

# Intramembrane Electrostatic Interactions Destabilize Lipid Vesicles

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**ABSTRACT** Membrane stability is of central concern in many biology and biotechnology processes. It has been suggested that intramembrane electrostatic interactions play a key role in membrane stability. However, due primarily to a lack of supporting experimental evidence, they are not commonly considered in mechanical analyses of lipid membranes. In this paper, we use the micropipette aspiration technique to characterize the elastic moduli and critical tensions of lipid vesicles with varying surface charge. Charge was induced by doping neutral phosphatidylcholine vesicles with anionic lipids phosphatidylglycerol and phosphatidic acid. Measurements were taken in potassium chloride (moderate ion-lipid binding) and tetramethylammonium chloride (low ion-lipid binding) solutions. We show that inclusion of anionic lipid does not appreciably alter the areal dilation elasticity of lipid vesicles. However, the tension required for vesicle rupture decreases with increasing anionic lipid fraction and is a function of electrolyte composition. Using vesicles with 30% charged (i.e., unbound) anionic lipid, we measured critical tension reductions of 75%, demonstrating the important role of electrostatic interactions in membrane stability.

## INTRODUCTION

As self-assembled structures, the mechanical properties of membranes are derived from noncovalent forces such as the hydrophobic effect, steric forces, and electrostatic interactions. The electrostatic force has drawn considerable attention, as most biological membranes are rich in anionic lipids and are therefore charged in aqueous solution. Plasma membranes of mammalian cells often consist of 10–20% anionic lipid (Yeagle, 1992), whereas bacterial membranes contain as much as 80% (Kates, 1964; for reviews on membrane electrostatics, see Cevc, 1990; Langner and Kubica, 1999).

Modulating the electrostatic interactions can tip the careful balance of forces in the bilayer and thus have implications on the mechanical properties of lipid membranes. For example, several authors have considered the effect of electrostatics on the various elastic moduli of lipid membranes both experimentally (Song and Waugh, 1990) and theoretically (Bivas and Hristova, 1991; Kozlov et al., 1992; Lekkerkerker, 1989; May, 1996). Of special interest is a series of papers regarding the rupture of red blood cell membranes placed in low ionic media (Betterton and Brenner, 1999; Cortez-Maghelly and Bisch, 1995; Gallez and Coakley, 1986). Betterton and Brenner (1999) described this using an electrostatic argument: as the salt concentration is lowered, the surface charges in the membrane are less screened. Eventually, the repulsive nature of the charge-charge interactions overpowers membrane cohesive forces, and the cell ruptures. Their conclusions are contrasted by the findings of Diederich et al. (1998). These authors, using an induced

tension argument, expected a reduction in the stability of charged black lipid membranes (BLMs) to electroporation. However, their experimental findings were that BLM stability is not affected by surface charge or electrolyte concentration. Clearly, additional experimental work is needed, preferably using spherical lipid vesicles that are structurally more relevant to cellular membranes than BLMs.

In this paper, we use the micropipette aspiration technique to determine the mechanical properties of charged lipid vesicles. Our results demonstrate that the introduction of surface charge has little effect on bilayer elasticity but dramatically lowers the tension that can be applied to vesicles before rupture. This effect is dependent on the fraction of charged lipid present in the bilayer, with critical tension reductions up to 75%. Similar results are seen for the anionic lipids phosphatidic acid (PA) and phosphatidylglycerol (PG). Data show the effect of electrolyte identity as higher stabilities are measured in moderately binding potassium chloride (KCl) than in poorly binding trimethylammonium chloride (TMA-Cl). We hypothesize that the reductions in mechanical stability are due to electrostatic interactions and demonstrate that the destabilization scales with an electrostatically induced tension. Finally, we comment on key experimental issues, especially regarding glass surface coatings, that must be addressed for the micropipette technique to be confidently used in the mechanical characterization of charged lipid membranes.

## MATERIALS AND METHODS

### Vesicle preparation

Giant unilamellar vesicles (GUVs) were created using a modification of the electroformation method (Angelova and Dimitrov, 1987; Longo et al., 1997). Neutral palmitoylcholine phosphatidylcholine (POPC) was combined with anionic lipids palmitoylcholine phosphatidylglycerol (POPG), or palmitoylcholine phosphatidic acid (POPA) (Avanti Polar Lipids, Alabaster, AL) to make 0.5 mg/ml solutions in chloroform with the desired anionic lipid

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fraction. 50  $\mu\text{l}$  of lipid solution was spread on platinum electrodes that were held 5 mm apart in a Teflon/glass cell. Films were dried under vacuum overnight to remove trace solvent. Vesicle interior solution was added to the cell, and vesicles were formed by the application of a 1.0-V sine wave across the electrodes. Interior solutions consisted of 150 mM sucrose, 1 mM electrolyte (KCl or TMA-Cl), and 10  $\mu\text{M}$  EDTA and were titrated to pH 7.4 with base (KOH or TMA-OH).

