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Determination of the Rate of Rapid Lipid Transfer Induced by Poly(ethylene glycol) Using the SLM Fourier Transform Phase and Modulation Spectrofluorometer

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Rate constants were determined for the transfer of the fluorescent lipid probe 1-palmitoyl-2-[[[2-[4-(6-phenyl-trans-1,3,5-hexatrienyl)phenyl]ethyl]oxy]carbonyl]-3-*sn*-phosphatidylcholine (DPHpPC) between large, unilamellar extrusion vesicles composed either of dipalmitoyl phosphatidylcholine (DPPC) or of DPPC mixed with a small amount (0.5 mol%) of lyso phosphatidylcholine (Lyso PC). Transfer of the lipid probe in the presence of varying concentrations of poly(ethylene glycol) (PEG) was monitored using the SLM 48000-MHF Multi-Harmonic Fourier Transform phase and modulation spectrofluorometer to collect multifrequency phase and modulation fluorescence data sets on a subsecond time scale. The unique ability of this instrument to yield accurate fluorescence lifetime data on this time scale allowed transfer to be detected in terms of a time-dependent change in the fluorescent lifetime distribution associated with the lipid-like DPHpPC probe. This probe demonstrates two short fluorescence decay times (*ca.* 1.1–1.4 and 4.3–4.8 ns) in a probe-rich environment but a single long lifetime (*ca.* 7 ns) in a probe-poor environment. A simple two-state model for initial lipid transfer was used to analyze the multifrequency data sets collected over a 4-s time frame to obtain the time rate of change of the concentrations of donor and acceptor probe populations following rapid mixing of vesicles with PEG. The ability to measure fluorescence lifetimes on this time scale has allowed us to show that the of rate of lipid transfer increased dramatically at 35% PEG in both fusing and nonfusing vesicle systems. These results are interpreted in terms of a distinct interbilayer structure associated with intimate bilayer contact induced by high and potentially fusogenic concentrations of PEG.

KEY WORDS: Phase fluorometry; lipid exchange; membrane fusion; kinetics; poly (ethylene glycol); diphenyl hexatrient.

INTRODUCTION

Recently, we showed that vesicles composed of pure phosphatidylcholine did not fuse in the presence of PEG[4][1]. However, introducing a small amount of a monoacyl phosphatidylcholine allowed vesicles to fuse [2]. In both cases, we observed the transfer of

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[4] Abbreviations used: PEG, poly(ethylene glycol); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid, tetrasodium salt; $C_{12}E_8$, dodecyloctaethylene glycol monoether; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DPHpPC, 1-palmitoyl-2-[[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl) carbonyl]-3-*sn*-phosphatidylcholine; LUVET, large, unlamellar vesicles made by rapid extrusion technique; Lyso PC, 1-oleoyl-*sn*-glycero-3-phosphocholine.

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lipids between membranes, even in the absence of fusion. In addition, we have measured the interbilayer distance between aggregated membranes for both lipid systems and found that under the same aggregating conditions, both fusing and nonfusing membranes come to the same distance of separation [3]. This suggests that a distinct interbilayer structure might exist which allows transfer of lipids between membranes in intimate contact but that another structure might be necessary for the fusion event. In order to establish the existence of a common interbilayer structure which supports lipid transfer and then to distinguish this from other structures which support fusion, it was necessary to compare the rates of lipid transfer between fusing and nonfusing membranes.

