Hydration Dynamics of Probes and Peptides in Captivity

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Abstract Water confined on nanometer-length scales is found in many physical and biological environments. Confinement induces special dynamics in liquids, different from that of their bulk counterparts. Reverse micelles, formed by the self-assembly of amphiphilic surfactants in nonpolar solvents, have emerged as an appropriate molecular assembly to monitor the property of water upon confinement due to a number of reasons. A unique advantage of reverse micelles is that molecular dynamics can be monitored with varying states of hydration that is difficult to achieve with assemblies, such as membranes. In this article, we focus on the change in confined hydration dynamics accompanied with increasing hydration, monitored by red edge excitation shift (REES). REES can be effectively used to directly monitor the environment and dynamics around a fluorophore in a molecular assembly utilizing slow solvent relaxation around an excited state fluorophore. It is apparent from the examples discussed that change in solvent relaxation with hydration is complicated and could depend on a number of factors, such as the location of the probe in the reverse micelle and the structure and compactness of the fluorophore involved. We conclude that care should be exercised in interpreting such results.

Abbreviations

12-AS	12-(9-Anthroyloxy)stearic acid
2-AS	2-(9-Anthroyloxy)stearic acid
6-AS	6-(9-Anthroyloxy)stearic acid
AOT	Sodium bis(2-ethylhexyl)sulfosuccinate

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EGFP	Enhanced green fluorescent protein
GFP	Green fluorescent protein
NBD	7-Nitrobenz-2-oxa-1,3-diazol-4-yl
NBD-cholesterol	25-[*N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino]-27-norcholesterol
NBD-PE	*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine
REES	Red edge excitation shift

1 Introduction

Although water is the most abundant liquid on the surface of the earth, it is often found in captivity [44]. There are numerous examples in geochemistry, tribology, nanofluidics, and biology, where water is often found in confinement. In fact, cellular water in biology has been referred to as *tamed hydra* [51]. Confinement brings about interesting properties in bound water in terms of organization and dynamics that are very different from that experienced in bulk water [9, 28].

Reverse micelles have emerged as an appropriate molecular assembly to monitor the property of water upon confinement. Amphiphilic surfactants self-assemble to form reverse (or inverted) micelles in nonpolar solvents in which the polar head groups of the surfactant monomers cluster to form a micellar core and are directed toward the center of the assembly and the hydrophobic tails extend outward into the bulk organic phase [46, 47]. Reverse micelles can solubilize an appreciable amount of water to form a spherical pool in the center. They are optically transparent, nanometer-sized water droplets of various size surrounded by a layer of surfactant molecules dispersed in nonpolar solvents. Studies on reverse micellar organization and dynamics are relevant since the general principle underlying their formation (the hydrophobic effect) is common to other related organized assemblies, such as micelles, bilayers, liposomes, and biological membranes [37, 73–75]. Reverse micelles provide an attractive model system for biomembranes since they mimic a number of important and essential features of biological membranes although lacking much of the complexity associated with them.

The water pools entrapped in reverse micelles have been extensively used as micromedia for chemical and biochemical reactions. The nature of water in reverse micelles, especially at low water content, has been studied extensively and is believed to be different from that of bulk water. Both experimental [36, 38, 79] and theoretical [25] approaches have shown that the crucial structural parameter of reverse micelles is [water]/[surfactant] molar ratio (w_o) that determines the micellar size as well as the extent of deviation of the properties of the entrapped water from those of normal bulk water [53, 60]. Reverse micelles, therefore, represent a type of organized molecular assembly which offer the unique advantage of monitoring dynamics of molecules with varying degrees of hydration. A number of physicochemical properties of the entrapped water, such as micropolarity, dielectric constant, microviscosity,

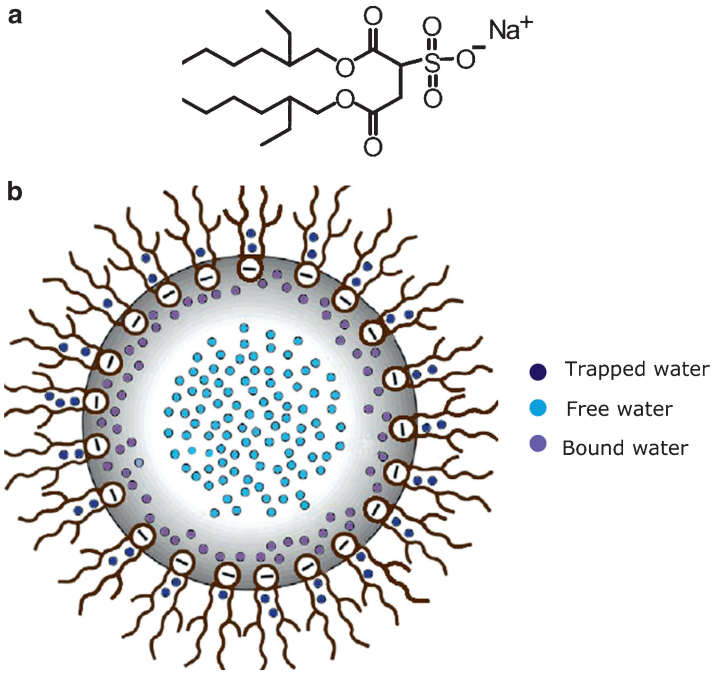

Fig. 1 (**a**) Chemical structure of AOT. (**b**) A schematic diagram of a reverse micelle showing highly structured yet heterogeneous water pools of graded dynamics: trapped water, bound water, and free water molecules. The crucial parameter is the [water]/[surfactant] molar ratio (w_o) which determines the relative proportions of these three types of water pools and the micellar size. Adapted and modified from Chattopadhyay [12]