Similar to previous studies (Akashi et al., 1996; Needham and Hochmuth, 1989), a small amount of anionic lipid (at least 4%) was required to form well-behaved GUVs in electrolyte solutions. Neutral POPC vesicles did form in electrolyte solutions; however, they frequently had nonlinear stress/strain curves and were therefore deemed unsuitable for mechanical testing. Vesicles at low to moderate anionic lipid fractions had extremely high yields, with vesicles numbering in the tens or hundreds of thousands. At larger anionic fractions, yields decreased dramatically, limiting the experimentally accessible range. Additionally, yields of GUVs dropped rapidly with increasing electrolyte concentration, limiting experiments to  $\sim 1$  mM salt. Before micromanipulation, vesicles were mixed with an equal volume of vesicle exterior solution (170 mM glucose, 1 mM matching electrolyte, 10  $\mu\text{M}$  EDTA, pH 7.4). The osmotic imbalance causes vesicles to slightly deflate, aiding aspiration. Using glucose improves optical contrast and forces vesicles to sink, resulting in an accumulation of vesicles on the bottom of the sample chamber.

## Determination of vesicle mechanical properties

The micropipette technique was used to determine the elasticity and critical tension of charged vesicles. Briefly, suction pressures were applied with a glass micropipette to individual GUVs, creating an isotropic membrane tension. Vesicle deformations from increased suction pressures allow calculation of vesicle elasticity. The applied areal strain at rupture is defined as the critical strain (for a general review of the technique, see Needham and Zhelev, 1996).

Using the concepts of Helfrich (Helfrich and Servuss, 1984), the relationship between stress,  $\tau$ , and strain,  $\alpha$ , for vesicles under aspiration is (Rawicz et al., 2000):

$$\alpha = \left( \frac{kT}{8\pi K_b} \right) \ln \left( 1 + \frac{c\tau A}{K_b} \right) + \tau/K, \quad (1)$$

where  $A$  is the total membrane area,  $K_b$  is the elastic bending modulus,  $K$  is the elastic dilation modulus,  $k$  is Boltzmann's constant,  $T$  is absolute temperature, and  $c$  is a constant,  $\sim 0.1$ . At low tensions, the logarithmic term dominates, and the change in membrane area is due to the smoothing of thermal undulations. At larger tensions, the linear term dominates and the vesicle approaches the expected elastic behavior described by  $\tau = K\alpha$ . However, even at the largest tensions, there is still a small contribution from the logarithmic term (Rawicz et al., 2000). As a result, linear fits to the high-tension regime commonly reported in micropipette aspiration studies overestimate  $K$  by 10–20%.

In this paper, we follow common convention and report the slope of stress versus strain in the high-tension regime ( $\tau > 0.5$  mN/m) as the elastic dilation modulus. One must use caution here, as changes in the bending modulus (which may occur with changing surface charge) may manifest themselves in changes in the apparent dilation modulus. As it was difficult to experimentally determine  $K_b$  for highly charged vesicles, we performed calculations to assess this effect. Using experimental results (Song and Waugh, 1990) or theoretical predictions (May, 1996), electrostatically induced changes in bending moduli do not alter fits to high-tension stress/strain data (i.e.,  $K$  for both charged and neutral membranes will be similarly overestimated). We therefore neglect this effect.

Proper pipette and cell preparation protocol was critical in obtaining reproducible results (see below). Borosilicate capillaries (0.9 mm o.d., 0.5 mm i.d.; Friedrich and Dimmock, Millville, NJ) were pulled to a fine point with a Kopf model 730 puller (Tujunga, CA) and forged to  $\sim 5$ – $7$   $\mu\text{m}$  with

a Narishige MF 830 microforge (Micron Optics, Cedar Knoll, NJ). Tips were then immersed in exterior solution doped with 1 wt % bovine serum albumin (BSA, 98% by electrophoresis; Sigma Chemical Co., St. Louis, MO) for 30 min. After incubation, the BSA solution in the tip was discharged and the tip was rinsed several times by aspirating and discharging water to insure removal of any nonadsorbed protein. The tip was then filled with water and flushed for at least 5 min by aspiration in the sample chamber. To reduce the possibility of artifacts, each vesicle batch was examined with at least two pipettes. The results of the two pipettes were in every case statistically identical.

Glass used for the sample chamber was coated with a self-assembled monolayer (SAM) of 2-[methoxy(polyethylenoxy)propyl] trimethoxysilane (Gelest, Tullytown, PA). For deposition, glass was immersed for 1 min in a 1 wt % SAM solution (95% ethanol, 5% water, to pH 5 with acetic acid), rinsed in ethanol, and then cured in a 110°C oven for 15 min. Air/SAM/water contact angles consistently measured 20°–25° with a Rame-Hart goniometer (Mountain Lakes, NJ). Chambers were manufactured by gluing two SAM-treated glass pieces to a 2.0-mm Teflon spacer with RTV sealant. Superior optical resolution was achieved by using a 1.0-mm-thick microscope slide as the top of the chamber and a 1½ coverslip for the bottom. Chambers had one side open to the atmosphere for micromanipulation and were held constant at 25.0°C by a circulating bath.

