Hydration at the membrane protein-lipid interface

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ABSTRACT Evidence has been found for the existence water at the protein-lipid hydrophobic interface of the membrane proteins, gramicidin and apocytochrome C, using two related fluorescence spectroscopic approaches. The first approach exploited the fact that the presence of water in the excited state solvent cage of a fluorophore increases the rate of decay. For 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl) phenyl]ethyl]carbonyl]-3-*sn*-PC (DPH-PC), where the fluorophores are located in the hydrophobic core of the lipid bilayer, the introduction of gramicidin reduced the fluorescence lifetime, indicative of an increased presence of water in the bilayer. Since a high protein:lipid ratio was used, the fluorophores were forced to be adjacent to the protein hydrophobic surface, hence the presence of water in this region could be inferred. Cholesterol is known to reduce the water content of lipid bilayers and this effect was maintained at the protein-lipid interface with both gramicidin and apocytochrome C, again suggesting hydration in this region. The second approach was to use the fluorescence enhancement induced by exchanging deuterium oxide (D_2O) for H_2O. Both the fluorescence intensities of trimethylammonium-DPH, located in the lipid head group region, and of the gramicidin intrinsic tryptophans were greater in a D_2O buffer compared with H_2O, showing that the fluorophores were exposed to water in the bilayer at the protein-lipid interface. In the presence of cholesterol the fluorescence intensity ratio of D_2O to H_2O decreased, indicating a removal of water by the cholesterol, in keeping with the lifetime data. Altered hydration at the protein-lipid interface could affect conformation, thereby offering a new route by which membrane protein functioning may be modified.

INTRODUCTION

Water plays a fundamental role in cell membrane structure in that it drives the formation of the lipid bilayer, with a polar surface facing the aqueous environment and a hydrophobic interior containing the fatty acyl chains and transmembrane proteins. In general, the structure and dynamics of proteins are also to a large extent governed by interactions with water (Teeter, 1991). Cell membrane proteins, such as ion channel, enzyme, and receptor systems, differ from soluble proteins in having a transmembrane region in which the folding directs nonpolar amino acid side chains to face outward into the hydrophobic region of the lipid bilayer. Water associates with the head group region of phospholipids via hydrogen bonding (Boggs, 1987) and there is considerable evidence that water penetrates into lipid bilayers at least as far as the glycerol backbone and also deeper between fatty acyl chain packing defects. While the penetration of water into the "hydrophobic" region of lipid bilayers is firmly established (Griffith et al., 1974; Worcester and Franks, 1976; Simon et al., 1982; Smaby et al., 1983; Simon and McIntosh, 1986; Blume et al., 1988) hydration at the protein-lipid hydrophobic interface has hardly been explored, although the possibility of water occurring at the relatively hydrophobic surfaces of proteins is not entirely unknown (Teeter, 1991). Also, recent studies have shown that the introduction of small peptides, consisting of three amino acids, can cause a shift of water deeper into the bilayer (Jacobs and White, 1989).

The varying degree of water penetration into the lipid bilayer sets up a dielectric constant gradient from the membrane surface, where the value is ~70 to ~5 at the bilayer center, and this sets up a "dielectric constant gradient" across the membrane. The steepness of the gradient depends on the degree and depth of the water penetration. Various methods have been used to probe the dielectric constant gradient including neutron diffraction, electron spin resonance, and capacitance techniques (Griffith et al., 1974; Worcester and Franks, 1976; Simon et al., 1982; Smaby et al., 1983; Flewelling and Hubbell, 1986; Simon and McIntosh, 1986; Blume et al., 1988). More recently, it has been shown that information on the penetration of water into lipid bilayers can be obtained by time-resolved fluorescence spectroscopy (Zannoni et al., 1983; Fiorini et al., 1987).

