University of Groningen

Asymmetric fusion of Di-n-alkylphosphate vesicles

Fonteijn, Theodorus Adrianus Antonius

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1992

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
Fonteijn, T. A. A. (1992). *Asymmetric fusion of Di-n-alkylphosphate vesicles*. s.n.

Chapter 2

Asymmetric Fusion of Di-*n*-dodecylphosphate Vesicles with Phospholipid Vesicles

2.1 Introduction

Phospholipid bilayers have extensively been used as model systems for the much more complex biomembranes[62,63]. For example, fundamental aspects of membrane fusion can be conveniently studied by using artificial membrane vesicles, composed of either (acidic) phospholipid[40,42,62,63] or charged synthetic amphiphiles[25,58,59,64,65,66,67,68,69]. In most of those studies symmetric fusion has been investigated, i.e. fusion between alike vesicles. Upon addition of multivalent cations (symmetric) membrane merging takes place between vesicles formed from either negatively charged lipids or synthetic amphiphiles. Positively charged synthetic amphiphiles fuse under the influence of divalent anions[25,59]. On the basis of these studies, it has been proposed that ion-induced dehydration of the polar head groups leads to (local) packing defects in the bilayer, which act as nucleation sites for membrane fusion. Depending on the nature of the amphiphile (phospholipid or synthetic amphiphile), these fusion events can be accompanied by a transition of the bilayer to non-lamellar structures[70,71,72]. Untill recently, it was thought that di-*n*-dodecylphosphate (DDP) vesicles, after Ca^{2+}-induced fusion, exclusively transform into a hexagonal H_{II} phase. At equilibrium conditions however, the tubular structures that are formed after symmetric DDP fusion are organized in lamellar crystals rather than in a hexagonal phase[73]. Yet, these lamellar crystals may be the result of initial processes that involve nonbilayer structures (see chapter 6).

In this chapter a study is described that involves the interactions between synthetic di-*n*-alkylphosphate (DAP) vesicles and various phospholipid membranes. Special attention is focussed on the question whether DDP vesicles are capable to engage in *asymmetric* vesicle fusion and, if so, if the characteristics of asymmetric fusion bear any resemblance to those of the symmetric DDP system.

Vesicles composed of synthetic amphiphiles, may have considerable potential as a delivery system for macromolecules into the plasma membrane and/or the cytoplasm of eukaryotic cells[27,28,29,30]. Efforts to employ (fusogenic) phospholipid vesicles for this purpose were frustrated by the occurrence of extensive symmetric fusion among the phospholipid vesicles themselves, rather than asymmetric fusion with the cell membrane. Therefore it was also of interest to examine whether conditions could be found at which the DDP vesicles preferentially fuse with phospholipid vesicles (asymmetric fusion).

In this chapter, the influence of various fusogenic agents (Ca^{2+} and Mg^{2+}), temperature, and membrane fluidity, on asymmetric fusion of DAP vesicles with various phospholipid liposomes wil be described. Vesicle fusion was monitored using a fluorescence assay for lipid mixing. The kinetics of asymmetric vesicle aggregation

have also been studied. The results are compared to those of the corresponding symmetric aggregation processes and those of the asymmetric lipid mixing assay. The fusion products are characterized by transmission electron microscopy (TEM) and sucrose gradient analysis.

PS   DOPC   CL
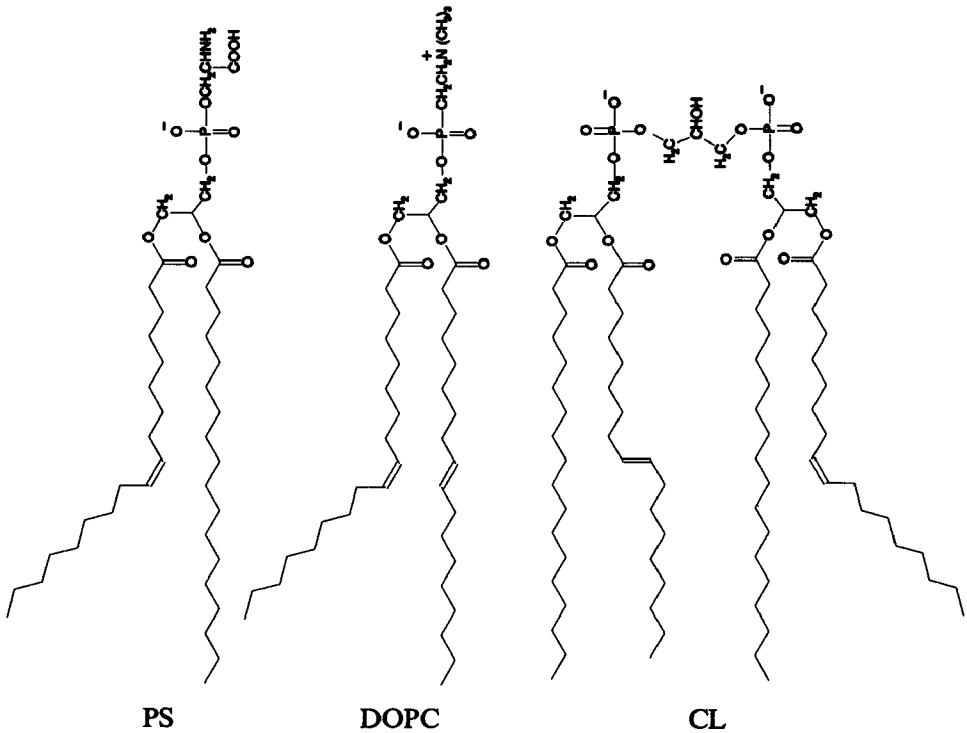
The phospholipids used in this study are phosphatidylserine (PS), cardiolipin (CL) and dioleoylphospatidylcholine (DOPC). The natural abundance of PC in most biomembranes is much higher than that of PS. CL figures least in biomembranes, however, the amounts of each specific phospholipid depend highly on the nature and function of the membrane. PS and CL are negatively charged whereas DOPC bears no net electric charge at physiological pH. DOPC exhibits no symmetric Ca^{2+}-induced fusion[74,75,76], whereas PS and CL do. DOPC and PS are not capable to form non-bilayer structures under normal conditions; at extreme conditions (high temperature, low pH) PS membranes can transform into a hexagonal H_{II} phase[77]. Upon addition of Ca^{2+}, CL membranes may readily adopt the tubular hexagonal H_{II} state. These differences presumably arise from Ca^{2+}-induced changes in the packing constraints, as expressed in the packing parameter (see section 1.6).

16

2.2 Symmetric vesicle fusion

To be able to compare the results of asymmetric fusion with those of symmetric fusion, the Ca^{2+}-induced symmetric fusion reactions were investigated first. The results obtained for DDP, PS and DOPC membranes at 25°C and pH 7.4, as a function of the Ca^{2+} concentration, are shown in Figure 2.1.

PS liposomes show rapid lipid mixing upon Ca^{2+} addition, provided that the Ca^{2+} concentration exceeds the threshold concentration for fusion (*ca.* 1 mM). From the final extent of NBD-fluorescence, considering the ratio of labeled to unlabeled vesicles (1:1), it can be calculated that approximately 30% of all PS vesicles take part in the symmetric fusion process.

As expected, DOPC liposomes do not fuse under the conditions that were employed[74, 75, 76].

At 25°C, which is below the gel-to-liquid crystalline phase-transition temperature, (T_c, 29°C)[66], DDP vesicles do not fuse symmetrically. Consequently, no probe dillution is observed even after several hours. Interestingly, although symmetric DDP vesicle fusion does not take place at 25°C, Ca^{2+}-induced aggregation does occur. DDP vesicles have been reported to indulge in Ca^{2+}-induced symmetric fusion, but only above T_c[66, 77].

2.3 Ca^{2+}-induced asymmetric fusion of DDP vesicles with liposomes

2.3.1 Phosphatidylserine (PS)

Upon Ca^{2+} addition to an equimolar mixture of labeled PS liposomes (0.8 mole % each of N-NBD-PE and N-Rh-PE) and unlabeled DDP vesicles at 25°C, lipid mixing takes place (Figure 2.1). Thus, in spite of the solid nature of the DDP membranes, which refrains them from fusing symmetrically, they *do* merge with fluid PS membranes.

These results indicate that DDP vesicles must be involved in cross-fusion reactions, since relief of resonance energy transfer is only established upon merging of labeled PS vesicles with unlabeled DDP vesicles. The symmetric fusion of PS vesicles that might take place, will go unnoticed since this process does not result in probe dillution. The Ca^{2+} threshold concentration for symmetric and asymmetric PS fusion is almost the same. Therefore, the initial events of the asymmetric PS-DDP fusion reaction may involve symmetric (PS-PS) and asymmetric (PS-DDP) fusion products (dimers, oligomers) or the interaction of single DDP vesicles with symmetric PS fusion products.