Vesicle aspiration tests were conducted using an inverted optical microscope fitted with differential interference contrast optics (Nikon TE200, Micron Optics). A Narishige MHW-3 micromanipulator (Micron Optics) was used for pipette manipulation. Digital images taken with a Kodak ES310 CCD camera were directly acquired on PC using a PIXCI-D imaging board (EPIX, Buffalo Grove, IL). (Capturing digital images directly provides greater image acquisition speed and resolution compared with an analog data source such as a VCR.) Both vesicle and pipette features were measured optically using the Subpixel Edger tool in the XCAP software package (EPIX). Suction pressure applied to vesicles was measured with Validyne pressure transducers (Advanced Controls, Warmminster, PA) and recorded along with vesicle images. Pressure was stepwise increased to give membrane stress rates of  $0.9 \pm 0.1$  mN m<sup>-1</sup> min<sup>-1</sup> until vesicle rupture. Mechanical properties reported are the averages of  $\sim 20$  vesicles.

## Chemicals and reagents

Unless otherwise stated, all chemicals were from Sigma, of the highest grade available, and used as received. Water used was produced by a Milli-Q UF unit (Millipore, Bedford, MA) and had a resistivity of 18.2 megohm-cm.

## Micromanipulation of charged lipid vesicles

In this work, we used the micropipette aspiration method to assess the effect of electrostatic interactions on the mechanical properties of lipid membranes. Although this technique has become somewhat routine in the characterization of neutral membranes, we found alterations in standard micropipette protocols were essential to determine the mechanical properties of charged vesicles. Given the growing popularity of this versatile technique, we report on these new protocols here.

The most important factor in the success of charged membrane aspiration involves proper preparation of the pipette tip and sample chamber. Vesicles, both charged and uncharged, adhere to bare glass. This results in very irreproducible stress/strain curves and extremely low lysis tensions when vesicles are examined by micropipette aspiration. To alleviate this problem, most micropipette experimenters use BSA, a globular protein that adheres strongly to glass, either as a precoating on the chamber and tip or in the sample solution itself.

We found that this protocol is not applicable to the micropipette aspiration of charged vesicles in either electrolyte or nonelectrolyte solutions. The presence of even trace amounts of unadsorbed BSA in the sample chamber results in degraded vesicles, as evidenced by extremely irreproducible mechanical data or even the complete dissolution of GUVs (data not shown). This is consistent with experiments that show BSA in solution causes leakage of anionic lipid vesicles (Wu and Fletcher, 2000; Yokouchi et al., 2001). Although we assume the concentration of unadsorbed BSA remaining in the sample chamber is markedly lower than the 0.1 mg/ml Yokouchi et al. used to induce leakage from PG vesicles (Yokouchi et al., 2001), it is quite possible that even when present at lower concentrations, BSA structurally perturbs anionic lipid membranes.

To eliminate unadsorbed BSA from the sample, we have adopted a protocol in which the pipette tip is thoroughly rinsed with water after BSA incubation and the sample chamber is coated with a self-assembled monolayer (SAM) instead of BSA. (It would clearly be desirable to eliminate BSA altogether by SAM-coating the pipette tip. To this end, we have screened SAMs with different terminal moieties (methyl, methoxyl, and chlorodimethylsiloxy) but have yet to find one that allows reproducible determination of POPC mechanical properties (unpublished results). Fortunately, it seems far more important to avoid BSA treatment of the cell (as compared with the tip), as the cell has a large surface area and many nooks that make rinsing of unadsorbed BSA difficult.) We emphasize the removal of unadsorbed BSA, because once adsorbed to glass, BSA does not appreciably desorb into aqueous solutions (Zhelev, 1998). We have conducted fluorometric and contact angle studies that suggest that BSA also does not desorb onto POPG/POPC vesicles in 1 mM electrolyte (data not shown). Therefore, minimization of BSA-coated surfaces and copious rinsing should result in BSA-free solutions.

Finally, although the problems with the standard BSA protocol are exacerbated when charged vesicles are examined, we strongly recommend that experimenters exercise caution even when using BSA in membrane tests on neutral vesicles. It has been shown that BSA binds to (Wu and Fletcher, 2000) and causes aggregation of (Sato et al., 1999; Schenkman et al., 1981) neutral phosphatidylcholine vesicles. This suggests that the use of BSA could result in experimental artifacts when neutral membranes are examined.

## RESULTS AND DISCUSSION

We used the micropipette aspiration technique to determine the mechanical properties of lipid vesicles of varying surface charge. According to Gouy-Chapman-Stern (GCS) electrostatic theory, surface charge is set by the fraction of anionic lipids in the membrane and the extent of ion-lipid binding (Cevc, 1990). We therefore measured the elastic modulus,  $K$ , and the applied tension required to rupture,  $\tau_c^{\text{mech}}$ , for vesicles as a function of both anionic lipid fraction and electrolyte composition. Two different anionic lipids, POPG, ( $pK_a^0 = 2.9$ ) and POPA ( $pK_a^0 = 3.5$ ;  $pK_a^{2-} = 9.5$ ) (Cevc, 1990) were examined. We also analyzed vesicles in two different electrolytes, KCl and TMA-Cl. These salts were chosen because they bind to anionic lipids with differing affinities; KCl is considered a moderately binding salt, whereas TMA-Cl binds poorly to lipid membranes (Eisenberg et al., 1979).

