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THEORY OF SELF-ASSEMBLY OF LIPID BILAYERS AND VESICLES

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Summary

A simple theory is developed that explains the formation of bilayers and vesicles and accounts quantitatively for many of their physical properties: Properties including vesicle size distributions and bilayer elasticity emerge from a unified theory that links thermodynamics, interaction free energy, and molecular geometry. The theory may be applied to the analysis of more complicated membrane structures and mechanisms.

Introduction

Any fundamental theory of self-assembly of small molecules, such as phospholipids, into large well-defined structures, such as bilayers, must involve (1) the interaction free energies of the molecules, (2) the geometry of the molecules and (3) a correct thermodynamic treatment of the whole system.

The thermodynamics of self-assembly of lipid molecules into micelles has long been well understood [1—4], though only recently has the role of molecular geometry been appreciated [3—6]. On the other hand, in the area of bilayer and vesicle research, while the importance of the geometry (or packing) of lipids has been recognized [5,6], the role of thermodynamics in self-assembly has been ignored. Theories of micelles use thermodynamics together with the ideas of "opposing forces" and "hydrophobic forces", with emphasis on such micellar properties as the critical micelle concentration and micelle aggregation numbers [3]. By contrast, theories of bilayers and membranes have been modelled on certain a priori assumptions involving such concepts as "fluid mosaics" [7], "lipid packing" [5,6] or "bilayer elasticity" [8—10], with the emphasis on bilayer and membrane structure. Yet the only difference between bilayers and micelles is that lipids containing two (or more) hydrocarbon chains form bilayers, whereas single chained lipids form micelles. A single theory should describe both.

We have shown [4] that when thermodynamics, interaction free energies, and molecular geometry are taken together a unified theory emerges that accounts for many previously unexplained properties of micelles (both spherical and cylindrical), and also explains why diacyl chained lipids form into extended bilayers and vesicles. Here we develop the theory further for diacyl chained lipids, and consider both one- and two-component bilayer vesicles. For a more detailed account of the theoretical background, see ref. 4.

Mechanism of self-assembly

The assembly of lipids into well defined structures such as micelles and bilayer vesicles derives from the hydrophobic interaction between the hydrocarbon tails, which induces the molecules to congregate, and the hydrophilic nature of the head groups, which imposes the requirement that the head groups remain in contact with water. These two effects compete to give rise to the idea of two "opposing forces" [3]: the one tending to decrease and the other tending to increase the head group area in contact with water. These interactions lead to the concept of an "optimal surface area" per head group at which the total interaction free energy per lipid molecule is a minimum.

Geometric considerations can now be applied to establish the possible structures. These depend on the optimal surface area and the hydrocarbon chain volume, and are limited by the maximum length that the hydrocarbon chains can extend. Such packing considerations illustrate why single-chained lipids (e.g. lysophosphatidylcholine) can assemble into small micelles while double-chained lipids (e.g. diacyl phosphatidylcholines) cannot. However, geometry does allow double-chained lipids to form into bilayers and vesicles, as indeed they do. But so can the single-chained lipids, which do not in practice. The deciding factor is entropy, which tells us that those structures with large lipid aggregation numbers, while possible on energetic and geometric grounds, are improbable entropically.

Thus the formation of bilayers from lysophosphatidylcholine does not occur because of entropy, whereas the formation of micelles from diacyl-phosphatidylcholine does not occur because of energy and geometry. Diacyl lipids form vesicles of homogeneous size: larger vesicles are disallowed because of entropy while smaller vesicles are disallowed because of energy and packing.

The concepts of interaction free energies, molecular geometry and entropy, when taken together, furnish a framework for a theory of self-assembly.

Thermodynamics, interaction energies and packing constraints of lipids in aggregated structures

Thermodynamics of self-assembly. Equilibrium thermodynamics demands that the chemical potential of all molecules in a system of aggregated structures (micelles, bilayers, etc.) will be the same. This may be expressed as [1–4]

$$\mu_N^0 + \frac{kT}{N}\ln(X_N/N) = \text{const.} \quad N = 1, 2, 3, \ldots, \tag{1}$$

where X_N is the mol fraction of molecules incorporated into micelles of aggregation number N (X_N = concentration in mole fraction units, i.e. in Mol/

55.5, for an aqueous solvent), μ_N^0 is the free energy per molecule in the micelle, k is Boltzmann's constant and T is the temperature. $N = 1$ corresponds to isolated molecules or monomers in solution. Eqn. 1 can be written more conveniently in the form

$$X_N = N(X_M/M)^{N/M}\, e^{N(\mu_M^0 - \mu_N^0)/kT} \tag{2}$$

where M is any arbitrary reference state of micelles with aggregation number M. Eqn. 2 is the basic thermodynamic equation for any self-assembly analysis. It shows, for example, that in the absence of the free energy contributions small aggregates are entropically favoured over larger ones; in fact, when $\mu_M^0 = \mu_N^0$, most of the molecules will be in the monomer state ($N = 1$). Little more can be said without spelling out the form and magnitude of the free energies μ_N^0.

Interaction free energies. We shall assume that the temperature of the hydrocarbon chains is above the melting temperature. The interaction forces between lipids in an aggregate will therefore arise mainly from the hydrocarbon-water interface and polar head group region. Contributions to the interaction free energy μ_N^0 will be of two kinds: (1) an attractive interaction arising from attractive hydrophobic or interfacial tension forces, and (2) a repulsive interaction arising from electrostatic head-group repulsion, steric head-group repulsion and steric hydrocarbon side-chain repulsion.

(1) The attractive contribution can be represented by an interfacial free energy per unit area, $\gamma \approx 50$ erg/cm^2, characteristic of a liquid hydrocarbon-water interface [4]. The hydrophobic free energy contribution to μ_N^0 will therefore be γa, where a is the molecular area measured at the hydrocarbon-water interface.

(2) The repulsive contributions are more difficult to formulate explicitly, and we adopt an expression of the form C/a, where C is a constant. For zwitterionic and ionic head-groups an electrostatic repulsive energy of the form C/a is appropriate. This choice for the repulsive energy is not critical [4]; all the subsequent analysis can be carried out with other repulsive forms, e.g. C/a^2.

