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New fluorescent lysolipids: Preparation and selective labeling of inner liposome leaflet

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Abstract

Two new fluorescent lysophosphatidylcholine probes have been synthesized for use as a donor–acceptor pair in fluorescence resonance energy transfer (FRET): 9-anthrylvinyl (LAPC) as donor and 3-perylenoyl (LPPC) as acceptor. The partition coefficients between membrane and aqueous phases were 8.3×10^5 and 10.5×10^5 for LAPC and LPPC, respectively. The inner leaflets of unilamellar lipid vesicles were labeled with these probes to assess conservation of membrane sidedness after membrane fusion. After medium-sized unilamellar vesicles (MUV) were prepared with a probe in both leaflets, probe in the outer leaflet was removed by repeatedly washing with an excess of unlabeled giant unilamellar vesicles (GUV). MUV and GUV were separated by centrifugation. The probes did not flip-flop across bilayers at 25°C for at least 12 h. MUV containing the ganglioside G_{T1b} were labeled with the LAPC/LPPC pair in the inner leaflet and incubated for 30 min at neutral pH with influenza virus. Fusion was triggered by acidification to pH 5.0 and was monitored by an increase in donor fluorescence in a FRET assay. When the inner leaflets of MUV were labeled by LAPC only, its fluorescence did not change after fusion. However, the fluorescence decreased by 60% when the LAPC was removed from

Abbreviations: APC, 1-acyl-2-[12-(9-anthryl)-11-*trans*-dodecanoyl]-*sn*-glycero-3-phosphocholine; EPC, egg yolk phosphatidylcholine; FRET, fluorescence resonance energy transfer; GUV, giant unilamellar vesicles; HA, hemagglutinine; LAPC, 2-[12-(9-anthryl)-11-*trans*-dodecenoyl]-*sn*-glycero-3-phosphocholine; LMV, large multilamellar vesicles; LPPC, 2-[9-(3-perylenoyl) nonanoyl]-*sn*-glycero-3-phosphocholine; MUV, medium-sized unilamellar vesicles; PPC, 1-acyl-2-[9-(3-perylenoyl)nonanoyl]-*sn*-glycero-3-phosphocholine; SDS, sodium dodecyl sulfate

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the outer leaflets of the fused membranes by repeated washings with GUV. We conclude that the lipids of the inner and outer leaflets of the fused MUV/virus complexes intermixed. © 1997 Elsevier Science B.V.

Keywords: Fluorescent probe; Lipid mixing; Membrane asymmetry; Membrane fusion; Resonance energy transfer

1. Introduction

The various types of phospholipids contained by cell membranes are distributed asymmetrically between inner and outer leaflets [1]. When membranes fuse, the lipid asymmetry should be maintained. However, when leakage of aqueous contents accompanies membrane fusion, the membrane organization can be disturbed [2]. For example, the asymmetrical distribution of phosphatidylserine in erythrocyte membrane is lost when fusion is induced by osmotic swelling or by electric pulses [3]. The fusion of virus with host membranes has been shown to increase membrane permeability in a variety of systems [4–6], and molecules encapsulated in liposomes leak when the liposomes fuse with influenza virus [7]. It is therefore not clear that lipid asymmetries would be maintained after fusion in all systems.

Membrane fusion is often studied by following the redistribution of fluorescent lipid incorporated into both leaflets or only into outer leaflets. In this study, we have labeled liposomes with fluorescent lipids in the inner leaflet only and followed their movement as a result of fusion. For this purpose, we synthesized two new lysophosphatidylcholines labeled with fluorescent residues, anthrylvinyl or 3-perylenoyl, at the end of the acyl chain. These lysolipids form a pair of probes suitable for fluorescence resonance energy transfer (FRET) measurements. Utilizing the ability of lysolipids to spontaneously transfer between membranes through an aqueous phase [8,9] we removed a probe from the outer leaflets by incubating uniformly labeled giant unilamellar vesicles (GUV) with unlabeled medium-size unilamellar vesicles (MUV). Following their separation, GUV were recovered with only their inner leaflets labeled. Alternatively, MUV were labeled with probes and the label from the outer leaflet was removed by washing with unlabeled GUV. Asymmetrically-labeled vesicles were used to determine whether or not the lipid asymmetry was maintained after fusion of vesicles with influenza virus. A report of some aspects of this paper has been published in Russian [34].

2. Materials and methods

2.1. Materials

Lipids, cholesterol, ganglioside G_{t1b} and detergents were obtained from Sigma, bovine serum albumin from Calbiochem, Tris-HCl and lipase from *Rhizopus delamar* (activity 20 units/mg) from Serva (Germany); silica gel 60 (40–63 μm) and precoated silica gel TLC plates (Kieselgel 60, aluminium sheets) were from Merck (Germany).

