Bubble nucleation in lipid bilayers: A mechanism for low frequency ultrasound disruption

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Abstract

Recent experiments have shown that low frequency ultrasound (LFUS) induces leakage from lipid vesicles. However, the mechanism by which LFUS disrupts the lipid bilayer structure is not clear. In this paper we develop a theoretical model to test the possibility that gas molecule partitioning from the aqueous media into the lipid bilayer core can lead to the nucleation of microscale gas bubbles. If these can, indeed, form, then their presence in the lipid bilayer and interactions with an ultrasound field can cause bilayer disruption and leakage. The model derived here for the nucleation of stable bubbles accounts for the ‘surface tension’ that the lipid bilayer exerts on the bubble, a result of the associated disruption of the lipid packing. The model predicts that the probability of bubble nucleation is highly sensitive to the bilayer thickness, and largely insensitive to the bilayer phase. The probability of stable bubble formation is shown to correlate with experimentally measured sensitivity of lipid bilayers to LFUS, suggesting that membrane disruption may be due to embedded bubbles that nucleated in the bilayer.

1. Introduction

Lipid bilayers, or membranes, are a highly effective barrier to transport; in cells, exchange between the cell interior and the surroundings must be conducted through protein-lined channels [see, for example, 1], while release from synthetic liposomal carriers requires an environmental trigger to disrupt the bilayer [2,3].

One such trigger is ultrasound (US) [4]. The – largely irreversible – mechanism of membrane disruption under high frequency US was linked to heating effects [5–8]. In contrast, low frequency US (LFUS) has been found to increase the permeability of lipid membranes in a transient manner through non-thermal effects [9,10]. The rate of release was linked to US characteristics (frequency, intensity), as well as liposome size, number of lamellar layers in the lipid shell, and lipid bilayer properties (composition, phase) [11–13].

Analysis of LFUS-induced release from synthetic liposomes suggests that it is due to some diffusive process that is linked to changes in lipid packing in the bilayer [14,13]. Indeed, early studies have shown that US disrupts the packing of the hydrophobic lipid tails in the bilayer core [15] and create structural defects [16]. More directly, LFUS was found to induce transient structural deformations resulting in the formation of transient pores [9,17–19].

The mechanism by which US affects lipid packing is not, however, clear. It has been shown that US is hardly absorbed by the membrane below the solid ordered–liquid disordered transition temperature [20,21], thereby suggesting that the packing disruption may not be due, at least in such systems, to direct interactions between the lipids and US. One potential indirect mechanism is a gradient in the pressure field, induced by US: when the liposome dimensions are smaller than the US wavelength, the pressure field acting on the liposome is relatively uniform and no deformation occurs. In contrast, liposomes whose size is comparable to the US wavelength experience a pressure gradient that causes a shear force, which can disrupt the bilayer structure. However, due to the incompressible nature of the water inside and outside the liposome, it is not clear how such deformations affect permeation and transport.

Another mechanism that is frequently cited in connection with increased permeability in both cellular and liposomal lipid membranes is that of inertial cavitation [22–24]. In this process, gas-filled bubbles are destroyed by an oscillating US-induced pressure field [25], thereby damaging nearby assemblies such as the lipid bilayers. The bubbles may pre-exist or form in the medium when the US-induced pressure decreases below the liquid’s vapor pressure. The damage from inertial cavitation may cause irreversible damage or cause temporary poration of the membrane [26–28].

It is not clear that inertial cavitation in the bulk medium plays a significant role in vitro, where bubbles are not intentionally introduced and the pressure induced by LFUS is too low to induce formation of vapor pockets. However, the suggested formation of a gas compartment in the hydrophobic core of echogenic liposomes [29] has led Schroeder et al. [12] to propose a modified model for enhanced bilayer permeability: They hypothesize that the oscillating
US field nucleates gas bubbles in the hydrophobic region of the lipid bilayer. These grow, thereby causing pores to form. Pore lifetime, and thus transport, depends on the pore structure — hydrophobically lined or hydrophilically-lined. Most pores would be transient, allowing some encapsulant transport before healing, but some pores may grow to a critical size whereby they cause liposome destruction [4].

