Asymmetric Fusion between Phospholipid Vesicles and Vesicles Formed from Synthetic Di(*n*-alkyl)phosphates†

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ABSTRACT: We have investigated the fusion behavior of a mixed vesicle system consisting of vesicles prepared from the simple synthetic surfactants di(*n*-dodecyl)phosphate (DDP) or di(*n*-tetradecyl)phosphate (DTP) and vesicles prepared from the phospholipids phosphatidylserine (PS) or dioleoylphosphatidylcholine (DOPC). Fusion between the vesicles, induced by Ca^{2+}, was determined by a resonance energy transfer assay for lipid mixing, sucrose density gradient analysis, and electron microscopy. We demonstrate that synthetic surfactant vesicles can specifically engage in asymmetric fusion events, provided that the incubation temperature is kept *below* the gel–liquid crystalline phase-transition temperature (T_c) of the synthetic amphiphile (29 and 48 °C for DDP and DTP, respectively) and that the physical state of the target membrane is fluid. Asymmetric fusion of DDP or DTP vesicles was most efficient with PS vesicles, but it also occurred with zwitterionic PC vesicles. In the latter case, fusion proceeded spontaneously, but the process was markedly accelerated upon addition of Ca^{2+}. Furthermore, in contrast to a massive transformation of bilayer into nonbilayer hexagonal H_{II} tubular structures, as occurs upon symmetric Ca^{2+}-induced fusion of DDP vesicles, asymmetric fusion with phospholipid bilayers predominantly leads to the formation of larger vesicles. This indicates that both PS and DOPC stabilize the DDP bilayer structure in the fusion product.

Fundamental aspects of membrane fusion can be conveniently studied by using artificial membrane vesicles, composed of either (acidic) phospholipids (Düzgünes, 1985; Wilschut & Hoekstra, 1986; Bentz & Ellens, 1988; Hoekstra & Wilschut, 1989) or charged synthetic amphiphiles (Rupert et al., 1985, 1986, 1987a, 1988; Beigel et al., 1988; Düzgünes et al., 1989). Commonly, in these studies *symmetric* fusion is investigated, i.e., fusion between alike vesicles. Membrane merging takes place when divalent or trivalent cations are added to lipid vesicles or divalent anions and cations to vesicles consisting of positively and negatively charged synthetic amphiphiles, respectively. On the basis of these studies, it has been proposed that ion-induced dehydration of the polar headgroups leads to (local) packing defects in the bilayer, which act as nucleation sites for membrane fusion. Depending on the molecular structure of the phospholipids or synthetic amphiphiles involved, such fusion events may be accompanied by a transition of the bilayer from a lamellar to a defined nonlamellar structure (Cullis & De Kruijff, 1979; Siegel, 1986a,b). Recently, we have shown that Ca^{2+}-induced fusion of di(*n*-dodecyl)phosphate (DDP)[1] vesicles (average diameter 900 Å) leads to the formation of large vesicles with diameters up to 7000 Å, which is *followed* by a transformation of the latter vesicles into extended tubular structures, which display the hexagonal H_{II} phase (Rupert et al., 1987a). On the basis of a model proposed by Siegel (1986a,b), these observations have been rationalized by taking into account the transient formation of nonbilayer structures, the so-called inverted micellar intermediates (IMI's). According to this model, IMI's may transform into fusogenic structures, provided that they remain isolated and that their number in the contact area is low. If not, IMI's can assemble and collapse, which results in a transformation into a hexagonal phase. Close interbilayer contacts between two (alike) membranes is essential for IMI formation (Siegel, 1986a).

Within this context, it appeared interesting, therefore, to examine whether DDP vesicles would be capable of fusion with non-alike (phospholipid) vesicles and, if so, whether the characteristics of *asymmetric* fusion (including polymorphism) bear any resemblance to those of the symmetric DDP system.

In a recent study, it was shown that vesicles composed of synthetic amphiphiles may have great potential as a delivery system for macromolecules into the cytoplasm of eukaryotic cells (Felgner et al., 1987; Pinnaduwage et al., 1989). Efforts to employ (fusogenic) acidic phospholipid vesicles for this purpose were frustrated by the occurrence of extensive fusion of the vesicles among themselves (upon addition of Ca^{2+}) rather than with the cell surface [see, for example, Struck et al. (1981)]. Another aim of this study was, therefore, to examine whether conditions could be found at which fusion of the amphiphilic vesicles would take place preferentially with the lipid vesicles ("asymmetric fusion") rather than among themselves ("symmetric fusion"). In this report we describe conditions at which Ca^{2+}-induced asymmetric fusion can occur between vesicles consisting of di(*n*-dodecyl)phosphate and vesicles composed of various phospholipids.