2.2. Spectral measurements

Fluorescence measurements (using an Hitachi F-4000 fluorimeter) were made with continuous stirring at 37°C. Anthrylvinyl-labeled probes were excited at 370 nm and the emission was recorded at 430 nm with a 250–400 nm filter (Corning 9863) placed between the cuvette and excitation monochromator to eliminate any stray light passed by the excitation monochromator. The excitation and emission for the perylenoyl-labeled probes was 450 and 515 nm. UV spectra were recorded with a LKB Ultrospec II spectrophotometer.

2.3. Synthesis of probes

Fluorescent probes 1-acyl-2-[12-(9-anthryl)-*trans*-11-dodecanoyl]-*sn*-glycero-3-phospho-choline (APC) and 1-acyl-2-[9-(3-perylenoyl)nonanoyl]-*sn*-glycero-3-phosphocholine (PPC) were synthesized as described earlier [10,11]. Enzymatic hydrolysis of the phosphatidylcholine probes APC and PPC was performed in accordance with the method of Slotboom et al. [12].

2.4. 2-[12-(9-Anthryl)-trans-11-dodecanoyl]-sn-glycero-3-phosphocholine (LAPC)

APC (10 mg, 11.4 mmol) and sodium deoxycholate (3 mg) were dispersed in 2 ml of 0.1 M

borate buffer supplemented with 5 mM $CaCl_2$, pH 6.5. Delipidated bovine serum albumin (4.5 mg) was added to bind free fatty acid, followed by addition of lipase (6 mg, 120 unit) dissolved in 0.3 ml of borate buffer. The mixture was incubated with vortexing at 30°C for 6 h and then evaporated under vacuum. The residue was applied on a silica gel column (0.5×20 cm), eluted with chloroform-methanol-water mixtures, 65:25:1 followed by 65:25:4. The eluates were checked by TLC in a chloroform-methanol-water (65:25:4) system and visualized with molybdenum blue and independently under UV illumination. LAPC (amount, 2 mg; yield, 29%) was obtained as a yellowish gum; its UV (λ_{max} 257, 350, 367 and 387 nm, in ethanol) and fluorescence (λ_{max} 412 and 432 nm, in ethanol) spectra were the same as that of APC [10].

2.5. 2-[9-(3-Perylenoyl)nonanoyl]-sn-glycero-3-phosphocholine (LPPC)

PPC (10 mg, 10.6 mmol) and 20 mg of sodium taurodeoxycholate were dispersed in 2 ml of 0.1 M borate buffer with 5 mM $CaCl_2$, pH 6.5. Then 9 mg of bovine serum albumin and lipase (30 mg, 600 units) dissolved in 1 ml of borate buffer were added and the mixture incubated for 24 h at 35°C, with vortexing. After purification (see Section 2.4) LPPC (amount, 6 mg; yield, 86%) was obtained as a red gum. Its UV (λ_{max} 260 and 447 nm, in ethanol) and fluorescence (λ_{max} 525 nm, in ethanol) spectra were the same as those of PPC [11]. Both probes, LAPC and LPPC, migrated nearly identically on TLC with lysophosphatidylcholine prepared by the action of phosphalipase A_2 on egg yolk phosphatidylcholine (EPC).

2.6. Vesicle preparation

Medium-size unilamellar vesicles (MUV) were prepared by sonicating 1 mg/ml of dried lipids and (including the ganglioside G_{T1b}) in Tris-buffered saline (10 mM Tris-HCl in 0.15 M NaCl, pH 7.4) with a titanium tip (ultrasonicator UZDN-100, Russia, 100 W, 22 kHz) for three 2 min periods. The sonication was performed under an argon atmosphere in a tube immersed in a water bath at room temperature. The MUV were centrifuged at $12\,000 \times g$ for 10 min to remove titanium dust and large aggregates. The size of the vesicles was determined by recording the 90° scattering of the 632.8 nm line of a helium-neon laser of a Coulter N4MD laser analyzer. The mean diameter of 95% of the vesicles was within 100–200 nm.

Very giant unilamellar vesicles (GUV) were prepared by modifying [13] the procedure of Kim and Martin [14] using 8 mg of egg PC and 2 mg of cholesterol. Two vials were made, one marked 'inside' and the other 'outside,' each containing 5 mg aliquots of lipids in a chloroform stock solution at 1 mg/ml. Both aliquots were evaporated under vacuum. To the 'inside' vial, chloroform was added to bring the volume to 0.5 ml. A water-in-chloroform emulsion was made in this vial by carefully adding, dropwise, 0.5 ml of Tris-buffered saline followed by vortexing at a moderate rate for 30 s. The lipid mixture in the 'outside' vial was dried under vacuum and resuspended in 0.5 ml of fresh distilled diethyl ether. Then 2.5 ml of Tris-buffered saline supplemented with 0.7 M sucrose was added to this vial and vortexed for 30 s. The emulsion of the 'inside' vial was injected into the 'outside' one. This double emulsion was vortexed for 30 s at moderate setting and then transferred to a round-bottom flask. The organic solvent was removed by rotary evaporation. The vesicles were transferred to a 1.5 ml conical tube and pelleted by centrifugation at $10\,000 \times g$ for 5 min. After the supernatant was aspirated, Tris-buffered saline (without sucrose) was added. The washing procedure was repeated twice. Finally, the liposomes were resuspended in Tris-buffered saline (without sucrose) to a concentration 10 mg/ml and stored at 4°C no more than 24 h.