Several membrane fusion studies have reported rates of lipid transfer or fusion [4,5]. Most of these studies have relied on quantitating changes in fluorescence intensity, an extrinsic property of a membrane-associated probe, and relating that quantity to the amount of probe which had transferred. The problems associated with these measurements are, first, that probe transfer may not accurately reflect bulk lipid transfer, and, second, that quantitating probe transfer is often difficult. These problems are, to some extent, relieved by monitoring fluorescence lifetime changes of the probe DPHpPC to quantitate lipid transfer [6,7]. However, the assumptions made in interpreting probe lifetime in terms of lipid transfer make our past analysis [7] useful mainly for quantitating the final extents rather than the initial rates of lipid transfer. This was appropriate considering that lipid transfer in the presence of fusing concentrations of PEG was too rapid to be followed by lifetime measurements made even by phase shift and modulation ratio methods. We present here results obtained with a new generation of phase/modulation fluorometers that make possible detection of lipid transfer on a subsecond timescale. To accompany this new technology, an alternative analysis is also developed that should be valid in the limit of initial exchange and, under certain conditions, up to completion of the lipid transfer process.

Transfer of lipids between contacting vesicles at high concentrations of PEG is quite rapid, with transfer being essentially complete in the first several seconds following mixing of vesicles and PEG. Since the current generation of SLM phase fluorometer (the 48000) has a minimum phase/modulation data acquisition time of 1 s, such a fast process would press the limits of its capabilities to resolve the kinetics of a change in even a single fluorescence lifetime. However, DPHpPC transfer involves changes in a three-component lifetime distribution, the rigorous resolution of which requires a multifrequency analysis. Even with the simplifications that we have adopted (see Results), the experimental uncertainties associated with making measurements in high concentrations of PEG demand that several data points be averaged and that data from several modulation frequencies be analyzed in order that reasonably accurate estimates of probe populations can be obtained. This is a task that is very difficult, if not impossible, to accomplish through acquisition of data at a single modulation frequency with the current generation of phase/modulation fluorometers (e.g. the SLM 48000). A new generation of spectrofluorometer, the SLM 48000-MHF Fourier Transform Spectrofluorometer, allows multifrequency phase and modulation data sets to be acquired on a submillisecond timescale. While we do not utilize the submillisecond acquisition feature of the instrument for this study (the acquisition time appropriate for the present application was either 15 or 50 ms), we show here that the rates of rapid probe movement between membranes can be easily and accurately determined using fluorescence lifetime measurements made on this instrument. Thus, the Fourier transform feature of this new instrumentation has allowed us to develop new techniques that promise to be an important tool in eludicating additional structural requirements necessary for the fusion process, both in model membranes and in cellular fusion events such as viral fusion.

We have utilized this new technology to measure the rate of transfer of DPHpPC from probe-rich to probe-free vesicles and report here measurements made on two phospholipid vesicle systems which vary in their ability to fuse in response to aggregation by PEG. The ability to fuse in the presence of high concentrations of PEG appeared not to correlate with enhanced rates of interbilayer lipid transfer also observed at high PEG concentration. The results suggest the existence, at high PEG concentrations, of unique interbilayer structures that may aid in fusion but that are not sufficient to induce fusion.

EXPERIMENTAL PROCEDURES

Materials

Chloroform stock solutions of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) and 1-palmitoyl-2-[[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl] oxy]carbonyl]-3-*sn*-phosphatidylcholine (DPHpPC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL).

1-Oleoyl-*sn*-glycero-3-phosphocholine (Lyso PC) was obtained from Sigma. Lipids were verified to be greater than 98% pure by thin-layer chromatography [8]. Plates were developed in a 65:25:4 (v/v/v) $CHCl_3$:CH_3OH:H_2O mixture and were stained with iodine vapors. DPHpPC was also viewed under near-UV light. Poly(ethylene glycol) (Carbowax PEG 8000; average MW 8000) was obtained from Fisher Scientific (Fair Lawn, NJ; Lot No. 874229) and purified as described previously [2]. *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) was purchased from Calbiochem (San Diego, CA). Buffer solutions were filtered prior to use through a 115-ml Nalgene disposable filter (Nalge Company, Rochester, NY) to remove dust which might interfere with the fluorescence measurements. All other chemicals were of the highest quality available.