Experimental Procedures

Materials. Di(*n*-dodecyl)hydrogenphosphate was purchased from Alpha Chemicals. Sodium di(*n*-dodecyl)phosphate

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[1] Abbreviations: DDP, di(*n*-dodecyl)phosphate; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; PS, phosphatidylserine; DMPS, dimyristoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; DTP, di(*n*-tetradecyl)phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LUV, large unilamellar vesicles; CTAB, cetyltrimethylammonium bromide; IMI, inverted micellar intermediate; REV, reverse-phase evaporation vesicles; ILA, interlamellar attachment; EDTA, ethylenediaminetetraacetic acid.

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(DDP) was prepared by dissolving the acid in warm ethanol (50 °C) and by subsequent addition of an equimolar amount of sodium ethanolate, followed by crystallization. Sodium di(*n*-tetradecyl)phosphate (DTP) was synthesized according to Bauman (1974). *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (*N*-NBD-PE), *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE), dioleoylphosphatidylcholine (DOPC), phosphatidylserine (PS, bovine brain), and dimyristoylphosphatidylserine (DMPS) were obtained from Avanti Polar Lipids, Inc. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was from Sigma Chemical; sodium acetate and calcium chloride were purchased from Merck.

Vesicle Preparation. Vesicles consisting of DDP, DTP, and DMPS were prepared by the ethanol injection method (Rupert et al., 1987a). Briefly, 10 mg of the amphiphile was dissolved in 100 μL of ethanol. Of this solution, 80 μL was injected into 2 mL of 5 mM sodium acetate/5 mM HEPES, pH 7.4, with an Exmire microsyringe. The temperature of the buffer solution was kept above the phase-transition temperature of the individual amphiphiles, i.e., vesicle preparation temperatures were 55, 70, and 60 °C for DDP, DTP, and DMPS, respectively.

Large unilamellar vesicles (LUV) composed of DOPC or PS were prepared by reverse-phase evaporation (Szoka & Papahadjopoulos, 1978; Hoekstra & Düzgünes, 1986). The vesicles, made in 5 mM sodium acetate/5 mM HEPES, pH 7.4, were sized by extrusion through polycarbonate Unipore membranes (pore size 0.1 μm, Bio-Rad).

Vesicle Aggregation. Aggregation of vesicles, induced by the addition of Ca^{2+}, was followed by monitoring turbidity changes at 250 or 400 nm with a Perkin-Elmer lambda 5 UV–Vis spectrophotometer equipped with a thermostated cell holder and a magnetic stirring device. Initial rates of aggregation were calculated from the slope (at time zero) of the curve describing the change in turbidity as a function of time.

Vesicle Fusion. Membrane fusion was followed by monitoring lipid mixing, with an assay based on resonance energy transfer (Struck et al., 1981; Hoekstra, 1982). Vesicles containing 0.8 mol % each of *N*-NBD-PE and *N*-Rh-PE were mixed with an equimolar amount of unlabeled vesicles. Fusion was induced by the addition of Ca^{2+}. Continuous monitoring of the relief of energy transfer, as reflected by an increase of NBD fluorescence, was performed on an SLM-Aminco SP-F-500 C spectrofluorometer, equipped with a thermostated cell holder, a magnetic stirring device, and a chart recorder. Samples were excited at 465 nm, and the emission was followed at 530 nm. The fluorescence scale was calibrated by adjusting the initial fluorescence of the (nonfused) labeled vesicles to 0% fluorescence. The level of infinite dilution (100% fluorescence) was determined after disruption of the phospholipid vesicles in Triton X-100 (1% v/v) and of the synthetic amphiphile vesicles in cetyltrimethylammonium bromide (CTAB, 1% w/v). In both cases, corrections were made for sample dilution and for effects of the detergents on the quantum yield of NBD. In some cases, the degree of lipid dilution was verified by reading the fluorescence of mock fusion products, containing 0.4 mol % each of the fluorescent probes and the appropriate amounts of lipid and synthetic amphiphile. All fusion experiments were carried out in a 5 mM sodium acetate/5 mM HEPES buffer, pH 7.4.

Sucrose Gradient Analysis of Vesicles and Fusion Products. Linear sucrose density gradients of 5–25% (w/w) were prepared in 5 mM sodium acetate/5 mM HEPES buffers at pH 7.4. The vesicles (2 mM lipid) were incubated with Ca^{2+} (10 mM) at 37 °C for 10 min. The reaction was arrested by addition of a 4-fold molar excess of EDTA. The samples were then layered on top of the gradient (13 mL). After centrifugation, carried out at room temperature at 30 000 rpm for 3 h in an SW41 Beckman rotor, fractions (ca. 200 μL) were collected from the bottom of the tube, and the densities were determined by measuring the refractive index. NBD fluorescence (λ_{ex} = 465, λ_{em} = 530 nm) in each fraction was measured after addition of Triton X-100 (1% v/v). The lipid composition of the fractions was determined by extraction, followed by HPTLC analysis, with $CHCl_3$/MeOH/25% NH_3/H_2O (70/30/4/1, v/v) as the running solvent. The composition was quantified by a phosphorus determination, after scraping the spots from the plate.