2.3.2 Dioleoylphosphatidylcholine (DOPC)

On incubating DDP vesicles with labeled DOPC liposomes, membrane merging was also observed. Spontaneous DOPC-DDP lipid mixing, taking place in the absence of Ca^{2+}, occurs very slowly but to a final extent comparable to that of Ca^{2+}-induced DOPC-DDP fusion (Figure 2.1). At 4 mM Ca^{2+}, a distinct maximum in the initial rate

17
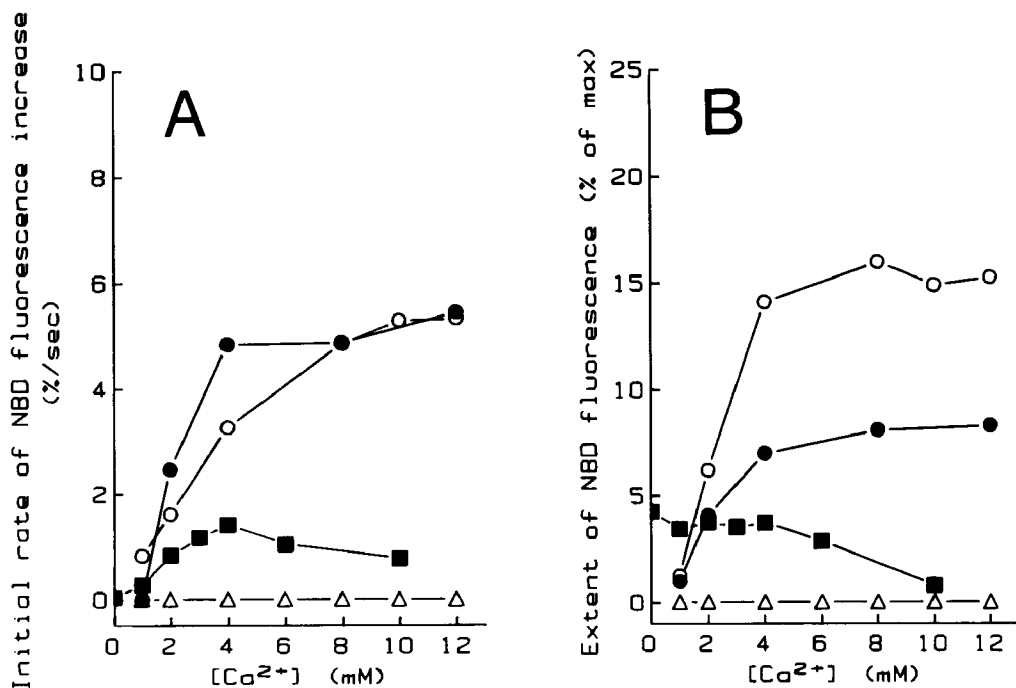
Figure 2.1. Initial rates (A) and extents (B) of lipid mixing as a function of the Ca^{2+} concentration for symmetric and asymmetric vesicle fusion. (△): DDP-DDP and DOPC-DOPC, (○): PS-PS, (●): PS-DDP, (■): DOPC-DDP. Experimental conditions: 25°C; pH 7.4; ratio of labeled to unlabeled vesicles 1:1; total lipid concentration 50 μM. In the asymmetric fusion experiments the PS and the DOPC vesicles were labeled.

of fusion is found. At Ca^{2+} concentrations higher than 4 mM, both the initial rate and extent of fusion decreased.

Since both DOPC and DDP vesicles do not fuse symmetrically under the conditions employed, it was anticipated that if fusion was to take place, this could only be asymmetric fusion. The initial events of the asymmetric fusion process will therefore consist of interactions between "single" DOPC and DDP vesicles, resulting in the formation of asymmetric dimers.

2.3.3 Cardiolipin (CL)

Cardiolipin is a remarkable lipid with a high fusion capacity, which is attributed[78,79,80] to its polymorphic behaviour in the presence of Ca^{2+}. No asymmetric CL-DDP fusion could be detected on the basis of lipid mixing experiments, irrespective of whether the CL liposomes or the DDP vesicles were labeled with the fluorescent probes. However, large increases in fluorescence are observed upon addition of Ca^{2+}

ions to a mixture of labeled CL liposomes and DDP vesicles. The extremely fast increase in fluorescence completely disappears when EDTA, a Ca^{2+}-complexing agent, is added.

CL liposomes are known for their capacity to transform into an inverted hexagonal (H_{II}) structure in the presence of Ca^{2+}. This transformation is accompanied by a change in the packing of the fluorescent probes in the CL matrix, resulting in an increase in the quantum yield of the N-NBD-PE fluorescence. The values of the observed fluorescence increase after Ca^{2+}-induced asymmetric CL-DDP interaction are much larger than those reported in literature[81] for the formation of a H_{II} phase: 60% *vs.* 10-15%, respectively. Although the system refrains from lipid mixing, the presence of DDP does appear to exert an influence on the Ca^{2+}-induced changes in the packing of the CL molecules in the newly formed phase. Further studies are necessary to elucidate the origin of this effect. It is concluded that the lipid mixing assay is not a reliable method when CL liposomes are involved; an assay based on the mixing of the internal contents of the vesicles should give more relevant information[82].

2.4 Effects of Mg^{2+} on the Ca^{2+}-induced PS-DDP fusion

In general, Mg^{2+} ions are less efficient fusogenic agents than Ca^{2+} ions are[68,83,84]. This has been attributed to the inferior dehydrating properties of Mg^{2+} ions[85], not allowing the formation of a Mg^{2+} trans complex between the apposed bilayers. The Mg^{2+} ion only binds in a so-called cis complex[85]. Under the influence of Mg^{2+} both DDP vesicles and large unilamellar PS vesicles (LUV) do not fuse, they merely aggregate massively[83]. Remarkably, although Mg^{2+} is not capable of inducing symmetric fusion either between DDP vesicles or PS (LUV), it can induce asymmetric PS-DDP fusion (Figure 2.2). Since Mg^{2+} has a smaller dehydrating capacity than Ca^{2+}, this suggests that the formation of the asymmetric fusion intermediate requires less dehydration than the formation of both symmetric fusion intermediates. Mg^{2+}-induced fusion does only take place to a limited extent if the DDP membrane is fluid. Below T_c (29°C), no fusion is detected (not shown). Mg^{2+}-induced PS-DDP fusion is less effective than Ca^{2+}-induced asymmetric fusion. This result is consistent with reports on symmetric phospholipid membrane fusion and reflects the importance of head-group dehydration and alkyl chain mobility in the fusion process.

In a way the situation is akin to the observations by Bentz et al.[86] who studied the destabilization of H_{II} phase-forming phosphatidylethanolamine containing liposomes. They hypothesized that, when the lipids of two dissimilar liposomes (A and B), are H_{II} competent, the asymmetric bilayer destabilization (AB) is more important than either of the two symmetric destabilization processes (AA and BB). Taking the possibility into account that DDP could form a H_{II} phase upon transformation into the lamellar $Ca(DDP)_2$ phase, this transient H_{II} phase formation might destabilize the asymmetric DDP-PS system sufficiently to fuse in an asymmetric fashion.

DDP vesicles exhibit a slightly higher binding constant for Mg^{2+} than for Ca^{2+} ions[68]. In contrast, PS vesicles display a larger binding constant for Ca^{2+} than for Mg^{2+}
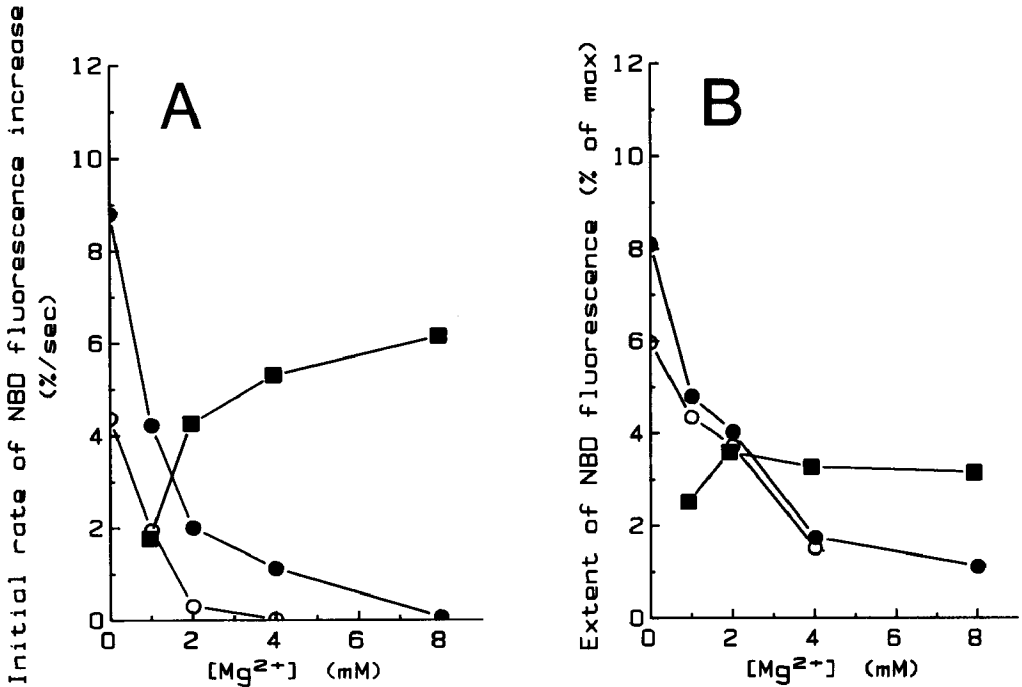
Figure 2.2. Initial rates (A) and extents (B) of lipid mixing as a function of the Mg^{2+} concentration for asymmetric PS-DDP vesicle fusion. (■): 0 mM Ca^{2+} at 37°C. The circles represent experiments where immediately after addition of 4mM Ca^{2+}, Mg^{2+} was added both above and below T_c of DDP. (○): 25°C, (●): 37°C. Experimental conditions: pH 7.4; ratio of labeled to unlabeled vesicles 1:1; total lipid concentration 50 μM.

ions[85,87]. Therefore it can be expected that in a competition experiment, the preferences of DDP and PS for Mg^{2+} and Ca^{2+}, respectively, will become apparent.

