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A closer look at calcium-induced interactions between phosphatidylserine-(PS) doped liposomes and the structural effects caused by inclusion of gangliosides or polyethylene glycol- (PEG) modified lipids

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ABSTRACT

The effects of polyethylene glycol- (PEG) modified lipids and gangliosides on the Ca^{2+} induced interaction between liposomes composed of palmitoyl-oleoyl phosphatidylethanolamine (POPE) and palmitoyl-oleoyl phosphatidylserine (POPS) was investigated at physiological ionic strength. Förster resonance energy transfer (FRET) studies complemented with dynamic light scattering (DLS) and cryo-transmission electron microscopy (Cryo-EM) show that naked liposomes tend to adhere, rupture, and collapse on each other's surfaces upon addition of Ca^{2+}, eventually resulting in the formation of large multilamellar aggregates and bilayer sheets. Noteworthy, the presence of gangliosides or PEGylated lipids does not prevent the adhesion-rupture process, but leads to the formation of small, long-lived bilayer fragments/disks. PEGylated lipids seem to be more effective than gangliosides at stabilizing these structures. Attractive interactions arising from ion correlation are proposed to be a driving force for the liposome-liposome adhesion and rupture processes. The results suggest that, in contrast with the conclusions drawn from previous solely FRET-based studies, direct liposome-liposome fusion is not the dominating process triggered by Ca^{2+} in the systems studied.

1. Introduction

The aggregation and fusion of liposomes induced by Ca^{2+} has been a subject of study for several decades [1–6]. The phenomenon has been used to mimic lipid-mediated cell and viral fusion events and explain the role that Ca^{2+} and other divalent ions play in these (e.g. [3,6]). In several cases, addition of Ca^{2+} has also been used in order to facilitate the formation of supported lipid bilayers (SLBs) via liposome adhesion and spreading on a variety of surfaces, e.g., mica [7], silica [8], indium-tin oxide [9], and titanium dioxide [10]. Although anionic (particularly phosphatidylserine- (PS) based) liposomes are considered to be more sensitive to the presence of Ca^{2+}, addition of the ion has been suggested to improve and/or trigger fusion events and formation of SLBs also for liposomes built from zwitterionic lipids alone [11,12].

The molecular mechanism by which Ca^{2+} facilitates the aggregation and fusion of liposomes has been discussed extensively. In the case of PS-containing liposomes, it is generally agreed that a high affinity of Ca^{2+} for the PS headgroup leads to liposome aggregation and fusion [3,13]. A similar, although weaker, affinity has been observed between Ca^{2+} and other negatively charged (e.g. phosphatidylglycerol, PG) and zwitterionic (e.g. phosphatidylcholine, PC) lipid headgroups [14,15], accounting thus for the widespread effect of Ca^{2+}. In the case of liposome rupture and spreading on surfaces, it is generally believed that Ca^{2+} serves to screen the electrostatic repulsion between the surfaces and the liposomes, as well as to trigger liposome fusion events at the surface, which can facilitate the formation of SLBs [7,10].

In previous studies the most used approach to characterize liposome-liposome fusion (and the role of Ca^{2+}) has been the monitoring of either the lipid mixing or the intermixing of the aqueous contents [16]. For the former, fluorescence assays based on Förster resonance energy transfer (FRET) between labeled lipids incorporated in part of the total liposome population are often used [17], whereas for the latter liposomes filled with an encapsulated fluorescent dye are mixed with liposomes filled with an appropriate quencher [18]. Alternatively, the two populations of liposomes can be filled with complementary reagents that give a fluorescence response upon mixing [19]. In all cases, the changes in fluorescence intensity over time are then related to the degree of liposome-liposome fusion. It is to be noted, however, that the results

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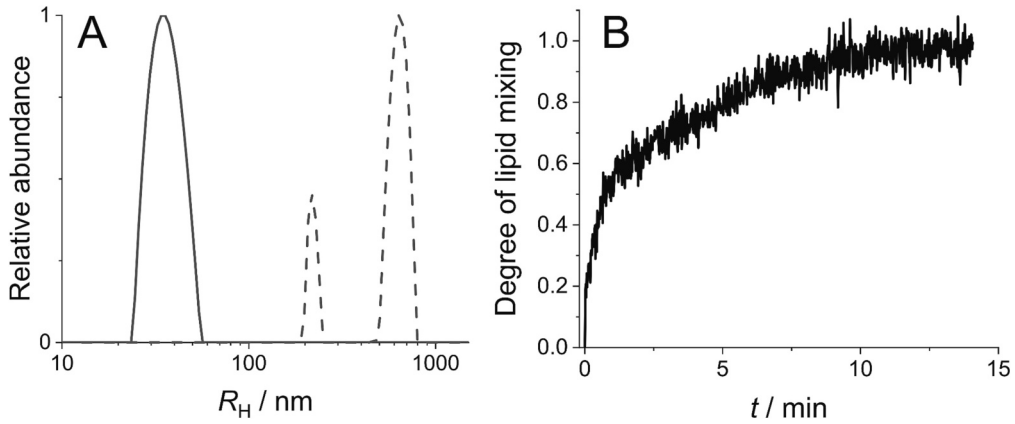

Fig. 1. A: Intensity weighted hydrodynamic radius (R_H) distribution of POPE:POPS 75:25 liposomes before (solid line) and 30 min after (dashed line) the addition of 10 mM Ca^{2+}. B: Determined degree of lipid mixing between POPE:POPS 75:25 liposomes as a function of time after Ca^{2+} addition.

from studies examining lipid mixing do not always agree with the conclusions from studies based on monitoring the intermixing of the aqueous contents [20], implying that the results from these kind of experiments need to be interpreted with caution.

