Theory of Tunable pH-Sensitive Vesicles of Anionic and Cationic Lipids or Anionic and Neutral Lipids

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ABSTRACT The design of vesicles that become unstable at an easily tuned value of pH is of great interest for targeted drug delivery. We present a microscopic theory for two forms of such vesicles. A model of lipids introduced by us previously is applied to a system of ionizable anionic lipid and permanently charged cationic lipid. We calculate the pH at which the lamellar phase becomes unstable with respect to an inverted hexagonal one, a value that depends continuously on the system composition. Identifying this instability with that displayed by unilamellar vesicles undergoing fusion, we obtain very good agreement with the recent experimental data of Hafez, Ansell, and Cullis, (2000, *Biophys. J.* 79:1438–1446) on the pH at which fusion occurs versus vesicle composition. We explicate the mechanism in terms of the role of the counterions. This understanding suggests that a system of a neutral, nonlamellar-forming lipid stabilized by an anionic lipid would serve equally well for preparing tunable, pH-sensitive vesicles. Our calculations confirm this. Further, we show that both forms of vesicle have the desirable feature of exhibiting a regime in which the pH at instability is a rapidly varying function of the vesicle composition.

INTRODUCTION

The creation of liposomes that become unstable in response to changes in their environment has been the object of longstanding interest in connection with applications to drug delivery (Yatvin et al., 1980). One particularly interesting environmental cue is the relatively low pH found in tumor tissue (Tannock and Rotin, 1989) and in endosomes (Tycko and Maxfield, 1982). In the latter case, the rapid acidification that occurs in the endocytic vesicle would bring about the instability of the liposome, resulting either in the release of its contents within the endosome itself or in liposome-mediated destabilization of the endocytic vesicle with consequent release of the liposome's contents to the cytoplasm (Straubinger, 1993).

There exist various strategies for producing pH-sensitive liposomes (Thomas and Tirrell, 1992; Torchilin et al., 1993; Straubinger, 1993; Chu and Szoka, 1994; Sorgi and Huang, 1996). One method is to combine a lipid that does not form bilayers under physiological conditions with an ionizable anionic amphiphile or lipid. The latter, when sufficiently charged, stabilizes a bilayer of the combined system. The mechanism of this stabilization, as we argue below, is the attraction of counterions and their associated waters of hydration to the vicinity of the headgroups, which effectively increases their size. As the pH is reduced, so is the fraction of anionic amphiphiles that are ionized. Therefore, there are fewer counterions near the headgroups to stabilize them. Thus the reduction in pH eventually triggers an instability of the vesicle; the lipids revert to their more stable phase, usually an inverted hexagonal (H_{II}) one. Most commonly, the nonlamellar-forming lipid is a phosphatidylethanolamine (PE), such as dioleoylphosphatidylethanolamine (DOPE) (Cullis and de Kruijff, 1978).

One problem with this strategy is that the pH at which the instability occurs is determined by the pK of the single ionizable component, and is therefore not easily tuned. Discrete tuning can be obtained by using different ionizable components (Collins et al., 1989). An alternative method, which results in a vesicle exhibiting an instability at a value of pH which can be tuned continuously, has recently been demonstrated (Hafez et al., 2000). They use a vesicle with an ionizable anionic lipid, cholesteryl hemisuccinate (CHEMS), and a permanently charged cationic lipid *N*,*N*-dioleoyl-*N*,*N*-dimethylammonium chloride (DODAC). The pH at which the vesicle becomes unstable is a monotonically increasing function of the DODAC concentration, and is therefore easily, and continuously, tuned. We understand this result as follows. Vesicles consisting only of CHEMS are unstable with respect to formation of an H_{II} phase at pH less than 4.2 (Hafez and Cullis, 2000). This implies that a sufficient number of CHEMS must be ionized to stabilize such a vesicle. These ionized headgroups attract counterions to their vicinity. It is these counterions, enlarged by their waters of hydration, that stabilize a system that would otherwise tend to an H_{II} phase. The addition of cationic DODAC to such a vesicle at any pH, causes a decrease in the number of counterions in the vicinity of the headgroups. This decrease tends to destabilize a previously stable vesicle. To restore the number of counterions near the headgroups, and the vesicle's stability, more counterions must be attracted to the headgroups, which can be done by ionizing more of the CHEMS; i.e., by increasing the pH. Hence the

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value of the pH at the instability is an increasing and continuous function of the concentration of DODAC.

In this paper, we apply our model to the system of mixed, ionizable, anionic lipid and fully charged, cationic lipid and solvent such as that examined by Hafez and Cullis (2000). We identify the pH at which bilayer vesicles become unstable as the pH at which the lamellar phase becomes unstable to the inverted hexagonal phase, a reasonable assumption supported by much experimental data (Hope et al., 1983; Ellens et al., 1986). With this identification, we indeed find that the pH at which an instability occurs is a monotonically increasing function of the cationic lipid concentration, or equivalently, a monotonically decreasing function of the anionic lipid concentration. Our results fit the experimental data very well.

The above reasoning indicates that tunable, pH-sensitive liposomes should also be formed from a mixture of a neutral lipid that favors a nonlamellar phase, such as PE, and an anionic lipid that stabilizes the liposome by attracting counterions to it. Such stabilization is well known using various anions, such as palmitoylhomocysteine (Yatvin et al., 1980; Connor and Huang, 1985), oleic acid (Straubinger et al., 1985; Wang and Huang, 1987), or CHEMS (Straubinger, 1993; Ellens et al., 1984). Because the concentration of the stabilizing counterions clearly depends on both the concentration of the anionic lipid and the pH, the value of the latter at which the vesicle becomes unstable will be tunable, depending continuously on the concentration of anionic lipid. To test this hypothesis, we apply our model to a system of mixed, ionizable, anionic lipid and neutral lipid. We again find that the pH at which an instability occurs is a monotonically decreasing function of the anionic lipid concentration. Our results are in accord with experiments on the pH sensitivity of vesicles composed of 1-palmitoyl-2-oleoyl-phosphatidyletanolamine stabilized with tocopherol hemisuccinate (Jizomoto et al., 1994). Last, our results show that both forms of vesicle have the desirable property of exhibiting a regime in which the pH at the instability is very sensitive to the concentration of the anionic lipid. This will always be the case whenever a minimum amount of one lipid is required to stabilize the formation of vesicles by the mixture.

