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Bending rigidity of mixed phospholipid bilayers and the equilibrium radius of corresponding vesicles

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In spite of the large mean bending moduli observed for phospholipid bilayers, stable vesicle phases were recently observed for dilute solutions of charged phospholipids. A correspondingly large negative Gaussian bending modulus associated with charged membranes results in an overall curvature energy that is so low that entropic stabilization is possible. The mean bending modulus determines the membrane persistence length and therefore it is reasonable that there is a correlation between the membrane rigidity and the size of the lipid vesicles. Here we show that in mixtures of the anionic phospholipid dioleoylphosphatidylglycerol and the zwitterionic phospholipid dioleoylphosphatidylcholine the radius of vesicles produced by repetitive freeze-thaw cycles is considerably smaller than expected from the rigidities of the corresponding pure lipid bilayers. Self-consistent field calculations indicate that the changes in the equilibrium radius of mixed bilayers can be attributed to the dependences of the mean bending modulus k_c on lipid mixing and the average surface charge density.

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INTRODUCTION

Mixtures of anionic and cationic surfactants form spontaneous vesicles in a certain composition and concentration regime. It has been argued that this spontaneous vesicle phase represents thermodynamic equilibrium and is not just a kinetically trapped metastable state [1–3]. It is, however, not always *a priori* clear how these vesicles are stabilized against the formation of multilamellar sheets.

There are, in principle, two scenarios for vesicles to be in thermodynamic equilibrium; they can either be stabilized by entropy on a mesoscopic scale (undulations, translation, polydispersity) [4–7], or they can have a nonzero spontaneous curvature [1]. Both stabilization mechanisms give rise to vesicles of a particular size and size distribution. Membranes composed of a single lipid species are generally not believed to exhibit a spontaneous curvature, as the two monolayers that make up the bilayer are oppositely curved. Vesicles composed of one surfactant species can therefore only be stabilized against the formation of lamellar phases by entropic contributions on mesoscopic length scale.

Phospholipid bilayers are reported to have a mean bending modulus k_c that is of order $10k_BT$ [8–10]. Such large k_c values are often thought to prevent the entropic stabilization of phospholipid vesicles [11,12]. In the absence of a spontaneous curvature the energy needed to curve a bilayer into a closed vesicle, E_{ves}, is given by $E_{ves}=4\pi(2k_c+\bar{k})$, where $\bar{k}$ is the Gaussian bending modulus. Although the value of k_c is positive for a phospholipid bilayer, the sign of $\bar{k}$ can, in principle, be either positive or negative. However, it is usually assumed that it is negative for bilayer membranes. The negative Gaussian bending modulus stabilizes vesicles because it reduced the curvature energy of the vesicles E_{ves}. A low value of the curvature energy means that, in spite of the high k_c values measured, entropic stabilization of phospholipid vesicles is possible. This occurs, in particular, when the values of k_c and $-\bar{k}$ are of the same order of magnitude or when the $-\bar{k}$ exceeds k_c.

Self-consistent-field (SCF) calculations have shown that for charged phospholipid bilayers at low ionic strength, k_c and $-\bar{k}$ are indeed of the same order of magnitude [13,14] and that in extreme cases, i.e., at very low ionic strength E_{ves} approaches zero. Moreover, a stable vesicle phase has been observed experimentally over a range of phospholipid and salt concentrations for charged bilayers composed of dioleoylphosphatidylglycerol (DOPG) or dioleoylphosphatidylcholine (DOPC) [13,14]. It was found that the average radius of these vesicles scales with the lipid concentration c as $R \propto c^{0.15}$, which suggested that translational entropy and undulations are large enough for vesicle stabilization [6,7]. Even though the vesicle size was found to respond to changes in the lipid concentration, we have argued before [13,14] that in first order the vesicle size should be correlated to the membrane persistence length. The membrane persistence length is a strong function of the membrane rigidity (k_c). Indeed, in previous work we have shown that there is a strong correlation between the predicted values of the membrane rigidity (as resulted from SCF calculations) and experimentally found vesicle sizes of vesicles produced by the freeze-thaw cycling method.