In this report, the effect of Ca^{2+} on liposomes composed of palmitoyl-oleoyl phosphatidylserine (POPS) and phosphatidylethanolamine (POPE) is explored by FRET measurements complemented with dynamic light scattering (DLS) and cryo- transmission electron microscopy (Cryo-EM) determinations. A mixture of PS and phosphoethanolamine (PE) lipids was chosen as this mixture is among the most commonly used in previous FRET-based studies on Ca^{2+} induced liposome-liposome fusion (e.g. [2,4,5,21]). The results indicate that, in contrast with the conclusions drawn from previous FRET-based studies, liposome-liposome fusion is only one of two main processes triggered by the presence of Ca^{2+}, the other one being the adhesion, rupture, and collapse of liposomes on each other.

In the present study we moreover explore how incorporation of lipids modified with polyethylene glycol (PEGylated lipids) and gangliosides affect the liposomes' response to Ca^{2+}. The presence of PEGylated lipids has been reported to inhibit the fusion process, likely by preventing close contact between the liposomes [21]. Our results indicate, however, that neither PEG nor gangliosides prevent liposome-liposome aggregation, adhesion nor rupture. Instead, their presence result in the formation of open bilayer structures with good colloidal stability.

2. Materials and methods

2.1. Materials

Total ganglioside extract (porcine brain, ammonium salt, >99 %), chloroform (pro analysis) and methanol (pro analysis) were purchased from Merck KGaA (Darmstadt, Germany). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000, 98 %) ammonium salt was obtained as a kind gift from Lipoid GmbH (Ludwigshafen, Germany). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-750] (DSPE-PEG750), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-1000] (DSPE-PEG1000) were bought from Avanti Polar Lipids (Alabaster, USA). Lissamine™ Rhodamine B 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine (rhodamine-PE) and N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine (NBD-PE) were purchased from Thermo Fischer Scientific.

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, >99.5 %), calcium chloride, and sodium chloride were purchased from Sigma Aldrich (St. Louis, Missouri, US).

All aqueous solutions were prepare using deionized water (18.2 MΩ cm) from a Milli-Q plus system from Millipore (Bedford, USA).

2.2. Preparation of liposomes

Liposome components were weighed or pipetted from stock solutions in chloroform or methanol. A lipid film was obtained by evaporation of the organic solvent, first with a gentle stream of nitrogen followed by removing the residual solvent in a vacuum oven (Lab instruments IL, USA) overnight. The dried lipid film was suspended in HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4). The lipid dispersion was then subjected up to 10 freeze-thawing cycles (freezing with liquid Nitrogen and thawing with a water bath at 50 °C) to ensure complete lipid mixing. Unilamellar liposomes were then prepared by extruding the sample 31 times through a 100 nm pore size filter from Whatman plc (Kent, UK).

2.3. Dynamic light scattering (DLS)

DLS experiments were performed using an ALV/CGS-3 compact goniometer system and an ALV/LSE-5004 light scattering electronics and multiple tau digital correlator from ALV-GmbH (Langen, Germany). The laser beam had a wavelength of 532 nm with vertically polarized light. All measurements were performed in triplicate for each sample at room temperature with a run time of 2 min and at least two runs. Data acquisition and fitting was performed with the ALV-correlator software (version 3.0). The regularized fit model with the (g2(t)) DLS-exponential was used.

2.4. Cryo-transmission electron microscopy (cryo-EM)

Specimens for cryo-EM investigations were prepared at controlled temperature (25 °C) and humidity (>90 %) within a custom-built environmental chamber. A small (~1 μL) drop of sample was placed onto a copper grid (300 mesh, Agar scientific) coated with a custom-made holey polymer (cellulose acetate butyrate) film, and excess liquid was blotted away with a filter paper. The sample was then vitrified by plunging the grid into liquid ethane held at a temperature just above its freezing point. Thereafter the grid was mounted in a Gatan CT3500 sample holder and transferred to a Zeiss TEM Libra 120 transmission electron microscope (Carl Zeiss AG, Oberkochen, Germany) for viewing. The sample was kept at a temperature below −160 °C during the transfer and viewing processes. The microscope was operating at 80 kV and the observations were made in zero-loss bright-field mode. Digital images were recorded under low dose conditions with a Bio-Vision Pro-SM slow scan CCD camera from Proscan elektronische Systeme GmbH (Scheurig, Germany). For the samples treated with Ca^{2+}, 1 μL of 1 M $CaCl_2$ was added to 100 μL of the liposome dispersion and

