# Formation of Giant Liposomes Promoted by Divalent Cations: Critical Role of Electrostatic Repulsion

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ABSTRACT Spontaneous formation of giant unilamellar liposomes in a gentle hydration process, as well as the adhesion energy between liposomal membranes, has been found to be dependent on the concentration of divalent alkali cations, $Ca^{2+}$ or $Mg^{2+}$, in the medium. With electrically neutral phosphatidylcholine (PC), $Ca^{2+}$ or $Mg^{2+}$ at 1–30 mM greatly promoted liposome formation compared to low yields in nonelectrolyte or potassium chloride solutions. When negatively charged phosphatidylglycerol (PG) was mixed at 10%, the yield was high in nonelectrolytes but liposomes did not form at 3–10 mM $CaCl_2$. In the adhesion test with micropipette manipulation, liposomal membranes adhered to each other only in a certain range of $CaCl_2$ concentrations, which agreed with the range where liposome did not form. The adhesion range shifted to higher $Ca^{2+}$ concentrations as the amount of PG was increased. These results indicate that the divalent cations bind to and add positive charges to the lipids, and that membranes are separated and stabilized in the form of unilamellar liposomes when net charges on the membranes produce large enough electrostatic repulsion. Under the assumption that the maximum of adhesion energy within an adhesive range corresponds to exact charge neutralization by added $Ca^{2+}$, association constants of PC and PG for $Ca^{2+}$ were estimated at 7.3 $M^{-1}$ and 86 $M^{-1}$, respectively, in good agreement with literature values.

## INTRODUCTION

Giant unilamellar liposomes, which are readily observed and manipulated under a microscope, serve as useful cell models (see, e.g., Hotani, 1984; Needham and Hochmuth, 1989; Itoh et al., 1990; Farge and Devaux, 1992; Miyata and Kinosita, 1994; Stoicheva and Hui, 1994a, b; Evans et al., 1995; Lipowsky, 1995; Elbaum et al., 1996). Preparation of giant liposomes is apparently straightforward under no (or low) salt conditions, where liposomes form spontaneously upon hydration of lipid (Reeves and Dowben, 1969; Mueller et al., 1983; Needham and Evans, 1988). The spontaneous formation implies that lipid bilayers tend to separate from each other under these conditions, and thus the membranes tend to be unilamellar. In the presence of salts, however, the yield of giant liposomes is often low or negligibly small (e.g., Mueller et al., 1983). In a previous paper (Akashi et al., 1996), we have shown that if a charged lipid such as phosphatidylglycerol (PG) is included, giant unilamellar liposomes with diameters of several tens of micrometers are formed by the hydration method even in physiological salt solutions (e.g., 100 mM KCl plus 1 mM $CaCl_2$). This last result points to the importance of electrostatic repulsion in separating and keeping lipid membranes apart.

The electrostatic interaction between lipid membranes is modulated extrinsically by mobile ions in the surrounding medium (Israelachvili, 1992). Divalent alkali cations such as $Ca^{2+}$ and $Mg^{2+}$, in particular, bind to phospholipids and provide positive charges on membranes (Bangham and Dawson, 1962; McLaughlin et al., 1978). Thus, if electrostatic repulsion is the key for membrane separation, the divalent cations may have significant effects on the formation of giant liposomes.

In this paper we show that $Ca^{2+}$ and $Mg^{2+}$ greatly promoted spontaneous formation of giant unilamellar liposomes from electrically neutral phospholipids, as expected. For negatively charged lipids from which liposomes formed readily, $Ca^{2+}$ reduced the yield. The inhibitory effect was, however, reversed at higher concentrations of $Ca^{2+}$, suggesting that the membranes acquired a net positive charge and hence repelled each other. To confirm the interpretation in terms of electrostatic repulsion, we measured the adhesive energy between two liposomes by the micropipette aspiration method (Evans and Metcalfe, 1984). Adhesion occurred only under those conditions where unilamellar liposomes failed to form. Thus, electrostatic repulsion is crucial both for separation of unilamellar membranes from amorphous lipid (liposome formation) and for prevention of membrane adhesion. The repulsive force derives from the net charge on the membranes, intrinsic charges of the lipid plus extrinsic charges conferred by divalent cations. The force is modulated by the concentration of divalent cations in the medium.

## MATERIALS AND METHODS

### Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-3-[phospho-rac-(1-glycerol)] (POPG), egg phosphatidylcho-

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line (egg-PC) and phosphatidylglycerol (egg-PG), all sealed in an ampule, were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Bovine serum albumin (BSA) and poly-L-lysine (mol wt 3970) were from Sigma Chemical Co. (St. Louis, MO). Nile Red was from Molecular Probes, Inc. (Eugene, OR). *N*-trimethoxysilylpropyl-*N*,*N*,*N*-trimethylammonium chloride (TMA-silane) was from Chisso K. K. (Tokyo, Japan). Anhydrous chloroform was from Wako Chemical Co. (Tokyo, Japan), and anhydrous methanol from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of analytical grade. Deionized water (Mill-Q system, Millipore, Tokyo, Japan) was used in all experiments.

The lipids were used without further purification, after checking their purity as described (Akashi et al., 1996). Lipids that exhibited contaminant spots, e.g., lyso compounds, in thin layer chromatography on silica gel with chloroform/methanol/water (65:25:4) as solvent were not used. The degree of lipid oxidation was estimated from UV absorbance (New, 1990); the ratio between the diene peak at 230 nm to the monoene peak around 200 nm was typically $\sim$0.05 and lipids with a ratio $>$0.1 were not used.

## Preparation of giant liposomes

The protocol described in Akashi et al. (1996) was slightly modified to obtain a higher yield. Lipids were each dissolved at 7.5 mg/ml in chloroform/methanol (2:1 by volume) and stored under a blanket of argon at $-25$°C. Eighty microliters of the solution (0.6 mg lipid) in a 10-ml glass test tube (ID $\sim$1.5 cm) was dried at 45°C with a rotary evaporator to form a thin lipid film on the bottom surface (2–3 cm high). The tube was subsequently placed in vacuo for $>$6 h to remove the last trace of the organic solvent. The completely dried lipid film was then prehydrated at 45°C with water-saturated $N_2$ for 30$-$60 s until the film became transparent. Five milliliters of an aqueous solution containing 0.1 M sucrose and appropriate salts, which had been $N_2$-purged, was added gently to the tube. The tube was sealed under argon and incubated at 37°C for $\sim$2 h, and then gently rocked to disperse the lipid film uniformly in the solution. After further overnight incubation at room temperature (22–24°C), we found, in successful cases, an almost transparent bulky white cloud floating in the middle of the solution, which contained giant liposomes. Otherwise, the solution contained only small particles at the top.

## Observation of liposomes

Liposomes were observed on an inverted phase-contrast microscope (ICM-405, Carl Zeiss Inc., Tokyo) as described (Akashi et al., 1996). To estimate the yield of giant liposomes, the materials in the preparation tube were gently dispersed to ensure unbiased sampling where the lipid concentration became 0.12 mg/ml in all samples, and then an appropriate amount (10, 50, or 100 $\mu$l) of the resulting liposome suspension was introduced into an observation chamber ($1\times1$ cm$^2$ wide and 3 mm high) containing the same medium as in the liposome suspension except that 0.1 M sucrose was replaced with 0.1 M glucose. The total volume in the chamber was 300 $\mu$l. The replacement with glucose allowed sedimentation of liposomes and also enhanced the image contrast. After 20 min, when all liposomes settled down on the bottom surface, the whole area was scanned manually and the number and sizes of giant unilamellar liposomes were scored. Here we define a "giant" liposome as one with a diameter (along a scale inserted in the field of view) exceeding 25 $\mu$m as in the previous study (Akashi et al., 1996). To prevent medium flow due to evaporation, the observation chamber was covered with an inverted petri dish moistened with wet filter paper so that most liposomes appeared almost circular in the image plane.