Measured area dilation elastic moduli for POPG/POPC vesicles in 1 mM KCl are shown in Fig. 1. Within the margin of error, we detected no measurable change in elasticity as the anionic lipid fraction,  $\lambda$ , is increased. The average value, 143 mN/m, was significantly different than

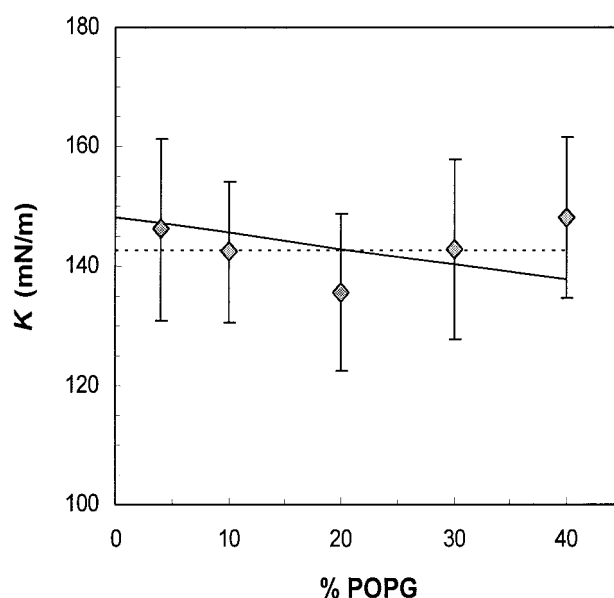


FIGURE 1 Measured areal dilation elasticities for POPG/POPC vesicles in 1 mM KCl. Symbols are experimental values (average  $\pm$  SD). The solid line is the prediction of Eq. 7 with  $K^N$  fitted to 148 mN/m. The dashed line is a constant value  $K = 143$  mN/m, the average of all points.

the 178 mN/m found in our lab for neutral POPC vesicles in nonelectrolyte (Shoemaker and Vanderlick, 2002). Because  $K$  is constant with increasing anionic lipid content, this difference is not electrostatic in origin but might rather reflect the difference in membrane hydration of electrolyte and nonelectrolyte solutions.

Data for POPG/POPC vesicles in 1 mM TMA-Cl and POPA/POPC vesicles in 1 mM KCl shown in Table 1 also show a lack of dependence on anionic lipid fraction. There is little experimental work on charged vesicles in the literature for comparison. Akashi et al. (1996) reported elastic

TABLE 1 Mechanical properties for anionic lipid vesicles in 1 mM electrolyte solution

Lipid components	Salt	Anionic lipid fraction, $\lambda$	Elastic modulus, $K$ (mN/m)	Critical tension, $\tau_c^{\text{mech}}$ (mN/m)
POPG/POPC	KCl	0.04	146 $\pm$ 15	7.1 $\pm$ 1.6
		0.1	142 $\pm$ 12	5.5 $\pm$ 0.9
		0.2	135 $\pm$ 13	2.9 $\pm$ 0.9
		0.3	143 $\pm$ 15	2.4 $\pm$ 0.8
		0.4	148 $\pm$ 13	2.3 $\pm$ 0.9
POPG/POPC	TMA-Cl	0.04	145 $\pm$ 10	7.2 $\pm$ 1.0
		0.1	148 $\pm$ 16	5.2 $\pm$ 0.7
		0.2	140 $\pm$ 12	2.2 $\pm$ 0.7
		0.3	NA*	1.7 $\pm$ 0.7
POPA/POPC	KCl	0.04	156 $\pm$ 13	7.3 $\pm$ 1.2
		0.1	156 $\pm$ 7	6.1 $\pm$ 0.7
		0.2	145 $\pm$ 11	5.0 $\pm$ 1.1
		0.3	154 $\pm$ 17	2.9 $\pm$ 1.1

\*Vesicles ruptured too low to accurately measure  $K$ .

moduli for 10% charged vesicles equal to neutral membranes. Using osmotic swelling, Haines and co-workers (Haines et al., 1987) demonstrated a large effect of surface charge on elasticity but later retracted that result (Rutkowski et al., 1991).

We can compare our elasticity results to a simple continuum model. The free energy of a neutral monolayer,  $f^N$ , is commonly described by (Israelachvili et al., 1980):

$$f^N = \gamma a + \gamma a_0^2/a, \quad (2)$$

where  $\gamma$  is the interfacial tension,  $a_0$  is the optimal area per lipid headgroup, and  $a$  is the instantaneous lipid area. In this model, the energetic cost of small deformations can be found as (Israelachvili et al., 1980):

$$\frac{f^N(a)}{a_0} = 2\gamma + \frac{1}{2}(2\gamma)\alpha^2, \quad (3)$$

where  $\alpha$  is membrane areal strain,  $(a - a_0)/a_0$ . Because, by definition, mechanical free energy  $\sim \frac{1}{2}K\alpha^2$ , we see that the areal dilation elasticity for a neutral lipid bilayer,  $K^N$ , is  $4\gamma$  (where the additional factor of 2 is to account for the two monolayers in a bilayer) (Israelachvili et al., 1980).