Methods

Vesicle Preparation. Large, unilamellar extrusion vesicles (LUVET) were prepared by the method of Hope *et al.* [9]. Lyophilized lipid samples were suspended in a buffer at a temperature above the gel to liquid–crystalline phase transition. The large, multilamellar vesicles which formed were allowed to equilibrate and hydrate fully above their phase transition for approximately 30 min. The vesicles were then forced seven times through a 0.1-μm polycarbonate filter (Nucleopore, Pleasanton, CA) above their phase transition under a pressure of approximately 200 psi of argon. For both DPPC and DPPC/Lyso PC vesicles, this procedure yielded a fairly homogeneous population of unilamellar vesicles with a mean diameter determined by quasielastic light scattering of ~1200 Å. The concentrations of all vesicle samples were determined by phosphate analysis using a modification of the procedure of Chen *et al.* [10]. Vesicles were prepared in a buffer containing 100 m*M* NaCl, 2 m*M* TES, 1 m*M* EDTA, pH 7.4. Osmolarities of all buffers were monitored using a μOsmette freezing-point depression microosmometer (Precision Systems, Sudbury, MA).

Fluorescence Lifetime Measurements to Detect Lipid Transfer on a Millisecond Time Scale. Mixing of membrane components induced by PEG was demonstrated by using the DPHpPC fluorescent lifetime lipid mixing assay [6,7]. A detailed description of the assay and its intricacies is given by Burgess and Lentz [11].

We have utilized a recently developed, multiharmonic, Fourier transform spectrofluorometer (SLM 48000-MHF) [12,13] located in the laboratories of SLM-Aminco, Inc., in Urbana, IL, to measure the rate of transfer of DPHpPC from probe-rich to probe-free vesicles. This instrument allows acquisition of complete multifrequency, phase/modulation data sets on a submillisecond timescale. Multifrequency acquisitions at millisecond rates were performed by digitizing the detector signal using a sampling interval which is the reciprocal of the cross-correlation frequency offset. The offset and time-domain averaging aperture were varied to optimize the signal-to-noise ratio for the number of frequencies collected at a given time resolution. In these experiments, we used a base frequency of 6 MHz with a correlation frequency offset of 20 Hz for the DPPC:Lyso PC vesicle system and a 7-MHz base frequency with a 65-Hz correlation frequency offset for the DPPC vesicle system. These settings yield time-domain impulses every 50 and 15 ms, respectively. The Fourier transform of an impulse (or averaged series of impulses) waveform yielded the phase shift and demodulation information.

Details of Fluorescence Measurements. Excitation of DPHpPC for lifetime measurements was with a He-Cd laser (Liconix Model 4240NB; 13-mW multimode output at 325 nm), while emission was monitored through stacked 3-mm high-pass KV-390 and KV-410 filters (50% transmittance at 390 and 410 nm, respectively; Schott Optical Glass, Duryea, PA). Vertically polarized and modulated light from the Pockel cell was rotated to 35° from vertical by placing a Soliel-Babinet compensator (Karl Lambrecht, Chicago, IL) rotated 17.5° from vertical and set for half-wave at 325 nm in the excitation path. Scattering from unlabeled vesicles was used to establish a zero lifetime reference. A 3-mm UG-11 filter (Schott Optical Glass, Duryea, PA) was placed in the emission path to block all fluorescence intensity and pass only zero lifetime scattered light.

Time-domain data were collected and transformed using an analysis window of 50 to 500 ms over which collected response waveforms were averaged. Discrete multifrequency measurements were collected at a chosen impulse frequency in the time domain, e.g., every 50 ms. This information was translated to the frequency domain based on the size of the window selected. An analysis window of 50 ms would result in each individual 50-ms pulse being transformed into phase and modulation data. If the analysis window was set to 500 ms, then all the information contained within the 10 impulse waveforms in the 500-ms window was averaged and the average phase and modulation data were transformed to the frequency domain. Figures 1 A and B show phase and modulation data for the DPPC vesicle system using different analysis windows. Data shown were obtained using a pulse interval of 15 ms and an analysis window