Bilayers composed of more than one surfactant or lipid species open at least, in principle, the possibility to obtain membranes with a spontaneous curvature. These mixed systems have therefore been a subject of both theoretical [4,5,15,16] and experimental investigations [1,3]. These studies have mainly focused on mixtures of cationic and anionic surfactant species for which spontaneous equilibrium vesicles were first observed [17]. In (cationic-anionic) surfactant mixtures complexation and nonideal behavior may allow for the formation of bilayers of which the two monolayers differ strongly in surfactant composition. This composition difference can, in principle, result in monolayers with opposite spontaneous curvature [4,5]. At sufficient nonideal mixing, e.g., when there is a miscibility gap, one leaflet of the bilayer can be populated by one amphiphilic species and the other leaflet by the second one. Then the spontaneous curvature can be controlled by the lipid composition. In the absence of such a miscibility gap, however, it is generally accepted that the asymmetry between the inner and outer layers is very small, especially for vesicles that are large compared to the bilayer thickness. These minor composition differences do not lead to a spontaneous curvature and such vesicles can only be entropically stabilized [18]. The same conclusion is also reached from symmetry arguments. As long as the flat bilayer has leaflets with similar composition, the bilayer can bend equally probable to one side or the other and thus there is no spontaneous curvature. For systems in which the flat bilayer has already two leaflets with different composition, which is possible for mixtures that have a solubility gap, the curvature in one direction differs from that in the other direction and a spontaneous curvature is expected. Below we focus on systems in which the lipid miscibility is complete.

There exists yet another subtle effect that must be expected for vesicles composed of a mixture of lipids. As shown below and consistent with intuition we should expect a lowering of the bilayer bending rigidity as compared to the membrane rigidities expected from the interpolation of the values of the pure bilayers. This relatively low value of the bending modulus results in a correspondingly lower value for the membrane persistence length which, as we argued above, will result in a lowering of the equilibrium size of the vesicles. The work presented in this paper focuses on this effect.

As in previous work [13,14] we present both experimental observations for the size of vesicles formed by the freeze-thaw method and confront these results with predictions from a molecularly realistic self-consistent field analysis. In the latter calculations we focus on the mechanical properties. More specifically we have used mixtures of the bilayer forming phospholipids DOPC and DOPG and studied the radii of entropically stabilized vesicles as a function of the bilayer composition.

MATERIALS AND METHODS

Experiments

The anionic phospholipid dioleoylphosphatidylglycerol (DOPG) is obtained as a sodium salt, and the zwitterionic phospholipid dioleoylphosphatidylcholine (DOPC) as a chloroform solution, from Avanti Polar lipids. Both phospholipids are used without any further purification. Analytical grade sodium bromide (NaBr) is bought from J.T. Baker Chemicals.

Mixtures of DOPG and DOPC are obtained from chloroform solutions. The molar fraction of DOPC, $\phi_{DOPC} = c_{DOPC}/(c_{DOPC}+c_{DOPG})$, in these mixtures is varied between 0 and 1. After mixing, the chloroform is removed by evaporation under a stream of nitrogen. The last traces of solvent are removed under vacuum. The dry lipid film is rehydrated in a NaBr solution of the desired ionic strength to a phospholipid concentration of 2.5 mM. The resulting DOPG/DOPC vesicles are subjected to 15 freeze-thaw cycles as described before [13,19,20]. Multilayered phospholipid vesicles are known to fragment when the solution is subjected to successive cycles of freezing and thawing [19]. As seen for the pure lipid case, also for the lipid mixtures the size and size distribution relaxes in the order of ten freeze-thaw cycles. The method of freezing and thawing probably leads to fast vesicle equilibration, in spite of the low solubility of phospholipids.

The average hydrodynamic radius R of the resulting vesicle solutions is analyzed using an ALV light-scattering apparatus with an argon ion laser as a light source. The data is collected at a scattering angle of 90° at a wavelength of 514 nm. During the measurements the temperature is kept at 293 K. The hydrodynamic vesicle radius is obtained from a cumulant analysis of the intensity autocorrelation function.

