Dynamics of Polar Solvation in Lecithin/Water/Cyclohexane Reverse Micelles

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Abstract: The solvation dynamics of water in lecithin/cyclohexane reverse micelles have been determined via ultrafast time-resolved fluorescence studies. At hydration levels $w_0 \leq 4.8$, the reverse micellar samples are nonviscous. Here a single relaxation time is observed that is much longer than the response of free or bulk water. In contrast, small additions of water to the samples produces a viscous gel, referred to by others as an organogel or "living polymer". At hydration levels of $w_0 \geq 5.8$, three relaxation times are observed with approximate time constants of 0.5, 15, and 200 ps, the shortest of which correlates to free water motion. The dynamics reveal no evidence of micelle crossover or branch points associated with gel formation. In comparison to Aerosol OT reverse micelles of similar hydration, the water in the lecithin reverse micelles is significantly more restricted. It is proposed that lecithin sequesters significantly more water than previously predicted precluding formation of distinct core water pools in the micelles. The results are also compared to models for aqueous structure and dynamics near phospholipid membranes and to bulk water dynamics.

I. Introduction

When surfactants are dispersed in nonpolar solvents, reverse (or inverted) micelles can form spontaneously.[1] If water is present, the inverted portion of the micelle can swell, creating dispersed water droplets delineated by the layer of surrounding surfactant molecules.[2] The polar end of the surfactant, or headgroup, points into the aqueous pool, while the hydrophobic alkyl chains are directed into the continuous organic phase. These aqueous pools are thermodynamically stable, and their sizes and shapes can often be controlled.[3] The micellar matrix can be controlled by adjusting variables such as water content, surfactant concentration, and temperature. Reverse micelles can deliver polar solutes to nonpolar solutes present in the organic phase allowing cosolvation of both polar and nonpolar reactants in a single phase.[4] Molecules trapped in the reverse micelles have shown enhanced behavior. For example, pharmaceuticals have displayed enhanced structural and conformational stability and slow diffusivity,[5] and certain enzymes manifest enhanced activity at certain hydration levels.[5]

Reverse micelles have been used as biological membrane models to aid in understanding membrane chemistry.[2] Biomembranes are composed, in part, of lipid bilayers that consist of two sheets of aggregated lipids arranged so that the polar headgroups face out into the aqueous environment while the hydrophobic alkyl chains face into one another. Reverse micelles, therefore, represent a simplified lipid membrane containing only one water/lipid interface. At the same time, some features of the interfaces bear resemblance to biological membranes. For instance, water solubilized in reverse micelles has restricted mobility and a depressed freezing point and lacks the normal hydrogen-bonded structure present in bulk water.[6] Because interfacial interactions should be similar for both reverse micelles and lipid bilayers, studies of one system are frequently compared to studies of the other.[7]

The water solubilized within reverse micelles influences micelle radius, shape, and polar headgroup packing.[2] As a result, micelle properties are often characterized using w_0

$$w_0 = \frac{[H_2O]}{[\text{surfactant}]} \tag{1}$$

the ratio of the molarity of water to the molarity of the surfactant. Additionally, the lipid interface influences the water sequestered in the micellar interior. A variety of methods have been used to interrogate this perturbed water structure.[8] Vibrational and NMR spectroscopy have revealed distinct water environments based on their proximity to the surfactant interface. Finer[9] formally proposed that water shells are sequentially filled as hydration of lipid bilayers increases. Each shell contains a water type that becomes progressively more bulklike as hydration increases. The first water type is considered to be strongly bound to the surfactant headgroup, while the final type is considered to be bulklike. Although results vary, several studies have found the existence of three water types in various reverse micellar systems.[6,10]

Two very different surfactants commonly used in the formation of reverse micelles are Aerosol OT (AOT) and lecithin.

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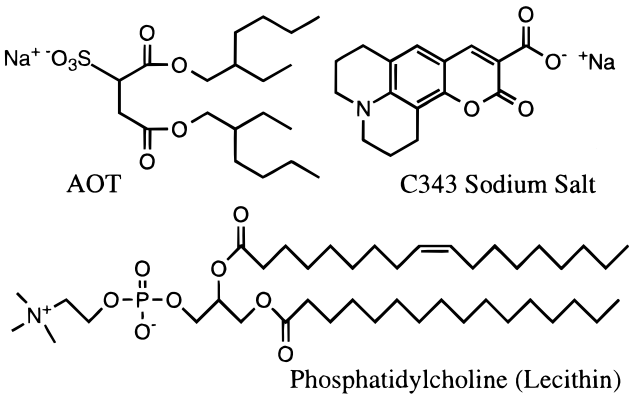

Figure 1. Chemical structures of the two surfactants and solvation dynamics fluorescent probe molecule used in this work.

The chemical structures of each are displayed in Figure 1. AOT is a synthetic anionic surfactant that forms spherical micelles and can solubilize large amounts of water, as high as $w_0 = 60$.[8,11] On the other hand, lecithin is a naturally occurring phospholipid that can be purified from cellular membranes. Figure 1 shows phosphatidylcholine which is the main component of lecithin. Unlike AOT, lecithin has a zwitterionic headgroup.

Lecithin reverse micelles in many nonpolar solvents possess the unusual property of forming cylindrical tubes when small amounts of water are added to the microemulsion. At a constant surfactant concentration with increasing water loading, these tubular reverse micelles extend into wormlike structures crossing one another and forming a transient entangled network.[12,13] Macroscopically, the system appears as a viscous gel, often termed an organogel.[14] The gel is thermostable, thermoreversible, and isotropic.[13] If viscosity of a lecithin/organic solvent sample is measured vs water addition, a maximum viscosity is reached at a given hydration level, sometimes called the percolation threshold. Further hydration results in a gradual decrease in viscosity until a phase separation point is reached.[13] While organogels may be formed in many organic solvents, cyclohexane allows greater hydration of the lecithin micelles before phase separation occurs than either linear or branched alkanes.[4]

Lecithin organogels have recently attracted attention for several reasons. Because lecithin is a natural phospholipid, it can be used in biological applications. In this role, it can be used to deliver pharmaceuticals and cosmetics and is preferable to bioincompatible surfactants such as AOT.[2] Direct comparison between lecithin reverse micelles and lipid bilayers can be drawn since the water-headgroup interactions should be very similar.[15] Furthermore, the role of molecular structure in macroscopic gel formation remains a particularly challenging question.[2,16] Current theory juxtaposes the static properties of the transient network to semidilute solutions of flexible polymers,[17,18] hence the term "living polymers". However, details of the microscopic interactions of the cylindrical micelles remains a point of contention.[16,19]

The role of water in organogel formation is not well understood. Indeed, lecithin hydration has been studied via a variety of techniques. It is well-known that the structure of water at phospholipid interfaces differs significantly from bulk water. In addition, the dynamics of water also differ from bulk water. Several studies have investigated the dynamical nature of the water/lecithin interface. For example, using ^{2}H NMR, several groups have probed the mobility of water at phosphatidylcholine bilayer surfaces.[20,21] Whaley Hodges et al. investigated the relaxation of water in aqueous palmitoyloleoylphosphatidylcholine vesicles using proton NMR with nitroxide spin labels.[22] Enders and Nimtz[23] and Klosgen et al.[24] probed the dielectric relaxation of lecithin bilayers at microwave frequencies. Dynamical properties of various phosphatidylcholine lipid−water interfaces have also been studied via spectroscopy of fluorescent probe molecules.[25] The major finding of all these studies is that water mobility is reduced at the lipid interface and increases with increasing hydration. However, none of these techniques are capable of measuring the ultrafast motion of water, hence they are incapable of distinguishing water interacting with the interface unless its dynamical response is slowed by orders of magnitude.