Figure 2.2 shows the influence of a short preincubation of the PS-DDP vesicle mixture with Mg^{2+}, prior to the addition of Ca^{2+} on the asymmetric lipid mixing. Little difference is observed for the asymmetric fusion experiments performed either above or below T_c of DDP: a profound decrease in activity is the result of preincubation with Mg^{2+}. The presence of Mg^{2+} inhibits the Ca^{2+}-induced fusion. No synergistic effects, like partial dehydration of the membrane surfaces and reduction of the surface charge are observed, as for the fusion of PS SUVs[88].

2.5 Effect of the pH on Ca^{2+}-induced asymmetric PS-DDP fusion

Figure 2.3 shows the effect of the pH on the initial rate and the extent of PS-DDP vesicle fusion both above (37°C) and below (25°C) the T_c of the DDP membrane. The initial rates of fusion experience marked differences under both conditions. At 37°C,

an increase in the initial rate of fusion on lowering the pH is observed, until pH 5 is reached. Below pH 5.0 the initial rate of fusion decreases. At 25°C, the opposite effect, an increase in the initial rate of fusion below pH 5.0, is observed. At both temperatures the extents of fusion are comparable and are almost pH independent.

Fusion activity can be modulated by physical changes in the bilayer. Alterations in pH affect both the hydrocarbon region of the membrane and the bilayer-water interface of the membrane. As a result, changes in the morphology of the aggregates of both phospholipids and synthetic amphiphiles[89,90,91,92] may occur.

Since the PS membranes are in a fluid phase both at 25 and 37°C, the influence of the temperature on the PS membranes is not likely to affect the pH profile of the asymmetric fusion dramatically. Therefore the difference between the fusion activity at 25 and 37°C mainly has to find its origin in a change in the DDP bilayer properties.

The DDP alkyl chain mobility will be much less at 25°C than at 37°C, explaining the reduced initial rates of fusion: the hydrocarbon chains in the membrane have to be able to reorient themselves, which is a likely prerequisite for the occurrence of fusion. In the asymmetric fusion process, the influence of pH on the PS bilayer also has to be taken into account. PS has a pK_a of about 4.0[93], whereas the pK_a of DDP is 5.2[65].
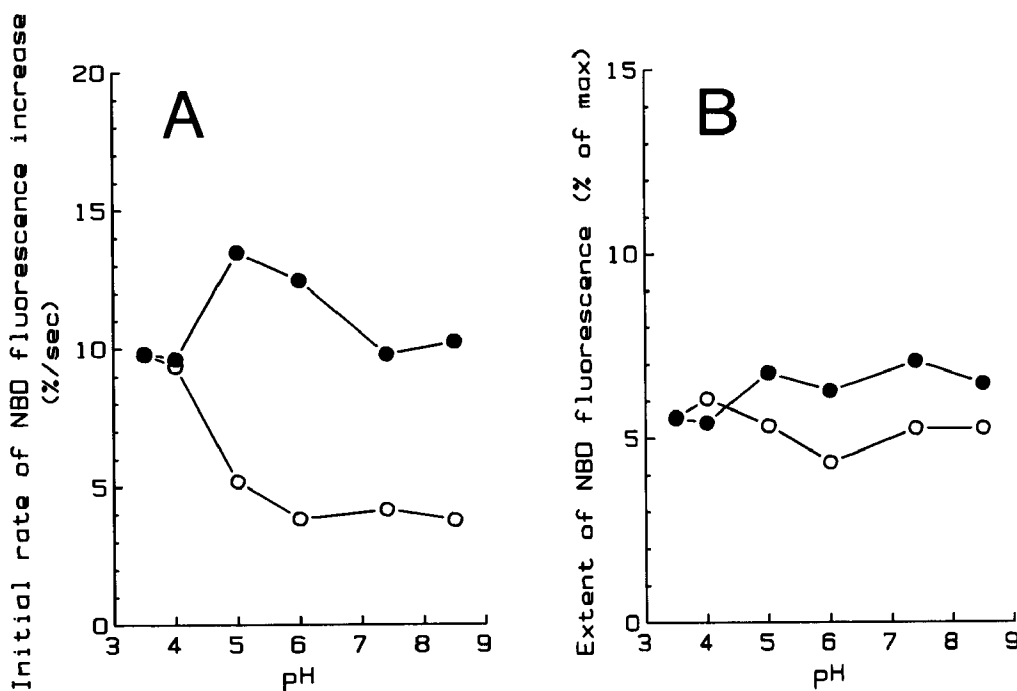
Figure 2.3. Initial rates (A) and extents (B) of lipid mixing as a function of the pH for asymmetric PS-DDP vesicle fusion at 25 (○) and 37°C (●). Experimental conditions: $[Ca^{2+}]$= 4 mM; ratio of labeled to unlabeled vesicles 1:1; total lipid concentration 50 μM.

21

Lowering the pH has a positive effect on the symmetric Ca^{2+}-induced PS fusion. The sole addition of H^+ ions to PS vesicles does lead to vesicle aggregation and fusion, but only at very low pH (pH 2). PS membranes are capable of forming non-bilayer H_{II}structures[77], but only at elevated temperatures (>50°C) or under quite acidic conditions (pH≤3.5). The capacity to form non-bilayer structures has been suggested as aprerequisite for lipids to be fusogenic[18,19]. The increased activity of PS in symmetric fusion reactions at low pH, could find its origin in the formation of these non-bilayer phases.

The profile of the initial rate of fusion versus pH at 37°C completely opposes the changes in the membrane fluidity of DDP bilayers as a function of pH[18,65]: increases in fusion rates correlate with decreases in bilayer fluidity.

The results of the pH-dependent asymmetric PS-DDP fusion may be explained in terms of the influence of the pH on the symmetric DDP vesicle fusion. The symmetric fusion reactions (DDP-DDP and PS-PS) are competing with the asymmetric fusion process. The DDP fusion activity above T_c is profoundly affected by changes in the pH[18,65]. Below pH 7.4 a large decrease in symmetric DDP fusion occurs. Since asymmetric PS-DDP fusion is accompanied by the symmetric DDP side reaction, a decrease in symmetric DDP fusion would favor asymmetric fusion. The decrease in fusion rate below pH 5.0 at 37°C is likely to be caused by the increase in the symmetric DDP fusion reaction at low pH[18,65].

Lowering of the pH raises the phase transition temperature of acidic phospholipids: for PS membranes, when the pH decreases from 7.4 to 3.5, T_c raises from 7°C to about 22°C[94]. However, the membrane fluidity of DAP membranes is optimal around the pK_a of the membrane as reflected by the bell shaped curves of membrane fluidity and T_c versus pH[18,65,95]. Therefore, the increase in asymmetric fusion activity at low pH is likely to be a combination of increased mobility of the DDP molecules, possibly coupled to an increase in fusogenic activity of the PS membranes. Changes in the DDP bilayer, like clustering of the protonated phosphate headgroups, which is known to induce disorder even in Ca(DAP) complexes[96], may also have an influence on the steep increase in fusion activity.

