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Novel inner monolayer fusion assays reveal differential monolayer mixing associated with cation-dependent membrane fusion

Paul Meers *, Shaukat Ali, Ravi Erukulla, Andrew S. Janoff

The Liposome Company, Princeton, NJ, USA

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Abstract

The ability to specifically monitor the behavior of the inner monolayer lipids of membranous vesicles during the membrane fusion process is useful technically and experimentally. In this study, we have identified *N*-NBD-phosphatidylserine as a reducible probe particularly suitable for inner monolayer fusion assays because of its low rate of membrane translocation after reduction of the outer monolayer probes by dithionite. Data are presented on translocation as a function of temperature, vesicle size, membrane composition, and serum protein concentration. Translocation as a result of the fusion event itself was also characterized. We further show here that a second membrane-localized probe, a long wavelength carbocyanine dye referred to a diI(5)C18ds, appears to form a membrane-bound resonance energy transfer pair with *N*-NBD-PS, and its outer monolayer fluorescence can also be eliminated by dithionite treatment. Lipid dilution of these probes upon fusion with unlabeled membranes leads to an increase in NBD donor fluorescence, and hence is a new type of inner monolayer fusion assay.

These inner monolayer probe mixing assays were compared to random lipid labeling and aqueous contents mixing assays for cation-dependent fusion of liposomes composed of phosphatidylserine and phosphatidylethanolamine. The results showed that the inner monolayer fusion assay eliminates certain artifacts and reflects fairly closely the rate of non-leaky mixing of aqueous contents due to fusion, while outer monolayer mixing always precedes mixing of aqueous contents. In fact, vesicle aggregation and outer monolayer lipid mixing were found to occur over very long periods of time without inner monolayer mixing at low cation concentrations. Externally added lysophosphatidylcholine inhibited vesicle aggregation, outer monolayer mixing and any subsequent fusion. The state of vesicle aggregation and outer monolayer exchange that occurs below the fusion threshold may represent a metastable intermediate state that may be useful for further studies of the mechanism of membrane fusion.

Abbreviations: tPE, phosphatidylethanolamine derived from transesterification of egg PC; PS, bovine brain-derived phosphatidylserine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-PE (transesterified from egg PC); *N*-NBD-PE, *N*-7-nitro-2,1,3-benzoadiazol-4-yl phosphatidylethanolamine (derived from transesterified egg PC); *N*-NBD-PS, 1,2-dioleoyl-*sn*-glycero-phospho-L-serine-(*N*-7-nitro-2,1,3-benzoadiazol-4-yl); *N*-NBD-DOPE, 1,2-dioleoyl-*sn*-glycero-phosphoethanolamine-(*N*-7-nitro-2,1,3-benzoadiazol-4-yl); *N*-NBD-DPPE, 1,2-dipalmitoyl-*sn*-glycero-phosphoethanolamine-(*N*-7-nitro-2,1,3-benzoadiazol-4-yl); diI(5)C18, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate; diI(5)C18ds, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine-5,5′-disulfonic acid; diI(3)C18, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-tPE; *N*-glutaryl-DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine *N*-glutaryl; lyso-PC, 1-oleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylylenebis(pyridinium bromide); DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; EDTA, ethylenediaminetetraacetate; TES, *N*-tris(hydroxylmethyl)methyl-2-aminoethanesulfonic acid; $C_{12}E_8$, octaethyleneglycol monododecyl ether

* Corresponding author. Fax: +1-609-951-0259; E-mail: pmeers@lipo.com

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1. Introduction

Membrane fusion is an important cellular phenomenon for changing the compartmentalization of molecules. The process ultimately involves the mixing of bilayer lipids from separate membranes. The current model for lipidic fusion suggests an initial coalescence of outer monolayers, perhaps in a stalk-like intermediate, followed by the mixing of the inner monolayers so as to form an aqueous fusion pore through which the originally delimited aqueous compartments can communicate. Sensitive fluorescent assays have been developed on the basis of some of these phenomena to monitor membrane fusion. The most stringent assays require the fusion-dependent mixing of aqueous contents to generate a fluorescent signal [1–4]. However, aqueous contents mixing assays can suffer from induced leakage and require the often non-trivial loading of probes into the vesicles or cells of interest.

Lipid probe dilution fusion assays are more convenient as they require the lipid labeling of only one population of vesicles, but have a number of potential artifacts associated with exposure of fluorophores on the outer monolayer of the membranes. For instance, exposure of the fluorophores to the extravesicular aqueous milieu in standard lipid dilution assays allows membrane binding of proteins or ions and/or induction of lipid lateral phase separation to potentially modify the fluorescence emission intensity of the externally exposed probes without any movement of lipid between membranes.

One fluorescent assay that depends on the dilution of membrane-associated phospholipid-derived probes (*N*-NBD-PE and Rh-PE) into unlabeled target membranes has been very popular and useful for many years [5]. In these assays, an increase of the intensity of the NBD probe indicates fusion as a result of the dilution-related relief of energy transfer. While these phospholipid probes do not appear to readily exchange between vesicles in the absence of fusion [5–7], there are demonstrations of apparent lipid dilution without aqueous contents mixing under certain conditions. For instance, apparent hemifusion has been observed in some systems, such that only the outer monolayers of vesicles fuse or mix lipids without mixing of aqueous contents [4,8,9]. It has become increasingly accepted that such hemifusion events with stalk-like intermediates may represent an important step in at least one type of fusion process [10–12]. As such, these events are of great interest, and the isolation of the behavior of inner and outer monolayers during fusion is an important goal.

Specific fluorescent labeling of the inner monolayer of liposomes has been previously studied. The use of dithionite-mediated reduction of NBD labeled lipids as a means of creating a transmembrane asymmetry in fluorescent labeling was introduced by McIntyre and Sleight [13] for the purpose of studying the transmembrane movement of these lipids. Shortly thereafter, the concept of utilizing the same reduction method to create inner monolayer NBD labeled vesicles for lipid dilution fusion assays (using NBD-PE and Rh-PE probes) was introduced [14]. The fidelity of the fusion assays was monitored in the latter study by comparing the fluorescence response of randomly labeled vesicles to vesicles in which the outer monolayer NBD-lipid donor probe had been reduced before fusion assays. Ideally, the observed rate of NBD dilution would be the same in the two assays if no outer monolayer exchange had occurred.

A true inner monolayer fusion assay requires that both energy transfer donor and acceptor reside only in the inner monolayer. The R_0 for any energy transfer pair that can be used at less than several mole% of the total lipid must, by definition, be greater than the thickness of the bilayer, so that transbilayer energy transfer would occur [15]. Therefore, it is necessary to also eliminate the fluorescence of the outer monolayer energy transfer acceptors, such as Rh-PE, for a true inner monolayer fusion assay.

Another crucial requirement for inner monolayer fusion assays is that the labeling must remain asymmetric, i.e. fluorescent lipids on the inner monolayer should not translocate to the outer monolayer before or during the membrane fusion experiments.

We present here an approach to inner monolayer fusion assays introducing two new probes, including

an energy transfer acceptor, demonstrations of the advantages of such assays and provide information on some of the basic factors that govern bilayer translocation of relevant probes. The assay was then used to study polyvalent cation-induced inner monolayer fusion of liposomes composed of phosphatidylserine and phosphatidylethanolamine with particular attention to the conditions that promote outer monolayer versus inner monolayer coalescence. Portions of this work have been presented previously in abstract form [16,17].

2. Materials and methods

N-(lissamine rhodamine B sulfonyl)-PE (Rh-PE, transesterified from egg PC), *N*-7-nitro-2,1,3-benzoxadiazol-4-yl phospatidylethanolamine (*N*-NBD-PE, derived from transesterified egg PC), 1,2-dioleoyl-*sn*-glycero-phospho-L-serine-(*N*-7-nitro-2,1,3-benzoxadiazol-4-yl) (*N*-NBD-PS), 1,2-dioleoyl-*sn*-glycero-phosphoethanolamine-(*N*-7-nitro-2,1,3-benzoxadiazol-4-yl) (*N*-NBD-DOPE), 1,2-dipalmitoyl-*sn*-glycero-phosphoethanolamine-(*N*-7-nitro-2,1,3-benzoxadiazol-4-yl) (*N*-NBD-DPPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), egg phosphatidylethanolamine transesterified from phosphatidylcholine (tPE), bovine brain phosphatidylserine (PS), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine *N*-glutaryl (*N*-glutaryl-DOPE) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (all > 99%) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). 1-Hexadecanoyl-2-(pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (pyrene-PC), 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), *p*-xylylenebis(pyridinium bromide) (DPX), 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindodicarbocyanine (diI(5)C18), 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindocarbocyanine (diI(3)C18) and 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindodicarbocyanine-5,5′-disulfonic acid (diI(5)C18ds) were obtained from Molecular Probes (Eugene, OR, USA). Octaethyleneglycol monododecyl ether ($C_{12}E_8$, > 98%) and sodium dithionite were from Fluka (Ronkonkona, NY, USA). $CaCl_2$ (> 99%), $MgCl_2$ and NaCl (> 99%) and TES (98%) were from Fisher (Pittsburgh, PA, USA). $TbCl_3 \bullet 6H_2O$ (99.9%) was obtained from Alfa (Danvers, MA, USA). Dipicolinic acid (DPA) (99%) and nitrilotriacetic acid (99%) were obtained from Sigma (St. Louis, MO, USA).