One method that has successfully tracked water motion is solvation dynamics measurements. In these experiments, ultrafast time-resolved spectroscopies, such as time-resolved fluorescence,[26−28] time-resolved optical Kerr effect,[29,30] and photon-echoes,[31,32] measure the dynamic response of solvent molecules to an instantaneous electric perturbation. Solvation dynamics have been studied for a wide range of bulk liquids and for some restricted environments. For all solvents, there exist two components to the solvation dynamics: an ultrafast, sub 100 fs inertial component and slower diffusive components.[33] Solvation dynamics of water have been measured[34] and show a large (>50%) sub 50 fs inertial component. Two additional diffusive components comprise the dynamics, with time constants of ∼200 and ∼600 fs. The solvation dynamics can be characterized by a solvent correlation function

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$$C(t) = \frac{\nu(t) - \nu(\infty)}{\nu(0) - \nu(\infty)} \quad (2)$$

where $\nu(t)$, $\nu(\infty)$, and $\nu(0)$ are related to a spectral feature of the system, such as the peak fluorescence frequency of a probe molecule, at time *t*, at equilibrium, and instantaneously after the perturbation.

While the large fraction of past studies have concentrated on dynamics in bulk solutions,[26−29,35−38] a few recent studies have probed solvation dynamics in restricted environments. Yanagimachi et al.[39] measured the dynamics of 1-butanol at a sapphire surface, while Bessho et al.[40] studied the fluorescence decays of 1,8-anilino-8-naphthalenesulfonic acid at the water−heptane interface. In both these studies, the polar solvent response observed was slower at the interface than in bulk. Sarkar et al. have probed microemulsion systems with oil-in-water micelles[41] and water-in-oil reverse micelles.[42] Their experiments revealed additional long time, nanosecond components to the water dynamics. In addition, water solvation dynamics in zeolites measured by coumarin 480 has been attributed to the motion of the mobile sodium ions and/or the motion of the probe molecules and is similar to ionic solvation dynamics significantly slower than bulk water.[43] While the observed dynamics in all these studies were attributed to solvation dynamics, the time resolution was >50 ps, hence the normal ultrafast water response would not be observable. However, Vajda et al. have measured the dynamics of water solvation within cyclodextrin cavities using subpicosecond resolution.[44] At short times, <0.2 ps, the solvation dynamics in the cyclodextrin cavity is similar to that in bulk water. At longer times, ≥0.2 ps, the solvation dynamics in the cyclodextrin cavity is observed to be significantly slower compared to bulk water displaying additional long time components to the relaxation. These were attributed to probe molecule motion in and out of the cavity as well as water molecules constrained by binding to the cyclodextrin.[44,45]

Of particular relevance to these studies are recent investigations into solvation dynamics in AOT reverse micelles. We have used ultrafast time-resolved fluorescence techniques to probe the motion of water and formamide in AOT reverse micelles.[46−48] These experiments show that the water inside the reverse micelles is significantly immobilized compared to the bulk solvents. Mittleman et al.[49] have probed the dielectric properties of water in Aerosol OT reverse micelles using subpicosecond terahertz spectroscopy. Both the time scale and amplitude of the relaxation they observed had significantly smaller amplitude than bulk water and was attributed to reduced long-range collective behavior resulting from the small water pool in the reverse micelles.

In this paper, we report the first ultrafast measurements of the aqueous solvation dynamics in lecithin reverse micelles using the fluorescence upconversion technique. By comparing the dynamics within reverse micelles comprised of surfactants of different molecular structures, that is lecithin and AOT, we probe the surfactant interactions that dictate the dynamics of the solubilized water. In addition, we probe the dynamics of lecithin reverse micelles as a function of hydration. The dynamics are correlated with present knowledge of water structure in micelles based primarily on FTIR and NMR techniques. We also explore the relation between the aqueous dynamics and lecithin organogel formation. Finally, we compare our results for water dynamics within lecithin reverse micelles to studies of aqueous structure and dynamics near phospholipid bilayers.

II. Experimental Section

A. Sample Preparation. Lyophilized egg phosphatidylcholine (lecithin) was purchased from Avanti Polar Lipids, Inc. A typical lecithin structure is shown in Figure 1. Samples were stored at −10 °C prior to use and were used without further purification. Lecithin samples were always prepared from newly opened bottles to minimize water adsorption. Using the method of standard additions, ^{1}H NMR analysis (Bruker AM·500 MHz Spectrometer) revealed 1.8 ± 0.2 mol H_2O per mol lecithin. Aerosol-OT (AOT, sodium bis-2-ethylhexylsulfosuccinate, Aldrich) was dried over molecular sieves (Grade 522 5 Å, Mallinckrodt) in diethyl ether, filtered, and stored as a precipitate under nitrogen gas. Analysis by Karl-Fisher titration showed the dried AOT contained 0.037 ± 0.002 mol H_2O per mol AOT. All reported w_0 values for both lecithin and AOT samples include this intrinsic water, that is, the w_0 values refer to dry surfactant. Lecithin micelles were prepared with cyclohexane (spectrophotometric grade, 99+%, Acros), while AOT micelles were prepared with isooctane (2,2,4-trimethylpentane, HPLC grade, Aldrich). Cyclohexane and isooctane were used without further purification. Added water was of high purity (Milli-Q filtered, 18.2MΩ/cm^2 resistivity).

Coumarin 343 (C343, Exciton, Inc.) was used as the fluorescent probe molecule. Coumarin dyes are commonly used solvation dynamics probes because of their rigid structure, nanosecond lifetimes, significant Stokes shift, and absorption and emission spectra that show solvent polarity dependence.[36,50] We use C343, in particular, because it is more readily solvated in aqueous environments than other dyes. The aqueous solubility of C343 was enhanced via the sodium salt, shown in Figure 1, prepared by titrating an aqueous solution of C343 with sodium hydroxide to pH = 7, evaporating the excess water, and storing under nitrogen. When placed in either a pure cyclohexane or water/cyclohexane biphasic solution, the sodium salt form of C343 showed no detectable UV/vis absorbance or fluorescence in cyclohexane.