The value of the fluorescence lifetime (for a discrete analysis) or the fluorescence lifetime center (for a lifetime distribution) yields information on the average environment of the fluorophore. The presence of water decreases this value due to the higher dielectric constant, a property which was exploited in this study to yield information on the presence of hydration in the lipid bilayer at the protein-lipid interface. If fluorophores are distributed across a region with varying dielectric constant values, that is across the gradient, then a range of decay rates will result (Fiorini et al., 1987). This and other factors governing fluorophore environmental heterogeneity have been discussed (Williams and Stubbs, 1988). It has also been shown that fluorophore environmental heterogeneity arises from the presence of membrane proteins, although the underlying cause was not found (Williams and Stubbs, 1988; Williams et al., 1990; Ho et al., 1992). One method of analyzing a range of decay rates is to model the data as a continuous fluorescence lifetime distribution. In this approach the range or diversity of decay

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rates is modeled as a continuous distribution (in this work a bimodal Lorentzian) with a characteristic full width at half maximum peak height (FWHM) which is related to the diversity of decay rates. A diverse range of decay rates yields a broad width, while a narrow range yields a narrow width. Thus, information on the structural diversity in the lipid bilayer can be determined from the value of distributional width, recovered from this type of analysis.

The effect of water on the fluorescence lifetime of 1,6-diphenyl-1,3,5-hexatriene (DPH) type fluorophores, and the intrinsic fluorophore tryptophan, was used as a test for the presence of water at the protein-lipid interface of gramicidin and apocytochrome C. Also, the ability of cholesterol to reduce the amount of water associating with the lipid bilayer was exploited (see review in Yeagle, 1985) as a further test for the presence of water. The use of DPH fluorophore lifetimes to demonstrate the dehydrating effect has been previously shown in lipid bilayers (Straume and Litman, 1987) and in this study the approach is extended to the study of proteins. The results provided evidence for hydration of the protein-lipid interface. The significance of this result is that water in this region could influence the protein structure and therefore ultimately its function.

MATERIALS AND METHODS

Materials

DPH, trimethylammonium-DPH (TMA-DPH) and 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl) phenyl]ethyl]carbonyl]-3-*sn*-PC (DPH-PC) were obtained from Molecular Probes (Eugene, OR). Phospholipids were from Avanti Polar Lipids (Birmingham, AL). Cholesterol, gramicidin D, and cytochrome C was from Sigma Chemical Co. (St. Louis, MO). Other general reagents were from Fisher Scientific (Pittsburgh, PA), trifluoroethanol was from Aldrich (99%+; Milwaukee, WI). D_2O (99.9%) was from Cambridge Isotope Laboratories (Woburn, MA).

Lipid vesicles

Large unilamellar vesicles (LUV) (1–200 μM lipid, in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4) were prepared as previously described (Hope et al., 1985) using a Lipex Extruder (Lipex, Vancouver, BC).

Incorporation of gramicidin and apocytochrome C

1-palmitoyl, 2-oleoyl-phosphatidylcholine (POPC) bilayers with gramicidin (channel form) incorporated (1:12, gramicidin:phospholipid molar ratio) were prepared as described previously (LoGrasso et al., 1988) from trifluoroethanol. The amount of incorporation of the gramicidin into the vesicles was verified by measurement of the lipid phosphorus (Bartlett, 1959) and of the gramicidin by absorption at 280 nm. Apocytochrome C was prepared as described (Hennig and Neupert, 1983; Rietveld et al., 1986) and was incorporated directly into preformed POPC/brain-phosphatidylserine (brain-PS) (9:1) LUV.

Fluorescence measurements

Steady-state fluorescence spectra (uncorrected) were obtained using either SLM 48000 or PTI spectrofluorimeters. For tryptophan excitation was at 285 nm. Fluorescence lifetime data were obtained using the multifrequency phase-modulation technique, using the SLM 48000 (Gratton and Limkemann, 1983; Lakowicz and Maliwal, 1985), with a Liconix model 4210NB HeCd laser as the excitation source. Emission was observed through a 420 nm red-pass filter and a Glan-Thompson polarizer, set at the magic angle.

Data analysis

Phase and modulation data were analyzed using the GLOBALS UNLIMITED analysis software (Laboratory of Fluorescence Dynamics, University of Illinois, Urbana-Champaign, IL) (Beechem and Gratton, 1988; Beechem, 1990). The fluorescence decay was modeled as a bimodal Lorentzian distribution (taken to 10 half-widths). The parameters recovered included the lifetime, fractional intensities (relating to the fraction of molecules associated with the lifetime center), and the distributional width at half peak maximum (proportional to the degree of fluorophore environmental heterogeneity) as previously described (e.g., Alcala et al., 1987*a, b, c;* Fiorini et al., 1987; Williams and Stubbs, 1988). Each measurement was repeated three times on separate vesicle preparations and the results are the mean of three determinations (±SD).

