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Fluorescence Lifetimes of Diphenylhexatriene-Containing Probes Reflect Local Probe Concentrations: Application to the Measurement of Membrane Fusion

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An important process in the life of a cell is fusion between cellular membranes. This is the process by which two cellular compartments surrounded by different membranes join to become a single compartment surrounded by a single membrane, without significant loss of compartment contents. To demonstrate fusion, the cell biophysicist must demonstrate all three critical aspects of the process: (1) mixing of membrane components, (2) mixing of compartment contents; and (3) retention of compartment contents. Most commonly, accomplishing this involves the use of fluorescence probes. The general theme to the methods described involves some form of concentration-dependent quenching. An unique method developed in our laboratory utilizes the concentration dependence of the fluorescence lifetime of a phosphatidylcholine containing carboxyethyl diphenylhexatriene at position 2 and palmitic acid at position 1 of glycerol (DPHpPC). The fluorescence lifetime of this molecule and that of its parent fluorophore diphenylhexatriene (DPH) shorten dramatically as their two-dimensional concentrations in a membrane increase. This "lifetime quenching" can be described by dimer formation that reduces the symmetry of the DPH excited state. This phenomenon allows one to use the fluorescence lifetime to gain insight into the local concentration of probe in microscopic regions of a membrane. One application of this is in distinguishing lipid transfer between the outer leaflets of two contacting membrane bilayers from fusion between these membranes that leads to mixing of lipids in both the inner and outer leaflets of the membrane bilayers. This allows a single measurement to demonstrate fusion between membrane pairs.

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

INTRODUCTION

What Is Fusion?

Fusion is the process by which two cellular compartments surrounded by different membranes join to become a single compartment surrounded by a single membrane, without significant loss of compartment contents. This process is crucial to such essential cellular processes as endocytosis, exocytosis, protein sorting and transport, and cell division. Despite the importance of the fusion process to cell function, the molecular mechanism by which this process is accomplished by the cell remains one of the key unsolved mysteries of modern

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[2] Abbreviations: PEG, poly(ethylene glycol); Na_2EDTA, ethylenediamine-tetraacedic acid, disodium salt; LUV, large, unilamellar vesicles made by rapid extrusion technique; DPH, 1,6-diphenyl-*trans*-1,3,5-hexatriene; 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; PA, palmitic acid; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE; Rh-PE, *N*-(lissamine Rhodamine B sulfoyl)-PE; R_{18}, octadecyl Rhodamine B chloride; ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; DPX, *N,N'-p*-xylylene-bis(pyradinium bromide).

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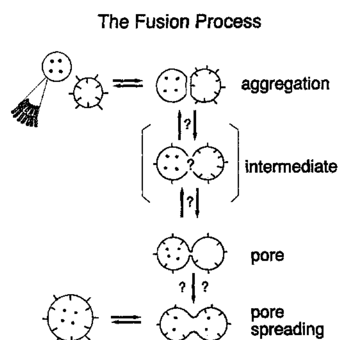

Fig. 1. Illustration of the presumed steps of the fusion process in terms of the expected redistribution of outer and inner leaflet lipids (respectively outward- and inward-oriented lines emanating from the membrane) or aqueous compartment probes (dots) at each of the steps.

molecular biology. Some basic features of the fusion process are well established, both in model membranes and in cellular systems (see Fig. 1). First, it is accepted that two membranes must be brought into contact in order that they might fuse; this is the aggregation step. It remains an issue exactly how close the contact must be; apparently, it must be closer for model membrane fusion [1] than for biomembrane fusion [2]. The next step of the fusion process is less well defined, but is the step of greatest interest. Somehow the bilayer structure must be destabilized, represented by "?" in Fig. 1. Then this unknown structure must decay to formation of an initial pore. It is not known whether these steps are reversible. The formation of a "single-bilayer septum" or "stalk" is a currently popular view of the destabilization step [3,4]. The outer leaflets of the contacting membrane bilayers become continuous and should interchange their components if the single-bilayer septum is the destabilized intermediate (illustrated by movement of outward-facing lines in Fig. 1). This condition is often referred to as *hemifusion*. The initial pore is thought to be very small (<<20 nm [5,6]), rapidly forming (opening in ca. 1 ms [2]), and reversible [2]. While the pore may be too small to allow mixing of compartment contents (dots in Fig. 1), it should allow complete interchange of membrane components (inward- and outward-facing lines in Fig. 1). It is currently thought that this flickering pore must expand over a time of tens to hundreds of milliseconds to yield the final fused state in which the contents of two compartments are free to mix.