2.6 Effect of temperature on asymmetric vesicle fusion

DDP bilayers are characterized by two phase transitions[68]. A main transition between 22 and 33°C (T_c *ca.* 29°C) and a so-called pretransition between 18 and 22°C, with a midpoint at 20°C. As noted above (Figure 2.1), DDP vesicles do not fuse symmetrically below T_c. Figure 2.4 shows the temperature dependence of Ca^{2+}-induced asymmetric PS fusion with DDP and di-*n*-tetradecylphosphate (DTP) vesicles. Asymmetric PS-DDP fusion occurs even at temperatures as low as 1°C. Below 10°C however, the fusion activity is almost negligible. Raising the temperature leads to a gradual increase of both the initial rate and the extent of asymmetric fusion. The extent of fusion steeply increases between 15 and 20°C, a temperature range that coincides with the pretransition temperature (T_{pre}) region of the DDP bilayers[68,59]. A comparison of the asymmetric fusogenic properties of DTP and DDP vesicles with
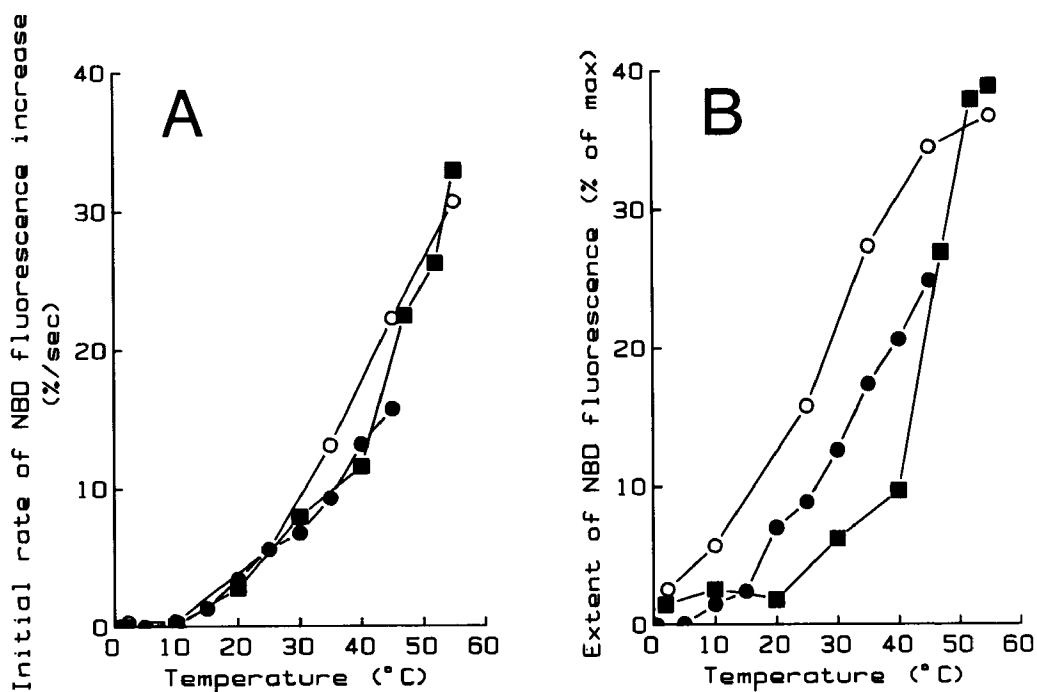
Figure 2.4. Initial rates (A) and extents (B) of lipid mixing as a function of temperature for fusion between PS vesicles and vesicles composed of synthetic amphiphiles. The vesicles were mixed and equilibrated at the indicated temperatures. (O): PS-PS, (●): PS-DDP, (■): PS-DTP. Experimental conditions: $[Ca^{2+}]$= 10 mM; pH 7.4; ratio of labeled to unlabeled vesicles 1:1; total lipid concentration 50 μM.

PS membranes (Figure 2.4), shows a remarkable similarity between the initial rates of asymmetric fusion for both DAPs as a function of temperature. These results indicate that mere elongation of the alkyl chain has little effect on the overall fusion proper ties. This implies that the fusogenic activity may be more dependent on changes in the headgroup area. Indeed, modification of the headgroup of an amphiphilic molecule, with unaltered alkyl chain length does markedly affect its fusogenic properties[25,59]. Similar conclusions can be drawn from the fusogenic activities of various phospholipids[40]. The fusion of DTP with PS also seems to be facilitated around T_{pre} of DTP, which is centered around 30°C[97]. As for DDP-PS fusion this is especially reflected by an increase in the extent of fluorescence.

Remarkable is the similarity of the initial rates of fusion for DDP and DTP with PS between 30 and 45°C. In this temperature range the DDP bilayers are fluid while the DTP membranes are in a solid state (T_c= 48°C). From this and the observation that the fluid DTP bilayer (above 48°C) fuses to a higher extent than the fluid DDP bilayer (above 30°C), it can be concluded that the DTP membranes exhibit a higher

23

susceptibility towards asymmetric fusion with PS than DDP membranes. These observations bear analogy to the spontaneous fusion of sonicated DPPC and DSPC vesicles[98,99,100].

In the case of the DOPC-DDP system, no increase in fusion activity around T_{pre} of DDP (20°C) was observed (Figure 2.5). Instead, a large increase of the initial rate and the extent of fusion is located around 30°C, which corresponds to the T_c of DDP.
Lipid mixing that takes place in the absence of Ca^{2+} ions occurs slowly and without a sudden increase in either initial rate or extent of fluorescence (Figure 2.5).
The Ca^{2+} dependence of asymmetric DOPC-DDP lipid mixing shows a distinct maximum around 4 mM Ca^{2+}, irrespective of whether the DDP membrane is in the solid or the fluid state (not shown). However, the lipid mixing was faster and took place to a higher extent when the DDP bilayer was fluid (above 29°C). The fact that in both cases, a maximum is found at a Ca^{2+} concentration of 4 mM, suggests that a similar fusion mechanism in the DOPC-DDP system is operating both above and below T_c.
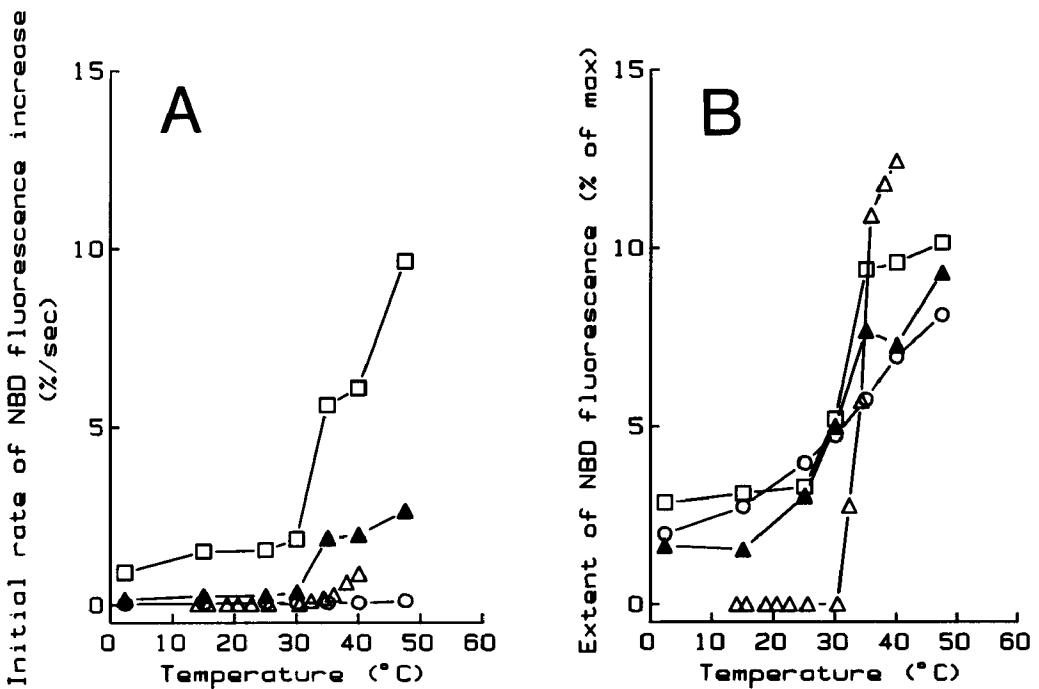
Figure 2.5. Initial rates (A) and extents (B) of lipid mixing as a function of temperature and Ca^{2+} concentration for fusion between DOPC vesicles and DDP vesicles. The vesicles were separately equilibrated at the indicated temperatures. $[Ca^{2+}]= 0$ mM (○), 1 mM (▲) and 4 mM (□). For comparison, symmetric DDP-DDP fusion at 4 mM Ca^{2+} is also shown (△). Experimental conditions: pH 7.4; ratio of labeled to unlabeled vesicles 1:1; total lipid concentration 50 μM.

Since DOPC cannot bind Ca^{2+}, the mechanism for fusion does not involve the formation of a trans Ca^{2+}/lipid complex, as proposed for the Ca^{2+}-induced fusion of acidic phospholipid vesicles[101]. The spontaneous nature of DOPC-DDP fusion indicates that Ca^{2+} is not absolutely required, therefore it might be better to refer to this fusion process as Ca^{2+}-facilitated, rather than Ca^{2+}-induced. Since the presence of Ca^{2+} will affect the DDP vesicles more than the DOPC liposomes, it is tempting tosuggest that Ca^{2+} creates certain structural defects in the DDP membrane that causes them to fuse with the DOPC bilayers. Recently the influence of ionic strength has been postulated to affect the headgroup packing of PC bilayers[102], the effect of Ca^{2+} could therefore also involve changes in the PC membrane.