The repulsive free energy contribution to μ_N^0 is therefore taken as C/a, where C is a constant, into which all repulsive interactions are incorporated. C may be expected to be larger for larger head-groups and, in the case of anionic head-groups, should increase with increasing pH and decreasing ionic strength [4,11]. Note that the area a in the repulsive energy term C/a is not necessarily measured at the hydrocarbon-water interface, but may be slightly above it (if the head-group repulsion dominates) or below it (if the side-chain repulsion dominates). This effect is important when curvature corrections are introduced into the free energy but can be ignored in the present case.

The free energy per molecule is thus

$$\mu_N^0 = \gamma a + C/a\,. \tag{3}$$

The minimum free neergy $\mu_N^0(\min)$ is given when

$$\frac{\partial \mu_N^0}{\partial a} = \gamma - C/a^2 = 0 \quad \text{i.e. } a = a_0 = \sqrt{C/\gamma} \tag{4}$$

whence

$$\mu_N^0(\min) = 2\gamma a_0 . \tag{5}$$

a_0 will be referred to as the optimal surface area per molecule (defined at the hydrocarbon-water interface), being that area at which the free energy per molecule in a micelle or bilayer is a minimum. The free energy per molecule may now be expressed in the convenient form

$$\mu_N^0 = \gamma(a + a_0^2/a) = 2a_0\gamma + \frac{\gamma}{a}(a - a_0)^2 . \tag{6}$$

Eqn. 6 shows that μ_N^0 has a parabolic (elastic-type) variation about the minimum energy, as expected for any type of interaction. If phospholipids can pack into a variety of structures in which their surface areas remain equal or close to a_0, it follows from Eqn. 2 that entropy will favour the structure with the smallest aggregation number. We must therefore now consider how the molecular geometry of lipids determine the sort of structures they can pack into such that their surface areas are close to a_0 and at the same time the aggregation number N is a minimum.

Packing constraints. Given that the hydrocarbon interior of a phospholipid in a bilayer is in a liquid-like state, we may derive geometric expressions that relate the hydrocarbon-water interfacial area a, the hydrocarbon chain volume v, the hydrocarbon thickness l, and the radius of curvature R of the structure (bilayer or micelle) measured at the interface (Fig. 1).

For a spherical micelle of radius $R = l$, we have $4\pi R^3/3v = 4\pi R^2/a = N$, i.e., $v/a = l/3$. For a cylindrical micelle of radius $R = l$, we obtain $v/a = l/2$.

For a spherical bilayer vesicle (Fig. 1) of outer radius R, the geometric "packing equation" for the outer layer molecules is [4]

$$\frac{v}{a} = l\left(1 - \frac{l}{R} + \frac{l^2}{3R^2}\right) \tag{7}$$

from which, in the limit of large $R(R >> l)$, we obtain the obvious result for a planar bilayer: $v/a = l$, where l is the bilayer half-thickness. Note that in going from spherical micelle → cylindrical micelle → spherical vesicle → planar bilayer the aggregation number increases.

Our packing constraint is that the hydrocarbon region l must not exceed some maximum length l_c [3,4]. The desired structure is then that which has the smallest aggregation number N and l equal to or less than l_c.

One-component vesicles

Consider a 12-carbon single-chained anionic lipid for which typically [4] a_0 = 70 Å^2, v = 350 Å^3. Thus v/a_0 = 5 Å. For this lipid to pack into a spherical micelle with $a = a_0$ the micellar radius would have to equal $l = 3v/a \approx 15$ Å. This would only be possible if the hydrocarbon chain can extend this far, which for a 12-carbon chain may just be possible [3,4]. Such lipids would therefore form into spherical micelles of radii ≈ 15 Å. They could also form into bilayers, with their areas remaining equal to a_0; but since the spherical micelle has a lower aggregation number than a bilayer the micelle would be the

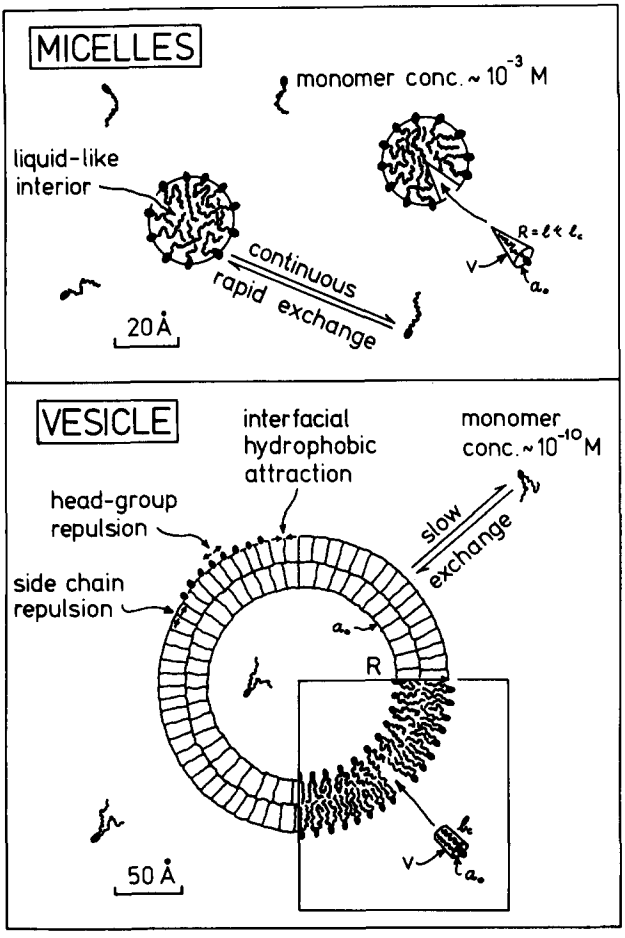

Fig. 1. Schematic illustration of micelle and vesicle formation in water. Entropy favours structures with small aggregation numbers. Single-chained lipids can form micelles. Double-chained lipids cannot always do this due to geometric packing constraints. These form bilayers and vesicles.