Large multilamellar vesicles (LMV) were prepared by adding Tris-buffered saline to dried EPC. After vortexing the emulsion for 20 s, the liposomes was washed three times with Tris-buffered saline by centrifugation.

2.7. Determination of partition coefficients of fluorescent lysolipids

Partition coefficients (K_p) of the fluorescent probes LAPC and LPPC between aqueous and lipid phases were determined by titrating the number of liposomes against a constant concentration of LAPC and LPPC [15]. An aliqout of a 0.1 mM probe stock solution in

ethanol was diluted with Tris-buffered saline to 5 or 10 μM in the fluorimeter cuvette and aliquots of MUV made from EPC (0.1 mg/ml) were then added. After a 10 min incubation, the fluorescence was measured at 447 nm for LAPC (λ_{ex} 370 nm) and at 510 nm for LPPC (λ_{ex} 450 nm). The fluorescence intensity F was defined as:

$$F = F_0\left[L/\left(55.6/K_p + L\right)\right]$$

where F_0 is the maximal fluorescence resulting from total probe incorporation into membranes, L is the phospholipid molar concentration and 55.6 M is the molar concentration of water. (The probes aggregate in aqueous solution and self-quench.) The double reciprocal plot ($1/F$ versus $1/L$) of fluorescence and lipid concentration yields the linear curve:

$$1/F = \left[55.6/\left(K_p F_0\right)\right](1/L) + 1/F_0$$

K_p was calculated as -55.6($1/L$ intercept).

2.8. Determination of CMC

Critical micelle concentrations (CMC) were measured for LAPC and LPPC by methods previously employed [9]. After drying the probes under vacuum, 10 ml and 3 ml of 150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 were added to 100 nmol of LPPC and 300 nmol of LAPC, respectively. The solutions were sonicated and fluorescence intensities were measured for each probe in the range of 10 nM to 10 μM at 37°C. At low concentrations, for both probes the fluorescence increased linearly with concentration. When micelles formed, fluorescence no longer increased linearly due to self-quenching. The CMC was taken as the concentration when fluorescence ceased to increase linearly.

2.9. Asymmetrically labeled vesicles

Fluorescent MUV (probe/lipid molar ratio, 1:250) and unlabeled GUV were mixed in the ratio 1:10 (w/w) at a total lipid concentration of 1.1 mg/ml and incubated for 40 min at 37°C. MUV and GUV were separated by centrifugation (5 min, 12 000 $\times g$). The supernatant containing the fluorescent MUV was removed from the centrifuge tube, taking care not to disturb the pellet consisting of GUV. The fluorescence of the MUV were measured and again washed with GUV in the same manner. The washings were repeated 5–6 times to remove fluorescent lysolipids from the outer leaflets and produce MUV with probe in the inner leaflet only.

GUV with fluorescent lysolipids in the inner leaflets only were prepared by incubating labeled GUV with unlabeled MUV (1:10, w/w) and separating by centrifugation. The GUV were washed at least 5 times with fresh MUV, gradually increasing the centrifuge speed from 5000 $\times g$ at the first washing up to 10 000 $\times g$ at the 5th one. This assured a sufficient quantity of GUV for experiments, with losses of GUV in the high-speed supernatant of less than 3%.

2.10. Fusion measurements

Fusion between virus and labeled liposomes was measured by FRET [16] using anthrylvinyl as donor and perylenoyl as acceptor (APC/PPC or LAPC/LPPC). Twice as much lysoprobes than PC probes, on a molar basis, were used to label MUV. After lysoprobes were removed from outer monolayers, equal amounts of lyso- and non-lysoprobes remained associated with MUV (i.e. 0.2 mol% of both donor and acceptor was incorporated into liposome membrane). Influenza virus A/Duck/Czech/56 (H4N6) was kindly provided by Professor N.V. Kaverin from the Ivanovsky Institute of Virology (Moscow).

The fluorescence scale was calibrated by setting the initial residual fluorescence of the liposomes to 0% and the fluorescence at infinite probe dilution to 100%, obtained by adding 1 mol% of SDS. The value for the initial fluorescence was corrected for PPC or LPPC fluorescence at 447 nm.