RESULTS AND DISCUSSION

To determine if water penetrates into the protein-lipid interfacial region, its ability to shorten the lifetime of the excited state of the fluorophore DPH, which locates in the central region of the bilayer (Davenport et al., 1985), was exploited. In a previous study it was found that if the protein:lipid ratio is low enough then there will be a population of "bulk lipid fluorophores" which are too distant to be under protein influence during the excited state (Ho et al., 1992). This means that, in general, the lifetime analysis would require three distinct lifetime centers, for bulk lipid and protein influenced fluorophore populations and also one for the minor component (~2–5%) of ~$1–3 \times 10^{-9}$ s, considered to be due to a combination of the photophysical properties of DPH and/or a photodegrative product (Lentz, 1988; Lentz and Burgess, 1989; Parasassi et al., 1991). In this study, to simplify the analysis, a relatively high protein:phospholipid ratio of 1:10–12 was used, allowing at most 2–3 lipids between adjacent proteins. This meant that most of the lipids, and therefore the fluorophores, were forced to be adjacent to the protein, at least for a major part of the excited state lifetime, thus eliminating the bulk fluorophore population.

The introduction of gramicidin caused a decrease in the fluorescence lifetime (biexponential analysis) from 8.22 to 7.65×10^{-9} s (see Table 1), indicating a higher dielectric constant in the fluorophore locality (i.e., increased hydration). In the lipid bilayer without protein, it was found that the inclusion of cholesterol increased the fluorescence lifetime of DPH from 8.22 to 8.90×10^{-9} s (see Table 1). It is well known that cholesterol dehydrates lipid bilayers as shown by a number of techniques (Yeagle, 1985), as demonstrated using DPH fluorophore lifetimes (Straume and Litman, 1987). Therefore, the effect can be ascribed to a reduction in the

TABLE 1 **The effect of cholesterol on fluorescence decay of DPH and DPH-PC in lipid bilayers**

	τ_1	w_1	f_1	τ_2	w_2	f_2	χ^2
DPH							
POPC							
2exp	8.22 ± 0.03		0.92 ± 0.01	2.42 ± 0.06		0.08 ± 0.01	1.12
POPC + cholesterol (75:25)							
2exp	8.90 ± 0.22		0.97 ± 0.01	2.50 ± 0.76		0.03 ± 0.01	1.27
POPC + gramicidin (90:10)							
2exp	7.65 ± 0.22		0.92 ± 0.01	2.05 ± 0.12		0.08 ± 0.01	1.81
POPC + cholesterol (90:10) + gramicidin (8.3% molar)							
2exp	9.26 ± 0.18		0.93 ± 0.01	3.27 ± 0.55		0.07 ± 0.01	1.39
2Lor	9.15 ± 0.12	0.88 ± 0.04	0.94 ± 0.02	4.27 ± 0.36	0.97 ± 0.78	0.06 ± 0.02	1.08
POPC + BPS (90:10)							
2exp	8.26 ± 0.04		0.94 ± 0.01	1.91 ± 0.31		0.06 ± 0.01	1.57
POPC + BPS + cholesterol (65:10:25)							
2exp	9.95 ± 0.16		0.97 ± 0.01	2.96 ± 0.29		0.03 ± 0.01	0.90
POPC + BPS (90:10) + apocytochrome C (10% molar)							
2exp	8.14 ± 0.03		0.92 ± 0.02	1.99 ± 0.24		0.08 ± 0.02	1.24
2Lor	8.12 ± 0.06	0.57 ± 0.05	0.92 ± 0.01	2.09 ± 0.15	0.74 ± 0.38	0.08 ± 0.01	0.98
POPC + BPS + cholesterol (65:10:25) + apocytochrome C (10% molar)							
2exp	9.58 ± 0.03		0.94 ± 0.01	2.18 ± 0.28		0.06 ± 0.01	1.99
2Lor	9.51 ± 0.14	1.00 ± 0.34	0.94 ± 0.01	2.88 ± 0.57	2.00 ± 0.78	0.06 ± 0.01	1.21
DPH-PC							
POPC							
2exp	7.06 ± 0.12		0.92 ± 0.01	2.54 ± 0.03		0.08 ± 0.01	0.37
POPC + cholesterol (75:25)							
2exp	7.77 ± 0.13		0.91 ± 0.05	3.53 ± 0.72		0.09 ± 0.05	1.41
POPC + gramicidin (90:10)							
2exp	6.88 ± 0.04		0.94 ± 0.01	1.98 ± 0.16		0.06 ± 0.01	1.84
POPC + cholesterol (90:10) + gramicidin (8.3% molar)							
2exp	7.30 ± 0.09		0.96 ± 0.02	2.50 ± 0.51		0.04 ± 0.02	1.26