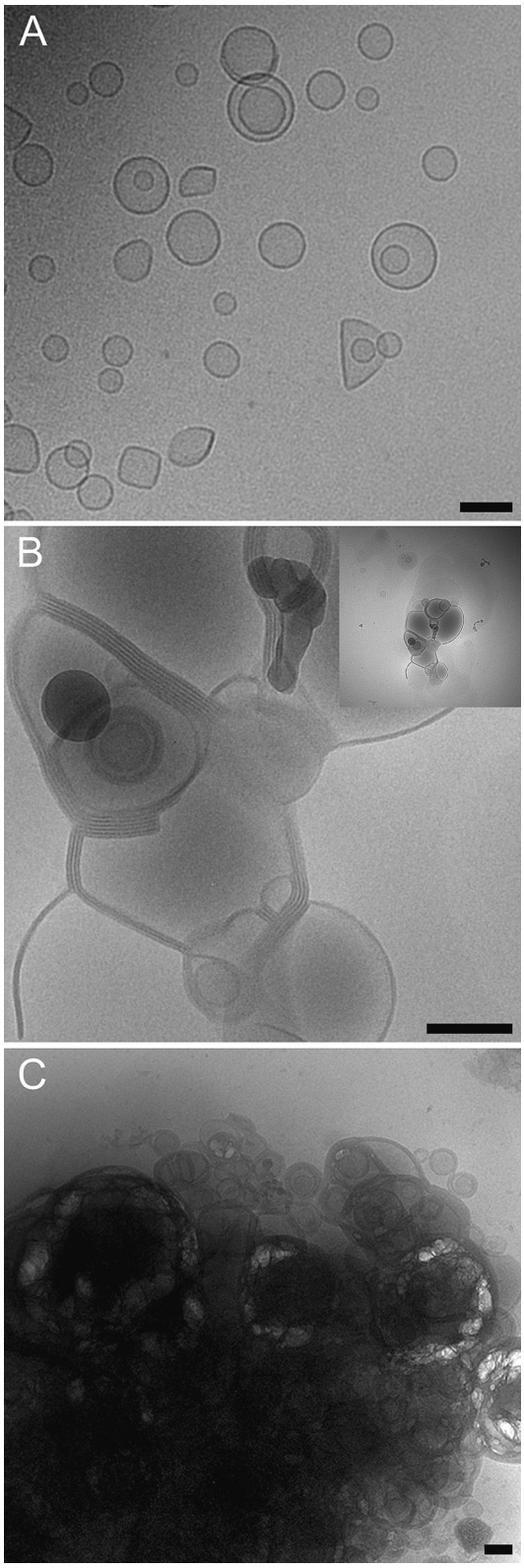

Fig. 2. Cryo-EM micrographs of liposomes composed of POPE:POPS with a molar composition of 75:25. A) Liposomes dispersed in buffer; B) In the presence of 10 mM Ca^{2+} after 30 s (inset, same picture at lower magnification, showing the formation of large bilayer sheets); C) In the presence of 10 mM Ca^{2+} after 20 min. Scale bar = 100 nm. Note that the size of the scale bar is unique for each image.

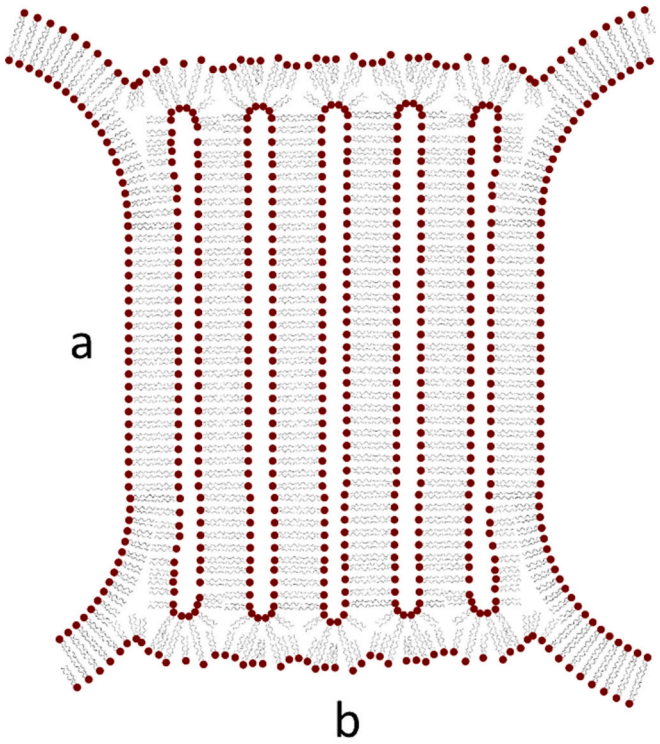

Fig. 3. Schematic representation of the structures formed after addition of Ca^{2+} to naked POPE:POPS (75:25) liposomes. Lipid mixing between ruptured (a) and collapsed (b) vesicles is observed. The open structures on either side can aggregate with similar structures forming open bilayer sheets or large vesicles, in agreement with the Cryo-EM images.

mixed before applied on the grid and blotted. The total time between mixing and plunging into ethane is the one used in results.

2.5. Lipid mixing assay

Lipid mixing was followed using the Förster resonance energy transfer assay developed by Struck et al. [17] as described in Duzgunes et al. [16]. Briefly, liposomes containing both acceptor, rhodamine-PE, and donor, NBD-PE, fluorescent lipids with a proportion of 0.8 mol% each (labeled liposomes) were mixed with liposomes devoid of fluorescent lipids (unlabeled liposomes) at a ratio of 1:9 (labeled:unlabeled) to a total concentration of 50 μM lipid. Calcium chloride was then added from a stock solution to a final concentration of 10 mM. Upon lipid mixing, the average separation between donor and acceptor molecules increases, resulting in a decrease in the energy transfer. The fluorescence signal from the donor thus increases. The measurements were performed at a temperature of 25° with constant stirring set to 500 rpm in a quartz cuvette (Quartz SUPRASIL ®, Hellma Analytics, Mühlheim, Germany). The fluorescence signal from the donor molecule (NBD-PE lipids) was monitored with a Fluorolog®-3 from Horiba (Kyōto, Japan) in the right-angle mode. The fluorescence emission intensity was measured at 530 nm with excitation at 460 nm [22,23].

The maximal fluorescence (I(max)) was determined by measuring the fluorescence intensity of a dispersion containing 50 μM of calibration liposomes, i.e. liposomes containing 0.08 mol% of NBD-PE and rhodamine-PE, mimicking thus the composition expected at 100 % lipid mixing. The degree of lipid mixing over time (M(t)) was calculated by the following equation [22,23]:

$$M(t) = \frac{I(t) - I(0)}{I(max) - I(0)}$$

where $I(t)$ is the fluorescence intensity at time t and $I(0)$ is the

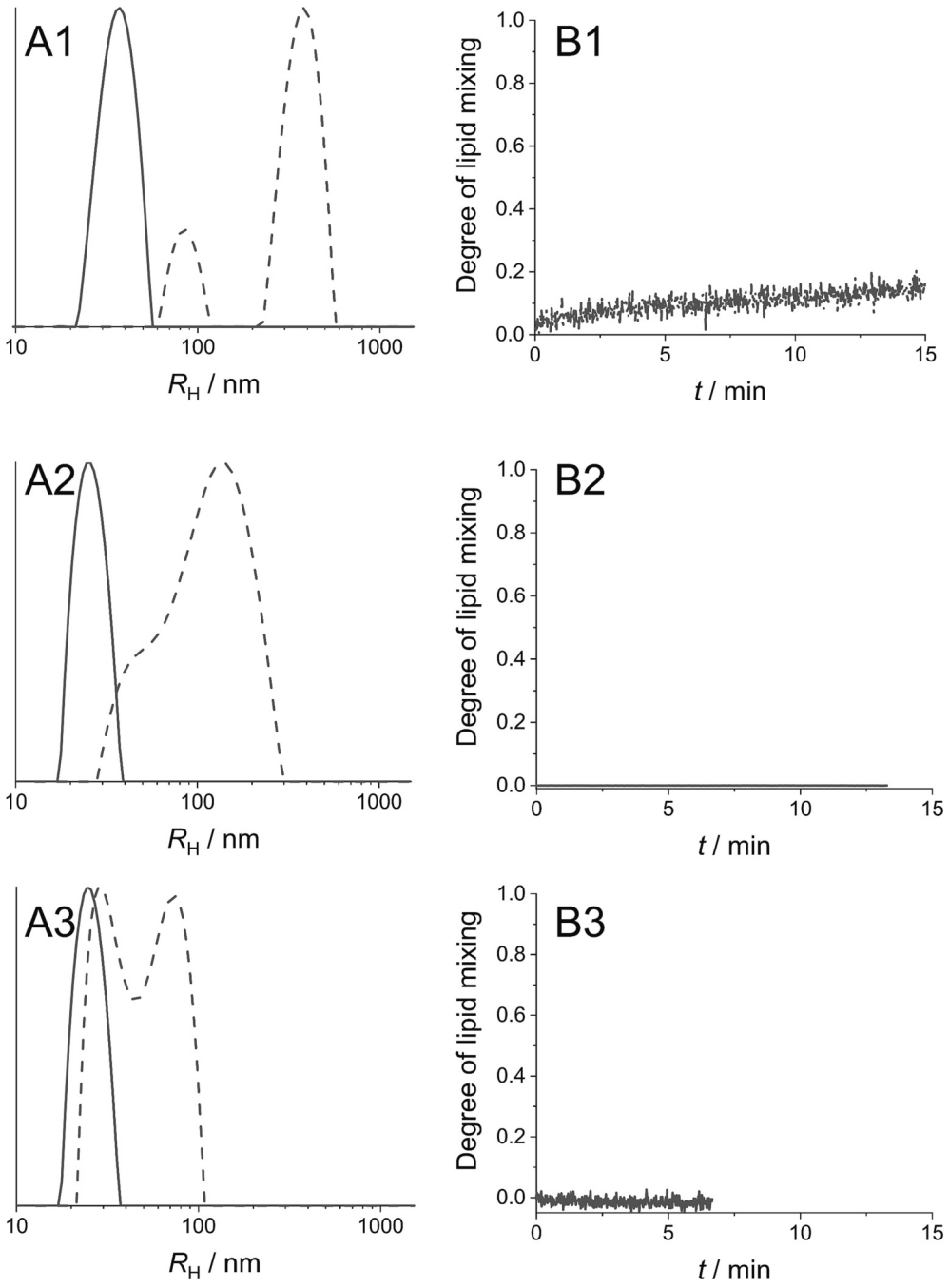

Fig. 4. A1-A3: Intensity weighted hydrodynamic radius (R_H) distribution of POPE:POPS liposomes modified with 4 mol% (final molar composition POPE:POPS: DSPE-PEG 71:25:4). A1) DSPE-PEG750, A2) DSPE-PEG1000 and A3) DSPE-PEG2000 liposomes before (solid line) and 30 min after (dashed line) the addition of 10 mM Ca^{2+}. B1-B3: Calculated degree of lipid mixing for the corresponding PEGylated liposomes as a function of time after Ca^{2+} addition.

fluorescence intensity before the addition of $CaCl_2$. Lipid mixing results in an increased fluorescence intensity as the fluorescent lipids in the labeled vesicles are diluted upon mixing with lipids in the unlabeled liposomes [16,17,22,23].

3. Results and discussion

3.1. Naked and PEG-stabilized POPE:POPS liposomes

Liposomes composed of POPE:POPS 75:25 were first characterized using DLS. The results shown in Fig. 1A indicate that the sample is dominated by liposomes with a hydrodynamic radius between 30 and 50 nm. Cryo-EM experiments suggest that the liposomes are mostly spherical and unilamellar (Fig. 2A). Upon addition of $CaCl_2$ to a concentration of 10 mM, the original population of liposomes disappears and is replaced by two populations with radius ~ 200 nm and ~ 650 nm, respectively, indicating liposome aggregation and/or fusion. FRET experiments (Fig. 1B) confirm that full mixing of the lipid components is achieved within minutes after the addition of $CaCl_2$. These results are in agreement with previous studies concerning the Ca^{2+}-induced fusion behavior of PE:PS liposomes [4,5,20]. Addition of extra 40 mM NaCl instead of 10 mM $CaCl_2$ did not induce any changes in the data retrieved from DLS measurements (see Fig. S1 in the supplementary material), suggesting that no aggregation or fusion occur even though the ionic strength was higher than in the case of $CaCl_2$ addition. This observation is also in agreement with the results from previous publications [20],