Indeed, the hypothesis that gas may preferentially partition into the lipid bilayer in concentrations that may nucleate into bubbles is supported by data for gas/bilayer interactions. For example, the partition coefficient for O₂ or NO gas between a membrane and water is of order 3, depending on the bilayer type and system conditions [30]. N₂ is at least 5 times more soluble in lipid bilayers than in water [30]. The partition coefficients for some (less common) gases such used for anesthesia, between water and lipid bilayers, can reach values of order 100–1000 [31]. Furthermore, simulations show that hydrophobic compounds sequestered in the lipid bilayer tend to concentrate in specific regions, in particular the midplane between the monolayers [32,33]. Therefore, even if the average concentration of gases solubilized in the bilayer is low, the concentration in regions such as the midplane may be sufficient for bubble nucleation.

In this paper we develop a theoretical model for the nucleation of gas bubbles in lipid bilayers, as a function of bilayer properties. The growing bubble induces a deformation in the packing of the surrounding lipids, which is associated with an energetic penalty that could be expressed as an effective surface tension. Thus, as in any nucleation and growth process, bubble formation will depend on the relative magnitude of the penalty due to surface tension (namely, lipid deformation) and the gain due to the gas association energy, expressed as a function of the degree of supersaturation [34].

2. Model derivation

The process of bubble nucleation and growth in a lipid bilayer is expected to follow classical nucleation and growth [34], a function of the clustering molecules’ properties and concentration (or supersaturation), and the surface energy which defines the interaction energy between cluster molecules and the medium. However, in lipid bilayers, the surface energy depends on the deformation of lipid packing induced by the growing cluster. In Section 2.1 we derive the surface energy of a spherical inclusion embedded in a lipid bilayer, as a function of the inclusion size and the lipid properties. This surface energy is then used, in Section 2.2, to calculate gas bubble nucleation and growth.

2.1. Surface tension acting on a spherical inclusion embedded in a lipid bilayer

The presence of an inclusion in a lipid bilayer or monolayer disrupts the local packing of the lipids due to an angular of thickness mismatch (see Fig. 1). The result is an energetic penalty that is composed of two contributions [35]: (i) a penalty due to the perturbation of the local packing density from the equilibrium preferred value, and (ii) a curvature energy penalty that arises from the development of curvature in the interface between the hydrophobic and hydrophilic regions.

Here we adapt our previous analysis for inclusion-induced membrane perturbation [35]: The lipid bilayer is taken to be locally flat and composed of two identical monolayers. The unperturbed thickness of the hydrophobic monolayer region, $h_0$, is coupled to an optimal surface density $\Sigma_0$ (area per molecule) through an equation of state. For an incompressible molecule this means that $h_0 \Sigma_0 = V$, where $V$ is the volume. Thus, a perturbation in local thickness is translated to the packing density, and vice versa. We define the local deformation therefore as $\Delta(z) = (h(z) - h_0)/h_0 - 1$, where $h(z)$ is the thickness of the perturbed monolayer at distance $z$ from the inclusion boundary (see Fig. 1). The perturbation free energy of the membrane, per unit length, is given by [35]

$$\gamma_M = \int_0^\infty \left[ B \Delta^2 + K h_0^2 \left( \frac{d^2 \Delta}{dz^2} \right)^2 \right] dz$$

(1)

where we assume that inclusions are widely dispersed so that their perturbation profiles do not overlap. $B$ is the monolayer compressibility (or area) modulus, which defines resistance to changes in the area per lipid, in units of energy per area [36]. $K$ is the mean bending modulus, which accounts for the penalty associated with curvature, in units of energy. (Here we use for energy units $kT$ where $k$ is the Boltzmann constant and $T$ temperature.)