Calculations

In general it is possible to impose some curvature onto an interface. Homogeneously curved interfaces can be parametrized by the mean curvature J $\left(J=\frac{1}{R_1}+\frac{1}{R_2}\right)$ and the Gaussian curvature K $\left(K=\frac{1}{R_1R_2}\right)$. Here R_1 and R_2 are the two radii of curvature. The expansion of the surface tension up to second order in the curvature is attributed to Helfrich [21].

$$\gamma = \gamma_0 + \frac{1}{2}k_c(J-J_0)^2 + \bar{k}K. \tag{1}$$

It is well known that this equation can be used for curved lipid bilayer membranes. Its application to bilayers is restricted, however, to bilayers that are not under some sort of lateral stress.

The self-consistent-field (SCF) theory can be used to evaluate the distribution of surfactant or lipid molecules into association colloids (spherical micelles, cylindrical micelles, bilayers, cylindrically curved bilayers, or spherically curved bilayers). Structural parameters (such as the distributions of heads and tails of the lipids) are computed using molecular input parameters such as the lipid architecture and the relevant interactions (solvency parameters and electrostatics). In addition, it is possible to accurately evaluate corresponding thermodynamic data. For example, we can compute the chemical potential of the lipids that correspond to tensionless bilayers. We have shown before that the mechanical parameters of the lipid bilayers can be determined unambiguously from SCF calculations [22]. In particular, the curvature dependence of the grand potential of cylindrically curved vesicles (which are allowed to freely adjust the membrane area) gives access to the mean bending modulus k_c. The grand potential Ω of cylindrically curved membrane $K=0$, $J=\frac{1}{R}$, and $A=2\pi R$ (we consider the grand potential per unit length along the long axis of the cylinder) can be interpreted with Eq. (1) as

$$\Omega = \gamma A = -2\pi k_c J_0 + \pi k_c J. \tag{2}$$

The value of the radius of the curved bilayer is found by the first moment over the radial distribution function of the lipid molecules. The curvature of the vesicles can be varied by changing the number of surfactant or lipid molecules in this system.

For a spherical interface $J^2=4K=\frac{4}{R^2}$, and $A=4\pi R^2$. Again, using Eq. (1) we find for the grand potential Ω of the vesicle

$$\gamma A = -8\pi k_c J_0 R + 4\pi(2k_c + \bar{k}). \quad (3)$$

Note that in the SCF calculations the radius of the vesicles is not an input for the calculations, but it results from the calculations. The area is adjusted automatically such that the membranes are free of lateral stress.

As told, k_c follows from calculations in the cylindrical geometry by varying J (through the change of the number of lipids in the system). This k_c value can subsequently be used to obtain $\bar{k}$ from the grand potential data for the spherical geometry. The existence of a spontaneous curvature J_0 is evaluated by extrapolating the intercept of the $\gamma A(J)$ data to $J=0$. A nonzero spontaneous curvature is characterized by a nonzero value of γA for $J\rightarrow 0$.

Even though the details of the SCF calculations have been reported before in the literature (see, e.g., [13]), it is necessary to briefly mention a few of its characteristics. In this method the statistical weight of a chain (in a particular configuration) is evaluated using the Boltzmann weight for this chain (in this configuration). The Boltzmann weight features the self-consistent potential felt by this entity. The value of the potential reflects the surroundings of the molecule and contains contributions due to the electrostatic interactions—as in the Poisson Boltzmann equation—the short-range nearest-neighbor interactions (solvency effects; hydrophilic or hydrophobic specified using Flory-Huggins parameters) and a contribution that is linked to the incompressibility constraint. The evaluation of the spatial arrangements of the molecules is facilitated using a lattice. The lattice is a set of coordinates onto which the segments of the molecules are placed. The lattice sites are arranged in layers. Within the layers the positional correlations of the segments are ignored (mean-field approximation). When the layers are flat we automatically focus on flat bilayers and for cylindrically or spherically curved bilayers we need to arrange the lattice layers in corresponding cylindrical or spherical order. The equilibrium structure of the (curved) bilayers follows from the combined statistical weights of all possible and allowed configurations of the chain molecules. These configurations are generated using a freely jointed chain approximation. A numerical iteration procedure is used to find the (radial) volume fraction profiles and the corresponding potential profiles that are mutually consistent. For such a solution the Helmholtz energy is available. From this all other thermodynamical parameters (such as the grand potential) follow in high precision (at least five significant digits).