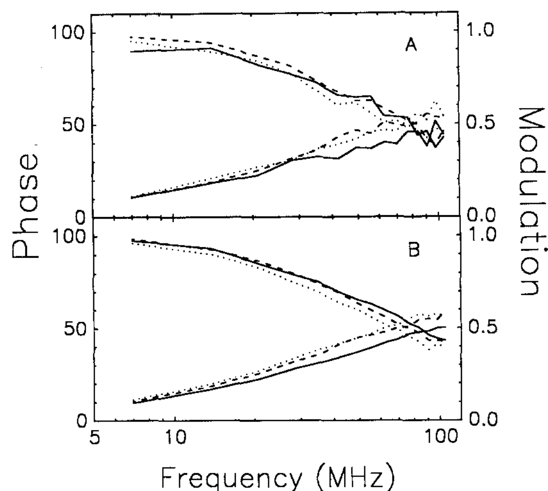

Fig. 1. Raw phase and modulation data. Phase shift (lower curves) and modulation ratio (upper curves) data are presented for DPPC (A and B) vesicles in 35% (w/w) PEG at 50°C. Data shown are for t = 0 (solid line), t = 2 s (dashed line), and t = 4 s (dotted line). The averaging window was set to ~50 ms for frame A and ~500 ms for frame B.

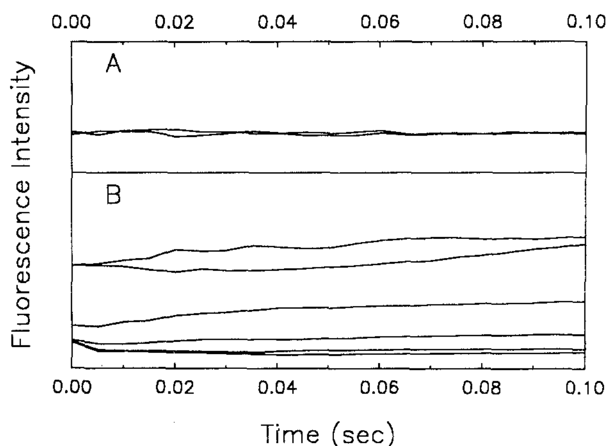

Fig. 2. Mixing times for PEG. The characteristic mixing times for (A) two aqueous solutions at 3 and 6 bar and 50°C and (B) 50% (w/w) PEG pushed with an aqueous solution at 3, 4, 5, 6, 7, and 8 bar (top to bottom on right side of graph, respectively) at 50°C [final PEG concentration, 25% (w/w)]. The mixing times were determined using 20 μM ANTS in one solution pushed with 20 μM DPX in the other solution and by monitoring the quenching of ANTS fluorescence due to mixing.

of ~50 ms (A) or ~500 ms (B). This figure illustrates the expected playoff between high data precision and high time resolution, with data averaging yielding the most significant enhancement of precision in the range of modulation frequencies beyond 30 MHz.

Rapid Mixing Methods. Certain modifications were necessary in order to adapt the DPHpPC transfer assay [6,7] to the stopped-flow measurement of rapid rates of lipid mixing. The stopped-flow device used for this study was the Stopped Flow Accessory from Applied Photophysics (Model RX1000; Leatherhead, UK) fitted with a pneumatic driver. The RX1000 was adapted to push PEG by enlarging the bore of one of the valves and by using larger-diameter tubing to connect the modified valve to the mixing chamber. Experiments with PEG were performed at 50°C in order to reduce the viscosity of the PEG solution and allow better mixing. The syringes used for these experiments had volumes of 1.0 ml for vesicles and 2.5 ml for PEG. Observed mixing times for PEG were generally ~40 ms (see Fig. 2) when pushing with a pressure of 8 bar. After adding the deadtime of the RX1000 (17 ms), the total mixing time is of the order of 55–60 ms. Incomplete mixing of solutions occurred at a 35% (w/w) PEG final concentration. At this concentration, two phases resulted, an upper phase of approximately 34% (w/w) PEG and a lower phase of approximately 36% (w/w) PEG. Fluorescence emission from the lower phase was selected by adjusting the modulated light beam so it would pass through only the lower portion of the observation window. Vesicles containing 10 mol% DPHpPC (10:1 lipid-to-probe ratio) and probe-free vesicles were combined at a 10:1 ratio of blank vesicles to probe vesicles and loaded into a stopped flow syringe. Vesicles and PEG were allowed to equilibrate for 5 min at 50°C before each push.