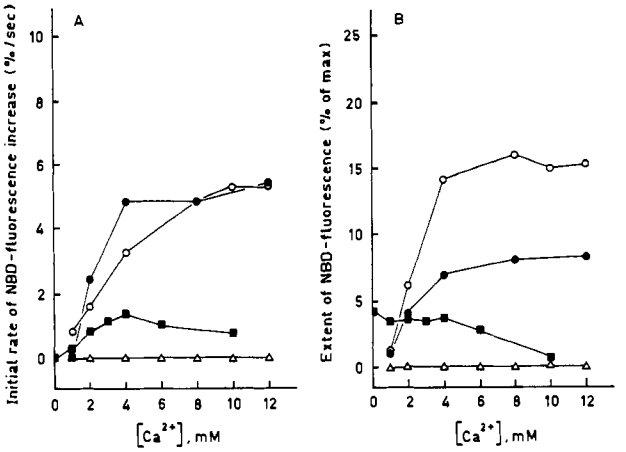

FIGURE 1: Symmetric and asymmetric fusion of PS, DOPC, and DDP vesicles as a function of the Ca^{2+} concentration. Labeled and unlabeled vesicles were mixed in equimolar amounts (the final lipid concentration was 50 μM) in 5 mM sodium acetate/5 mM HEPES, pH 7.4, at 25 °C. Fusion was initiated by injection of Ca^{2+} into the medium, and lipid dilution was continuously monitored as a function of time. The initial rates (A) were calculated and plotted as a function of the Ca^{2+} concentration. The final extent of NBD fluorescence (B) was determined after the fluorescence tracings reached a plateau value that did not further increase over a time interval of at least 30 min. In the asymmetric systems, the phospholipid vesicles were labeled with the fluorescent analogues. The systems were as follows: (○) PS–PS, (●) PS–DDP, (△) DDP–DDP and DOPC–DOPC; and (■) DOPC–DDP.

Electron Microscopy. Vesicles and fusion products were analyzed by transmission electron microscopy. Samples were prepared as described previously (Rupert et al., 1986, 1987a). The suspensions were stained with a 1% (w/v) solution of uranyl acetate, after mounting on carbon-coated Formvar grids, pretreated by glow discharge in *n*-pentylamine. The samples were examined in a Philips EM300 electron microscope, operating at 80 kV.

Results and Discussion

Symmetric Vesicle Fusion. Because DDP vesicles rapidly aggregate in the presence of salt, the fusion medium in the present work contained a relatively low sodium concentration (5 mM). The fusogenic properties at these conditions (25 °C, pH 7.4) of vesicles consisting of PS, DDP, or DOPC are shown in Figure 1.

PS vesicles show rapid lipid mixing upon addition of Ca^{2+}, the cation threshold concentration being centered around 1 mM. Given the ratio of labeled to unlabeled vesicles (1:1), the extent of NBD fluorescence increase indicates that less than half of the total fraction of PS vesicles is involved in fusion. As anticipated, DOPC vesicles do not fuse under these conditions. At 25 °C, where PS vesicles avidly fuse in the presence of Ca^{2+}, symmetric fusion of DDP vesicles is com-

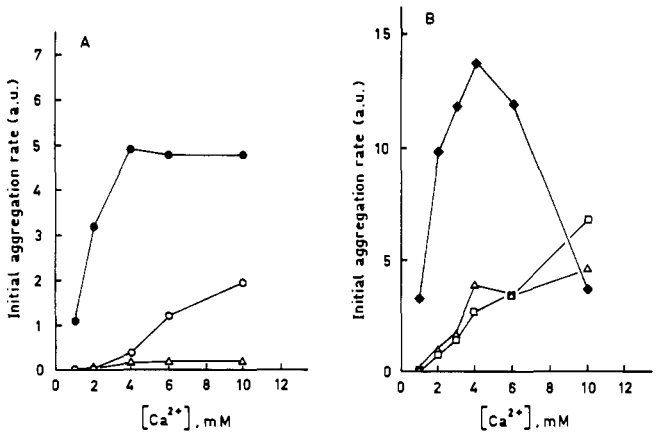

FIGURE 2: Ca^{2+}-induced aggregation of symmetric and asymmetric vesicle systems. Ca^{2+}-induced aggregation of the vesicles was monitored as described under Experimental Procedures. The initial rates were calculated and plotted as a function of the Ca^{2+} concentration. Incubation conditions were as described in the legend to Figure 1. (A) Symmetric aggregation of DDP (△) and PS (○) vesicles (total lipid concentration 25 μM) and the calculated curve (●) for asymmetric PS–DDP vesicle aggregation (eq 2, the total lipid concentration was 50 μM). (B) Symmetric aggregation as in A, for DDP (△) and DOPC (□) vesicles (the total lipid concentration was 25 μM). (◆) Calculated cross aggregation at a final lipid concentration of 50 μM.