favoured structure entropically. If now the ionic strength of the aqueous solvent were increased, the electrostatic head-group repulsion would decrease and the optimal area would fall below 70 Å^2 to, say, 60 Å^2. Thus now $v/a \approx 5.8$ Å, and the radius of a spherical micelle would now have to be equal to $l \approx 17.5$ Å. But the hydrocarbon chains cannot extend more than 15 Å; hence they can no longer pack into spherical micelles. To form cylindrical micelles, the radius would only have to be $l = 2v/a \approx 11.6$ Å, so that we may conclude that the chains could comfortably pack into such cylindrical micelles and this they can do at elevated ionic strengths even though the aggregation number is higher than for a spherical micelle [12,13]. A rigorous analysis of the transition from spherical to (finite) cylindrical micelles is complicated [4], but the thread of the argument outlined above will be a recurring theme in all that follows: i.e. lipid structures are determined by a subtle interplay of entropy which favours small structures, and packing constraints which energetically resist the packing of molecules into arbitrarily small structures.

If we double the number of chains of our lipid, we shall have $v = 700$ Å^3, but the optimal area a_0 would remain largely unchanged and still close to 60 Å^2. (This is an important supposition, and is a consequence of assuming that the chains are in a liquid-like state. The optimum area a_0 will remain the same if the interfacial tension γ and repulsive coefficient C in Eqns. 3 and 4 do not change. The value of the interfacial tension ($\gamma \approx 50$ erg/cm^2) of a hydrocarbon-water interface is clearly not dependent on the number of chains and so may safely be assumed to remain constant. If the chains are in a liquid-like state, the repulsive coefficient C, arising from head-group repulsion, should likewise remain constant. The optimal area a_0 should not be much affected by doubling or extending the hydrocarbon chains. This is borne out by experiments: both single-chained lipids, which form into micelles, and double-chained lipids, which form into bilayers, have very much the same surface areas [4], normally in the range 50—70 Å^2. Further, the surface areas of lipids measured at the oil-water interface have been found to be insensitive to chain length for areas above the transition area (ref. 14 and Mingins, J., personal communication.) Thus for the diacyl chained lipid we have $v/a \approx 700/60 \approx 12$ Å. Since the fully extended length of the chain is still the same as before (close to 15 Å) neither spherical micelles nor cylindrical micelles may be packed, since their radii would have to be 36 Å or 24 Å, which is far beyond the fully extended length of 15 Å. However, planar bilayers can easily be packed since these would have a half-thickness $v/a \approx 12$ Å. But the aggregation number would now be very large. Alternatively, the lipids could pack into spherical vesicles which, from Eqn. 7, putting $a = a_0 = 60$ Å, v 70 Å^3, $l = 15$ Å, would have an outer radius of $R_1 \approx 62$ Å. Such a structure would have the lowest possible aggregation number consistent with the free energy per molecule being a minimum and at the same time satisfying the packing constraints.

Theoretical properties of vesicles and bilayers. Certain lipids cannot pack into small micelles or even small vesicles since this would require their hydrocarbon chain region to be longer than the hydrocarbon chains can extend. This fully extensible hydrocarbon region is denoted as the "critical length" l_c, and we expect l_c to be somewhat less than the fully extended molecular length of the hydrocarbon chains [3,4]. Rearranging eqn. 7 we obtain for the outer radius R of a spherical vesicle

$$R = \frac{l[3 + \sqrt{3(4v/al - 1)}]}{6[1 - v/al]} \approx \frac{l}{(1 - v/al)} \tag{8}$$

If the surface area a is to remain equal to the optimal area a_0, and if the hydrocarbon thickness l is not to exceed l_c, the vesicle radius R cannot be less than a certain radius, the "critical radius" R^c, given by Eqn. 8 putting $l = l_c$, $a = a_0$. For example, if $v = 1{,}000$ Å^3, $a_0 = 65$ Å^2, $l_c = 18$ Å, we obtain $R^c = 118$ Å (the approximate equation gives $R^c = 124$ Å).

For vesicles that are larger than the critical radius R^c there are no packing restrictions on any of the molecules, which can assume their minimum energy configuration with $a = a_0$. Thus when $R > R^c$ we have $a = a_0$, and for all the molecules

$$\mu_N^0 = 2a_0\gamma\,. \tag{9}$$

Note that the inner layer molecules are always in a favourable packing state whatever the value of R; their hydrocarbon chains simply fill up the inner volume with their inner surface areas remaining at their optimal value a_0 [4], i.e. packing constraints only affect the outer layer molecules. On the other hand, for $R < R^c$ packing constraints have to be taken into account. We therefore have, from Eqn. 6,

$$\mu_N^0 = 2a_0\gamma \text{ for the inner layer molecules} \tag{10}$$

$$\mu_N^0 = 2a_0\gamma + \frac{\gamma}{a}[a - a_0]^2 \text{ for the outer layer molecules} \tag{11}$$

Thus the mean μ_N^0 per molecule in a vesicle of aggregation number N is

$$\mu_N^0 = 2a_0\gamma + \frac{\gamma}{a}[a - a_0]^2 \frac{n}{N}, \tag{12}$$

where $n = 4\pi R^2/a$ is the number of molecules in the outer layer. Now using Eqn. 8 we obtain to a sufficient degree of approximation [4] that, for $R < R^c$,

$$\mu_N^0 = 2a_0\gamma + \frac{4\pi l_c^2\gamma}{N}\left(1 - \frac{R}{R^c}\right)^2 \tag{13}$$

To obtain the vesicle size distribution it is necessary to substitute Eqns. 9 and 13 into Eqn. 2. We take our arbitrary reference state as that when $R = R^c$ ($M = N^c$ and $\mu_M^0 = 2a_0\gamma$) and finally obtain the distributions:

$$X_R = N\left(\frac{X_{R^c}}{N^c}\right)^{N/N^c} \quad \text{for } R > R^c \tag{14}$$

and

$$X_R = N\left(\frac{X_{R^c}}{N^c}\right)^{N/N^c} e^{-4\pi l_c^2\gamma[1-R/R^c]/kT} \quad \text{for } R < R^c, \tag{15}$$

where $N \approx 4\pi[R^2 + (R - t)^2]/a_0$. In these expressions N/N^c is the ratio of the aggregation numbers of vesicles with radii R and R^c, and is given by

$$\frac{N}{N^c} = \frac{[R^2 + (R - t)^2]}{[(R^c)^2 + (R^c - t)^2]}, \tag{16}$$

where t is the bilayer hydrocarbon thickness. This may be shown to be effectively constant and equal to that of the planar bilayer $t = 2v/a_0$.