RESULTS

We analyzed the phase and modulation data obtained from the time-domain impulse for three lifetime components. This analysis was based on our previous detailed analysis of the concentration dependence of the DPHpPC fluorescence lifetime [14] and on a two-state lipid transfer model (Fig. 3), where the probe initially resides in a probe-rich vesicle population where it has two lifetimes (τ_1 and τ_2) and transfers to a probe-poor vesicle population where it has a single lifetime (τ_3) different from the other lifetimes in the system [15]. The lifetimes τ_1 and τ_2 appear to result from the excited-state dynamics of a probe dimer at high probe concentrations, while τ_3 is presumably the fluorescence lifetime of the probe in the monomer form [14]. From our previous studies, τ_3 was fixed at 7.1 ns. During the analysis of our current multifrequency data sets, due to the large number of parameters being fit, we fixed τ_2 and τ_3 and

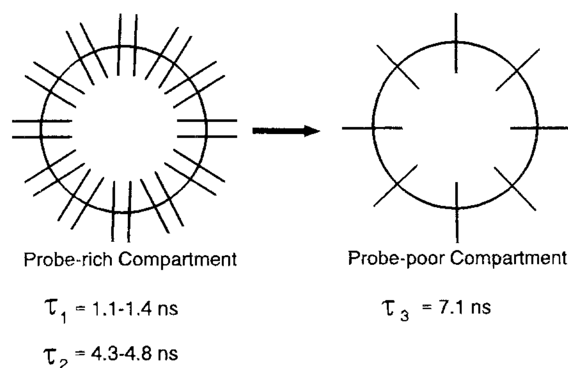

Fig. 3. Two-state lipid transfer model. Probe initially resides in a probe-rich vesicle population where it exists predominantly as a probe dimer having a two-component lifetime (τ_1 and τ_2). After the probe transfers to a probe-poor vesicle population, it exists predominantly in the probe monomer form having a single lifetime component (τ_3) longer than the other lifetimes in the system.

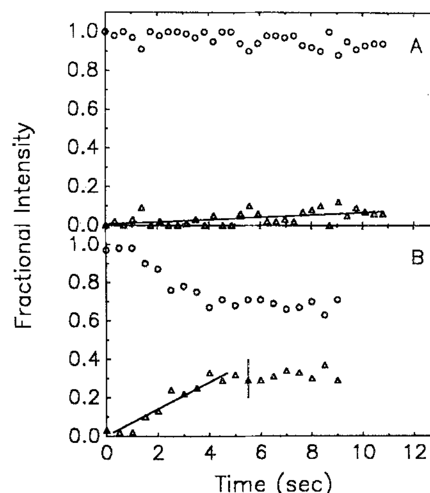

Fig. 4. Fractional intensities recovered from analysis of phase and modulation data. Fractional intensities are shown for DPPC vesicles in (A) 10% (w/w) PEG and (B) 35% (w/w) PEG. Fractional intensity, f_p (open triangles), corresponds to the appearance of probe in the probe-poor vesicle population, while fractional intensity f_r (open circles), which is the sum of the fractional intensities for lifetimes τ_1 and τ_2 (probe-rich compartment), reflects the behavior of probe in the probe-rich vesicle population. Linear regression lines show the range of data used for further analysis to obtain initial rates of transfer, as illustrated in Fig. 5.