Fluorescence Fusion Assays

To demonstrate fusion, the cell biophysicist must demonstrate all three critical features of the process: (1) mixing of membrane components, (2) mixing of compartment contents, and, at the same time, (3) minimal leakage of compartment contents. These features are most often demonstrated using any of a variety of fluorescence assays based on some form of concentration-dependent quenching or enhancement of a fluorescence signal (for a comprehensive review, see Ref. 7). There are two basic types of fusion assays. The first detects the *intermixing of membrane components*. Such an assay is illustrated in Fig. 2 for the case of a very popular lipid intermixing assay that utilizes a fluorescence resonance energy transfer pair, NBD-PE (donor) and Rh-PE (acceptor) [8].[2] A major advantage of this assay is its ease of use. However, it also has several disadvantages. First, results obtained with this assay are very difficult to interpret quantitatively, since it is hard to know exactly how much energy transfer results from how much fusion. Second, the probes are exposed to the aqueous environment and thus are easily quenched by added fusing agents. Third and most importantly, because the results are not quantitatively interpretable, it is not possible to distinguish fusion, which produces complete intermixing, from outer leaflet transfer, which produces only partial intermixing (see Fig. 2). A recent variation on this assay involves reduction of exposed NBD by dithionite (Fig. 2); this allows fusion and lipid transfer to be distinguished, but requires much more work [9]. Another important member of this class of assays utilizes self-quenching of R_{18} incorporated into one membrane population at high concentration, resulting in an increase in fluorescence once dilution into another membrane is accomplished [10]. The mechanism of quenching in this case involves energy transfer to a nonfluorescent dimer [11]. The important feature of the R_{18} probe is the ease with which it can be incorporated into biomembranes via simple diffusion through the aqueous phase. Unfortunately, it is exactly this property that also accounts for the greatest problem with this probe, namely that it cannot be used to distinguish simple lipid transfer from fusion. Clearly, there is a need for a lipid intermixing assay capable of distinguishing between these two processes.

Because of this general failure of most lipid mixing fluorescence assays, it is necessary to perform a different type of experiment to confirm the occurrence of fusion, namely the measurement of the *mixing of trapped membrane compartments*. Such an assay is illustrated in Fig. 3 for the specific case of ANTS fluorescence quenched by DPX [12]. An essential feature of all such assays is the need to determine and correct for the *leakage of vesicle contents* stimulated by procedures that lead to fusion. The protocol for detecting the leakage of contents using the ANTS/DPX pair is illustrated in the lower por-

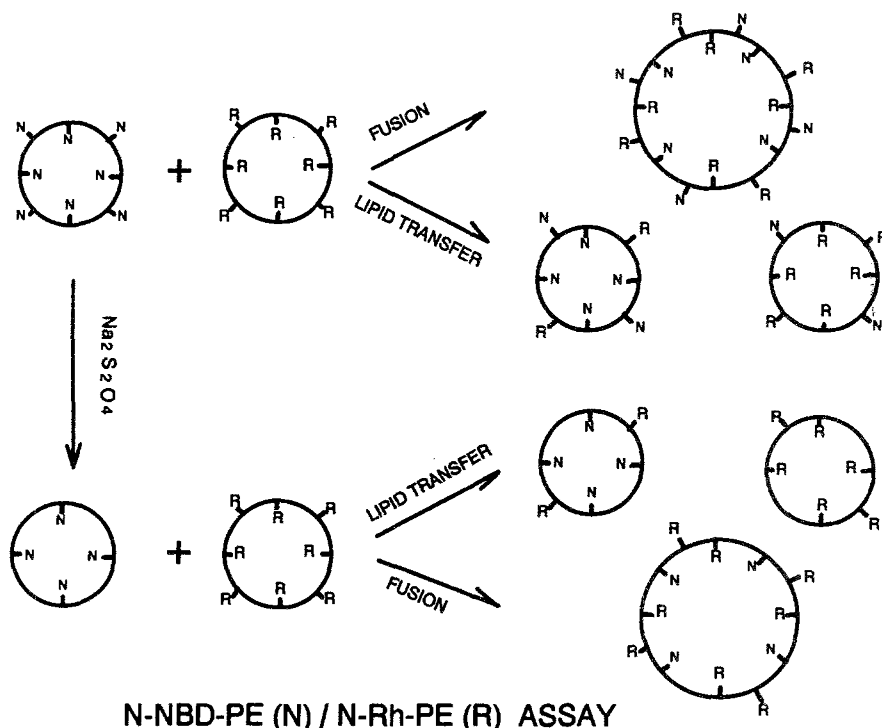

Fig. 2. Illustration of the assay for lipid transfer utilizing energy transfer from NBD-PE (N) to Rh-PE (R). The upper scheme illustrates that fusion, in which both inner and outer bilayer leaflets mix, should result in twice the quenching resulting from outer leaflet lipid transfer. The bottom scheme illustrates the expected results for these two processes if outer leaflet NBD-PE is reduced by sodium dithionite prior to the fusion or transfer experiment [9].

tion of Fig. 3. The big advantage of assays in this class is that they unambiguously demonstrate fusion. The major disadvantage is that they are difficult to perform even in simple model membranes and very difficult if not impossible to perform in cell systems. For this reason, these assays are usually used in conjunction with and to confirm conclusions reached with simpler membrane mixing assays. Development of a membrane mixing assay that could demonstrate fusion as distinct from outer leaflet lipid transfer would avoid the need to use these complex contents mixing and leakage assays, especially in systems where their implementation is very difficult. This paper reviews the properties of a probe, DPHpPC (see Fig. 4), that I believe has the potential to accomplish this.