In the following section we briefly review our model of charged lipids (Li and Schick, 2000a) and of lipid mixtures (Li and Schick, 2000b,c). We then present our results. First, we show the phase diagram of a single, ionizable, anionic lipid, solvent, and counterions, a diagram that shows the transition from a lamellar phase (L_α) to an inverted hexagonal one (H_{II}) as a function of pH. We then consider the mixed system of ionizable anionic lipid and completely charged cationic lipid, and show the phase behavior. There is a transition between L_α and H_{II} phases, which occurs at a value of the pH that is a function of the concentration of the ionizable anionic lipid. We also show the spatial distribution of the various mass and charge densities in the coexisting phases. Last, we consider the system of ionizable anionic and neutral lipids, and present its phase behavior. It also shows a transition between L_α and H_{II} phases, which occurs at a value of pH that depends on the concentration of the ionizable lipid.

THE MODEL AND ITS SELF-CONSISTENT FIELD SOLUTION

We consider a system of volume, V, consisting of anionic lipids, cationic lipids, counterions, and solvent whose densities are controlled by the fugacities z_1, z_2, z_c, and z_s, respectively (Li and Schick, 2000a). By taking the charge of the cationic lipid to zero, we can also describe a mixture of anionic and neutral lipids. The counterions are positively charged. Because of overall charge neutrality, the average amount of charge on the anionic lipids is related to the density of cationic lipids and the density of counterions. Hence we use the fugacity z_c to control the charge on the anionic headgroups, which is equivalent to controlling the pH.

With the exception of their Coulombic properties, the two lipids are modeled identically. They each consist of headgroups of volume v_h, and two equal-length, completely flexible tails each consisting of N segments of volume v_t. Each lipid tail is characterized by a radius of gyration $R_g = (Na^2/6)^{1/2}$, with a the statistical segment length. The counterions are characterized by their charge, $+e$, and their volume, v_c, whereas the neutral solvent particles are characterized by their volume v_s.

There are eight local densities that specify the state of the system. We measure them all with respect to the convenient density v_h^{-1}. They are the number density of the headgroups of the anionic lipids, $v_h^{-1}\Phi_h^{(1)}(\mathbf{r})$, and of the cationic lipids, $v_h^{-1}\Phi_h^{(2)}(\mathbf{r})$; the number density of the tail segments of each lipid, $v_h^{-1}\Phi_t^{(1)}(\mathbf{r})$ and $v_h^{-1}\Phi_t^{(2)}(\mathbf{r})$; the number density of the solvent, $v_h^{-1}\Phi_s(\mathbf{r})$ and of the counterions $v_h^{-1}\Phi_c(\mathbf{r})$; and the local charge density of the headgroup of the anionic lipid, $ev_h^{-1}P_h^{(1)}(\mathbf{r})$ and of the cationic lipid, $ev_h^{-1}P_h^{(2)}(\mathbf{r})$. The local charge density of the positive counterions is simply $ev_h^{-1}\Phi_c(\mathbf{r})$. Note that all functions $\Phi(\mathbf{r})$ and $P(\mathbf{r})$ are defined to be dimensionless.

The interactions among these densities are of two kinds. First, there is a repulsive, contact interaction between headgroups and tail segments, and also between solvent and tail segments. The strength of this interaction is $kTv_h\chi$, where k is Boltzmann's constant and T the absolute temperature. Second, there is the Coulomb interaction between all charges. The energy per unit volume of the system, expressed in the natural units kT/v_h, can be written

$$\frac{v_h}{VkT}E[\Phi_h^{(1)}, \Phi_h^{(2)}, \Phi_t^{(1)}, \Phi_t^{(2)}, \Phi_s, \Phi_c, P_h^{(1)}, P_h^{(2)}]$$

$$= 2\chi N \int \frac{d\mathbf{r}}{V} \left[\sum_{L=1}^{2} \Phi_h^{(L)}(\mathbf{r}) + \Phi_s(\mathbf{r}) \right] \sum_{M=1}^{2} \Phi_t^{(M)}(\mathbf{r}) + \frac{\beta^*}{8\pi} \int \frac{d\mathbf{r}}{V} \frac{d\mathbf{r}'}{R_g^2} \left[\sum_{L=1}^{2} P_h^{(L)}(\mathbf{r}) + \Phi_c(\mathbf{r}) \right] \times \frac{1}{|\mathbf{r} - \mathbf{r}'|} \left[\sum_{M=1}^{2} P_h^{(M)}(\mathbf{r}') + \Phi_c(\mathbf{r}') \right], \quad (1)$$

where

$$\beta^* \equiv \frac{4\pi e^2 R_g^2}{v_h \epsilon kT} \quad (2)$$

is a dimensionless measure of the strength of the Coulomb interaction, and ϵ is the dielectric constant of the solvent. In addition to these interactions, we impose a local incompressibility constraint on the system, which models the hard core interactions between all particles. Upon defining the volume ratios $\gamma_s \equiv v_s/v_h$, $\gamma_c = v_c/v_h$, and $\gamma_t = 2Nv_t/v_h$, the incompressibility constraint that the sum of the volume fractions of all components must be unity everywhere takes the form

$$\gamma_s \Phi_s(\mathbf{r}) + \gamma_c \Phi_c(\mathbf{r}) + \sum_{L=1}^{2} [\Phi_h^{(L)}(\mathbf{r}) + \gamma_t \Phi_t^{(L)}(\mathbf{r})] = 1. \quad (3)$$