Reverse micellar samples were prepared as follows: lecithin was weighed into 10 mL of cyclohexane to make 5% (w/v) solutions. Assuming a lecithin density of 0.95 g/cm^3,[51] this corresponds to a volume fraction of ca. $\phi = 0.047$.[52] Water was added with a microliter syringe (Hamilton) to make a $w_0 = 3.8$ solution. Next, excess C343 sodium salt was added to the prepared solutions. Samples were briefly sonicated and then shaken for at least 12 h. Samples were then heated to 35 °C and subsequently filtered through 0.1 μm syringe filters (PTFE,

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Whatman). Differing quantities of water were added to the samples with the microliter syringe to create the final samples. Samples were again shaken for at least 12 h before use. The AOT $w_0 = 5$ sample was prepared in a similar fashion except the AOT was weighed into isooctane to create 10% (w/v) solutions, corresponding to a volume fraction of ca. $\phi = 0.10$.[47]

B. Sample Characterization. Absorption spectra were recorded with a Cary 2400 UV−vis−NIR spectrophotometer. Samples exhibited absorbances between 0.22 and 0.35 at 410 nm, measured in a 1 mm quartz cell. While the absorbance of the C343 dye in the samples dropped as much as 46% during the course of upconversion measurements, no shift of the absorbance or fluorescence spectra was observed indicating that the chromophore of the dye was unchanged.

Using $\epsilon_{C343} = 1.99 \times 10^{-4}$ L/mol·cm[53] and $N_{agg} = 3000$ as the average micellar aggregation number of $w_0 = 4.8$ lecithin micelles,[54] we estimate that there are seven C343 molecules per micelle. This would correspond to an aqueous concentration of about 3 mM within the micellar interior. At this concentration, it is conceivable that C343 could be aggregating. However, there are no reports of coumarin aggregation in the literature. Moreover, absorption spectra for C343 sodium salt in bulk water over a concentration range from 0.025 to 2.5 mM revealed no evidence of aggregating species.

Fluorescence spectra were collected with a home-built fluorometer.[55] Fluorescence lifetimes were determined by time-correlated single-photon counting (TCSPC) measurements using a home-built instrument.[56] Lifetime measurements of C343 within reverse micelles relative to bulk water showed no significant quenching. Lifetimes obtained from TCSPC were used as fixed parameters in the fits of upconversion transients.

Because dryness, purity, and temperature are variables that can influence the point at which gel formation is seen from researcher to researcher, we performed viscosity measurements on the different lecithin samples for comparison. Viscosity measurements were made with a home-built viscometer. Depending on the sample's viscosity, stainless steel or glass balls were timed as they dropped down a glass tube filled with a given sample. Time measurements were converted to viscosity by calibrating the apparatus with solvents of known viscosity.[57] At 25 °C, steel balls were calibrated with glycerol, while glass balls were calibrated with cyclohexane. Sample measurements were performed at 21.5 °C. The viscosity of the different samples we analyzed were as follows: $w_0 = 4.8$ measured 0.1 cP, $w_0 = 5.8$ measured 5000 cP, and $w_0 = 6.8$ measured 20 000 cP. In comparison, lecithin in isooctane is reported to produce an organogel with a viscosity of 10^6 cP,[58] while the viscosity of pure cyclohexane is 0.09 cP.[57] These measurements clearly show that macroscopically the samples change from a very fluidlike state at $w_0 = 4.8$ to a viscous gel state at $w_0 = 6.8$.

When placed on a glass slide and observed through a Zeiss Axiophot compound microscope fitted with cross polarizers, samples showed no birefringence. The isotropic nature of these reverse micelles is in accord with previous characterizations.[13,59]

C. Femtosecond Fluorescence Upconversion Apparatus. The fluorescence upconversion spectrometer used in these experiments has been described in detail previously.[47] Briefly, a mode locked Ti:sapphire laser produced output pulses centered at 820 nm, with a duration of 75−90 fs (fwhm assuming Gaussian pulse shape), at 100 MHz repetition rate, and with energies of 6.5 nJ/pulse. Frequency-doubled laser pulses served to excite the sample, while the residual fundamental light was used to gate the sample fluorescence in a nonlinear BBO crystal. The sum-frequency of the fluorescence and the gate pulse was detected as a function of the time delay between excitation and gate pulses. The upconverted light passed through a monochromator and was detected with a photomultiplier tube. Signals were collected via photon counting interfaced to a computer. Samples were circulated with a peristaltic pump (Masterflex, Model 7553-70) fitted with PTFE tubing to ensure the integrity of the samples. The sample reservoir was submerged in a water bath kept at 21.5 ± 0.5 °C.

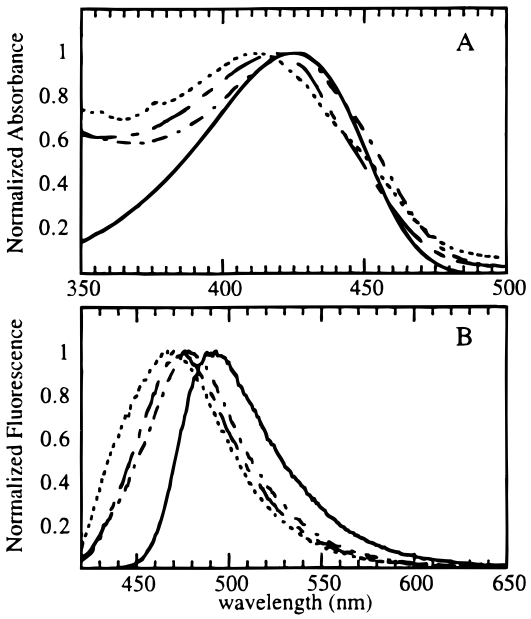

Figure 2. Absorption (A) and emission (B) spectra of coumarin 343 sodium salt in $w_0 = 4.8$ (···), $w_0 = 5.8$ (- − - −), $w_0 = 6.8$ (- · -) lecithin reverse micelles, and bulk water (—).

III. Analysis and Results

We have measured the spectral response of C343 as a probe of solvation dynamics in lecithin reverse micelles. An ideal solvation dynamics probe should exhibit a solvent dependence of the fluorescence Stokes shift.[50] In other words, in polar solvents the fluorescence spectrum of a solvated probe should shift to longer wavelengths because the solvent has a greater capacity to stabilize an excited probe molecule via reorganization.[36,50] Our previous studies have shown that C343 dissolved in various solvents displays the correct spectral shifting.[52] We have also shown that C343 dissolved in AOT reverse micelles shows a continuous spectral shift to longer wavelengths as hydration is increased, indicating the probe responds to the increased micelle polarity as the water content increases.[47] Figure 2 reports the absorption and emission of C343 in the lecithin micelles as a function of hydration in comparison to C343 solvated in bulk water. As seen with AOT, the absorption and emission both shift to longer wavelengths with increasing hydration. Because the emission spectrum shifts with hydration, we conclude that C343 probes the aqueous interior of the lecithin reverse micelles and is, therefore, sensitive to the aqueous dynamics within them.