pletely inhibited (Figure 1). Neither does lipid dilution occur when these vesicles are incubated in the presence of Ca^{2+} for prolonged incubation times (Figure 1B). These results are consistent with previous observations showing that Ca^{2+}-induced fusion of DDP vesicles does not take place below the gel–liquid crystalline phase-transition temperature (29 °C; Rupert et al., 1988). It should be noted that, although refractory to fusion at these conditions, vesicle aggregation does occur albeit fairly moderately (see Figure 2).

Asymmetric Vesicle Fusion. When PS vesicles containing 0.8 mol % each of *N*-NBD-PE and *N*-Rh-PE are mixed with an equal amount of unlabeled DDP vesicles followed by addition of Ca^{2+}, lipid mixing does occur at 25 °C (Figure 1). Thus, although the DDP bilayer is in the solid state, preventing symmetric fusion, merging of the vesicles with the fluid PS vesicles is not inhibited. Membrane merging was also seen when DDP vesicles were incubated with labeled DOPC vesicles (Figure 1). In that case, fusion takes place even in the absence of Ca^{2+}, though very slowly but to a final extent comparable to that of Ca^{2+}-induced fusion. At 4 mM Ca^{2+}, a distinct maximum in the initial rate of DOPC–DDP fusion was observed. At Ca^{2+} concentrations higher than 4 mM, both the initial rate and extent of fusion decreased (Figure 1; see also Figure 6).

These results indicate that the DDP vesicles must be involved in cross-fusion reactions, since relief of energy transfer will only occur when the labeled DOPC or PS vesicles merge with the unlabeled DDP vesicles. Since symmetric and asymmetric fusion of PS vesicles display approximately the same Ca^{2+} threshold concentration, the initial fusion events in the mixed PS/DDP system may involve the formation of symmetric (PS) and asymmetric (PS/DDP) dimers. However, it may also be possible that during early events single DDP vesicles fuse with symmetric PS vesicle fusion products. In the case of DOPC–DDP vesicle fusion, only asymmetric fusion will take place, since symmetric fusion of either vesicle type cannot occur under these conditions. Therefore, in contrast to events in the mixed PS/DDP system, the initial fusion events in the DDP/DOPC system are likely to consist of the exclusive formation of asymmetric dimers. To further analyze these possibilities, we next examined the Ca^{2+}-induced aggregation of DDP, PS, and DOPC vesicles separately, as well as the aggregation in the mixed DDP/PS and DDP/DOPC systems.

Symmetric and Asymmetric Aggregation of Vesicles. Vesicle aggregation is a second-order process (Nir et al., 1983), and the rate equation for asymmetric aggregation [i.e., aggregation between PS (or DOPC) and DDP vesicles] is given by

$$V_{aggr} = k_1[PS]^2 + k_2[DDP]^2 + k_3[PS][DDP] \quad (1)$$

in which V_{aggr} represents the *overall* rate of aggregation, while k_1, k_2, and k_3 are the respective rate constants. The term $k_3[PS][DDP]$ indicates the rate for asymmetric aggregation V_{aggr}^{asym}, which, by rearranging eq 1, can be expressed as

$$V_{aggr}^{asym} = V_{aggr} - k_1[PS]^2 - k_2[DDP]^2 \quad (2)$$

Experimentally, it can be readily shown that Ca^{2+}-induced vesicle aggregation is a second-order process. After aggregation is established at a given vesicle concentration, a doubling of this concentration should lead to a 4-fold increase in the rate of aggregation. In case of Ca^{2+}-induced aggregation of a mixture of DDP and either PS or DOPC vesicles, this is exactly what is seen (not shown). From these curves, V_{aggr}^{asym} (eq 2) can be obtained by subtracting the symmetric rates of aggregation for PS (DOPC) and DDP vesicles. The curves thus obtained show that asymmetric PS–DDP aggregation is almost three times faster than symmetric aggregation of PS vesicles and almost an order of magnitude faster than DDP–DDP vesicle aggregation. For DOPC–DDP aggregation, a distinct maximum is observed at 4 mM Ca^{2+}. Note that this aggregation maximum correlates very well with that for fusion (Figure 1). Furthermore, the rate of asymmetric aggregation at 4.0 mM Ca^{2+} is 3.5 times faster than that for symmetric aggregation of DOPC and DDP (Figure 2). At 10 mM Ca^{2+}, the initial rate for asymmetric aggregation is slower than the rates observed for symmetric aggregation. In this context, it is finally relevant to note that, for acidic phospholipid bilayers, almost complete charge neutralization is achieved at 4 mM Ca^{2+} (Lansman & Haynes, 1975). This appears to be the case for the symmetric DDP system as well, as is reflected by a leveling off of Ca^{2+}-induced aggregation of the DDP vesicles at 4 mM Ca^{2+} (Figure 2B). The observation that both aggregation and fusion are inhibited above 4 mM Ca^{2+} may then be explained by assuming that the net surface charge of the DOPC–DDP fusion product becomes positive, occurring when the ratio of Ca^{2+} to DDP binding becomes 1 as a result of randomization of DDP in the DOPC bilayers. Electrostatic repulsion could thus prevent additional fusion between a DOPC–DDP fusion product and DDP vesicles, while potential fusion nucleation sites created by DDP have been eliminated by mixing with DOPC (which stabilizes the bilayer structure of DDP, see next paragraph). This would preclude any subsequent fusion between the fusion product and additional DOPC vesicles.