water activity, freezing point, proton transfer efficiency, and the hydrogen-bonding potential, can be experimentally varied with w_o, thereby providing a unique and versatile reaction medium.

The double-chain anionic surfactant AOT has been extensively used to form reverse micelles in nonpolar solvents [21]. A major advantage with AOT is that reverse micelles formed by AOT can solubilize a large quantity of water in a nonpolar solvent *without the use of any cosurfactant* [47]. In addition, reverse micelles formed by AOT retain a spherical shape over a wide range of w_o. As a result of this, the radius of the entrapped water pool can be linearly related to w_o [24, 50]. Three types of water populations (pools) have been shown to coexist in reverse micelles (see Fig. 1). These are bound water, trapped water, and free water [34, 36, 38]. The relative proportions of these three types of water pools are determined by w_o. The properties of water in reverse micelles of AOT at low w_o values are rather different from those of bulk water [36, 38, 79]. Even at higher water content ($w_o = 50$), the apparent microviscosity is 6–9 times greater than that of free aqueous solutions [5]. The trapped water pool is characterized by the fact that this population of water molecules does not hydrogen bond to other water molecules [25, 38]. The various types

of water pools in reverse micelles, characterized by graded dynamics, represent interesting models for water present in biological systems, such as membranes (see Fig. 1). The physical and chemical properties of the entrapped water are markedly different from the properties of bulk water but similar in several aspects to those of biological interfacial water as found in membrane or protein interfaces [10, 36, 38, 79]. For example, the dielectric constant of the aqueous phase in reverse micelles has been estimated to be ~60–70 [19]. This review is focused on the application of the wavelength-selective fluorescence approach [12, 22, 23, 56, 65] as a powerful tool to monitor the organization and dynamics of probes and peptides inside reverse micelles.

2 Red Edge Excitation Shift

Fluorescence techniques have been widely used to characterize reverse micellar organization and dynamics due to suitable timescale, noninvasive nature, and intrinsic sensitivity [7, 45]. Reverse micelles offer certain inherent advantages in fluorescence studies since they are small and optically transparent, have well-defined sizes, and are relatively scatter-free. They, therefore, represent model systems appropriate for the study of probes, peptides, and proteins in membrane-mimetic, hydration-controlled environment [62, 72, 78]. Reverse micelles are highly cooperative, organized molecular assemblies of amphiphilic surfactants and are dynamic in nature. A direct consequence of such organized systems is the restriction imposed on the dynamics and mobility of their constituent structural units. We have previously shown that the microenvironment of molecules bound to such organized assemblies can be conveniently monitored using wavelength-selective fluorescence as a novel tool [12]. Wavelength-selective fluorescence comprises a set of approaches based on the red edge effect in fluorescence spectroscopy, which can be used to directly monitor the environment and dynamics around a fluorophore in a complex system [12, 22, 23, 56, 65, 68]. A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed red edge excitation shift (REES). This effect is mostly observed with polar fluorophores in motionally restricted media, such as very viscous solutions or condensed phases, where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime. REES arises due to slow rates of solvent relaxation (reorientation) around an excited state fluorophore, which depends on the motional restriction imposed on the solvent molecules [or the dipolar environment, as in green fluorescent protein (GFP) ([29]; see later)] in the immediate vicinity of the fluorophore. Utilizing this approach, it becomes possible to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) using the fluorophore *merely* as a reporter group.

Furthermore, because the ubiquitous solvent for biological systems is water, the information obtained in such cases comes from the otherwise "optically silent" water molecules. The unique feature of REES is that while fluorescence techniques, such as fluorescence quenching, energy transfer, and anisotropy measurements, yield information about the fluorophore itself (either intrinsic or extrinsic) REES provides information about the relative rates of solvent relaxation dynamics, not possible to obtain by other techniques. This makes REES in particular and the wavelength-selective fluorescence approach in general extremely useful since hydration plays a crucial modulatory role in a large number of important cellular events, such as protein folding, lipid–protein interactions, and ion transport [33, 82]. As mentioned above, reverse micelles represent a type of organized molecular assembly which offers the unique advantage of monitoring dynamics of molecules with varying states of hydration. This feature, along with the confinement imposed on reverse micellar water, makes reverse micelles a spatiotemporally suitable system for the application of the wavelength-selective fluorescence approach.