allowed τ_1 and the fractional intensities f_1, f_2, and f_3 to vary (subject to the constraint $\Sigma f_i = 1$) to minimize the squared difference between calculated and observed data. A grid search over the values of τ_2 was then performed (0.1-ns steps) to obtain a best fit of the data. Occasionally it became necessary to fix τ_1 as well to obtain a proper fit, and in this case, a similar grid search was performed on values of τ_1. From our previous results [14], τ_1 and τ_2 were constrained to values in the ranges 1.1–1.4 and 4.3–4.8 ns, respectively. This procedure was followed for each multifrequency data set during our kinetic analysis.

Figure 4 shows the time-resolved fractional intensity contributions of the three lifetime species to the overall fluorescence decay of the probe incorporated into DPPC vesicles exposed to 10 wt% PEG (Fig. 4A) and 35 wt% PEG (Fig. 4B). From this analysis, we observe that the rate of probe transfer between probe-rich and probe-poor vesicles increased with increasing PEG concentration, as evidenced by the increased rate of appearance of f_p ($f_p = f_3$), the fractional intensity of probe-poor vesicles. Concomitantly, the fractional intensity f_r ($f_r = f_1 + f_2$), which reflects the fractional intensity of the probe-rich vesicles, decreased with time in the presence of PEG. While f_1 and f_2 cannot separately be assigned clear physical interpretations, the sum of f_1 and f_2 represents in a complex fashion the behavior of probe dimers in the probe-rich vesicles. The fractional intensity f_3 represents, in the context of our dimer model for DPHpPC concentration-dependent photophysics [14], the contribution of probe monomers in the probe-poor vesicles.

Since we could not determine directly the rates of lipid transfer from the fractional intensity, we had to convert the observed fractional intensities to mole fractions of probe in the probe-poor and probe-rich vesicles [15]. The data were transformed using the equation [15]

$$\frac{f_a}{f_b} = \frac{\tau_a\alpha_a}{\tau_b\alpha_b} = \frac{X_a\,F_a}{X_b\,F_b} \tag{1}$$

where the α's are the preexponential factors associated with either the probe-rich or the probe-poor compartments, the X's are the mole fractions of probe in each compartment, and the F's are the fluorescence intensities for each compartment. τ_a is the average lifetime of the donor compartment (calculated from the fractional intensities, f_1 and f_2, and lifetimes, τ_1 and τ_2, of the individual components; see Ref. 16), and $\tau_b = \tau_3$ is the lifetime of the acceptor population.

The data in the linear regions of time courses such as shown in Fig. 4 were analyzed as described above to obtain the time variation of the mole fraction of probe in the acceptor population. The initial rate of DPHpPC (probe) transfer was taken as the slope of the least-squares line through the mole fraction data (constrained to intercept at the origin) in the initially linear region of the data. The rates of DPHpPC transfer were calculated for each lipid system and are plotted in Fig. 5 as a function

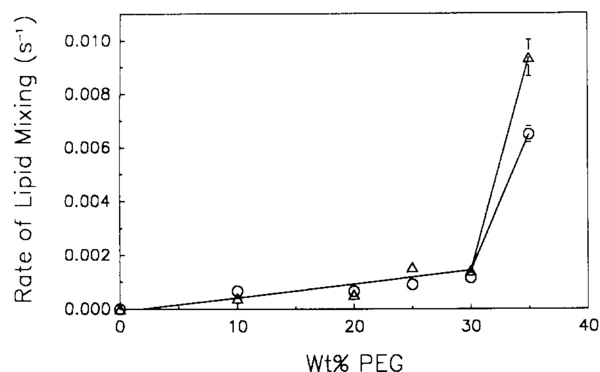

Fig. 5. *Rate constants for lipid exchange.* Rate constants for lipid exchange as a function of PEG concentration were derived as described under Results. Results of this analysis are presented for DPPC:Lyso PC (open triangles) and DPPC (open circles) vesicles at 50°C. Error bars represent the error in the slope of the function used to fit the data presented in Fig. 4. Error bars for PEG concentrations ≤30% (w/w) were smaller than the size of the symbol.