The lamellarity of liposomes was estimated by eye in the phase-contrast images: those liposomes that showed the thinnest contour were judged unilamellar. The judgment by trained eye has been shown to be reliable in the previous study (Akashi et al., 1996). Of those judged unilamellar, a few percent might be bilamellar, but none should be trilamellar or higher whose contrast clearly differs from the contrast of unilamellar ones.

## Treatment of glass surface

When an untreated coverslip constituted the bottom of the observation chamber, liposomes tended to adhere to and collapse on the surface. To prevent this, the surface was coated with BSA, poly-L-lysine, or TMA-silane (positively charged). BSA and poly-L-lysine were dissolved in pure water at 10 mg/ml, and the solution was spread on a coverslip. The solution was removed after 5 min and the surface was dried with argon or $N_2$ gas. For coating with TMA-silane, coverslips cleaned with a saturated solution of KOH (Merck) were dipped in 1% aqueous solution of TMA-silane and then dried. The tips of micropipettes were coated with TMA-silane in the same way.

To select an appropriate coat, we made fluorescent liposomes by mixing Nile Red in the starting lipid solution (0.3 wt % of the lipid). In this way, collapsed liposomes on the glass surface could be clearly observed under a fluorescence microscope. We adopted the following coatings: poly-L-lysine or TMA-silane for PC liposomes in 1–30 mM $CaCl_2$ or 1–30 mM $MgCl_2$, and BSA for all others. For PC liposomes in pure water, an untreated glass surface was equally successful. In the adhesion test below, untreated coverslips were used except for $\leq$3% PG in $>$ 1 mM $CaCl_2$ where coverslips were coated with TMA-silane, and for 10% PG with BSA. Micropipettes were coated with TMA-silane for PC liposomes, BSA for all others.

## Adhesion test

To test whether the liposomes adhere to each other, two liposomes were brought into contact by manipulating them independently with two sets of the micropipette aspiration system described by Akashi et al. (1996). The observation chamber in this experiment had two openings on opposite sides, allowing medium exchange and insertion of two micropipettes. Liposomes were prepared in a medium that warranted a high yield (see Fig. 5 legend), and resuspended in a test solution in the observation chamber. To make liposomes sufficiently flaccid, the osmolarity of the test solution was made slightly higher by adjusting the concentration of glucose. After gentle but complete mixing with a pipette, two liposomes were aspirated by micropipettes and brought into contact. Subsequent release of the aspiration pressure in one micropipette revealed whether the two liposomes adhered to each other.

When adhesion occurred, the adhesion energy $\gamma$ between the two membranes was estimated from their contact angle using the Young-Dupré equation (Evans and Needham, 1987):

$$\gamma = \tau(1 - \cos\theta_o). \quad (1)$$

Here, the strongly aspirated liposome was regarded as rigid, and $\tau$ represents the tension in the membrane of the other liposome which was partially released. The contact angle, $\theta_o$, between the two membranes is defined in Fig. 4 *A* below. The tension $\tau$ was calculated from the dimensions (Fig. 4 *A*) of the released liposome as

$$\tau = P \cdot D_p/4(1 - D_p \cdot C) \quad (2)$$

where $C = [D_o - D_1 \cos(\theta_1/2)]/(D_o^2 - D_1^2)$ is one-half the mean curvature of the unsupported portion of the partially released liposome (Evans, 1980), and $P$ is the pressure difference between the external medium and the micropipette interior. For each pair of liposomes, $\theta_o$ was measured at various aspiration pressures, $P$, and $\gamma$ was estimated from a slope of $1/\tau$ vs. $(1-\cos\theta_o)$ (see Fig. 4 *C* below).

# RESULTS

## Formation of giant liposomes promoted by divalent cations

In the presence of millimolar concentrations of $CaCl_2$ or $MgCl_2$, many giant unilamellar liposomes were formed

from electrically neutral phospholipids. Fig. 1 *A* shows a phase-contrast image of POPC liposomes prepared in 3 mM $CaCl_2$. To demonstrate the density of liposomes in a successful preparation, this sample was taken directly from the white cloud in the preparation tube and diluted 3× in the observation chamber. The bottom of the 3-mm high chamber was fully crowded with giant liposomes.

The yields of liposomes prepared under various ionic conditions are summarized in Table 1. For unbiased comparison the contents of the preparation tube were dispersed uniformly, and then unilamellar liposomes exceeding 25 $\mu$m in diameter were scored under the phase-contrast microscope. Typical images for samples diluted 3× in the observation chamber are shown in Fig. 1, *B–J*.

With the neutral lipid POPC, preparation in the absence of salts yielded some, but not many, giant unilamellar liposomes (Fig. 1 *B*). The yield was variable, and often no giant liposomes were found. Smaller liposomes (~10 $\mu$m) were more abundant. All preparations at no salts contained large lipid aggregates from which thin tubelike structures extended occasionally. Presence of 1–30 mM $CaCl_2$ in the preparation medium dramatically increased the yield of giant liposomes by two orders of magnitude (Table 1 and Fig. 1 *C*). Binding of $Ca^{2+}$ to the neutral POPC and the resultant electrostatic repulsion can account for the high yield. $MgCl_2$ at 1–30 mM also promoted the formation of giant unilamellar liposomes (Fig. 1 *D*; not shown in Table 1). In contrast to the case of $CaCl_2$, the yield in $MgCl_2$ was higher at 10 and 30 mM than at 1 mM. Potassium chloride was not effective and was rather inhibitory (Fig. 1 *E*). With 1–100 mM KCl, we found mainly lipid debris (and small liposomes as rare exceptions, e.g., a few 10-$\mu$m unilamellar liposomes in some preparations at 1 and 10 mM KCl). No liposomes were found in 5 mM $K_2SO_4$; the divalent anion $SO_4^{2-}$ did not assist the formation of liposomes. Mere increase in the ionic strength, whether by monovalent KCl or by divalent $SO_4^{2-}$, did not help, although the increase is expected to reduce the van der Waals attraction between membranes (Israelachvili, 1992).