Liposomes were prepared in the appropriate buffer by extrusion ten times through 0.1 μm polycarbonate membranes as previously described [18]. Unless otherwise indicated buffer A (100 mM NaCl, 10 mM TES, 0.1 mM EDTA, pH 7.4) was used for all experiments.

Phospholipid concentrations were determined using a phosphate assay as described in Kingsley and Feigenson [19] modified from Chen et al. [20], Bartlett [21] and Morrison [22].

2.1. Synthesis of N-NBD-N,N-dimethyl-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine

N,*N*-dimethyl-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (25 mg, 0.034 mmol), dissolved in 10 ml anhydrous methylene chloride, was stirred at room temperature with NBD chloride (22 mg, 0.1 mmol) in presence of triethylamine (0.1 mM, 10 μl). After 48 h of reaction, the solvent was evaporated under vacuum and the residue was purified by preparative thin layer chromatography on silica using $CHCl_3$:MeOH:H_2O (60:30:4) to yield 18 mg of dark orange product ($R_f = 0.67$).

2.2. Aqueous contents mixing assays

Liposomes were prepared for the Tb^{3+}/dipicolinic acid (DPA) membrane fusion assay as previously described [1,2], except that the multiple extrusion method [18] was used to initially swell and load the liposomes. 100% fusion was calibrated using liposomes encapsulating both Tb^{3+} and DPA at half the concentration present in the separate Tb^{3+} and DPA loaded populations of liposomes. Zero fusion was taken as the fluorescence level of the original mixture of Tb^{3+} containing and DPA containing liposomes. Fluorescence was monitored on a Photon Technology Alphascan instrument with single excitation and emission monochromators, both excitation slits at 4 nm and both emission slits at 15 nm. A 500 nm longpass filter was used on the emission side of the cuvette. Excitation and emission monochromators were set at 276 and 540 nm respectively. Leak-

age was monitored using the liposomes (see above) containing both Tb^{3+} and DPA. The decrease in fluorescence due to leakage and concomitant dissociation of this complex was monitored. In all cases, negligible leakage was observed below approximately 25% maximal fusion. Therefore Tb/DPA assays were corrected for leakage essentially as described by Nir et al. [23] applying the equation $I(t) = I_m(t) + L(t)$, where $I_m(t)$ is the measured fraction of maximal fluorescence and $L(t)$ is measure fractional leakage of the Tb–DPA complex at time t. As expected, this correction only significantly affected the curves at the later stages of fusion, above approximately 25–30% maximal fluorescence.

The ANTS/DPX fusion assay was performed essentially as previously described [4].

2.3. Standard lipid dilution fusion assays

Lipid dilution fusion assays were performed essentially as previously described [5]. Liposomes contained 0.75 mole% each of an NBD probe and a rhodamine probe. The probes used were *N*-NBD-PE (from egg PC) or *N*-NBD-dioleoyl PS and lissamine rhodamine PE (from egg PC). All assays were performed in buffer A (see above). 100% fusion was calibrated by the fluorescence of liposomes with 0.375 mole% of each probe. All fusion assays were performed at a 1:1 ratio of labeled–unlabeled vesicles to match the conditions of the aqueous mixing assays.

2.4. Inner monolayer labeling of liposomes

Inner monolayer labeled vesicles were produced by adding the reducing agent, sodium dithionite, to preformed vesicles and removing it after reduction of the outer monolayer probes. A typical reduction is shown in Fig. 6. Reduction of NBD or diI(5)C18ds by dithionite was found to generally proceed to completion rapidly with little or no leakage to the interior of vesicles as previously reported for NBD lipids [13]. It was previously shown that this reduction does not functionally modify unlabeled lipids [14]. Furthermore, at only 0.75 mole% the reduced probe is not expected to significantly affect the bulk properties of the vesicle bilayers.

For lipid mixing fusion assays, liposomes contained 0.75 mole% each of an NBD probe and rhodamine-tPE or diI(5)C18ds. For latency of reduction assays, liposomes contained either 0.075 or 0.75 mole% of the NBD probe and 0.075 mole% of rhodamine-tPE, such that the NBD probe was not strongly quenched by the presence of the rhodamine probe. This was not a consideration for the latency of reduction of the diI(5)C18ds probe. Comparisons between samples labeled with 0.75 and 0.075 mole% of the *N*-NBD-PE probe showed no difference in apparent translocation rates (data not shown).

Stock reducing solutions were made up shortly before use by dissolving sodium dithionite to 200 mM in 100 mM Tris, pH 10. For direct observation of reduction, this stock solution was added to a stirred cuvette at 25°C to a final concentration of 10 or 20 mM. For stock solutions of reduced liposomes devoid of dithionite, a different procedure was used. All liposome compositions, except positively charged liposomes, were reduced by incubating equal volumes of liposomes (usually approximately 10 mM total lipid) with either 80 mM sodium dithionite and 40 mM Tris or 100 mM sodium dithionite and 50 mM Tris for approximately 30–45 min on ice. Dithionite was then removed by rapid centrifugation of 100 μl aliquots through 0.8 ml spin columns with BioGel A-30 (Bio-Rad).

Reduction of positively charged liposomes was a special case. The polyvalent anion dithionite can cause aggregation of such liposomes. However, because of concentration of the dithionite in the double layer near the positively charged surface, much less can be used to effect reduction. For DOTAP/tPE liposomes, a 100 μl stock of 10 mM total lipid was reduced with 4 μl of the 200 mM dithionite stock described above by incubation at room temperature for 5 min. The sample was then immediately placed on a spin column for removal of dithionite.

The correspondence of rhodamine fluorescence in a reduced and untreated sample to the total phospholipid phosphate was also tested. The ratio of rhodamine fluorescence to total phospholipid concentration was $1.85(\times 10^4) \pm 0.06$ fluorescence units ($n = 4$) per nmol lipid in untreated phosphatidylserine (PS) liposomes versus $1.78(\times 10^4) \pm 0.12$ ($n = 4$) for a dithionite treated sample, indicating no effect of reduction on rhodamine fluorescence. Therefore the total rhodamine fluorescence of the sample in deter-

gent could be used to calibrate the total amount of phospholipid present. Similar experiments with a pyrene-PC probe as a marker for total phospholipid showed similar results. Therefore pyrene-PC was used as a total lipid marker for the experiments in which both NBD and diI(5)C18ds probes were used.

The product of dithionite treatment also does not appear to significantly alter the fluorescence of other NBD fluorophores. For instance, the percent remaining NBD fluorescence after reduction of one particular sample of egg PC liposomes was 47.5 ± 3.4% in vesicles versus 42.8 ± 1.6% in 0.2% C12E8 micelles, where each micelle contains on average less than one probe, either reduced or fluorescent.

2.5. Measurement of the rate of probe translocation by latency of dithionite reduction

Samples that had been inner monolayer labeled were incubated under the desired conditions for the appropriate amount of time. They were then kept on ice for a short amount of time before dilution into 2 ml of room temperature buffer in a cuvette. Most experiments generated a final phospholipid concentration in the cuvette of 125 μM. Typically a final concentration of 20 mM dithionite was added to the stirred sample. The initial and final fluorescence values (typically approximately 60 s after dithionite addition) were then recorded. The amount of time required for the reduction to reach completion varied with sample composition, qualitatively corresponding to the expected Guoy Chapman double layer distribution of ions. The more negatively charged the liposome, the longer it took to reach a flat baseline indicating complete reduction. In all cases the time required for reduction was insignificant with respect to the half-time for translocation of the probe.