In the following SCF calculations DOPG-like molecules are modeled as linear chains with segment sequence $C_{18}X_2C_2X_2C_{18}$. The two C_{18} groups correspond to the hydrophobic tails, whereas the hydrophilic headgroup is represented by the segment sequence $X_2C_2X_2$, in which each X carries a charge of $-\frac{1}{4}$e. The zwitterionic phospholipid DOPC shows electrophoretic mobility at intermediate NaBr concentrations [14]. The DOPC-like surfactants are therefore modeled as slightly charged linear chains with segment sequence $C_{18}Y_2C_2Y_2C_{18}$. Each Y segment is a segment that carries a charge of $-\frac{1}{10}$e. Water is represented by a simple solvent monomer W. The ionic strength of the solution is determined by cations and anions with the size of water molecules, and with valency +1e and −1e, respectively. Ion hydration is taken into account by the choice of the Flory-Huggins interaction parameters $\chi_{\text{cation-}W}=-2$ and $\chi_{\text{anion-}W}=-1$. The more negative value for the cation reflects its stronger hydrogen-bonding properties in comparison with the anion, which typically tends to destroy water structure. The surfactant headgroup is made water soluble by choosing $\chi_{X\text{-}W}=-2$. The $C_{18}X_2C_2X_2C_{18}$ and $C_{18}Y_2C_2Y_2C_{18}$ differ only in headgroup charge; the critical micelle concentrations of the two surfactants are similar. The mixing ratio of the phospholipidlike molecules is therefore comparable to the lipid ratio in the bilayer. The molar surfactant fraction of $C_{18}Y_2C_2Y_2C_{18}$ is denoted ϕ. In all calculations the following dielectric constants are used: $\varepsilon_C=2$, $\varepsilon_X=\varepsilon_{\text{cation}}=\varepsilon_{\text{anion}}=5$, $\varepsilon_W=80$.

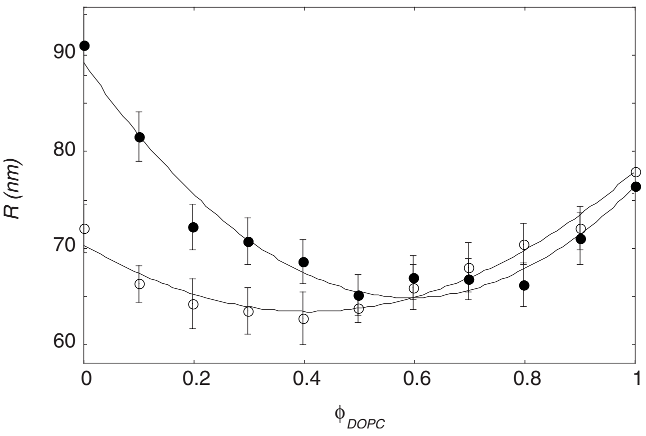

FIG. 1. The radius of mixed DOPG-DOPC vesicles in 300 (●) and 50 (○) mM NaBr after 15 freeze-thaw cycles as a function of the fraction of DOPC in the mixture. The total lipid concentration during the freeze-thaw procedure was 2.5 mM.