3 Fluorescent Probes in Reverse Micelles: Depth-Dependent Solvent Relaxation

The applicability of the wavelength-selective fluorescence approach to reverse micellar systems has been tested using the interfacial fluorescence probe NBD-PE incorporated in AOT reverse micelles. The NBD group possesses some of the most desirable properties for serving as an excellent probe for both spectroscopic [57] as well as microscopic [61] applications. It is very weakly fluorescent in water and upon transfer to hydrophobic media, it fluoresces brightly in the visible range and shows a large degree of environmental sensitivity [14, 27, 43, 55]. This large degree of environmental sensitivity of NBD fluorescence is very useful in monitoring various types of reverse micellar organizations formed under conditions of varying [water]/[surfactant] molar ratio. NBD-labeled lipids are widely used as fluorescent analogues of native lipids in biological and model membranes and in membrane-mimetic assemblies to study a variety of processes [8, 11, 49]. In NBD-PE (see Figs. 2 and 3a), the NBD group is covalently attached to the head group of a phosphatidylethanolamine molecule. The NBD group in NBD-PE has earlier been shown to be localized in the interfacial region of the membrane ([4, 13, 14, 52, 81]; see Fig. 2) and its location in the reverse micellar environment is most likely to be interfacial. The emission maximum of NBD-PE in AOT reverse micelles displays a progressive red shift with increasing w_o, implying that the polarity inside reverse micelles gradually increases with increasing w_o. NBD-PE was found to exhibit REES when incorporated in AOT reverse micelles [18]. Interestingly, the extent of REES was found to decrease with increasing w_o indicating that REES is sensitive to changing hydration dynamics (see Fig. 3a) and it is possible to detect differences in

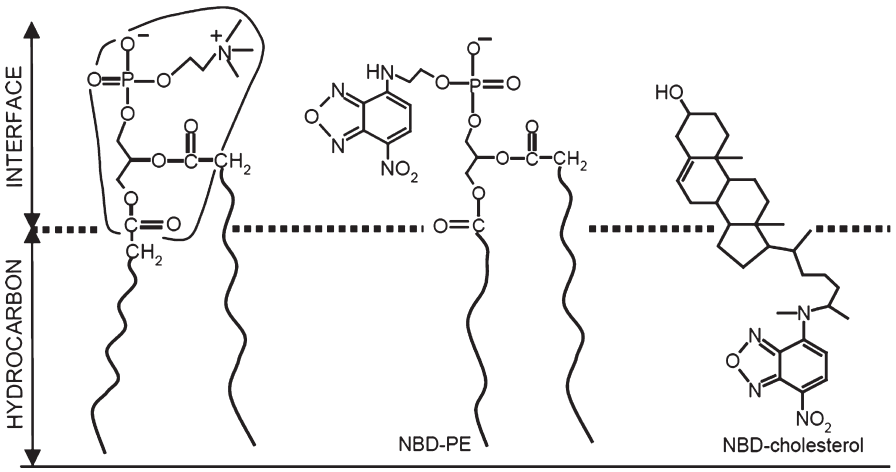

Fig. 2 A schematic representation of a leaflet of the membrane bilayer showing the localizations of the NBD groups of NBD-PE and NBD-cholesterol in phosphatidylcholine bilayer. The NBD group of NBD-PE localizes at the interfacial region while that of NBD-cholesterol resides at the nonpolar hydrocarbon region. These are time-averaged locations of the NBD group (in ns timescale) and fluctuations at shorter timescales are possible. The *horizontal line* at the *bottom* indicates the center of the bilayer. Adapted and modified from Mukherjee et al. [58]

water dynamics that is accompanied with increasing water content. The choice of a suitable probe is of considerable importance in wavelength-selective fluorescence studies of organized molecular assemblies [15–17]. NBD-PE is a suitable probe since the location of the NBD group in NBD-PE is expected to be interfacial, a region in the reverse micellar organization that is important from structural and functional aspects and is characterized by unique dynamics of water molecules. More importantly, we have earlier shown using solvatochromic and quantum chemical approaches that the dipole moment of the NBD group changes by ~4 D upon excitation [55], an important criterion for a fluorophore to exhibit REES effects [12]. These results show that REES is sensitive to the changing dynamic hydration profile (Fig. 3a) and can be conveniently used to probe dynamics of molecules in various states of hydration.