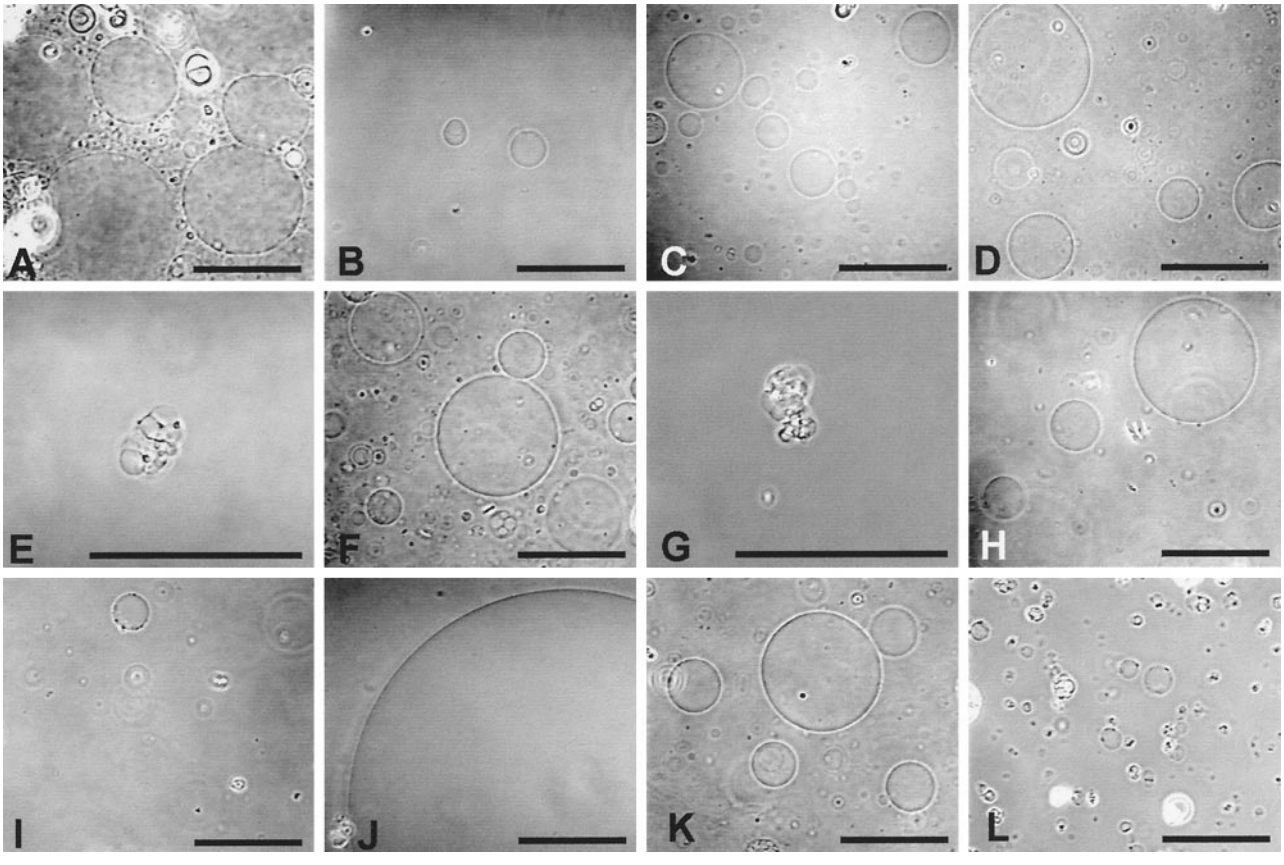



FIGURE 1 Phase-contrast images of giant liposomes prepared under different ionic conditions. Images were captured through a 20× objective with a CCD camera and contrast-enhanced (Inoué and Oldenbourg, 1995). Samples except for *A* were taken from the preparation tube after careful mixing, and diluted 3× in the observation chamber. For *A*, the white cloud in a preparation tube was diluted in the chamber in the same way. Lipid compositions and ionic conditions in the preparation solution (all contained 0.1 M sucrose) are: (*A*) POPC in 3 mM $CaCl_2$; (*B*) POPC without salts (a rare, unilamellar giant shown on the right); (*C*) POPC in 3 mM $CaCl_2$; (*D*) POPC in 10 mM $MgCl_2$; (*E*) POPC in 10 mM KCl (unsuccessful); (*F*) 90% POPC/10% POPG in 1 mM $CaCl_2$; (*G*) 90% POPC/10% POPG in 3 mM $CaCl_2$ (unsuccessful); (*H*) 90% POPC/10% POPG in 30 mM $CaCl_2$; (*I*) 90% POPC/10% POPG in 100 mM $CaCl_2$ (decoration with many lipid particles); (*J*) POPC in 1 mM $CaCl_2$ (the largest ever found); (*K*) POPC in 3 mM $CaCl_2$ plus 10 mM KCl; (*L*) POPC in 3 mM $CaCl_2$ plus 30 mM KCl (unsuccessful). In general, a clean background, as in *B, E, G*, (and *I, L*), indicates that the lipid was not dispersed and is therefore correlated with a low or negligible yield. Temperature, 22 ± 2°C. Scale bars, 100 $\mu$m.

**TABLE 1 Yields of giant liposomes prepared under different ionic conditions**

| | $CaCl_2$ (mM) | | | | | | | KCl (mM) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Lipid | 0 | 1 | 3 | 10 | 30 | 50 | 100 | 1 | 10 | 100 |
| POPC | 7 ± 8 (4) | 1095 ± 67 (2) | 611 ± 280 (5) | 221 ± 147 (6) | 299 ± 63 (2) | 85 ± 21 (2) | 0.5 ± 0.7 (2) | 0 (2) | 0.1 ± 0.3 (4) | 0 (2) |
| POPC/POPG (9:1) | 2480 (1) | 910 ± 332 (2) | 0 (2) | 0 (2) | 146 ± 62 (2) | 56 ± 0 (2) | 0.5 ± 0.7 (2) | | 775 (1) | 391 ± 343 (2) |

Numbers of unilamellar liposomes ≧25 $\mu$m in diameter in 50 $\mu$l of preparation suspension (0.12 mg lipid/ml) are listed. Values represent the averages and standard deviations, over one to six preparations. The number of preparations is shown in the parentheses. For each preparation, repeated sampling gave consistent results (within ~10%).

Inclusion of the negatively charged POPG in POPC greatly enhanced the liposomal yield at no salts (Table 1). Up to 100 mM KCl or up to 1 mM $CaCl_2$ (Fig. 1 *F*) did not severely impair the yield, as in the previous report (Akashi et al., 1996). However, $CaCl_2$ at 3 and 10 mM was totally inhibitory. Samples prepared at these concentrations did contain liposomal structures, but they were small and formed aggregates (Fig. 1 *G*). These aggregates were distinguished from amorphous lipid debris in that liposomal structures were evident at the edges of the aggregates. A reasonable yield of giant unilamellar liposomes was recovered at 30 mM $CaCl_2$ (Fig. 1 *H*).

At >50 mM $CaCl_2$, the yield of giant liposomes became lower irrespective of lipid composition, and liposomal membranes were often decorated with small lipid particles (Fig. 1 *I*). At 100 mM $CaCl_2$, <100 giant unilamellar liposomes were found per 50 $\mu$l of the preparation suspension, and most were heavily contaminated with lipid particles. Dirty liposomes such as one in Fig. 1 *I* are not counted in Table 1. The lower yields at high $CaCl_2$ concentrations are accounted for by ionic shielding of the electrostatic repulsion.

The preparations shown in Table 1 were made in unbuffered solutions of the stated salts containing 0.1 M sucrose. The trend in Table 1 was not changed when 0.1 mM EDTA was added to the $CaCl_2$ solutions (buffered with 1 mM Tris/HCl at pH 7.2). Egg-PC and egg-PC/egg-PG gave results similar to POPC and POPC/POPG, respectively.

Under conditions where many giant liposomes formed, their diameters were distributed approximately exponentially (Fig. 2 *A*). Of the giant unilamellar liposomes defined here (>25 $\mu$m), 3–6% were >100 $\mu$m. In favorable cases, up to nine liposomes that were >200 $\mu$m were found in 50 $\mu$l of the final suspension. The largest ever observed measured 500 $\mu$m in diameter (Fig. 1 *J*). The average diameter, determined as the exponential decay constant, is plotted in Fig. 2 *B* for different preparation conditions. As seen, the average diameter, and thus the size distribution, was not sensitive to the ionic conditions (as long as the yield was not too low). Ions affect the stability, but not the size, of unilamellar structures.

