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A Method for Quantitative Interpretation of Fluorescence Detection of Poly(ethylene Glycol)-Mediated 1-Palmitoyl-2-[[[2-[4-(phenyl-*trans*-1,3,5-hexatrienyl) phenyl]ethyl]oxyl]carbonyl]3-*sn*-Phosphatidylcholine (DPHpPC) Transfer and Fusion Between Phospholipid Vesicles in the Dehydrated State

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A method has been developed for calculating the expected fluorescence lifetime of the DPH_pPC probe distributed between different membrane environments. We show how this method can be used to distinguish between lipid transfer and fusion between large unilamellar vesicles occurring in the presence of poly(ethylene glycol) (PEG). This application of the calculation took into consideration the heterogeneity of microenvironments experienced by the probe in a sample containing vesicle aggregates of different sizes. Assuming that the aggregate size distribution was a delta function of the aggregate size, comparison of the calculated and observed lifetimes yielded an estimate of the vesicle aggregate size. For vesicles of varying compositions in the presence of dehydrating concentrations of PEG, this method suggested that only small aggreggates formed. For vesicles that could be demonstrated by other means not to have fused, the data were consistent with lipid transfer occurring only between the outer leaflets of two to four vesicles, even at high PEG concentrations. For vesicles that could be demonstrated to fuse by contents mixing and size changes, the fluorescence lifetime data were consistent with lipid transfer between both the inner and the outer leaflets of two to four fused vesicles. At very high PEG concentrations, where extensive rupture and large, multilamellar products were previously observed, the lifetime data were consistent with much more extensive lipid transfer within larger aggregates. The agreement of predictions made on the basis of lifetime measurements with other observations attests to the validity of the fluorescence lifetime method. In addition, the model and data presented here provide evidence that fusion occurs between small numbers of PEG-aggregated vesicles before the removal of PEG.

KEY WORDS: Fusion; poly(ethylene glycol); fluorescence; DPH.

INTRODUCTION

Model membrane fusion induced by poly(ethylene glycol) (PEG[3]) has been investigated extensively in re-

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[3] Abbreviations used: PEG, poly(ethylene glycol); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Na_2EDTA, ethylenediaminetetraacetic acid, disodium salt; LUVET, large, unilamellar vesicles made by rapid extrusion technique; DPHpPC, 1-palmitoyl-2-[[[2-[4-(-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]oxy] carbonyl]3-*sn*-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DLPE, 1,2-dilauroyl-3-*sn*-phosphatidylethanolamine; DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; LPC, L-α-lysopalmitoylphosphatidylcholine; PA, palmitic acid.

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cent years [2–10]. Earlier studies have focused largely on defining the PEG concentration required to induce increases in light scattering and/or lipid transfer as indicators of fusion. Unfortunately, because unambiguous interpretation of these measurements in terms of fusion was often not possible in the presence of PEG, conclusions were often obscured. For instance, aggregation cannot be distinguished from fusion on the basis of light scattering alone, and we have shown that PEG causes extensive transfer of lipids between vesicles even in the absence of a fusion event [11]. Our approach to these complexities has been to require that three criteria be satisfied to conclude that fusion has occurred: (1) mixing of membrane components, (2) mixing of internal compartments, and (3) an increase in vesicle diameter [12,13]. The latter two approaches, while capable of clearly demonstrating fusion, require the removal or dilution of high PEG concentrations before a measurement can be made. The first approach, demonstration of the mixing of membrane components, was based on the observation that the lifetime of the fluorescent membrane probe, DPHpPC, is a sensitive function of the local concentration of probe in a membrane [13]. This approach has the advantage that it can be carried out in concentrated PEG solutions but, as outlined below, has been limited by the availability of an appropriate theoretical framework for making detailed mechanistic interpretations.

In our previous studies, interpretation of DPHpPC lifetime data was crude: Essentially, average fluorescence lifetimes were assumed to reflect uniform microenvironments with a DPHpPC concentration determined from a calibration curve produced using uniformly dispersed DPHpPC/phospholipid vesicles [12]. This crude model made it impossible to interpret small changes in observed lifetime in terms of the molecular events involved in the lipid transfer and fusion processes. The basic problem with this crude interpretation is that, in a real fusing vesicle system, the DPHpPC probe will experience a broad range of microenvironments. For instance, fusion might occur between two, three, four, or even a large number of monomer vesicles, with the local DPHpPC concentration being a function of the aggregate size, j. In addition, even different j-mers can contain different local DPHpPC concentrations. A trimer, for instance, may contain one probe and two blank vesicles, or two probe and one blank vesicle, or three blank or three probe vesicles. Each of these situations leads to a different lipid/probe ratio and so to different lifetimes. In the present study, we have modified our crude model to account for such heterogeneity in probe microenvironments. Others have treated the problem of heterogeneous aggregation of unlike vesicles using combinatorial methods [14,15]. Our present study applies this combinatorial approach to heterogeneous aggregation, in conjunction with knowledge of the photophysical behavior of DPHpPC [16], to model the DPHpPC fluorescence lifetime behavior resulting from PEG-induced lipid transfer and fusion between phospholipid vesicles. This more detailed treatment has allowed us to interpret DPHpPC lifetime changes as reflecting either lipid transfer or fusion between small numbers of vesicles aggregated in the presence of PEG.