RESULTS

Experiments

Entropically stabilized vesicles (using the freeze-thaw method) can be obtained from both dilute DOPG and DOPC solutions at intermediate NaBr concentrations [13,14]. Mixed DOPG/DOPC bilayers also form a rather monodisperse population of stable vesicles. At intermediate NaBr concentrations the hydrodynamic radius of these mixed phospholipid vesicles is significantly smaller than the radii of vesicles obtained from a single phospholipid species (Fig. 1). In 300 mM NaBr the absolute decrease in vesicle radius is as much as 25 nm compared to DOPG and 10 nm compared to DOPC vesicles, corresponding to a relative size decrease of 10–30 %. The minimum in the hydrodynamic radius does not occur at exactly equimolar composition. At high ionic

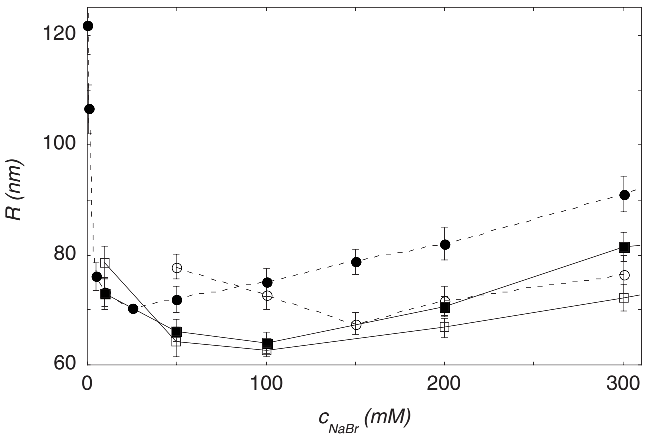

FIG. 2. The radius of stable DOPG (●), DOPC (○), and mixed DOPG/DOPC vesicles (ϕ_{DOPC}=0.1, ■; ϕ_{DOPC}=0.2, □) as a function of the NaBr concentration.

strength pure DOPG vesicles are larger than DOPC vesicles and the minimum is shifted toward the DOPC-rich mole fractions, whereas at low ionic strength DOPC vesicles are larger than DOPG and the minimum in R is on the DOPG rich side.

The phospholipid mixtures show a dependence of R on the NaBr concentration that is comparable to what is observed for the pure components (Fig. 2). As described before an initial decrease in R is followed by a slight increase of the vesicle radius with c_{NaBr} [13,14]. In phospholipid mixtures the minimum in R is observed at NaBr concentrations that lie between those seen for pure DOPG and DOPC vesicles. The shift in the position of the minimum in $R(c_{\mathrm{NaBr}})$ probably results from changes in the surface charge density with composition.

Calculations

As expected it is not possible to find a regime in which mixed $C_{18}X_2C_2X_2C_{18}/C_{18}Y_2C_2Y_2C_{18}$ bilayers exhibit a nonzero spontaneous curvature. In all cases the surface tension $\gamma(J,K)$ follows Eq. (2) or (3) with J_0=0. Interestingly, even in the absence of a spontaneous curvature the distribution of lipids over the membrane is asymmetric (Fig. 3, inset). Not only is the distribution of the $C_{18}Y_2C_2Y_2C_{18}$ lipid in the bilayer membrane a little bit broader, the $C_{18}Y_2C_2Y_2C_{18}$ lipid is also slightly more concentrated in the outer monolayer of the membrane.

Similar to what is observed for the equilibrium radius of entropically stabilized DOPG/DOPC vesicles mixing of $C_{18}X_2C_2X_2C_{18}$ and $C_{18}Y_2C_2Y_2C_{18}$ results in bilayers with a decreased k_c (Fig. 3). The position of minimum in k_c does not always occur at equimolar composition but depends on the ionic strength. At low salt concentrations the minimum in k_c is shifted toward the $C_{18}X_2C_2X_2C_{18}$ lipid, and with increasing ionic strength the minimum in k_c moves toward surfactant species carrying less charge.

