Vibrational Sum Frequency Spectroscopy of Surfactants and Phospholipid Monolayers at Liquid-Liquid Interfaces

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ABSTRACT

Work from our laboratory on vibrational sum frequency spectroscopic investigations of molecular ordering at the carbon tetrachloride-water interface is reviewed. Simple charged surfactants adsorbed at the liquid-liquid interface are seen to induce alignment of interfacial water molecules to a degree which is dependent on the induced surface potential. Saturation of water molecule alignment occurs at a surfactant surface concentration corresponding to a calculated surface potential of approximately 160 mV. In complementary studies, the relative degree of hydrocarbon chain ordering within monolayers of symmetric phosphatidylcholines of different chain lengths is inferred by the relative signal contributions of the methyl and methylene symmetric stretch modes. The degree of hydrocarbon chain disorder observed depends strongly on the method of monolayer preparation. By one method, a decrease in hydrocarbon chain order is seen with increasing chain length. Another method of monolayer formation yielded very well ordered hydrocarbon chains for the longest chain phosphatidylcholine studied, and showed much greater disorder in shorter chain species which was comparable to the other preparation method. These studies are a foundation for further work with this technique geared towards understanding molecular-level structural features in membrane-like assemblies and surface biochemical interactions of relevance to biomedical research.

keywords: liquid-liquid interface, vibrational sum frequency spectroscopy, phosphatidylcholine, sodium dodecyl sulfate, hydrocarbon chain ordering, water orientation

1. INTRODUCTION

Surfactants, of which phospholipids form a subclass, have been widely investigated for their many uses in scientific research and industrial processes, and for a multitude of current and potential consumer product applications. A main structural component in the membranes of all living cells, phospholipids are of particular interest for biomedical scientists, where the large variety of phospholipid types allows for an even larger variety of biomembrane compositions and functions. Surfactants, in general, are molecules with both a hydrophobic and hydrophilic regions such that adsorption at the boundary between hydrophobic and hydrophilic phases is favored. Surfactants in aqueous solutions can thereby surround and solvate oil droplets in a typical cleaning process, or, by their presence at an interface between liquids, enable transport or catalyze a chemical reaction which would not occur in the absence of these molecules. In a similar way, the biomembrane bilayer environment is necessary for integral membrane proteins to hold their functional form in vivo, and the integrity of the membrane itself permits tight control of transport across it. On a molecular level, however, the features of the interface formed by these biological surfactants are not well understood structurally. Phospholipid monolayers have been well studied as model membrane systems at the air-water interface due to the ease of controlling various thermodynamic parameters in this well-defined experimental system. Until very recently, however, phospholipid monolayers at the buried oil-water interface, which more closely model a membrane bilayer, have been experimentally inaccessible to many techniques otherwise capable of providing molecular-level information. We have employed the interface-specific nonlinear optical technique of vibrational
sum frequency spectroscopy (VSFS) to examine surfactants and phospholipids adsorbed at the oil-water interface to gain new insight into this molecular-level ordering.

This article describes experiments performed in our laboratory studying the structure of surfactants adsorbed at a liquid-liquid interface using surface specific VSFS. Original work on the study of simple charged surfactants investigated both the hydrocarbon chain ordering of the adsorbed surfactants and the orientational ordering of water molecules induced by the charged surface layer. Further work has included studies of symmetric chain phosphatidyicholines (PC's) adsorbed from the aqueous phase to the carbon tetrachloride (CCl4)-deuterium oxide (D2O) interface. The zwitterionic PC head group, although net neutral, has also been shown to promote the ordering of interfacial water molecules\cite{1,2} as has been previously observed only indirectly with the aid of molecular modeling techniques. Majority emphasis is given to VSFS of symmetric chain PC hydrocarbon chain ordering in relationship to chain length and method of sample preparation, including new observations of possible crystalline multilayer formation at the oil-water interface by the longest chain PC studied. This work provides a foundation for further investigations into the structural aspects of phospholipid membrane organization in biomimetic systems.