The ability of a fluorophore incorporated in a reverse micellar assembly to exhibit red edge effects depends on a number of factors, such as its polarity, as well as the effective polarity of its immediate environment, and its fluorescence characteristics (e.g., lifetime). Since all these properties of a probe are a function of its location in the reverse micelle, the extent of REES would depend on its location in the reverse micelle. In order to test this, we carried out REES experiments with NBD-cholesterol in which the NBD group is covalently attached to the flexible acyl chain of the cholesterol molecule (see Figs. 2 and 3b). The NBD group of this molecule has been found to be localized in the hydrocarbon region of the membrane [13, 14, 57, 67]

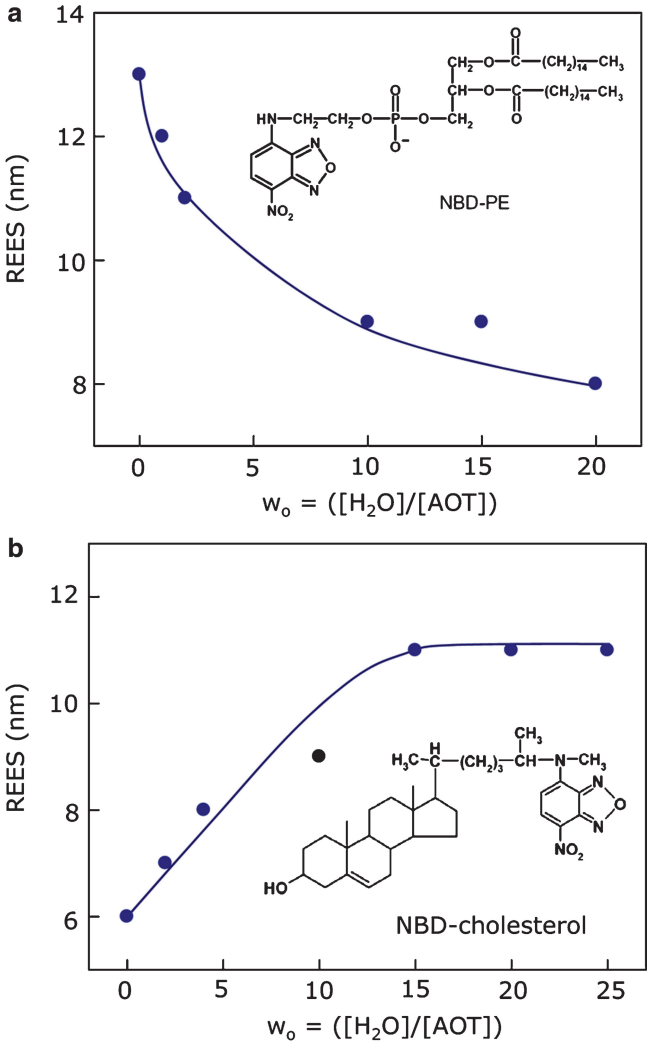

Fig. 3 Probe depth-dependent REES in reverse micelles. Effect of increasing amounts of water on the magnitude of REES of (**a**) NBD-PE and (**b**) NBD-cholesterol in reverse micelles of AOT. The *insets* show the chemical structures of NBD-PE and NBD-cholesterol. Adapted and modified from Chattopadhyay et al. [18] and Kelkar and Chattopadhyay [39]

and membrane-mimetic media, such as micelles [66]. Due to its deeper location, the NBD group of NBD-cholesterol is capable of reporting solvation dynamics in the deeper regions of the organized molecular assembly in which it is incorporated. This is important since unlike bulk solvents, compartmentalized molecular assemblies are anisotropic in nature and therefore display differential solvent relaxation

rates in different regions of the assembly [16]. In our previous work, we utilized the spatial localization of NBD-cholesterol in the membrane bilayer to monitor water penetration in the deeper hydrocarbon region of the membrane [17].

In contrast to what was observed in case of NBD-PE, the emission maximum of NBD-cholesterol in AOT reverse micelles does not exhibit any dependence on hydration (w_o), thereby confirming its deeper localization in the reverse micellar interior, a region characterized by reduced water penetration. Interestingly, REES of NBD-cholesterol incorporated in AOT reverse micelles exhibits increase with increasing water-to-surfactant ratio (w_o) (see Fig. 3b) [39]. This difference in the pattern of change of REES with w_o for NBD-PE and NBD-cholesterol could possibly be due to the deeper location of the NBD group in the reverse micellar assembly in the case of NBD-cholesterol. This implies that the rate of solvent relaxation (reorientation) varies with probe location. We have earlier shown such depth-dependent solvent relaxation with NBD-labeled lipid probes [17] and anthroyloxy-labeled fatty acid probes [16] incorporated in membranes. The above results bring out the important point that increasing w_o in an organized anisotropic molecular assembly, such as a reverse micelle, does not necessarily imply uniform polarity shifts in all regions of the reverse micelle. In other words, the change in polarity experienced in an anisotropic assembly is dependent on the position (location) of the incorporated probe.

