Effect of Tension on Pore Formation in Drug-Containing Vesicles

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Received: August 23, 1994; In Final Form: October 4, 1994[⊗]

Pore formation in unilamellar lipid vesicles is believed to occur when the concentration of membrane-bound drug molecules exceeds a certain value. We treat this phenomenon in analogy with that of the micellization of surfactant in bulk aqueous solutions, thereby relating the threshold concentration of drug molecules to the free energy associated with transferring a molecule to a pore from its uniformly-dispersed state in the membrane. Incorporating the effects of lateral tension induced by osmotic pressure, we calculate the lowering of the pore-formation threshold with increasing tension. These predictions are tested by direct measurements on liposomal dispersions involving the antifungal drug amphotericin B.

I. Introduction

Recently a great deal of progress has been made on the formulation of stable dispersions of drug-containing, unilamellar, lipid vesicles—liposomes—in aqueous solution.[1] These systems are of widespread medical interest because they allow for the administering of drugs with significantly reduced toxicities[2] and enhanced site specificity of action.[3] In one particular system of this kind the following have been shown: below a certain drug-to-lipid ratio, the drug is uniformly distributed throughout the vesicle bilayer as a dilute "monomer"; above this threshold, the drug molecules aggregate into transmembrane "pores".[4] Some of the experimental details of this study are reviewed in section III, where we report the results of similar measurements *in the presence of osmotically-induced lateral tension.* First, however, we present in section II a simple statistical thermodynamic theory in which *the appearance of pores at a critical drug-to-lipid ratio is treated as a two-dimensional analogue of the formation of micelles at the critical micelle concentration (cmc) in bulk solutions of surfactant in water.* The (inverse of this) threshold for pore formation is shown to depend exponentially on the free energy change accompanying the transfer of a membrane-bound drug molecule into the "lip" of a pore. In the presence of lateral tension induced by an osmotic concentration gradient, this transfer free energy is significantly *increased*, resulting in a *lowering* of the drug-to-lipid ratio required for pore formation. Our predictions are tested against experiment via studies of liposomal dispersions of amphotericin B (henceforth AmB) in which sucrose is used as the osmotically active agent (section III).

II. Micellization Theory

A. Tension-Free Membranes. Absorption spectroscopy measurements[4] on the AmB/liposome system[5] indicate that the drug molecules (D) do not dissociate from the bilayers, i.e., move into the aqueous phase, even at very low concentrations: upon lowering [D] by almost 2 orders of magnitude in the micromolar range, at fixed drug-to-lipid ratio ([D]/[L]), the intensity of the absorption peaks associated with the monomeric, membrane-bound drug decreases linearly with [D]. Similar results are found for fixed [D]/[L] values ranging from 1/20 to 10^{-3}, from which we conclude that essentially all of the drug molecules can be considered to reside in the bilayer walls of the vesicles rather than in the aqueous medium. Accordingly, the only changes that need to be accounted for upon an increase in the concentrations of drug, relative to lipid, are structural reorganizations *within the membranes.*

In particular, we shall be concerned with the structural transformation whereby monomeric drug molecules, uniformly dispersed throughout the bilayer, aggregate into *pores.* These pores are transmembrane holes in which the water channels are protected from the lipid hydrocarbon chains by a "rim" of drug molecules which are lined up with their hydrophilic sides facing the aqueous medium.[6] In order for the system to organize itself in this way at sufficiently high drug-to-lipid concentrations, the energy of the drug molecules in a pore must be lower than that for monomers dispersed throughout the bilayer. We shall show, in fact, that it is this energy difference (lowering) which drives the aggregation/pore formation process.

We pursue this point by explicitly modeling the pore formation phenomenon in analogy with micellization in bulk surfactant solution. The analogues of monomers-in-water and of micelles are the bilayer-dispersed-drug molecules and the transmembrane pores, respectively. At low concentrations of drug in lipid, where entropy of mixing is dominant, the drug molecules are uniformly dispersed throughout the bilayer: each has an energy which is higher than what it would have if it were forming part of the lip of a pore. As the relative number of drug molecules is increased, a kind of cmc is reached, at which point monomers (*remaining within the bilayer*) aggregate into, i.e., "microphase separate" to form, "pore-type micelles": now they have given up their entropy of dispersion in favor of lower energy.