PROPERTIES OF DPHpPC

DPHpPC as a Lipid

We have investigated the behavior of DPHpPC in synthetic, multilamellar DPPC vesicles [13]. This fluorescent phospholipid has photophysical properties similar to its parent fluorophore, DPH. DPHpPC preferentially partitioned into fluid phase lipid ($K_{f/s}$ = 3.3), and reported a lower phase transition temperature as detected by fluorescence anisotropy than that observed by differential scanning calorimetry. Calorimetric measurements of the bilayer phase transition in samples having different phospholipid-to-probe ratios demonstrated very slight changes in membrane phase transition temperature (0.1–0.2°C) and showed no measurable change in transition width. Temperature profiles of steady-state fluorescence anisotropy, limiting anisotropy, differential tangent, and rotational rate were similar to those of DPH below the main lipid phase transition, but indicated more restricted rotational motion above the lipid phase transition temperature. As for DPH, the fluorescence decay of DPHpPC could be described by either a single or double exponential both above and below the DPPC phase transition. A strange feature of the DPHpPC lifetime was that it decreased with decreasing temperature in the membrane solid phase. These data indicate that the DPH moiety of DPHpPC is oriented with the bilayer acyl chains at all temperatures and is fairly re-

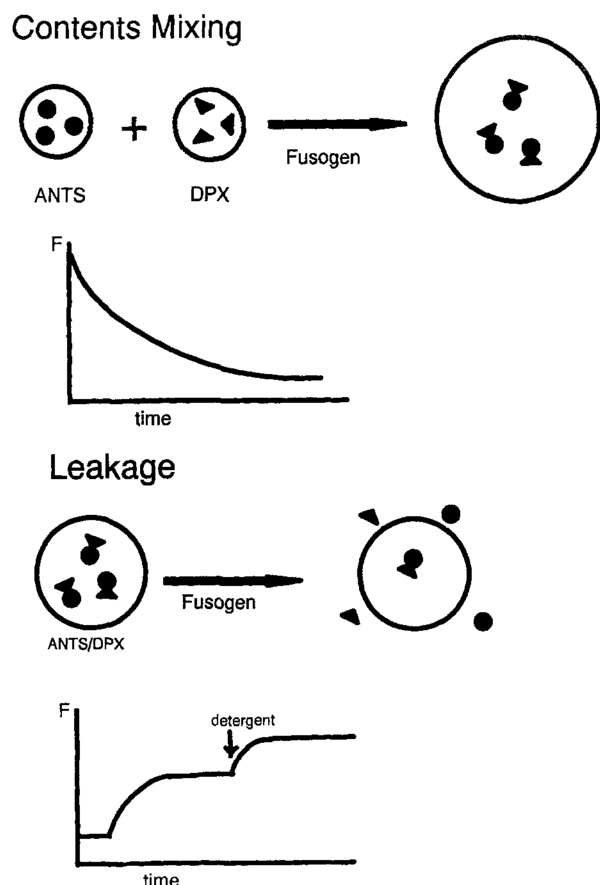

Fig. 3. Illustration of the use of the ANTS/DPX pair (fluorophore/quencher) to report mixing of trapped contents as a clear indication of fusion. Essential to the proper interpretation of such an assay is a related measure of leakage of vesicle contents, as illustrated in the lower half of the figure.

stricted in its motion, reflecting molecular order near the glycerol-acyl chain linkage. Nonetheless, measurements of probe fluorescence properties suggested that DPHpPC disrupts its local environment in the membrane and may even induce perturbed probe-rich local domains below the phospholipid phase transition, possibilities that must be kept in mind when using this or any other fluorescent membrane probe. Overall, however, the measured properties of DPHpPC and its lipid-like structure (see Fig. 4) make it a powerful probe of membrane structure and dynamics.

Anomalous Photophysical Properties of DPHpPC

Our earlier observation that DPH fluorescence lifetime decreased with concentration [14] as well as the observation that the DPHpPC fluorescence lifetime decreased with decreasing temperature below the phase transition led us to examine in more detail the photophysical properties of DPHpPC. We found that, as for DPH, the fluorescence lifetime decreased dramatically at high concentrations of DPHpPC in a membrane [15]. We have investigated the reason for this sensitivity of DPHpPC fluorescence excited-state lifetime to its concentration in DPPC and other membranes [16]. We have interpreted self-quenching data (see Fig. 5), the concentration dependence of excitation spectra (see Fig. 6), emission spectra, as well as phase and modulation lifetime data in terms of a model that envisions dimerization of these probes in a membrane bilayer as the essential feature responsible for the anomalous photophysical properties of DPH-related probes [16]. It was proposed that dimerization alters the symmetry of the DPH excited state so as to allow more rapid decay via the normally symmetry-disallowed route from the $^1A_g{}^*$ state (Fig. 7). We note that the dimerization model derives from and was formulated to be consistent with the known photophysical behavior of DPH and other *trans* polyenes in organic solvents (e.g., see Refs. 17 and 18). Global analysis of fluorescence phase shift and modulation ratio data for DPHpPC in terms of the dimerization model provided a good fit of these data as a function of both modulation frequency and probe concentration. Global analysis of a similar set of data for a phosphatidic acid (a charged phospholipid) containing DPH made the physically reasonable prediction that this molecule was much less prone to dimerize than was the uncharged DPHpPC. We concluded from these studies that the dimerization model allowed rationalization of many of the anomalous photophysical properties of DPHpPC in membranes [16].[3]