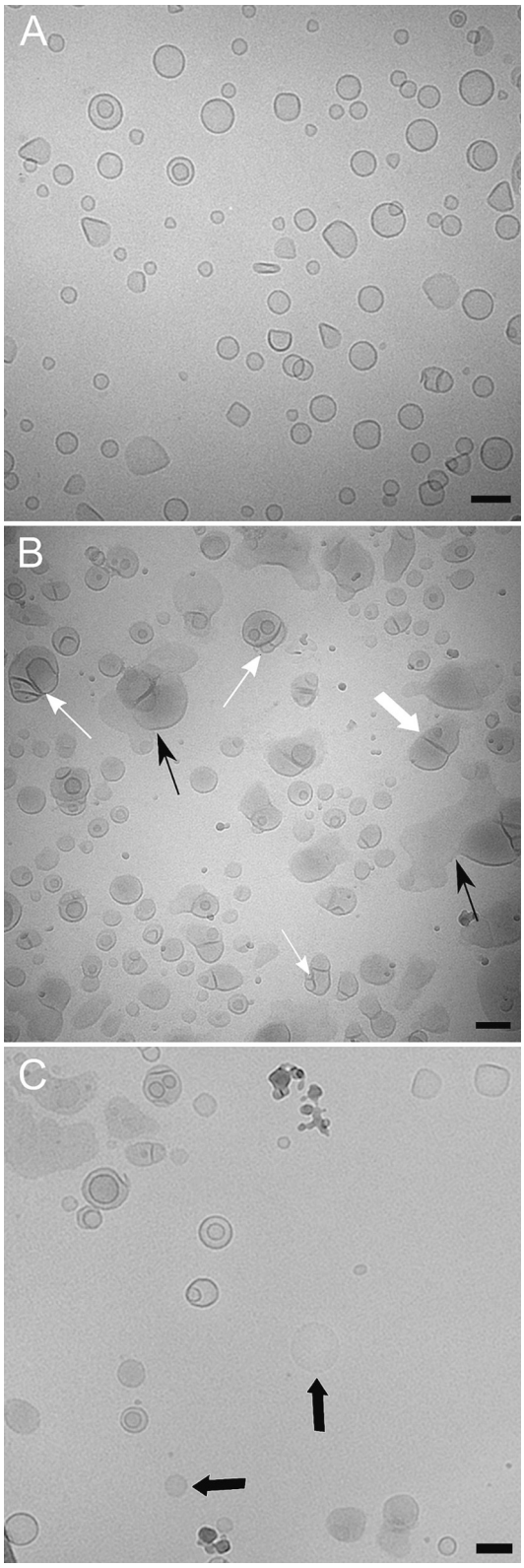

Fig. 5. Cryo-EM micrographs of liposomes composed of POPE:POPS:DSPE-PEG1000 with a molar composition of 71:25:4. A) Liposomes dispersed in buffer; B) In the presence of 10 mM Ca^{2+} after 30 s; C) In the presence of 10 mM Ca^{2+} after 20 min. White thin arrows: Two or more liposomes in close contact with each other. Thick white arrows: Two liposomes in close contact in the process of rupturing and opening. Black thin arrows: open bilayer structures attached to partially open liposomes. Thick black arrows: Open bilayer structures (lipodisks). Scale bar = 100 nm. Note that the size of the scale bar is unique for each image.

and suggest that the fusion-inducing effect of Ca^{2+} is not limited to a decrease in the repulsion between the charged lipid headgroups.

Cryo-EM images of the sample 30 s after addition of $CaCl_2$ show the presence of large aggregates in which closed large liposomes are "coated" and connected to each other by multiple bilayers (Fig. 2B). The large spacing and general appearance of the latter suggest that they may represent stacks of collapsed but still intact liposomes. Large open bilayer sheets are also observed. The formation of these structures suggests that liposomes do not only fuse with each other, but also rupture, collapse and spread on each other's surfaces. This phenomenon has been observed previously in preparations of bovine brain PS liposomes treated with Ca^{2+}, leading to structures similar to the one depicted in Fig. 2B [1,2]. The collapse and rupture of liposomes in contact with each other is believed to arise from a strong attractive inter-liposome interaction leading to a fast and extensive deformation of the liposomes [1,2]. It has been argued, however, that such attractive interactions become relevant only when the ionic strength in the medium is very low (only 2 mM TES in the cited publications) leading to a strong adsorption of Ca^{2+} on the liposome surface [3] and facilitating the formation of inter-liposomal PS-Ca^{2+}-PS complexes [1]. It has indeed been shown more recently that the binding of Ca^{2+} to PS-based membranes [13] is greatly diminished by increasing the ionic strength, with no significant adsorption/binding of Ca^{2+} being observed at NaCl concentrations above 100 mM. Since the NaCl concentration in the present case is 150 mM, the strong attraction leading to liposome rupture cannot be explained by Ca^{2+} adsorption and complex formation. An alternative explanation is that the strong attraction between liposomes in our system arises from ion correlation forces [24] caused by the accumulation of Ca^{2+} in the electric double layer. This potential explanation is discussed in more detail in the following sections.

20 min after the addition of Ca^{2+} the dispersion consists mainly of large clusters formed by multilamellar structures (Fig. 2C), although in some cases large bilayer sheets were also observed (not shown). The fact that complete lipid mixing is observed in the FRET experiments even though individual small structures can still be identified in Cryo-EM suggest that bilayer rearrangements take place in which lipids are exchanged. These rearrangements are likely to be similar to the multilayer stacking mechanism proposed by Rand et al. [1] to account for the formation of structures resembling those shown in Fig. 2B and C. The proposed resulting structures are illustrated in Fig. 3.

Inclusion of 4 mol% PEGylated lipids did not prevent aggregation of the liposomes, as illustrated by the DLS data obtained with lipids modified with three different PEG molecular weights (Fig. 4 A1-A3). In all cases, it is observed that addition of Ca^{2+} results in the formation of a population with much larger hydrodynamic radius than the parent liposomes. This effect is most obvious for the low molecular weight PEG chains, and is reduced as the PEG length increases. In spite of aggregation taking place, the presence of PEG with molecular weights 1000 and 2000 prevented the mixing of the lipid contents (Fig. 4 B2-B3), in agreement with previous reports [21]. For the shortest PEG-chain studied (PEG750), mixing of the lipids was still observed (Fig. 4 B1), although at a drastically reduced rate compared to what was observed with the naked liposomes.