The statistical thermodynamic formulation of self-assembly (micellization) theory—applied to the situation described above—requires chemical/exchange equilibrium between the drug molecules in their dispersed (monomer) and aggregated (pore) states.[7] (Henceforth, "drug molecules" will be referred to simply as "surfactants", and "pores", as "micelles".) This

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⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1995.

0022-3654/95/2099-7694$09.00/0

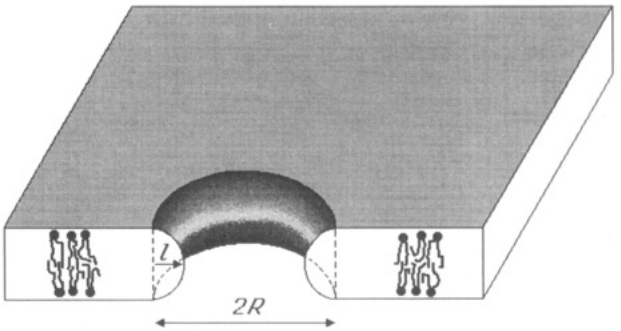

Figure 1. Schematic, geometric model of a transmembrane pore formed by drug molecules in a lipid bilayer. l is the length of a lipid (and drug) molecule, and $R - l$ is the radius of the aqueous region of the pore.

equilibrium situation can be described by the condition $\mu_1 = \mu_n/n$, where μ_1 is the chemical potential of a dispersed monomer and μ_n is that of a pore of size n, i.e., consisting of n surfactants. Because the ratio of drug to lipid ([D]/[L]) is small throughout the micellization region (see below and section III), μ_1 and μ_n can be taken to have their ideal solution form: $\mu_1 = \mu_{1,o} + k_BT \ln(X_1)$ and $\mu_n = \mu_{n,o} + k_BT \ln(X_n/n)$, where X_1 and X_n are the mole fractions of the surfactant (drug) in the bilayer and in n-sized micelles (pores), respectively. k_B is the Boltzmann constant, T is the absolute temperature, and $\mu_{1,o}$ and $\mu_{n,o}$ are the standard chemical potentials of monomer and aggregate.

Application of the equilibrium condition $\mu_1 = \mu_n/n$ leads immediately[7] to the micellar version of "the law of mass action":

$$X_n = n\{X_1 \exp[-((\mu_{n,o}/n) - \mu_{1,o})/k_BT]\}^n \qquad (1)$$

The driving force for micellization (pore formation) is seen directly to be the energy difference Δ_n between a surfactant in bilayer ($\mu_{1,o}$) and one in a pore ($\mu_{n,o}/n$). Mass conservation requires that the total mole fraction of surfactant, X, be given by $X = X_1 + \sum X_n$, and the cmc can be identified, for example, as the X for which $X_1 = \sum X_n$. It remains only to relate the energy difference Δ_n to physical properties of surfactant drugs in lipid bilayers.

Because the radius of a typical vesicle (liposome) is large compared to its bilayer thickness, the vesicle bilayer can be treated as a flat two-dimensional system for purposes of assigning energies to surfactant molecules in their monomeric ($e_1 = \mu_{1,o}$) and pore ($e_n = \mu_{n,o}/n$) forms. Suppose we model the pore by the "inside" half of a torus: then $e_n = \mu_{n,o}/n$ is the energy of a molecule in a half-toroidal rim, one of whose principal radii of curvature is simply the (fixed) molecular length (l) and the other of which (R) depends linearly on n (see Figure 1):

$$R = R(n) = \frac{\nu n + {}^4/_3\pi l^3}{\pi^2 l^2} \qquad (2)$$

(Here ν is the volume of a single molecule.) To evaluate e_n, and in particular to determine its dependence on n, we model the energy of the toroidal rim via a *one*-dimensional (1D) theory of curvature elasticity:

$$e_n = e^* + \zeta K\left(\frac{1}{R} - \frac{1}{R_o}\right)^2 \qquad (3)$$

ζ is the length per molecule in the rim of the pore (assumed to be independent of n: it is estimated by dividing the diameter of a "head group" by the number of molecules in a cross section of the half-toroidal rim), K is a (1D) bending constant—related by $K = kl$ to the usual (2D) curvature elastic energy k [8] and the molecular length l, and R_o is the *spontaneous* radius of curvature.[9] Note that e^* is the *optimum* energy per molecule, associated with the pore rim whose radius is $R = R_o$, and it is referenced to the zero of energy $e_1 = \mu_{1,o}$.