We now assume that we may approximate the free energy of a charged monolayer,  $f^C$ , by adding the free energy of electrostatic interactions,  $f^{el}$ , to that of a neutral monolayer (i.e.,  $f^C = f^N + f^{el}$ ). We can again calculate the cost of small derivations from  $a_0$ :

$$\frac{f^C(a)}{a_0} = 2\gamma + \frac{f^{el}(a_0)}{a_0} + f^{el'}(a_0)\alpha + \frac{1}{2}(2\gamma + a_0 f^{el''}(a_0))\alpha^2 \quad (4)$$

(Note that because we are interested in the coefficient of the quadratic term, we have neglected the small change in  $a_0$  due to electrostatic interactions.) Using the definition of elasticity and accounting for the two monolayers in the bilayer we see:

$$\begin{aligned} K^C &= 4\gamma + 2a_0 f^{el''}(a_0) \\ &= K^N + 2a_0 f^{el''}(a_0) \end{aligned} \quad (5)$$

To evaluate  $K^C$ , the areal dilation elasticity of the charged bilayer, we need only to calculate the free energy of electrostatic interactions from Gouy-Chapman theory. We use the derivation given by May (1996) following the method of Lekkerkerker (1989):

$$f^{el} = 2kT\lambda \left[ \frac{1-q}{p} + \ln(p+q) \right] \quad (6)$$

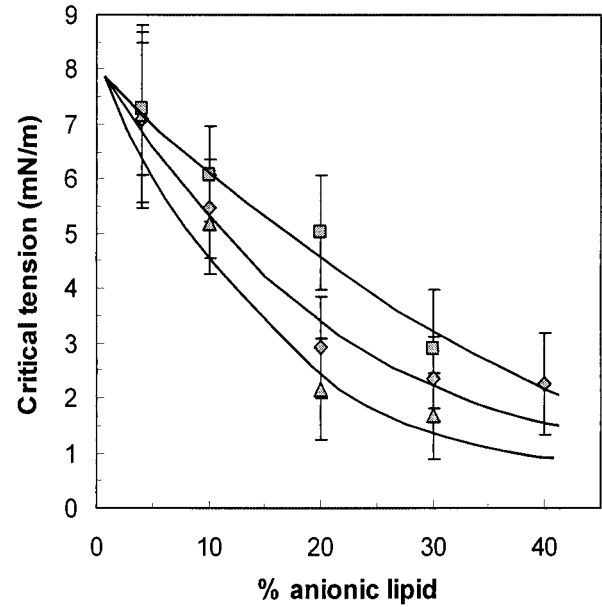


FIGURE 2 Measured critical stresses for anionic vesicles in 1 mM electrolyte. Triangles, diamonds, and squares correspond to POPG/POPC in TMA-Cl, POPG/POPC in KCl, and POPA/POPC in KCl, respectively. Data are averages  $\pm$  SD, and lines are drawn to aid the eye.

with

$$p = \frac{\lambda e^2}{2\kappa a \epsilon \epsilon_0 kT}$$

and

$$q = \sqrt{p^2 + 1},$$

where  $e$  is the elementary charge,  $\kappa$  is the inverse Debye length,  $\epsilon$  is the dielectric constant, and  $\epsilon_0$  is the permittivity of vacuum. We can now calculate the expected effect of electrostatic interactions on lipid membrane elasticity as:

$$K^C = K^N - \frac{4kT\lambda p}{aq} \quad (7)$$

Predictions from Eq. 7 are plotted along with the experimental data in Fig. 1. Clearly, the introduction of electrostatic interactions has little effect on lipid elasticity, as the predicted total change in  $K$  is within the size of the experimental error bars. Therefore, the lack of a strong dependence of  $K$  on  $\lambda$  for each of our experimental systems is not surprising; in fact it suggests our data are well behaved and reproducible.

Fig. 2 shows the applied tension that results in vesicle rupture,  $\tau_c^{\text{mech}}$ , for lipid vesicles of varying anionic content (data are also tabulated in Table 1). At low anionic fraction ( $\lambda = 0.04$ ), vesicles rupture at  $\sim 7$  mN/m, similar to uncharged POPC vesicles in nonelectrolyte solutions (Shoemaker and Vanderlick, 2002). There is little in the literature



on charged vesicles for comparison. Akashi et al. (1996) did not report a critical strain for 10% anionic vesicles, but implied that vesicles frequently ruptured at tensions less than 1 mN/m. This is well below our results for our 10% POPG vesicles, and likely results from their use of BSA as a surface coating (see Materials and Methods).

As shown in Fig. 2, we measure a clear change in  $\tau_c^{\text{mech}}$  as the fraction of anionic lipid is increased. This effect is seen for both POPG and POPA and in the presence of different electrolytes, KCl and TMA-Cl. Although there appear to be subtle differences between the lipid/salt systems, in all cases the lysis tensions steeply decrease with increasing anionic fraction. At 30% anionic lipid, the decrease in  $\tau_c^{\text{mech}}$  is 60–75%.