Latency of dithionite reduction was expressed as 'percent remaining' which refers to the percentage of the original fluorescence that remained after reduction. Latency was also expressed as 'percent randomization' in some cases, which was defined as

$$\%\text{random} = 100(\%\text{out} - \%\text{out}_{\min})/(\%\text{out}_{\max} - \%\text{out}_{\min})$$

Minimum and maximum %out are from dithionite reduction values for freshly reduced samples (normally approx. 0) and original samples (normally approx. 50), respectively. %out is determined directly from the fractional reduction of a sample.

For latency measurements made in samples with a high PE content or incubated with serum or associated with fusion, a correction for leakage of dithionite was made as follows:

$$\%\text{out} = (100 - \%\text{rem}_{\text{red,ex}} - 100(x))/(1-x)$$

$$\%\text{out}_{\min} = (100 - \%\text{rem}_{\text{red,ice}} - 100(x))/(1-x)$$

where %rem = percent remaining and 'x' gives the leakage correction.

$$x = (\%\text{rem}_{\text{orig,ice}} - \%\text{rem}_{\text{orig,ex}})/(\%\text{rem}_{\text{orig,ice}}) \text{ for } \%\text{rem}_{\text{orig,ice}} > \%\text{rem}_{\text{orig,ex}}$$

ex = incubation under experimental conditions; ice = sample kept on ice; orig = original sample, both monolayers fluorescent; red = reduced outer monolayer sample.

In short, the correction for leakage is made by checking the amount of dithionite-mediated reduction of the original unreduced sample after incubation under the experimental conditions. Any increase in reduction over the roughly 50% value for the original sample kept on ice is taken as leaked dithionite, and a correction is made. For non-leaky systems, $x = 0$ and the equation simplifies.

Rate constants for translocation were analyzed in terms of a spontaneous 'flip-flop' mechanism by a formalism similar to that presented by Kornberg and McConnell [24]. It is assumed in this case that the rate constants for inward and outward movement are equal. If n is the fraction of fluorescent probe on the outer monolayer of the membrane, n is defined as 0 at $t = 0$ and as 0.5 at $t = \infty$. Then $\log[1-2n] = -kt$ where k is the sum of individual rate constants for inward and outward translocation. Since dithionite reduction data are expressed normally as the fraction of fluorescence remaining (= percent remaining/100), rate constants are obtained from the slopes of log-[2(fraction remaining)−1] versus t. This analysis assumes unilamellar vesicles. In general, dithionite reduction of original non-reduced samples revealed that all compositions used were close to unilamellar.

2.6. Some characteristics of the N-NBD-PS probe in various vesicles

The effect of liposome size on translocation rate was investigated using POPC liposomes extruded through 0.8, 0.1 or 0.03 μm polycarbonate membranes. Essentially no difference in the rate of translocation was observed over a 21 h period (data not shown). Therefore, it is unlikely that a certain size subpopulation of a liposomal preparation will undergo probe translocation after reduction of outer monolayer fluorescence. Consequently this probe can be used with liposomes of any nominal average size, with little concern about translocation under most conditions.

POPC vesicles with the *N*-NBD-PS probe were also allowed to incubate in 50% rat plasma for various periods of time and the latency of dithionite reduction was then tested. Leakage was monitored and used for correction of the translocation data as described in Section 2. While translocation is relatively minor on a short time scale, over a period of several hours, serum interactions can destroy the inner monolayer labeling (data not shown). After 65 min, 17% randomization occurred as a result of translocation and 5% as a result of leakage. The ability of serum to induce translocation and leakiness to dithionite may be useful as a tool to study serum protein interactions with liposomes, but also may limit inner monolayer fusion assays under these conditions.

Translocation of *N*-NBD-PS in lipids that prefer non-bilayer phases, i.e. that contain a relatively high proportion of PE, was studied near the inverted hexagonal phase transition temperature for the PE component after a 3 h incubation. The major component of these liposomes is PE transesterified from egg PC, which, as a pure multilamellar dispersion, enters the inverted hexagonal phase above approximately 37°C at neutral pH [25]. The percent randomization for DOTAP/tPE 1/3, DOTAP/egg PC 1/3 and PS/tPE 1/3 liposomes was 9.5 ± 1.5, 9.2 ± 5.5 and 0.3 ± 1.5 at 25°C and 30.1 ± 1.0, 1.8 ± 3.5 and 7.0 ± 1.5 at 37°C, respectively. When the temperature was increased to 43°C, 20.8 ± 6.0 percent randomization was observed in the PS/tPE. The *N*-NBD-PE probe was completely randomized at 37°C after 3 h in the DOTAP/tPE liposomes. The data indicate a possible correlation between bilayer to hexagonal II transition temperature and probe translocation rate. The rapid translocation in DOTAP/tPE was not apparently due to collisions between vesicles, as the rate did not depend on vesicle concentration over a 50-fold range (data not shown), so actual formation of inverted hexagonal phase is not responsible for the translocation.

3. Results

3.1. Examples of artifactual effects on the fluorescence of outer monolayer labeled vesicles

When fluorescent fusion probes are exposed on the surface of a liposomal membrane, there are potential interactions with components of the external milieu that can change fluorescence. One example is an effect of ion binding on outer monolayer fluorescence. When Ca^{2+} was added to liposomes composed of

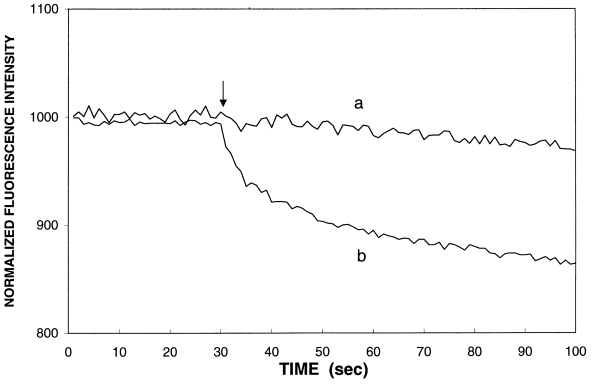

Fig. 1. Effect of Ca^{2+} binding on the fluorescence of outer monolayer *N*-NBD-PE. Samples consisted of PS/tPE (1/3) liposomes at 50 μM total phospholipid in 154 mM NaCl, 10 mM TES, 0.1 mM EDTA, pH 7.4. The liposomes contained 0.75 mole% each of *N*-NBD-PE and Rh-PE. A final concentration of 3 mM Ca^{2+} was added to a stirred cuvette as an aliquot from a 100 mM stock $CaCl_2$ solution at the arrow. The time course of NBD fluorescence was monitored. Inner monolayer labeled liposomes (curve a) were prepared by dithionite treatment and removal as described in Section 2. Data from untreated liposomes (both monolayers labeled) are shown in curve b. Concentrations were adjusted from the total rhodamine fluorescence of each sample. Excitation and emission were monitored at 450 nm and 530 nm respectively with 4 nm slit widths. The data were normalized to make starting baseline values the same for both samples. No significant Ca^{2+} effect is seen with unlabeled liposomes (data not shown).

brain phosphatidylserine (PS) and phosphatidylethanolamine (transesterified from egg PC, tPE) in a 1/3 ratio and also containing the *N*-NBD-PE and Rh-PE probes (0.75 mole%), a small but significant artifactual decrease in fluorescence was observed (Fig. 1). This could be the result of any of several factors discussed below. For comparison, liposomes with fluorescent NBD probes only on the inner monolayer were needed.

Reduction of NBD probes by dithionite has become a well-established method for generation of liposomes labeled only on inner monolayers. Typically, when liposomes are prepared by the methods used here (see Section 2), with *N*-NBD-PE as a fluorophore, addition of dithionite rapidly reduces the fluorescent signal by approximately 50%, corresponding to reduction of the outer monolayer probes. When detergent is subsequently added, the remaining fluorescence disappears due to the exposure of the remaining NBD probes to dithionite. Samples can be prereduced in bulk and separated from dithionite by column chromatography, as described in Section 2. By comparing untreated and prereduced samples, artifactual effects specific to the outer monolayer lipids can be detected [14].

For instance, when the fluorescent NBD probe was present only on the inner monolayer of the same liposomes in Fig. 1, there was essentially no effect of Ca^{2+} binding, except for a slow downward drift of the baseline. Therefore inner monolayer labeling appears to eliminate a significant ion binding artifact in this case.