Using characteristic values for ζ, k, l, and R_o, the cmc can be evaluated as a function of the phenomenological parameter e^*: we simply substitute from (3) for $e_n = \mu_{n,o}/n$ in (1), using $R = R(n)$ from (2), and determine the value of X_1 for which $X_1 = \sum X_n = {}^1/_2$cmc. For example, for the particular choice of $\zeta = 1$ Å/molecule, $k = 10k_BT$, $l = 10$ Å, and $R_o = 30$ Å, we find cmc's of about 0.2, 0.02, 0.003, and 0.001 for $e^* = -2.0$, -4.0, -6.0, and -7.0 (in units of k_BT), respectively, indicating a strong (approximately exponential) dependence of pore-formation threshold on the monomer-to-pore transfer free energy. Note that, in our language of the preceding paragraph, and in the numerical estimates just provided, we have had in mind the situation of "normal" surfactants (e.g., anions of fatty acids), rather than AmB molecules, since we want to emphasize the analogy with familiar (3D, aqueous solution) micellization and are not concerned with quantitative details. As discussed in section III, experiments on the AmB/mixed lipids system suggest a critical drug-to-lipid ratio of 0.001, from which we conclude that e^* must be approximately $-7.0k_BT$ in this case.

It is interesting that, when pores *first* appear (at X = cmc), there is *on the average* approximately only one pore per vesicle: the typical liposome in the experimentally studied system contains about 16 000 lipids and (at threshold) only 0.001 times this many (i.e., 16) drug molecules. It is hard to imagine significantly *fewer* than 16 drug molecules being necessary to comprise a transmembrane pore of AmB.[4] Accordingly, *if* the cmc were as much as 10 times lower, or *if* the vesicles were 10 times larger, the average number of pores per liposome would be 10 times smaller; i.e., only about one in ten vesicles would have a pore! In these cases, then, one would expect some interesting (slow!) kinetics of pore formation, because the drug molecules "start out" uniformly dispersed throughout the bilayer of *all* of the vesicles, and at the critical concentration there are not enough of them in an average *single* liposome (only "1.6"!) to form a pore. Accordingly, the drug molecules from (on the order of) ten liposomes need to diffuse through the aqueous medium and "collect" in sufficient numbers in *one* vesicle. (Only if the lipids are organized in a single, infinite bilayer can micellization at *arbitrary* cmc's proceed without any drug molecules needing to leave the lamellar membrane.) Of course, there is always a nonnegligible fraction of vesicles whose size is significantly larger than the average and/or which contain more than the mean number of drug molecules. In any case, it appears, coincidentally, that in the AmB liposomal system which we study, we are poised quite near the limit of the cmc and vesicle size being consistent with the minimum-pore aggregation number.

B. Osmotic-Induced Tension. Now consider the case where an osmotic concentration gradient gives rise to a lateral tension in the vesicle membranes. For an osmotic pressure difference Π (energy per unit volume), the lateral tension σ (energy per unit area) is given by $2\sigma = \Pi r$, where r is the vesicle radius. Accordingly, we argue that the energy of a surfactant (drug) molecule dispersed as monomer in the membrane is increased from its value $e_1 = \mu_{1,o}$ by an amount σA, where A is the area per head group; the energy per molecule $e_n = \mu_{n,o}/n$ in the rim of a pore is assumed *not* to be affected by the lateral tension, since the tension is released throughout the bilayer upon pore formation (i.e., upon attendant equilibration of "inside" and "outside" concentrations). Now, "typical" lipid bilayers—

without any curvature-loving surfactant present—have been found to rupture when their lateral tension exceeds values on the order of $\sigma_{crit} = 40$ ergs/cm^2;[10] these tensions correspond, in turn, for vesicles with radii $r = 200$ Å, to concentration differences of 1600 mM ($\Delta c = \Pi/R_{gas}T$). For tensions of about half the limiting value σ_{crit}, then, the energy of a surfactant molecule (with head group area of 50 Å^2) is increased by roughly $(2-3)k_BT_{room}$, significantly increasing the energy lowering associated with pore formation *and thus decreasing (by roughly an order of magnitude) the effective cmc.*