of PEG concentration. In the absence of PEG, the rate of DPHpPC transfer was too small to be quantitatively estimated in the time frame of the measurements reported here. We have estimated this in another paper to be $3 \times 10^{-5}\ s^{-1}$ [15], which is effectively zero on the scale of rates measured here in the presence of PEG. For both lipid systems, PEG increased substantially the rate of lipid exchange, and this effect was linear in PEG concentration up to PEG concentrations of ≤30 wt%. In this PEG concentration range, the rates of lipid transfer were identical for nonfusing and fusing vesicles (DPPC and DPPC/Lyso PC, respectively). At 36 wt% PEG, the rate of transfer increased dramatically for both lipid systems. The rate was slightly faster for the fusogenic DPPC:Lyso PC vesicle system, although more data will be needed before this rate enhancement could be taken as significant and reflective of the fusion process.

DISCUSSION

We have developed a new procedure to determine rates of rapid lipid transfer between vesicles aggregated in the presence of PEG. This procedure uses an assay for lipid transfer which directly measures the concentration of probe in the membrane using the fluorescence lifetime quenching of DPHpPC [6] as well as a new generation of Fourier transform phase/modulation fluorometers. Two features of this new instrumentation have made possible the rapid measurements reported here. First, it was not possible to acquire lifetime measurements with greater than a 1-s resolution using the SLM 48000. Therefore, determination of lipid transfer rates where a significant amount of transfer occurs in the first second was extremely difficult or impossible. Other single-frequency instruments allow measurements with a 0.2- to 0.4-s resolution, however, they, like the SLM 48000, still suffer from an additional limitation. This second limitation is that conventional phase/modulation fluorometers acquire data at a single modulation frequency. If data are to be acquired at additional frequencies, the frequency must be reset and the experiment repeated, a process that would introduce considerable experimental variation into kinetic measurements such as we report here. The advantages of multifrequency data are twofold. First, data from a single frequency will not yield sufficient information to resolve even two lifetime components unless at least one of the lifetime values is fixed. For our application, only one of the three lifetime components present (τ_3) could be fixed; the other two could only be constrained to reasonable ranges. Even if additional values could have been fixed, a second and more significant advantage exists for multifrequency measurements. Highly viscous PEG solutions induce substantial uncertainties and variations in measurements which create inaccuracies in the multicomponent lifetime analysis. These inaccuracies are greatly diminished by multifrequency acquisition due to the additional information provided by data obtained at more than one frequency. The effect of having available both phase shift and modulation ratio data from several frequencies is illustrated in Fig. 1A; phase shift data were most useful from 7 to 30 MHz, while modulation ratios were reasonably reproducible from 30 to 70 MHz. Further improvement in data quality can be attained by collecting data over short intervals and then averaging several points (Fig. 1B). We consider the ability to collect data simultaneously at several modulation frequencies to be the main advantage of the new Fourier transform instrument over existing instrumentation. Without this ability, the measurements presented here would probably not have been possible at sufficient accuracy to allow the analysis and interpretation we have made.