Very interestingly, cryo-EM analysis of samples containing PEG1000 (Fig. 5) showed that the aggregates detected by DLS do not consist of intact and separate liposomes, but of planar bilayer fragments often attached to vesicular structures (Fig. 5B). 20 min after exposure to Ca^{2+}, also some isolated large bilayer fragments can be observed (Fig. 5C), although they are found only in a few selected areas of the sample. Some structures similar to hemifused liposomes are also identified in the pictures (Fig. 5B and C). However, as indicated by the FRET experiments, it is likely that these structures consist of two (or more) liposomes that have attached to each other without mixing of the lipids. The fact that the separation between bilayers at the contact area cannot be resolved in the images, suggests that PEGylated lipids are excluded from this region leading to very close contact. This would result in

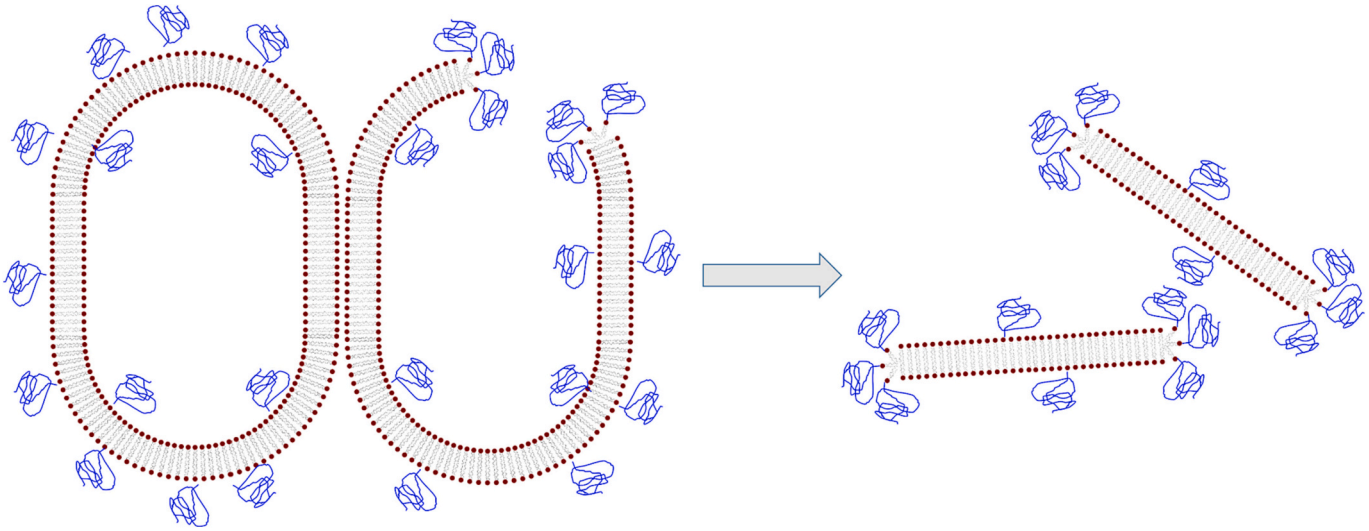

Fig. 6. Schematic representation of the structures formed after addition of Ca^{2+} to PEGylated POPE:POPS-based liposomes. PEG is excluded from the contact area and accumulates at the points of vesicle rupture, stabilizing thus the formed open structures and resulting in the formation of disks.

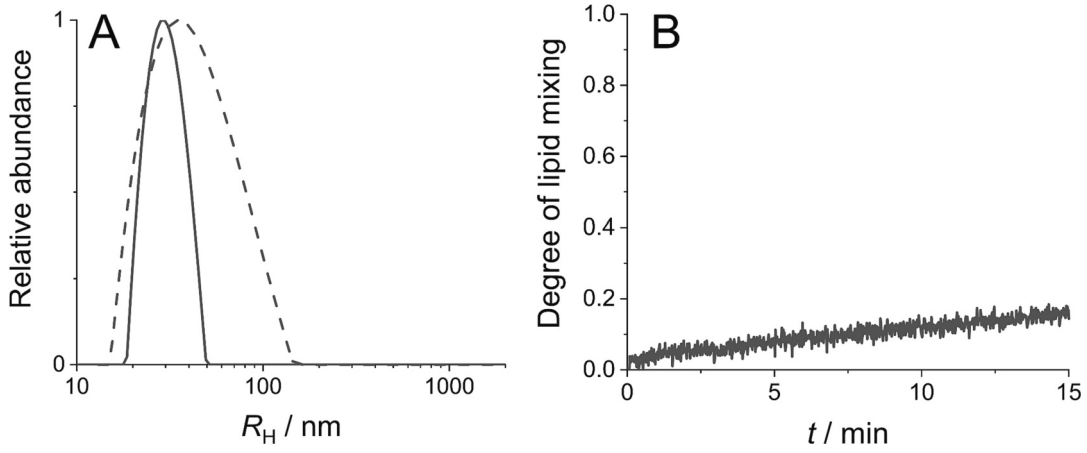

Fig. 7. A: Intensity weighted hydrodynamic radius (R_H) distribution of POPE:POPS:gangliosides 65:25:10 liposomes before (solid line) and 30 min after (dashed line) the addition of Ca^{2+}. B: Calculated degree of fusion between POPE:POPS:gangliosides 65:25:10 liposomes as a function of time after Ca^{2+} addition.

accumulation of PEGylated lipids in other parts of the membrane. As the lipid membrane is already close to the PEGylated lipid saturation limit (~5 mol% according to Edwards et al. [25]), this segregation of PEGylated lipids would lead to the formation of open structures stabilized by a PEGylated rim (see Fig. 6), in a similar fashion to the formation of PEGylated lipodisks described in previous publications [26]. This explains also why most of the observed adhered liposomes seem to have ruptured upon contact.