2exp, biexponential analysis; 2Lor, bimodal Lorentzian; t, lifetime centers ($\times 10^{-9}$ s); w, widths at half height of distribution ($\times 10^{-9}$ s); f, fractional intensities; χ^2, chi-squared. The average errors in the phase and modulation were 0.2° and 0.002, respectively. Data were collected for 10–12 frequencies for 5–120 MHz.

amount of water in the vicinity of the fluorophore. In the presence of gramicidin, inclusion of cholesterol still led to an increase in the DPH fluorescence lifetime from 7.65 to 9.26 $\times$ 10^{-9} s. Thus, the dehydration effect of cholesterol was fully maintained adjacent to the protein hydrophobic surface. From this it can be inferred that water is present in this region, i.e., at the protein-lipid interface.

Fluorophores will locate in environmentally distinct regions, such as found in a lipid bilayer, and decay from the excited state with a range of decay rates reflecting the degree of heterogeneity. In this situation it may be appropriate to analyze the fluorescence decay modeled as a range of decay rates rather than using a discrete exponential decay. In common with other studies (Alcala et al., 1987*a–c;* Fiorini et al., 1987, 1988, 1989; Hermetter et al., 1988, 1989; Schroeder et al., 1988; Valentino et al., 1988; Nemecz and Schroeder, 1988; Kalb et al., 1989) we have used a bimodal Lorentzian continuous distribution, although the choice of the form of the distribution is somewhat arbitrary and alternate forms, such as a Gaussian distribution may be equally valid (Lakowicz et al., 1987). The important point is to compare the relative effects on the distributional width since this gives an indication of the degree of fluorophore environmental heterogeneity of the underlying structural diversity "sampled" by the fluorophore. Whereas in this work the major conclusion of hydration at the protein-lipid interface does not require a lifetime distributional analysis and a discrete analysis adequately reveals effects on the lifetime center reflecting changes in hydration, nevertheless, the possibility that useful information could still be gained from analysis as a range of decay rates was investigated.

In general, distributional widths in the range of 0–1 $\times$ 10^{-9} s were found, however; in most cases there was little if any improvement in χ^2 for the bimodal Lorentzian as compared to the double exponential for narrow widths

(especially $\sim$ <0.5 $\times$ 10^{-9} s). It is difficult to assign a distributional width with certainty when the values are in this region (Lakowicz et al., 1987). For bilayers of POPC alone a bimodal Lorentzian distribution did not yield an improved χ^2 and the recovered width of the major lifetime component was zero. This would be in keeping with a DPH location in an environmentally homogeneous region, below the steep part of the dielectric constant gradient. In the presence of gramicidin a distributional width for the major lifetime center of 0.55 $\times$ 10^{-9} s was recovered by the analysis. However, this was still too small to result in an improved χ^2 for the bimodal Lorentzian analysis as compared with a discrete analysis. The bimodal Lorentzian fit has therefore to be rejected. This result means that if the DPH is in fact in an environmentally heterogeneous region at the protein-lipid interface, the data are not of high enough quality to distinguish the heterogeneity from a homogeneous bulk lipid environment. This does not mean that any environmental heterogeneity, such as may nevertheless be present at the protein-lipid interface, has no significance, but only that the method is not able to demonstrate its presence with the required reliability. In the presence of cholesterol, the distributional width recovered from a bimodal Lorentzian analysis was 0.88 $\times$ 10^{-9} s. In this case the χ^2 was somewhat improved over the discrete analysis (see Table 1). The result may be interpreted as indicative of environmental heterogeneity at the protein-lipid interface. This contrasts with the effect of cholesterol in lipid bilayers without protein (Fiorini et al., 1988; Hermetter et al., 1988; Nemecz and Schroeder, 1988; Fiorini et al., 1989; Kalb et al., 1989; Fiorini et al., 1988), where it was shown that the distributional width narrows (indicating decreased environmental heterogeneity), in this instance due to the dielectric constant gradient set up by water penetration being pushed up towards the bilayer surface and away from the region accessible to DPH. It is possible that water may be only partly responsible for the fluorophore heterogeneity at the protein-lipid interface and that other factors such as amino acid side chains may also contribute and be under the influence of cholesterol.