MATERIALS AND METHODS

Materials

Chloroform stock solutions of 1,2-dipalmitoyl-3-*sn*-phospatidylcholine (DPPC), 1,2-dilauryl-3-*sn*-phosphatidylethanolamine (DLPE), 1,2-dioleoyl-3-*sn*-phospatidylcholine (DOPC), and DPHpPC were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Lipids were verified to be greater than 98% pure by thin-layer chromatography on Analtech (Newark, DL) GHL silicic acid plates developed in a 65:25:4 (v/v/v) $CHCl_3$:CH_3OH:H_2O mixture and stained with iodine vapors. DPHpPC was also viewed under near-UV light. Poly(ethylene glycol) (PEG) (average molecular weight, 8000) was obtained from Fisher Scientific (lot No. 874229) and was purified by a procedure described elsewhere [13]. Solution concentrations of PEG are expressed as weight percentage (g PEG/100 ml buffer or water). *N*-[Tris(hydroxymethyl)-2-aminoethanesulfonic acid (TES) was purchased from Calbiochem (LaJolla, CA). DPPC stocks were filtered over Norit A neutral activated charcoal to remove trace fluorescent contaminants. L-α-Lysopalmitoylphosphatidylcholine (LPC) was purchased from Sigma Chemical Company (St. Louis, MO). Palmitic acid (PA) was purchased from Nu Chek Prep, Inc. (Elysian, MN). All other reagents were the highest quality available. Buffer solutions were filtered prior to use through a Nalgene disposable filter (Nalge Company, Rochester, NY) to remove dust which might interfere with the fluorescence measurements.

Methods

Vesicle Preparation. Large unilamellar extrusion vesicles (LUVET) were prepared by the method of Hope *et al.* [17] as described in detail elsewhere [13]. This procedure yielded a fairly homogeneous, stable population of unilamellar vesicles with average diameters of 1300 Å [13]. The concentrations of all vesicle samples

were determined by phosphate analysis using a modification of the procedure of Chen *et al.* [18].

Fluorescence Lifetime Measurements. Measurements of single-frequency fluorescence lifetimes were made on an SLM 48000 spectrofluorometer (SLM-Aminco, Urbana, IL) at a modulation frequency of 30 MHz, as described in detail elsewhere [11,12]. Calibration curves were generated for different concentrations (wt%) of PEG at a lipid concentration of 0.25 m*M* by measuring the DPHpPC lifetime at a minimum of three different lipid/probe ratios, as described by Burgess and Lentz [12]. Calibration data were used to determine the parameters of an empirical equation using a Simplex curve fitting routine [19,20]. Once the calibration curves were established for a particular lipid system, probe-containing (donor) vesicles and probe-free (acceptor) vesicles were added to the buffer solution with a certain amount of PEG, resulting in a final phospholipid concentration of 0.025 and 0.25 m*M* for the donor and acceptor vesicles, respectively. For most samples, the phase shift fluorescence lifetime at a modulation frequency of 30 MHz (averaged over 50 data points) was collected. The phase shift of an isochronal reference fluorophore (23) at 23°C [DPH in heptane; τ(30 MHz, phase) = 6.75 ns, 2×10^{-1} *M*) was measured before and after each phase shift measurement to determine the relative phase angle of the sample. The sample relative phase angles were converted to average lifetimes using the method of Spencer and Weber [24]. Standard deviations in the fluorescence lifetime values obtained in this way were commonly of the order of 20–30 ps but seldom greater than 50 ps.

We have shown previously [9,21,22] that the single exponential fluorescence lifetime of DPHpPC is well approximated by the 30-MHz phase lifetime. For many samples, the single-exponential lifetime is a good representation of the decay characteristics of DPHpPC, although for samples with low lipid/probe ratios, this is a less satisfactory approximation. To test how this approximation might affect our results, a complete set of calibration curves and a set of data on DPHpPC transfer were collected using the average lifetime instead of the 30-MHz lifetime. Average DPHpPC fluorescence lifetimes were obtained using an SLM 48000MHF spectrofluorometer (SLM-Aminco) with vertical excitation in a region from 351.1 to 363.8 nm via a Coherent Inova 90 Argon-Ion laser (Coherent Auburn Group, Auburn, CA) and with emission detected at an angle of 54.7° to the vertical using a KV-450 filter (50% transmission at 450 nm; Schott Optical Glass, Duryea, PA). The Dynamic Data Aquisition routine of the SLM MHF Spectroscopy software package was used to collect phase shifts and modulation ratios at 50 frequencies (base frequency = 4 MHz) using a 10-s aquisition time and a 200-aquisition average.

Treatment of Fluorescence Lifetime Data in Terms of Lipid Transfer

Approximate Description of the Aggregation Process. A mass action kinetic model has been applied previously to give the time course of aggregation of colloidal particles in general and of phospholipid vesicles in particular [14,15]. As applied to vesicle fusion, this model has viewed mass action-driven aggregation as a precursor to fusion. The treatment of this model has recognized that aggregation must proceed in a complex fashion, involving the formation of a wide variety of aggregated species [15], as summarized below:

$$V_1 + V_1 \underset{D_{11}}{\overset{C_{11}}{\leftrightarrows}} V_2 \xrightarrow{f_{11}} F_2$$

$$V_1 + V_2 \underset{D_{12}}{\overset{C_{12}}{\leftrightarrows}} V_3 \xrightarrow{f_{11}} V_1F_2$$

$$V_1 + F_2 \underset{D'_{12}}{\overset{C'_{12}}{\leftrightarrows}} V_1F_2 \xrightarrow{f_{12}} F_3$$

and so on, for aggregate-fusion products composed of four, five, and larger numbers of vesicles [15]. Here V_1 denotes the original vesicle monomer and V_j denotes an aggregate of j unfused vesicles. F_2 denotes a fused doublet and F_j denotes the fusion product of j vesicles. Likewise, V_1F_2 denotes an intermediate product of a monomer adhering to a fused doublet.