As shown earlier (Li and Schick, 2000a), the partition function of the system can be written in the form in which the eight fluctuating densities, instead of interacting directly with one another, interact indirectly via eight fluctuating fields, here denoted $W_h^{(L)}$, $W_t^{(L)}$, $U_h^{(L)}$, with $L = 1$, 2, and W_s, U_c. Self-consistent field theory results when the fluctuating fields and densities are approximated by those values that minimize the free energy, Ω, of the system in the presence of these fields. The free energy to be minimized has the form

$$\frac{v_h}{kTV}\Omega = -\frac{1}{V}\sum_{L=1}^{2} z_L \mathcal{Q}_L[W_h^{(L)}, W_t^{(L)}, U_h^{(L)}] - z_c\frac{\mathcal{Q}_c[U_c]}{V} - z_s\frac{\mathcal{Q}_s[W_s]}{V} + \frac{v_h}{kTV}E - \int \frac{d\mathbf{r}}{V}\left[W_s(\mathbf{r})\Phi_s(\mathbf{r}) + U_c(\mathbf{r})\Phi_c(\mathbf{r}) + \sum_{L=1}^{2}\left(W_h^{(L)}(\mathbf{r})\Phi_h^{(L)}(\mathbf{r}) + W_t^{(L)}(\mathbf{r})\Phi_t^{(L)}(\mathbf{r}) + U_h^{(L)}(\mathbf{r})P_h^{(L)}(\mathbf{r}) \right) \right] - \int \frac{d\mathbf{r}}{V}\left[\Xi(\mathbf{r})\left(1 - \gamma_s\Phi_s(\mathbf{r}) - \gamma_c\Phi_c(\mathbf{r}) - \sum_{L=1}^{2}\Phi_h^{(L)}(\mathbf{r}) - \sum_{L=1}^{2}\gamma_t\Phi_t^{(L)}(\mathbf{r}) \right) \right]. \quad (4)$$

Here $\mathcal{Q}_c[U_c]$ is the partition function of a single counterion of unit positive charge in an external potential U_c,

$$\mathcal{Q}_c[U_c] = \int d\mathbf{R}_c \exp[-U_c(\mathbf{R}_c)], \quad (5)$$

$\mathcal{Q}_s[W_s]$ is the partition function of a single solvent molecule in an external field W_s,

$$\mathcal{Q}_s[W_s] = \int d\mathbf{R}_s \exp[-W_s(\mathbf{R}_s)], \quad (6)$$

and $\mathcal{Q}_L[W_h^{(L)}, W_t^{(L)}, U_h^{(L)}]$, given below, is the partition function of a single lipid of type L in external fields $W_h^{(L)}$, $W_t^{(L)}$, and $U_h^{(L)}$. Note that a Lagrange multiplier $\Xi(\mathbf{r})$ has been introduced to enforce the incompressibility constraint of Eq. 3. The functions $W_h^{(L)}$, $\Phi_h^{(L)}$, etc., which extremize this free energy, will be denoted by their corresponding lower case letters $w_h^{(L)}$, $\phi_h^{(L)}$, etc.

It is not difficult to see from the form of the free energy Ω and that of the energy E of Eq. 1 that the fields acting on the different heads and extremizing the free energy are equal, $w_h^{(1)}(\mathbf{r}) = w_h^{(2)}(\mathbf{r}) \equiv w_h(\mathbf{r})$, that the fields acting on the different tails and extremizing the free energy are equal $w_t^{(1)}(\mathbf{r}) = w_t^{(2)}(\mathbf{r}) \equiv w_t(\mathbf{r})$, and that the fields acting on all charge densities and extremizing the free energy are related, $u_h^{(1)}(\mathbf{r}) = u_h^{(2)}(\mathbf{r}) = u_c(\mathbf{r}) - \gamma_c\xi(\mathbf{r}) \equiv u(\mathbf{r})$. Thus there are only five independent functions, $w_h(\mathbf{r})$, $w_t(\mathbf{r})$, $w_s(\mathbf{r})$, $u(\mathbf{r})$, and $\xi(\mathbf{r})$, and these are obtained from the five equations

$$w_h(\mathbf{r}) = 2\chi N \sum_{L=1}^{2} \phi_t^{(L)}(\mathbf{r}) + \xi(\mathbf{r}), \quad (7)$$

$$w_t(\mathbf{r}) = 2\chi N\left[\phi_s(\mathbf{r}) + \sum_{L=1}^{2} \phi_h^{(L)}(\mathbf{r}) \right] + \gamma_t\xi(\mathbf{r}), \quad (8)$$

$$w_s(\mathbf{r}) = 2\chi N \sum_{L=1}^{2} \phi_t^{(L)}(\mathbf{r}) + \gamma_s\xi(\mathbf{r}), \quad (9)$$

$$u(\mathbf{r}) = \frac{\beta^*}{4\pi}\int \frac{d\mathbf{r}'}{R_g^2} \frac{\Sigma_{L=1}^{2}\rho_h^{(L)}(\mathbf{r}') + \phi_c(\mathbf{r}')}{|\mathbf{r} - \mathbf{r}'|}, \quad (10)$$

$$1 = \gamma_s\phi_s(\mathbf{r}) + \gamma_c\phi_c(\mathbf{r}) + \sum_{L=1}^{2}[\phi_h^{(L)}(\mathbf{r}) + \gamma_t\phi_t^{(L)}(\mathbf{r})]. \quad (11)$$