Leakage of liposomal contents associated with fusion was determined by a calcein-Co^{2+} assay [17]. MUV were loaded with a buffer solution that contained 0.8 mM calcein with 1.0 mM $CoCl_2$ to quench the fluorescence of the calcein. External dye and quencher were removed by passing the liposomes through a Sephadex-50 column and the liposome/virus suspension was incubated for 30 min at 4°C in a buffer containing 20 mM EDTA prior to triggering fusion. When liposomes leaked contents,

the diluted calcein-Co^{2+} complex dissociated, the fluorescence of the calcein increased and Co^{2+} complexed with EDTA.

3. Results and discussion

3.1. Lysolipid synthesis

The synthetic lysophosphatidylcholines LAPC and LPPC were prepared by enzymatic hydrolysis of previously synthesized phosphatidylcholine probes APC and PPC [10,11,18] as outlined in Fig. 1. The microbial lipase splits off only esters of the primary hydroxy groups and thus removes the unlabeled fatty acid residue from the *sn*-1 position of a phosphatidylcholine. This method is suitable for lipase hydrolysis of phospholipids as has been elaborated [12]. APC was solubilized with sodium deoxycholate to promote hydrolysis. The deoxycholate did not appear to solubilize PPC; excess sodium taurodeoxycholate was used for this purpose.

The resulting lysoprobes LAPC and LPPC have the labeled fatty acid in the *sn*-2 position of the glycerol backbone. In natural lysophosphatidylcholine, produced from phosphatidylcholine by hydrolysis with phospholipase A_2, the fatty acid is in the *sn*-1 position. We consider this difference of position to be insignificant for our physical studies: both acyl chains of phosphatidylcholine *sn*-1 and *sn*-2 behave similarly in membranes, differing only in their depth of immersion into the bilayer [19]. The similar physical properties of our fluorescent 1-lysolipids and natural 2-lysolphosphatidylcholine (number, by convention, is position of hydrolysis) is underlined by the nearly identical chromatographic mobility of LAPC, LPPC and natural lyso-EPC.

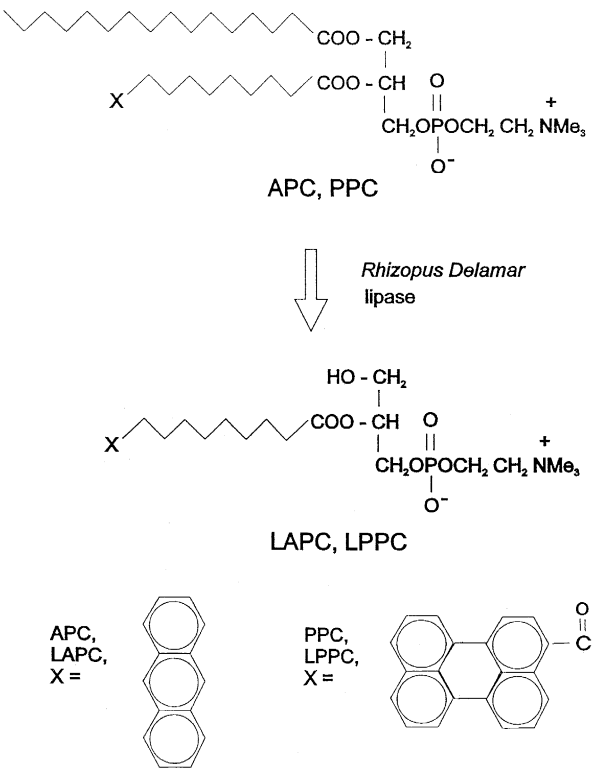

Fig. 1. Preparation of LAPC and LPPC.

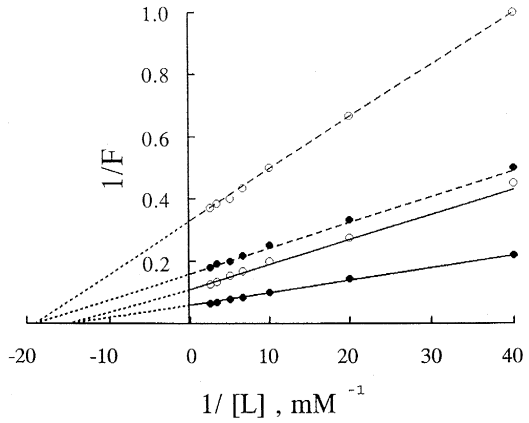

Fig. 2. EPC-liposome titration against 5 (○) and 10 (●) μM of LAPC (solid line) and LPPC (dotted line), double reciprocal plots of fluorescence versus lipid concentration. Water phase: 10 mM Tris-HCl in 0.15 M NaCl, pH 7.4; temperature 37°C.

3.2. CMCs and partition coefficients of LAPC and LPPC

When in solution, the fluorescence of LAPC and LPPC increased linearly with concentrations in the range of 10 nM to 1 μM and 10 to 200 nM, respectively. Above 1 μM for LAPC and above 200 nM for LPPC, fluorescence of probes began to saturate with increasing concentration. We thus conclude that the CMCs are 1 μM for LAPC and 200 nM for LPPC.