To further explore depth-dependent polarity in reverse micelles, we designed experiments using the well-known series of anthroyloxy fatty acid probes. The anthroyloxy fatty acids in which an anthracene group is attached by an ester linkage to various positions of an alkyl chain have been extensively used as fluorescent probes of micellar and bilayer structures [3, 16, 80]. The anthroyloxy fatty acids are well-designed to monitor such position-dependent effects since they locate at a graded series of depths in the bilayer (see Fig. 4a). It has been shown that the depth of the anthroyloxy group in organized assemblies, such as a membrane bilayer, is almost linearly related to the number of carbon atoms between it and the carboxyl group [3, 80]. The fluorescence emission maximum of the shallow interfacial probe 2-AS is found to be extremely sensitive to a change in w_o from 0 to 25 which is accompanied by a red shift of the emission maximum by 15 nm (see Fig. 4b). Interestingly, the sensitivity of the emission maximum of the anthroyloxy probes appears to reduce as the position (location) of the anthroyloxy group increases from the water pool. In support of this, the red shifts in emission maximum of 6- and 12-AS are reduced to 6 and 2 nm, respectively, upon changing w_o from 0 to 25. The sensitivity of the emission maximum to hydration, therefore, appears to be dependent on the position of the fluorophore in the reverse micelle and decreases with increasing distance of the fluorophore from the water pool, that is, 2-AS > 6-AS > 12-AS.

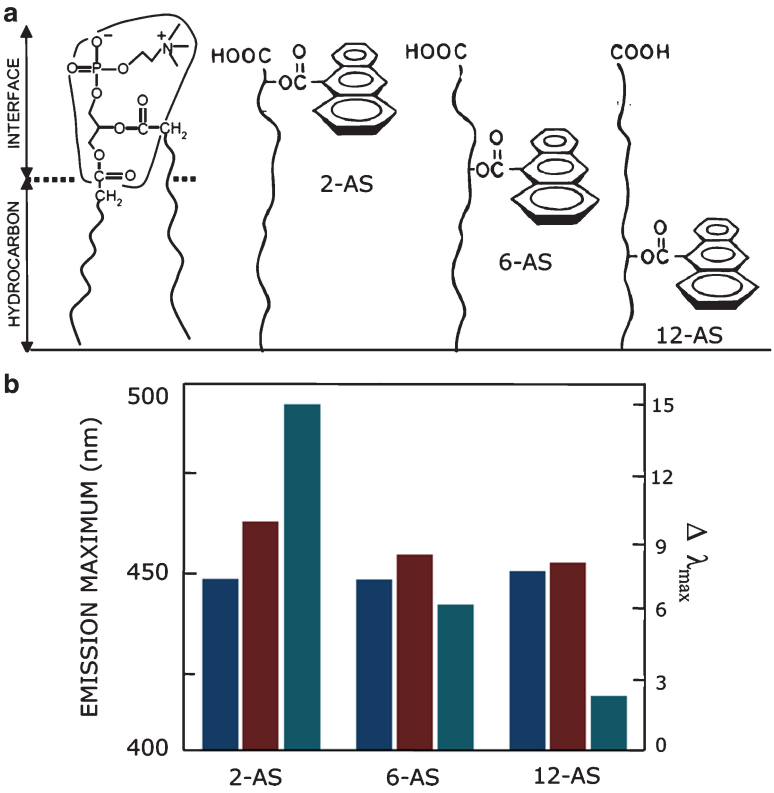

Fig. 4 (**a**) A schematic diagram of a leaflet of the membrane bilayer showing the localizations of the anthroyloxy groups of 2-, 6-, and 12-AS in phosphatidylcholine bilayers. The horizontal line at the bottom indicates the center of the bilayer. Adapted and modified from Chattopadhyay and Mukherjee [16]. (**b**) Effect of water on the wavelength of maximum emission of differentially localized anthroyloxy probes (2-, 6-, and 12-AS) in AOT reverse micelles. The *blue bars* represent emission maximum recorded at $w_o = 0$ and the *maroon bars* correspond to $w_o = 25$. The *cyan bars* represent the difference in emission maximum ($\Delta\lambda_{max}$) in these two cases. Adapted and modified from Kelkar and Chattopadhyay [39]

4 Peptides in Reverse Micelles: Effect of Depth Heterogeneity in Solvent Relaxation

Water has a crucial role in determining the folding, structure, dynamics, and, in turn, the function of proteins and peptides [20, 26, 48, 76, 83]. It is estimated that a threshold level of hydration (less than 0.4 grams of water per gram of protein) is required to fully activate the dynamics and function of globular proteins [6]. Knowledge of the dynamics of hydration at the molecular level is of considerable importance in understanding cellular structure and function since water plays a crucial role in the formation and maintenance of organized molecular assemblies,

such as proteins and membranes [51]. In particular, hydration has been shown to be a crucial parameter in protein folding and it has been suggested that water-mediated interactions could guide the folding process even before the formation of native contacts [59]. Interestingly, water has been shown to act as a catalyst for hydrogen bond exchange in protein folding, thereby acting as a "foldase" [83]. Reverse micelles represent popular assemblies for studying hydration effects on peptide and protein structure and dynamics [21, 46, 47]. Proteins trapped in reverse micelles are widely utilized in protein biotechnology [50]. As mentioned above, the entrapped water in reverse micelles has properties that are markedly different from the properties of bulk water but similar in several aspects to those of biological interfacial water as found in membranes or protein interfaces [10, 36, 38, 79]. The interfacial water is crucial for the induction of secondary structure in peptides and proteins when bound to surfaces, such as membranes or micelles, as well as for variation of their local internal motion. Confinement of a peptide chain within a restricted environment has been reported to increase the relative stability of the folded state against unfolded states [2]. This observation has potential biological relevance since intrinsically disordered proteins could be structured in their native cellular environment. In such a scenario, it becomes important to monitor the effects of hydration on the conformation and dynamics of proteins and peptides. Small peptides (such as melittin and gramicidin) are particularly suitable for such hydration studies due to high surface/volume ratio.