Eqns. 14 and 15 give the concentration of molecules X_R in vesicles of radius R; the vesicle concentration being given by X_R/N.

The results strictly apply only to low lipid concentrations where vesicle-vesicle interactions may be ignored. At higher concentrations these interactions become important and modify the free energy μ_N^0, and can lead to vesicle aggregation and the formation of ordered mesomorphic phases [4,15—20].

Comparison with experiments. As an example we consider the theoretical predictions as they apply to egg phosphatidylcholine vesicles (see also ref. 4):

putting [16,17,4] $v = 1063$ Å^3, $a_0 = 71.7$ Å^2, $t = 2v/a_0 = 29.6$ Å, $l_c = 17.5$ Å (giving $R^c = 108$ Å), $\gamma = 50$ erg/cm^2, the distribution is plotted in Fig. 2 ($D = 0$ curve) for a total lipid concentration of $X_R = 10^{-5}$ (≈ 0.5 mg phospholipid/ml water, or ≈ 0.5 mM). The distribution peaks at about $R_{peak} \approx 105$ Å [21].

The main features revealed by the theory are:

(1) The distribution of vesicles has a near-Gaussian profile $exp(R - R_{peak})^2/2\sigma^2$ which peaks at an outer radius R_{peak} close to, but slighly smaller than, the critical packing radius R^c. The standard deviation

$$\sigma = \frac{R_{peak}}{l_c}\sqrt{\frac{kT}{8\pi\gamma}} \tag{17}$$

is of the order of a few per cent of R (for $R = 105$ Å, $l_c = 17.5$ Å, $\gamma = 50$ erg/cm^2, we obtain $\sigma \approx 3.5$ Å). The distribution is therefore found to be narrowly peaked (homogeneous), in agreement with observation [4,21].

(2) The distribution shifts to lower radii as the total phospholipid concentration is lowered, but the magnitude of this shift is small. A million-fold reduction in concentration shifts the peak radius by only a few Angstrom units.

(3) The surface areas of the inner molecules should be the same as in a planar bilayer a_0, while the outer areas should be slightly greater. The outside-inside ratio of molecules is given by $R^2/(R - t)^2$, where t is the hydrocarbon bilayer thickness and not that of the whole bilayer. For egg lecithin we obtain a ratio of ≈ 2.0 [4].

(4) The factors that are expected to affect vesicle size are changes in the hydrocarbon volume v, the critical length l_c and the optimal surface area a_0. for anionic lipids the optimal area a_0 may be expected to decrease with decreased pH and increased ionic strength [11], resulting in larger R^c values and correspondingly larger vesicles [22]. Zwitterionic lipids should be less sensitive to such changes, as observed [22].

Increased unsaturation should change the critical length l_c but should not much affect the hydrocarbon volume v (cf. the densities of liquid hexadecane and hexadecene differ by only 1%). We estimate from a bond-length and bond-angle analysis that a *cis* double bond shortens l_c by about 0.9 Å, while a *trans* double bond leaves l_c practically the same. From Eqn. 8 we find that the addition of a *cis* bond (to both chains) should increase the vesicle radius, assuming a_0 remains unchanged, and lower the outside-inside ratio, $r \approx [R/(R - t)]^2$, while the addition of a *trans* bond should have little effect on the size and ratio as the critical length l_c varies. Expressing this ratio as

$$r \approx [R^c/(R^c - t)]^2 \tag{18}$$

where R^c is given by Eqn. 8 and $t = 2v/a_0 \approx$ constant, we obtain by differentiation the variation in r as l_c changes:

$$\frac{\Delta r}{\Delta l_c} \approx \frac{2t}{l_c^2}\left(\frac{t}{l_c} - 1\right) r^{3/2}. \tag{19}$$

We can see that for typical values $t \approx 30$ Å, $l_c \approx 17.5$ Å, for a 16-carbon or 18-carbon diacyl chain, we have $\Delta r/\Delta l_c \approx 0.14\, r^{3/2}$. De Kruijff et al. [23] have

found that the outside-inside ratio of phosphatidylcholine vesicles, at 30°C, increased from $r = 1.75$ for a 18:1 *cis* diacyl-chain to $r = 2.0$ for a 18:1 *trans* phosphatidylcholine, i.e. r increased by 0.25 on replacing a *cis* bond by a *trans* bond in each chain. The theoretical estimate using $r \approx 1.85$ and $\Delta l_c = +0.9$ Å, is $\Delta r \approx +0.41\ (1.85)^{3/2}\ 0.9 \approx 0.3$. The theoretical estimate for Δr of 16:0 and 16:1 *cis* phosphatidylcholine vesicles also agrees with De Kruijff et al.'s [23] results ($\Delta r = 0.4$) but not with their 18:0 *cis* results ($\Delta r = 0.05$). These measurements, however, were made at very different temperatures so that a comparison with theory is not strictly possible.

On the other hand, increasing the chain length should leave v/l_c unchanged since both v and l_c are roughly proportional to the number of carbons per chain [3,4]. Eqn. 8 shows that for $v/l_c \approx$ constant, and $a_0 \approx$ constant, R^c is proportional to l_c. Thus, increased chain length should lead to an increased vesicle radius. However, the outside-inside ratio r will not change since the bilayer thickness $t \approx 2v/a_0$ is also proportional to the number of carbon atoms, leaving $r \approx [R^c/(R^c - t)]^2$ practically unchanged. This conclusion is not in accord with observation: De Kruijff et al. [23] found that the outside-inside ratio of saturated diacyl-chained phosphatidylcholines 18:0, 16:0 and 14:0 were equal to 1.7, 2.2 and 2.65 respectively, though the measurements were made at different temperatures (60°, 50° and 30°C). For 16:1 *cis* and 18:1 *cis* vesicles, both measured at 30°C, the ratios were about the same (1.8 and 1.75). Further, they found that for chains shorter than 14:0 no stable vesicles could be prepared, in agreement with earlier work [23,24]. This, too, is not borne out by the theory, which allows for the existence of vesicles even for very short chains. We return to this matter in the next section.