## Direct evidence of the prevention of membrane adhesion by divalent cations

Electrostatic repulsion between membranes, modulated by binding of $Ca^{2+}$ or $Mg^{2+}$, appears to be the key to a high liposomal yield. For the neutral phosphatidylcholines, binding of these cations produces a repulsive force. When the negatively charged phosphatidylglycerol is included, the lipid by itself contributes to the electrostatic repulsion. Divalent cations at low concentrations cancel the negative charge and diminish the repulsion; at higher concentrations the cations render the membranes positively charged, and repulsion ensues. The repulsion assists the formation of unilamellar membranes and of unilamellar liposomes by counteracting inherent attractive interactions such as the van der Waals and hydrophobic interactions (Bailey et al., 1990).

To confirm this interpretation, we directly manipulated two liposomes into contact and tested whether the liposomes

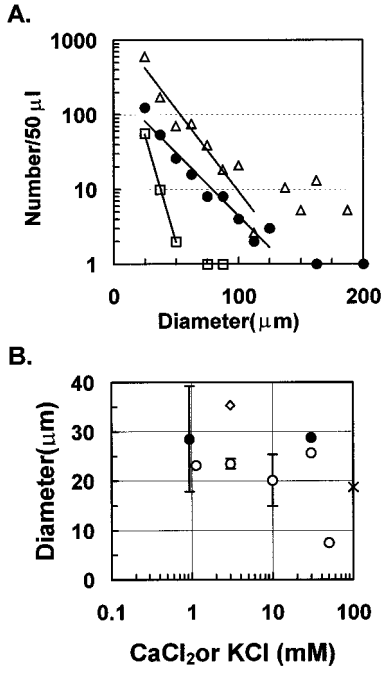



FIGURE 2 (*A*) Size distribution of giant unilamellar liposomes. △, POPC liposomes prepared in 1 mM $CaCl_2$ and 0.1 M sucrose; ●, POPC in 30 mM $CaCl_2$ and 0.1 M sucrose; □, POPC in 50 mM $CaCl_2$ and 0.1 M sucrose. Solid lines show fit with an exponential function, $C\exp(-D/M)$, where $D$ is the diameter, $M$ its average, and $C$ a constant. (*B*) Average diameters of giant unilamellar liposomes prepared under different ionic conditions. ○, POPC in 0.1 M sucrose plus indicated concentrations of $CaCl_2$; ●, POPC/POPG in 0.1 M sucrose plus $CaCl_2$; ◇, POPC in 0.2 M sucrose plus 3 mM $CaCl_2$; ×, POPC/POPG in 0.1 M sucrose plus 100 mM KCl. The diameters were determined as the decay constant ($M$) in the exponential fit. Error bars indicate standard deviations for 2–3 determinations.

adhere to each other. In Fig. 3 *A*, two POPC liposomes made in 3 mM $CaCl_2$ and 0.1 M sucrose were each held with a micropipette and immersed in 3 mM $CaCl_2$ and 0.12 M sucrose. After the suction pressure was reduced to allow relatively free movement of the liposomes, as evidenced by their Brownian motions, the two liposomes were brought into contact (Fig. 3 *B*). The two membranes did not adhere to each other and remained fluctuating. When $Ca^{2+}$ was diluted by exchanging the surrounding medium, the two liposomes made extensive contact and stopped fluctuating, as seen in Fig. 3 *C*, demonstrating the presence of an attractive interaction between the two. The suction pressure was increased again to separate the two liposomes (Fig. 3 *D*), and $Ca^{2+}$ was re-introduced; then the two membranes no longer adhered to each other (Fig. 3 *E*). When $Ca^{2+}$ was added without prior separation of two adhering liposomes, the adhesive area shrank slowly from the periphery, indicating that $Ca^{2+}$ should reach the membrane surfaces to separate them.

After the reintroduction of $Ca^{2+}$, there often remained a point of contact (Fig. 3 *F*) between two liposomes. It became a thin lipid tether when the two liposomes were moved farther apart. Tether formation was also observed after forced contact (by pipettes) in the presence of $Ca^{2+}$. The tether did not affect the basic adhesion characteristics: the initial contact in the absence of the tether was always indistinguishable from later contacts, with a tether connecting the two, repeated under the same ionic conditions.

These observations show that membranes composed of electrically neutral phospholipids tend to adhere to each other, that a proper amount of $Ca^{2+}$ prevents the adhesion, and that the effect of $Ca^{2+}$ is reversible.

## Estimation of the adhesion energy

To quantify the $Ca^{2+}$-induced prevention of membrane adhesion, we estimated the adhesion energy from the contact angle between two membranes. As shown in Fig. 4, *A* and *B*, two liposomes were allowed to adhere to each other head to head. The upper liposome was held with a high

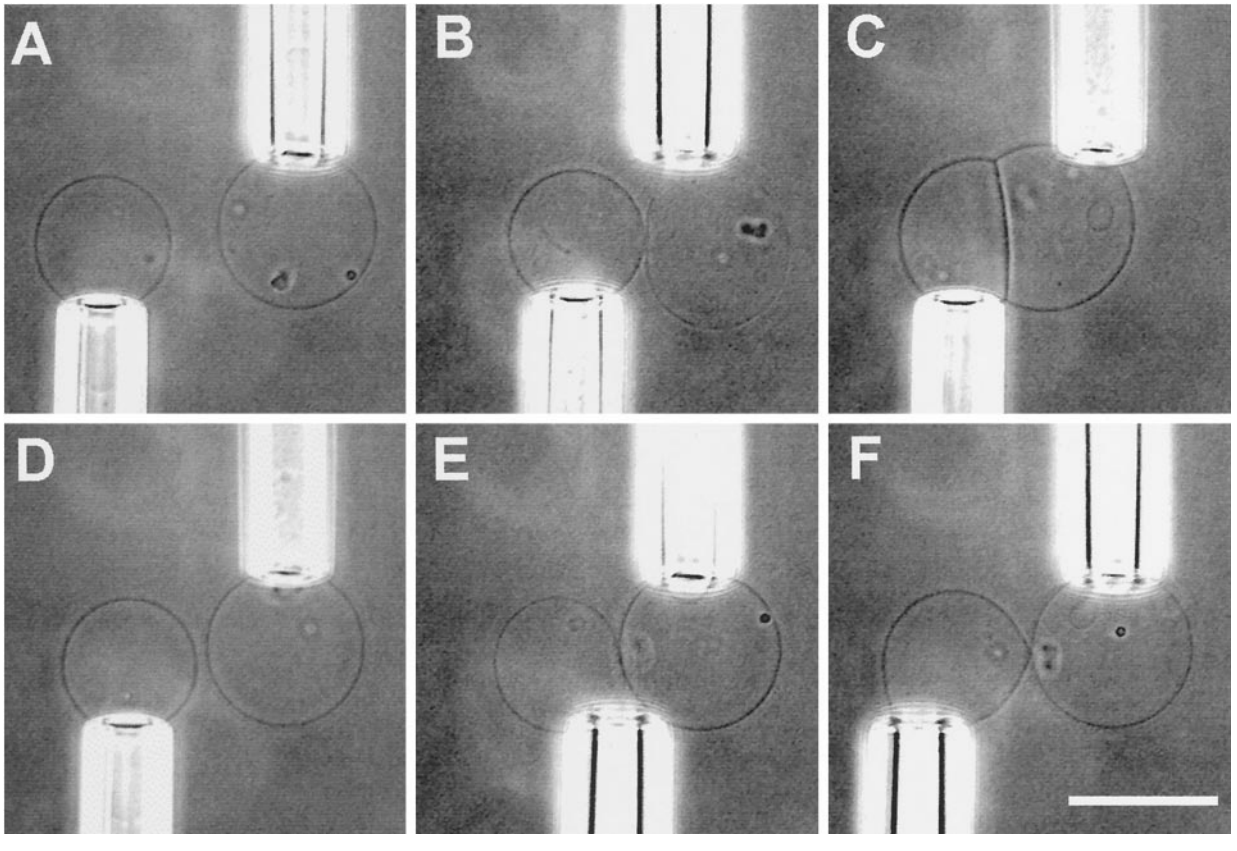
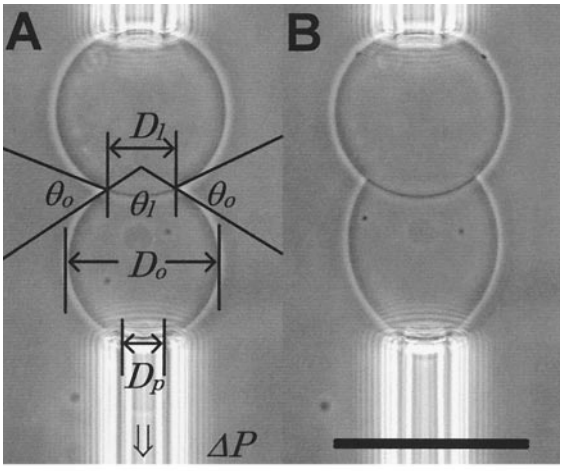