The solvation dynamics for water in $w_0 = 4.8$, $w_0 = 5.8$, and $w_0 = 6.8$ lecithin in cyclohexane reverse micelles and $w_0 = 5$ AOT in isooctane reverse micelles are reported here. The time-resolved fluorescence measurements require significant analysis, and, although the methods used to determine the dynamics are very similar to those described in detail elsewhere,[47] we outline the data analysis used here as we report the results.

We obtained single-wavelength fluorescence transients over a wavelength range from roughly 440 nm to 530 nm in 10 nm increments. These decays were acquired over various time

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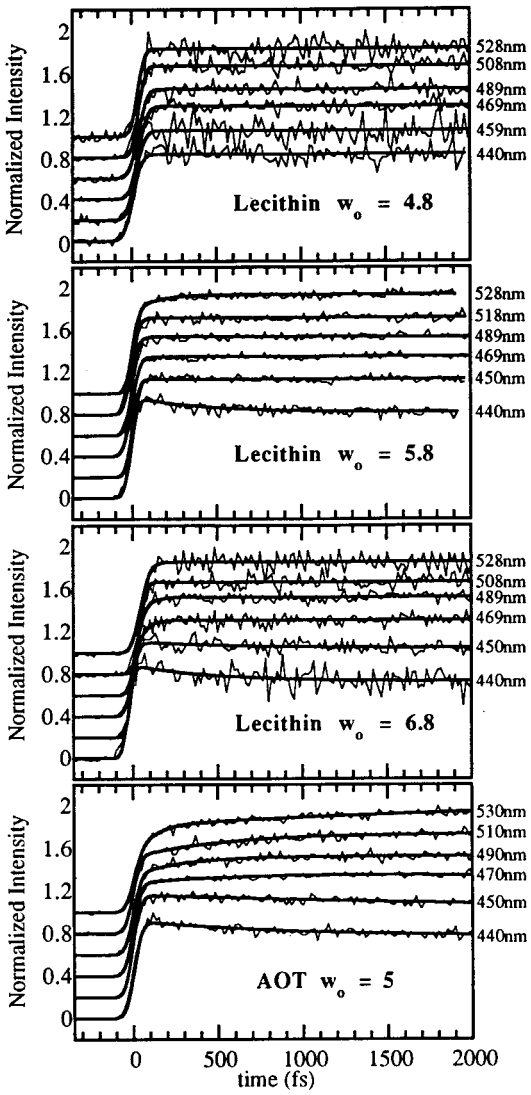

Figure 3. Short time (2 ps) fluorescence transients for different reverse micellar environments with multiexponential fits.

periods, that is, 2 and 477 ps. The time step size was adjusted to total approximately 250 time steps per scan. Thirty-three ps scans were also acquired for selected wavelengths of $w_0 = 6.8$ lecithin and $w_0 = 5$ AOT micelles in order to resolve dynamics on the 2−15 ps time scale. From all the fluorescence transients, we generate time-resolved spectra. If the fluorescence spectrum shifts to longer wavelengths in time, then the individual fluorescence transients at wavelengths shorter than the fluorescence maximum reveal a decay while those at longer wavelengths display an initial rise before eventually decaying. Figure 3 shows 2 ps fluorescence transients at selected wavelengths for each micellar sample. The responses for $w_0 = 4.8$ lecithin micelles are flat, revealing no solvent relaxation processes over this time period. In contrast, the transients for the AOT sample, and to a lesser extent the $w_0 = 5.8$ and $w_0 = 6.8$ lecithin samples, show significant relaxation on this time scale.

The individual fluorescence transients are fit to multiexponential functions. For the 2 ps scans the instrument response must be accounted for because components of the solvent response occur on a time scale similar to the laser pulse duration. This is accomplished by deconvoluting the instrument response function from the multiexponential transient fit using an iterative reconvolution analysis program. A Gaussian function fit to the cross-correlation signal of the pump and gate pulses is used as the instrument response function. Transients in Figure 3 include the fits obtained by this method. Longer time scans are fit to a multiexponential decay using KaleidaGraph software (Synergy Software) without the instrument response deconvolution because the time step size is too large to define the instrument response accurately. Fits are normalized at the 477 ps time delay with respect to their individual steady-state fluorescence spectra shown in Figure 2. The time-resolved fluorescence decays obtained over shorter periods of time are normalized to reconstructed fluorescence spectra obtained from the next longest transient set. These normalized fits are then used to reconstruct the time-resolved fluorescence spectra at selected times, shown as insets in Figure 4. By definition, the 477 ps spectra are the same as the steady-state spectra displayed in Figure 2.

Time-dependent fluorescence maxima ($\nu_{max}(t)$) were found by fitting each reconstructed spectrum to a log-normal line shape. The $\nu_{max}(t)$ values were used in eq 2 to obtain the time correlation function, C(t). Figure 4 shows the C(t) points fit with multiexponential decays. Table 1 summarizes the numerical results of the fits displayed in Figure 4. For comparison, Table 1 also displays the spectral response of C343 in bulk water that we have previously obtained.[47]

The results from the solvation dynamics experiments show a number of interesting features. First, as the hydration of the lecithin micelles increases, the number of distinct decay components increases. For $w_0 = 4.8$ lecithin micelles, there exists only a single relaxation. In contrast, both $w_0 = 5.8$ and $w_0 = 6.8$ lecithin micelles display three components to the decay. Second, while the amplitudes for each decay component change with hydration, the time-constants remain largely constant. Third, the relative relaxation for similarly hydrated lecithin and AOT micelles reveal significant departure from each other. Finally, all micellar samples show additional relaxation components with longer time-constants that are not present in bulk water. Each of these points is discussed and interpreted in detail in the following section.

IV. Discussion

Lecithin Hydration. While a variety of techniques have been used to correlate microscopic structure with macroscopic viscosity associated with organogel formation, several questions remain unanswered.[60] The experiments reported here present a unique perspective on the mechanism of the organogel formation. However, while we employ a unique technique, we find several direct comparisons to the current view of organogel structure and dynamics. Before comparing our results to previous studies, we review the most applicable points.

Many studies have attempted to correlate properties of phospholipid components with the onset of organogel formation. While the choline moiety of the headgroup shows insignificant changes upon hydration,[61] the properties of the phosphate groups, carbonyl groups, and alkyl chains have shown progressive trends correlating with organogel formation. The phosphate atom NMR line width has been shown to reach a maximum at the percolation threshold.[60,61] Similarly, the frequency of the P═O stretching vibration, measured using FTIR, shifts to lower energies until the percolation threshold is reached and then levels off.[19,62] These findings have been interpreted as the phosphate

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Table 1. Multiexponential Fits of the Time Correlation Functions, $C(t)$, for Coumarin 343 Solubilized in Various Aqueous Environments[a]

sample[b]	a_1	τ_1 (ps)	a_2	τ_2 (ps)	a_3	τ_3 (ps)
bulk water[c]	0.3 ± 0.3	0.2 ± 0.1	0.7 ± 0.3	0.6 ± 0.1		
AOT $w_0 = 5$[c]	0.19 ± 0.03	0.3 ± 0.1	0.19 ± 0.03	6 ± 2	0.63 ± 0.02	130 ± 10
lecithin $w_0 = 4.8$[d]	1.00 ± 0.01	219 ± 7				
lecithin $w_0 = 5.8$[d]	0.100 ± 0.003	0.42 ± 0.03	0.17 ± 0.03	17 ± 7	0.74 ± 0.07	340 ± 100
lecithin $w_0 = 6.8$[d]	0.13 ± 0.01	0.57 ± 0.07	0.25 ± 0.01	14 ± 1	0.62 ± 0.02	320 ± 40

[a] $C(t) = \sum_i a_i \exp(-t/\tau_i)$. [b] Uncertainties refer to standard error associated with each parameter obtained from a single multiexponential fit of the time correlation function. [c] Results taken from ref 47. [d] Longest relaxations were not fully complete by 477 ps. Amplitudes have been normalized by taking this into account.