In our studies, the kinetics of aggregation are not of interest, since even low concentrations of PEG produce aggregation at rates much faster than we can measure. In addition, high concentrations of PEG produce lipid transfer on a subsecond scale that can be measured only with a very fast-response phase/modulation fluorometer [25]. For this reason, we have measured only the final or pseudoequilibrium lifetimes (after 30 min of mixing). Even though we are not concerned with kinetics, the counting procedures of the treatment of Bentz *et al.* [15] are useful to describe the distributions of species to be expected in our PEG-aggregated samples. Under these conditions, the vesicle system may contain a variety of aggregates, as determined by the initial kinetics of rapid aggregation in the presence of PEG. Since we have no information about these kinetics, we have simplified the problem by assuming that the size distribution of aggregates is a δ function of the average size.

That is, when treated with PEG, each vesicle system is considered to be dominated by one average-sized aggregate [14]. We also consider only multimers of the original monomer vesicles, since aggregation in PEG apparently takes place much more rapidly than fusion or intervesicle lipid transfer. That is, species of the sort V_j (which could fuse to form F_j) are considered, but species of the sort V_iF_j are ignored. This simplifying approximation is consistent with our earlier observation that lipid exchange or fusion occurred on a very limited basis unless PEG was removed and vesicles were dispersed and then reaggregated with PEG [20].

Even with these approximations, there are several ways to obtain an aggregate V_j or a fusion product F_j. Thus, of the j original vesicles which went into forming an F_j vesicle, the number of probe-containing vesicle could be any number between 0 and j. Like Bentz *et al.* [15], we assume that the vesicles aggregate randomly and that the probability (P_{ij}) that an F_j vesicle derived from i probe-containing vesicles and j-i probe-free vesicles is given by the combinatorial expression

$$P_{ij}=R^i(1-R)^{j-i}\frac{j!}{i!(j-i)!} \tag{1}$$

in which R is the ratio of probe-containing vesicles to total vesicles used in a given experiment. Each subspecies of fused j-mer, $F_j(i, j-i)$, or of unfused aggregate $j-$mer, $V_j(i, j\text{-}i)$, has its own lifetime related to the surface concentrations of probe present in the j-mer [20]. In the following sections, we show how to calculate the fluorescence lifetime of these j-mers.

Calculation of Fluorescence Lifetime Expected for a Fused j*-mer.* Figure 1 illustrates the DPHpPC redistribution thought to take place during formation of a j-mer. If no fusion occurs in the j-mer, lipid molecules are expected to redistribute only between the outer leaflets of aggregated vesicles. On the other hand, if fusion occurs, lipid molecules should distribute between all the bilayer leaflets (inner and outer) present in the aggregate. Each j-mer subspecies will then contribute a lifetime determined by the surface concentration of DPHpPC in that j-mer, which, in turn, will depend on whether fusion or only lipid transfer occurred in the j-aggregate. The average lifetime observed for a sample containing j-mers will be the average of these j-mer subspecies lifetimes:

$$\tau_j^{av}=\sum_i f_{ij}\tau_{ij}^{av} \tag{2}$$

where τ_{ij}^{av} is the *average lifetime* of the ith j-mer subspecies and f_{ij} is the fractional intensity contributed by this subspecies. Because Eq. (2) is formulated in terms of average lifetime in a j-mer, it is valid even if DPHpPC shows significant nonexponential behavior in a j-mer. This is appropriate since our purpose here is not to analyze the fundamental nature of DPHpPC fluorescence decay but rather to estimate how contributions from different environments contribute to the overall observed lifetime. The fractional intensity from the ith component is related to the preexponential factors in a multiexponential treatment of the fluorescence decay, α_{ij} [$=P_{ij}$ in Eq. (1)]:

$$f_{ij}=\frac{\alpha_{ij}\tau_{ij}^{av}}{\sum_i\alpha_{ij}\tau_{ij}^{av}} \tag{3}$$

As we have shown previously [20,22], the fluorescence lifetime of DPHpPC is sensitive to its concentration in the bilayer, a sensitivity that is reasonably well described by the empirical relation

$$\tau_{ij}=c_1-(c_1-c_2)e^{-c_3(LP_{ij}-c_4)} \tag{4}$$

where c_1, c_2, c_3, and c_4 are constants obtained from calibration experiments for any given system and LP_{ij} is the lipid/probe molar ratio in the bilayer of a fused j-mer, which is related to the initial lipid-to-probe ratio, LP_0, in the probe-containing vesicles by

$$LP_{ij}=LP_0\left(\frac{j}{i}\right) \tag{5}$$

We have found that the functional form of Eq. (4) holds for both the average lifetime and the 30-MHz phase lifetime, although its parameterization differs for these two types of measurements. Combining Eqs. (1) to (5),

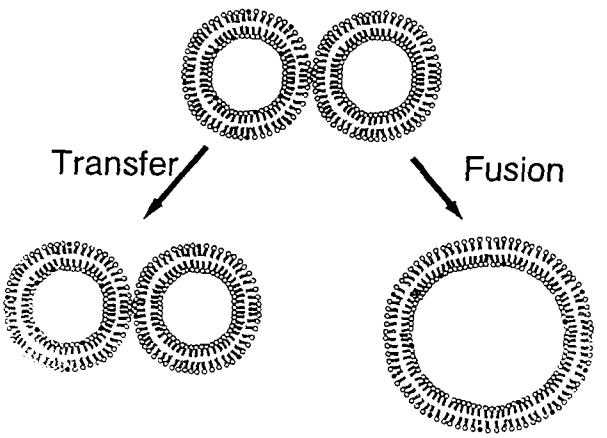

Fig. 1. Schematic diagram illustrating the distribution of DPHpPC probe between PEG-aggregated vesicles for the two possible situations considered: (1) transfer of probe between vesicle outer leaflets and (2) full vesicle fusion.