Because the field ξ can be easily eliminated, one deals essentially with four equations. The eight densities are all functionals of the above fields except ξ and, therefore, close the cycle of self-consistent equations:

$$\phi_h^{(L)}(\mathbf{r})[w_h, w_t, u] = -z_L \frac{\delta \mathcal{Q}_L[w_h, w_t, u]}{\delta w_h(\mathbf{r})}, \quad L = 1, 2, \quad (12)$$

$$\phi_t^{(L)}(\mathbf{r})[w_h, w_t, u] = -z_L \frac{\delta \mathcal{Q}_L[w_h, w_t, u]}{\delta w_t(\mathbf{r})}, \quad L = 1, 2, \quad (13)$$

$$\rho_h^{(L)}(\mathbf{r})[w_h, w_t, u] = -z_L \frac{\delta \mathcal{Q}_L[w_h, w_t, u]}{\delta u(\mathbf{r})}, \quad L = 1, 2, \quad (14)$$

$$\phi_s(\mathbf{r})[w_s] = -z_s \frac{\delta \mathcal{Q}_s[w_s]}{\delta w_s(\mathbf{r})} = z_s \exp\{-w_s(\mathbf{r})\}, \quad (15)$$

$$\phi_c(\mathbf{r})[u_c] = -z_c \frac{\delta \mathcal{Q}_c[u_c]}{\delta u_c(\mathbf{r})} = z_c \exp\{-u_c(\mathbf{r})\}. \quad (16)$$

Note that one of the self-consistent equations, Eq. 10, is the nonlinear Poisson–Boltzmann equation, and $u(\mathbf{r})$ is the electric potential.

With the aid of the above equations, the self consistent, or mean field, free energy, Ω_{mf}, which is the free energy function of Eq. 4 evaluated at the self-consistent field values of the densities and fields, can be put in the form

$$-\Omega_{mf} = \frac{kT}{v_h}\left(\sum_{L=1}^{2} z_L \mathcal{Q}_L[w_h, w_t, u] + z_c \mathcal{Q}_c[u_c] + z_s \mathcal{Q}_s[w_s]\right) + E[\phi_h^{(1)}, \phi_h^{(2)}, \phi_t^{(1)}, \phi_t^{(2)}, \phi_s, \phi_c, \rho_h^{(1)}, \rho_h^{(2)}], \quad (17)$$

where we have chosen $\int \xi(\mathbf{r})d\mathbf{r} = 0$ for convenience. All of the above is a simple extension of the procedure in our earlier paper (Li and Schick, 2000a) with one exception: previously, we assumed the counterion to have negligible volume and included the interaction between its charge and the dipole of the solvent so that it would attract waters of hydration and gain an effective volume. Here we simply assign the counterion a volume, v_c, so that we need not use the interaction between charges and solvent dipoles.

The fact that the anionic lipids are ionizable has the consequence that the fields w_h, w_t, and u, acting on that lipid, can be replaced (Borukhov et al., 1998; Li and Schick, 2000a) by $w_{h,eff}^{(1)}$, w_t, and 0, where

$$w_{h,eff}^{(1)}(\mathbf{r}) = w_h(\mathbf{r}) - \ln[1 + p(\exp\{u(\mathbf{r})\} - 1)]. \quad (18)$$

The parameter p is related to the pK of the headgroup and can therefore be related to the fugacity z_c of the counterions by means of the condition of charge neutrality

$$\int d\mathbf{r}\left[\sum_{L=1}^{2} \rho_h^{(L)}(\mathbf{r}) + \phi_c(\mathbf{r})\right] = 0. \quad (19)$$

In practice, we use this fugacity to control the fractional charge on the anionic lipid and therefore the pH.

In the system in which the cationic lipid is fully charged, the fields w_h, w_t, and u, acting on it, can be replaced by $w_{h,eff}^{(2)}$, w_t, 0 with

$$w_{h,eff}^{(2)}(\mathbf{r}) = w_h(\mathbf{r}) + u(\mathbf{r}). \quad (20)$$

Note that, from Eqs. 12 and 14, it immediately follows that the number density of the headgroup and its charge density, in units of e, are identical,

$$\rho_h^{(2)}(\mathbf{r}) = \phi_h^{(2)}(\mathbf{r}), \quad (21)$$

as they should be because the cationic lipid is always fully charged.

In the other system that we consider, the second lipid is neutral so that

$$w_{h,eff}^{(2)}(\mathbf{r}) = w_h(\mathbf{r}), \quad (22)$$

and

$$\rho_h^{(2)}(\mathbf{r}) = 0. \quad (23)$$

There remains only to specify how the partition function of the lipids is calculated. As in our earlier study (Li and Schick, 2000a), one defines the end-segment distribution function $q^{(L)}(\mathbf{r}, s)$, which satisfies the equation

$$\frac{\partial q^{(L)}(\mathbf{r}, s)}{\partial s} = 2R_g^2 \nabla^2 q^{(L)}(\mathbf{r}, s) - [w_{h,eff}^{(L)}(\mathbf{r})\delta(s - \tfrac{1}{2}) + w_t(\mathbf{r})]q^{(L)}(\mathbf{r}, s), \quad (24)$$

with initial condition

$$q^{(L)}(\mathbf{r}, 0) = 1. \quad (25)$$

From this function, one obtains the partition functions of the lipids,

$$\mathcal{Q}_L = \int d\mathbf{r} q^{(L)}(\mathbf{r}, 1), \quad (26)$$

the head and tail densities,

$$\phi_h^{(L)}(\mathbf{r}) = \exp\{-w_{h,eff}^{(L)}(\mathbf{r})\}q^{(L)}(\mathbf{r}, \tfrac{1}{2}-)q^{(L)}(\mathbf{r}, \tfrac{1}{2}-), \quad L = 1, 2, \quad (27)$$

$$\phi_t^{(L)}(\mathbf{r}) = \int_0^1 ds q^{(L)}(\mathbf{r}, s)q^{(L)}(\mathbf{r}, 1 - s), \quad L = 1, 2, \quad (28)$$

and the charge density of the anionic lipid head,

$$\rho_h^{(1)}(\mathbf{r}) = -\frac{p(z_c)\exp\{u(\mathbf{r})\}}{1 + p(z_c)(\exp\{u(\mathbf{r})\} - 1)}\,\phi_h^{(1)}(\mathbf{r}). \quad (29)$$