When the EPC-liposome concentration was increased against 5 and 10 μM fluorescent lysolipids dispersed in Tris-buffered saline, the fluorescence increased in a saturating manner. The corresponding double reciprocal plots (Fig. 2) show good linearity and the intercepts of the $1/L$ axis, -15 mM^{-1} for

LAPC (solid line) and $-19\ mM^{-1}$ for LPPC (dashed line), are the same for 5 (open circles) and 10 μM (filled circles) of probe. The partition coefficients, K_p, of LAPC and LPPC are thus $8.3 \cdot 10^5$ and $10.5 \cdot 10^5$, respectively. The fraction of probe associated with the membrane depends on the amount of lipid, $[L]$, not the probe concentration provided $[L] \gg$ [probe]. The values of K_p were used to determine lipid concentrations which minimize the amount of free probe within the aqueous phase. The lipid concentration for EPC liposomes needed for one-half the probe to incorporate within the membranes is $55.6/K_p$: 67 mM for LAPC and 53 mM for LPPC.

3.3. LAPC and LPPC transfer between vesicles

We incubated MUV containing fluorescent lysolipids with an excess of unlabeled GUV for varied times. After indicated times we separated MUV and GUV by centrifugation and the fluorescence of the supernatant (MUV) was measured. This allowed us to observe the intervesicular transfer of LAPC (Fig. 3, filled circles) and LPPC (open circles). The transfer had similar time courses, $\tau_{1/2} = 5$ min, for the two fluorescent lysolipids at 37°C. Phospholipid transfer through the aqueous phase has been extensively studied [20]. The exchange of lysopalmitoylphosphatidylcholine between phospholipid vesicles is fast, with $\tau_{1/2} < 2$ min at room temperature [21]. Our probes may transfer more slowly because they have larger acyl chains (26 carbons for LAPC and 30 carbons for LPPC) or because of differences in vesicle compositions [22,24].

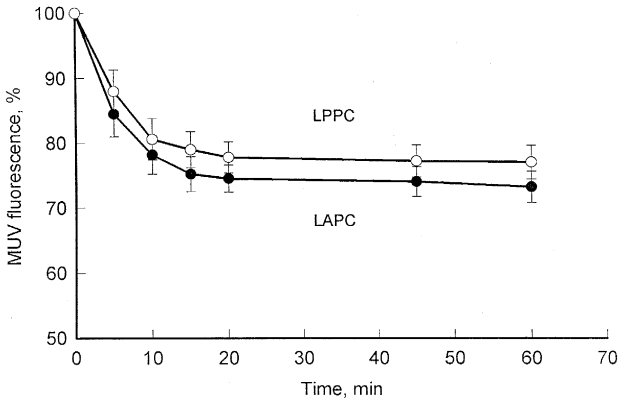

Fig. 3. The time course of fluorescent lysolipid transfer between vesicles. MUV labeled with 0.4 mol% LAPC (●) or LPPC (○) were incubated with unlabeled GUV composed of EPC (1:10, w/w) at 37°C.

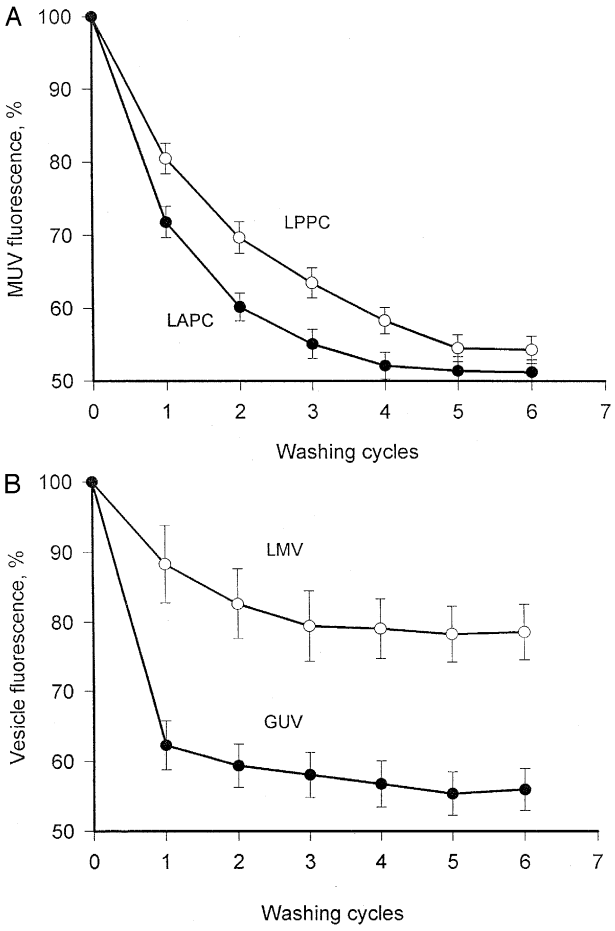

Fig. 4. (A) Washing out of lysoprobe from the outer leaflets of MUV labeled with 0.4 mol% of LAPC (●) or 0.4 mol% of LPPC (○) by incubation with unlabeled GUV. (B) Washing out of lysoprobe LAPC from outer leaflets of GUV (●) and LMV (○) labeled with 0.4 mol% of LAPC by incubation with unlabeled MUV.