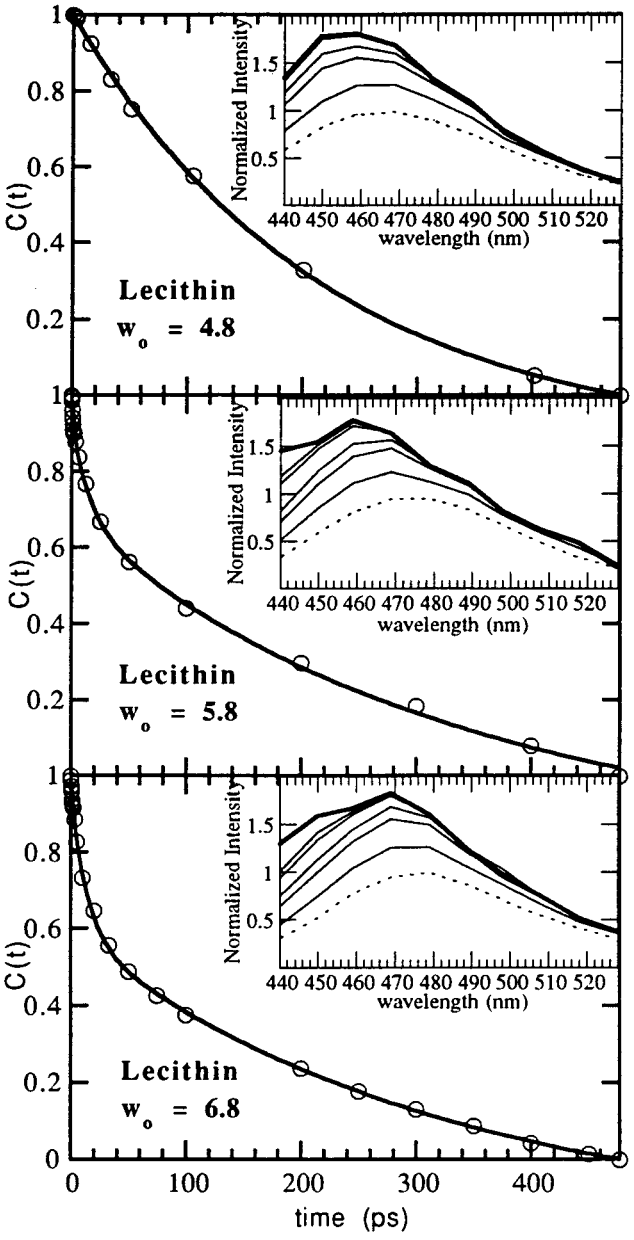

Figure 4. Time-correlation functions, C(t), for the different lecithin samples. Insets: Reconstructed fluorescence spectra for each lecithin sample.

moiety becoming increasingly hydrogen bonded with increased hydration that culminates in a decrease in local motion at the percolation point. In contrast, the carbonyl moiety seems to only show water interactions at elevated hydration levels. Like P═O, the frequency of the C═O stretching vibration also shifts to lower energies but only at hydration levels higher than the percolation threshold.[62] The lipid chain C−H symmetric and antisymmetric stretching vibrations have shown evidence of increased lateral packing order with increasing hydration. However, the increasing order shows no correlation with gel formation.[63]

Previous studies have explored the behavior of water at the various hydration stages leading to organogel formation using NMR and FTIR techniques.[60,61,63,64] While these studies reveal the expected trend that water becomes less restricted with increasing hydration, they do not correlate with organogel formation. These studies suggest that the first 1−2 water molecules added to the system become strongly coordinated to the phosphate moiety. Further hydration, even past the percolation threshold, results in the aqueous environment becoming progressively more bulklike in nature. While there is evidence for the existence of three water types in the spherical nonorganogel forming lecithin/benzene reverse micelles,[10] studies of lecithin/cyclohexane reverse micelles detect only two types, strongly bound and free. In contrast, by accounting for solvated monomers, Maitra et al.[64] find that for micelles between $w_0 =$ 4.8 to 6.8, the hydration range we have studied, half the water population is bound and the other half free. From these studies it is clear that (1) two water types are detected for the entire hydration level we have probed, and (2) the water structure shows no correlation with the onset of organogel formation.

While there are several microscopic trends that coincide with the formation of the organogel, there is little proof for tubular micelle crossover or branch points that should be prevalent at the percolation threshold.[16,19] According to the organogel model proposed by Schurtenberger et al.,[58,65] upon increasing hydration, the mean length of the wormlike micelles should reach a threshold value at which a transient network is formed and the system undergoes a macroscopic phase transition to the gellike form. It is hypothesized that at the percolation threshold, wormlike micelles pass through each other, creating structural anomalies at crossover points not seen at lower hydration levels. These crossover points should be evident in the motion of the CH_2 groups of the lipid tails. However, Arcoleo et al. argue that no discontinuity in the CH_2 region of the FTIR spectrum is observed and question whether crossover points are the source of the increased viscosity of the lecithin system.[16] We note that microscopic anomalies have been seen at the percolation threshold in a series of papers by Kumar et al.[66−69] However, since their organogel was described as anisotropic while our organogel and all others reported in this paper have been shown to be isotropic, direct comparisons are not warranted.

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In comparison to the current structural view of organogel formation, our results reveal some interesting points. From our dynamical results, we find that faster relaxation processes appear sequentially with increasing hydration in the same manner different water types appear structurally from FTIR and NMR techniques. We therefore attribute the different time constants to different water types and the relative amplitudes of the components to the amount of each water type. While previous studies have detected only strongly bound and free water in water/lecithin/cyclohexane reverse micelles, our results clearly show the existence of three distinct water types. We attribute the three relaxation times to strongly bound, bound, and free states. The single 220 ps relaxation that we observe for $w_0 = 4.8$ is attributed to very slow, hindered water relaxation. In contrast, diffusive relaxation in bulk water occurs on the 0.2 and 0.6 ps time scales (see Table 1). Since this 200 ps relaxation is so much slower than free water, it is unlikely to be due exclusively to water motion. Furthermore, because there are 4.8 waters per headgroup, it is just as unlikely that the observed dynamics arise exclusively from headgroup motion. Rather, the observed dynamics most likely reflect coupled motion of the water and headgroup. We therefore assign the water populations with relaxation time components longer than 200 ps to strongly bound water. At hydration levels above $w_0 = 4.8$, we observe additional relaxation components. For the $w_0 = 5.8$ and $w_0 = 6.8$ micelles, we see two additional relaxation processes with time constants of about 15 and 0.5 ps. We attribute the 15 ps population to bound water because it displays intermediate relaxation between strongly bound and free water. We attribute the 0.5 ps population to free water since the relaxation time is similar to bulk water (see Table 1).