The average fractional charge, f_c, on the anionic lipid headgroup follows,

$$f_c(z_c) \equiv -\frac{\int \rho_h^{(1)}(\mathbf{r})\,d\mathbf{r}}{\int \phi_h^{(1)}(\mathbf{r})\,d\mathbf{r}}, \quad (30)$$

from which the pH relative to the pK of the anionic lipid headgroup is obtained,

$$\mathrm{pH} = \mathrm{pK} + \log_{10}\left(\frac{f_c}{1 - f_c}\right). \quad (31)$$

To summarize, there are five self-consistent equations to be solved for the five fields $w_h(\mathbf{r})$, $w_t(\mathbf{r})$, $w_s(\mathbf{r})$, $u(\mathbf{r})$, and $\xi(\mathbf{r})$. They are Eqs. 7–11. The fields depend on the eight densities $\phi_h^{(L)}(\mathbf{r})$, $\phi_t^{(L)}(\mathbf{r})$, $\rho_h^{(L)}(\mathbf{r})$ with $L = 1, 2$, $\phi_s(\mathbf{r})$, and $\phi_c(\mathbf{r})$, which depend, in turn, on these fields. The densities are given by Eqs. 15, 16, 21 or 23, 27, 28, and 29. Once the fields and densities are obtained, the free energy follows from Eq. 17.

Instead of solving these equations in real space, we do so in Fourier space in such a way as to guarantee that our solution has the symmetry of either the lamellar or inverted hexagonal phases (Matsen and Schick, 1994). Comparison of the free energies of these phases tells us which is the globally stable one. We do this for different temperatures, lipid concentrations, and pH, and thereby map out the phase diagram.

RESULTS

We first consider the system of the single anionic lipid in solvent. The architecture of this lipid is characterized by γ_t, the ratio of the volume of its tail groups to the volume of its head. In choosing the value of this parameter, we have been guided first and foremost by the requirement that our model lipid exist in the inverted hexagonal phase when its head group is neutral and the system is hydrated so that it correctly models the behavior of CHEMS (Hafez and Cullis, 2000). The value we have chosen, $\gamma_t = 2.5$ does indeed produce a model lipid that exists in the inverted hexagonal phase over a large region of phase space when it is neutralized, as is seen below. We note, in passing, that this is not an unreasonable value when compared to that which follows from volumetric data on the nonlamellar forming lipid DOPE (Rand and Fuller, 1994), $\gamma_t = 2.94$, a value that most likely assigns some of the volume of the waters of hydration, those most tightly bound, to the headgroup. However, in our model, some of the volume of water of hydration should be included in the volume of the head group because the only interaction it has with water occurs when it has a net charge. The model interactions, therefore, neglect those waters attracted via their dipole moment to the charges of a physical, neutral, head group.

The solvent is characterized by its relative volume $\gamma_s \equiv v_s/v_h = 0.1$, close to the ratio of 0.096 appropriate to water and a PE headgroup (Rand and Fuller, 1994; Kozlov et al., 1994). The counterions are modeled as $H_9O_4^+$, a reasonable choice (Bell, 1959), and are therefore characterized by their relative volume $\gamma_c \equiv v_c/v_h = 0.4$. The strength of the Coulomb interaction is, again, given by the parameter β^*. Eq. 2, which can be written as $\beta^* = \xi/L_1$, where $\xi \equiv e^2/\epsilon kT$ is the Bjerrum length, and $L_1 \equiv v_h/4\pi R_g^2$ is a length characterizing the architecture of lipid 1. Using the value of R_g found earlier (Li and Schick, 2000a) to be appropriate to DOPE and a Bjerrum length of 7 Å appropriate for water, we obtain $\beta^* = 27$ and have used this value. The phase diagram of the system is shown in Fig. 1 as a function of the effective temperature $T^* \equiv (2\chi N)^{-1}$ and the pH relative to the pK of the lipid headgroup. We observe the characteristic transition between the inverted hexagonal and lamellar phases as the pH is increased (Hope and Cullis, 1980; Bezrukov et al., 1998). The fugacity of solvent here is $z_s = 3.2$. At this value, the system is not in the presence of excess water. When completely neutralized, the two phases coexist at $T^* = 0.065$ with the H_{II} phase containing a volume fraction of solvent $\gamma_s\phi_s = 0.063$, and, the lamellar phase, a volume fraction $\gamma_s\phi_s = 0.087$. For a given temperature, the transition between phases occurs at a given pH. If this value is not a biologically useful one, as it is not for PS, which undergoes a phase transition at the very acidic value pH $\approx$ 3 (Hope and Cullis, 1980), then a vesicle made from this lipid is not applicable for drug delivery.