we obtain for the observed average lifetime for fused j-mer vesicles,

$$\tau_j^{av} = \sum_i \frac{R^i(1-R)^{j-i}\frac{j!}{i!(j-i)!}\left(c_1 - (c_1 - c_2)e^{-c_3(LP_0(\frac{j}{i}) - c_4)2}\right)}{\sum_i R^i(1-R)^{j-i}\frac{j!}{i!(j-i)!}\left(c_1 - (c_1 - c_2)e^{-c_3(LP_0(\frac{j}{i}) - c_4)}\right)} \quad (6)$$

For any given set of experimental conditions (i.e., R, LP_0, c_1, c_2, c_3, c_4), this expression was used to generate expected observed average lifetimes values corresponding to fused vesicles as a function of j, i.e., of aggregate size. Note that this equation holds whether fluorescence lifetimes are measured as average values or as 30-MHz phase lifetimes, as long as the appropriate empirical form [Eq. (4)] is used to relate observed lifetime to lipid/probe ratio in either case. Examples of the dependence of calculated lifetimes are given in Fig. 2 (filled symbols) under experimental condition with probe-free to probe-containing vesicles of 10:1 (circles). For any given experiment, such a curve is used to interpret an observed lifetime in terms of an average aggregate size for that experiment (i.e., locate the observed lifetime on the ordinate and read the predicted aggregate size from the abscissa).

Calculation of Fluorescence Lifetime Expected for an Aggregated j*-mer.* So far, we have developed a model to calculate the observed lifetime expected from fused vesicles in terms of the number of original vesicles that aggregate to form a fused product in the presence of PEG. However, we have shown [11] that lipid transfer can and did occur between PEG-aggregated DPPC or DOPC vesicles without the occurrence of fusion. To distinguish between lipid transfer and fusion, a model taking into account lipid transfer without fusion was developed. The fact that no more than 50% of potential lipid transfer occurred between probe-rich and probe-poor DOPC vesicles during 120 h of incubation at subrupturing PEG concentrations indicated that DPHpPC in the inner leaflet of the donor vesicle population was not available for transfer to probe-poor vesicles on this time scale [20]. In other words, probe "flip-flop" was very slow. This observation allows us to treat lipid transfer as occurring only between the outer leaflets of bilayers in an aggregate (see Fig. 1). Thus, for lipid transfer without fusion, two independent probe environments, inner leaflet and outer leaflet, must be considered. The fluorescence lifetime of probe in the inner leaflet environment (τ_{inr}) is expected to be the same as the original lifetime characteristic of the probe-containing vesicles. The calculation of the lifetime for the outer-leaflet probe population (τ_{out}) is similar to the calculations for fused vesicles described above. The average lifetime is expressed as

$$\tau_j^{av} = f_{out}\tau_{out}^{av} + f_{inr}\tau_{inr}^{av} \quad (7)$$

The f_{inr} and f_{out} values were calculated according to Eq. (3), with $\alpha_{inr}/\alpha_{out} = 1.0$ for 1300-Å LUVET, as estimated from the arguments of Sheetz and Chan [26]. By this procedure, the expected DPHpPC fluorescence lifetime can be calculated as a function of aggregate size for lipid transfer without fusion, just as described above for transfer with fusion.

Lifetimes calculated as described above for fused (open symbols) and unfused (filled symbols) aggregates are shown as a function of aggregate size in Fig. 2. This figure illustrates the sensitivity of our method, i.e., its ability to distinguish between fusion and transfer and its ability to predict aggregate size. It is clear from this figure that, if a definition of aggregate size is desired, a larger ratio of probe-free to probe-containing vesicles is preferable. Indeed, a small ratio (e.g., see triangles in Fig. 2) will result in the inability to distinguish aggregate sizes of three and above. However, the ability to distinguish the appropriateness of a fused versus a nonfused model is clearly greater for $R = 0.5$. Smaller LP values also enhance the ability to resolve different aggregate sizes, although one worries that too small a value may distort the properties of the probe-containing membranes. It is worth noting that the precision of our data (20–30 ps) along with our common experimental conditions ($R = 0.09$; $LP = 25$) is sufficient to allow distinguishing between aggregation sizes of 2 to 6, but that aggregate sizes beyond 10 are not distinguishable. Our conditions and data precision also allow the fusion and transfer models to be distinguished on the basis of DPHpPC lifetime measurements as long as aggregate

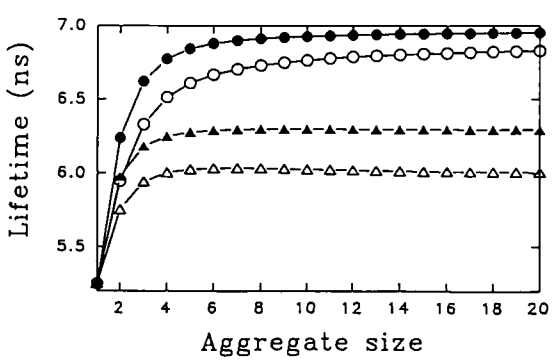

Fig. 2. DPHpPC fluorescence lifetime (ns) calculated as described under Methods using the transfer (open symbols) or fusion (filled symbols) models is plotted as a function of vesicle aggregate size, with $LP = 25/1$ and $R = 0.09$ (circles) and $R = 0.5$ (triangles).

sizes are not too large (below 15–20; see Fig. 2). Large aggregate sizes would require $R = 0.5$ to distinguish between these two events.