2.7 Influence of membrane fluidity on asymmetric fusion

Comparing the temperature-dependent asymmetric fusion of DOPC and PS with DDP membranes indicates that DOPC-DDP fusion occurs over the whole temperature range tested (between 1 and 55°C), while PS-DDP fusion becomes significant only above *ca.* 10°C. The T_cs of DOPC and PS are -22 and +7°C, respectively. This might suggest that solid DDP vesicles can only fuse with fluid target membranes (DOPC and PS). To support this notion further, asymmetric fusion experiments were carried out between LUV's (labeled with N-NBD-PE and N-Rh-PE) of the synthetic amphiphile di-*n*-tetradecylphosphate (DTP, T_c= 48°C) and synthetic di-myristoylphosphatidylserine (DMPS, T_c= 38°C) with unlabeled DDP vesicles as a function of temperature. The threshold temperature for lipid mixing (the temperature at which the initial rate of

Table 2.1. 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
DOPC	-22	+ 1[c]	[d]
PS	+ 7	+ 7	+ 7
DDP	+29	+29	+29
DMPS	+38	+38[e]	+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]. [c] Lowest temperature that was measured. [d] Not determined. [e] Vesicles of the phospholipid DMPS cannot fuse while in the gel-phase with the fluid DDP membranes. This typical behaviour of phospholipid vesicles emphasizes the remarkable property of the DAP vesicles.

NBD-fluorescence increase exceeds 0.1 %s^{-1}) was determined for the various target membranes. The results, summarized in Table 2.1, support the view that synthetic DAP vesicles (DDP, DTP) only fuse with target membranes in the liquid crystalline state, irrespective of the physical state of the DAP membrane. In view of this property, DAP vesicles distinguish themselves from phospholipid vesicles, that have to be in an overall fluid state to engage in fusion processes[83,103]. It also provides a tool to design specifically asymmetric fusing systems.

2.8 Asymmetric aggregation behaviour

Ca^{2+}-induced vesicle aggregation is a reversible, second order process[104], that pre-ceeds the actual fusion step. This fusion step is normally rate determining in the overall fusion process of vesicles[105]; aggregation usually happens on a much faster time-scale. Studies of the initial aggregation behaviour of the asymmetrical fusion processes (PS-DDP and DOPC-DDP) can yield information concerning the events that occur at the beginning of the asymmetric fusion process. The rate equation for the second-order reaction of asymmetric aggregation between DDP vesicles and phospholipid vesicles (DOPC or PS), is given by :

$$V_{aggr} = k_1[\text{Phospholipid}]^2 + k_2[\text{DDP}]^2 + k_3[\text{Phospholipid}][\text{DDP}] \qquad (2.1)$$

in which V_{aggr} represents the overall initial aggregation rate and k_1, k_2 are the rate constants for the respective symmetric aggregation processes. The term $k_3[\text{Phospholipid}][\text{DDP}]$ indicates the initial rate of asymmetric aggregation: V_{asym}. By rearranging equation (2.1), V_{asym} can be expressed as:

$$V_{asym} = V_{aggr} - k_1[\text{Phospholipid}]^2 - k_2[\text{DDP}]^2 \qquad (2.2)$$

Experimentally, it can easily be verified that Ca^{2+}-induced aggregation is indeed a second-order process (V_{aggr} can be determined experimentally). After having estab-lished aggregation at a given concentration, a doubling of this concentration should lead to a 4-fold increase in the rate of aggregation. In the case of Ca^{2+}-induced aggregation of either PS or DOPC with DDP vesicles, this is exactly what is observed (Figure 2.6).

From these curves, the initial rate of asymmetric aggregation, V_{asym} (eq.(2.2)) can be obtained by subtracting the symmetric rates of aggregation for PS (or DOPC) and DDP vesicles. The curves thus obtained show that asymmetric PS-DDP aggregation is almost three times faster than symmetric aggregation of PS liposomes and more than an order of magnitude faster than DDP-DDP vesicle aggregation (Figure 2.6a). For DOPC-DDP aggregation a distinct maximum is observed at 4 mM Ca^{2+} (Figure 2.6b). Note that this maximum correlates very well with that of Ca^{2+}-induced DOPC-DDP fusion (Figure 2.1). At 4 mM Ca^{2+}, the asymmetric aggregation is 3.5 times faster than either of the two symmetric aggregation processes (DOPC-DOPC and DDP-DDP, Figure 2.6b). At higher Ca^{2+} concentrations, the rate of asymmetric aggregation
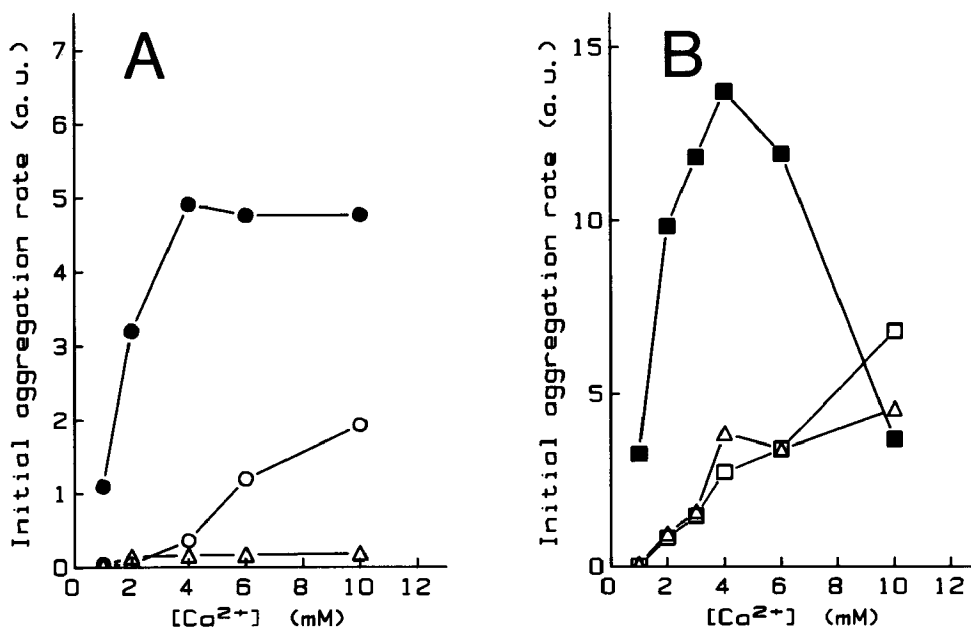
Figure 2.6. Ca^{2+}-induced aggregation of vesicle suspensions containing either one or two vesicle species. The initial rates of asymmetric aggregation were calculated according to eq.(2.2) and plotted as a function of the Ca^{2+} concentration. Symmetric aggregation rates were obtained experimentally.

(A) Symmetric aggregation of DDP ($\triangle$) and PS ($\bigcirc$) vesicles (total lipid concentration 25 μM) and the calculated curve ($\bullet$) for asymmetric PS-DDP vesicle aggregation (eq. (2.2), the total lipid concentration was 50 μM). (B) Symmetric aggregation as in (A), for DDP ($\triangle$) and DOPC ($\square$) vesicles (the total lipid concentration was 25 μM). ($\blacksquare$): calculated DOPC-DDP cross aggregation at a total lipid concentration of 50 μM. Experimental conditions: pH 7.4; 25°C; ratio of phospholipid to DDP vesicles 1:1. The differences in the symmetric aggregation rates are due to the different wavelengths at which the turbidity was monitored: (A) at 400 nm, (B) at 250 nm.

decreases: at 10 mM Ca^{2+}, the initial rate of asymmetric aggregation is slower than the initial rates of symmetric aggregation.

It is relevant to note that at 4 mM Ca^{2+} almost complete charge neutralization is achieved for acidic phospholipid bilayers[106]. This may also occur for DDP vesicles, explaining the levelling-off of the Ca^{2+}-induced aggregation of the DDP vesicles at 4 mM Ca^{2+}. The distinct maximum in both the Ca^{2+}-induced aggregation and fusion at 4 mM Ca^{2+} then can be ascribed to a change in the net surface charge of the fusion products. Above 4 mM Ca^{2+}, the net surface charge of the DOPC-DDP fusion product becomes positive, as a consequence of the randomization of DDP in the DOPC bilayers and the binding of one Ca^{2+} ion per DDP molecule in these randomized bilayers. The resulting electrostatic repulsion could prevent additional fusion between DDP vesicles and the DOPC-DDP fusion products. Since potentially "active" DDP fusion sites in the fusion product, are eliminated by mixing and randomizing of

27

DDP with the DOPC (a process that stabilizes the bilayer structure of DDP), any fusion between fusion products and DOPC liposomes might be prevented.

These results indicate that Ca^{2+}-induced aggregation of DDP vesicles with PS liposomes results in preferential asymmetric PS-DDP aggregation, which is followed by asymmetric fusion. In the case of the DOPC-DDP system, the occurrence of preferential asymmetric aggregation depends on the Ca^{2+} concentration.