The precise factor by which tension reduces the threshold value of drug-to-lipid ratio for pore formation will of course depend on the actual lateral tension operative in the liposomal bilayers, which in turn will be determined by the local concentration gradients and vesicle radii. We discuss these considerations in more detail, as well as the simplifying approximations on which the above prediction is based, in the following section where the relevant experimental studies are presented.

III. Experiments

A. Tension-Free Membranes. Here we summarize the fluorescence studies of membrane leakage, upon addition of AmB to lipid vesicles in the absence of tension in the bilayers.

Liposomes were prepared as follows. Lipid powders containing hydrogenated soy phosphatidylcholine (HSPC), cholesterol (Chol), distearoylphosphatidylglycerol (DSPG), and amphotericin B (AmB) were dissolved in chloroform to create solutions having compositions. HSPC:Chol:DSPG:AmB = 2:1:0.8:X with X varying so that the drug-to-lipid (plus cholesterol) ratio ranged from 10^{-6} to 10^{-2}. For each composition of interest, a thin homogeneous film is formed at the bottom of a glass tube, upon evaporation of solvent under a stream of nitrogen gas at 338 K followed by pumping under vacuum for at least 8 h. Preparation of the liposomes was accomplished by hydration of 250 mg of the lipid films with 2.5 mL of 25% sucrose/10 mM pH 5.5 (sodium) succinate buffer (SSB)/0.1 mM pyranine dye, and incubation of this mixture for 5 min at 338 K. The lipid suspension was then probe sonicated at the same temperature for 3 min until a transparent solution was obtained. After cooling to room temperature, the pyranine was removed from the "external" aqueous solution via passage through size-exclusion columns, and the liposomes were passed through 0.22-μm pore size filters. Concentrations of the lipids and AmB were determined by HPLC and the liposomes sized by inelastic light scattering with a Microtrac ultrafine particle analyzer.

In the first series of experiments, a 0.5-mL sample of the above liposome dispersion (i.e., unilamellar lipid vesicles each containing—*and* surrounded (minus dye) by—25% sucrose/pH 5.5 (sodium succinate) buffer/pyranine dye aqueous solution) was first diluted 10-fold with a 25% sucrose/pH 7.0 (sodium phosphate) buffer solution with matching osmolarity (to maintain zero tension in the membranes), and the pyranine excitation fluorescence spectrum measured as a function of time. The intensity peak of pyranine at 450-nm excitation wavelength has been shown to increase linearly with pH in the range 5.0–7.4.[11] $F(0)$ denotes the intensity of this peak measured in the above experiment at time $t = 0$, i.e., when the dispersion of liposomes with an "internal" pH of 5.5 is first diluted by the 7.0 buffer. As "leakage" occurs through the liposome membrane, the pH inside will increase and hence, correspondingly, the fluorescence intensity. After 5 min we record the intensity of the same peak in the pyranine fluorescence excitation spectrum, denoted $F(5)$. The fractional increase in fluorescence intensity