In some fusion systems, there is a large discrepancy between the rates reported by standard lipid dilution fusion assays and aqueous contents mixing assays. This has been reported previously for pure phosphatidylserine vesicles [6,9]. In Fig. 2, we compare the apparent Mg^{2+}-induced fusion of phosphatidylserine/phosphatidylethanolamine (PS/tPE) liposomes using a standard lipid dilution assay and aqueous mixing assays. It is clear that the mixing of aqueous contents is much slower than the fusion reported by the probes in the standard lipid dilution assay, suggesting that the outer monolayer probes may be exchanging before actual fusion. When the outer monolayer *N*-NBD-PE probe was reduced by dithionite treatment, the lower rate of probe mixing more closely resembled the results from the aqueous

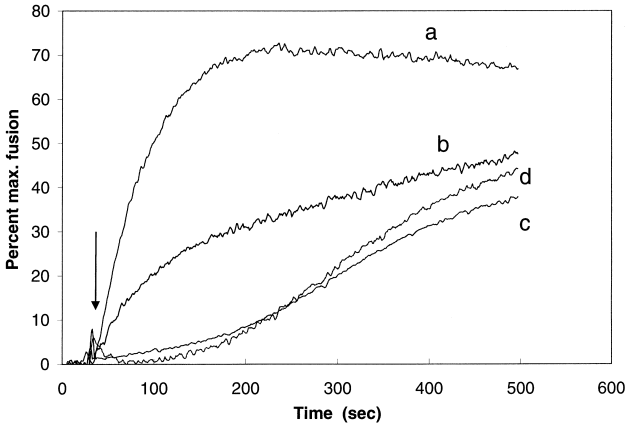

Fig. 2. Comparison of the observed rates of Ca^{2+}-dependent fusion of PS/tPE liposomes as monitored by different fusion assays. All samples contained 20 μM total phospholipid in buffer A at 22°C, and fusion was induced by addition of 3 mM Ca^{2+} at the arrow. Assays were the standard randomly labeled lipid dilution assay, i.e. time course of NBD fluorescence (a), an assay in which only the outer monolayer NBD probes were reduced (b) or the Tb/DPA (c) or ANTS/DPX (d) aqueous mixing assays (see Section 2). Fusion of a 1:1 ratio of labeled and unlabeled liposomes was measured in the case of the lipid dilution assays and a 1:1 ratio of Tb^{3+} and DPA containing liposomes in the Tb/DPA assay or ANTS and DPX containing liposomes in the ANTS/DPX assay. Assays were run in triplicate and curves shown are representatives.

mixing assays, but still appeared to be much faster. As we have discussed and will show below, this is at least partially due to the remaining fluorescence of the outer monolayer acceptor fluorophores and transbilayer energy transfer.

3.2. *Stability of inner monolayer labeling; importance of overall membrane composition*

The first important step in designing an inner monolayer fusion assay is to determine the conditions that would allow sufficiently stable labeling of the inner monolayer so that fusion assays could be performed, i.e. does excessive probe translocation take place after inner monolayer labeling? We initially focussed our attention on the *N*-NBD-PE probe. The transbilayer movement was assessed by latency of dithionite reduction as discussed in Section 2. Liposome samples were prereduced with dithionite, separated from the dithionite solution, incubated varying lengths of time and then treated again with dithionite while observing fluorescence. We found,

not surprisingly, that the translocation rate of the probe was dependent on the characteristics of the predominant lipids in the membrane, as though probe translocation in one direction (flip) was dependent on the translocation in the other direction (flop) of one of the bulk lipids [26]. For instance, when the translocation rate of the probe was tested at 37°C in liposomes composed of POPS, PS/tPE 1/3, POPC, POPS/POPC 2/8, or *N*-glutaryl-POPE/POPC 1/9, 30% randomization was observed at 45 min for all but the POPS liposomes, which took 6 h to reach this point. In particular, triplicate measurements at 45 min showed 82.0 ± 4.0 and 83.3 ± 1.0% remaining fluorescence for PS/tPE and POPC samples respectively, while after 3 h 66.3 ± 1.5 and 69.8 ± 1.2% remained in these samples respectively. Half-maximal randomization was at about 2 h for all but the POPS composition. These results indicate the potential deficiency of the *N*-NBD-PE probe in all but the most highly charged liposomes. Because relatively long term experiments may be relevant to the study of liposomal fusion with live cell membranes it was of interest to study other potential NBD probes.

3.3. *Probe structure; effect of acyl chain unsaturation and headgroup on translocation*

In an attempt to find improved NBD probes for inner monolayer fusion assays, several structures were evaluated. The importance of the acyl chain of the probe molecule was investigated by measuring the transbilayer diffusion rate of *N*-NBD-dioleoyl-PE, *N*-NBD-dipalmitoyl-PE and *N*-NBD-transesterified egg PE (from egg PC) in POPC membranes. In Fig. 3A, the data are plotted in a form consistent with a first order 'flip-flop' mechanism (see Section 2). It is clear from the data that the latency of dithionite reduction as a function of time is nearly identical for the three probes. In agreement with these data, dithionite reductions in triplicate gave 88.2 ± 0.8, 89.8 ± 1.3 and 85.8 ± 2.0% remaining fluorescence after 45 min incubation and 76.4 ± 1.1, 76.8 ± 2.2 and 72.2 ± 1.5% at 3 h for *N*-NBD-tPE, *N*-NBD-DPPE and *N*-NBD-DOPE, respectively. Hence the rates of transbilayer movement are nearly identical. Apparently these small changes in acyl chain length and unsaturation have little effect.

Three different headgroup derivatives were also

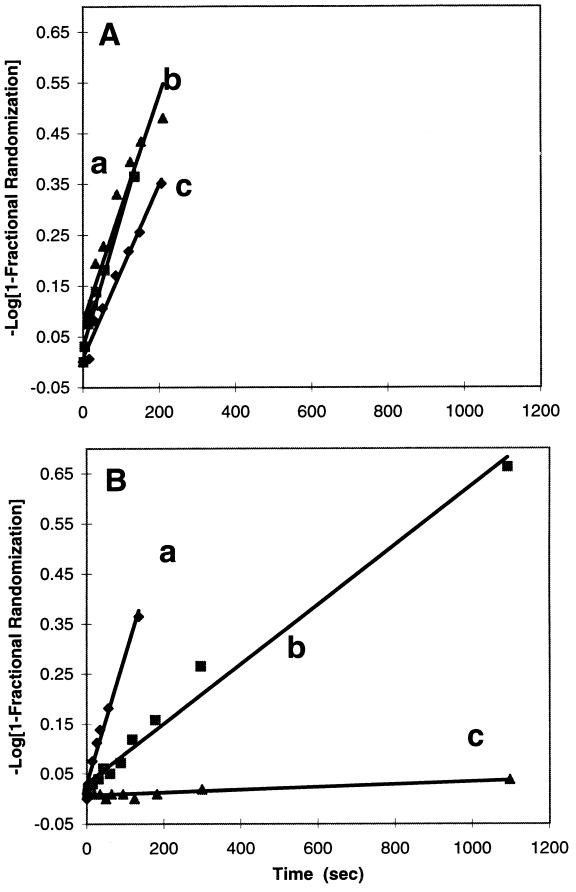

Fig. 3. Effect of NBD probe structure on the bilayer translocation rate. Liposomes were composed of POPC with 0.075 mole% each of an NBD probe and *N*-Rh-PE. The randomization of each probe after reduction of outer monolayer fluorescence was monitored as described in Section 2. In panel A, variations on the acyl chain structure were explored. The NBD probes and corresponding rate constants for randomization were *N*-NBD-DOPE (a) 2.2×10^{-3} min^{-1}, *N*-NBD-tPE (b) 2.6×10^{-3} min^{-1}, *N*-NBD-DPPE (c), 1.7×10^{-3} min^{-1}. In panel B, the different NBD headgroup probes were *N*-NBD-tPE (a) 2.6×10^{-3} min^{-1}, *N*-NBD-*N*,*N*-dimethyl-PE (b) 6.0×10^{-4} min^{-1}, and *N*-NBD-PS (c) 3×10^{-5} min^{-1}.

tested. *N*-NBD-dioleoyl PS, *N*-NBD-*N*,*N*-dimethyl-DPPE and *N*-NBD-transesterified egg PE were studied in POPC liposomal membranes. As shown in Fig. 3B, the three headgroup analogs varied greatly in their spontaneous rates of transbilayer movement. Both *N*-NBD-DOPS and *N*-NBD-*N*,*N*-dimethyl-DPPE were significantly less prone to transbilayer movement than the original probe used for inner monolayer labeling, *N*-NBD-transesterified PE. These rates were obviously not simply related to overall probe charge, but may possibly be related

to the overall hydration and/or volume of the various headgroup structures (see Section 4). Clearly, the new probes are potentially much better than the original (*N*-NBD-PE) for preparation and maintenance of inner monolayer labeled membranes for fusion assays.