The rapid transfer kinetics of fluorescent lysolipids between vesicles and the slow translocation of phospholipids from one leaflet of a bilayer membrane to the other (flip-flop) [24] allows removal of all the outer monolayer lysolipid. The method of removing labeled lipid from outer vesicle monolayers with excess unlabeled vesicles was first used by Pagano et al. [16] with NBD-labeled phosphatidylcholine. Apparently, the somewhat higher polarity of the N-NBD residue caused them to transfer between vesicles faster than the unlabeled lipids. In contrast, anthrylvinyl and perylenoyl fluorophores are quite apo-

lar; the rapid interbilayer exchange is caused by lysolipids having only a single acyl chain. The washing cycle (incubation of labeled and unlabeled vesicles and separation of MUV and GUV by centrifugation) had to be repeated at least 5–6 times to assure that nearly all the fluorescent lysolipid present in the external leaflet was extracted (Fig. 4A).

We established that only lysolipids located within the outer monolayers exchanged between vesicle populations. GUV and LMVs uniformly labeled with LAPC were incubated with unlabeled MUV (Fig. 4B). For GUV, 55–60% of the fluorescent lysolipid remained associated with the initially labeled vesicles. In contrast, for multilamellar vesicles (LMV), about 80% of the lysolipid remained after washing. This is consistent with the expectation that all the lysolipids in the interior bilayers as well as the lysolipids in the inner leaflet of the external bilayer of LMV are hidden from unlabeled MUV. The 20% removal indicates that our method of producing LMV generated oligolamellar vesicles rather than vesicles with tens to hundreds of lamellae. The fact that somewhat less than half of fluorescent lysolipids (about 40%) could be washed out from GUV is accounted for by the presence of a small population of oligolamellar liposomes in the GUV preparation [13].

After the MUV containing fluorescent probes in the inner leaflet were prepared, they were stored at 25°C for 12 h. Unlabeled GUV were then added and the washing procedure with GUV was performed. The GUV-pellet was not fluorescent, testifying that flip-flop did not occur across the MUV membrane during the 12 h incubation time. Thus, the method of generating vesicles labeled by lysoprobes in only the inner monolayer is general: both GUV and MUV could be asymmetrically labeled.

3.4. Energy transfer in asymmetric liposomes labeled with both LAPC and LPPC

LAPC, excited at 370 nm, served as the energy donor for the acceptor, LPPC in MUV. The efficiency of energy transfer, E, is independent of the surface density of donor, dependent only on the acceptor density [25]. It is defined as:

$$E = 1 - F/F_0$$

where F and F_0 are the fluorescence intensities of the donor in the presence and in the absence of the acceptor, respectively. F_0 was obtained by solubilizing the liposomes by addition of detergent which greatly increases the donor–acceptor distance, thereby abolishing FRET.The dependence of E on the acceptor LPPC concentration (Fig. 5, inset) allowed the LPPC concentration in membranes to be determined after washings. Energy transfer was diminished after each washing cycle (Fig. 5) because intervesicular lipid transfer reduced the surface density of LPPC. We used fluorescent probes that had rather long labeled acyl chains, with the fluorophores located close to the middle of the bilayer. Therefore, FRET between donor and acceptor should have occurred not only within the plane of one leaflet but also between fluorophores located in opposite leaflets. The situation may be approximated by assuming all the probes lie on a single surface in the middle of the bilayer [19].

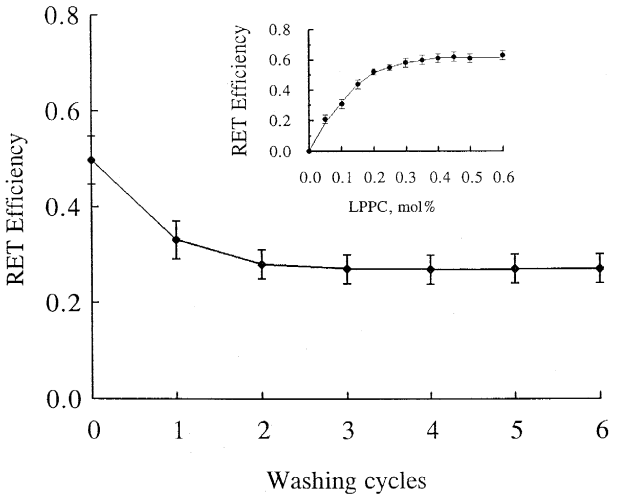

Fig. 5. Energy transfer in MUV containing both LAPC (0.2 mol%) and LPPC (0.2 mol%) after incubation with unlabeled GUV. Inset: Dependence of energy transfer between LAPC (0.2 mol%) and LPPC incorporated in MUV on the LPPC concentration.