It is worth noting here that real vesicles exposed to PEG might experience both DPHpPC transfer and fusion in the aggregated state. Naturally, the aggregate size that would be predicted by a model that combined these two processes would be intermediate between the aggregate sizes predicted by the two limiting models. For this reason, it seemed unnecessary to complicate our treatment by formally developing such a combination model.

RESULTS

Limitations of the Method

Using Eq. (1), we have calculated and plotted in Fig. 3 the probabilities, P_{ij}, that aggregates of size "j" contain "i" probe-containing vesicles under a variety of experimental conditions. It can be seen from these plots that the 1:1 ($R = 0.5$) experimental setup (filled symbols, solid lines) leads to much higher probabilities that small aggregates will contain some probe-containing vesicles combined with probe-free vesicles. These are all aggregates that can produce dilution of DPHpPC surface concentration and, therefore, increases in observed lifetimes. On the other hand, the 10:1 ($R = 0.0909$) design produces a large number of aggregates containing no probe-containing vesicles (open symbols and dashed curves in Fig. 3); these aggregates can produce no probe dilution and no increase in observed lifetime. For simplicity, our calculation of predicted DPHpPC lifetime assumed the distribution of aggregate sizes to be a δ distribution (see Methods). This assumption of an "average aggregate size" may overweight in favor of larger aggregates for a 10:1 relative to a 1:1 experimental design to include species that result in probe dilution. This would have the effect of making our estimates of aggregate size somewhat dependent on experimental design. That this dependence on experimental design was observed can be seem from the results summarized in Table I. Here we have listed aggregate sizes of pure DPPC LUVET vesicles at different PEG concentrations from 0 up to 30% (w/v) obtained from observed lifetimes according to the procedures described under Methods. If all size aggregates were allowed in our distribution of aggregate sizes, i.e., a broad distribution containing both small and large aggregates was allowed, the same distribution of aggregate sizes should produce, assuming that other assumption did not interfere, the lifetimes actually observed with different experimental designs. To avoid the ambiguity associated with the assumption of a single aggregate size, all experiments were performed at $R = 0.0909$ (10:1), allowing comparisons of aggregate sizes estimated from different experiments. Previous studies performed in our laboratory [11,13] also used this experimental design, since it produces larger lifetime changes associated with aggregation or fusion.

In addition to testing for the effect of varying the

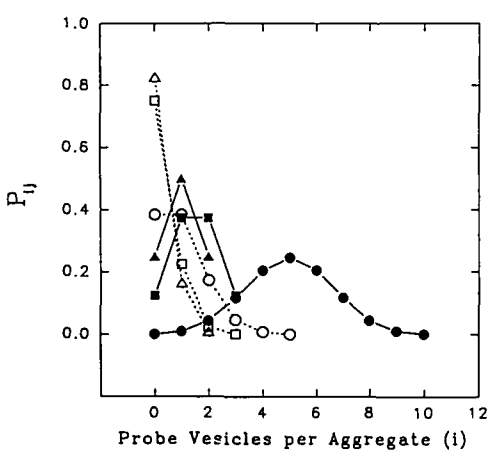

Fig. 3. The probability, P_{ij}, that, in an aggregate containing j vesicles, i will be probe-containing vesicles is plotted as a function of i. Shown are values calculated using Eq. (1) for probe-free/probe-containing vesicle ratios of 10/1 (open symbols, dashed lines) and 1/1 (filled symbols, solid lines) with $j = 2$ (triangles), 3 (squares), or 10 (circles).

Table I. Aggregate Size of DPPC LUVET Vesicles Under Different Experimental Protocols

PEG (w/v)	Aggregate size at r; LP[a]			
	10/1; 1:1	10/1; 2:1	10/1; 10:1	25/1; 10:1
0	1.0	1.0	1.0	1.0
5	nd[b]	nd	nd	3.2
10	1.6	1.5	4.9	2.5
15	2.2	2.2	4.1	nd
20	1.3	1.6	4.1	3.3
25	1.8	1.6	4.1	2.5
30	1.5	1.3	6.8	2.4
35	nd	nd	nd	3.4 (2.6[c])

[a]Measurements were performed using probe containing vesicles at different lipid-to-probe ratios (LP) mixed with probe-free vesicles in different ratios (r = probe-free/probe-containing). The 30-MHz phase lifetime was used.
[b]Not determined.
[c]Calculated with the fusion model, as opposed to the transfer model.

probe-free to probe-containing vesicle ratio, we examined the effect of varying the probe concentration within probe-containing vesicles (lipid/probe ratio). The results are also recorded in Table I. It can be seen that changing this experimental condition also affected the estimated vesicle size. There are at least two possible reasons for this effect. It may be that vesicles rich in DPHpPC (10:1 lipid/probe ratio) actually do aggregate more readily than do vesicles containing a lower concentration of this perturbing probe. Alternatively, it must be recognized that the expression used to relate the lipid-to-probe ratio to the observed lifetime [Eq. (5)] is an approximate functional form that does not fit the calibration data perfectly (see, e.g., Fig. 2 in Ref. 20). Small deviations from this equation, especially at a low lipid-to-probe ratio, could account for the effects recorded in the last two columns in Table I.

Despite the limitations of our model, it is clear that the approximate methods developed here offer a greatly improved interpretation of DPHpPC lipid mixing data relative to the crude approximations made previously [12,13] but that calculated aggregate sizes are only approximate and can be compared only between experiments performed under common conditions. In the next section, we compare the predictions made by this method with observations from entirely different types of measurements.