3.4. *N-NBD-PS as a probe for fusion; comparison to N-NBD-PE*

We chose to focus on *N*-NBD-PS as a stable probe for inner monolayer assays. It was considered possible that the *N*-NBD-PS probe, because of its doubly negative charge, might interact with cations or other lipids in such a way as to report fluorescence changes not related to fusion. The *N*-NBD-PS probe should report fusion in a manner similar to the originally characterized *N*-NBD-PE probe under conditions where neither lipid exchange nor translocation of these probes is a factor. One test of this was to compare observed rates of fusion for *N*-NBD-PS versus *N*-NBD-PE labeled liposomes. Calcium-dependent fusion of PS/tPE liposomes was tested as shown in Fig. 4. In this case the acceptor probe was Rh-PE (see [14]). When both monolayers were labeled there was a probe-dependent high rate of fluorescence evolution. The small difference between the probes in this case is consistent with small differences in outer monolayer mixing rate, which could be influenced by probe structure. By contrast, the apparent rates of fusion of samples in which the outer monolayer probes were reduced are essentially independent of the probe structure, suggesting that a true probe-independent fusion rate is reported by the assays. Similar results were observed under other fusion conditions. Clearly, the *N*-NBD-PS does not interact with Ca^{2+} in a way that significantly changes the observed rate of fusion in the inner monolayer labeled assays. Because *N*-NBD-PS was the most suitable probe for maintenance of inner monolayer labeling, its translocation characteristics were further studied in various potential assay conditions.

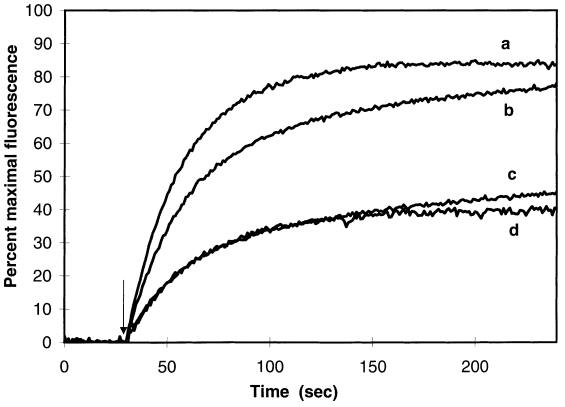

Fig. 4. Effect of probe structure on reported rate of fusion; comparison of *N*-NBD-PS and *N*-NBD-PE. All liposomes were composed of brain PS/tPE 1/3 as described in Section 2. Labeled liposomes (10 μM total lipid) containing 0.75 mole% each of *N*-NBD-PS and Rh-PE (a,c) or 0.75 mole% each of *N*-NBD-PE and Rh-PE (b,d) were added to unlabeled liposomes (10 μM total lipid) in a stirred cuvette in buffer A at 25°C. In some samples (c,d) the outer monolayer NBD fluorescence was prereduced by dithionite treatment (see Section 2). To this mixture was added a final concentration of 3 mM $CaCl_2$ at the arrow. The time course of NBD fluorescence was monitored as described in Section 2. Triplicate experiments for each condition gave curves essentially superimposable on those shown.

Table 1
Translocation of *N*-NBD-PS as a result of fusion of PS/tPE liposomes

Conditions[a]	% Accessible (no leak correction)	Normalized per % maximal fusion
3 mM Ca^{2+}, isoosmotic	8.0 ± 2.2	0.43
3 mM Ca^{2+}, 80% osmolarity	17.9 ± 4.8	0.52
3 mM Ca^{2+}, 60% osmolarity	24.8	0.49
no Ca^{2+}, isoosmotic	1.1 ± 3.9	no fusion
no Ca^{2+}, 80% osmolarity	2.2 ± 2.1	no fusion
no Ca^{2+}, 60% osmolarity	2	no fusion

[a]Samples contained 10 μM (total phospholipid) of vesicles composed of PS/tPE (1/3) labeled with 0.75 mole% each of *N*-NBD-PS and *N*-Rh-PE and reduced by dithionite to generate inner monolayer labeled vesicles (see Section 2). This was mixed with 90 μM unlabeled PS/tPE vesicles. Either 3 mM or 0 mM $CaCl_2$ was added and each sample was incubated for 240 s. 10 mM EDTA was then added to the Ca^{2+} containing samples. These were then tested for dithionite accessibility, i.e. percent of total fluorescence signal eliminated, as in Section 2. Note that values are not corrected for leakage.

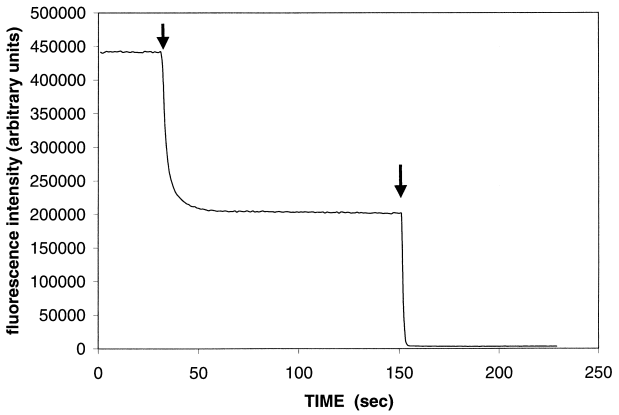

Fig. 5. Apparent reduction of diI(5)C18ds by dithionite. Liposomes composed of POPC containing 0.32 mole% *N*-NBD-PS and 0.75% diI(5)C18ds were dispersed in buffer A at a total lipid concentration of 50 μM in a stirred cuvette and treated with a final concentration of 10 mM sodium dithionite at the first arrow (30 s). A final concentration of 0.2% of the detergent $C_{12}E_8$ was added at 150 s. Fluorescence was monitored with excitation at 650 nm and emission at 670 nm with 4 nm slits.

3.5. Translocation of N-NBD-PS during fusion

One question of interest regarding the assay is whether *N*-NBD-PS translocates as the result of the fusion process itself. One intriguing possibility is that bilayer translocation may reflect the structure of fusion intermediates. In Table 1 is shown the accessibility of the *N*-NBD-PS probe after fusion of PS/tPE liposomes induced with Ca^{2+}. Very little accessibility of the probe was seen after fusion under isoosmotic conditions. However, there was considerable probe accessible to dithionite after fusion under hypoosmotic conditions. This accessibility was not the result of the hypoosmotic conditions alone, as incubation in the absence of Ca^{2+} did not result in any significant probe accessibility. Therefore, translocation under these conditions may result from some osmotically induced rearrangement of the membrane during fusion to relieve osmotic stress or possibly, but less likely, an induced permanent leakiness to dithionite (since leak was not tested in these particular experiments). Results similar to these, originally reported by Meers et al. [16], were also observed later in PEG-induced fusion [27]. In practical terms, they suggest that osmolarity must be carefully controlled to accurately monitor inner monolayer fusion.

3.6. An energy transfer acceptor for inner monolayer fusion assays, diI(5)C18ds

A true inner monolayer fusion assay requires that all fluorescence emanates only from inner monolayer fluorophores. This is because any energy transfer pairs that can be used at a concentration below a few mole percent in the membrane must also exhibit significant transbilayer energy transfer due to the high R_0 value necessary for energy transfer at this low probe concentration. Because of transbilayer energy transfer, any exchange of outer monolayer energy transfer acceptors would increase the fluorescence of inner monolayer donors without fusion.

Dithionite is known to reduce many types of organic molecules, including cationic ring systems, such as nicotinamide adenine dinucleotide and indigo carmine [28,29]. We have discovered that dithionite can also influence the fluorescence of the conjugated cationic system in a certain carbocyanine dye which we refer to as diI(5)C18ds (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine- 5,5′-disulfonic acid, excitation maximum 650 nm, emission 670 nm; see below). As in the case of *N*-NBD-PS, addition of dithionite to liposomes containing diI(5)C18ds reduced its fluorescence to approximately the 50% range as shown in Fig. 5. This reduction is followed by complete elimination of fluorescence after addition of a detergent exposing the inner monolayer. The light absorption giving a strong blue color to the probe completely disappears under these conditions. Presumably, the change in fluorescence is due to chemical reduction of the probe, though this has not been actually verified. The key to the appropriate reduction potential is known to be an extended π system, as is the case for this molecule. Interestingly, we found that the fluorescence of an analog with a less extended system, diI(3)C18 (1,1′-dioctadecyl-3,3,3′,3′- tetramethylindocarbocyanine perchlorate) was not affected by dithionite under similar conditions (data not shown).

