 1
The fluorescence anisotropy, $r(t)$ and solvation correlation function, $C(t)$ of LDS in different restricted media

Medium	$r(t)$		$C(t)$	
	τ (ns)	Offset (%)	τ_1 (ns)	τ_2 (ns)
BSA	2.88 (11%)	89	0.147 (52%)	0.696 (48%)
SDS micelles	1.37 (67%)	33	–	–
CTAB micelles	1.25 (45%)	55	0.08 (80%)	0.660 (20%)
TX-100 micelles	1.48 (34%)	66	0.08 (82%)	0.620 (18%)
RM, $w_0 = 2.5$	1.92 (39%)	61	0.110 (50%)	0.700 (50%)
RM, $w_0 = 5$	1.51 (35%)	65	0.110 (55%)	0.500 (45%)
RM, $w_0 = 10$	1.21 (37%)	63	0.070 (66%)	0.230 (34%)
RM, $w_0 = 20$	0.56 (42%)	58	0.02 (100%)	–

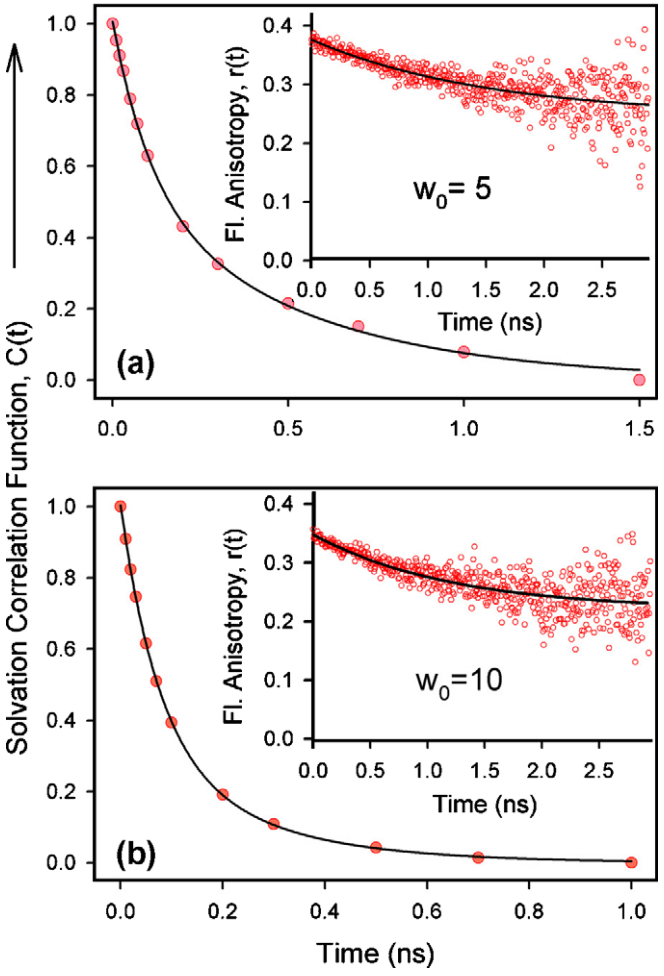

Fig. 3. The temporal decay of solvation correlation functions and fluorescence anisotropies (corresponding insets) of LDS in RM of different w_0 values.