As discussed earlier, the addition of anionic lipid adds an electrostatic component to the membrane free energy. This by itself might be expected to alter membrane stability. However, we must consider other possible effects of anionic lipid inclusion. Surface hydration, a key consideration for membrane mechanics (Cevc, 1990), increases with anionic lipid fraction. This, however, should serve to increase stability (Kraayenhof et al., 1996), contrary to our experimental results. Another potential effect involves the different headgroup size of the PA or PG lipid molecules. This is unlikely to be key, as micropipette studies suggest no effect from the inclusion of the small headgroup phosphatidylethanolamine (PE) on PC membrane stability (Evans and Needham, 1987). Finally, because our charged membranes are two-component systems, there is the possibility of phase separation, which could impact mechanical properties. Differential scanning calorimetry has shown that in the absence of calcium, PG/PC (Findlay and Barton, 1978) and PA/PC (Graham et al., 1985) membranes are in a single fluid phase at room temperature. We have performed all experiments in the presence of EDTA to remove divalent impurities, meaning that phase separation is not a likely cause for the lowered stability of anionic vesicles. We therefore conclude that electrostatic interactions are the most probable explanation for our experimental data.

Additional evidence directly implicating electrostatic interactions is the similar behavior of POPA- and POPG-doped vesicles. PA and PG headgroups have identical charge states at neutral pH and are therefore electrostatically identical. Otherwise, the two lipid molecules are somewhat dissimilar. PG lipids contain a glycerol moiety linked to the phosphate group in the head region. This not only alters the size of the headgroup, it sterically impedes interaction with either molecules in solution or other lipids as compared with the sterically unhindered PA structure (Langner and Kubica, 1999). Thus, the lipids, for example, show different binding characteristics (Cevc, 1990), gel transition temperatures (Silvius, 1982), and phase behavior (Findlay and Barton, 1978; Graham et al., 1985).

If indeed the reduction in stability is solely due to electrostatic interactions, electrolyte composition and concen-

tration should be important parameters. For example, higher salt concentrations should screen headgroup charges, reducing the effect. Unfortunately, unlike previous techniques (Akashi et al., 1996), our formation method requires low (<10 mM) electrolyte concentration, making concentration effects impossible to detect. We can, on the other hand, examine vesicle mechanical stability in the presence of different electrolytes. Anionic lipids bind electrolytes from solution, causing the surface charge to partially neutralize. The extent of this neutralization is both lipid and salt dependent, leading to the term specific binding (Eisenberg et al., 1979). In the GCS framework, this is usually described by a Langmuir-type equilibrium:

$$\lambda^{\text{eff}} = \frac{\lambda}{1 + BC \exp(-e\psi_0/kT)}, \quad (8)$$

where  $\lambda$  is the anionic lipid fraction in the bilayer and  $\lambda^{\text{eff}}$  is the effectively charged lipid fraction after binding.  $B$  is a first-order binding constant and the exponential function accounts for the accumulation of cations at the interface due to electrostatic attraction. As alluded to earlier, the glycerol moiety of PG headgroups gives the lipid a lower binding affinity relative to PA lipids (Langner and Kubica, 1999). The bulky TMA<sup>+</sup> ion binds less efficiently than K<sup>+</sup>, also presumably for steric reasons (Eisenberg et al., 1979). Thus, binding constants follow the pattern PA<sup>-</sup>:K<sup>+</sup> > PG<sup>-</sup>:K<sup>+</sup> > PG<sup>-</sup>:TMA<sup>+</sup>. Common values found in the literature range from 1.1 M<sup>-1</sup> for K<sup>+</sup> binding to the monomethyl ester of phosphatidic acid to negligible binding of TMA<sup>+</sup> to phosphatidylserine (Cevc, 1990; Eisenberg et al., 1979; Kraayenhof et al., 1996; Langner and Kubica, 1999).

Fig. 2 shows that, although all three lipid/salt systems show similar monotonic decreases in lysis tension, there appear to be small systematic differences. We now may understand this based on differing cation binding levels; because higher binding constants result in greater anionic charge neutralization, lipid stabilities should fall in the same order. This trend is seen in Fig. 2, further suggesting that the stability reduction is electrostatic in nature.

Defining the mechanistic cause of this electrostatic stability reduction is more difficult. One approach, following the suggestion of Diederich et al. (1998), is to consider the electrostatic interactions in terms of an induced membrane tension. As mentioned earlier, neutral POPC vesicles can support mechanical tensions up to ~7 mN/m before rupture (Shoemaker and Vanderlick, 2002). If other forces generate a membrane tension, this may reduce the mechanical tension that the vesicle can withstand before the membrane is burst.

Electrostatic interactions force lipid films to dilate (Jahnig et al., 1979), creating such an effective membrane

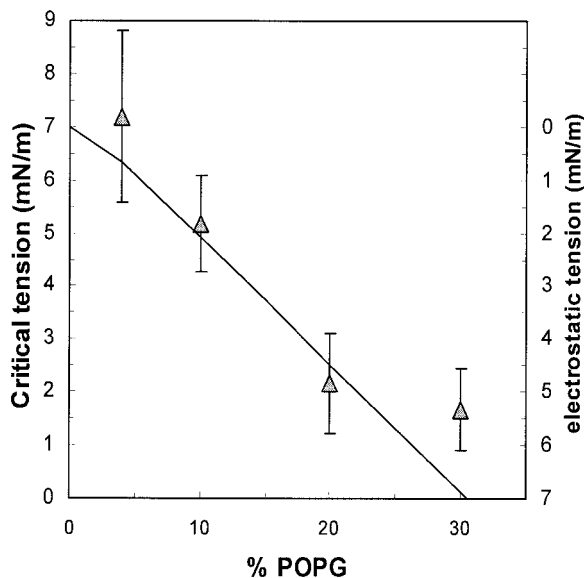


FIGURE 3 Symbols are measured critical tensions for POPG/POPC vesicles in 1 mM TMA-Cl (*left axis*). The solid line is  $\tau^{el}$  calculated from Eq. 9 assuming negligible ion binding (*right axis*).