2. THEORY

Vibrational sum frequency spectroscopy, a nonlinear optical technique with an inherent sensitivity for surfaces, has been widely applied in the study of physical chemical phenomena at many types of gas, liquid, and solid interfaces, including buried interfaces.\cite{3,4} Under the electric dipole approximation, the second order process of VSF generation is symmetry forbidden in isotropic media and is therefore allowed only at interfaces where inversion symmetry is broken between two bulk phases. At a liquid-liquid interface, information specific to the surface region may therefore be gained even when interfacial adsorbates of interest are in equilibrium exchange with one or both bulk phases.

When two intense optical fields, $\omega_1$ and $\omega_2$, are overlapped both spatially and temporally at a surface, a polarization, $P_{SF}$, at the sum frequency, $\omega_{SF} = \omega_1 + \omega_2$, is induced in the surface region of the interface. The generated SF field, $E_{SF}$, is proportional to the product of the amplitudes of the electric fields, and to the nonlinear susceptibilities, $\tilde{\chi}^{(2)}$ and $\tilde{\chi}^{(3)}$, of the surface region being sampled.

$$E_{SF} \propto P_{SF} = \tilde{\chi}^{(2)} E_{VIS}E_{IR} + \tilde{\chi}^{(3)} E_{VIS}E_{IR} \Phi_0 \quad [1]$$

For our purposes, the more intense field, $\omega_1 = \omega_{VIS}$, is in the visible (or very near IR) region, and the second field, $\omega_2 = \omega_{IR}$, is tunable in the IR region. As $\omega_{IR}$ comes into resonance with allowed molecular vibrational transitions a sum frequency signal is produced, generated both in transmission and reflection from the sample surface, as shown in Figure 1. The second order nonlinear susceptibility, $\tilde{\chi}^{(2)} = \tilde{\chi}^{(2)}_{NR} + \tilde{\chi}^{(2)}_{R}$, contains a resonant component when $\omega_{IR}$ is resonant with an allowed vibrational transition, and a small nonresonant component, or background signal, which is negligible in our experiments. The resonant component, $\tilde{\chi}^{(2)}_{R} = N_s \langle \alpha^{(2)} \rangle$, is proportional to the surface number density, $N_s$, and the molecular hyperpolarizability, $\alpha^{(2)}$, of the adsorbed molecules. The brackets refer to an averaged molecular orientational distribution within the surface layer. The frequency dependence of $\alpha^{(2)}$ is described by Equation 2.

$$\alpha^{(2)} = \sum_n \frac{A_n}{\omega_n - \omega_{IR} - 3/2} \quad [2]$$
where $\omega_n$ is the frequency of the $n$th vibrational mode, $\Gamma_n$ the line width, and $A_n$ is an amplitude coefficient proportional to the product of the Raman and IR transition moments. In order for a vibrational resonance to be sum frequency active, therefore, it must be both Raman and IR active.

The third order nonlinear susceptibility, $\chi^{(3)}$, is nonzero only when a significant surface potential, $\Phi_0$, is present, such as might be induced by charged interfacial adsorbates. A monolayer of charged surfactants, for example, produces an electric field at the oil-water interface which is on the order of $10^7$ V/m. Water and other polar molecules near the interface in either liquid phase will be aligned in the electric field and potentially contribute to the measured SF intensity from the surface. In the unaligned state the molecules are in a centrosymmetric environment and do not contribute to VSF generation from the interface. The magnitude of the surface potential, and therefore the degree of molecular alignment induced by the surface charge, depend on the density of surface charge and on the ionic strength of the bulk solution. Nonresonant contributions to $\chi^{(3)}$ from alignment of water molecules near charged surfactant monolayers at air-water and solid-liquid interfaces have been investigated using second harmonic generation. However, for studies of the simple charged surfactants, we have employed VSFS in the O-D and O-H IR stretching regions to probe resonant $\chi^{(3)}$ contributions. In these studies, nonresonant contributions to both $\chi^{(2)}$ and $\chi^{(3)}$ are insignificant.