We conclude that Ca^{2+}-induced aggregation in the mixed PS/DDP system occurs preferentially for the asymmetric process. In the case of the DOPC/DDP system, the extent of asymmetric aggregation is dependent on the Ca^{2+} concentration.

Characterization of Asymmetric Fusion by Electron Microscopy. Symmetric fusion of DDP vesicles is characterized by the initial formation of large fused vesicles (diameter ca. 7000 vs 700 Å of the original vesicles), followed by their transformation into long (up to several microns) hexagonal H_{II} tubular structures (Rupert et al., 1987a). By contrast, the fusion event occurring in the DOPC/DDP system, as judged

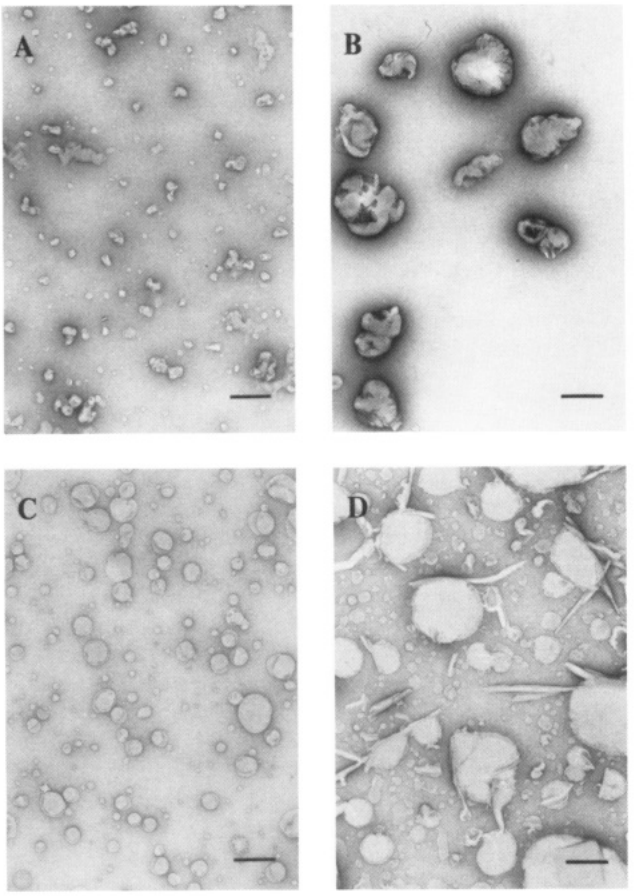

FIGURE 3: Electron micrographs of asymmetric PS–DDP and DOPC–DDP fusion products. The samples were prepared and examined as described under Experimental Procedures. A and B show the asymmetric PS/DDP vesicle system, before (A) and after (B) addition of Ca^{2+}. (C) DOPC/DDP vesicles before addition of Ca^{2+}. Electron micrograph D is obtained after addition of Ca^{2+}. The bar is 300 nm.

by lipid mixing, appears to result in the formation of larger vesicles and fairly small rods, as revealed by electron microscopy. In other words, the formation of the hexagonal tubes is severely inhibited in the asymmetric system. This result supports the occurrence of true asymmetric fusion, which results in a stabilization of the DDP bilayer by DOPC. Very similar results were obtained for Ca^{2+}-induced fusion occurring in the asymmetric PS/DDP system (Figure 3).

Effect of Temperature on Asymmetric Vesicle Fusion. In previous work (Rupert et al., 1987b), we have shown that DDP bilayers display a main transition between 22 and 33 °C (T_c is ca. 29 °C) and a pretransition between 18 and 22 °C with the midpoint at 20 °C. As indicated above, symmetric fusion of DDP vesicles does not take place below T_c (Figure 1). As shown in Figure 4, asymmetric fusion between DDP and PS vesicles takes place at tempertures as low as 1 °C, although the initial rate is almost negligible below 10 °C (Figure 4A). When the temperature is subsequently raised, both the initial rate and extent of fusion gradually increase. Between 15 and 20 °C the extent of asymmetric fusion steeply increases. Note that the onset of this increase coincides with the pretransition temperature region of the DDP bilayers (Rupert et al., 1986, 1987a).