At a fixed Ca^{2+} concentration, the asymmetric aggregation behaviour of the PS-DDP system was studied and V_{asym} was determined as a function of temperature (Figure 2.7). A gradual increase of the symmetric aggregation rates with temperature is observed. The asymmetric aggregation rate, however, exibits a steep decline above T_c of DDP (29°C). An increase in the aggregation rate with temperature is expected[84] since it is also found for the symmetrically aggregating systems. The large decrease in
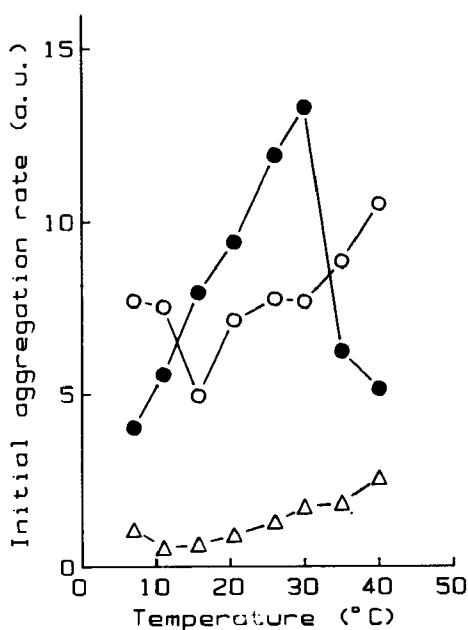
Figure 2.7. Ca^{2+}-induced aggregation of vesicle suspensions containing either one or two vesicle species. The initial rates of asymmetric PS-DDP aggregation were calculated according to eq.(2.2), and plotted as a function of the temperature (●), the total lipid concentration was 50 μM. Symmetric aggregation rates of DDP (△) and PS (○) vesicles (total lipid concentration 25 μM) are experimentally determined. $[Ca^{2+}]$= 4 mM, pH 7.4.

asymmetric aggregation rate appears to be related to the phase transition of the DDP membranes. The transition from a liquid crystalline state to a gel state will affect the hydration of the bilayer, since the headgroup packing will change as a consequence of the change in the hydrocarbon packing.

Repulsive protrusion and undulation forces can become involved and the repulsive hydration force increases as the membrane becomes more fluid[107,108]. Since symmetric DDP aggregation is hardly affected around T_c, there must also be other contributions to explain the sudden decrease in asymmetric aggregation rate. These contributions may find their origin in the so-called structural forces, that operate at short distances ("the last 30 Å"[48,109]).

28

The reduced asymmetric aggregation can be a result of either an increased symmetric aggregation rate constant k_2, or a decrease in k_3, the asymmetric aggregation rate constant (eq.(2.2)). Since the symmetric aggregation reactions do not reveal a sudden increase, a decrease in k_3 appears to account for the abrupt decrease in initial asymmetric aggregation at 30°C.

2.9 Characterization of the asymmetric fusion process and its fusion products

2.9.1 Electron microscopy

Symmetric fusion of DDP vesicles results in the formation of large, fused vesicles (diameter 700 nm *vs.* 70 nm of the original vesicles), which transform into long tubular structures. Initially, these tubular structures have erroneously been characterized as an inverted hexagonal H_{II} packing[69]. Recently, it has been established that these tubular structures are lamellar $Ca(DDP)_2$ crystals[73]. The formation of these lamellar crystals can readily be seen with both fluorescence and electron microscopy, and is indicative for the occurrence of symmetric DDP fusion.

As shown in Figure 2.8, symmetric DDP fusion is substantially reduced during asymmetric fusion with either PS or DOPC. In the DOPC-DDP system, Ca^{2+}-induced fusion results in the formation of larger vesicles and fairly small rods (Figure 2.8d), which contrasts with the long tubules that form upon symmetric DDP fusion. This result supports the occurrence of true asymmetric fusion, since DOPC liposomes resist Ca^{2+}-induced symmetric fusion. The PS-DDP system revealed a complete absence of symmetric DDP fusion products (tubes). Therefore it can be concluded that both DOPC and PS stabilize the DDP bilayer structure.

The difference between PS and DOPC in this stabilizing ability, reflects the extent of
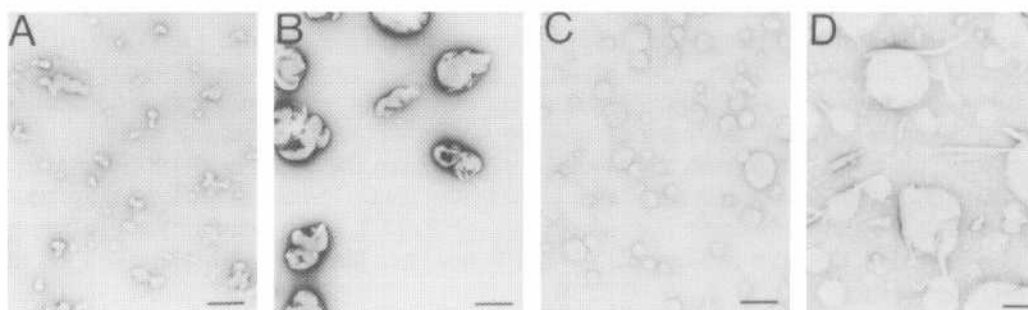
Figure 2.8. Electron micrographs of asymmetric PS-DDP and DOPC-DDP fusion products. A and B show the asymmetric mixture of PS and DDP vesicles (ratio 1:1), before (A) and after (B) addition of Ca^{2+}. (C) A 1:1 mixture of DOPC and DDP vesicles before addition of Ca^{2+}. Electron micrograph D is obtained after addition of Ca^{2+}. The bars represent 300 nm.

fusion taking place for both asymmetric systems. These results may be rationalized by a difference in Ca^{2+}-induced interactions. PS-DDP can interact in a trans Ca^{2+} complex, whereas DOPC-DDP is less prone to do.

2.9.2 Sucrose gradient analysis

The complexity of the DDP-PS system will increase upon passing the T_c of DDP. Above T_c, one asymmetric and two symmetric fusion reactions take place simultaneously between *all* vesicles and any other vesicle or fusion product. In comparing the fusion processes above and below T_c, it was observed that there is a marked difference in aggregation behaviour (see section 2.8). Below 29°C, the asymmetric aggregation is several times faster than any of the symmetric aggregation processes, while above T_c the asymmetric aggregation rate is smaller -by a factor 2- than the symmetric rate of PS aggregation (Figure 2.6). This change in aggregation kinetics might well result in the formation of fusion products of varying nature above and below T_c.

Therefore the composition of the fusion product(s) of asymmetric PS-DDP fusion was studied above T_c, using continuous sucrose gradient analysis[110]. After fusion was induced by the addition of Ca^{2+}, the samples were left to fuse for 10 minutes at 37°C. Then a fourfold molar excess (based on Ca^{2+}) of EDTA was added, prior to the layering of the samples on top of the gradients.

Non-fused vesicles of DDP and PS remained near the top of the gradient (not shown). After symmetric fusion of both DDP and PS a shift to higher densities was found (Figure 2.9). About 40% of the DDP vesicles was recovered near the top of the gradient at a position similar to that of non-fused DDP vesicles. The majority of the DDP fraction (60%) was found at a density of approximately 18% (w/w) sucrose. Symmetric fusion of PS vesicles -under identical conditions- revealed similar characteristics: a small fraction of unfused vesicles centered near the top of the gradient, and the major PS fraction at a density of *ca.* 16% (w/w) sucrose.

The remarkable distinctions in density of the DDP and PS symmetric fusion products then allowed a similar analysis for Ca^{2+}-induced asymmetric PS-DDP fusion. The results, shown in Figure 2.9, indicate that asymmetric fusion is incomplete, in accord with the symmetric fusion which was also far from complete. Surprisingly, the asymmetric fusion product has a lower density (*ca.* 14% sucrose (w/w)) than both symmetric fusion products. After asymmetric PS-DDP fusion, none of the symmetric fusion products could be detected, indicating that fusion finally leads to a randomization of lipids and that the number of pure symmetric fusion products must be quite small. This is consistent with the results obtained from the TEM analysis (Figure 2.8). Analysis of the peak fractions of the asymmetric fusion products by lipid extraction, high performance thin layer chromatography (HPTLC), and phosphorous determination revealed that the ratio of DDP to PS was 1:1, identical with the ratio of the starting vesicles.