DiI(5)C18ds is an apparent energy transfer acceptor for NBD. When these two probes are incorporated into the same vesicles, classical behavior of resonance energy transfer pairs is observed (Fig. 6). Fluorescence spectra were obtained for POPC liposomes containing *N*-NBD-PS and varying amounts of diI(5)C18ds. The *N*-NBD-PS fluorescence in the

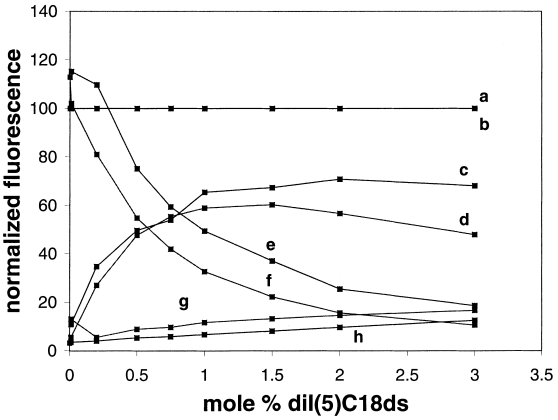

Fig. 6. Apparent energy transfer between *N*-NBD-PS and diI(5)C18ds probes in POPC membranes. Liposomes composed of POPC, 0.32 mole% of *N*-NBD-PS and varying amounts of diI(5)C18ds were prepared (a,b,f,h) and an aliquot was prereduced with dithionite as in Section 2 (b,c,e,g) and compared to the untreated liposomes. Fluorescence spectra of samples were measured at a total lipid concentration of 50 μM in buffer A (c–f) or in buffer A with 0.2% $C_{12}E_8$ present (a,b,g,h). Excitation was at 450 nm with 4 nm slits, while emission was monitored at the maximum for the NBD fluorophore, 530 nm (a,b,e,f), or at the maximum for the diI(5)C18ds at 670 nm (c,d,g,h), with 4 nm slits. Peak heights were used to estimate the total fluorescence. For each sample the value of the *N*-NBD-PS fluorescence in detergent was set to 100 and the other readings normalized to this value.

presence of detergent was taken as a standard for each sample. Increasing amounts of the diI(5)C18ds in POPC liposomes decreased the fluorescence of the NBD donor and showed increasing acceptor fluorescence when excited at the donor excitation maximum. It is clear from these data that *N*-NBD-PS and diI(5)C18ds can form an acceptable energy transfer pair for fusion assays at approximately 0.5–1 mole%, even after pretreatment of the vesicles with dithionite to eliminate the outer monolayer fluorescence (Fig. 6B). Although such curves are never linear, the response of the NBD probe to dilution of the diI(5)C18ds probe is relatively close to linear in the region, as is the case for the NBD, rhodamine energy transfer pair [5].

The emission spectra of the probes at 0.75 mole% each in fusogenic vesicles composed of PS/tPE are shown in Fig. 7. As in the PC vesicles, energy transfer is observed between the probes. Prereduction of a concentrated stock of liposomes reduces the fluorescence emission intensity of both probes, but energy transfer is maintained. Dispersal in detergent eliminates the energy transfer. The effect of removal of transbilayer energy transfer on the NBD fluorescence is of particular interest. While the comparison of samples solubilized in detergent shows that dithionite initially reduced approximately 50% of NBD fluorescence, the inner monolayer NBD fluorescence of the dithionite treated sample still in liposomal membranes is greater than 50% of the original sample. This is because of the concomitant elimination of quenching by the diI(5)C18ds probes from the outer monolayer.

The effect of dithionite on the fluorescence of the diI(5)C18ds acceptor probe when excited at its maximum absorption wavelength and normalized for total phospholipid is also shown. It should be noted that, perhaps because of the operant reduction potentials, the final apparent reduction of the diI(5)C18ds probe in these concentrated liposome stock preparations, and especially in negatively charged membranes, did not quite reach the final level of reduction of the NBD probe. This can be seen in detergent solubilized samples before and after reduction. In general, approximately 50% reduction of the NBD probe could be obtained when only approximately 40–45% of the diI(5)C18ds probe was reduced. This was not the case in dilute liposome

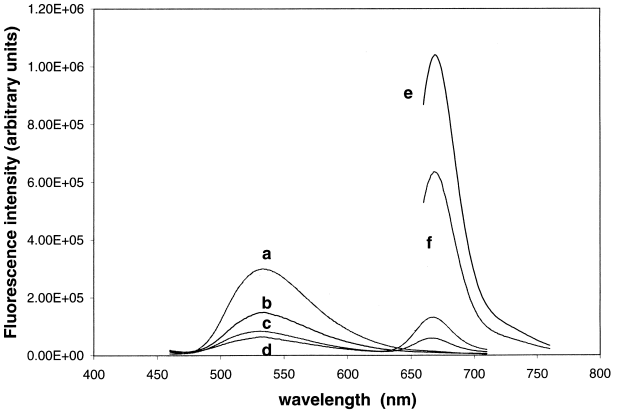

Fig. 7. Fluorescence spectra of *N*-NBD-PS and diI(5)C18ds showing energy transfer between the probes and the effect of dithionite treatment. Liposomes composed of PS/tPE 1/3 and 0.75 mole% each of *N*-NBD-PS and diI(5)C18ds were studied. Some samples were treated with sodium dithionite (b,d,f) and/or dissolved in 0.2% $C_{12}E_8$ (a,b,e,f) as described in Section 2. All samples were normalized to equal total lipid. Spectra were obtained by excitation at 450 nm (a–d) or 650 nm (e,f).

solutions, such as those used for observation of the dithionite effect directly in the cuvettes, where almost exactly 50% reduction of this probe could be obtained on a short time scale (see Fig. 5). The lower level of apparent reduction of the acceptor probe in the stock preparations was considered acceptable as it would be sufficient to eliminate most of the contribution of outer monolayer exchange (80–90%).

3.7. Translocation of diI(5)C18ds and comparison to N-NBD-PS in fusogenic liposomes

A limited characterization of the translocation properties of diI(5)C18ds was also performed. We initially tested the probe diI(5)C18 (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate), a version that does not contain the sulfonate groups. Its membrane translocation was very rapid (data not shown). Because of the two sulfonate groups appended to diI(5)C18ds, its rate of translocation across the membrane is apparently relatively slow on the time scale of the data in Fig. 5. In preparation for the use of this probe as an acceptor for *N*-NBD-PS in fusion assays, its translocation characteristics were also tested and compared to *N*-NBD-PS in liposomes containing PS and tPE. The rate of randomization of the two probes is shown in Table 2. In addition experiments similar to those in Table 1 have also shown a lack of translocation of diI(5)C18ds induced by fusion of these liposomes (data not shown). It is clear that in this particular system, these two probes are suitable for short term (< hours) fusion assays in buffer without significant translocation from the inner monolayer.

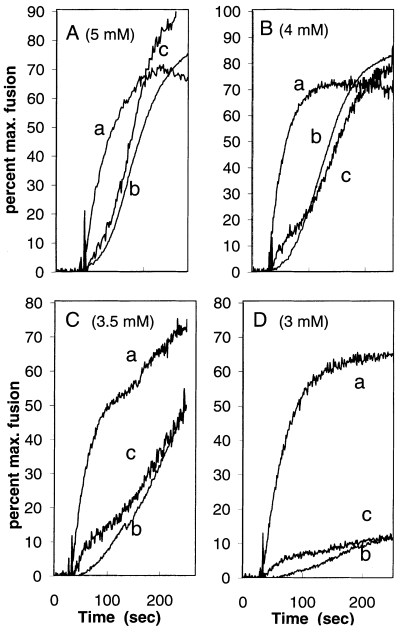

Fig. 8. Calcium-dependent fusion of PS/tPE liposomes as monitored by several fluorescent assays. The percent maximal fusion, as defined in Section 2, was monitored versus time using the standard lipid mixing assay (a), the Tb/DPA aqueous contents mixing assay (b) or the inner monolayer fusion assay (c). Liposomes were composed of PS/tPE (1/3) and were at a total lipid concentration of 100 μM in all cases. A concentrated stock solution was added to a stirred cuvette at 30 s (arrow) giving a final Ca^{2+} concentration of 5 (panel A), 4 (panel B), 3.5 (panel C) or 3 mM (panel D).