head groups influences the excited state stability of the probe. The attachment of the probe to TX-100 micelles is borne out by the geometrical restriction (the rotational time constant = 1.48 ns) to the rotational motion of the probe in TX-100 micelles. The observed time constant is close to the value of 1.80 ns reported for the dye Nile Red in TX-100 micelles [30]. Note that the overall tumbling motion of the TX-100 micelles remains as an offset in our experimental time window. The fast decay in the blue end and rise in the red end of the emission spectrum of the probe in TX-100 micelles (data not shown) suggests stabilization of the excited state dipole in the micellar environment. The $C(t)$ (Fig. 4a) decays with time constants of 80 ps (80%) and 660 ps (20%). The observed time scales are faster than that in the environmental dynamics reported by Coumarin 153, known to reside in the palisade layer of TX-100 micelles [31]. The observation suggests that LDS is located at the periphery of the micelle, in close association with the polar head groups of the surfactants and free type water molecules [32]. Fig. 4b and the corresponding inset show the temporal decay of the solvation correlation function and rotational anisotropy of LDS in CTAB micelles. The slow component (1.25 ns) in the fluorescence anisotropy suggests the association of the probe with CTAB micelles, whereas environmental dynamics suggests significant contribution from ionic head groups and free type water molecules, similar to the results obtained for neutral TX-100 micelles. The geometrical restriction to rotational motion (evidenced by a slower component in the decay of fluorescence anisotropy, Table 1) indicates that the probe interacts with SDS micelles. However, the

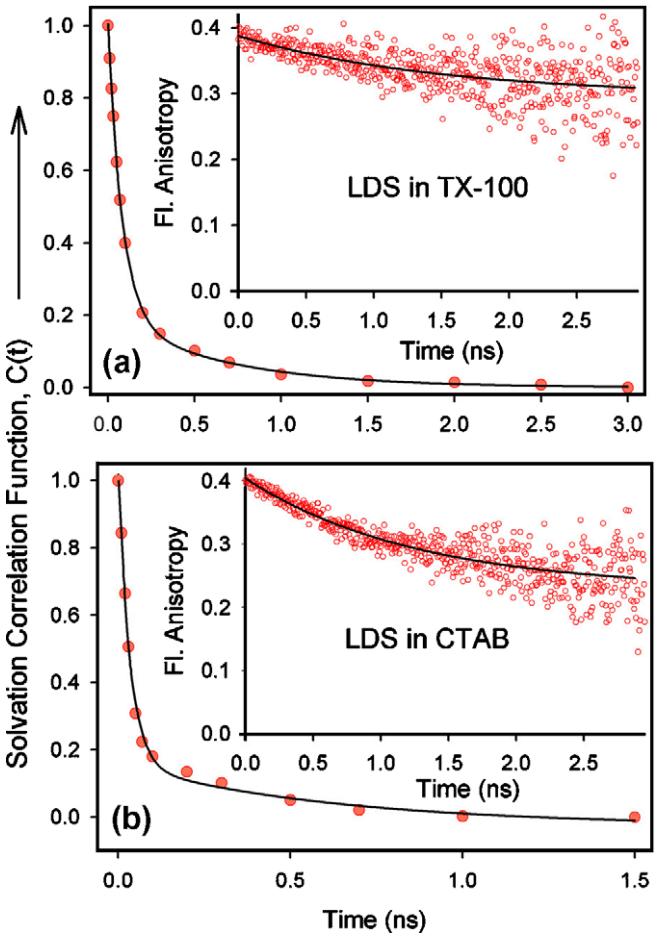

Fig. 4. The temporal decay of the solvation correlation functions and fluorescence anisotropies (corresponding insets) of LDS in micelles.

similarity of the fluorescence transients at the blue and red ends of the emission spectrum suggests the absence of solvation stabilization in our experimental window. The absence of any change may be due to the very fast environmental relaxation of the ionic environment, which is not detectable in our experimental window.