The observed behavior of k_c with bilayer composition is expected to result from lipid mixing, and changes in surface charge density. The effect of surface charge density can be understood by investigating k_c as a function of lipid charge in a bilayer composed of a single surfactant species. When the charge on the surfactant headgroup is decreased from −1e to $-\frac{2}{5}$e a maximum in k_c is observed (Fig. 4). At low ionic strength the maximum occurs at a high surface charge, but with increasing ionic strength the maximum is observed to shift to lower surface charges. The difference between the two calculated k_c curves (Fig. 3 and Fig. 4) is expected to be related to mixing of lipids within the bilayer. Subtraction of the values for k_c at equal average surfactant charge results in a curve with a minimum at equimolar composition (Fig. 4, inset). The depth of this minimum decreases with increasing ionic strength. When the charges are completely screened, the $C_{18}X_2C_2X_2C_{18}$ and $C_{18}Y_2C_2Y_2C_{18}$ lipids are effectively

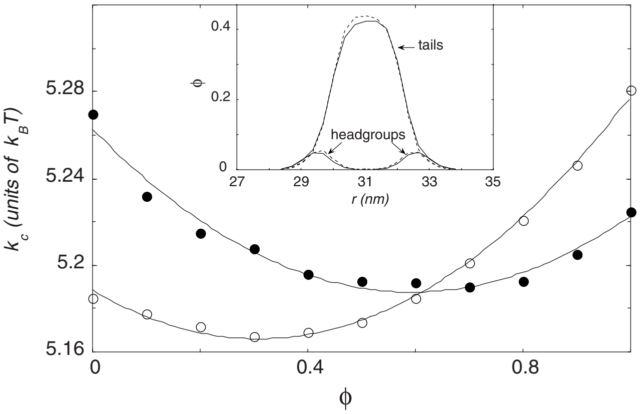

FIG. 3. The mean bending moduli k_c of a mixed lipid bilayer as a function of composition $\phi \equiv n_X/(n_X+n_Y)$. SCF calculations are performed at volume fractions of salt, ϕ_s=0.005 (○), and ϕ_s=0.01 (●). The charge on the molecule $X \equiv C_{18}X_2C_2X_2C_{18}$ lipid is −1e, the $Y \equiv C_{18}Y_2C_2Y_2C_{18}$ lipid carries a charge of $-\frac{2}{5}$e. Inset: Volume fraction profile of a lipid bilayer consisting of 50% $C_{18}X_2C_2X_2C_{18}$ (solid line) and 50% $C_{18}Y_2C_2Y_2C_{18}$ (dashed line), ϕ_s=0.01.

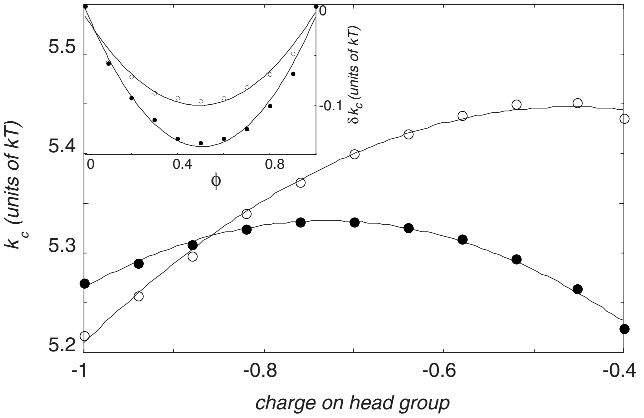

FIG. 4. The mean bending modulus k_c of a charged $C_{18}X_2C_2X_2C_{18}$ bilayer at ϕ_s=0.0025 (○) and ϕ_s=0.01 (●) as a function of the charge (in units of e) on the headgroup. The total charge on the headgroup was varied between −1e and $-\frac{2}{5}$e. Inset: The dependence of k_c that results from lipid mixing as a function of the molar fraction of $C_{18}Y_2C_2Y_2C_{18}$ in the bilayer.

indistinguishable, and therefore mixing of the two differently charged lipids equals mixing of lipids of the same species.