(5) The theory allows us to calculate values for the elastic moduli of bilayers. For a planar bilayer under compression or tension the free energy of a molecule in the bilayer is given by Eqn. 6. For small expansions or compressions in area $\Delta a = (a - a_0)$ about the equilibrium optimal area a_0 the energy change per unit area of bilayer is

$$\frac{\Delta(2\mu_N^0)}{a} = 2\gamma(\Delta a/a)^2 = \tfrac{1}{2}k_s(\Delta a/a)^2 \ . \tag{20}$$

An energy dependence of the form $\frac{1}{2}k_s(\Delta a/a)^2$ is the same as that of an elastic membrane of elastic stretch modulus k_s equal to 4γ, or $k_s \approx 200$ erg/cm^2. This value agrees well with measured values on lipid bilayers and red cell membranes [4,25,26]. This elasticity is only for planar bilayers under vertical compression or tension, or lateral stretch and does not extend to the bending of bilayers. On the contrary, the analysis has shown that the bending of a bilayer is favoured down to the critical packing radius R^c if the lipids in each layer can freely move or slide past each other), and that "bending elasticity" sets in only for radii smaller than R^c.

The theory as it stands appears to account fairly well for many but not all of the observed physical properties of vesicles. The development so far is model-independent and relies only on thermodynamics, simple packing considerations and the notion of opposing forces. We shall now extend the analysis by considering curvature corrections to the free energy μ_N^0.

Effects of finite head-group on vesicle properties

So far, the forces between adjacent lipids have all been assumed to occur at the hydrocarbon-water interface, at which the surface area a per molecule has been defined. This is likely to be true for the interfacial tension, but not necessarily for the head group repulsive forces which are more likely to be centred above this interface. If the head group region is of length D, then the centre of the repulsion will be located at a distance $D/2$ above the hydrocarbon-water interface.

The repulsive energy C/a of Eqn. 3 must now be redefined such that its area a is located at a distance $D/2$ away. This area is equal to $a(1 + D/R)$, where R is the radius of curvature of the surface. Thus for a spherical surface the repulsive energy C/a of Eqn. 3 must now be replaced by $C/(a\{1 + D/R\})$. This equation has the same form as that of the electrostatic repulsion between zwitterionic head-groups of dipole length D [4]. We may now write for the free energy (cf. Eqn. 3)

$$\mu_N^0 = a\gamma + \frac{C}{a[1 + D/R]} \quad . \tag{21}$$

The magnitude of D will depend on factors like the head-group length, size and conformation, and for ionic head-groups on the ionic strength [4]. The free energy per molecule in a vesicle can now be shown to be [4]:

$$\mu_N^0 = 2a_0\gamma - \frac{4\pi\gamma Dt}{N} \quad , \quad \text{for } R > R^c \, . \tag{22}$$

and

$$\mu_N^0 \approx 2a_0\gamma + \frac{4\pi l_c^2\gamma}{N}[1 - R/R^c]^2 - \frac{4\pi Dt\gamma}{N} \quad , \quad \text{for } R < R^c \tag{23}$$

which reduces to Eqns. 9 and 13 when $D = 0$. Thus, a finite head-group repulsion results in a small additional term to the free energies which favours smaller vesicles. Its effect may be obtained by minimizing Eqn. 23 with respect to R. Assuming that $N \propto R^2$, we obtain

$$R_{\text{peak}} \approx R^c[1 - Dt/l_c^2] \tag{24}$$

which gives a good estimate for the peak radius R in terms of R^c and D.

The effect of a finite head-group does not modify the qualitative picture of vesicles already obtained, but certain quantitative aspects are significantly altered:

(1) The peak radius R_{peak} is appreciably shifted below R^c even for fairly small values of D. For egg phosphatidylcholine, using t = 29.6 Å, l_c = 17.5 Å, R^c = 108 Å, D = +4 Å, the peak radius shifts from about 105 Å down to about 70 Å. Thus for eggg phosphatidylcholine vesicles to peak at $R \approx 105$ Å [21] with D = +4 Å, the value of l_c must be less than 17.5 Å (and closer to 16.5 Å).

When we consider lipids of different chain lengths we find that, since both t and l_c are roughly proportional to the number of carbons per chain, the factor

$[1 - Dt/l_c^2]$ in Eqn. 24 falls rapidly for progressively shorter chains. As the chain lengths are shortened we now expect the vesicle radii to fall, and consequently the outside-inside ratio $r \approx [R/(R - t)]^2$ to increase. This effect, which relies entirely on the existence of a positive non-zero D, explains why shorter chained lipid vesicles have higher outside-inside ratios [23]. Further, Eqn. 24 shows that for sufficiently short chains, such that $D \approx l_c^2/t \approx l_c/2$, no stable vesicles should exist. We conclude that once the hydrocarbon chain length falls to about twice the head-group length no stable vesicles should form; instead, micelles should be formed. For phosphatidylcholine no stable vesicles have been reported for chains with less than 14 carbons [23,24]. For a 12-carbon chain we may expect l_c to be in the range 10—14 Å, yielding an empirical value for the "effective length" of the phosphatidylcholine head-group of 5—7 Å.

(2) The vesicle distribution still has a near-Gaussian profile. The standard deviation in the radius being now

$$\sigma \approx \frac{R_{peak}}{l_c} \left(\frac{1 - Dt/2l_c^2}{1 - Dt/l_c^2}\right) \sqrt{\frac{kT}{8\pi\gamma}} . \tag{25}$$

The standard deviation of egg phosphatidylcholine vesicles ($R \approx 105$ Å) should therefore rise from $\sigma \approx 3.5$ Å (for $D = 0$) to $\sigma \approx 5.0$ Å for $D = +4$ Å, and to $\sigma \approx 8$ Å for $D = +6$ Å, as shown in Fig. 2. Further, for progressively shorter chain lengths vesicles should become not only smaller but also progressively less homogeneous.