In the case of asymmetric DOPC–DDP fusion, no inflection around 20 °C was observed (Figure 5A). Rather, a steep increase of the initial rate and extent of fusion is located around 30 °C, which corresponds with the T_c of DDP. Lipid mixing occurring in the absence of Ca^{2+} takes place gradually, i.e.,

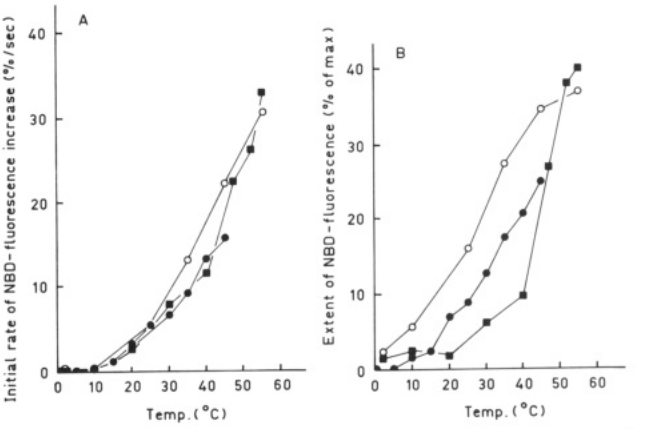

FIGURE 4: Ca^{2+}-induced asymmetric fusion between PS vesicles and vesicles composed of synthetic amphiphiles as a function of temperature. Vesicles were mixed and equilibrated at the indicated temperatures. Fusion was induced by injecting Ca^{2+} (10 mM final concentration) into the medium. Equimolar amounts of vesicles were used (the final lipid concentration was 50 μM), and in all cases the PS vesicles were labeled with *N*-NBD-PE and *N*-Rh-PE. Initial rates (A) and final extent (B) were determined and plotted as a function of temperature. Symbols: (○) PS–PS, (●) PS–DDP, and (■) PS–DTP.

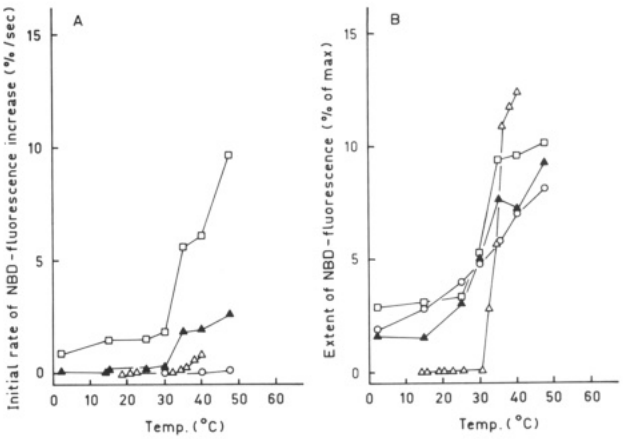

FIGURE 5: Ca^{2+}-induced asymmetric fusion of DOPC and DDP vesicles, as a function of temperature and Ca^{2+} concentration. Vesicles were separately equilibrated at the indicated temperatures. In the asymmetric systems, DOPC vesicles were labeled with *N*-NBD-PE and *N*-Rh-PE. The total lipid concentration was 50 μM. Asymmetric fusion between DOPC and DDP vesicles was triggered by mixing the vesicles followed by injection of buffer (○) or 1 (▲) and 4 mM Ca^2 (□) into the cuvette. For comparison, symmetric DDP–DDP fusion at 4 mM Ca^{2+} (△) is also shown.

without a sudden increase in both the initial rate (≤0.1%/s) and extent of fluorescence (Figure 5). The Ca^{2+} dependence of asymmetric DOPC–DDP fusion showed very similar patterns, irrespective of whether fusion was induced below or above the phase-transition temperature of DDP (not shown, see Figure 1). Quantitatively, the initial rates and the extent of lipid dilution were higher at all-fluid conditions, but in both cases an optimum in the Ca^{2+} concentration around 4 mM was observed, suggesting a similarity in the fusion mechanism of this system at either temperature. Obviously, this mechanism cannot involve the formation of a so-called "trans" Ca^{2+}/lipid complex, as postulated for Ca^{2+}-induced fusion of acidic phospholipid vesicles (Ekerdt & Papahadjopoulos, 1982). Moreover, since fusion between DOPC and DDP vesicles occurs spontaneously, the results indicate that Ca^{2+} is not directly required for triggering the fusion reaction. Rather, it is tempting to suggest that its effect only involves a facilitation of the creation of certain structural defects in