Melittin is the principal toxic component in the venom of the European honey bee *Apis mellifera* and is a cationic, hemolytic peptide (for a review, see [64]). It is a small, linear peptide composed of 26 amino acid residues (sequence shown in Fig. 5) in which the amino-terminal region is predominantly hydrophobic, whereas the carboxy-terminal region is hydrophilic due to the presence of a stretch of positively charged amino acids. This amphiphilic property of melittin makes it water soluble, and yet it spontaneously associates with natural and artificial membranes and membrane mimetics. Melittin adopts predominantly random coil conformation as a monomer in aqueous solution [63]. Interestingly, melittin adopts an α-helical conformation when bound to membranes, micelles, or reverse micelles [30, 62, 64]. Under conditions of low w_o in reverse micelles, increasing hydration causes an increase in helicity of melittin [62]. Melittin is intrinsically fluorescent due to the presence of a single tryptophan residue, Trp-19, which makes it a sensitive probe to study the interaction of melittin with membranes and membrane-mimetic systems.

Melittin, when incorporated in reverse micelles of AOT, exhibits REES implying that localization of the peptide in reverse micelles results in considerable restriction to the reorientational motion of the solvent dipoles around the excited state tryptophan ([62]; see Fig. 5). The interfacial region of reverse micelles is associated with bound water with characteristic dynamics [10, 36, 38, 79]. Since REES arises because of the rate of reorientation of solvent molecules, these results therefore assume significance in the context of reports of slow (ns) water relaxation in reverse micelles [9, 34]. Interestingly, the extent of REES decreased with increasing w_o (see Fig. 5). Figure 5 shows that as the water content of the reverse micellar system increases, the magnitude of REES decreases gradually until $w_o = \sim 8$ is reached.

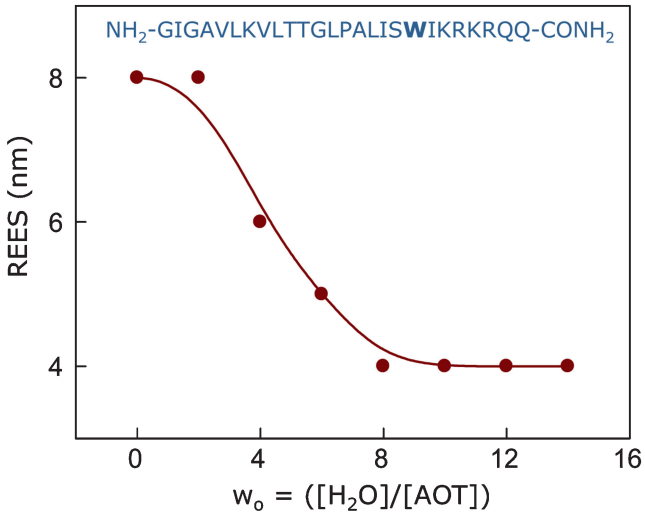

Fig. 5 Hydration-dependent REES of a surface-active hemolytic peptide. Effect of increasing amounts of water on the magnitude of REES of melittin in reverse micelles of AOT. The *inset* shows the amino acid sequence of melittin (the sole tryptophan residue is highlighted). Adapted and modified from Raghuraman and Chattopadhyay [62]

At $w_o > 8$, REES attains a more or less steady value and becomes less sensitive to further addition of water into the system. This essentially means that there is a reorganization of water molecules in the reverse micellar assembly upon increasing w_o from 0 to 8. This is in excellent agreement with earlier reports in which it was shown that water relaxation rates in reverse micelles become faster with an increase in w_o [69]. This suggests that the overall motional restriction experienced by the reorienting solvent molecules is reduced as more water is added to the reverse micelles. Similar results have previously been obtained for amphiphilic probes, such as hemicyanine dye [35] or fluorescent phospholipid ([18]; Fig. 3a), incorporated in reverse micelles.