(3) Both the outer and inner surface areas should be roughly the same and slightly greater than the optimal area a_0 in lamellar bilayers [27,28]. Due to the different packing boundaries of the inner and outer hydrocarbon chain regions we should also expect these to have slightly different chain configurations [29].

The agreement of some of these theoretical predictions with the limited

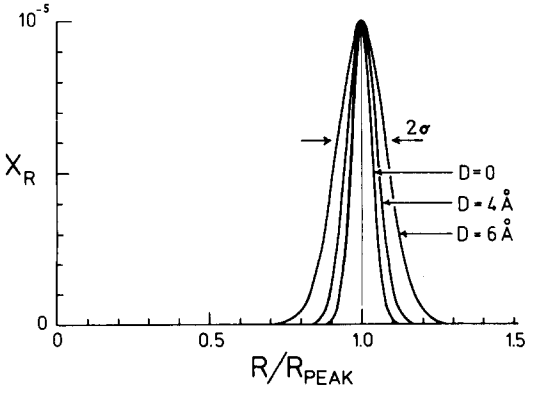

Fig. 2. Theoretical concentration distribution X_R in mol fraction units of lipid in vesicles of normalized outer radius R/R_{peak} (theoretical parameters taken for egg phosphatidylcholine). The distributions are near Gaussian with a standard deviation σ that depends on the "effective" head-group length D.

experimental data available gives support to the notion that the repulsive forces are located within the polar head-group region (at least for phosphatidylcholine), and are therefore due more to head-group repulsion than to side-chain repulsion.

Two-component vesicles

In a two-component system the competing forces of entropy and curvature effects, and packing constraints, are still operating, but two additional factors must be taken into account. First, the molecules may distribute asymmetrically and still satisfy the packing constraints. This can result in a vesicle of lower aggregation number than in the symmetrically distributed vesicle. Since a lower aggregation number is favoured both entropically and energetically we conclude that in general all two-component vesicles should be asymmetrical (Fig. 3). Opposing this asymmetry is an additional entropic force: the entropy of mixing, and the problem reduces to one of establishing the balance of these competing effects.

In extending the theory to two-component vesicles, we shall assume that there are no specific interactions between the different lipids, so that they mix ideally. To include such effects as phase separations arising from specific interactions would certainly be desirable but would require a much deeper theoretical understanding of head-group interactions than we have at present. We shall also assume that the fully extensible "critical length" l_c of the hydrocarbon region is the same for both lipids. This considerably simplifies the geometric packing analysis, but is not otherwise justified.

To analyse a two-component vesicle we first determine how two lipids with different geometric packing constraints assemble to form a vesicle. Such an analysis is purely geometric. Second, we determine the free energy for a two-component lipid system. Finally, we determine the properties of the "equilib-

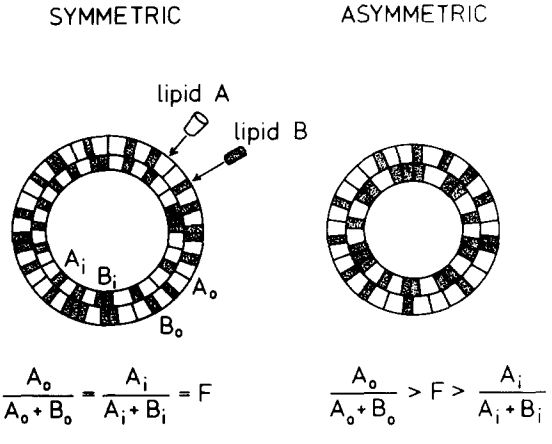

Fig. 3. Symmetric and asymmetric vesicles composed of a mixture of lipids A and B. The packing of the two lipids are the same in both vesicles, and the total mol fractions of lipids A and B are also the same. Asymmetric vesicles are favoured due to head-group repulsion contributions to the free energy, but are opposed by the unfavourable entropy of demixing. The equilibrium asymmetrical vesicle is determined by the balance of these two effects.

rium" vesicle, and compare the results with experimental data. We cannot assume a priori that the two lipids distribute asymmetrically on the inner and outer layers of the vesicle. Any asymmetry, if it exists, must emerge naturally in the determination of the equilibrium vesicle.

The different lipids will be denoted by A and B. For a vesicle of outer and inner radii R_o and R_i (measured at the hydrocarbon-water interfaces as before) we define: A_o and B_o (A_i and B_i) = number of A and B lipids in the outer (inner) vesicle layer; $N = A_o + B_o + A_i + B_i$; $F = (A_o + A_i)/N$; $X_o = A_o/(A_o + B_o)$; $X_i = A_i/(A_i + B_i)$; $f = (A_o + B_o)/N$. Note that for no asymmetry, $X_o = X_i = F$ (Fig. 3).

Packing equation of a two-component vesicle. We denote the hydrocarbon volumes of lipids A and B as v_A and v_B, and their optimal surface areas as a_A and a_B. The critical hydrocarbon length, l_c, is assumed the same for both lipids. The equation for the critical outer radius R_o^c is still given by Eqn. 8, i.e.

$$R_0^c = l_c \left\{3 + \sqrt{3\left(4\frac{v}{al_c} - 1\right)}\right\} \Big/ 6\left\{1 - \frac{v}{al_c}\right\} \tag{26}$$

but v and a are now the mean outer layer values:

$$v = X_o v_A + (1 - X_o)v_B, \qquad a = X_o a_A + (1 - X_o)a_B. \tag{27}$$

We remark that an alternative way of analysing the packing of a mixed lipid system, based on a solid-angle approach [6], yields values of vesicle radii that differ by 1% or less. We adopt the present approach for convenience only.