tension. This effective electrostatic tension,  $\tau^{el}$ , can be calculated using the definition of tension and Eq. 6:

$$\tau^{el} = -df^{el}/da = 2 \frac{2kT\lambda^{eff}}{a} \left( \frac{q-1}{p} \right), \quad (9)$$

where the factor of 2 is to account for the two monolayers in a bilayer. In Fig. 3, we use this equation to calculate electrostatically induced tension in the absence of ion binding (that is,  $\lambda^{eff} = \lambda$ , shown on the right axis) along with the critical tension data from the POPG/TMA-Cl system (shown on the left axis). Noting that the scales of the two axes are identical, the reduction in measured critical tension equals the tension from electrostatic interactions. This suggests that the electrostatic and mechanical tensions are simply additive; as the electrostatic tension is increased, the mechanical tension that may be applied before the critical point is reached is diminished. This is summarized in the equation:

$$\tau_c^{mech} = \tau_c^{total} - \tau^{el} = \tau_c^{total} - \frac{4kT\lambda^{eff}}{a} \left( \frac{q-1}{p} \right), \quad (10)$$

where  $\tau_c^{total}$  is the total tension that is required for membrane rupture. This quantity is defined as the tension required to rupture a neutral membrane and is assumed to be a constant with increasing anionic lipid content.

Equation 10 can be used to fit our experimental data using the binding constant  $B$  as a fit parameter. Fig. 4 shows the results of this procedure for each of the lipid/salt systems. The data are fairly well described using binding constants of  $0.8 \text{ M}^{-1}$ ,  $0.4 \text{ M}^{-1}$ , and  $0.0 \text{ M}^{-1}$  for  $\text{PA}^-:\text{K}^+$ ,  $\text{PG}^-:\text{K}^+$ , and  $\text{PG}^-:\text{TMA}^+$ , respectively. Numerical com-

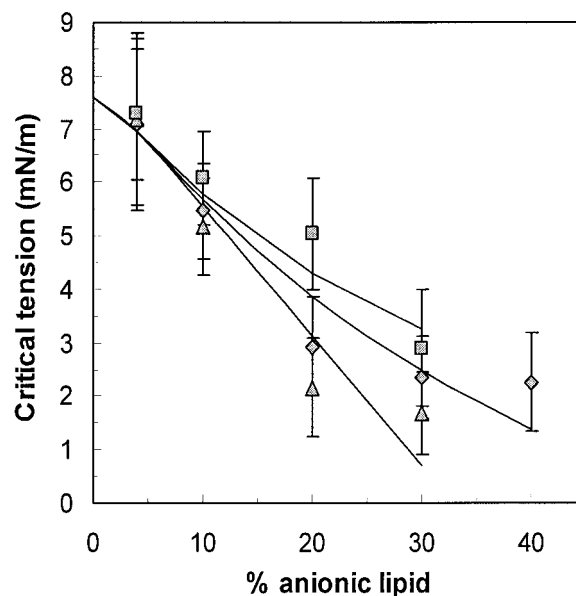


FIGURE 4 Model fits (shown in *solid lines*) to experimentally measured anionic vesicle critical stresses. Symbols match those in Fig. 2. Fits were obtained using Eq. 10 and binding constants of  $0.8 \text{ M}^{-1}$ ,  $0.4 \text{ M}^{-1}$ , and  $0.0 \text{ M}^{-1}$  for  $\text{PA}^-:\text{K}^+$ ,  $\text{PG}^-:\text{K}^+$ , and  $\text{PG}^-:\text{TMA}^+$ , respectively.

parison with literature values is difficult, as  $B$  is sensitive to salt concentration (Kraayenhof et al., 1996), and to the best of our knowledge no binding studies have been conducted in 1 mM electrolyte. Instead, we point out that we have correctly captured the relative order of the binding constants and the resulting  $B$  values are within the range normally reported ( $0.0\text{--}1.1 \text{ M}^{-1}$ ) (Cevc, 1990; Eisenberg et al., 1979; Kraayenhof et al., 1996; Langner and Kubica, 1999).

The data from Fig. 4 are replotted in Fig. 5 as scaled critical tension (critical tension divided by that of a neutral POPC membrane) versus the effective charged lipid fraction  $\lambda^{eff}$ . As expected, the data collapse to the line predicted by Eq. 10. In addition, this plot indicates the large magnitude of this effect, as an effectively charged lipid fraction of 0.3 reduces the tension required to rupture by 75%.