FUSION AND LIPID TRANSFER ASSAYS BASED ON DPHpPC

Essential Features of the Fusion/Transfer Assay

We have taken advantage of this anomalous photophysical behavior of DPHpPC to develop methods for

[3] It should be noted that the proposed DPHpPC photophysical model has been criticized for its lack of symmetry in that the $A_g{}^*$ state of the dimer form of DPHpPC does not interconvert to a $B_u{}^*$ state as occurs for the monomer (E. Gratton, personal communication). Such a symmetric model retains the essential dimerization feature, but might better account for the fact that the excitation spectrum is concentration dependent while the emission spectrum is not. Additional measurements of the concentration dependence of the DPHpPC fluorescence lifetime at several excitation and emission wavelengths would be necessary to test this more complex model.

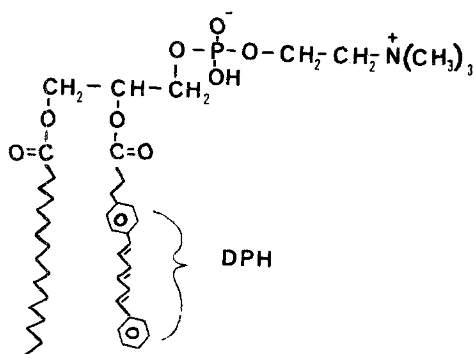

Fig. 4. Schematic model of DPHpPC, illustrating the similarity to a native phosphatidylcholine molecule.

detecting lipid transfer and fusion between membrane vesicles [15]. The details of this assay have been described elsewhere [16]. The fusion/transfer assay takes advantage of the observation that plots of lifetime versus lipid/probe ratio could be represented reasonably by an exponential form described by four easily determined parameters (Fig. 8). This allows the lifetime of DPHpPC to be estimated for any environment within which it is located with a known two-dimensional probe concentration [16].

The basic idea of the fusion/transfer assay is also illustrated in Fig. 8. One vesicle population containing a fairly high concentration of probe (e.g., 2–5 mol %) is combined with an excess (often tenfold) of a second, nonfluorescent vesicle population, as indicated by the left-hand arrow in Fig. 8. Bilayer mixing induced by the addition of a known fusogenic agent is expected to result in a reduction of the probe concentration in the newly formed vesicles and thus an increase in lifetime to roughly 7.5 ns (right-hand arrow in Fig. 8). Errors in our lifetime measurements were typically in the range of 0.05 ns, which means that this technique was sensitive to small changes in the probe environment. Parente and Lentz [15] demonstrated that this method could detect fusion of phosphatidylserine vesicles induced by addition of Ca^{2+}. Their results are shown in Fig. 9. The diagram above the data summarizes the interpretation of how the observed lifetime changes reflect not only the Ca^{2+}-induced fusion, but also a subsequent Ca^{2+}-induced phase separation that occurs in such systems. Thus, the initial increase in lifetime is seen as reflective of dilution of DPHpPC into the probe-free vesicles due to fusion. The decline in lifetime seen thereafter (from 10 to 20 min) is attributed to a separation of a fluid DPHpPC phase from a solid phosphatidylserine phase induced by Ca^{2+} [19], which is reversible by Na_2EDTA addition.

The simplest treatment of data from experiments such as that illustrated in Fig. 9 assumes that 100% fusion would result in complete intermixing of all lipids between all vesicles. From calibration curves such as shown in Fig. 8, it is easy to determine the lifetime that would be observed if probe molecules were diluted into all the other lipids in the sample (i.e., 100% fusion). In our studies of poly(ethylene glycol) (PEG)-mediated fusion of model membrane vesicles, lifetimes reflective of complete lipid intermixing are never observed, a situation that is described by assigning values of less than 100% fusion to such observations (e.g., see Ref. 16). Unfortunately, this method for describing the data from DPHpPC fusion/transfer assays provides little or no insight into the molecular events responsible for the observed change in lifetime. This point was driven home to us by observations we made while studying the mechanism of PEG-mediated lipid transfer between vesicles [20]. PEG is a polymer that, due to its solvation properties, dehydrates and aggregates membrane vesicles or cells. We found that PEG caused rapid but limited lipid exchange between vesicles, i.e., DPHpPC fluorescence lifetime increased rapidly to an asymptotic value well short of that expected for complete intermixing between vesicles. Only when the vesicles were removed from and then retreated with PEG and this process was repeated many times did the DPHpPC lifetime increase in a stepwise fashion to a final value roughly characteristic of 50% lipid transfer, a value that would be expected for mixing of lipids between the outer leaflets of all vesicle bilayers [20]. The simple assumption of uniform intermixing of all lipids between all vesicles offered no reasonable interpretation for these observations. This forced us to develop a treatment of DPHpPC lifetimes based on the assumption of lipid exchange or fusion between limited numbers of vesicles.