Using the unique capabilities of the SLM 48000 MHF spectrofluorometer, we were able to determine initial rates of lipid transfer between contacting vesicles in the presence of PEG. Our findings show that vesicles aggregated in the presence of PEG transfered lipids at a rate that is proportional to the concentration of PEG present in the solution. Transfer occurred at a rate much greater than occurred in the absence of PEG. Although we could not estimate the rate in the absence of PEG by the methods presented here, Wu and Lentz [15] reported

values for DOPC LUVET at 22°C and 3.3×10^{-5} s^{-1} in the absence of PEG and 5.5×10^{-4} s^{-1} in the presence of 10% (w/w) PEG. The values for the initial rate of transfer measured here for DPPC and DPPC/LysoPC at 50°C in the presence of 10% (w/w) PEG (6.7×10^{-4} and 4.0×10^{-4} s^{-1}, respectively) are consistent with the rate obtained by Wu and Lentz [15] on the basis of much longer-term experiments. We show elsewhere [15] that the enhancement of the rate of lipid transfer is due to aggregation rather than due to the effect of PEG on the dielectric properites of bulk water or to the action of PEG as a carrier of phospholipids. In addition, transfer between aggregated vesicles apparently involves diffusion through an altered interbilayer water space [15]. The linear dependence of transfer rate on PEG concentration is also consistent with this mechanism, as both the thickness of the interbilayer water space [3] and the dielectric properties of water [18] vary inversely with PEG concentration in the range of 10 to 40 wt%.

At 36% (w/w) PEG, the rate of lipid transfer increased dramatically for both DPPC and DPPC/Lyso PC vesicles. However, DPPC/Lyso PC vesicles show initial indications of fusion when treated with only 30 wt% PEG and clearly fused in the presence of 35 wt% PEG [1]. This makes it clear that the dramatic increase in the rate of lipid transfer seen at 36 wt% PEG is not due solely to the fusion process. It was also not due to a "monolayer fusion" or "hemifusion" [19,20] involving lateral diffusion between fused outer monolayer leaflets of PEG-aggregated vesicles. We conclude this because, even in the presence of 36 wt% PEG, the observed time required for lipid transfer was 5–7 orders of magnitude longer than it would have taken for probe to diffuse laterally from one vesicle to another joined by fused monolayers, based on lateral diffusion coefficients of typical phospholipids [21]. It should be noted that lateral diffusion is not slowed by the dehydration induced by 10% PEG [Wu and Jacobson, unpublished observations]. While the rate of intervesicle lipid transfer in the presence of 36 wt% PEG is too slow to reflect "hemifusion," the marked increase in the transfer rate between 30 and 36 wt% PEG suggests that an unique interbilayer structure forms at 36 wt% PEG concentrations and permits rapid movement of lipids between vesicles. Burgess *et al.* [3] showed that the interbilayer separation between contacting vesicles composed of phosphatidylcholine in 35 wt% PEG solutions is 5 Å. McIntosh *et al.* [22] have noted that membranes at somewhat larger interbilayer distances experienced hydration repulsion, whereas at smaller interbilayer separations, steric overlap of phosphatidylcholine headgroups was proposed. Burgess *et al.* [3] have termed this critical bilayer separation "near-molecular contact." At these separations, much of the bulk water between the membranes has been removed, leaving mainly water molecules involved in maintaining membrane structural integrity. This dehydrated structure may be sufficiently distinct from the normal water–bilayer interface structure to promote rapid transfer of lipids between apposed bilayers. The lack of water molecules in this structure may allow more extensive excursions of lipid molecules in direction normal to the bilayer.

If such a special, dehydrated structure exists, it is apparently not required for membrane fusion, as rapid lipid transfer appears to be independent of fusion. The question arises, then, as to whether the special interbilayer structure involved in rapid lipid transfer might contribute to fusion of DPPC/Lyso PC vesicles even though rapid lipid transfer is not evident until 35 wt% PEG and fusion was first detected at 30 wt% PEG. At 30 wt% PEG, the vesicle membranes are on average 5.5–6 Å apart. While this separation is not considered molecular contact [3], thermal fluctuations within apposed bilayers could bring local membrane areas of aggregated vesicles into molecular contact. Thus, it may be necessary only to approach this special dehydrated structure in order to obtain fusion. The methods presented here offer an approach to testing this hypothesis by providing a new method of defining the properties of bilayers in intimate, near-molecular contact.

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