Although a simple tension additivity model appears to follow the data well, we must ask how physically reasonable the approach is. The model relies on two basic assumptions: first, that electrostatic and mechanical tensions (or electrostatic and mechanical free energies) are additive, and second, that the critical tension needed to lyse the membrane is not a function of anionic lipid content. The first assumption seems logical. As supporting experimental evidence, Needham and Hochmuth (1989) showed that the effects of electrocompression and mechanical deformation were additive when electroporating tense lipid vesicles (these authors used additive strains rather than stresses, an equivalent argument when  $K$  is a constant). Additionally, NMR (Pott et al., 1995) and Raman spectroscopy (Jahnig et al., 1979) show lipid tail ordering is nearly independent of headgroup charge. This

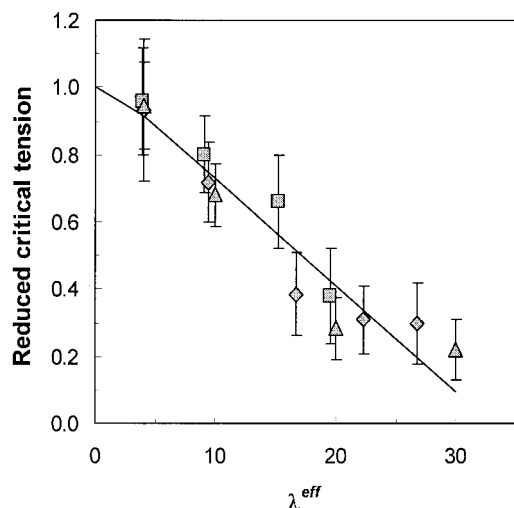


FIGURE 5 Scaled critical tensions ( $\tau_c^{mech}/\tau_c^{total}$ ) versus  $\lambda^{eff}$ , the effectively charged lipid fraction. Plotted in this way, experimental data (shown in symbols defined as in Fig. 2) collapses on the predictions of Eq. 10, shown by the solid line.

suggests that the mechanical properties of the neutral bilayer, which are dominated by tail interactions (Needham and Zhelev, 1996), are relatively unaffected by electrostatics. Thus, to first order, electrostatic interactions may be assumed additive to neutral bilayer interactions.

The second assumption is more problematic. Rupture is usually described by the propagation of unstable pores. If membrane energy decreases with increasing pore radius, the membrane is said to be unstable and will lyse (Zhelev and Needham, 1993). Therefore, our assumption of constant  $\tau_c^{total}$  implies the energetics of the pore are essentially unchanged by the addition of anionic lipid. Electroporation studies have suggested that the effective line energy of charged bilayers is higher than their neutral analogs, meaning charged bilayers possess a higher energy barrier to pore growth (Genco et al., 1993). This indicates that  $\tau_c^{total}$  would increase with  $\lambda$ , offsetting a portion of the expected stability reduction.

We can rationalize electrostatic effects on pore energetics in the spirit of Gouy-Chapman-Stern theory. If we assume the total area of the membrane constant, opening a pore of radius  $r_p$  forces charged lipids into a closer configuration, which is electrostatically unfavorable. However, as Betterton and Brenner have pointed out, if the pore is sufficiently small ( $r_p \ll \kappa^{-1}$ ), the screening cloud of double-layer charges effectively spill over and fill the pore. Thus, the volume of the double layer is essentially unchanged, and the contribution of electrostatic interactions to pore energetics is vanishingly small (Betterton and Brenner, 1999). The radius for an unstable pore in a neutral membrane can be approximated as the ratio of line energy,  $10^{-11}$  N (Zhelev and Needham, 1993), and the tension required to rupture,  $\sim 7$  mN/m. Comparing the result of 1.4 nm to the Debye

length in 1 mM electrolyte, 9.7 nm, shows that we may indeed be in the small pore limit and  $\tau_c^{total}$  is approximately constant. Improved calculations will be needed to further evaluate this result.

## CONCLUSIONS

Using the micropipette aspiration technique, we have shown that anionic lipids do not alter the elasticity of lipid vesicles but substantially reduce their mechanical stability. This destabilization, measured as the decrease in mechanical tension that induces vesicle rupture, is a function of the anionic lipid fraction in the bilayer and the choice of electrolyte. Similar results are seen using two different anionic lipids, POPG and POPA. We hypothesize that the reductions in stability are due to the presence of electrostatic interactions in the lipid membrane. We can fit our stability data with a simple model in which membranes rupture at a fixed sum of electrostatic and mechanical tensions. The large ( $\sim 75\%$ ) reductions in membrane stability dictate that this effect be considered whenever charged membrane mechanics are examined.

Our results contrast those of Diederich et al. (1998) who examined the stability of charged BLMs to electropermeabilization. Contrary to their expectations, the authors did not see a reduction in BLM stability with increased electrostatic interactions. The reasons for the discrepancy between their results and ours are not yet clear. We postulate that the difference may stem from the experimental systems used: vesicles are closed systems, whereas BLM lipid molecules may exchange between the bilayer and the Plateau-Gibbs reservoir (Picard et al., 1991). As a result, the elastic moduli and interfacial tension of BLMs and vesicles differ markedly (Picard et al., 1991), which could give rise to the discrepancy between our results and those of Diederich and colleagues.

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