To vary continuously the value of the pH at which the lamellar phase becomes unstable for a fixed temperature,

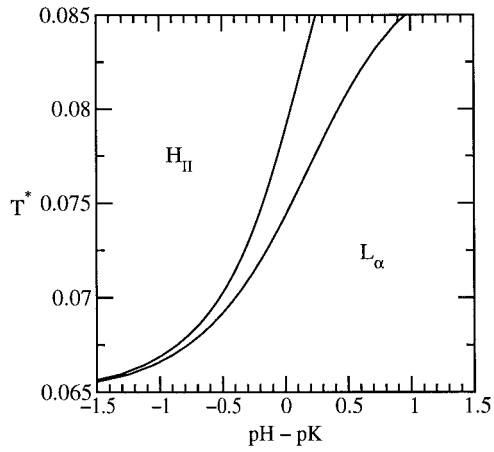

FIGURE 1 Phase diagram in the temperature, T^*, pH plane for a system of a single anionic lipid, solvent, and counterions. The pK is that of the anionic headgroup. The volume of the headgroup relative to that of the entire lipid is 0.286, similar to that of DOPE, the relative volume of the solvent is close to that of water, and the relative volume of the counterions is that of $H_9O_4^+$.

one can add an additional lipid. We now consider the case when this additional lipid is cationic and fully charged, as in the experiment of Hafez et al. (2000). The phase diagram we obtain for this system is shown in Fig. 2 as a function of the fractional concentration Θ of the ionizable anionic lipid

$$\Theta = \frac{\phi_h^{(1)} + \gamma_t \phi_t^{(1)}}{\int_{L=1}^{2} [\phi_h^{(L)} + \gamma_t \phi_t^{(L)}]}, \tag{32}$$

versus pH − pK, where the pK is that of the anionic lipid headgroup. Our results for the phase coexistence are shown in solid lines. We have chosen the solvent activity $z_s = 3.425$ and the effective temperature to be $T^* = 0.079$. Under these conditions, the system of pure anionic lipid is, when completely neutralized, just at phase coexistence between L_α and H_{II} phases. Therefore, our curves, in the limit of very large negative pH, asymptote to $\Theta = 1$. This coexistence is characterized by a volume fraction of solvent $\gamma_s \phi_s = 0.087$ in the inverted hexagonal phase and $\gamma_s \phi_s = 0.116$ in the lamellar phase. Because the phase boundary curves are flat near $\Theta = 1.0$, our results are not very sensitive to different choices of temperature and solvent chemical potential, provided, of course, that we remain in the same general region of phase behavior. The experimental results of Hafez et al., which show the pH at which vesicle fusion occurs (their pH_f), are shown by the solid dots. To obtain the best fit, we have taken the pK of CHEMS to be 5.5. The actual value of the pK of CHEMS in the DODAC/CHEMS system has not been measured. It has been measured in a large unilamellar vesicle composed of CHEMS and DOPE and a value of 5.8 obtained (Hafez and Cullis, 2000). Thus the value of 5.5 we have used in our fit to the data is not unreasonable. We note that our results fit the data rather well except for the very largest value of pH. Given the assumptions in the modeling, and the assumption that the architecture of the two lipids is the same, this agreement is gratifying. We also restate the interesting point made by Hafez et al. that the preferred phase of the lipid mixture can be inverted hexagonal even though both of the lipids in isolation adopt a lamellar organization. They do so, in our view, because, in isolation, their headgroups are sufficiently charged to attract stabilizing counterions, which increase the effective size of their headgroups. The transition to the inverted hexagonal phase comes about because, as the lipids are mixed, the number of those stabilizing counterions is reduced until eventually the lamellar phase becomes unstable.

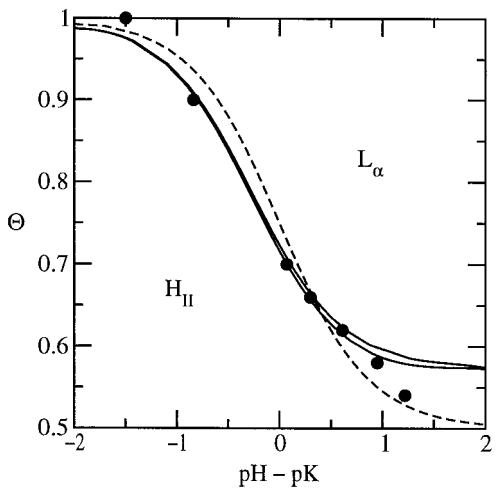

FIGURE 2 Phase diagram, at fixed temperature, $T^* = 0.079$ and solvent activity, $z_s = 3.425$, of a mixture of ionizable, anionic lipid, and fully ionized cationic lipid. The volume of the headgroup of each lipid relative to that of the entire lipid is the same as that in Fig. 1. The relative concentration of the ionizable lipid is denoted Θ. The solid lines show the coexistence obtained from our calculation, the solid circles show the data from Hafez et al. (2000), and the dashed line their criterion of vanishing counterion density, Eq. 33, with a shift of 0.3 to the right to account for their choice of pK = 5.8.

A somewhat different point of view is taken by Hafez et al. They assume that, for fusion to occur, i.e., the instability, the surface charge on the vesicle must be zero, permitting close contact. Therefore, the proportion of CHEMS that is negatively charged must equal the DODAC content of the membrane. Equivalently, the number of counterions must be identically zero. This condition is expressed in the equation

$$\mathrm{pH} - \mathrm{pK} = \log_{10}\left(\frac{1-\Theta}{2\Theta - 1}\right), \tag{33}$$

which is plotted in Fig. 2 as the dashed line with a shift of 0.3 to the right. This shift is made to account for their choice of pK = 5.8 as opposed to ours of 5.5.