FIGURE 3 The action of $Ca^{2+}$ upon liposomal membranes. Liposomes were prepared from POPC in 3 mM $CaCl_2$ and 0.1 M sucrose. The bottom coverslip and the tips of micropipettes were coated with TMA-silane. The experiment was made as follows. (*A*) Two unilamellar liposomes were held with the micropipettes in 3 mM $CaCl_2$ and 0.1 M glucose, at a sufficiently high suction pressure that allowed firm holding, and then the external medium was replaced with 3 mM $CaCl_2$ and 0.12 M sucrose by flowing three volumes of the solution through the observation chamber (all medium changes below were made in this way). (*B*) The suction pressures were reduced and the liposomes were brought into contact. The membranes started to fluctuate, but the two did not adhere to each other. (*C*) The suction pressures were increased again and the external medium was replaced with 0.12 M sucrose. When the two liposomes were brought into contact and the suction pressures were reduced, the two adhered to each other. (*D*) An increase in the suction pressures resulted in separation of the liposomes. (*E*) The medium was replaced with 3 mM $CaCl_2$ and 0.12 M sucrose, whereupon the situation in *B* was restored. (*F*) When the two pipettes were moved apart in the same medium, a connection at a singular point remained. Further separation of the pipettes resulted in the formation of a very thin tether between separated liposomes (not shown). Temperature, 22 ± 2°C. Scale bar, 50 $\mu$m.



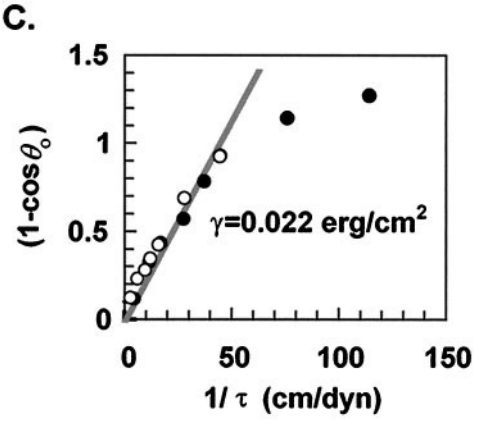




FIGURE 4 (*A*) and (*B*) Phase contrast images of giant unilamellar liposomes under the adhesion test. The suction pressure for the lower liposome was changed stepwise, while that for the upper one was kept high to form a rigid surface. In (*A*), the contact angle $\theta_o$ and parameters for the estimation of the membrane tension $\tau$ (Eq. 2) are shown. Values for $\theta_o$ measured on opposite sides were averaged, the difference being at most ~5°. The membrane tension $\tau$ was 0.06 dyn/cm for (*A*) and 0.02 dyn/cm for (*B*). Temperature, 22 ± 2°C. Scale bar, 50 $\mu$m. (*C*) A typical relation between the membrane tension $\tau$ and the contact angle $\theta_o$. Closed circles were obtained for decreasing tension and open circles for increasing tension.

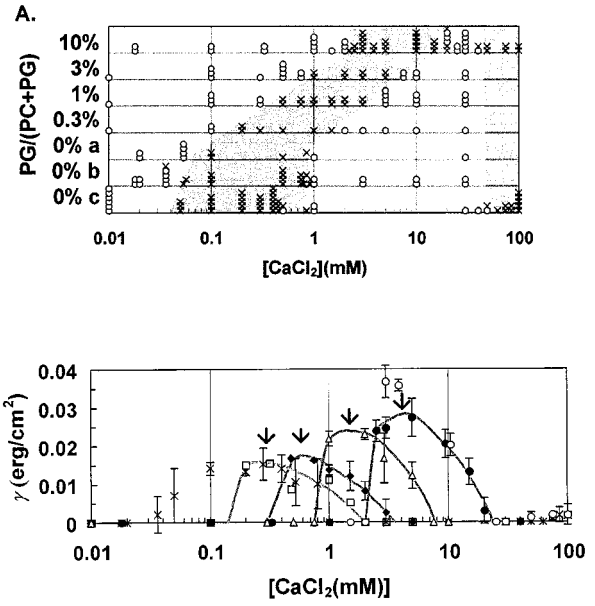



FIGURE 5 (*A*) Summary of the adhesion test. Each cross shows a sample in which liposomes adhered to each other, and each circle a sample in which adhesion was not observed. The adhesive regions are shaded. The abscissa shows the $CaCl_2$ concentration in the test medium. For the POPG content of 0% (pure POPC), three different lots of lipids were used and are separately displayed (*a, b,* and *c*). The liposomes were prepared in 0.1 M sucrose solution containing $CaCl_2$ at the following concentrations: for 0% PG, 0.5 or 1 mM, and for tests at >30 mM $CaCl_2$, 30 mM; for 0.3% PG, 0.01 mM; for 1% PG and 3% PG, 0.1 mM; for 10% PG, 1 mM, and for tests at >30 mM $CaCl_2$, 30 mM. (*B*) The adhesion energy $\gamma$ between liposomal membranes at various $CaCl_2$ concentrations. ×, 0% PG; □, 0.3% PG; ◆, 1% PG; △, 3% PG; ●, 10% PG; ○, 10% PG prepared in 30 mM $CaCl_2$. Plots indicate the average values and error bars standard deviations for 2–6 samples. The solid curves were drawn freehand. Arrows indicate peaks, judged by eye, from which the association constants $K_{pc}$ and $K_{pg}$ were estimated as explained in text. Tests for the 10% PG prepared in 30 mM $CaCl_2$ (○) were made at an osmolarity different from the others and are ignored in drawing the solid line.

suction pressure so that it remained spherical, whereas the pressure for the lower liposome was partially released. When the pressure, and thus the membrane tension $\tau$, in the lower liposome was decreased, the contact angle $\theta_o$ increased as seen in Fig. 4 *B* (Evans and Metcalfe, 1984; Evans, 1990). A linear relation between $1/\tau$ and $(1-\cos\theta_o)$ was obtained for $1/\tau <\sim 50$ cm/dyn (Fig. 4 *C*), from which the adhesion energy $\gamma$ was estimated (Eq. 1). The nonlinearity at low $\tau$ may arise from membrane undulations that oppose adhesion (Servuss and Helfrich, 1989). At high $\tau$, precise estimation of small $\theta_o$ was difficult. The case of no adhesion was easily distinguished because the lower liposome exhibited fluctuation and because the edge of the contact area remained round even when the two liposomes were pushed against each other (Fig. 3 *B*).