Prior to washing, $E = 0.5$. After removing both donor and acceptor from outer leaflets, $E = 0.3$. From the calibration curve of E versus concentration (Fig. 5, inset), we determined that the decrease in E corresponds to the removal of approximately $1/2$ of the acceptor by washing. This is in good agreement with the experiments that directly measured fluorescence intensity when only a single probe was incor-

porated into membranes (Figs. 3 and 4). This demonstrates in an alternate fashion that about 50% of the lysolipids are removed from MUV by our washing procedure. In other words, repetitive washings yielded asymmetrically labeled liposomes with donor and acceptor within inner leaflets only. As is the case with phosphatidylcholine, LPC does not flip-flop [26].

3.4.1. Lipid flip-flop is associated with virus induced leakage of liposomes

We used the liposomes with fluorescent probes located within their inner monolayers only to determine whether or not lipid asymmetry was maintained after membrane fusion. The best characterized fusion protein is the influenza virus hemagglutinin (HA) [27]. We therefore fused influenza virus to liposomes containing G_{T1b}, an effective receptor for HA [28].

EPC/G_{T1b} (10:1, mol/mol) liposomes (MUV) labeled with LAPC and LPPC in only inner monolayers were mixed with virus at a ratio of 1:1 (by lipid phosphorus), incubated for 30 min at 4°C to allow binding and fusion triggered by lowering pH to 5.0 at 37°C [29]. The donor fluorescence intensity increased (Fig. 6), indicating that the probes diluted as a result of liposome-virus membrane fusion. Reneutralization of the suspension to pH 7.4 did not cause the fluorescence of LAPC to change significantly. The LAPC/LPPC pair yielded the same kinetics of fusion as did the APC/PPC pair: MUV containing G_{t1b} were labeled with the phospholipids APC and PPC in both leaflets. The increase in donor, APC, fluorescence following acidification after mixing with virus

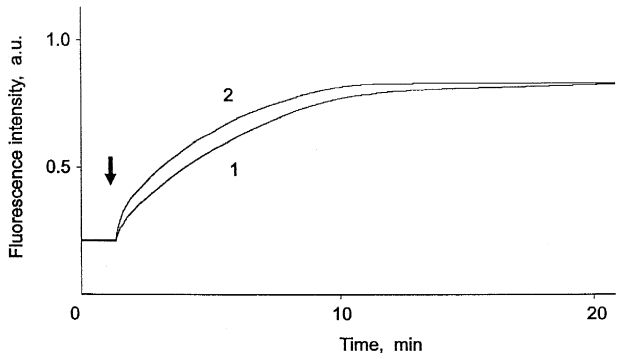

Fig. 6. Liposome-virus fusion detected by FRET. Time dependent fluorescence of donor (LAPC, curve 1; APC, curve 2) in the presence of acceptor (LPPC or PPC) in MUV incubated with virus. The arrow indicates the pH change. Vesicles and virus particles were taken in the same amount by phosphorus (5 μg).

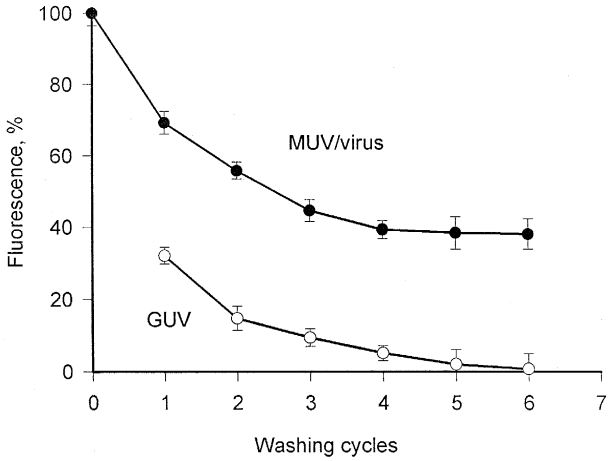

Fig. 7. Washing out of probe from fused liposome-virus aggregates of MUV, labeled with LAPC in inner leaflets, and influenza virus by incubation with a 10-fold (w/w) excess of GUV. (1) supernatant (MUV/virus); (2) pellet (GUV). Initial LAPC fluorescence in MUV/virus mixture after fusion and reneutralization was taken as 100%.

was the same as for LAPC (Fig. 6). As a control, we determined that in the absence of virus there was no changes in donor fluorescence after acidification or after reneutralization for MUV labeled by either pair of probes.