Table I: Effect of Membrane Fluidity on Asymmetric Fusion of Vesicles Composed of Synthetic Amphiphiles[a]

target membrane	T_c (°C)	fusion threshold temperature (°C)[b] DDP	DTP
PS	+7	+7	+7
DDP	+29	+29	+29
DMPS	+38	+38	+38
DTP	+48	+29	+48

[a] Various target membranes (labeled with *N*-NBD-PE and *N*-Rh-PE) were incubated with DDP or DTP vesicles as a function of temperature. Ca^{2+} (4 mM) was injected to trigger fusion. [b] This temperature is defined as the temperature at which the initial rate of fluorescence increase was greater than 0.1% s^{-1}.

the DDP bilayers that cause them to fuse with the DOPC bilayers.

Effect of Membrane Fluidity on Asymmetric Fusion. The temperature-dependent fusion experiments between DDP and PS or DOPC vesicles (Figure 4) indicated that asymmetric fusion with DOPC vesicles occurred over the entire temperature range tested (i.e., between 1 and 55 °C). On the other hand, with PS vesicles significant fusion became apparent only above ca. 10 °C. Since the transition temperatures for DOPC and PS LUV are −22 and +7 °C, respectively, this might suggest that the solid DDP vesicles only fuse with fluid target membranes. To further substantiate this notion, LUV containing *N*-NBD-PE and *N*-Rh-PE were prepared from synthetic dimyristoyl-PS (DMPS, T_c = 38 °C) and the synthetic amphiphile di(*n*-tetradecyl)phosphate (DTP, T_c = 48 °C). Lipid mixing between (unlabeled) DDP and DMPS or DTP vesicles was then monitored as a function of temperature, and the threshold temperature for fusion (i.e., the temperature at which the initial rate was greater than 0.1% s^{-1}) was determined. A similar set of experiments was carried out, with unlabeled DTP vesicles. The results, summarized in Table I, support the suggestion that vesicles composed of the synthetic amphiphiles (DDP or DTP) fuse with the target membrane only when the physical state of the latter is liquid crystalline, irrespective of the physical state of the former.

Asymmetric Fusion of DDP versus DTP Vesicles. It is known that modification of the headgroup of an amphiphilic molecule, with unaltered alkyl chain length, markedly affects its fusogenic properties (Rupert et al., 1985, 1986). A comparison of the asymmetric fusogenic activity of DDP and DTP vesicles with PS vesicles (Figure 4) shows that the initial fusion rates of both systems are remarkably similar, when monitored as a function of temperature. Thus, the present results suggest that mere elongation of only the alkyl chain does not cause major alterations in overall asymmetric fusion properties.

As noted above for DDP vesicles, an apparent facilitation of fusion may occur around the pretransition temperature of the amphiphile. This notion seems to be supported by the observation that a significant facilitation of fusion, particularly the extent of fusion, can also be seen around the pretransition temperature of DTP, which is centered around 30 °C (Wagenaar et al., 1989). Interestingly, between 30 and 40 °C the rates for asymmetric fusion of PS–DDP and PS–DTP are virtually indistinguishable, in spite of DDP bilayers being fluid and those of DTP being solid in this temperature range. In conjunction with the observation that at all-fluid conditions (i.e., above 48 °C for DTP and above 30 °C for DDP) DTP vesicles fuse to a higher extent with PS than DDP, the results suggest that DTP bilayers possess a higher susceptibility for asymmetric fusion than DDP bilayers. These observations thus bear analogy to the spontaneously induced fusion of sonicated DPPC and DSPC vesicles (Larrabee, 1979).

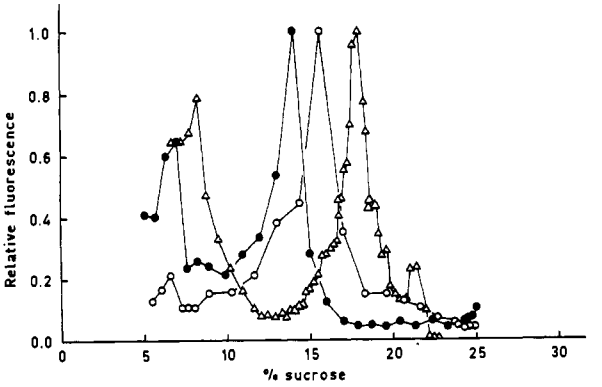

FIGURE 6: Sucrose density gradient analysis of PS and DDP vesicles after Ca^{2+}-induced symmetric and asymmetric fusion. Fusion was induced by incubating equimolar amounts of vesicles (2 mM final lipid concentration) with Ca^{2+} (10 mM) for 10 min at 37 °C. As a membrane marker, *N*-NBD-PE was used to label the vesicles (0.8 mol %). Prior to layering on a continuous sucrose gradient (0–25%), the vesicle mixtures were treated with 40 mM EDTA. The fractions were analyzed as described under Experimental Procedures. Symbols: (○) PS–PS, (△) DDP–DDP, and (●) PS–DDP.