To exclude the possibility that the fractions recovered at higher sucrose density merely reflect the presence of aggregates rather than fusion products, the experiments (summarized in Figure 2.9) were repeated with labeled PS liposomes (0.8 mole % N-NBD-PE and N-Rh-PE). By comparing the resonance energy transfer of the peak fraction of the Ca^{2+}-induced asymmetric fusion product with that of the starting
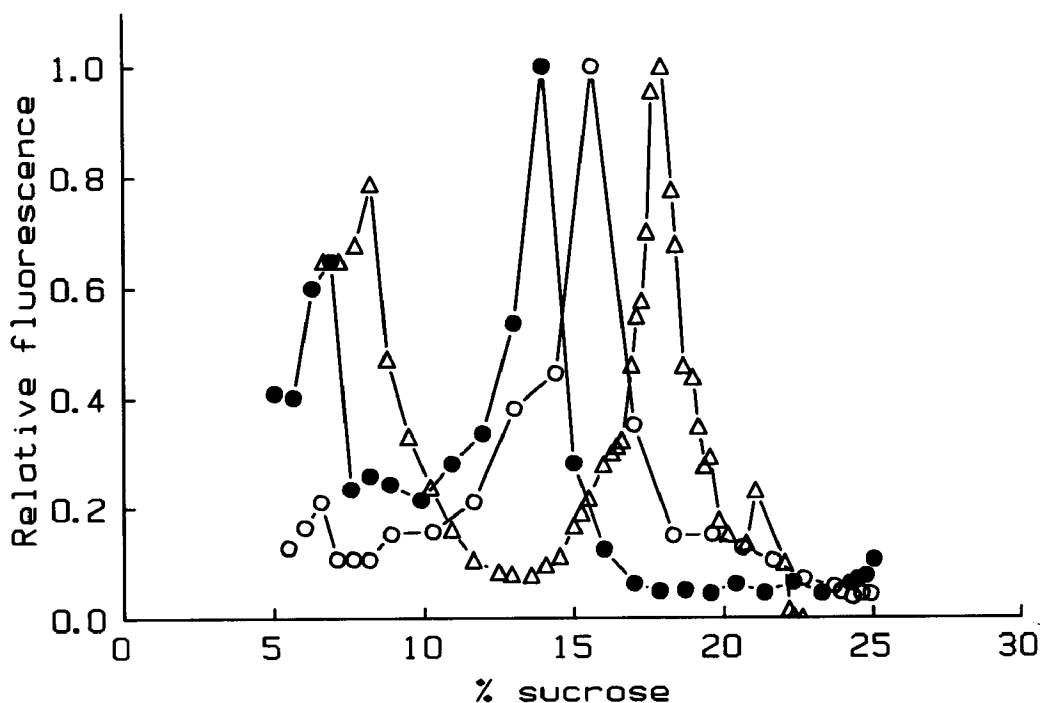
Figure 2.9. Sucrose density gradient analysis of PS and DDP vesicles after Ca^{2+}-induced symmetric and asymmetric fusion. Fusion was induced by incubating equimolar amounts of vesicles (final concentration 2 mM) with Ca^{2+} (10 mM) for 10 minutes at 37°C. N-NBD-PE (0.8 mole %) was used as a membrane marker to label the vesicles. After treatment with 40 mM EDTA, the vesicle mixtures were layered on top of the continuous sucrose gradients (0-25%). The fractions were analized as described under Experimental. (○): PS-PS, (△): DDP-DDP, and (●): PS-DDP.

vesicles an estimate could be made regarding the extent of lipid mixing. The observed increase of *ca.* 50% of N-NBD-PE fluorescence is consistent with a 1:1 probe dilution, as was found directly by analyzing the lipid composition of the peak fractions.

Although the physical basis for the separation of the fusion products on sucrose gradients is as yet unclear (it is not merely based on density), it does allow the conclusion that complete randomization occurs between PS and DDP during asymmetric fusion.

2.10 On the possible origin of asymmetric PS-DDP fusion

Some preliminary experiments were carried out to delineate the molecular mechanisms that may cause the DDP vesicles to fuse with PS liposomes below their phase transition temperature. Firstly, the influence of PS vesicles on the physical state of DDP vesicles was investigated. A mixture of DDP vesicles and PS liposomes (ratio

1:1) was incubated at various temperatures and subsequently the NMR spectra were recorded. On decreasing the temperature, the ^{31}P-NMR signal of the DDP vesicles did not show any anomalies compared to that of pure DDP vesicles: abrupt line broadening around 29°C, culminating at slightly lower temperatures in a completely vanished ^{31}P signal. These results reveal there is no macroscopic "long-range" effect of PS liposomes on the membrane fluidity of the DDP vesicles.

During collisions phopholipid vesicles are said to be capable of exchanging material both in the gel and the liquid crystalline state[111,112,113]. To investigate whether PS transfer to DDP bilayers may also occur, DDP-PS vesicles were prepared with varying PS contents (0-20%). The phase transition of the vesicles as a function of the PS content, was measured using differential scanning calorimetry (DSC)[114].

As is shown in Table 2.2, the DDP membranes are affected by the presence of very small amounts of PS. Already at 1 mole %, an amount that could have been transferred spontaneously during the incubation of the vesicle mixture during an asymmetric fusion experiment, pronounced effects are observed. Especially the packing of the molecules is influenced as reflected by the lowered ΔH. Extrapolating these data, it can be concluded that after asymmetric fusion of a single DDP vesicle with a PS liposome of comparable size, the resulting fusion product will be a vesicle with a T_c below 25°C.

In a separate RET fusion experiment, it was investigated whether the local packing of the bilayer or the macroscopic property, T_c was the dominant parameter in the symmetric fusion process. A symmetric fusion experiment was carried out with DDP vesicles containing 1 mole % of PS. The presence of 1% of PS in the DDP membrane was insufficient to render these "mixed" vesicles symmetric fusion ability at 25°C. Thus, neither the macroscopic influence of small amounts of PS on the fluidity of the DDP bilayer nor the microscopic effect of PS on the DDP membrane lead to sufficient perturbations in the DDP bilayer to make them susceptible towards symmetric fusion at 25°C.

The influence of the PS content of the DDP membrane was also studied with temperature dependent ^{31}P-NMR. DDP molecules packed in a bilayer above the T_c, possess many degrees of freedom (axial rotation, fast lateral diffusion in the plane of the bilayer), resulting in motions of the phosphate headgroup that are isotropic on the

Table 2.2. Effect of PS on the Phase Transition Temperature of DDP-PS Vesicles as Determined by DSC[a].

Vesicle Composition mole % of PS	Phase Transition Temperature, T_c (°C)	ΔH (J/g of lipid)
0	30.3	44.5
1	29.3	25.3
20	25.7	19.0

[a] Results obtained from cooling scans.
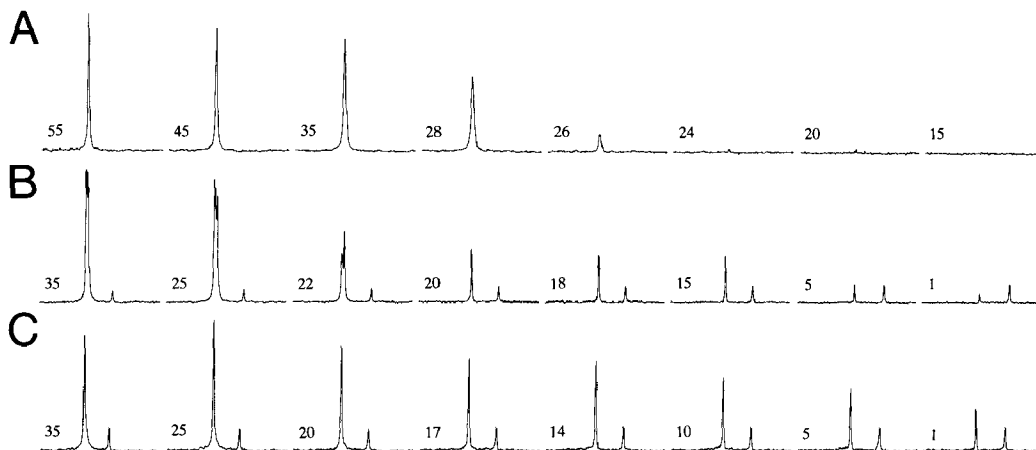
Figure 2.10. ^{31}P-NMR spectra of DDP vesicles with varying PS contents as a function of temperature (°C).

^{31}P-NMR time scale[115]. At temperatures below the T_c, the molecular motions are severely hindered due to the frozen state of the hydrocarbon interior of the bilayer. This will broaden the ^{31}P-NMR signal as a result of the chemical shift anisotropy[116].

Monitoring the ^{31}P-NMR line width of DDP vesicles as a function of decreasing temperature discloses a small decrease rather than in an increase in line width. The intensity of the DDP ^{31}P-NMR signal (the peak integral), also decreases below T_c on lowering the temperature. This results in a loss of information, the line width is no longer a reliable parameter of the headgroup mobility.

The presence of small amounts of PS in the DDP bilayer has consequences for the ^{31}P spectrum. A second ^{31}P resonance peak appears at high field (Figure 2.10). This smaller signal is almost insensitive towards temperature changes, with respect to both its line with and resonance integral. The large resonance peak, located at the DDP resonance frequency is strongly affected by the temperature. The signal almost completely vanishes for samples that contain no or only a small amount (0-10%) of PS, while for the samples with 20 and 40% PS the peaks do not disappear (Figure 2.11).