## 3. MATERIALS & METHODS

98% atom d-25 sodium dodecyl sulfate (d-SDS) was obtained from Cambridge Isotope Laboratories and used as received. d-SDS stock solutions were prepared by dissolving in HPLC grade water (H$_2$O) from Mallinckrodt, or ultrapure H$_2$O obtained from a Barnstead Nanopure purification system. d-SDS bulk concentration in the aqueous phase of the sample was increased by stepwise addition of the d-SDS stock solution followed by a suitable equilibration period. 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), purity greater than 99%, were obtained from Avanti Polar Lipids (Alabaster, AL) in powder form and used without further purification. Concentrated phospholipid solutions were prepared in 10 mM phosphate buffer, pH 7.0 in D$_2$O by one of two similar methods. Differences between the two preparation methods are pertinent to the later discussion. Walker, et al. prepared liposome solutions by bath sonication above the gel to liquid crystalline phase transition temperature for the particular phospholipid for a time sufficient to clarify the solution. The liposome solution was then allowed to sit overnight to allow surface active metastable PC assemblies, formed together with the liposomes during sonication, to form stable vesicles. Smiley, et al. prepared phospholipid solutions at concentrations less than 1 mM by prewarming the solution to at least 10°C above the gel to liquid crystalline phase transition temperature of the lipid and then sonicating the solution in a warm ultrasonic bath at least until a homogeneous solution was formed. The solution was used same day and for subsequent additions the following day. In both cases, the sample cell was prepared with carbon tetrachloride (CCl$_4$) from Sigma-Aldrich, 99.9+%, HPLC grade as the bottom phase, and 10 mM phosphate buffer, pH 7.0 in deuterium oxide (D$_2$O), either from Aldrich or Cambridge Isotope Laboratories, 99.9% HPLC grade, as the top phase. After preparation of the neat CCl$_4$-D$_2$O interface in the sample cell, the cell was then allowed to sit for a few hours to permit adsorption of any possible contaminants to the liquid-liquid interface. The sample cell was emptied and recleaned if contamination was evident at the neat sample interface (rare). A small volume of the concentrated phospholipid solution was then injected into the prepared sample cell and allowed to absorb at the liquid-liquid interface.
The two laser systems used in our laboratory for VSFS measurements have been described previously in separate works.11,12 Briefly, one system uses a regeneratively amplified, passively mode-locked Titanium:Sapphire 800 nm pump laser producing a 2 picosecond pulse of 750 µJ at 1 kHz. A portion of the 800 nm energy is directed at the sample interface along a path normal to the sample cell at the critical angle for the CCl₄-water interface, as shown in Figure 1. The remaining 800 nm energy is used to seed, by means of white light generation in an ethylene glycol cell, and pump a two stage optical parametric amplifier constructed from counter-rotating KTP crystals, producing between 2 and 9 µJ of IR in the 2.7-3.9 µm region. This IR beam is overlapped with the 800 nm beam, both temporally and spatially, at the sample surface. The sum frequency signal is measured in reflection using a PMT interfaced with gated detection electronics and a computer for automatic data acquisition and tuning control for the OPA. The bandwidth for this Ti:Sapphire laser system was calibrated at 18 cm⁻¹.

![Figure 1: Schematic diagram of VSFS at the CCl₄-water interface. Measurements were done in a total internal reflection geometry.](image)

The second laser system used for VSFS has a 6 cm⁻¹ bandwidth, but is limited in IR tuning range to 3.2-3.7 µm, and is therefore not able to access the O-H or O-D vibrational modes to either side of this wavelength region. In spite of the relatively narrow linewidth, this system is less sensitive than the picosecond Ti:Sapphire system because of a much greater pulse length. A portion of the output from a Nd:YAG laser with a 1064 nm, 12 nanosecond pulse at 10 Hz is used pump a LiNbO₃ optical parametric oscillator producing 1-3 mJ IR pulses which are directed to the sample surface as was shown in Figure 1. The 532 nm visible beam for VSFS is generated by frequency doubling the remaining 1064 nm light in a KDP crystal, and directing the green at the sample surface at the critical angle for the CCl₄-D₂O interface at this wavelength. The SF signal from the interface is measured in reflection similar to the case for the Ti:Sapphire system. The much greater pulse length (i.e. average energy per pulse) for this system requires that D₂O, rather than H₂O, be used as the aqueous phase in the cell, since absorption of the IR pulse energy by water at the sample interface causes boiling which disrupts the surface.
4. RESULTS & DISCUSSION