Although the relative amplitudes can be thought of as indicating ratios of the different water populations, they do not necessarily reflect the *actual* ratios at a given hydration level for a number of reasons. First, the presence of the probe molecule perturbs the environment. This is significant because our results are compared to FTIR and NMR techniques that do not require a probe molecule. Furthermore, some solutes present in the micellar interior have been shown to redistribute water organization.[15] However, C343 and related coumarins are not reported to have specific interactions with water.[50] Therefore, while the probe may perturb the aqueous environment to some degree, it is unlikely that this perturbation approaches that of the lecithin headgroup. Second, the position of the individual probe molecules within the micelles is not well defined; most likely, the molecules span a distribution of locations with preference near the interface. Time-resolved fluorescence anisotropy measurements of C343 in AOT reverse micelles show that the probe is preferentially located at the lipid interface.[47] While time constants are not influenced, it is probable that the spatial location of the probe molecules in the lecithin micelles influences the relative relaxation amplitudes. However, it is unlikely that the spatial distribution varies over the hydration range we have studied. Therefore, we are justified in comparing the relative amplitudes from one hydration level to the next. In Table 1, we see that the populations of both bound and free water increase, while the relative population of the strongly bound water decreases as hydration goes from $w_0 = 5.8$ to 6.8. As we would expect, the dynamics of the micellar interior become less restricted and more bulklike in nature as hydration increases.

It is possible C343 interacts with components of the surfactant headgroups such as the Na^+ counterion in AOT or the cationic quaternary ammonium on lecithin. Such interactions could conceivably account for relaxations that proceed in the picosecond regime. We have observed a 100 ps relaxation component for C343 in 1 M aqueous $NaSO_4$.[47] If the 15 ps component appearing at $w_0 \geq 5.8$ is due to such interaction, then it should be manifest at every hydration level decreasing in amplitude as the hydration increases. To the contrary, the 15 ps component appears only above $w_0 = 4.8$ and *grows* with increasing hydration. One could also argue that the 200 ps component observed arises exclusively from dye-headgroup interactions. Again this seems unlikely because that would preclude any aqueous dynamics in micelles containing 4.8 water molecules per headgroup.

The three distinct water types seen in these studies are not only a noteworthy finding in comparison to organogel hydration studies but also with respect to reverse micellar and lipid bilayer studies in general. The relaxation times reported here associated with strongly bound, bound, and free water vary little with changing hydration, while their relative populations do. This provides strong evidence that distinct water types exist and remain invariant as micelle hydration level changes. This is an important finding because there has been considerable debate over whether distinct water types actually exist since the model was formally introduced by Finer[9] in 1973. Several studies have interpreted results to conclude that the water properties evolve smoothly, or at least quasi-continuously, as hydration of a lipid bilayer[21,23,70,71] or reverse micelles[7,72] increases. If there truly is a quasi-continuous water organization, our data would show gradual shifting of relaxation times as hydration is increased. However, we see the *same* relaxation times at all hydration levels with only relative *amplitudes* changing.

The hydration levels at which the different water types appear in our studies disagree with previous lecithin organogel studies. We find that all water is strongly bound in the $w_0 = 4.8$ micelles. Furthermore, while we do observe free water in $w_0 = 5.8$ and $w_0 = 6.8$ micelles, it represents a relatively small population. Based on the relative amplitude of the free water signal, we estimate that the free water population in the $w_0 = 6.8$ micelles is, at most, one water per headgroup. Since the addition of the first few water molecules induces the tubular micelle structure, previous researchers have assumed these water molecules are incorporated into the lipid headgroup.[58,59,73−75] However, FTIR and NMR studies suggest that all further water addition is bulklike in nature. Clearly, our results reveal the aqueous interior of the lecithin organogel contains much less free water than has previously been predicted. As will be discussed in the next section, we believe this arises due to the lipid headgroups sequestering water to a greater extent than predicted from static studies.

We find that at least 4.8 water molecules per headgroup are strongly bound, while predictions state that only 1−2 waters are strongly bound. While it is possible that the first 1−2 water molecules are more strongly coordinated than the subsequent 3−4 water molecules comprising $w_0 = 4.8$, the time correlation function shows no evidence that part of the water is substantially more immobilized than the rest. Furthermore, the 200 ps relaxation observed is very slow for aqueous motion indicating that all water at $w_0 = 4.8$ is strongly bound. The apparent

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discrepancy between previous work indicating 1−2 strongly bound water molecules per lecithin headgroup and the work reported here most likely stems from the fact that structure and dynamics are not equivalent.[48] A significant water population may appear bulklike in FTIR and NMR studies but at the same time have hindered solvation dynamics due to its position, for example, intercalated between lipid headgroups. We have observed a similar effect for formamide sequestered in AOT reverse micelles.[48] In this case, the static structure indicated by FTIR studies reveals essentially bulklike characteristics, while dynamical measurements show that the solvent is completely immobilized. In addition, the lack of agreement can be attributed to the sensitivities of the detection method. FTIR analysis involves deconvolution of the different water types from a single peak.[10] If relative populations are small, as our results suggest for both bound and free water, analysis becomes difficult. Dielectric studies suffer a similar drawback requiring deconvolution of a small perturbation from a single spectroscopic anomaly.[19,23] Proton and deuterium NMR identify different types by deconvolving the separate time constants from a single T_1 relaxation study.[76] If different types have similar time constants, deconvolution becomes challenging. Moreover, if exchange between different types occurs on a time scale faster than the experiment, a single weighted average relaxation will be observed. The time-resolved fluorescence experiments reported here follow motion on a much faster time scale than diffusional exchange processes. Therefore, water populations are essentially frozen on the time scale of the experiment. Also, the technique is sensitive to motion of small populations as long as the relaxation is significantly different from others.