The introduction of apocytochrome C into the lipid bilayer (which included brain-PS [POPC:PS, 9:1], necessary for the apocytochrome C to incorporate) did not appreciably affect the fluorescence lifetime, contrasting with the effect of gramicidin. This indicated that apocytochrome C did not introduce further water into the bilayer than was already present. The fact that water was nevertheless accommodated at the apocytochrome C protein-lipid interface was suggested by the ability of cholesterol to increase the fluorescence lifetime from 8.14 to 9.58 $\times$ 10^{-9} s (see Table 1). In the presence of apocytochrome C, a bimodal Lorentzian analysis yielded a distributional width of 0.57 $\times$ 10^{-9} s (see Table 1). In this case the χ^2 was slightly improved over the discrete analysis so that the result is suggestive of underlying environmental heterogeneity at the protein-lipid interface. With cholesterol, the distributional width increased to 1.00 $\times$ 10^{-9} s, with a consequently greater improvement of the χ^2 as compared with the discrete analysis.

In addition to free DPH, which locates across the bilayer central region, the excited state properties of DPH anchored at a fixed depth in the bilayer, by attachment to the *sn*-2 position of phosphatidylcholine (1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]-carbonyl]-3-*sn*-PC or DPH-PC), was examined. Inclusion of gramicidin reduced the lifetime, although by less than with DPH. The addition of cholesterol caused the fluorescence lifetime to increase from 6.88 to 7.30 $\times$ 10^{-9} s (see Table 1) as found with DPH. Thus, hydration of the protein-lipid interface could again be inferred using this probe. The DPH in DPH-PC, being located at a fixed depth in the bilayer, does not allow it to sample across the dielectric constant gradient, even in mixed species phospholipids (Ho et al., 1992). Presumably, it also limits its ability to sample different regions of the protein-lipid interface as indicated the lack of an improvement in the χ^2 using bimodal Lorentzian fit, both with or without cholesterol (with cholesterol the distributional width was 0.51 $\times$ 10^{-9} s right at the margin of acceptability).

To provide further evidence for hydration of the hydrophobic surface of membrane proteins, deuterium isotope exchange was used. This leads to a higher fluorescence yield (when H_2O is replaced by D_2O) and can therefore be used to indicate the extent of water accessibility to a fluorophore (Stryer, 1966). Whereas the effect was too small to be useful with DPH, trimethylammonium-DPH (TMA-DPH) was found to be sensitive to the presence of deuterium. TMA-DPH had the added advantage that it provided information on hydration at the protein-lipid interface adjacent to the phospholipid head group region, thereby providing information complementing that provided by the DPH and DPH-PC on the deeper regions with the bilayer. The effect of replacing H_2O by D_2O on the fluorescence intensity can be expressed as the ratio of the fluorescence intensity in D_2O to that in H_2O, which should be greater than unity if the fluorophore is in contact with water. On the contrary, if the fluorophore is shielded from water, then the D_2O/H_2O fluorescence intensity ratio should equal one. The D_2O/H_2O fluorescence intensity ratio data for TMA-DPH in lipid bilayers with apocytochrome C or gramicidin, with and without cholesterol, are summarized in Table 2 and an example spectra are shown in Fig. 1. In the case of POPC bilayers, the ratio was greater than one, however, in the presence of gramicidin the ratio increased significantly. This indicated that gramicidin introduced more water into the locality probed by the TMA-DPH, (i.e., at the protein-lipid interface). Further, addition of cholesterol drastically decreased the ratio showing that its dehydrating effect was able to occur at the protein-lipid interface (in this case in the lipid head group region). The