The structure and bonding characteristics of interfacial water molecules near a charged surface have only recently become amenable to theoretical and direct experimental exploration. The spectroscopic complexity of the vibrational modes of bulk water, which arises as the result of coupling across hydrogen bonds and energetic coupling among numerous normal modes, has been extensively investigated with both IR and Raman spectroscopy. Although this complex vibrational structure makes peak identifications difficult, assignments based on the previous IR and Raman studies of bulk water have enabled meaningful interpretation of data on interfacial water structure obtained by VSFS. At the air-water interface in the presence of charged surfactants, there are two populations of water molecules with accessible vibrational resonances which are distinguishable based on their hydrogen bonding characteristics. An ice-like, symmetrically bonded symmetric OH stretch (OH-SS-S) appears between 3200 and 3250 cm$^{-1}$, and a more water-like asymmetrically bonded symmetric OH stretch (OH-SS-A) is present between 3400 and 3450 cm$^{-1}$. At the CCl$_4$-H$_2$O interface, however, only the OH-SS-S stretch is in evidence, as shown in Figure 2 at various aqueous concentrations of d-SDS. Adsorption of the charged deuterated surfactant to the liquid-liquid interface produces a static electric field at the surface which aligns interfacial water molecules. Deuterated surfactant was used to prevent interference between CH and OH stretching modes observed previously which is difficult to deconvolute. A shoulder in the 2900 to 3050 cm$^{-1}$ region to the lower energy side of the large OH-SS-S peak results from trace surface contaminants with CH stretching vibrations which could not be eliminated. No evidence of an OH-SS-A contribution is seen in the region to the right of the symmetrically bonded OH stretch. The intensity of the OH-SS-S peak increases with increasing d-SDS bulk concentration, achieving a maximum value at approximately 0.5 mM d-SDS.

The Gibbs equation applied to our surface tension data as a function of bulk d-SDS concentration was used to estimate the surface excess of surfactant at the liquid-liquid interface, which for totally charged d-SDS molecules corresponds directly to the surface charge density. The surface potential as obtained from Gouy-Chapman theory using the surface charge density and the experimental ionic strength was then estimated as a function of bulk d-SDS concentration. A surface potential of approximately 160 mV is induced at 0.5 mM d-SDS, but our surface tension measurements also indicated that maximum d-SDS surface concentration is not reached until 1-2 mM bulk d-SDS concentration, or 260 mV. These data and additional temperature dependent measurements (not discussed here) have shown that the observed enhancement of the SF intensity in the water region results from alignment of the permanent dipole moment of interfacial H$_2$O molecules due to the static electric field induced by surfactant adsorption. Water molecules contributing to this enhancement reside in the double layer region, which for an ionic strength of 10 mM is approximately 3 nm wide. Alignment of interfacial water molecules at the CCl$_4$-H$_2$O interface with d-SDS, as probed by VSFS, reaches a maximum at a surface potential of approximately 160 mV and is not further increased at higher surface potentials.

Given that natural biomembranes typically contain charged lipids, the influence of charged surfactants on the structural arrangement of H$_2$O (or D$_2$O) near a charged phospholipid monolayer at a liquid-liquid interface is very relevant to issues of biomembrane interactions and processes. These studies fall into the category of future work. The remaining results to be described here focus on VSFS investigations of hydrocarbon chain ordering of zwitterionic PC's, the major phospholipid component of natural biomembranes, without consideration of the effect their adsorption produces on the interfacial water (D$_2$O) structure.
Walker, et al. have shown clear differences in hydrocarbon chain ordering observed as a function of chain length and surface density for saturated, symmetric PC's adsorbed to the CCl₄-D₂O interface by transformation from liposomes injected into the aqueous phase. The rate of liposome to monolayer transformation and the physical state of the phospholipid monolayer formed at the liquid-liquid interface by this method are strongly dependent on both temperature and liposome concentration. Above the gel to liquid crystalline phase transition temperature, Tₘ, for the lipid, a condensed monolayer forms more quickly (hours). Below Tₘ, monolayer formation is very slow on the order of days, and even very high bulk liposome concentrations in the millimolar range may not produce tightly packed PC monolayers at the interface. Therefore, for PC's with Tₘ values above room temperature, tightly packed monolayers were produced by heating the sample above Tₘ for a period of time sufficient for monolayer adsorption to take place, and then allowing the sample to cool down to room temperature before VSFS measurements were taken. Monomers from these tightly packed monolayers do not desorb from the interface with this reduction in temperature. Differences in hydrocarbon chain ordering are evidenced in the VSFS data of Figure 3 for tightly packed DLPC, DMPC, DPPC and DSPC monolayers adsorbed at the CCl₄-D₂O interface. The respective Tₘ values for these PC's are -1, 23, 41 and 55°C.
Figure 3: VSFS spectra for monolayers of symmetric chain phosphatidylcholines at equilibrium surface tensions formed from monomer adsorption to the CCl4-D2O interface above their respective Tm's. 10 mM phosphate buffer, pH 7.0 was present in the aqueous phase. Ratio refers to the integrated intensity ratio of CH3-SS/CH2-SS.