Under the conditions used, no signal could be detected for pure PS liposomes in a separate experiment. We therefore presume that the two signals observed both originate from DDP. The appearance of two ^{31}P signals points towards two different types of DDP: "free" DDP and PS-modulated DDP. Vesicles containing two lipids with different headgroups often display separate ^{31}P resonances from the two components. This allows a determination of the chemical composition of both

33
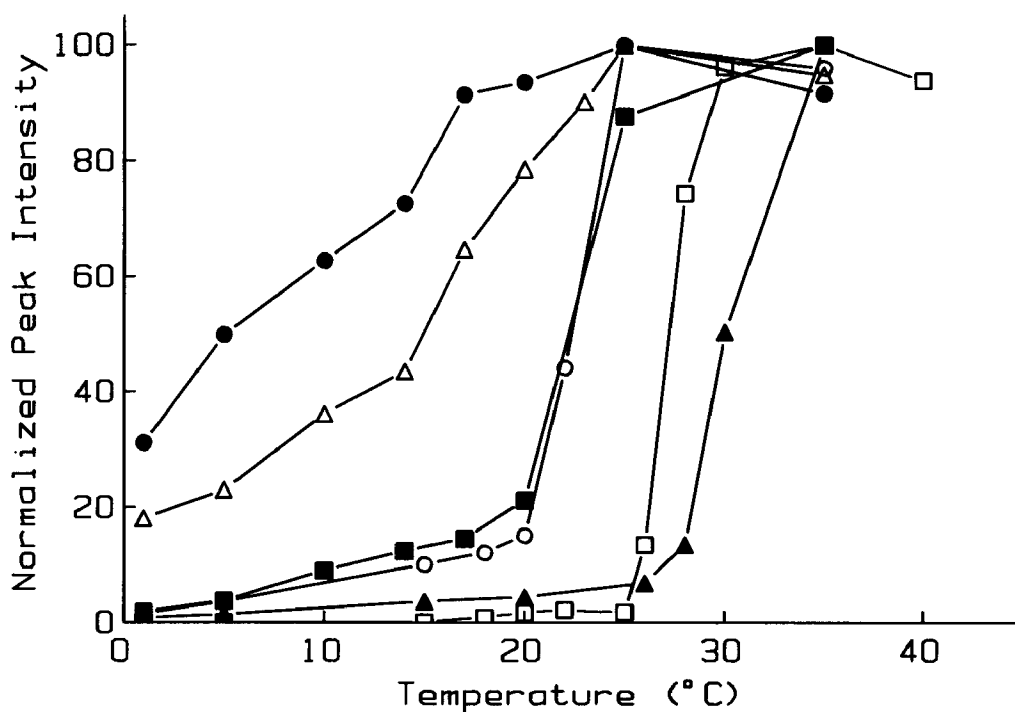
Figure 2.11. Relative ^{31}P-NMR line intensities of DDP/PS vesicles with various PS contents, as a function of temperature. The spectra were recorded during cooling experiments, the maximum intensity (integral) of the ^{31}P was taken as 100%. Mole percentages PS: (□): 0%, (▲): 1%, (○): 5%, (■): 10%, (△): 20%, (●): 40%.

monolayers[117]. Since DDP vesicles do not respond to shift reagents in a straight-forward manner, this method could not be used.

The smaller second peak does increase with higher contents of PS in the bilayer, but there is no obvious relation between the peak integral and the "PS-feeding ratio" of the vesicles. When non-distorted DDP is characterized by a cooperative decrease in intensity of the large ^{31}P-NMR signal, we can say that this decrease is getting less cooperative when more PS is incorporated in the bilayer. Between 10 and 20% of PS seems to be required to influence most of the DDP molecules. Addition of more PS might lead to phase separation phenomena (in mixed phospholipid membranes (PC/PS) phase separation only takes place upon addition of divalent cations[118]), possibly this is attended by an asymmetric distribution of PS over the two monolayers. These phenomena might be affected by changes in temperature and result in a quite complex system.

Phase separation processes have been proposed to be of importance during membra-

34

ne fusion of some membranes[94]. The patches enriched in some lipid are thought to be the active sites. However, it appeared that some membranes (PS, small unilamellar vesicles) revealed phase separation much slower than fusion [118]. Yet, the boundaries between these rich and poor patches have never been awarded a role in any fusion mechanism. It may rather be the nature of these phase boundaries than the phase separation itself that determines the fusion process.

Recently, the cooperativity of the gel-to-liquid crystal phase transition has been questioned, as distinct patches of amphiphiles might undergo the gel-to-liquid crystal transition as separate units[119]. It is tempting to suggest that the boundaries between these patches could then function as a nucleation point for fusion. Likewise, the PS-distorted and the undistorted DDP regions could form patches with the concomitant boundaries. However, since DDP vesicles containing 1% PS do not fuse symmetrically, the existence of these boundaries is not the only prerequisite for fusion to occur.

2.11 Conclusions

In this chapter it has been shown that simple synthetic DAP vesicles can fuse asymmetrically with phospholipid membranes. Asymmetric fusion is only established when the target membrane for DAP is in a fluid state. With this knowledge, specific asymmetrically fusing systems can be designed (DOPC/DDP, below T_c of DDP). However, even in systems where symmetric fusion of one or both populations of the asymmetrically fusing system may take place (PS/DDP), asymmetric fusion still occurs avidly.

An asymmetric fusion reaction may require a lower extent of dehydration of the head-groups than symmetric fusion reactions do, as illustrated by the fact that Mg^{2+} can induce asymmetric PS-DDP fusion (although only under all-fluid membrane conditions).

The property of these synthetic amphiphiles to indulge in asymmetric fusion may be relevant to enhance the efficiency of delivery of lipophilic and hydrophilic drugs or macromolecules into cells by specific asymmetric fusion of the vesicles with the target (cell)membrane. Fusogenic phospholipid vesicles hardly fuse with cell surfaces, due to extensive symmetric fusion of the phospholipid vesicles prior to their interaction with the cell. Eliminating all symmetric interactions will result in improved asymmetric fusion and vesicles prepared from synthetic amphiphiles may serve that purpose. The factors governing the asymmetric fusion properties need to be further investigated, in order to develop systems that exhibit improved specific asymmetric fusion activity.

2.12 Experimental procedures

Materials.
Di-*n*-dodecylhydrogen phosphate was purchased from Alpha Chemicals. Sodium di-*n*-dodecylphosphate (DDP) was prepared by disssolving the acid in warm ethanol (50°C) and by subsequent addition of an equimolar amount of sodium ethanolate, followed

by crystallization. Sodium di-*n*-tetradecylphosphate (DTP) was synthesized according to Bauman[120]. N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (N-NBD-PE), N-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (N-Rh-PE), phosphatidylserine (PS, bovine brain), dioleoylphosphatidylcholine (DOPC) and dimyristoylphosphatidylserine (DMPS) were obtained from Avanti Polar Lipids, Inc.. 4-(2-Hydroxyethylpiperazineethanesulfonic acid (HEPES) was purchased from Sigma Chemical; sodium acetate, ethylenediaminetetraacetic acid (EDTA) and calcium chloride were obtained from Merck. Water was distilled twice in an all quartz apparatus.

Vesicle preparation.
DDP, DTP and DMPS vesicles were prepared by the ethanol injection method[69]. Briefly, 10 mg of the amphiphile was dissolved in 100 μL of ethanol. 80 μL of this solution 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 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, PS or CL were prepared by reverse phase evaporation[121,122]. 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 continuous monitoring of turbidity changes at 250 or 400 nm (for the DOPC-DDP and the PS-DDP system, respectively) 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. The data presented in the figures represent average values of two independent experiments, each of which was performed in duplo. The reproducibility of the obtained initial aggregation rates was usually within 10%.

Vesicle fusion.
Membrane fusion was followed by monitoring lipid mixing, with an assay based on resonance energy transfer[52,118]. Vesicles (usually the phospholipid vesicles) containing 0.8 mole % each of N-NBD-PE and N-Rh-PE were mixed with an equimolar amount of unlabeled vesicles (mostly the DAP vesicles). Fusion was induced by the addition of Ca^{2+}. Continuous monitoring of the relief of energy transfer, as reflected by the increase of NBD fluorescence, was performed on a SLM-Aminco SPF-500C spectrofluorometer, equipped with a thermostated cell holder, a magnetic stirring device and a chart recorder. Samples were exited at 465 nm, the emission was followed at 530 nm. The fluorescence scale was calibrated by setting the initial fluorescence of the non-fused labeled vesicles equal to 0% fluorescence. 100% fluorescence (infinite dilution) 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 surfactants 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 mole % each of the fluorescent probes and the appropriate amounts of lipid and synthetic amphiphile. All fusion experiments were carries out in a 5 mM sodium acetate/5 mM HEPES buffer, pH 7.4.

Electron microscopy.
Vesicles and fusion products were analyzed by transmission electron microscopy. Samples were prepared as described previously[69]. The suspensions were stained with a 1% (w/v) aqueous 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 EM 300 electron microscope, operating at 80 kV.

Differential scanning calorimetry (DSC).
DSC-scans were obtained using a Perkin Elmer DSC 7. The vesicle samples were prepared according to Shavnin[114].

^{31}P NMR spectroscopy.
Spectra were recorded under carefully controlled conditions, as has been described previously[97]. The spectra recorded as a function of temperature always result from cooling experiments, the first spectrum being taken at the highest temperature. At each temperature the sample was equilibrated over a period of 15 minutes prior to data collection.

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 minutes. Addition of a 4-fold molar excess of EDTA paralyzed the fusion reaction. The samples were then layered on top of the gradients (13 mL). After centrifugation (room temperature, 30000 rpm, 3 hours in an SW41 Beckman rotor), fractions (*ca.* 200 μL) were collected from the bottom of the tube, and the densities were determined on the basis of their refractive indices. 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 high preformance thin layer chromatography (HPTLC) analysis, with $CHCl_3$/MeOH/25% NH_3/H_2O (70/30/4/1, v/v) as the running solvent. The lipid composition was quantified by a phosphorous determination, after scraping the spots from the HPTLC-plate.