Fig. 5 *A* summarizes whether or not adhesion occurred as a function of the lipid composition and $CaCl_2$ concentration. Each circle represents one sample in which adhesion was not observed, and each cross a sample in which adhesion occurred (the regions where adhesion occurred are indicated as gray zones). With the increase in the POPG content, the region of crosses (below 30 mM $CaCl_2$) moves toward higher $CaCl_2$ concentrations, which is consistent with the scenario that the adhesion resulted from the cancellation of the negative charge of POPG by bound $Ca^{2+}$.

The adhesion energy $\gamma$ is plotted in Fig. 5 *B*. Within the adhesive region for each lipid composition, $\gamma$ varied sharply with the $CaCl_2$ concentration, as indicated by the solid lines. If we assume that the attractive part of the intermembrane force does not vary significantly within each region, the change in $\gamma$ mainly represents $Ca^{2+}$-induced variation in the repulsive part. The repulsive force is minimal around the arrows where, presumably, the net charge on the membrane became zero. At >40 mM $CaCl_2$, $\gamma$ was too low to obtain the linear relation in Fig. 4 *C* (our system required $\gamma >0.002$ erg/cm$^2$), and therefore the highest measurement is shown. The increase in $\gamma$ in this region is ascribed to ionic shielding of the electrostatic repulsion (see below).

For 10% POPG, the nonadhesive ranges in Fig. 5 agree with the ranges of $CaCl_2$ concentration where the yield of giant unilamellar liposomes was high (Table 1) and, in the adhesive ranges, giant liposomes did not form. Good correspondence was also found for the case of pure POPC, where liposomes formed and did not adhere at $CaCl_2$ concentrations between 1 and 50 mM. The correspondence is expected, because spontaneous formation of giant unilamellar liposomes requires separation of membranes. The pure POPC liposomes adhered to each other below 1 mM $CaCl_2$ but, at very low $CaCl_2$ concentrations, adhesion was not observed. A probable explanation, deduced from Fig. 5 *A*, is that our "pure" POPC contained negatively charged impurities corresponding to ~0.1% POPG. The lower and upper borders of the adhesive region for the pure POPC were slightly different depending on the lot (0% a, b, and c in Fig. 5 *A*), which also points to the possibility of impurities. Correspondingly, one lot produced giant liposomes at 0.5 mM $CaCl_2$, but others required 1 mM. The yield of giant unilamellar liposomes at 0 mM $CaCl_2$, where $Ca^{2+}$-induced repulsion is not expected, was quite variable, as already noted. We suspect that a significant yield at 0 mM $CaCl_2$ is owed to the presence of charged impurities.

## Association constants of phospholipids for $Ca^{2+}$

From the results in Fig. 5 *B*, we estimate the affinity of POPC and POPG for $Ca^{2+}$. We assume that (*a*) POPG was fully dissociated before binding $Ca^{2+}$, (*b*) the net charge and therefore the electrostatic potential on the membrane surface was zero at the arrows in Fig. 5 *B*, and (*c*) for simplicity, $Ca^{2+}$ bound to phospholipids with the stoichiometry of 1:1. From (*c*) the association constants are defined as (Lau et al., 1981; Marra and Israelachvili, 1985)

$$K_{pc} = \frac{[CaPC]}{[Ca^{2+}]_s \cdot [PC_f]} \tag{4}$$

$$K_{pg} = \frac{[CaPG]}{[Ca^{2+}]_s \cdot [PG_f]} \tag{5}$$

where $[Ca^{2+}]_s$ is the $Ca^{2+}$ concentration on the membrane surface, and $[PC_f]$, $[PG_f]$, [CaPC], and [CaPG] are the surface densities of free and $Ca^{2+}$-bound forms of POPC and POPG, respectively. For mixtures of POPC and POPG, we define an apparent association constant $K_{app}$ by

$$K_{app} = \frac{[CaP]}{[Ca^{2+}]_s \cdot [P_f]} = \frac{\alpha}{[Ca^{2+}]_s \cdot (1-\alpha)}, \tag{6}$$

where $[CaP] = [CaPC] + [CaPG]$, $[P_f] = [PC_f] + [PG_f]$, and

$$\alpha = [CaP]/([CaP] + [P_f]) \tag{7}$$

is the fraction of $Ca^{2+}$-bound lipid in the total lipid.

If we denote the fraction of POPG in the total lipid by $\beta$:

$$\beta = ([CaPG] + [PG_f])/([CaP] + [P_f]) \tag{8}$$

$\beta$ equals $2\alpha$ at the arrows in Fig. 5 *B* by assumptions (*a*) and (*b*). $K_{app}$ at these points are therefore given by

$$K_{app} = \frac{\beta}{[Ca^{2+}]_s \cdot (2-\beta)} \tag{9}$$

where, by assumption (*b*), $[Ca^{2+}]_s$ equals the bulk concentration of $Ca^{2+}$ at the arrows. Experimental values of $K_{app}$ given by Eq. 9 for the four arrows in Fig. 5 *B* are plotted in Fig. 6 in solid circles. In this figure, the lowest $\beta$ of 0.3% has been shifted to 0.4% to account for the probable presence of negatively charged impurities above (points for 0.3% and 0.35% are also shown).

On the other hand, $K_{app}$ at zero charge, given by Eq. 9, is calculated as a function of $K_{pc}$, $K_{pg}$, and $\beta$ by eliminating $[Ca^{2+}]_s$ with the use of Eqs. 4, 5, and 8:

$$K_{app} = \frac{1}{2(2-\beta)}[(2-3\beta)K_{pc} + 2\beta K_{pg} + \{(2-3\beta)^2 K_{pc}^2 + 4\beta^2 K_{pg}^2 + (12\beta - 10\beta^2)K_{pc}K_{pg}\}^{1/2}] \tag{10}$$

By fitting the experimental $K_{app}$ with Eq. 10 (*thin solid line* in Fig. 6), we obtained $K_{pc} = 7.3\ M^{-1}$ and $K_{pg} = 86\ M^{-1}$. Because the positions of the arrows in Fig. 5 *B* were determined by eye, we also fitted experimental $K_{app}$ estimated at the lower and upper borders of the adhesion regions (*open circles* and *dashed lines*). The association constants obtained are listed in Fig. 6 on the right. The fitting results are consistent with literature values for $K_{pc}$ of 2.5–15 $M^{-1}$ (Cevc and Marsh, 1987; Marra and Israelachvili, 1985) and for $K_{pg}$ of 8.5–100 $M^{-1}$ (Lau et al., 1981; Marra, 1986), indicating that the adhesion characteristics in Fig. 5 are the results of the binding of $Ca^{2+}$ to phospholipids.