Fusion Versus Exchange Between Vesicles Within Limited Aggregates

The basic problem with the crude interpretation of DPHpPC lifetime data [16] 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 or among 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 vesicles and one blank vesicle, or three blank or three probe vesicles, each of which has a different lipid/

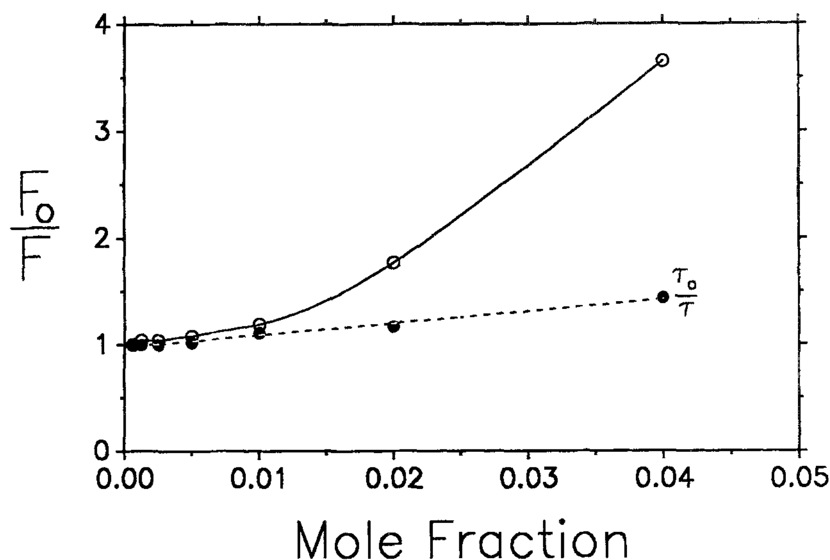

Fig. 5. Stern–Volmer plot of fluorescence intensities (open circles) and lifetimes (solid circles). Details of the measurement are given in the original publication [16]. The concave-up shape of the intensity quenching data implies that ground-state complex formation inhibits excitation. The linear lifetime quenching implies a concentration-dependent mechanism for enhancing decay of the excited state. (Reproduced with permission from Fig. 1 of Ref. 16.)

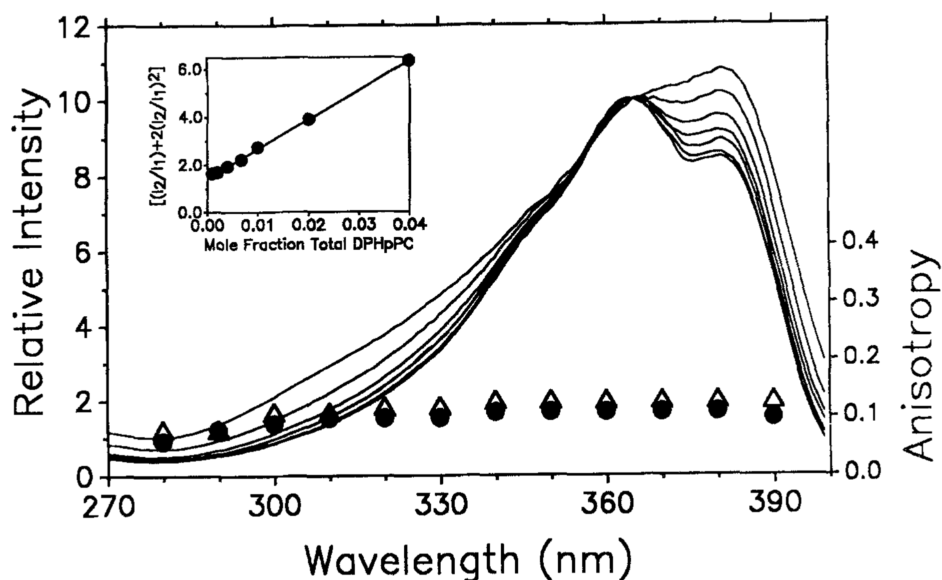

Fig. 6. Fluorescence excitation spectra of DPHpPC in DPPC multilamellar vesicles at 45°C. Spectra are presented for samples at lipid/probe molar ratios of 25:1, 50:1, 100:1, 150:1, 250:1, 500:1, and 1000:1 (in order from highest to lowest intensity at 383 nm). The relative intensities of the 365-nm and 383-nm peaks were used to determine the equilibrium constant for ground-state dimerization (illustrated in the insert). The constancy of the fluorescence anisotropy excitation spectra obtained at high (open triangles: 500/1) and low (solid circles: 25/1) lipid/probe ratios was used to suggest that wobbling diffusion of the dimer must be rapid. Experimental details as well as details of the equilibrium constant calculations are given in the original article [16]. (Reproduced with permission from Fig. 3 of Ref. 16.)