Finally, like previous studies, we find no evidence for crossover point anomalies as the percolation threshold is approached. It is likely that our $w_0 = 6.8$ micelles are not quite at the percolation threshold. We anticipate our samples would reach maximum viscosity at about $w_0 = 8$. Therefore these results only show the aqueous dynamics as hydration *approaches* the percolation threshold. As can be seen in Table 1, less restricted water types appear with increasing hydration in a very predictable way with relaxation times remaining constant, while the relative amplitudes change. The formation of crossover points at higher hydration levels should result in new and shifting relaxation processes. We see no evidence for this and conclude that macroscopic rheological properties have no apparent correlation with aqueous dynamics in agreement with others.[60,61,63,64]

Lecithin vs AOT Hydration. Comparison of relaxation in AOT and lecithin micelles shows that water in AOT reverse micelles approaches bulklike dynamics at significantly lower hydration levels than lecithin reverse micelles. Our previous work has shown a growing population of free water with increasing hydration of AOT micelles.[47] Although the degree of hydration possible in AOT micelles is significantly greater than lecithin, we observed the same general trend of increasing bulklike dynamics with hydration. However, the first appearance of free water in lecithin micelles appears as a faint relaxation at $w_0 = 5.8$, while in AOT micelles we detect a significant free water relaxation at $w_0 = 5$. As Table 1 indicates, even the dynamics of the $w_0 = 6.8$ lecithin micelles are slower than the dynamics of $w_0 = 5$ AOT micelles.

The relative increased water mobility in the AOT micelles over the lecithin micelles is, at first glance, counterintuitive. AOT should perturb the water strongly from ion−dipole interactions with the SO_3^- and Na^+ headgroup ions. In comparison, interactions with the phosphate and choline moieties of the zwitterionic lecithin headgroup should present a much softer ion−dipole interaction. FTIR and NMR studies suggest that the AOT headgroup should require a greater hydration to stabilize lipid−lipid interactions than the lecithin headgroup. Studies of AOT have concluded that 3−4 water molecules are strongly bound to the sulfate moiety.[77−79] In contrast, lecithin studies have found only 1−2 water molecules strongly bound to the phosphate moiety.[60,61,63,64]

To explain the discrepancy between our results and what would be expected, we propose that, at least at low hydration levels, lecithin does not form well-defined core water pools to the same extent AOT does. For AOT, the first few water molecules bind to the sulfonate and sodium ions.[78,79] FTIR studies have shown that the AOT carbonyl groups show no shifting attributed to water interactions as hydration increases.[78,79] After the first few water molecules pack around the sulfonate, additional water molecules become localized in core water pools rather than interacting with the AOT carbonyl groups or between the alkyl chains. Conversely for lecithin micelles, we propose that, at low hydration levels ($w_0 \leq 4.8$), water associates with the phosphate group and becomes sequestered in the headgroup region, unable to coalesce into core pools. Like the sulfonate group of AOT, the phosphate group is the initial site of hydration. However, the lecithin headgroup occupies significantly more volume than the AOT headgroup (see Figure 1). Therefore, water added once the phosphate group is hydrated can still hydrate the headgroup without coalescing to form core pools. Using FTIR, Maitra et al.[64] have shown that with increasing hydration the frequency of the P═O stretching vibration shifts up to hydration levels of about $w_0 = 4-5$. Further evidence suggesting that the hydration of the lecithin headgroup is more extensive than AOT comes from the behavior of the ester groups. Unlike AOT,[78,79] Maitra et al. also find that the C═O frequency shifts above hydration levels $w_0 = 4$, suggesting that the carbonyl moieties interact with the water.

Geometrical arguments based on small-angle neutron scattering (SANS) and dynamic light scattering (DLS) also suggest that lecithin lacks a defined water core at low hydration levels, while AOT does not. Drawing from SANS data of $w_0 = 1$ AOT micelles, Kotlarchyk et al.[80] find the micelle dimensions allow for the existence of a water core of one water molecule per headgroup. Additionally, Gorski et al.,[81,82] also interpreting SANS data, have found that AOT micelles in benzene and decane have spherical radii that are larger than would be expected based on accepted volumes of water and AOT molecules. They show that only at substantial hydration levels ($w_0 \geq 20$) do the measured and predicted radii agree. DLS data show the spherical radius of AOT micelles increases linearly with increasing hydration, even below $w_0 = 5$; the micellar radii increase from 15 Å at $w_0 = 0$ to ca. 100 Å at $w_0 = 50$.[11,52] These results show AOT micelles have a substantial aqueous core. In direct contrast, dimensions of lecithin reverse micelles suggest a lack of available space to form a defined

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aqueous core. SANS data of $w_0 = 1-3$ lecithin micelles in isooctane have shown that the cross-sectional radius is constant over this hydration range (ca. 30 Å). This led Schurtenberger et al.[13] to suggest that added water distributes radially throughout a very extended headgroup region. SANS data of lecithin in cyclohexane at higher hydration levels ($w_0 \geq 6$) revealed similar evidence that there is considerable penetration of the water molecules into the interfacial region, although a defined water core did seem evident at these hydration levels.[83] Wachtel et al.[59] have used DLS to investigate lecithin in cyclohexane reverse micelles. Assuming a uniform cylindrical reverse micelle shape, their data showed modest cross-sectional radius increases from $w_0 = 1$ to $w_0 = 5.4$ [While Wachtel et al. report hydration levels excluding the intrinsic one water molecule per lecithin molecule, we add this to the w_0 value listed here to make direct comparison with our work possible.] (ca. 110%).[59] To further illustrate, assuming the water molecular volume is about 30 $Å^3$, 1.7 lecithin molecules per angstrom align along the micellar contour,[83] and 1 water molecule per lecithin becomes bound to the phosphate moiety of the headgroup, we estimate the radius of Wachtel's cylindrical micelles[59] would increase 340% at the $w_0 = 5.4$ hydration level if the remaining 4.4 water molecules formed an aqueous core instead of incorporating into the headgroups.

In addition to experimental evidence, there is also a theoretical basis supporting the interpretation that a significant population of water exists radially distributed in the headgroup without forming a bulklike core pool. It has been established that lecithin micelles are transformed from spherical micelles to long cylindrical aggregates with small additions of water.[13,58] According to the theory of lipid packing parameters developed by Israelachvili et al.[3,84] in order for micelles to transform from spherical to cylindrical the phospholipid headgroup area must increase or the chain volume must decrease. Because water addition initiates the transformation, we can assume that the headgroup area increases due to water incorporation. If lecithin forms planar sheets, such as lipid bilayers, even greater headgroup hydration should be detected than in reverse micelles. While FTIR and NMR studies of lecithin reverse micelles find 1−2 bound water molecules, similar studies of lecithin in lipid bilayers suggest at least 6 and as many as 11 water molecules per headgroup are incorporated into the headgroup.[9,85,86] Also, simulations of lipid bilayers conclude that significant water populations become trapped within the headgroup region.[87,88] Koga et al. have argued this point suggesting that headgroup surface area of lecithin in small micelles in isooctane increases from 30 to 61−74 $Å^2$ when compared to the headgroup area of bilayer vesicles.[89] Because lipid morphology seems to control hydration, this analysis suggests that water incorporation into the lecithin headgroup region is thermodynamically driven by lipid chain interactions rather than specific water-headgroup interactions. In contrast, AOT reverse micelles show no morphological changes, remaining spherical at all hydration levels below $w_0 = 60$.[11] We argue that there is no thermodynamic driving force to incorporate water into the headgroup past 3−4 strongly bound water molecules. Therefore as would be expected, AOT shows only modest headgroup surface area increases as hydration increases.[90]