For any two-component system the value of F, the mole fraction of A lipids in the vesicle, is fixed. The mol fraction X_o of A lipids in the outer layer will be adopted as our yardstick of asymmetry. For $X_o = F$ there is no asymmetry. For any X_o the value of R_o^c is readily obtained from Eqns. 26 and 27. This in turn determines A_o and B_o, since, for the outer vesicle layer:

$$\frac{4\pi(R_o^c)^2}{[X_o a_A + (1 - X_o)a_B]} = A_o + B_o = \frac{A_o}{X_o} = \frac{B_o}{(1 - X_o)}. \tag{28}$$

Similarly, for the inner vesicle layer:

$$\frac{4\pi R_i^2}{[X_i a_A + (1 - X_i)a_B]} = A_i + B_i = \frac{4\pi[(R_o^c - l_c)^3 - R_i^3]}{3[X_i v_A + (1 - X_i)v_B]}. \tag{29}$$

Finally, A_i and B_i are related through $F = (A_o + B_o)/(A_o + B_o + A_i + B_i)$. Thus for a given total mole fraction F we can solve the above equations to obtain all the relevant parameters R_o^c, A_o, B_o, A_i, B_i, X_i, f and N, at any value of X_o.

As an example we take the case of an egg yolk phosphatidylcholine (lipid A)-phosphatidylethanolamine (lipid B) mixture, and use the following rough values for these lipids based on recent estimates [6,4]: $a_A = 72$ Å^2, $v_A = 1060$ Å^3, $a_B = 42$ Å^2, $v_B = 700$ Å^3, $l_c = 17$ Å. Berden et al. [30] have studied mixed phosphatidylcholine/phosphatidylethanolamine vesicles in which phosphatidyl-ehtanolamine/phosphatidylcholine = 1.08, i.e. $F = 0.48$. They found that the vesicles were asymmetric with (outside phosphatidylethanolamine)/(out-

side phosphatidylcholine) = 0.92, i.e. $X_o = 0.52$. Using $F = 0.48$, $X_o = 0.52$, and solving Eqns. 26 to 29 we obtain the following results and compare with experimental values (in brackets): $R_o = 175$ Å (≈180 Å, [31]), $B_i/A_i = 1.36$ (1.38), $B_o/B_i = 1.17$ (1.17), $A_o/A_i = 1.72$ (1.76), $(A_o + B_o)/(A_i + B_i) = 1.41$ (1.41), $N = 11\,400$ (not measured). The theoretical and experimental results all agree to within 2%.

Having established that the asymmetric distribution of lipids in a vesicle is well described by their packing properties, we have yet to predict the sign and magnitude of X_o. To do this we require the expression for the mean interaction free energy per molecule in a mixed system.

Free energy of a two-component vesicle. If we ignore any specific interactions between the lipids, it may be shown that their surface areas will remain close to their optimal areas (as in a one-component bilayer). Then the free energy per vesicle $N\mu_N^0$ is simply the sum of the separate contributions of the lipids, together with the entropy of mixing, i.e.

$$N\mu_N^0 = 2\gamma a_A A_o \left(1 - \frac{D_A}{2R_o}\right) + 2\gamma a_B B_o \left(1 - \frac{D_B}{2R_o}\right) + 2\gamma a_A A_i \left(1 + \frac{D_A}{2R_i}\right)$$

$$+ 2\gamma a_B B_i \left(1 + \frac{D_B}{2R_i}\right) - kT \ln \left(\frac{(A_o + B_o)!}{A_o! B_o!} \frac{(A_i + B_i)!}{A_i! B_i!}\right). \tag{30}$$

Eqn. 30 is valid in the absence of packing constraints and reduces to Eqn. 22 for a one-component vesicle. After some simple algebra, Eqn. 30 becomes

$$\mu_N^0 = 2\gamma a_A F + 2\gamma a_B (1 - F) + \frac{\gamma}{N}\left\{D_A a_A \left(\frac{A_i}{R_i} - \frac{A_o}{R_o}\right) + D_B a_B \left(\frac{B_i}{R_i} - \frac{B_o}{R_o}\right)\right\}$$

$$+ kT[f\{X_o \ln X_o + (1 - X_o) \ln(1 - X_o)\}$$

$$+ (1 - f)\{X_i \ln X_i + (1 - X_i) \ln(1 - X_i)\}]. \tag{31}$$

Eqn. 31 may be simplified by use of Eqns. 26 to 29, assuming that deviations from symmetry are small, i.e. that X_o is close to F. We may then express the free energy μ_N^0 in terms of the deviation $\Delta X_o = (X_o - F)$:

$$\mu_N^0 = \text{const.} - \frac{4\pi\gamma t}{N}\left(FD_A\left\{1 + (1 - F)\frac{(a_A - a_B)}{a_A}\right\}\right.$$

$$\left. + (1 - f) D_B \left\{1 - (1 - F)\frac{(a_A - a_B)}{a_B}\right\}\right)$$

$$- \frac{4\pi\gamma}{N}\{R_o(1 + R_o/R_i)(D_A - D_B)\}\Delta X_o + \frac{R_o^2}{R_i^2}\frac{kT(\Delta X_o)^2}{2F(1 - F)} \tag{32}$$

All that remains is to calculate the value of ΔX_o at which the mean molecular free energy μ_N^0 is a minimum. We differentiate Eqn. 32 and after some more simplifications we obtain the condition which gives a minimum μ_N^0:

$$\Delta X_o \approx \frac{\gamma F(1-F)}{R_o kT}\left\{[Fa_A + (1-F)a_B](D_A - D_B)\right.$$

$$\left. + \left(\frac{R_i}{R_o}\right)^2 [FD_A + (1-F)D_B]\left(\frac{a_A}{R_A} - \frac{a_B}{R_B}\right) t\right\}. \qquad (33)$$

Here R_o is the vesicle radius, R_A and R_B are the radii of the pure component vesicles as given by Eqn. 8, * and t the hydrocarbon thickness of the bilayer $(R_o - R_i)$.