The linear peptide gramicidin forms prototypical ion channels specific for monovalent cations and has been extensively used to study the organization, dynamics, and function of membrane-spanning channels [41]. Gramicidin is a multitryptophan protein (Trp-9, 11, 13, and 15) which serves as an excellent model for transmembrane channels due to its small size, ready availability, and the relative ease with which chemical modifications can be performed. This makes gramicidin unique among small membrane-active peptides and provides the basis for its use to explore the principles that govern the folding and function of membrane-spanning channels in particular and membrane proteins in general. The unique sequence of alternating L- and D-chirality (see Fig. 6) renders gramicidin sensitive to the environment in which it is placed. The head-to-head (amino terminal-to-amino terminal), single-stranded $\beta^{6.3}$ helical dimer form is the cation conducting channel conformation of gramicidin in membranes. In this conformation, the carboxy terminus is exposed to

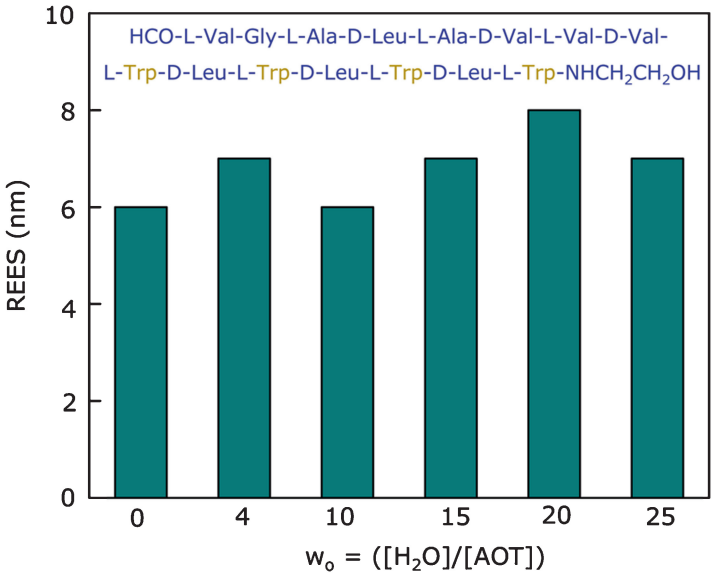

Fig. 6 Hydration-dependent REES of an ion-channel peptide. Effect of increasing amounts of water on the magnitude of REES of gramicidin in reverse micelles of AOT. The *inset* shows the amino acid sequence of gramicidin. Notice the unique alternating L- and D-chirality in gramicidin. Data taken from Kelkar and Chattopadhyay [40]

the membrane–water interface and the amino terminus is buried in the hydrophobic core of the membrane. This places the tryptophan residues clustered at the membrane–water interface at the entrance to the channel [42, 54].

Gramicidin assumes single-stranded $\beta^{6.3}$ helical conformation in AOT reverse micelles and the tryptophan residues experience motional restriction and exhibit REES [40]. This implies that the tryptophans in the gramicidin single-stranded $\beta^{6.3}$ conformation, on the average, are localized in a motionally restricted region of the reverse micelle. Interestingly, the magnitude of REES is found to be more or less independent of w_o (see Fig. 6). Gramicidin is a multitryptophan peptide, and therefore the REES may be indicative of the average environment experienced by the tryptophans. The locations of these tryptophans would, therefore, be heterogeneous in the reverse micelle. The presence of tryptophans at various locations would contribute to spectral heterogeneity (and also gives rise to spectral broadening).

The overall invariance of REES with water content in the reverse micelle is surprising. This is because, as mentioned above, the extent of REES has generally been shown to decrease with increasing w_o for probes and peptides incorporated at the reverse micellar interface ([18, 35, 62]; also see Figs. 3a and 5) and in the water pool [70]. This indicates that addition of water to the reverse micellar system in these cases leads to a reduction in the overall motional restriction experienced by the reorienting solvent molecules in the region of localization of the fluorophore. However, this has been shown to be not true for probes localized in the deeper acyl

chain regions of the reverse micellar assembly. Thus, in case of NBD-cholesterol in which the fluorescent NBD moiety is positioned at a deeper acyl chain location in the reverse micellar assembly, the extent of REES increases with increasing w_o ([39]; see Fig. 3b). *This implies that the rate of solvent relaxation (reorientation) varies with probe location in the reverse micellar assembly.* In the background of these results, the relative invariance of the magnitude of REES with increasing w_o in case of gramicidin in AOT reverse micelles (Fig. 6) presents an interesting case. Gramicidin is a multitryptophan protein (Trp-9, 11, 13, and 15) and the location of these tryptophan residues would be heterogeneous in the reverse micelle. While the carboxy-terminal tryptophan (Trp-15) would occupy an interfacial position, the tryptophan residue at position 9 (Trp-9) would be placed in a relatively deep acyl chain region of the reverse micelle in the single-stranded $\beta^{6.3}$ conformation. The overall variation in the extent of REES with increasing w_o would then be dependent on the average of the variations with individual tryptophans. This could explain the apparent insensitivity of the magnitude of REES to increasing w_o for gramicidin in AOT reverse micelles.