gramicidin tryptophans locate near the lipid head group region and protrude into the lipids (Cornell, 1987; Wallace, 1990) and were also used to detect the presence of water. When D_2O/H_2O fluorescence intensity ratio was examined an identical result to that for TMA-DPH was obtained, in this case showing hydration adjacent to the tryptophans. The addition of apocytochrome C to the bilayer did not affect the D_2O/H_2O ratio, a result in keeping with the lack of effect of apocytochrome C on the fluorescence lifetime of DPH in POPC/brain-PS bilayers (see above) showing that apocytochrome C did not increase the amount of water already in the bilayer. Inclusion of cholesterol again dehydrated the tryptophan locality, as shown by the considerable decrease in the D_2O/H_2O fluorescence intensity ratio almost to unity.

CONCLUSIONS

The presence of water at the protein-lipid interface of membrane proteins has not been previously described. The hydration of proteins in general has a profound and recognized role in directing the structure of the protein, mainly through the formation of hydrogen bond networks with water and amino acid side chains. Whereas membrane protein structure is directed to a large degree by ionic and hydrophobic interactions, the results of this study indicate that water at the protein-lipid interface is an additional factor involved in influencing the structure. The structure of a membrane protein is critical to its functioning and the conformation may alter as a part of this function. Therefore, changes in the degree of hydration brought about by altered levels of cholesterol or by the addition of lipophilic agents such as drugs, anesthetics and ethanol, for example, could exert a modulatory influence on the protein conformation, thus offering a new route whereby protein structure and hence function may be modulated.

TABLE 2 Summary of the fluorescence intensity ratio data (D_2O/H_2O)

		+cholesterol
TMA-DPH		
POPC	1.14 ± 0.03	1.01 ± 0.02
POPC:gramicidin (9:1)	1.23 ± 0.04	1.05 ± 0.04
POPC:brain-PS (9:1)	1.16 ± 0.03	1.01 ± 0.01
POPC:brain-PS:apocytochrome C (8:1:1)	1.15 ± 0.05	1.03 ± 0.02
TRYPTOPHAN		
POPC:gramicidin (9:1)	1.20 ± 0.04	1.08 ± 0.04

Vesicles (1,000 μM) were labelled with TMA-DPH and diluted (1:9) with either H_2O or D_2O. Fluorescence spectra were taken upon excitation at 360 nm. The ratio of the emission intensities in D_2O to H_2O at the emission maxima of 430 nm. For the vesicles with gramicidin, the intrinsic tryptophans were also monitored, by excitation at 280 nm and observation of the emission maxima at 330 nm. The results are expressed as the mean ± SD of three separate preparations.

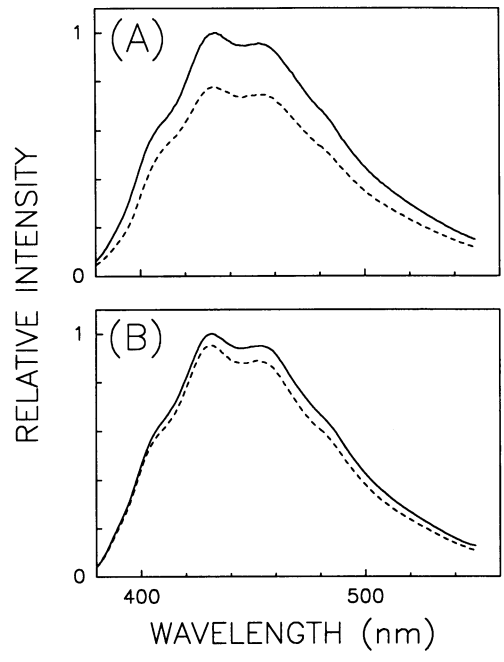

FIGURE 1 Fluorescence emission spectra of TMA-DPH in POPC bilayers obtained as described in Table 2. (*A*) POPC:gramicidin (9:1) in D_2O (——) or H_2O (- - -), (*B*) with 25 mol % cholesterol. Details are as described in Materials and Methods.

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