probe ratio and so lifetime. The fact that the DPHpPC excited-state fluorescence lifetime can be quantitatively related to two-dimensional probe concentration in a membrane makes it uniquely possible to estimate the DPHpPC fluorescence lifetime that would be expected for each of these trimers. In a recent paper, we have taken advantage of this unique property of DPHpPC to develop a new method for interpreting the DPHpPC fu-

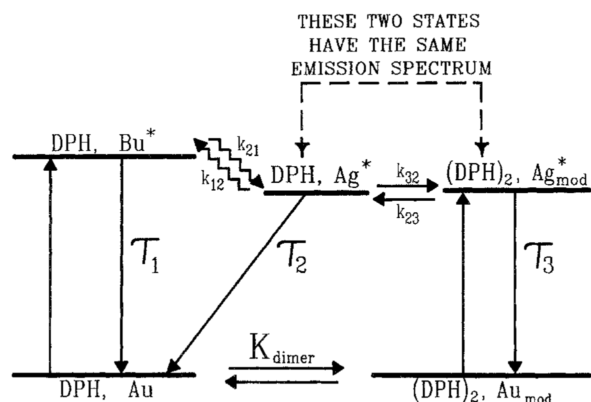

Fig. 7. Dimerization model to explain the anomalous lifetime and photophysical properties of DPHpPC in a membrane. τ_1 represents a rapid natural decay (about 1 ns) from the normally occupied ${}^1B_u{}^*$ state. τ_2 represents the much slower (8–12 ns) decay commonly observed for DPH and its derivatives in membranes. This decay is slow because the ${}^1A_u \rightarrow {}^1A_g{}^*$ transition is formally forbidden by symmetry. τ_3 represents a more rapid (2–5 ns) decay from an ${}^1A_g{}^*$ state whose symmetry properties are presumably altered due to dimer formation. We assumed that the monomer and dimer excited states had the same emission spectra, because we observed no shift in the emission spectrum of samples at high and low probe concentrations. This model is consistent with all the steady-state and phase-resolved dynamic data that we have published on this probe. (Reproduced with permission from Fig. 4 of Ref. 16.)

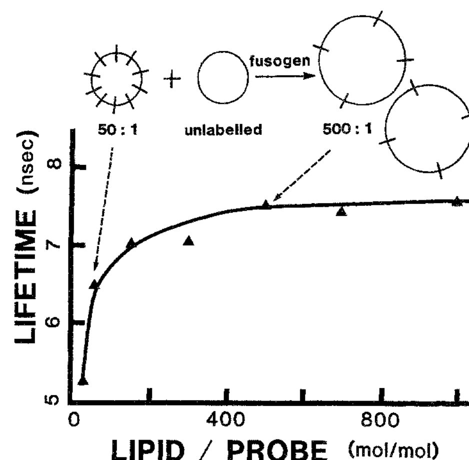

Fig. 8. The observed concentration dependence of the excited-state fluorescence lifetime of DPHpPC forms the basis for detecting fusion or lipid transfer. The data were obtained from DPPC multilamellar vesicles vesicles containing DPHpPC in various concentrations. The details of these measurements are given elsewhere [16]. The concentration dependence of the lifetime is reasonably well described by the empirical relation

$$\tau = C_1 - (C_1 - C_2)\exp[-C_3(LP - C_4)],$$

where c_1, c_2, c_3, and c_4 are empirical fitting constants and LP is the lipid–probe molar ratio in the bilayer. The diagram above these data illustrates how fluorescence lifetime changes can be used to monitor intervesicle lipid transfer during fusion. The arrows point to the expected lifetime values at the beginning and end of an "ideal" (completely random and uniform) fusion process. (Reproduced with permission from Fig. 1 of Ref. 15.)

sion/transfer assay that can account for such heterogeneity in probe microenvironments [21]. Others have treated the problem of heterogeneous aggregation of unlike vesicles using combinatorial methods [22,23]. Our effort to provide a more detailed interpretation of fusion/transfer data applied this combinatorial approach to heterogeneous aggregation, in conjunction with knowledge of the photophysical behavior of DPHpPC [24,16], 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.