## Effect of ionic strength

Both for POPC and for POPC/POPG, increase in the $Ca^{2+}$ concentration beyond 30 mM reduced the yield of giant

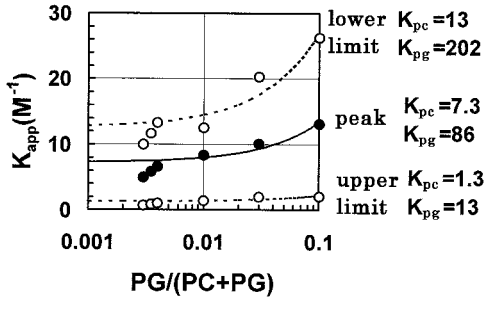



FIGURE 6 The apparent association constant $K_{app}$ as a function of the POPG content, $\alpha$. Closed circles show the experimental $K_{app}$ values (Eq. 9 in text) calculated from the $CaCl_2$ concentrations at the arrows in Fig. 5 *B*. The datum for 0.3% PG was also recalculated as for 0.35% and 0.4%, and all three results are plotted in the figure. The thin solid curve shows a least-square fit with Eq. 10 to data through 0.4% PG. The fit gave association constants, $K_{pc}$ and $K_{pg}$, shown on the right. Experimental $K_{app}$ values estimated at the borders of adhesion regions and corresponding fits are also shown in open circles and dashed lines.

unilamellar liposomes (Table 1) and induced membrane adhesion (Fig. 5), suggesting shielding of electrostatic repulsion at high ionic strengths. To see the effect of ionic strength more clearly, we tried to prepare giant liposomes (at least twice for each condition below) from POPC in solutions of 3 mM $CaCl_2$ and 0.1 M sucrose containing, in addition, KCl at 3, 10, 30, or 100 mM. Inclusion of KCl up to 10 mM did not significantly affect liposome formation: at 10 mM, the yield of giant unilamellar liposomes was 510 per 50 $\mu$l and 6% had a diameter $>100$ $\mu$m (Fig. 1 *K*). Further increase of KCl reduced the yield, and most liposomes produced were either multilamellar or heavily contaminated with lipid particles (Fig. 1 *L* for 30 mM KCl). The effect of ionic strength was also manifest in the adhesion test. With liposomes prepared in 3 mM $CaCl_2$ and 0.1 M sucrose, adhesion occurred at $>15$ mM KCl (Fig. 7). These effects of KCl are consistent with the importance of electrical repulsion in the formation of giant liposomes.

## DISCUSSION

### Comparison with previous studies

Modulation of intermembrane interactions by divalent cations has been documented. In x-ray studies (Inoko et al., 1975; Lis et al., 1981a, 1981b; Ohshima et al., 1982), interlamellar distances in multilamellar phosphatidylcholine were estimated at various concentrations of divalent cations. For $CaCl_2$ below 1 mM, the lamellae were closely packed and the interlamellar distance was independent of the $Ca^{2+}$ concentration. The distance, however, increased sharply at $\sim$1 mM $CaCl_2$, showing separation of lamellae. At or above 10–100 mM $CaCl_2$, the distance gradually decreased toward the no-salt value. $MgCl_2$ required $\sim$fivefold higher concentrations to induce similar effects (Inoko et al., 1975). These results are basically in agreement with our finding that $Ca^{2+}$ and $Mg^{2+}$ at 1–30 mM greatly promoted the formation of giant unilamellar liposomes from POPC and that the best yield was at $\sim$1 mM for $Ca^{2+}$ and $\sim$10 mM for $Mg^{2+}$. Direct measurement of forces between bilayers (Marra and Israelachvili, 1985) showed that $CaCl_2$ or $MgCl_2$ introduced a long-range repulsive force between PC membranes and the net interaction became repulsive at 1–30 mM, and that the force range became shorter at higher concentrations (shielding effect) indicating electrostatic nature of the repulsive force. This repulsive force must have played the major role in preventing the adhesion of liposomes in our adhesion tests (Fig. 3). The role of intrinsic charges has been shown in the x-ray measurements by Cowley et al. (1978) where the inclusion of negatively charged PG introduced repulsion between egg-PC membranes. Again, this result is consistent with our high yield of POPG-containing liposomes.

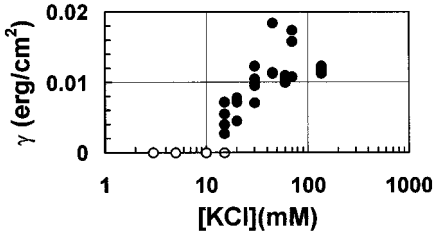



FIGURE 7 The adhesion energy $\gamma$ between liposomal membranes in 3 mM $CaCl_2$ plus KCl at indicated concentrations. Open circles show samples in which no adhesion occurred.

### Modulation of electrostatic repulsion by intrinsic and extrinsic charges

In this study we have further demonstrated an interplay between intrinsic and extrinsic charges in modulating the electrostatic interactions between membranes. With the mixture of POPC and POPG, liposomes adhered to each other in a certain range of $Ca^{2+}$ concentrations, in which no giant liposomes formed spontaneously (Table 1 and Fig. 5). Assuming that, within this range, the surface charge dominated by the intrinsic negative charge of POPG was reversed by binding of the extrinsic $Ca^{2+}$, we estimated the association constants $K_{pc}$ and $K_{pg}$ (Fig. 6). The successful fitting of four data points with the two association constants, with magnitudes consistent with literature values, supports the contention that the adhesion occurred around charge reversal points where electrostatic repulsion is expected to be minimal.

Charge reversal has directly been demonstrated by Lau et al. (1981), where a negative zeta potential of PG membrane became positive at the divalent cation concentrations of 0.1–0.2 M. Their value for $K_{pg}$ of 8.5 $M^{-1}$ in 0.1 M NaCl is lower than our value of 86 $M^{-1}$, but their $K_{pg}$ increased to 17 $M^{-1}$ in 0.01 M NaCl, suggesting that the discrepancy is due to ionic shielding. Direct force measurement (Marra, 1986) indicated a $K_{pg}$ for $Ca^{2+}$ of $\sim$100 $M^{-1}$ in $\sim$1 mM $CaCl_2$ plus 1.5 mM NaCl, closer to ours in the absence of monovalent ions.