The spatial distributions of the various components and of the charges is shown in Fig. 3 for the lamellar phase and Fig. 4 for the inverted hexagonal phase, which coexist near pH − pK = 0. In part (*a*) of each figure, the volume fractions of all elements are shown: of the headgroup of the anionic lipid, $\phi_h^{(1)}$, of the tails of the anionic lipid, $\gamma_t \phi_t^{(1)}$, of the headgroup of the cationic lipid, $\phi_h^{(2)}$, and of the tails of the cationic lipid, $\gamma_t \phi_t^{(2)}$, of the solvent, $\gamma_s \phi_s$, and of the counterions, $\gamma_c \phi_c$. In part (*b*) of each figure, the charge distributions, in units of $e v_h^{-1}$, are shown: that of the anionic lipid, $\rho_h^{(1)}$, of the cationic lipid, $\rho_h^{(2)}$, of the counterions, ϕ_c, and the total charge distribution. It should be recalled that these distributions are those of a lamellar phase, not an isolated lipid bilayer. In the latter, the volume fraction of headgroups would fall rapidly in the solvent-rich regions on either side of the bilayer. The points $x = 0$ and $x = D$ correspond to the centers of sequential solvent regions within the lamellae, with $D = 2.94 R_g$ being the lamellar period. In Fig. 4, $x = 0$ and $x = D$ correspond to the centers of adjacent tubes with $D = 3.03 R_g$ the lattice constant of the inverted hexagonal phase. As noted previously (Li and Schick, 2000a), a single dielectric constant has, for simplicity, been used for the entire system. Were a different dielectric constant used in the tail region, the distribution of counterions would change, with less of them in the tail

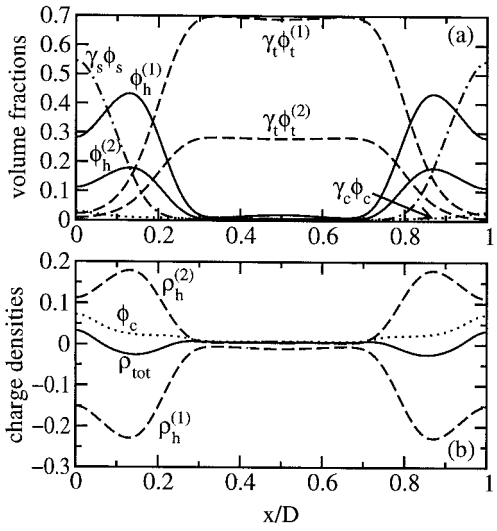

FIGURE 3 For the system of Fig. 2, the spatial distributions of the various components and of the charges is shown for the lamellar phase at coexistence with the inverted hexagonal phase near pH − pK = 0. (*a*) The volume fractions of the headgroup of the anionic lipid, $\phi_h^{(1)}$, of the tails of the anionic lipid, $\gamma_t\phi_t^{(1)}$, of the headgroup of the cationic lipid, $\phi_h^{(2)}$, and of the tails of the cationic lipid, $\gamma_t\phi_t^{(2)}$, of the solvent, $\gamma_s\phi_s$, and of the counterions, $\gamma_c\phi_c$. (*b*) The charge distributions, in units of ev_h^{-1}, of the anionic lipid, $\rho_h^{(1)}$, of the cationic lipid, $\rho_h^{(2)}$, of the counterions, ϕ_c, and the total charge distribution. The points $x = 0$ and $x = D$ correspond to the centers of adjacent solvent regions, with $D = 2.94R_g$ being the lamellar period.

region. However, their volume fraction in that region is already small due to their nonzero volume and the incompressibility constraint. Hence, any change in the distribution would probably be small.

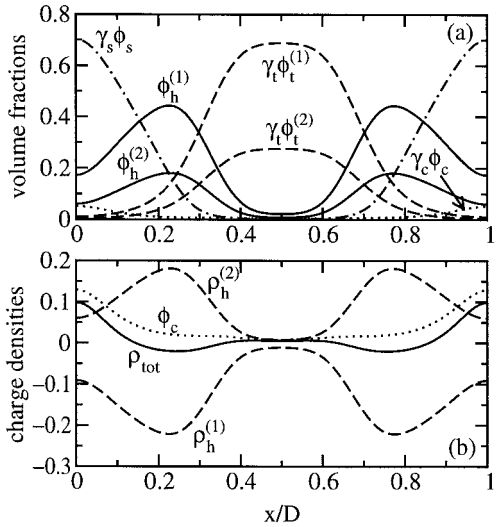

FIGURE 4 The same quantities shown in Fig. 3, *a* and *b*, for the lamellar phase are shown here for the inverted hexagonal phase with which the lamellar phase coexists. The points $x = 0$ and $x = D$ correspond to the centers of adjacent tubes, with $D = 3.03R_g$.

We turn now to the system of anionic and neutral lipids. The phase diagram is shown in Fig. 5. As in Fig. 2, Θ is the fractional composition of the anionic lipid. The temperature T^* and solvent fugacity are the same as in Fig. 2. The results for the phase coexistence of the anionic, neutral lipid system are shown with solid lines. They are compared to the calculated results for the anionic, cationic system shown previously in Fig. 2, and repeated here in dashed-dotted lines. We see again, in this system of anionic and neutral lipid, the characteristic transition from H_{II} to L_α phases with increasing pH. Again the pH at which the transition occurs is a continuous function of the system's composition, decreasing with an increase in the composition of the ionizable anionic lipid. This is in agreement with results on vesicles of 1-palmitoyl-2-oleoyl-phosphatidyletanolamine and CHEMS (Jizomoto et al., 1994). Equally important, we note a region in which a very small change in the composition of the system brings about a large change in the pH at which the instability of the lamellar phase occurs. We also note that a minimum fractional composition of anionic lipid, approximately 0.3, is necessary to stabilize the DOPE-like neutral lipid. This is similar to the experimental observation that a minimum molar composition of 0.2 CHEMS was necessary to stabilize vesicles of transesterified egg PE (Lai et al., 1985). It is easy to see from Fig. 5 that the existence of a minimum concentration of stabilizing anionic lipid guarantees a regime in which the pH at the instability changes rapidly with concentration. We understand the instability in this system in the same way as in the previous system. The neutral lipid prefers to be in an inverted hex-