Figure 10 illustrates how DPHpPC probe will be distributed differently depending on whether it transfers between probe-containing and probe-free vesicles via an outer-leaflet transfer or a fusion mechanism. We show elsewhere [21] that the average DPHpPC excited-state lifetime reported by a sample undergoing one of these processes can be calculated after making certain simplifying assumptions. First, one assumes that only one average aggregate size exists, i.e., a delta distribution of aggregate sizes is assumed to obtain in a sample. While this is certainly a rough approximation, it does capture the essence of a limited versus an extensive and random aggregation model. Second, one incorporates a combinatorial to account for the number of ways to make a j-size aggregate from probe-containing and probe-free vesicles. Third, one uses the empirical relationships obtained for lifetime versus lipid/probe ratio [16] to calculate the average lifetime expected for any aggregate size:

$$\langle\tau\rangle_j = \sum_i \frac{R^i(1-R)^{j-i}\{j!/[i!(j-i)!]\}\,[(C_1-(C_1-C_2)e^{-c_3[LP_0(j/i)-c_4)}]^2}{\Sigma_i R^i(1-R)^{j-i}\{j!/i!(j-i)![c_1-(c_1-c_2)e^{-c_3[LP_0(j/i)-c_4]}}$$

In this expression, R is the ratio of probe-containing to total vesicles used in a given experiment, LP_0 is the lipid/probe ratio in the probe-containing vesicles, the c values are coefficients for the empirical expression giving lifetime as a function of LP, and the sum is over all possible combinations of i probe-containing with $i - j$ probe-free vesicles to create a j-sized aggregate. For any given set of experimental conditions, this expression was used to generate expected observed average lifetime values corresponding to aggregated or fused j-mers of ves-

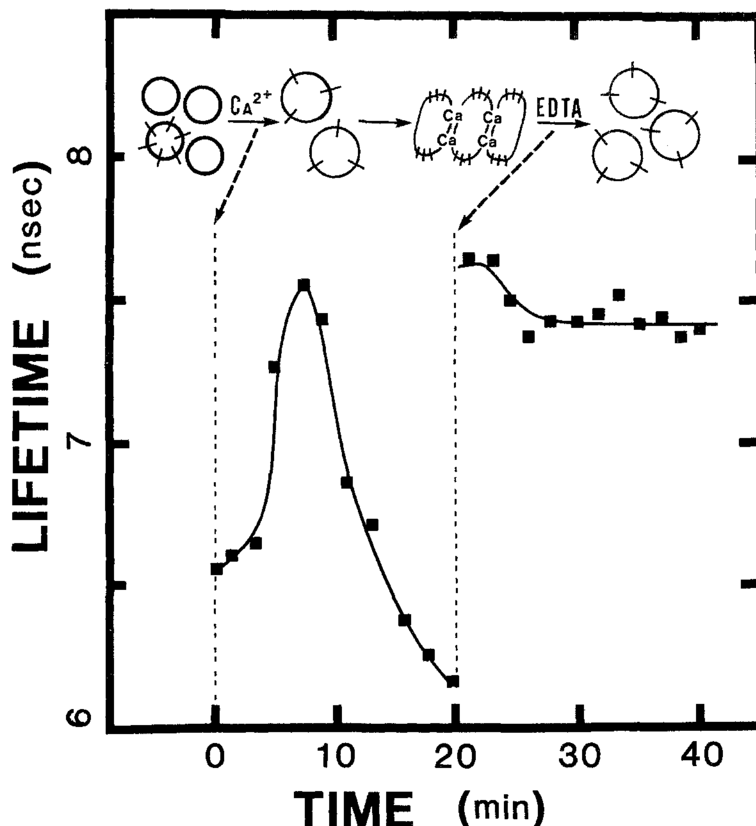

Fig. 9. DPHpPC lifetime *versus* time after Ca^{2+} addition to induce membrane fusion of phosphatidylserine vesicles. Details of the experiment are described in the original article [15]. Dashed lines indicate times of Ca^{2+} and Na_2EDTA addition, respectively. (Reproduced with permission from Fig. 4 of Ref. 15.)

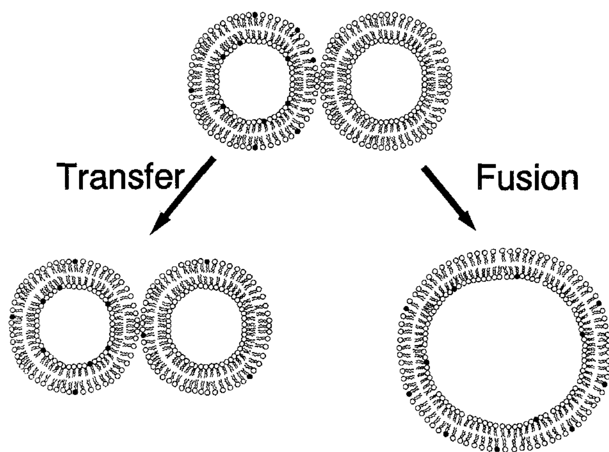

Fig. 10. The distribution of DPHpPC probe between PEG-aggregated vesicles for the two possible situations considered by Wu and Lentz [21]: (1) transfer of probe between vesicle outer leaflets and (2) full vesicle fusion. These two situations are distinguishable with careful fluorescence lifetime measurements, since appropriate experimental design will yield measurably different average lifetimes ($\Delta\tau \sim$ 0.2–0.4 ns) for these two situations. (Reproduced with permission from Fig. 1 of Ref. 21.)