3.8. Comparison of fusion assays

The favorable characteristics of *N*-NBD-PS and diI(5)C18ds in the PS/tPE system allowed us to study the inner monolayer labeled liposomes for comparison with other fusion assays. In particular we were interested in whether liposomes labeled in such a

Table 2
Translocation of probes for inner monolayer fusion assays in PS/tPE liposomes

Probe	Time of incubation (h)	Temperature (°C)	% Randomization[a]
N-NBD-PS	3	25	−0.288 ± 0.70
N-NBD-PS	3	37	7.02 ± 0.67
DiI(5)C18ds	1	25	2.4 ± 2.3
DiI(5)C18ds	1	37	4.6
DiI(5)C18ds	3	25	4.4
DiI(5)C18ds	3	37	6.9 ± 0.7

[a]Samples were incubated for the indicated amount of time at the indicated temperature after inner monolayer labeling as described in Section 2. Aliquots were removed and tested for probe exposure by addition of dithionite, and the percent randomization was calculated from the data as described in Section 2.

manner would show fusion kinetics similar to aqueous mixing assays. In Fig. 8 is shown a comparison of the Ca^{2+}-dependent fusion of PS/tPE liposomes as monitored by several assays at several Ca^{2+} concentrations. The rate of fluorescence increase is obviously much faster when both monolayers are labeled as opposed to the inner monolayer. In these particular cases the inner monolayer fusion assay reflects relatively closely the rate of fluorescence increase of the aqueous mixing assay, though somewhat faster. Of particular note is the fact that in each case outer monolayer mixing precedes actual fusion. These data suggested that it may be possible to generate conditions in which the outer monolayer exchange occurs in the absence of fusion, so that its characteristics and the various factors that can subsequently trigger inner monolayer mixing could be studied.

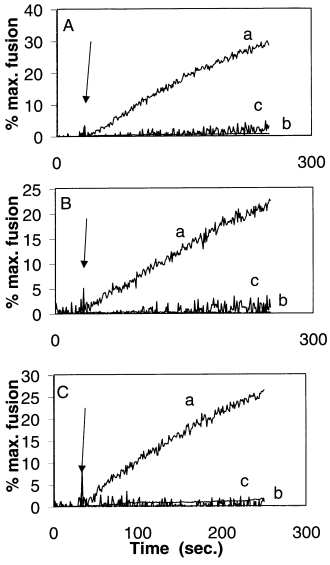

Fig. 9. Cation-dependent outer monolayer exchange at cation concentrations below the threshold for aqueous mixing. Liposomes and conditions were the same as in Fig. 8. At 30 s, stock solutions were added to stirred cuvettes to give 2 mM Ca^{2+} (A), 3 mM Mg^{2+} (B) or 420 μM spermine (C).

3.9. Lipid exchange in transient aggregates at polycation concentrations subthreshold for fusion

We next tested whether mixing of outer and inner monolayers could be essentially uncoupled on a long time scale at a cation concentration subthreshold for aqueous contents mixing. In Fig. 9A it can be seen that 2 mM Ca^{2+} is insufficient to induce any inner monolayer or contents mixing on the time scale of 5 min, but a substantial mixing of the outer monolayer can be observed. It was of further interest to determine whether this phenomenon was unique to an ion like Ca^{2+}, which can induce extensive contents mixing at slightly higher concentrations. Other cations, i.e. Mg^{2+} and the tetravalent cation, spermine, are known to interact very differently with PS, particularly in terms of dehydration of headgroups, and only weakly induce contents mixing for this PS/PE composition [30,31]. We compared the subthreshold concentration of Ca^{2+} to these ions. All three cations, at the chosen concentrations, displayed a similar significant rate of outer monolayer exchange, while no inner monolayer or aqueous contents mixing was observed (Fig. 9), indicating that this behavior is not dependent on ions that can strongly induce inner monolayer mixing.

In each case a slow but significant increase in turbidity and changes in 90° light scattering were also observed indicating formation of vesicle aggregates (not shown). These aggregates were completely reversible by addition of EDTA in each case, resulting in

Table 3
Size of PS/tPE liposomes before and after aggregation in Mg^{2+}

Sample[a]	Number weighted diameter ± S.D.	Intensity weighted diameter ± S.D.	Volume weighted diameter ± S.D.
Original ($n=6$)	85.6 ± 8.5	111.2 ± 1.0	110.4 ± 1.2
After 10 min in 3 mM Mg^{2+}, then 5 mM EDTA ($n=7$)	82.6 ± 9.2	112.5 ± 0.9	112.0 ± 1.0

[a]PS/tPE liposomes (100 μM total phospholipid) were mixed with 3 mM Mg^{2+} in a stirred cuvette. After aggregation (increased turbidity) for 600 s, a final concentration of 5 mM EDTA was added to the cuvette, reversing the turbidity. Nicomp dynamic light scattering measurements were made on the original samples and after addition of EDTA to estimate size.

vesicles indistinguishable from the original vesicles as measured by dynamic light scattering analysis (Table 3). Therefore, it appears that transient aggregates form which mix outer monolayer lipids, independent of the fusogenic characteristics of the particular cation used, but the average size of the vesicles does not grow under these conditions. One possibility is that these aggregates represent reversible hemifusion, an intermediate that may lead to fusion at higher cation concentrations. This would be consistent with the observation of outer monolayer exchange that precedes fusion at the higher cation concentrations.

We next tested the effect of addition of a lysophospholipid to the exterior of these vesicles, a lipid known to inhibit fusion in several systems. Fluorescent lipid mixing assays were performed with the same lipid composition at the same concentrations. When 2 mM Ca^{2+}, below the fusion threshold (see Fig. 9), was used to induced lipid mixing, 2 mole% lyso-PC added to the preformed vesicles was sufficient to completely inhibit all lipid probe mixing. At 3 mM Ca^{2+}, where both initial outer monolayer mixing and subsequent inner monolayer mixing occur (see Fig. 8), 2 mole% lyso-PC also inhibited all lipid mixing. In other words, when the observed outer monolayer mixing was eliminated, all true fusion was also inhibited. Interestingly, lyso-PC also inhibited the Ca^{2+}-dependent turbidity increase, suggesting that vesicle aggregation under these conditions may be supported by the same structural intermediates that mediate outer monolayer exchange. These data strongly suggest that outer monolayer mixing could be a precursor to true fusion in polyvalent cation-dependent events, suggesting a common mechanism with PEG and viral-mediated fusion. In this respect, the subthreshold polyvalent cation systems should be very useful for the study of the relationship between inner and outer monolayer mixing and the factors that trigger the mixing of the inner monolayers to complete the fusion process.

4. Discussion

The data presented have characterized an approach to a new type of assay for membrane fusion of vesicles in which only the mixing of the inner monolayer lipids of the fusing membranes is monitored. This method of monitoring membrane fusion provides many advantages over previous ones. Aqueous mixing assays have been considered the most stringent assays for membrane fusion, since full fusion of membranes requires eventual mixing of aqueous contents. However, these assays suffer from several major disadvantages. First, fluorescent aqueous mixing assays generally require loading high concentrations of quenchers or energy transfer acceptors into at least one of the fusing populations – a difficult task in many cases. Such assays generally require encapsulating material into each of the types of fusing vesicles. If one of the membrane vesicles is biologically derived (e.g. a whole cell or an organelle), it may be difficult to sufficiently load. Observation of dilution of the contents of a loaded liposome into an empty cellular membrane vesicle is not a solution to this problem, as it is very difficult to distinguish fusion-related dilution from leakage. Secondly, membrane fusion processes are occasionally leaky to encapsulated molecules. In the extreme case, rapid leakage of encapsulated material would make it impossible to monitor fusion.

Assays that rely on the dilution of lipid probes, from one population of vesicles into an unlabeled population, obviate some of these problems, but create new ones. One problem is that it is very difficult to prove that lipid dilution has occurred as a result of fusion rather than lipid transfer under any particular conditions. The only recourse is to verify with an aqueous mixing assay. Secondly, the exposure of lipid probes on the outer monolayer of the fusing vesicles allows them to interact with any number of components of the extravesicular milieu that may modify the probe fluorescence, even when there is no lipid mixing at all.