While there is ample evidence, there is no definitive proof that the lecithin headgroup is hydrated to a greater extent than the AOT headgroup. However, our results coupled with the supporting evidence make a strong case. We find that even though the AOT headgroup should have stronger water interactions than the lecithin, the water dynamics in AOT micelles become more bulklike at lower hydration levels. Therefore, the extent that surfactant headgroups perturb interfacial water depends not only on specific water-headgroup interactions but also on the interfacial interactions as a whole. In the case of lecithin, we suggest a greater population of water is perturbed because lipid chain interactions encourage increased hydration. This conclusion is not very surprising if we consider that previous studies have already concluded that the hydration capacities of phospholipids depend on the state of their lipid chains.[85] Whether the 4.8 strongly bound water molecules per headgroup are actually immobilized with specific headgroup interactions remains unanswered. It may be that the strongly bound water actually has loose headgroup interactions. But, because the water is incorporated between headgroups, the water is effectively hidden from the probe molecule. In any case, we conclude a solvated molecule will experience no solvation dynamics faster than 200 ps in a $w_0 = 4.8$ lecithin micelle. We also conclude that while techniques assessing the structural aspects of water intercalated into the headgroup region, i.e., FTIR and NMR, reveal only minor perturbations, our dynamical measurements show water motion is significantly impacted.

Implications. While many studies have probed the dynamics of water at lecithin interfaces in lipid bilayers, the results reported here are unique in that we probe the interface in reverse micelles. Unfortunately, there are no experiments with which we can compare our dynamics results directly. However, there are a plethora of computer simulations predicting the structure and dynamical behavior of water at a variety of lecithin interfaces. The bulk of these studies have focused on the structure of the model membrane, bilayer or lamellar interfaces.[91−93] A handful of reports discuss the dynamics of water at bilayer or lamellar interfaces. We are unaware of any simulations, structure or dynamics, of lecithin reversed micellar organogels. Again, the morphology difference between our micelles and the calculated lipid bilayers makes direct comparison of the results reported here with the computer simulations unclear. However, because the same water headgroup interactions persist, we can still mark the predicted trends to learn whether our observations are supported by the predictions.

The major finding of the computer simulations is similar to the predictions one would make from structural studies, that is, the water molecules bound to the surfactant interface suffer significant reduction in their mobility.[94−97] However, many details differ from our findings. For example, both Klose et al.[96]and Damodaran et al.[94,95] have predicted two hydration shells for water near phosphatidylcholine and phosphatidylethanolamine bilayers on the basis of calculated radial distribution

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functions. The dynamics of the water in each shell differs from bulk water. However, Damodaran et al. predict the water orientational correlation time, which is longer than measured solvation dynamics,[98] is ∼12 ps for "bound" water and ∼4 ps for "bulk" water. Klose et al. predict motion on the picosecond time scale due to an oscillation of water molecules about the preferred hydration site. The times reported for bound water in these simulations are significantly faster than motion we observe. However, the structure predicted in both these simulations entails two hydration shells, bound and free, differing from our interpretation of strongly bound, bound and free water. Raghavan et al.[97] have modeled reorientation times for water between phosphatidylethanolamine bilayers including no "bound" water. While their reorientation times are slightly slower for water molecules close to the bilayer surface, they are faster than bulk water in the middle of the bilayers. In molecular dynamics simulations of dimyristoylphosphatidylcholine monolayers, Alper et al.[99,100] have predicted three hydration shells about the lipid headgroups. They predict that the motion of the hydrating, or bound, water is about 10 times slower than bulk water. Often direct comparison between experiment and simulation is difficult or impossible because simulations cannot run long enough to model the observed dynamics. In our case, the time scale for our experiment is short enough to preclude this problem so it should be possible for structural molecular dynamics simulations to propagate dynamical information about the water motion for a direct comparison with our results. We find that, while these simulations do predict the general trends that motion of water near interfaces is reduced, the simulations do not seem to either agree on the time scale of bound water motion or predict the reorientation times even close to those that we observe. There exist several reasons for this discrepancy. First, lecithin is a mixture of a variety of phospholipid molecules with differing hydrophobic regions. Therefore, the mixture that we use may preclude direct comparison. Second, the morphology of our systems ranges from small quasispherical droplets to long tubular chains. All the simulations look at planar interfaces. So, while there exists hydrating water in both systems, the curvature of the micellar interface may impact the water dynamics. Finally, we note that our experiments might preclude observation of water that is sequestered in a cavity located some distance from the probe molecule. That is, our experiments might not reveal mobile water hidden deep within the alkyl chains.

One important application of lecithin reverse micelles is in the study of enzyme activity. There have been many studies done following the activity of enzymes sequestered in the interior of reverse micelles. These studies have shown that the enzymes show activity in these synthetic environments.[101−103] Interestingly, some enzymes display maximal activity at a relatively low water hydration level, with activity dropping for increasing hydration levels. For example, in the case of trypsin and α-chymotrypsin in lecithin reverse micelles, the optimal hydration level for maximal enzyme activity was found between $w_0 = 7$ and 10. Perhaps even more importantly, some enzymes display enhanced activity over the native state.[104] Hence, the reverse micellar organogels studied here could serve as excellent alternative environments for biocatalysis. In a theoretical study, Bru et al. suggest that bound water may be important to activate catalytic activity of enzymes in reverse micelles.[105] Luisi et al. have hypothesized that 1−2 molecules of the water available in a reverse micellar lecithin organogel are tightly bound to the phosphate moiety.[58] We find that at least 4.8 water molecules are involved in headgroup hydration. Clearly, to understand the observed superactivity of enzymes in micellar environments and tailor systems for biocatalysis requires an improved understanding of water dynamics in these reverse micellar systems.

V. Conclusions

This paper reports the first ultrafast aqueous dynamics measured near a phospholipid interface. By comparing the dynamics of sequential hydration levels, our results provide a unique perspective of the water structure and mobility within the lecithin organogel. Dynamics reveal three distinct water types that appear with increasing hydration. We show that the dynamics are much more restricted than has previously been predicted by FTIR and NMR spectroscopic investigations. More specifically, we find no free water at the $w_0 = 4.8$ hydration level and not more than one free water molecule per lecithin molecule at the $w_0 = 6.8$ hydration level. We also see no evidence for crossover or branch points in the measured aqueous dynamics, even though the sample exists macroscopically as a viscous gel. We find that lecithin perturbs the water core to a much greater extent than similar AOT reverse micelles. We explain these findings by proposing that lecithin, unlike AOT, sequesters water to a much greater degree than has previously been predicted.

Although we examined the dynamics within reverse micelles, our results also have significance for understanding the aqueous structure and dynamics near other lipid interfaces. For example, we have related the water that is incorporated into the headgroups to the water that others have concluded as trapped within the phospholipid interface. Comparison of our dynamical results to dynamics determined by computer simulations at lipid surfaces show that while the trends observed agree, the time scales we measure for water motion are substantially longer than what the simulations predict. We hope that our dynamical results of motion within reverse micelles can be used as a first approximation to predicting dynamics near a lipid interface.

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