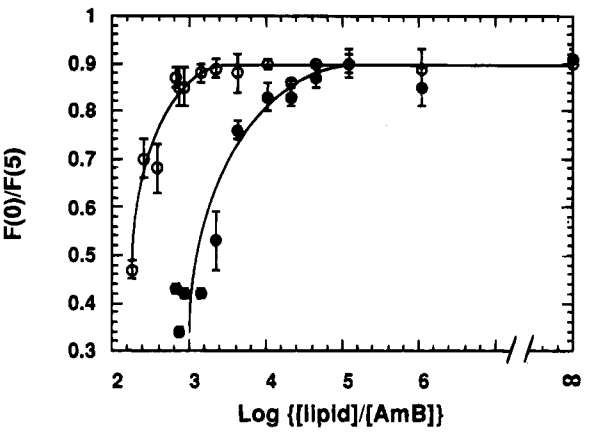

Figure 2. (Ordinate) An *inverse* measure of the liposome leakage rate—see discussion in text—plotted vs the log of the molar ratio of lipid to drug. Note the broken abscissa scale, after 7.0: the value of infinity corresponds to a total absence of drug molecules in the liposome. Open circles refer to the case of zero tension in the membranes, and filled ones, to a inside/outside osmolarity difference of approximately 700 mM, due primarily to a 25% sucrose solution inside.

$$\frac{\Delta F}{F(0)} = \frac{[F(5) - F(0)]}{F(0)} = \frac{1}{[F(0)/F(5)]} - 1 \qquad (4)$$

is then taken as a measure of the rate of leakage. As long as the drug-to-lipid ratio is low enough so that no pores form, we expect that the leakage rate will remain constant as this ratio (X) is increased. Above the threshold for pore formation, however (i.e., at a sufficiently high value of drug to lipid), the fractional rate of increase ($\Delta F/F(0)$) will begin to increase significantly.

Carrying out the fluorescence measurements described immediately above, for samples with successively larger values of lipid-to-drug ratio—ranging from 10^2 to 10^6—we find the plot of $F(0)/F(5) = 1/\{1 + (\Delta F/F(0)\}$ vs log {[lipid]/[drug]}, shown by the open circles in Figure 2. We see that the leakage rate (proportional to $\Delta F/F(0)$) is indeed essentially constant until a threshold value of the drug-to-lipid ratio of about 10^{-3} is reached; i.e., it begins to rise sharply only when log {[lipid]/[drug]} drops below 3. As discussed at length in the preceding section, we associate this threshold with the onset of pore formation and, hence, with a dramatic increase of the leakage rate and therefore of the internal pH (and concomitantly of the fractional increase over 5 min of the 450-nm excitation fluorescence intensity!).

B. Osmotically-Induced Tension. What happens to this phenomenon in the presence of osmotically-induced lateral tension in the vesicle membranes? To answer this question, we repeat the above series of measurements after first decreasing significantly the osmolarity of the pH 7.0 solution used to dilute each of the fixed [drug]/[lipid] liposomal dispersions. Specifically, we dilute with a sucrose-*free* pH 7.0 solution, so that the osmotic concentration difference (between aqueous media inside and outside the liposomes) is more than 700 mM, enough to give rise to a membrane tension of tens of dynes per centimeter in vesicles as large as 200 Å in radius. Our measurements of the fractional increase in fluorescence at a small [drug]/[lipid] ratio—see the three right-most *filled* circles in Figure 2, with abscissa values greater than 4—lying as they do on the same horizontal line, suggest that this tension is still small enough to leave the membranes intact when insufficient amounts of drug have been incorporated. More importantly, the sudden rise in $\Delta F/F(0)$ upon a decrease in [lipid]/[drug] is

seen to occur at values that are distinctly *smaller* (i.e., $<10^{-4}$) than in the tension-free case (10^{-3}), consistent with the theoretical predictions discussed in the preceding section (where we estimated a lowering of the cmc by roughly 1 order of magnitude). That is, *in the presence of lateral tension, the threshold for pore formation in the membranes, upon addition of drug, is significantly reduced.* (As in the case of the *zero*-tension experiments, we do not include data for [drug]/[lipid] ratios *far above* the threshold, because the leakage rates are so large there (due to the large number of pores) that a good measurement of $F(t=0)$ is no longer possible: the fluorescence intensity has already increased significantly by the time we get the sample in the spectrophotometer. This poses no difficulties for the present study, however, since our goal is only to establish the positions of the *thresholds*, in the absence and presence of membrane tension.)

Acknowledgment. W.M.G. acknowledges the National Science Foundation, through Grant CHE92-01166, for partial funding of this research. A.B.-S. would like to thank the Yeshaya Horowitz for their financial support of this work. Finally, C.L.W. is grateful to the University of California for sponsoring her postdoctoral research as a President's Fellow.

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JP942251Y