By contrast, inner monolayer labeling gives a probe that is protected from the extravesicular environment. Because of its location, it cannot undergo facile exchange into a second membrane without first undergoing translocation to the outer leaflet. Furthermore, because the probe is embedded in a membrane, it cannot leak from the vesicle of interest. Since inner monolayer fusion assays are lipid dilution assays, they can be performed by labeling only one population of vesicles, thus allowing the study of fusion of liposomes with unlabeled cellular membranes (e.g. [14]).

4.1. Probe translocation

Maintaining the interior location of the probe is an important aspect of inner monolayer assays, especially in cases where fusion is relatively slow. Assays of slow fusion of liposomes with cellular membranes are particularly relevant for liposomal drug delivery [32]. The data elaborated here [16] indicate that two new probes *N*-NBD-*N*,*N*-dimethyl-PE and *N*-NBD-PS have bilayer translocation characteristics that make them preferable for use in such assays by maintaining an interior localization after reduction of the outer monolayer. The relative rates of translocation of the *N*-NBD headgroup probes follow the relative rates for similar NBD-free phospholipids. The larger and potentially more hydrated headgroups undergo much slower translocation. The relatively hydrophobic NBD moiety probably does not contribute significantly to the rate-limiting step of translocation. While the *N*-NBD-PE probe has an overall net negative charge its translocation rate constant is still a factor of 5 higher than the zwitterionic PC-like *N*-NBD-*N*,*N*-dimethyl-PE. This is consistent with previous work showing that dimyristoyl-PG and PA translocation rate constants are 10-fold and 5-fold higher than dimyristoyl-PC, respectively [26]. The doubly charged *N*-NBD-PS was approximately 100-fold slower to translocate across the POPC membrane than *N*-NBD-PE. A similar analysis [27] subsequent to the data elaborated here [16] also qualitatively demonstrated this fact. *N*-NBD-PS has a relatively much slower rate of translocation than any naturally occurring phospholipid [26]. Because the *N*-NBD-PS probe undergoes almost no spontaneous translocation in the absence of significant bilayer disruption, it can provide an ideal probe to study translocation under bilayer disrupting conditions.

N-NBD-PS would probably also serve as a superior probe for other purposes. For instance the number of lamellae in a liposome preparation can be measured using the dithionite reduction of NBD probes [33]. Because of its tendency to remain in its original monolayer, it provides an ideal probe for measuring number of lamellae. We have found that it compares well with other assays for number of lamellae (data not shown).

Stably inner monolayer labeled vesicles will also provide opportunities for study of inner and outer monolayer coupling (e.g. lateral phase separation on each monolayer) and defining the interactions of external membrane binding proteins with the inner monolayer by effecting an environmental change for the probe or through tryptophan-NBD energy transfer.

4.2. Fusion assays

The main focus of this study has been the use of stable inner monolayer labeling for fusion assays. Such assays can also prove very useful for assessing the fusogenicity of liposome systems for the intracellular delivery of drugs or genes. In addition, specifically monitoring the behavior of the inner monolayer lipids can give important insights into the mechanism of fusion.

A number of other recent assays have improved on the NBD/rhodamine lipid dilution assay. For instance, new probes were synthesized that were not as sensitive as NBD to binding of ions to the exterior of the vesicle [34]. A pyrene probe was also introduced as an improvement on the basis of its localization in the hydrophobic interior of the membrane [35,36]. Similarly, probes based on a diphenylhexatriene chain derivative and measured by lifetimes obviate some of the potential problems [37]. While these probes decrease the incidence of artifacts they do not eliminate the possibility of fluorescence changes as a result of indirect interactions, such as lateral phase separation. For instance, binding of annexins to vesicles containing pyrene probes causes a substantial change in fluorescence in the absence of fusion [38,39]. Furthermore, localization on the outer monolayer allows mixing to occur without complete fusion. Therefore it is preferable to maintain protection of the probe on the interior monolayer of the vesicle of interest.

Dithionite-based reduction of NBD for inner monolayer labeling, originally proposed [14] as a modification of the *N*-NBD-PE/Rh-PE fusion assay [5], has also been used to develop a fluorescence lifetime-based assay using *N*-NBD-PS alone [40]. A key and unique aspect of our previously reported assay [17], elaborated here, is the discovery of an apparently reducible energy transfer acceptor for NBD [17] that allows fluorescent fusion assays without the

equipment for lifetime measurements. This method would allow continuous real-time assays, potentially on very short time scales. In addition, the magnitude of the changes observed in the resonance energy transfer assays tend to be greater than those observed in lifetime measurements, potentially enhancing the signal-to-noise ratio. For the lifetime-based assays, essentially self-quenching and potentially perturbing concentrations of *N*-NBD-PS were required for relatively small lifetime changes, e.g. 10 mole% probe gives a 50% increase in lifetime upon 10-fold dilution [40]. The assays reported here can be preformed with much lower probe concentrations, similar to the original resonance energy transfer fusion assays [5].

4.3. Monolayer mixing during fusion

The data presented here indicate the potential usefulness of steady state fluorescent inner monolayer fusion assays for the study of the fusion process itself. Previous work has demonstrated the importance of lipid structure for the initiation of outer monolayer coalescence or hemifusion [41]. The kinetically separate nature of hemifusion and fusion pore formation has been previously established for viral protein-mediated fusion systems [42,43], polymer-induced fusion [40] and fusion with planar bilayer membranes [41]. We show here for the first time that polyvalent cation-induced fusion of small vesicles has similar characteristics, at least for the particular lipid system studied. Specifically, the data suggest that an intermediate state may exist in which vesicles make contact and outer monolayer mixing occurs, but no inner monolayer mixing or aqueous contents mixing occurs.

The comparison between various cations is particularly interesting. Though a detailed analysis of the actual amount of ion bound versus rate of inner and outer monolayer mixing still must be performed, some general hypotheses can be advanced from these data. Spermine [31] and Mg^{2+} [30] are clearly very weak inducers of contents mixing, though fusogenic fatty acids can greatly enhance the rate [31]. Nonetheless, we have still observed strong outer monolayer mixing with these cations. This emphasizes the distinction between the mechanisms involved in the merging of outer monolayers and inner monolayers. How does external Ca^{2+} so strongly affect inner monolayer mixing? One possibility is that the well-known [44,45] dehydration of the outer surface of the PS containing membrane by Ca^{2+} may play an important role in the coalescence of inner monolayers. Lipids with dehydrated headgroups may be able to pack into a hemifusion septum with a smaller outer radius of curvature [46], allowing closer proximity between inner monolayers and/or a greater number of contacts in an intermediate hemifused state. This may allow inner monolayer coalescence to occur more easily. Certain free fatty acids [31,47] may also stabilize higher curvature in mediating closer contact between hemifused bilayers, even with a poorly dehydrating cation such as spermine or Mg^{2+}, thus enhancing contents mixing [31].

Inhibition of the formation of this intermediate state by lyso-PC also strongly inhibits inner monolayer mixing and aqueous contents mixing. Furthermore, the intermediate state always appears to precede fusion pore formation as outer monolayer mixing is always observed before aqueous mixing. These data suggest the hypothesis that this intermediate state may play a crucial role in many types of membrane fusion. It should be possible to use the subthreshold conditions for inner monolayer mixing to study the role of various factors that may affect the rate of this process. The outer monolayer mixing can be studied in isolation at these subthreshold conditions for possible kinetic analyses to determine whether intervesicular outer monolayer mixing is an all-or-none process or occurs incrementally. A particularly interesting experiment would be to test the effect of arachidonic acid [31]. Because it promotes hemifusion and inhibits fusion pore formation [41,48], it may further uncouple outer and inner monolayer mixing in this system. On the other hand, incorporating increasing amounts of lyso-PC may lead to a composition where inner and outer monolayer mixing occur at the same rates.

The ability to obtain a reliable fusion assay with labeling of only one of the interacting membranes is particularly important. This enhances the possibility of studying membrane fusion of model labeled liposomes with biologically derived membranes keeping in mind appropriate caveats [49]. This was demonstrated in the case of the fusion of inner monolayer labeled PS/tPE liposomes with human neutrophil-de-

rived plasma membranes and secretory granules [14,50]. It may also be useful for the study of the fusion of liposomes with whole cells as a mechanism of cytoplasmic drug delivery.

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