5 "Solvent" Relaxation in Green Fluorescent Protein Incorporated in Reverse Micelles

GFP from the jellyfish *Aequorea victoria* and its variants have become popular reporter molecules for monitoring protein expression, localization, and dynamics of membrane and cytoplasmic proteins in a relatively short span of time [31, 32, 77]. GFP possesses characteristics that are highly desirable for use as a reporter molecule. These include its intrinsic, cofactor-independent fluorescence which exhibits remarkable stability in the presence of denaturants and over a wide range of pH. GFP has a compact, barrel-shaped structure made of 11 β strands with an α helix running through the central axis of the cylindrical structure (see Fig. 7). The fluorophore of GFP responsible for its green fluorescence is localized at the center of the cylindrical structure and is formed spontaneously upon folding of the polypeptide chain by internal cyclization and oxidation of the residues Ser65-Tyr66-Gly67 in the α helix (Fig. 7). The fluorophore is in a highly constrained environment, protected from the bulk solvent by the surrounding β strands (see Fig. 7). This results in a small Stokes' shift and a high-fluorescence quantum yield. Although various photophysical aspects of GFP have been extensively studied, the phenomenon of solvent dipolar relaxation around the GFP fluorophore is only beginning to be addressed [1, 29].

Due to slow rate of solvent dipolar reorientation ("solvent" here refers to the dipolar protein matrix) around the excited state fluorophore, enhanced GFP (EGFP, a variant of GFP) exhibits REES in buffer and in glycerol ([29]; see Fig. 7). EGFP displays REES when incorporated in AOT reverse micelles. Interestingly, Fig. 7 shows that REES of EGFP in reverse micelles is completely independent of the reverse micellar hydration state (w_o). Neither entrapment in a reverse micelle nor

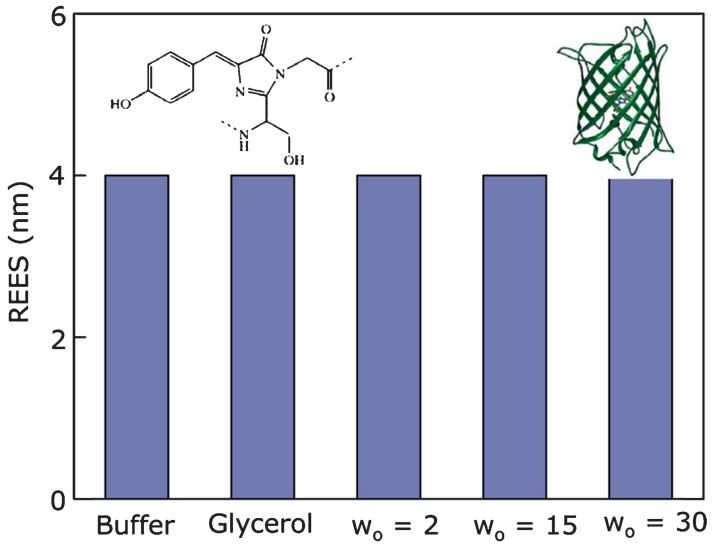

Fig. 7 Hydration-independent REES of a green fluorescent protein (GFP). Effect of increasing amounts of water on the magnitude of REES of EGFP in reverse micelles of AOT. The *inset* shows the GFP fluorophore (*p*-hydroxybenzylideneimidozolidinone) and the β-barrel structure of GFP. It turns out that the observed REES of EGFP is due to the constrained environment experienced by the EGFP fluorophore in the rigid protein matrix, rather than due to the dynamics of the host reverse micellar assembly. Adapted and modified from Haldar and Chattopadhyay [29]

the hydration state of the reverse micelle appear to influence the magnitude of REES of EGFP (see Fig. 7). This implies that the extent of REES of EGFP is independent of the viscosity and hydration of the surrounding medium, implying that the dynamics of the protein matrix, rather than the dynamics of the surrounding medium, plays an important role. Interestingly, it has been previously shown by measurement of kinetics of proton transfer in EGFP that the dynamics in the interior of the protein is very weakly coupled to viscosity changes of the bulk medium [71].

6 Conclusion

Water confined on nanometer-length scales is found in many physical and biological environments. As mentioned earlier, the dynamics of liquids in confined spaces is different from that of their bulk counterparts [10, 28, 44], and this constitutes one of the main reasons for the popularity that reverse micelles enjoy as a model system in studies of water dynamics. The highly structured yet heterogeneous water molecules in reverse micelles represent interesting models for water molecules present in biological systems, such as membranes, which are more difficult to analyze experimentally. Moreover, the dimension, shape, and overall charge of reverse micelles

can be conveniently modulated which make them particularly useful for monitoring dynamics of confined liquids. The interior dimensions of reverse micelles are believed to be similar to confined spaces found in cavities in biomolecules. These nanopockets of water are thought to be involved in folding and relaxation in proteins. In addition, reverse micellar structures are proposed to be formed during endosome formation [44].

The focus of this article is the change in confined hydration dynamics with increasing hydration, monitored by REES. We discuss here that while solvent relaxation for probes and peptides incorporated in reverse micelles is generally dependent on the extent of hydration (w_o), this effect is dependent on the position (location) of the probe in the reverse micellar assembly. For molecules with multiple fluorophores at various locations (such as gramicidin), positional heterogeneity could complicate the observed hydration effects. An interesting case is presented by EGFP, where the extent of REES appears to be independent of hydration, since the dynamics of the protein matrix is the important determinant in such a case. We conclude that change in solvent relaxation with hydration could be context dependent and care should be exercised in interpreting such results.

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