In order to remove the PEGylated lipids from the contact area, a strong attractive force between liposomes is required. It is also required that this attractive force has longer range than the steric repulsion provided by the PEG chains. Leckband et al. [27] have previously shown that the presence of Ca^{2+} can lead to attractive long range hydrophobic interactions between lipid bilayers due to induced membrane phase segregation. However, in contrast with our observations, such attraction was suggested to always lead to membrane fusion. Another plausible explanation for the observed behavior would be that the addition of Ca^{2+} results in strong attractive ion correlation forces [24]. Fig. S2 in the supplementary material shows the estimated strength of Ca^{2+} induced ion correlation forces between two spherical particles, with the same surface charge density as the liposomes used in this study, interacting in deionized water. The calculations are based on the simplified approach presented by Jönsson and Wennerström [28] complemented by the description of the repulsion between coaxial parallel uniformly charged disks described by Ciftja [29], and lipid structural and interaction parameters found in the literature [30–32]. Details about the estimations are found in the supplementary material. It is estimated that, at separations down to ~1 nm between the surfaces, the strength of the ion correlation attraction is enough to overcome both the electric double layer and the steric repulsions between liposomes (calculated, respectively, according to Engström and Wennerström [33] and Li and Pincet [34]). In the high ionic strength media used in our experiments, it is expected that the osmotic electric double layer repulsion will decrease much more than the attractive pressure from ion correlation forces, given the preferential accumulation of multivalent ions near the liposome surface. A roughly calculated total interaction (fig. S3 in the supplementary material) shows that under the experimental conditions a net attraction (slightly weaker than in the case of deionized water) can be expected at separations down to ~1 nm. In agreement with our discussion, it has been previously shown experimentally that inclusion of Ca^{2+} in the medium induces an attraction between phosphatidylglycerol membranes at separations of about 3 nm, and this attraction could also be attributed to ion correlation forces [35].

Even though the above estimations are based on rough

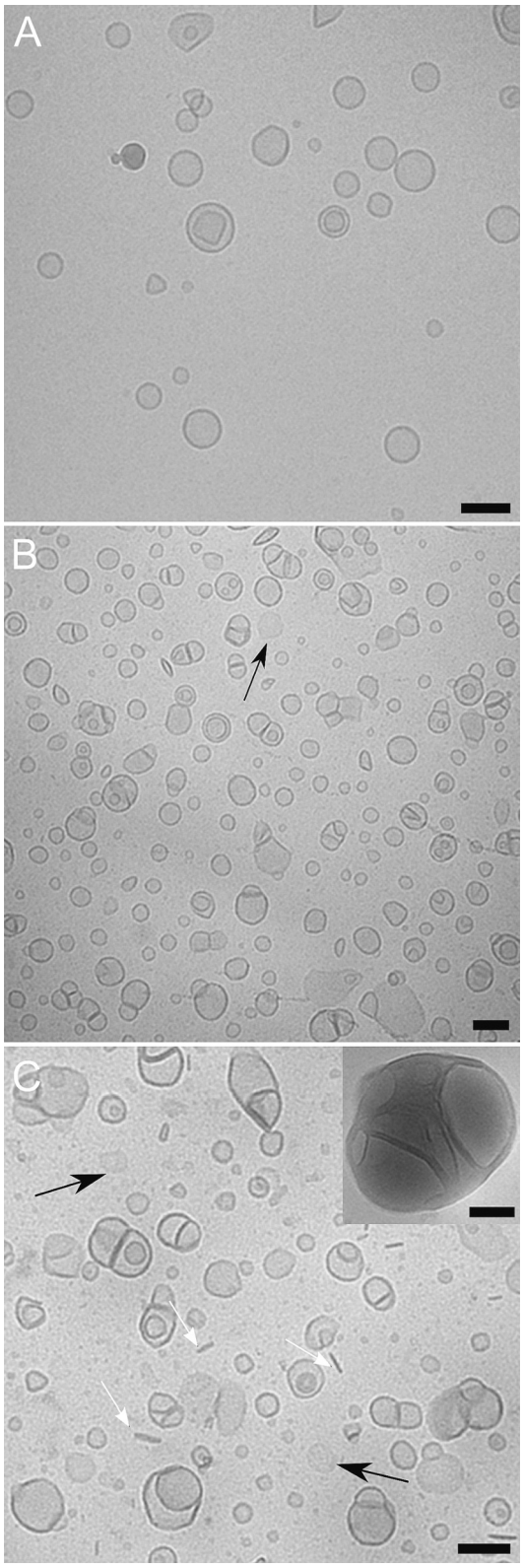

Fig. 8. Cryo-EM micrographs of liposomes composed of POPE:POPS:Gangliosides with a molar composition of 65:25:10. A) Liposomes dispersed in buffer; B) In the presence of 10 mM Ca^{2+} after 30 s; C) In the presence of 10 mM Ca^{2+} after 20 min. Small open bilayer structures can be observed face-on (black arrows) or edge-on (white arrows). Scale bar = 100 nm. Note that the size of the scale bar is unique for each image.

approximations, they show that it is indeed plausible that ion correlation forces can lead to close contact between liposomes. It is likely that the strong attraction will result in the segregation of PEGylated lipids from the area of contact. The cost in terms of mixing entropy when segregation takes place can be well accounted for by the gain in configurational entropy for the PEG chains if they leave the tight gap between the approaching surfaces. As mentioned before, this PEG-lipid segregation can lead to the formation of open structures. Thus, it is likely that the attractive ion correlation forces are the driving force behind the formation of the structures observed in Fig. 5B and C. Once open bilayer structures are formed, the accumulation of PEG on the bilayer edges would increase the range and strength of the repulsive steric forces, avoiding thus further aggregation and/or fusion. Open structures have indeed been shown to be stabilized by very low amounts of PEG lipids (down to 2 mol%) [26].