Sucrose Gradient Analysis of Symmetric and Asymmetric Vesicle Fusion Products. It is evident that above the phase-transition temperature of DDP vesicles (29 °C) fusion will be substantially more complex than below this temperature. Below 29 °C, symmetric fusion between DDP vesicles is completely inhibited; however, above 29 °C both symmetric and asymmetric fusion of *all* vesicles and fusion products involved can occur. Under these conditions, it was observed that the initial rate of symmetric aggregation of PS vesicles was approximately 2-fold *faster* than the initial rate of PS–DDP vesicle aggregation. This is in marked contrast with a similar analysis carried out below T_c of DDP (Figure 2), where the initial rate of symmetric aggregation was approximately 3-fold *slower* than the initial rate of asymmetric aggregation. Such distinct differences in rates of asymmetric vs symmetric aggregation might affect the nature of the fusion products formed. To obtain, therefore, more quantitative insight into the composition of the fusion product(s) formed at all-fluid conditions, an analysis on continuous sucrose gradients was carried out. All samples were treated with a 4-fold molar excess of EDTA (relative to Ca^{2+}) prior to their layering on top of the gradients.

Nonfused PS or DDP vesicles remained near the top of the gradient. However, after triggering fusion at 37 °C in a symmetric system, a shift to higher densities is observed in all cases (Figure 6). A significant fraction (ca. 40%) of the DDP vesicles is recovered near the top of the gradient at a position similar to that of nonfused vesicles. The major fraction (ca. 60%) peaks at a density around 18% (w/w) sucrose. Under identical conditions, the major PS fraction displayed a density of approximately 16% (w/w) sucrose. Also, in this case a small but significant fraction of unfused vesicles was recovered in the top fractions of the gradients. The remarkable distinctions in density of the fusion products in the symmetric systems then allowed us to carry out a similar analysis for fusion occurring in the asymmetric PS/DDP system. The results of this experiment are shown in Figure 6. Similarly, as observed for the symmetric system, asymmetric fusion is incomplete. The fusion product is centered around 14% (w/w) sucrose. Analysis of the peak fractions of the fused products by lipid extraction, TLC, and phosphorus determination revealed that the ratio of DDP to PS was 1:1, i.e., identical with the ratio of starting vesicles.

To exclude the possibility that the fractions recovered at higher sucrose density merely reflect the presence of aggregates rather than merged structures, the experiments summarized in Figure 6 were repeated, with mixtures of *N*-Rh-PE/*N*-NBD-PE labeled phospholipid vesicles and unlabeled DDP vesicles. Energy-transfer measurements were carried out of the fused peak fractions, and the efficiency was compared to the energy-transfer efficiency of the starting vesicles. The observed decrease of approximately 50% in energy-transfer efficiency of the peak fractions (not shown), relative to that of the starting vesicles, is consistent with a 1:1 dilution of the probes, as was also determined directly by analysis of the lipid composition of the peak fractions. Thus, at 37 °C there appears to be complete randomization of both the DDP and PS vesicles, suggesting that the number of (pure) symmetric fusion products must be relatively small. Indeed, a significant fraction of symmetric fusion products could not be detected (Figure 6). Finally, although sucrose gradient fractionation allows for a separation of the different vesicle populations, this separation is not merely based on density. Other factors involved are as yet unclear.

In summary, we have shown that asymmetric fusion between surfactant vesicles and phospholipid vesicles can be triggered under conditions where symmetric fusion between either vesicle type (DOPC and DDP, below T_c of DDP) does not occur. However, even in a system (PS and DDP) and under conditions (above T_c of DDP) where symmetric fusion of both vesicle populations may take place, extensive asymmetric fusion still occurs. We suggest that these asymmetric fusion properties of synthetic surfactants can be exploited to enhance the efficacy of delivery of lipophilic and hydrophilic drugs or macromolecules into cells by specific (i.e., "targeted") fusion of the vesicles with the cell surface only. With fusogenic phospholipid vesicles, this possibility is largely precluded due to massive fusion between the vesicles prior to their interaction with the cell surface. Current experiments, in which the interaction between surfactant vesicles and erythrocytes is examined, strongly support this suggestion (Fonteijn et al., 1990).

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Registry No. DDP, 7057-92-3; DMPS, 64023-32-1; DTP, 6640-03-5; Ca^{2+}, 7440-70-2; DOPC, 4235-95-4; sodium ethanolate, 141-52-6; di(*n*-dodecyl) hydrogen phosphate, 21302-09-0.

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