It is worthwhile at this stage to extend our studies on LDS to real biological systems. In a recent study [33] from our group on LDS–BSA interactions, we have shown that the dyes DCM and LDS compete for the same binding site in BSA. The dynamics of solvation of LDS has also been addressed in the study. However, the exact binding site(s) of the dye in the protein and the heterogeneity in the binding site(s), have not been ascertained therein. Here, we have used competitive binding studies with well-known BSA-binders warfarin and diflunisal to estimate the exact binding site of LDS in BSA. It is known that warfarin specifically binds in subdomain IIA [34] of the protein whereas diflunisal binds in both domain II and domain III in BSA [35]. It is important to mention here that for the competitive binding studies, the concentration of LDS is maintained an order of magnitude lower than that of BSA, while that of the competitive binder (warfarin/diflunisal) is in excess. The significant difference in the fluorescence lifetimes of LDS in buffer (<80 ps) and BSA (3 ns) makes it a very suitable parameter to study competitive binding. On addition of warfarin to LDS–BSA complex, there is no appreciable change in the observed fluorescence lifetime (Fig. 5a). This suggests that LDS does not bind to the warfarin-binding site in BSA. However, on the addition of diflunisal, the appearance of a faster component (<80 ps (50%)) suggests the presence of free LDS molecules in solution. The results indicate that diflunisal expels LDS from its binding site in domain III of the protein. To confirm the binding of LDS to a single site in BSA, TRANES is constructed for LDS–BSA complex. The absence of iso emissive point in TRANES (Fig. 5b) [23] clearly shows that LDS occupies a specific site in BSA, consistent with our earlier study [33] and hence rule out the possibility of heterogeneity in the dye–protein interactions.

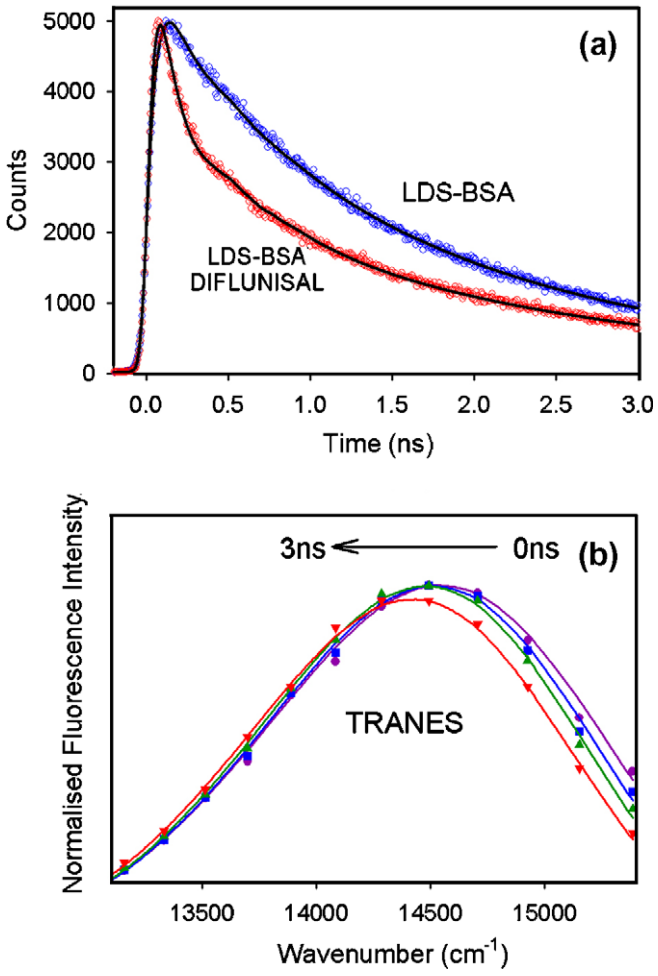

Fig. 5. (a) The temporal decay of fluorescence of LDS–BSA and LDS–BSA-Diflunisal complexes. (b) The TRANES of LDS in BSA.

4. Conclusion

In the present study, steady state, picosecond resolved fluorescence and polarization-gated anisotropy are used to characterize the binding of LDS in biomimetics and in the transporter protein BSA. The solvation dynamics studies using LDS as a fluorescence reporter in all the environments suggest that the probe can unambiguously report environmental dynamics in both biomimetics and real biological systems like proteins. The location of the probe at the periphery of micelles and water pool in RM makes it a suitable complement to other solvation probes, which reside in more hydrophobic regions in these biomimetics. Our studies are expected to find it is application in the use of the dye LDS as a efficient solvation probe for other biological macromolecules.

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