3.2. *Ganglioside stabilized PE:PS liposomes*

In order to compare the stabilizing effect of PEG and gangliosides, the effect of Ca^{2+} on POPE:POPS liposomes modified with 10 mol% gangliosides was studied. Inclusion of gangliosides slowed, but did not completely prevent, the lipid mixing process as illustrated in Fig. 7. DLS experiments (Fig. 7A) confirm the formation of larger structures after Ca^{2+} addition, although structures smaller than the parent liposomes are also identified. Cryo-EM investigations (Fig. 8) show that during the first seconds after Ca^{2+} addition ganglioside modified liposomes behave very similarly to their PEGylated counterparts, meaning that aggregation occurs almost immediately but does not lead to fusion (Fig. 8B), and that gangliosides appear to be excluded from the liposome-liposome contact area. The adhered liposomes appear quite resilient to rupture, but after 20 min open bilayer patches or fragments are frequently observed (Fig. 8C). These have a relatively small size, and can thus be an explanation for the small particles observed in DLS measurements. Similar open bilayer structures have previously been shown to form when mixing phosphocholine lipids with >20 mol% gangliosides [36] and to consist of a planar lipid bilayer disks stabilized by gangliosides accumulating at the edges. The fact that these structures form with only 10 mol% gangliosides after calcium-induced fusion suggest that gangliosides are segregated from the region where adhesion occurs, leading to the formation of high curvature patches that eventually become the edges of the open structures, similarly to what is believed to occur with PEGylated lipids. However, in contrast with the results from liposomes supplemented with DSPE-PEG1000 and DSPE-PEG2000, lipid mixing is not completely avoided (Fig. 7B). This is likely a consequence of the structural transformations resulting in open bilayer structures being slower than in the case of PEGylated liposomes. Also, it has been shown previously that gangliosides are not as effective as PEGylated lipids in generating open structures [36]. In agreement with the results from the FRET measurements, some large, partially fused structures were indeed found in the ganglioside-modified sample (see inset in Fig. 8C).

As in the case of PEGylated liposomes, it is likely that the driving force causing ganglioside segregation and leading to liposome aggregation, rupture and formation of open bilayer structures are ion correlation forces acting between liposomes. The fact that the formed open structures remain stable even in the presence of Ca^{2+} suggest that the stabilization provided by gangliosides has a strong non-electrostatic component. Although our results are not conclusive in this respect, it is plausible to think that the stabilization arises either from steric or hydration effects (or probably both) provided by the gangliosides.

The question remains, however, why the attractive forces leading to aggregation and rupture for both PEG- and ganglioside- decorated liposomes do not cause aggregation or stacking of the open bilayer structures formed. The open structures can be considered as charged planar surfaces and therefore it would be expected that the same attractive forces as discussed for liposomes would act. The fact that these structures remain dispersed as individual particles suggests that a strong

repulsive interaction arises upon their formation. Since the edge of these structures is rich in either PEGylated lipids or gangliosides, it is reasonable to assume that the ion correlation attraction between the planar surfaces is counteracted by a strong steric repulsion between the PEG or ganglioside coronas. The repulsion is expected to be stronger than that between the PEGylated surfaces of liposomes, as the local concentration of PEG or gangliosides is much higher in the case of the surrounding corona. Besides this effect, the fact that there is no possibility for further PEG or ganglioside segregation likely contributes to prevent the formation of stacks or other aggregated structures.

4. Conclusions

The results presented in this report suggest that Ca^{2+} can induce not only fusion of PE:PS based liposomes, but also the rupture and/or collapse of liposomes on each other's surfaces even at high ionic strengths. It is also shown that modifying the surface of the liposomes with PEGylated lipids or gangliosides can slow down, but not completely prevent, the adhesion and rupture of the liposomes. Indeed, once adhesion occurs, the segregation of the gangliosides or PEGylated lipids from the contact area serves to facilitate the formation of open bilayer structures.

In the case of PEGylated or ganglioside-modified liposomes, the adhesion and rupture processes can remain undetected by FRET measurements, as little or no lipid mixing occurs. In the case of "naked" vesicles, the bilayer fragments formed eventually fuse leading to an increase in the measured fluorescence signal. The signal increase due to fragment fusion can potentially be misinterpreted as evidence of direct liposome-liposome fusion. A scenario where a large proportion of the liposomes rupture before lipid mixing takes place would explain the discrepancies observed between the results from mixing-of-lipids and mixing-of-contents approaches to follow liposome-liposome fusion.

Our findings also suggest that the molecular mechanisms of Ca^{2+}–induced membrane fusion involve attractive forces strong enough to overcome the long-range repulsive forces provided by PEGylated lipids and gangliosides, and to induce segregation of the stabilizing lipids. These long-range forces cannot be explained by the previously proposed strong short-range attractive interactions between calcium and PS. Instead, it seems likely that ion correlation forces are involved. This would also explain the enhancement of the fusion behavior triggered by calcium in systems where PS is not present.

Gangliosides seem to have a similar inhibitory effect as PEGylated lipids concerning the Ca^{2+} induced fusion and/or adhesion and rupture. The fact that the open structures formed in the presence of ganglioside remain dispersed as single entities (i.e., without aggregation) indicates that there is a steric component in the stabilization provided by gangliosides.

It is our view that the results of this study merit reevaluation of the conclusions drawn from earlier FRET-based studies.

Declaration of competing interest

The authors declare no potential conflict of interest.

Data availability

Data will be made available on request.

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CRediT authorship contribution statement

Conceptualization: K.E. and V.A.H.; Methodology: P.G., K.E., L.G., and V.A.H.; Validation: P.G. and V.A.H.; Formal analysis: P.G., L.G., K.E. and V.A.H.; Investigation: L.G., P.G. and V.A.H.; Resources: V.A.H. and K.E.; Writing—original draft preparation: P.G. and V.A.H.; Writing—review and editing: P.G., L.G., V.A.H. and K.E.; Supervision: V.A.H. and K.E.; Project administration: K.E.; Funding acquisition: K.E. All authors have read and agreed to the published version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2023.184253.

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