In Figure 3, data for S-polarized sum frequency, S-polarized visible and P-polarized IR (i.e. SSP) conditions are shown for each sample prepared by the method described above, such that only SF-active vibrational modes normal to the plane of the interface are sampled. The peaks centered at 2850 cm\(^{-1}\) and 2875 cm\(^{-1}\) are assigned to the symmetric stretch modes of the methylene (CH2-SS) and methyl (CH3-SS) groups, respectively.\(^{22,23}\) The intensity peak centered at 2930 cm\(^{-1}\) has been assigned to the methylene asymmetric stretch (CH2-AS) and methyl Fermi resonance (CH3-FR) in different sources and probably contains contributions from both modes.\(^{22,23}\) The broad structure in the vicinity of 2905 cm\(^{-1}\) can be attributed to contributions from a methylene Fermi resonance (CH2-FR).\(^{22}\) The solid lines in each spectrum are provided as a guide to the eye. The ratio of the intensities from the CH3-SS relative to the CH2-SS for surfactants at an interface.
provides a measure of how well ordered the hydrocarbon chains are within a monolayer, since, for a well-ordered monolayer
with few gauche defects, the CH₂-symmetric stretches will be symmetry forbidden and therefore not contribute to SF
generation from the interface. A vanishing CH₂-SS intensity gives a large CH₃-SS/CH₂-SS ratio indicating a well-ordered
surface. Calculated ratios of integrated peak intensities are given to the right of each spectrum. The ratio is clearly largest for
the DLPC monolayer, and decreases with increasing hydrocarbon chain length, DPPC and DSPC being the least well ordered
with the lowest ratios. Greater disorder in the longer chain PC's is thought to result from the greater conformational disorder
in the hydrocarbon chains due to screening of chain-chain interactions by CCl₄.¹

Additional experiments with symmetric PC's adsorbed at the CCl₄-D₂O interface by a different preparation method
have given very different results, particularly for DSPC, the longest chain lipid studied. All samples were prepared at room
temperature. As noted in Methods, a second injection of sonicated phospholipids is made into the sample cell following an
overnight equilibration period subsequent to the first addition. Within a few hours following the second injection, a tightly
packed layer is formed at the liquid-liquid interface as evidenced by relaxation of the meniscus between the two liquids, and by
surface pressure measurements which give surface pressures of approximately 42 mN/m under the same experimental
conditions. For DSPC only, relaxation of the cell meniscus was pronounced but not complete, and the final surface pressure
at which VSFS measurements were performed may have been as much as 5 mN/m lower than for the other three samples.
The surface tension of the neat CCl₄-H₂O interface has been given as 45 mN/m.²⁷ VSFS results for DLPC resembled those
already illustrated in the previous experiments by Walker, et al. Ratios for DMPC and DPPC, of 1.07 ± 0.22 and 1.25 ± 0.31, respectively, were slightly higher than those measured by Walker, et al., indicating no significant change relative to
DLPC. However, the VSFS measurements of DSPC adsorbed at the interface, shown in Figure 4, clearly show a well-
ordered interfacial layer of hydrocarbon chains. The CH₃-SS/CH₂-SS ratio of 4.59 ± 1.38 is roughly four times larger than
for the other three PC's since the CH₂-SS is all but absent in this spectrum. The shoulder seen at approximately 2944 cm⁻¹
may be the CH₃-FR enhanced by mode mixing with the strong CH₃-SS vibration observed. The high degree of molecular
ordering is indicative of a crystalline state of the hydrocarbon chains expected for a highly condensed monolayer or multilayer
structure which could not reasonably be formed by simple adsorption from liposomes in the aqueous phase at room
temperature.