Dependence of Predictions on Methods for Measuring Lifetimes

The form of Eq. (6) implies that the proposed method for interpreting DPHpPC fluorescence lifetime changes should be independent of whether one records the average lifetime or 30-MHz phase lifetime, as long as the variation of both lifetime measures with lipid/probe ratio [i.e., the empirical relation of Eq. (4)] is similar for both lifetime measures. To test whether this might be the case, average DPHpPC lifetimes were measured for PA/DPPC (0.5/99.5) LUVET at different lipid/probe ratios to create calibration curves in the presence of varying concentrations of PEG. Each calibration curve fit the functional form shown in Eq. (4) (data not shown), but the relationship between curves obtained at different PEG concentrations was not exactly the same as obtained for the 30-MHz phase lifetime. To judge the exact effect of changing the method of lifetime measurement on the predictions of our model calculations, these calibration curves were used to interpret average DPHpPC lifetime measurements made on PA/DPPC LUVET at varying PEG concentrations. These measurements and their interpretation are summarized in Fig. 4. Similar experiments were performed using the 30-MHz phase lifetime to approximate the average DPHpPC lifetime, and these are recorded in Fig. 5. It is clear that the results obtained by these two methods and their interpretations were entirely consistent: Tetramers are predicted by both sets of measurements and the number of vesicles that appeared to be involved in fusion products remained fairly constant at four up to 30% PEG, beyond which it increased dramatically. Despite some small differences in the exact aggregate size predicted, the clear parallel between the predictions of these two sets of data makes it clear that the 30-MHz phase lifetime method can be used if more complete average lifetime data are not available. The relationship of these results to results obtained by other methods is discussed below.

Detection of Fusion and Lipid Transfer in Model Membranes: Comparison of DPHpPC Lifetime Measurements to Other Methods

Results obtained with several fusing vesicle systems offer confirmation of the validity of the proposed method

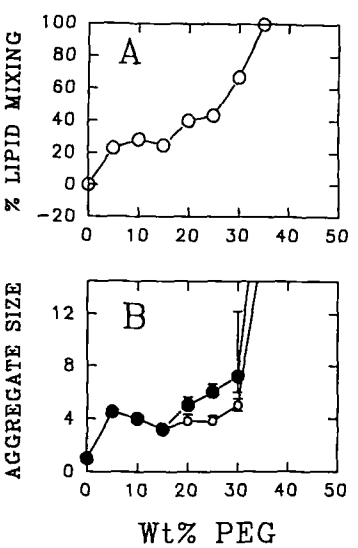

Fig. 4. (A) PEG-induced lipid mixing between DPPC LUVET containing 0.5mol% PA at 48°C (with LP = 25:1 and R = 0.09) is shown as a function of PEG concentration. Values were obtained from observed DPHpPC *average* fluorescence lifetimes in a manner similar to that described by Burgess and Lentz [12]. The slight increase in lipid mixing at 20% PEG correlates with the initiation of fusion as detected by other methods [1,13]. (B) The size of vesicle aggregates estimated as described under Methods using the "transfer" (filled circles) versus "fusion" (open circles) models is shown as a function of PEG concentration. Error bars were calculated based on the estimated standard error of our lifetime measurements (±30 ps).

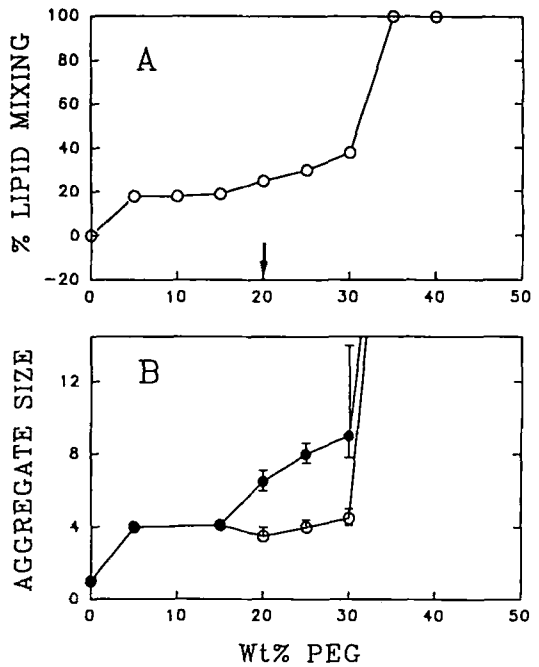

Fig. 5. (A) PEG-induced lipid mixing between DPPC LUVET containing 0.5mol% PA at 48°C (with LP = 25:1 and R = 0.09) is shown as a function of PEG concentration. Values were obtained from observed DPHpPC *30-MHz phase* fluorescence lifetimes as described by Burgess and Lentz [12]. The slight increase in lipid mixing at 20% PEG correlates with the initiation of fusion (indicated by an arrow) as detected by other methods [1,13]. (B) The size of vesicle aggregates estimated as described under Methods using the "transfer" (filled circles) versus "fusion" (open circles) models is shown as a function of PEG concentration. Error bars were calculated based on the estimated standard error of our lifetime measurements (±30 ps).

for interpreting DPHpPC lifetime data. Fatty acids, in particular, PA, render DPPC LUVET amenable to fusion by PEG at sub-rupture-inducing PEG concentrations, with vesicle contents observed to mix in the presence of only 20% PEG ([13); see arrow in Fig. 5A). The results described above for DPPC LUVET containing 0.5 mol% PA (Figs. 4A and 5A) confirm that lipid mixing assays performed with DPHpPC detected a slight increase in lipid mixing starting at 20% PEG. Figures 4B and 5B illustrate the interpretation of these data in terms of the variation of aggregate size with PEG concentrations (filled circles, transfer model; open circles, fusion model). Thus, the small increase in lipid mixing evident between 20 and 30% PEG in Fig. 5A can be interpreted as reflecting a shift from a "transfer" to a "fusion" model as applied to aggregates of a constant size ($j \approx 4$ for PA/DPPC). In this case, quasi-elastic light-scattering measurements on rehydrated vesicles detect an increase in vesicle diameter from roughly 1250 to 1900 Å, consistent with dimer or trimer formation [1]. Although our interpretation of the DPHpPC lifetime data predicts somewhat larger fusion products than observed by light scattering, the two methods are in essential agreement in reporting that the fusing aggregates are small.