DISCUSSION

Previously we showed that there is a range of NaBr and phospholipid concentrations in which both DOPG and DOPC vesicles are thermodynamically stable [13]. Mixtures of DOPC and DOPG also form stable vesicles at intermediate salt and phospholipid concentrations. The radius of these mixed vesicles is considerably decreased compared to vesicles consisting of one phospholipid species only. SCF calculations on mixtures of phospholipidlike molecules show that k_c of the mixed bilayer is also lowered. The similarity between the calculated k_c and measured R versus composition curves and the absence of a spontaneous curvature in the calculations strongly suggests that these vesicles are stabilized by entropy. The translational entropy will prefer many small objects, but the minimum size of these objects will be set by the persistence length of the bilayer ξ_p. Therefore, the vesicle radius R is expected to scale as $R \propto \xi_p \propto e^{k_c/k_BT}$. It is difficult to connect the measured values of R directly to the calculated values of k_c. The molecules in the calculations only mimic real phospholipid molecules, and various factors that influence k_c are neglected. The present SCF calculations neither correctly take into account the stiffness of the phospholipid tails, nor the collective modes of fluctuations in the bilayers. This indicates that one should not focus on the absolute values of the predicted bending moduli, but rather focus on the trends.

Nevertheless, both the calculations and the experiments show that the minima in the k_c and R versus composition curves occur at an off equimolar composition. The additional degree of freedom present in a phospholipid mixture results in a decrease of the mean bending rigidity. This effect is expected to be the largest at equimolar composition. The observed changes in bending rigidity are apparently not only determined by effects of lipid mixing. Upon mixing of two phospholipids with a difference in headgroup charge, the total surface charge density changes accordingly. This effect is studied separately in the calculations by putting different charges on the $C_{18}X_2C_2X_2C_{18}$ headgroup. Charge density changes result in a maximum of k_c as a function of the bilayer composition. In the literature k_c is predicted to scale with the surface charge density σ as $k_c \propto \sigma^2$ [23,24], which is not in agreement with our findings. The reason for this discrepancy can be found in the fact that changes in the headgroup area with surface charge are implicitly excluded in these literature predictions. Uncharged surfactants pack into thicker bilayers than charged ones, and this results in much stiffer membranes. As discussed before, k_c is determined by changes in the thickness of the diffuse double layer and coupled adjustments in bilayer thickness [13]. The maximum in the plots of k_c versus headgroup charge is caused by the opposing effects of electrostatics and membrane thickness. Although k_c tends to increase, with increasing lipid charge, adjustments in lipid packing result in thinner bilayers giving rise to a maximum in k_c at intermediate surfactant charge.

Evaluating the difference between Figs. 3 and 4, at equal surface charge and ionic strength, a curve is obtained with a minimum at the equimolar composition (Fig. 4, inset). This curve represents the effect of surfactant mixing on k_c. The fact that the minimum is found at equimolar compositions for all ionic strengths supports the view that lipid mixing and changes in surface charge density completely determine the behavior of k_c and R with composition. Although it is often stated that k_c of lipid films is dominated by the tails rather than by the heads of the constituent amphiphiles [24,25], both the SCF calculations and the experiments show that changes in the surface charge and headgroup area can result in measurable and significant changes in the radius of entropically stabilized vesicles. Although the contribution of the lipid headgroup to the mean bending modulus k_c is probably small, the exponential dependence of k_c on the radius of entropically stabilized vesicles makes it certainly not negligible.

CONCLUSIONS

Not all lipid vesicles are transient states of lipid self-assembly. We have argued that unilamellar vesicles can be the thermodynamically favored state. Especially for charged lipid vesicles it is possible that the curvature energy E_{ves} remains small because the negative Gaussian bending modulus almost completely compensates for the contribution of the mean bending modulus. As a result such vesicles can be stabilized by some translational entropy, most likely assisted by some undulation entropy. We collected evidence that the size of the vesicles as produced by repetitive freeze-thaw cycles of lipid (mixtures) is correlated to the membrane persistence length. The latter quantity is related to the mean bending modulus (membrane rigidity), which we have estimated by molecularly realistic self-consistent field calculations. Lipid mixtures have an extra degree of freedom of distributing the various lipids differently between inner and outer monolayers of the curved bilayers. This redistribution does not give rise to a nonzero spontaneous curvature J_0. The bilayers composed of a mixture of lipids typically do have a lower rigidity than expected from the rigidities of the pure lipid bilayers. This relatively low rigidity results in a relatively low membrane persistence length. We argue that this is why the sizes of the entropically stabilized vesicles composed of DOPC/DOPG mixtures are relatively small.

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