In explanation of these results, we hypothesize that the second sample injection, done somewhat vigorously so as to
perturb the liquid-liquid interface during the injection, induces collapse in the already partially formed monolayer by pushing
the adsorbates away from the injection site into the limited available surface area at the sample cell surface. With DSPC well
below its Tₘ of 55°C, it is not able to desorb from the interface as either a monomer or liposome component back into the
aqueous phase within the few seconds needed to make the second injection, and is not likely to desorb into the CCl₄ phase as
a monomer because of its zwitterionic, hydrated head group region. When pressed together more tightly than close chain
packing will allow, therefore, the monolayer collapses. This collapse phenomenon, in which the monolayer erupts out of the
surface plane and folds back onto itself, has been previously observed at the air-water interface,²⁸,²⁹ and also at the oil-water
interface,³⁰ and is thought to result in complete or partial surface multilayer formation. It is, however, possible that some of
the DSPC molecules are forming micelles, creating an oil-in-water emulsion in the aqueous phase, and/or a water-in-oil
emulsion of reverse micelles in the CCl₄ phase. Our VSFS measurements are not sensitive to either of these possibilities in
the bulk phases aside from adsorption of the IR pump beam by any PC's located in the beam path to the sample surface in the
CCl₄ phase which might reduce or eliminate the SF signal intensity. Another factor potentially responsible for the observed
differences between these results and those of Walker, et al. are differences in preparation of the stock phospholipid solution.
A relatively brief sonication time was used in the preparation of some samples such that closed vesicle structures may not have formed, increasing the tendency for these structures to transform into close packed monolayers due to a decreased stability. The absence of an overnight equilibration period prior to use of the stock phospholipid solutions also suggests the presence of metastable phospholipid aggregates with high surface activity relative to that of the liposomes. For monolayer adsorption below $T_m$, the ability of the CCl$_4$ molecules to penetrate between hydrocarbon chains may also be reduced.

![Figure 4: Measured VSFS intensity spectrum for DSPC at the CCl$_4$-D$_2$O interface prepared using the multiple injection technique at room temperature for S-polarised SF, S-polarized visible, and P-polarized IR. The cell aqueous phase contains 10 mM phosphate, pH 7.0. Peak identifications are as given in the text.](image)

Independent of whether a monolayer or surface multilayer is formed at the oil-water interface, a nonzero SF intensity is generated only from those components, or perhaps layers, of the interface structure which lack inversion symmetry. If a multilayer is formed, it can not be said with certainty from which layers of the surface structure the SF signal may originate. We can say with reasonable certainty, however, that the observed DSPC structure as a whole is extremely well ordered and certainly very different from the shorter chain PC assemblies we have investigated. The observed increase in hydrocarbon chain ordering for longer chain symmetric PC's is in agreement with studies at the air-water interface and reflects the increase in monolayer stability afforded by a thicker hydrophobic component of the interfacial layer.

5. CONCLUSIONS

A brief overview has been presented of research performed in our laboratory pertaining to surface structure deduced at the CCl$_4$-aqueous interface for zwitterionic phospholipids and a simple charged surfactant. Using surface specific VSFS we have selectively probed both the degree of hydrocarbon chain ordering and the organization of interfacial water molecules induced by the presence of a charged interfacial surfactant layer. In the water studies, alignment of water molecules by an
increasing static electric field with increasing surface density of surfactant appears to be primarily responsible for the increased signal intensity observed. This alignment appears to saturate beyond an induced interfacial potential of approximately 160 mV. Deduced hydrocarbon chain ordering of symmetric chain PC's at the CCl₄-D₂O interface was strongly dependent on sample preparation. While PC monolayers adsorbed from aqueous liposome solutions above the respective gel to liquid crystalline phase transition temperatures showed a decrease in chain ordering with increasing chain length from DLPC to DSPC, the DSPC monolayer prepared by a multiple injection technique at room temperature was clearly much more ordered than shorter chain samples. The latter results suggest that the history of the interface is an important factor in the observed monolayer characteristics for these samples. This work provides a foundation for further investigations of molecular level interfacial structure of membrane interactions and systems which mimic essential biological processes.

6. ACKNOWLEDGMENTS

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7. REFERENCES


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