The distribution of the lysoprobes after the virus-liposome fusion was determined by labeling MUV with the single probe LAPC in the inner leaflets only and inducing fusion with virus particles by acidification. The liposome-virus suspension was reneutralized to the initial pH 7.4 and any probe in the outer leaflet was removed by repeated washings with an excess of unlabeled GUV by the standard procedure. The probes redistributed from the inner leaflet to both leaflets at equal concentrations: the fluorescence decreased by 60% after probe was removed from outer leaflets of the fused membranes by repeated washings with GUV (Fig. 7). In parallel experiments we incubated the virus-liposome mixture without acidifying. As expected, for these experiments the probes did not flip-flop (results not shown).

In control experiments, we showed that repetitive washings with GUV did not cause a loss of fluorescence due to binding of GUV to MUV-virus complexes with consequent pelleting of fluorescent complexes during centrifugation. Fusion experiments were carried out exactly as above, except that MUV were labelled with APC rather than LAPC. Because APC

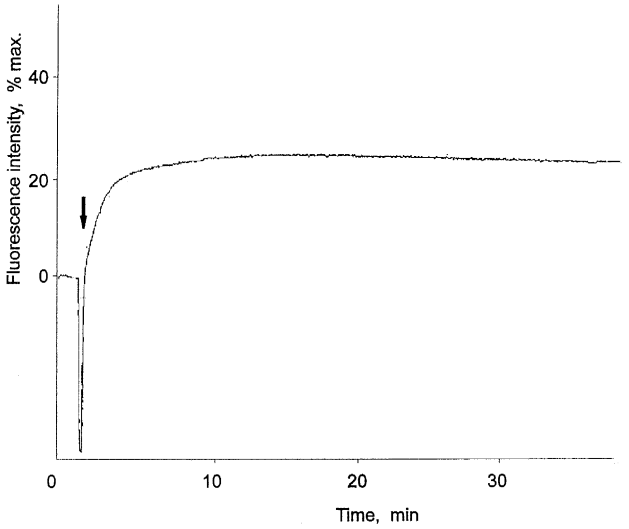

Fig. 8. Leakage of the calcein-Co^{2+} complex from MUV after fusion with virus. Vesicles and virus particles were taken in the same amount by phosphorus (5 μg). The arrow indicates the pH change. Fluorescence was calibrated to 100% after detergent addition when all incorporated calcein was free and Co^{2+} was bound by EDTA in solution.

does not transfer through the aqueous phase, the GUV should remain unlabeled and all the fluorescent label should remain in the supernatant. The fluorescence of the pellet was less than 4% (smaller than error bars) of the fluorescence associated with the MUV-virus complexes in the supernatant. Thus, the loss of LAPC from inner leaflets by washing after fusion was indeed due to flip-flop.

We determined that the lipid flip-flop is due to leakage pores formed within membranes. MUV loaded with calcein and Co^{2+} were bound to virus and fusion was triggered by acidification (Fig. 8). The increase in fluorescence shows that the calcein (and Co^{2+}) leaked from the liposomes and the quenching of calcein by Co^{2+} was relieved by dilution. The similar time courses of lipid mixing and contents leakage indicate that the two processes were correlated. Leakage of aqueous contents of liposomes upon fusion with influenza virus has been reported [7]. Similarly, electrical measurements show that the fusion of cells expressing influenza hemagglutinin to planar membrane can lead to conductance increases [30]. It is also well known that the fusion of influenza virus to cells causes increases in permeability of the target cell membrane [4–6]. Even the addition of isolated aggregates of hemagglutinin to cells leads to permeability increases [31]. In fact, the use of photoaffinity probes showed that simply binding influenza virus to the monomeric form of the ganglioside G_{T1b} in solution (i.e. without membranes) brought about significant rearrangements of lipids surrounding HA [28].

In contrast, fusion between virus and red blood cells [32] and virus and liposomes [33] has been reported in which lipids did not mix between inner and outer leaflets. In this study, we have shown that lipid flip-flop is associated with the permeability increases. If flip-flop of the probe was through pores that did not reseal, all the probe should have been removed by repetitive washings. Because only about one-half the probes flip-flopped (Fig. 7), the pores probably resealed with time. It remains to be determined whether the lipid rearrangements mediated by hemagglutinin that result in fusion are the same or similar to those that cause leakage.

In summary, this work reports the synthesis of two fluorescent lysolipids that can be used as donor–acceptor pairs in fluorescence FRET. They can be removed from outer leaflets of liposomes by washing which allows liposomes with fluorescent probes in only inner leaflets to be readily prepared. These liposomes can be used to determine whether lipid flip-flop occurs or whether lipid asymmetry is maintained in a variety of membrane processes, such as membrane fusion and fission.

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