We have shown [13] that pure DPPC LUVET did not fuse but that small amounts of LPC present in the vesicle bilayer made these uncurved vesicles susceptible to PEG-induced fusion. Figure 6A summarizes the results of DPHpPC transfer assays performed on DPPC LUVET containing 0.5 mol% LPC in the presence of varying concentrations of PEG. Lipid mixing, expressed as described by Burgess and Lentz [12], increased between 25 and 30% PEG (initial fusion was detected at 26–27%; see arrow in Fig. 6B [1]), which is the range of PEG concentration in which fusion occurs and rupture begins. Using the transfer-only model (Methods) to es-

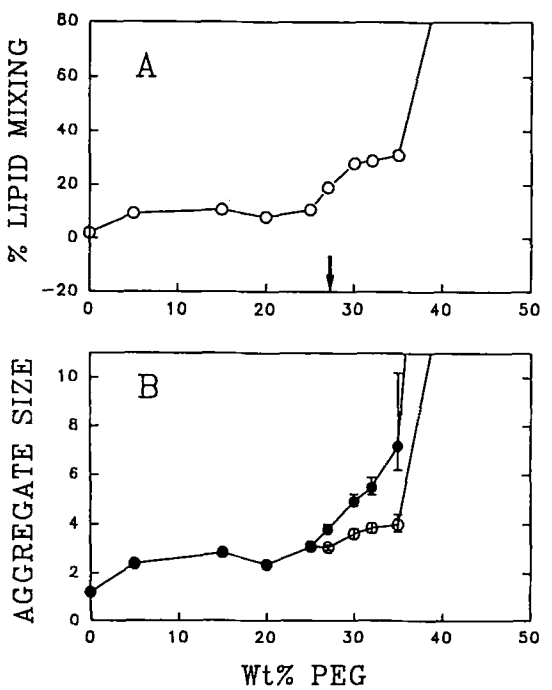

Fig. 6. (A) PEG-induced lipid mixing between DPPC LUVET containing 0.5 mol% LPC at 48°C (with LP = 25:1 and R = 0.09) is shown as a function of PEG concentration. Values were obtained from observed DPHpPC *30-MHz phase* fluorescence lifetimes as described by Burgess and Lentz [12]. The increase in lipid mixing at 27% PEG correlates with the initiation of fusion (indicated by arrow) as detected by other methods [1,13]. (B) The size of vesicle aggregates estimated as described under Methods using the "transfer" (filled circles) versus "fusion" (open circles) models is shown as a function of PEG concentration. Error bars were calculated based on the estimated standard error of our lifetime measurements (±30 ps).

timate aggregation number, we find that these vesicles form roughly dimers or trimers up to 25% PEG but that the aggregate size increases to roughly pentamers at 30% PEG (filled circles in Fig. 6B). However, if the fusion model (Methods) is used to estimate the aggregate size, a trimer is predicted at 27% PEG and trimers to tetramers at 30% PEG (open circles in Fig. 6B). Thus, the increase in lifetime observed between 25 and 30% PEG appears to reflect fusion rather than an increase in aggregate size. It is especially noteworthy that quasi-elastic light scattering measurements on rehydrated vesicles separated from PEG also showed that fusion involved only a slight increase in vesicle size for this system, consistent with fusion of a small number (approximately two) of aggregated vesicles [1,13]. The large increase in estimated aggregation number above 35% PEG correlates with substantial bilayer topological reorganization due to vesicle rupture [1]. We conclude that the proposed method for interpreting DPHpPC fluorescence lifetimes makes predictions entirely consistent with previous observations.

Vesicles with reduced interbilayer hydration repulsion have also been shown [15] to fuse in the presence of PEG. As the percentage of poorly hydrated DLPE increased in combination with DOPC, the concentration of PEG required to induce fusion decreased. Fusion of vesicles containing 85 mol% DOPC was clearly detected at 20% PEG (see arrow in Fig. 7A), as evidenced by the mixing of vesicle contents and increase in vesicle size [27]. Unlike the results for pure DOPC or DPPC vesicles, the extent of DPHpPC transfer increased continuously with increasing PEG concentration, but more rapidly so in the neighborhood of 20% PEG than at lower PEG concentrations (Fig. 7A). We applied our combined model to these DPHpPC transfer data to determine if the constancy of aggregate size during fusion as seen in the other fusing systems discussed above would extend to this system. Figure 7B shows the calculated aggregate size of 85/15 DLPE/DOPC LUVET as a function of PEG concentrations. Since fusion first started at 20% PEG, both the lipid transfer and the fusion models were applied at and above this PEG concentration. As shown in Fig. 7B, the aggregate size estimated by the fusion model at 20, 25, and 30% PEG (open circles) remained roughly that (dimers or trimers) calculated by the transfer model for 15% PEG (filled circles). As for the other systems described above, quasi-elastic light scattering subsequent to PEG removal detected vesicles that should have resulted from fusion of two vesicles, consistent with our interpretation of the DPHpPC lifetime data.