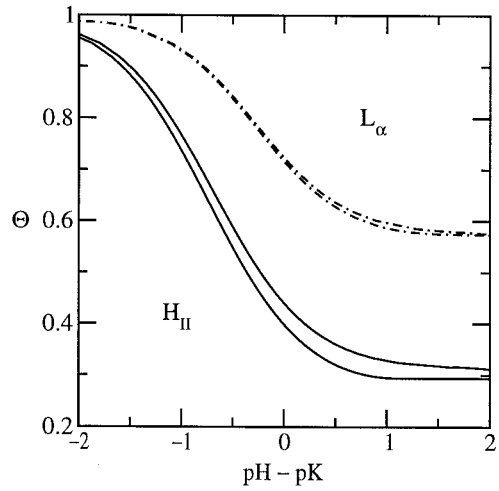

FIGURE 5 Phase diagram, at fixed temperature, $T^* = 0.079$ and solvent activity, $z_s = 3.425$, of a mixture of ionizable, anionic lipid, and neutral lipid. The volume of the headgroup of each lipid relative to that of the entire lipid is the same as that in Fig. 1. The relative concentration of the ionizable lipid is denoted Θ. The solid lines show the coexistence obtained from our calculation. For comparison, the dashed dotted lines show the calculated coexistence for the ionizable anionic and fully ionized cation lipid system, shown previously in Fig. 2 as solid lines.

agonal phase. The addition of an anionic lipid can stabilize a lamellar phase of the mixture if there is enough of it, and if these lipids are sufficiently charged. It does so by attracting a sufficient number of counterions that effectively increase the headgroup of the anionic lipid. When the pH is changed so that fewer counterions are attracted, the lamellar phase is less stable, and eventually becomes unstable with respect to the inverted hexagonal phase. Note that the criterion of Hafez et al. (2000), that the density of counterions be zero at the transition, yields no useful information for this system.

DISCUSSION

We have applied our model of lipids, introduced previously (Li and Schick, 2000a), to the systems of anionic lipids mixed either with a cationic lipid or a neutral lipid. We have solved the model within self-consistent field theory, and obtained the phase diagram for these systems showing the transition from the lamellar to inverted hexagonal phases, which occurs at a value of the pH that depends continuously on the membrane composition. Identifying the instability of unilamellar vesicles as evidenced by their fusion with the instability of the lamellar phase of the lipid mixture, we obtain good agreement between our calculations and the experimental data of Hafez et al. (2000) for the mixed anionic, cationic system they studied. We have interpreted the instability in terms of the counterions, which, by increasing the effective size of the anionic lipid, stabilize the lamellar phase of the mixture. As cationic lipids are added, counterions are subtracted. When their number gets too low, the vesicle becomes unstable, and the lipids try to revert to an inverted hexagonal phase. As noted by Hafez et al., the pH at which this instability occurs is a continuous function of the composition of the vesicle, and can therefore be readily tuned to occur at a biologically relevant value of pH. Hence such a vesicle has the possibility of being used for drug delivery. Applying similar reasoning, we suggested that a vesicle consisting of a neutral, nonlamellar-forming lipid, like PE, and stabilized by the presence of an anionic lipid could also be used to make pH-sensitive vesicles whose pH at the point of instability could also be readily tuned. We applied our model to such a system and found this to be true, and that both sorts of vesicles displayed a region in which the pH at instability was a sensitive function of composition, and thus readily tuned.

It might be argued that the instability of the mixed, charged, lipid vesicles prepared by Hafez et al. does not reflect the L_α-to-H_{II} phase transition of the bulk system, but rather is induced by a phase separation of the components. Such a scenario would be contrary to experimental evidence previously cited (Hope et al., 1983; Ellens et al., 1986) and seems to us most unlikely. Not only is the mixed system energetically favorable, due to the Coulomb attraction of the two components, but it is also entropically favorable because formation of the mixture liberates the counterions needed to neutralize the phase-separated charged components (Paulsen et al., 1988).

Throughout this paper, we have reiterated the view that the counterions, which we have treated as a proton with four waters of hydration, $H_9O_4^+$, play an important role in bringing about the transition with pH by effectively increasing the volume of the headgroup of the anionic lipid. This is reasonable given the well-known stabilization of the lamellar phase with respect to the inverted hexagonal one as the headgroup volume increases (Gruner, 1989). We further understand the importance of the counterions as follows. The Coulomb interaction in this system has several effects. One of them is that just noted; the charged headgroups attract counterions that have gained an effective volume due to their interaction with the dipoles of water, and these tend to stabilize the lamellar phase with respect to the inverted hexagonal. However, the Coulomb interaction has another effect, which is less appreciated. The Coulomb repulsion between the headgroups tends to separate the lipids, just as an increase in temperature does, and the consequence is the same; the tails have more room in which to move, and this tends to destabilize the lamellar phase with respect to the inverted hexagonal one. Thus there is a competition between these two effects, the outcome of which depends, among other things, on the effective volume of the stabilizing counterions. To confirm this picture, we have verified by explicit calculation that, if the effective volume of the counterions is too small, the destabilizing tendency of the repulsion between headgroups overcomes the stabilizing tendency of the added volume of counterions, with the result that the system makes a transition from lamellar to inverted hexagonal on increasing the pH. This is, of course, opposite to experimental observation and to our calculations using counterions of volume appropriate to those found in water. Nonetheless, the existence of this competition between different aspects of the Coulomb interaction emphasizes the importance of the counterions and their volume, and indicates at least one mechanism whereby the pH at which the lamellar phase becomes unstable should be expected to depend not only upon the system composition, but also upon the species of counterion and the nature of the solvent, as is observed (Seddon, 1990).

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