In Fig. 8, the average charge (disregarding its sign) per lipid calculated from $K_{pc}$ and $K_{pg}$ (see Appendix) is plotted

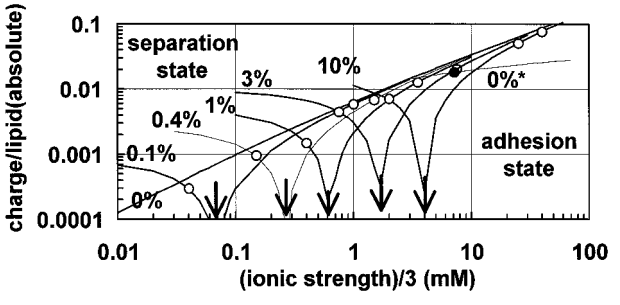



FIGURE 8 The average charge (without sign) per lipid calculated from the association constants of $K_{pc}$ = 7.3 $M^{-1}$ and $K_{pg}$ = 86 $M^{-1}$. The abscissa is one-third of the ionic strength (which equals the $CaCl_2$ concentration). Percentage values show the POPG content. The charge reversal points are indicated with arrows, and the borders of adhesion regions with open circles (pure POPC data in Fig. 5 are interpreted as 0.1% POPG). The curve labeled "0%*" is for POPC in 3 mM $CaCl_2$ plus KCl (the abscissa corresponds to 3 mM plus a third of KCl concentration), and the closed circle shows the onset of adhesion between 10 mM and 15 mM KCl in Fig. 7. See Appendix for the calculation.

in solid lines for several POPG contents. These curves indicate how the electrostatic repulsion between membranes, basically proportional to the square of the charge density, varies with $Ca^{2+}$ concentration. Open circles in this figure show the borders of adhesive regions outside of which the membranes did not adhere to each other (Fig. 5). Note that the calculated average charge decreases sharply in the adhesive region enclosed by the pair of circles on each curve. The figure also indicates that the average charge at the border increases monotonically with the ionic strength ($= 3 \times CaCl_2$ concentration). The closed circle shows the onset of adhesion for POPC liposomes when KCl was added to 3 mM $CaCl_2$ (Fig. 7). The point is almost in line with open circles. The region below the circles is of the adhesion state, and the region above of the separation state. Separation of membranes requires a higher charge density at higher ionic strengths, as anticipated for electrostatic repulsion, which is subject to shielding at high ionic strengths.

The adhesion energies $\gamma$ at the peaks in Fig. 5 *B* (and the maximal $\gamma$ in Fig. 7), where the electrostatic repulsion is expected to be absent, were $\sim 0.02$ erg/cm$^2$. Similar values have been reported for PC liposomes in 0.1 M NaCl (Evans and Needham, 1987). If we assume that the remaining attractive interaction is of van der Waals type, i.e., $\gamma = A_H/(12\pi \cdot s^2)$ where $A_H$ is the Hamaker constant and $s = 2$–$6$ nm is the distance between adhering membranes (Inoko et al., 1975; Marra and Israelachvili, 1985), our $\gamma$ suggests $A_H$ to be $2$–$40 \times 10^{-14}$ erg. Direct force measurements on PC membranes gave $A_H$ of $7 \times 10^{-14}$ erg (Marra and Israelachvili, 1985). To separate membranes, electrostatic repulsion has to counteract an attractive interaction of the order of 0.02 erg/cm$^2$.

### Importance of electrostatic repulsion in stabilizing unilamellar structures

Our results strongly indicate that electrostatic repulsion is of crucial importance in separating and keeping bilayer membranes apart. The yield of giant unilamellar liposomes was high and the membranes did not adhere to each other under conditions where the membranes were expected to bear non-zero charges. The effects of high ionic strengths in diminishing the liposome yield and enhancing membrane adhesion support this contention.

Another source of repulsive interaction has been proposed by Helfrich (1978): thermal fluctuation of membranes effectively introduced mutual repulsion between sugar-lipid bilayers and resulted in the separation of lamellae (Mutz and Helfrich, 1989). For neutral phospholipids without bound charges, this repulsive interaction would help produce unilamellar liposomes (Servuss and Helfrich, 1989). Our low but finite yield of giant POPC liposomes in the absence of salts (Table 1) may be explained by this effect. In our preparations, however, a more likely explanation is the inclusion of a small amount of negatively charged impurities (corresponding to $\sim 0.1\%$ PG), as already discussed. The trend shown in circles in Fig. 8 suggests that $<0.1\%$ would suffice for separating membranes in pure water where the ionic strength is zero. Previous reports on efficient formation of giant unilamellar liposomes from nominally neutral phospholipids might have possibly been due to the presence of trace impurities in the lipids or in solution.

Binding of divalent (or multivalent) cations produces effects other than electrostatic repulsion. For example, binding of multivalent cations to PC membranes has been reported to change the orientation of the headgroups from parallel to perpendicular with respect to the membrane surface (Brown and Seelig, 1977; Akutsu and Seelig, 1981; Altenbach and Seelig, 1984). Kataoka et al. (1985) reported that the fluidity of PC membranes was dependent on the concentration of $Ca^{2+}$ or $Mg^{2+}$. These effects occur on the concentration range of millimolar, and might play some role in the formation of unilamellar liposomes.

Fusion of vesicles made of negatively charged lipids is induced by millimolar $Ca^{2+}$ (Wilschut and Papahadjopoulos, 1979; Arnold, 1995). The fusion event is most likely to be related to the $Ca^{2+}$-induced adhesion shown in the present study.

## APPENDIX

### Average charge per lipid

From Eqs. 4 and 5, the fraction $\alpha$ of lipid binding $Ca^{2+}$ is calculated as

$$\alpha = \frac{(1-\beta)\cdot K_{pc}[Ca^{2+}]_s}{K_{pc}[Ca^{2+}]_s + 1} + \frac{\beta \cdot K_{pg}[Ca^{2+}]_s}{K_{pg}[Ca^{2+}]_s + 1} \quad \text{(A1)}$$

$\beta$ being the fraction of POPG in the total lipid. The concentration of $Ca^{2+}$ on the membrane surface is given by

$$[Ca^{2+}]_s = [Ca^{2+}]_o \cdot \exp(-2 \cdot e\psi/kT) \quad \text{(A2)}$$

where $[Ca^{2+}]_o$ is the bulk concentration of $Ca^{2+}$, $e$ the electronic charge, $kT$ the Boltzmann constant times the absolute temperature, and $\psi$ the surface potential. Thus, Eq. A1 is rewritten as

$$\alpha = \frac{(1-\beta)\cdot K_{pc}[Ca^{2+}]_o}{K_{pc}[Ca^{2+}]_o + \exp(2e\psi/kT)} + \frac{\beta \cdot K_{pg}[Ca^{2+}]_o}{K_{pg}[Ca^{2+}]_o + \exp(2e\psi/kT)} \quad \text{(A3)}$$

The net surface charge is the sum of the extrinsic charge of bound $Ca^{2+}$ and the intrinsic charge of POPG. Therefore the surface charge density $\sigma$ is given by

$$\sigma = (2\alpha - \beta)\sigma_o \quad \text{(A4)}$$

where $\sigma_o$ is the charge density expected when all lipid molecules have one electronic charge; $\sigma_o$ is $\sim 0.2$ Coulomb/m$^2$ in a fluid phase where area per lipid molecule is $\sim 0.7$ nm$^2$ (Marra and Israelachvili, 1985). Alternatively, the relation between $\sigma$ and $\psi$ is given by the Grahame equation:

$$\sigma = \sqrt{8\epsilon\epsilon_0 kT}\,\sinh(e\psi/2kT)\{[KCl] + [CaCl_2](2 + \exp(-\psi/kT))\}^{1/2} \quad \text{(A5)}$$

where $\epsilon\epsilon_0$ is the dielectric permittivity in water, and [KCl] and [$CaCl_2$] represent the bulk concentrations (Israelachvili, 1992).

Equations A3–A5 were solved numerically for $\sigma$ and $\psi$ for a given $\beta$ and ionic concentrations. The average charge per lipid, $\sigma/\sigma_0$, is plotted in Fig. 8. Note that Eq. A5 is valid for a single, isolated surface; corrections are required when two membranes approach each other.

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