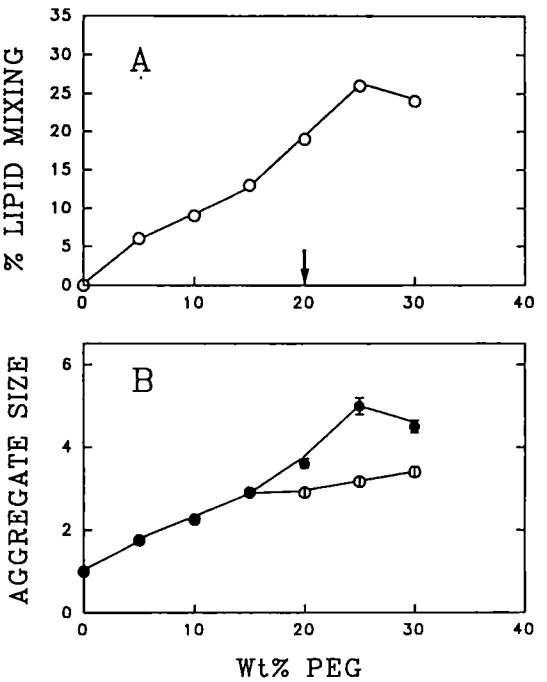

Fig. 7. (A) PEG-induced lipid mixing between 85/15 DLPE/DOPC LUVET at 40°C (with LP = 10:1 and R = 0.09) is shown as a function of PEG concentration. Values were obtained from observed DPHpPC *30-MHz phase* fluorescence lifetimes as described by Burgess and Lentz [12]. Initiation of vesicle fusion (indicated by the arrow) is reported to occur at 20% PEG [27]. (B) Size of vesicle aggregates estimated as described under Methods using the "transfer" (filled circles) versus "fusion" (open circles) models is shown as a function of PEG concentration. Error bars were calculated based on the estimated standard error of our lifetime measurements (±30 ps).

DISCUSSION

Validity of the New Method of Interpretation

Previous studies of PEG-mediated fusion from our laboratory have been able to document fusion (mixing of vesicle contents) and increase in vesicle size only after rehydration and removal of PEG. While lipid probes can be used to detect lipid transfer and mixing of membrane components, they cannot unambiguously demonstrate fusion. Nonetheless, the advantage of DPHpPC fluorescence lifetime measurements is that they can be made in the presence of even high concentrations of PEG. Unfortunately, the lack of a theoretical framework within which to interpret these measurements has limited their usefulness. In this paper, we have outlined a framework for interpreting DPHpPC lifetime measurements in terms of models assuming fusion or lipid transfer between aggregated vesicles. The close correspondence of predic-

tions from our model calculations and previously published observations obtained following rehydration and removal of PEG argues that the model calculations, while admittedly approximate, capture the essential features of the vesicle aggregation and lipid exchange processes.

Insights into PEG-Mediated Fusion

While the primary purpose of this paper has been to introduce a new method for interpretation of DPHpPC lifetime measurements, two significant conclusions result from the application of this analysis to existing data.

First, lipid transfer and fusion appear to occur between small numbers of aggregated vesicles. Under conditions where fusion occurs, this agrees with previous observations obtained using quasi-elastic light scattering [1,13,27]. In other studies under nonfusing conditions [20], the final extent of lipid transfer was found to be incomplete but could be increased by repeated disaggregation/aggregation cycles. The reason for this was unknown. DPHpPC lifetimes observed at subfusing concentrations of PEG are consistent with a model supposing transfer between the outer bilayer leaflets of only a small number of LUVET. The exact number of vesicles calculated with our model has been shown to vary with experimental design because of approximations in the treatment. However, the general conclusion of a small aggregate size is independent of ambiguities associated with these approximations and offers an explanation for our previous report of incomplete intervesicle lipid transfer [20]. Since PEG-aggregated vesicles produce four or five sharp peaks in X-ray diffraction patterns [27] and treatment with under 25% PEG does not produce multilayered structures [1], we must conclude that several vesicles are stacked in the aggregated state. The current interpretation of DPHpPC lifetime data (as well as our previous light-scattering data) suggests that only vesicle pairs (perhaps trimers or tetramers) within these aggregates experience lipid exchange and fusion. Thus, vesicle aggregates may consist of aggregate dimers, with interactions between the dimers being different from the interaction within a dimer that leads to fusion or lipid exchange.

A related interesting observation deriving from the current treatment is that the number of vesicles in a fusing or lipid-exchanging unit did not vary with PEG concentration. This follows from the observed constancy of DPHpPC lifetime at low PEG concentrations, at least for vesicles rich in DPPC (Figs. 4 and 5) or DOPC [27]. Since the sharpness of X-ray diffraction patterns did increase with PEG concentration [27], it must be that aggregation or stacking of vesicles in the presence of PEG is not sufficient to induce fusion or enhanced lipid transfer but that special interactions must exist between pairs (or small numbers) of vesicles in this aggregated state that encourage these processes.

Second, fusion occurs in the dehydrated state induced by PEG. The simple model presented here provides a framework within which to interpret the small changes in DPHpPC lifetime observed to correlate with detection of vesicle contents mixing. Because our fusion model (see Fig. 1) explains these changes without assuming any change in the number of interacting vesicles, this implies that fusion occurs between these already interacting vesicles in the presence of PEG, not just upon removal or dilution of this polymer. Thus, PEG-induced fusion seems to occur between vesicles gathered into small interacting units by the dehydrating influence of PEG.

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