icles [21]. These calculations have shown that, for a known aggregate size, it is possible to choose an appropriate experimental design so that a difference in average lifetime of ~0.2–0.4 ns is expected from outer-leaflet versus fusion probe transfer events (see Fig. 10). Uncertainties in average lifetime measurements of 0.05 ns are common, making this a measurable difference. Under conditions for which only one of these two events is known to occur (e.g., outer leaflet transfer is likely to be the only event at low PEG concentration), it is possible to use the observed lifetime to obtain a rough estimate of aggregate size (not to distinguish $j = 2$ from $j = 4$, but certainly to distinguish $j = 2$ from $j = 10$). Alternatively, if one can estimate the aggregate size, the observed lifetime can be used to distinguish the event leading to the dilution of probe molecules into probe-free membrane regions (i.e., fusion versus transfer).

An example from the work of Wu and Lentz [21] will illustrate the usefulness of this procedure. We have shown using the ANTS/DPX contents mixing assay de-

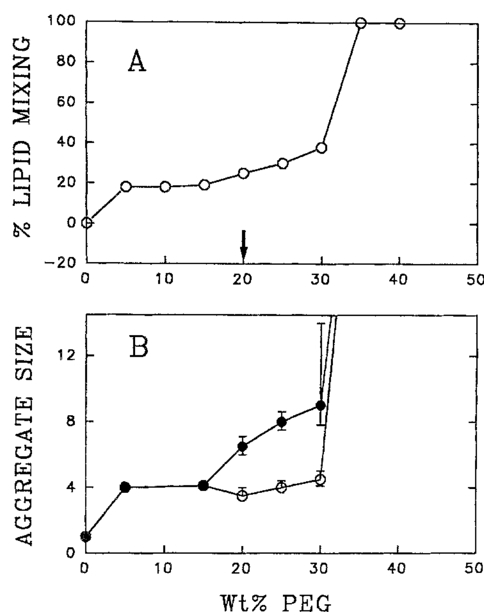

Fig. 11. (A) PEG-induced lipid mixing between DPPC LUV containing 0.5 mol% PA at 48°C under an experimental setup described by Wu and Lentz [21] is shown as a function of PEG concentration. Values were obtained from observed DPHpPC fluorescence lifetime as described by Burgess and Lentz [16]. The slight increase in lipid mixing at 20 wt% PEG correlates with the initiation of fusion (indicated by an arrow) as detected by other methods [25,26]. (B) The sizes of vesicle aggregates estimated using the "transfer" (closed circles) *versus* "fusion" (open circles) mechanisms as a function of PEG concentration. (Reproduced with permission from Fig. 5 of Ref. 21.)

scribed above that DPPC vesicles containing a small amount of palmitic acid (PA) first fused at 20 wt% PEG and then eventually ruptured as the PEG concentration added to the vesicles was increased beyond 30 wt% [25,26]. Since no fusion could be detected below 20 wt% PEG, we modeled DPHpPC lifetime data in terms of an outer-leaflet transfer mechanism below 20 wt% PEG [21]. The average aggregate sizes predicted from calculations based on the outer-leaflet transfer mechanism are recorded as a function of PEG concentration as closed circles in Fig. 11B. Since fusion had been observed at 20 wt% PEG [25,26], we considered both possible mechanisms at and above this concentration of PEG (open circles in Fig. 11 reflect the fusion process). Two aspects of this exercise require comment. First, the calculation shows that the observed DPHpPC lifetimes are consistent with formation of small aggregates of vesicles in the presence of PEG. Second, the aggregate size predicted by the outer-leaflet transfer mechanism below 20 wt% PEG was roughly the same as that predicted by the fusion mechanism at and above 20 wt% PEG. Similar results were obtained for two other lipid vesicle systems shown by other means to fuse in the presence of PEG [21]. The agreement of predictions made on the basis of lifetime measurements with other types of observations made on three different experimental systems attests to the validity of the method developed for interpreting DPHpPC lifetime measurements [21].

CONCLUSIONS

This method of interpreting our DPHpPC fusion/transfer assay observations has provided two important insights into the PEG-mediated membrane fusion process. First, lipid transfer and fusion appear to occur between small numbers of aggregated vesicles. Second, fusion occurs in the dehydrated state induced by PEG. This demonstrates how the unusual photophysics of DPHpPC can be used to extract details of the fusion and lipid transfer processes not obtainable by any other single measurement. Because the DPHpPC property being measured (fluorescence lifetime) is an intensive rather than extensive property of the probe, measurements can be made even in concentrated PEG, allowing characterization of membrane–membrane interactions and fusion even in the dehydrated state. The unique concentration-dependent photophysics of DPH-containing probes potentially can be used to explore other types of membrane microstructural heterogeneity or microdomain formation.

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