Properties of two-component vesicles. The main qualitative and quantitative aspects of two-component vesicles, as predicted by Eqn. 33, will now be summarized:

(1) In general, mixed lipid vesicles should always be asymmetrical. Those lipids which have a longer head group D and larger a/R will go preferentially to the outer layer. This is consistent with experimental studies on mixed phosphatidylcholine/phosphatidylethanolamine and phosphatidylcholine/cholesterol vesicles, as well as with the general conclusions of Berden et al. [30] that lipids with larger head groups tend to distribute preferentially in the outer layers.

(2) Asymmetries should be small at small mol fractions F (F close to 0 or 1). Maximum asymmetry should occur near $F = 0.5$ where $F(1-F)$ is maximum. But since the vesicle radius R_o also varies with F, this value could be appreciably shifted away from $F = 0.5$. With phosphatidylcholine/cholesterol vesicles, significant asymmetry has only been observed at mol fractions F above 0.3 cholesterol [32,33].

(3) For phosphatidylcholine/phosphatidylserine vesicles (phosphatidylserine being negatively charged), an increased pH will increase the head group area a_B (through an increased electrostatic repulsion) and thereby decrease R_B. At elevated pH we should therefore expect phosphatidylserine to go progressively more to the outer layer in a mixed phosphatidylcholine/phosphatidylserine vesicle, as observed [30].

(4) The first term in Eqn. 33 usually dominates; the head group areas a_A and a_B and head group lengths D_A and D_B being the major lipid properties that determine asymmetry. Variations in side chain composition and unsaturation should have a secondary effect (insofar as they vary R_A, R_B and R_o), in agreement with observation [34].

(5) Unless the asymmetry is large, the equilibrium vesicle radius is the same as if there were no asymmetry, given by Eqns. 26 and 27, or alternatively, as in a previous analysis [6]. Finally, large vesicles should have small asymmetries.

Eqn. 33 yields qualitative predictions which agree with the limited experimental data available at present. Quantitatively, it predicts equilibrium values for ΔX_0 that are close to those observed. Thus taking the phosphatidylcholine/phosphatidylethanolamine system as an example, using (roughly) $F = 0.48$, $\gamma = 50$ erg/cm^2, $a_A \approx 72$ Å^2, $a_B \approx 42$ Å^2, $R_A \approx 120$ Å, $R_B \approx 860$ Å, $t \approx 30$ Å, $R_o \approx 175$ Å, $D_A \approx D_B \approx 5$ Å, $D_A - D_B \approx 3$ Å, we obtain the measured value $\Delta X_o \approx$

* For a one-component vesicle Eqn. 8 gives $R \approx l_c/[1 - v/al_c]$. For some lipids, $v/al_c > 1$, and R takes a negative value. Such lipids, (e.g. cholesterol) cannot form vesicles or bilayers, and we have previously [6] termed such lipids "frayed" lipids.

+0.04 (i.e. phosphatidylcholine preferentially distributed in the outer layer). Unfortunately, the theoretical value for ΔX_0 depends critically on $(D_A - D_B)$ which depends on the relative head group conformations of phosphatidylcholine and phosphatidylethanolamine. This is still a matter of controversy. However, for values of D_A and D_B in the range 5—10 Å, the present theory would predict that the phosphatidylcholine head group is longer than the phosphatidylethanolamine head group by between 2 Å and 3 Å, in accord with an experimental measurement of 3 Å [35]. The values of D_A and D_B are not necessarily the same as the head group lengths. Strictly, they represent the length of the polar region above the hydrocarbon-water interface where the effective repulsive forces are operating.

Discussion

We have presented a general theory of lipid self-assembly in which the self-assembly mechanism involves the interplay of thermodynamics, interaction forces, and molecular geometry. We have shown how the theory yields simple analytical expressions for a number of vesicle and bilayer properties in terms of measurable parameters, and we have demonstrated how these predicted properties are in quantitative accord with experiments.

The theory also attempts to give a very definite picture of the mechanism of self-assembly. Vesicles emerge as thermodynamically stable, homogeneous structures, unaffected by drastic changes in the surrounding lipid concentration, so long as this is above the critical micelle concentration (CMC) of $\approx 10^{-10}$ M [3]. At high lipid concentrations, above about 10^{-3} M, vesicles begin to interact significantly with each other and form into ordered mesomorphic phases [15—20]. We stress that our treatment applies only to dilute systems, where vesicles are sufficiently far apart on average for these interactions to be ignored. Whereas we conclude that spherical vesicles, if allowed by packing, are thermodynamically favoured over isolated bilayers, we cannot say without further analysis whether they are generally more stable than multi-layered structures (liposomes) [4]. We note, however, that stable vesicles do form spontaneously free of multilamellar structures [36—38].

It is well known that the ability of vesicles to compartmentalize and segregate an inner region from the outside is utilised in many cellular transport processes. The existence of asymmetrical bilayer vesicles is now well established and we find theoretically that these too are thermodynamically stable and that the asymmetry is expected to be homogeneous. For asymmetric bilayer vesicles composed of charged and uncharged lipids, the different surface charge densities on the inner and outer vesicle surfaces would result in different surface potentials in the inner and outer surfaces depending on the magnitude of this asymmetry. The total membrane potential, as measured between the inner and outer bulk aqueous solutions, would be zero; but two different potentials would exist at each surface, these being compensated by a potential difference across the hydrocarbon region of the bilayer. * There is now compelling evi-

* For a calculation of these potentials for planar bilayers, equations 2 to 4 given by McLaughlin and Harary [41] may be solved assuming fixed surface charge densities σ_1 and σ_2 ($\sigma_1 \neq \sigma_2$), and putting $V = 0$.

dence which shows that these electrostatic potentials at membrane surfaces play a determining role in many transport and conduction processes [39,40]. We have only to take the argument one step further to conclude that a real membrane potential may be established if one of the membrane constituents is a molecule that selectively conducts cations or anions across the membrane. Such a membrane potential could form spontaneously without requiring a pump or metabolic energy. There is no violation of thermodynamics here since the system is always at thermodynamic equilibrium and the membrane potential can do no work. Indeed, the possibility that the inner and outer environments of vesicles may be asymmetric is conceptually no different from the equilibrium asymmetry in the membrane constituents.

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