To obtain the overall perturbation energy, $\gamma_M$ must be first minimized with respect to the perturbation profile $\Delta(z)$. We have shown that the optimal profile is not a simple exponential decay, as might have been expected, but an oscillating exponent whose properties depend on the magnitude of the perturbation, and the ratio of $K/B$ [35]. Once the optimal profile is obtained it is inserted back into Eq. (1) to yield the perturbation penalty per unit length. Finally, the overall penalty is obtained by multiplication of $\gamma_M$ by the circumference of the inclusion [35]. We have previously calculated the induced deformation energy for a symmetrical inclusion which imposes only a contact-angle perturbation (see Fig. 1) where the thickness is free to adjust so as to reduce the energetic penalty [35]. Expressed as a
‘surface tension’, or energy per unit surface area of the inclusion (in units of $kT$):

$$\sigma_m = \frac{2 l}{A} \approx \frac{2 l}{\sqrt{A h_0}}$$  \hspace{1cm} (2.a)

where $l$ is the circumference of the inclusion, $A$ is the contact area, and $\theta$ the contact angle between the inclusion and the bilayer (note that $\theta$ is the angular deviation from 90°, which is the preferred packing of lipids in a bilayer; $\theta = 0$ indicates no perturbation). For a spherical inclusion of radius $r$ that is completely submerged in the bilayer, $l = 2\pi r$ and $A = 4\pi r^2$. In the limit where $r < h_0$, $\theta$ is given by $r/(h_0 + r)$ and the surface tension

$$\sigma_m \approx \frac{\sqrt{2}}{h_0^{1/2}} K / \sqrt{B / \epsilon} \left( \frac{1}{1 + \epsilon/h_0} \right)^2 \left( \frac{r}{h_0} \right)$$  \hspace{1cm} (2.b)

so that in the limit where $r/h_0 \rightarrow 0$, namely, where the radius of the embedded sphere is much smaller than the bilayer thickness, the induced surface tension goes to zero.

2.2. Gas bubble nucleation and growth in a lipid bilayer

The process of nucleation and growth of a cluster from supersaturated medium is well understood. The free energy of any is set by a balance between the cluster molecules self-interactions – which favors cluster formation – and the penalty associated with the creation of an interface between the cluster and the medium [34]:

$$F = -\frac{4\pi r^3}{3\nu} \ln S + 4\pi \sigma r^2$$  \hspace{1cm} (3)

where $F$ is the cluster free energy (in units of $kT$), $r$ is the radius of the cluster, and $\nu$ the volume of a molecule (therefore, $4\pi r^3/3\nu$ denotes the number of molecules in the cluster). $\sigma$ is the surface energy (namely, the interaction energy between cluster molecules and the medium, per unit area, in units of $kT$). $S$, a measure of the supersaturation, defines the free energy gain due to cluster formation [34]:

$$\ln S = (\mu_m - \mu_c)$$  \hspace{1cm} (4)

where $\mu_c$ is the chemical potential of the molecule in the cluster, and $\mu_m$ the chemical potential in the medium (both in units of $kT$). Therefore, a positive value of $\ln S$ indicates that cluster formation is favorable (namely, $\mu_c < \mu_m$), and vice versa. The chemical potential of a gas in a bubble is set by the pressure in the bubble, given (in units of $kT$) by $\ln(P_0)$. Assuming one component environment (e.g. N$_2$), $P_0$ is equal to the gas pressure of the gas phase coexisting with the media, $P_g$. The chemical potential of the gas in the medium (assuming ideal solutions) is given by $\ln(x_g) = \ln(\alpha P_g)$, where $x_g$ is the concentration of gas dissolved, as a function of $\alpha$, the relevant partition coefficient. Thus, $\ln S = \ln(\alpha)$ [34].

The critical cluster size $r^*$ is the value that minimized the free energy; if the cluster size is smaller than $r^*$, the energetic penalty associated with the creation of the interface will dominate, and the cluster would dissolve. Above this value, the molecular interactions dominate, and the cluster would grow. $F(r^*)$ defines the ‘barrier energy’ or activation energy for cluster formation, and can therefore be used in an Arrhenius-type expression to calculate the rate of cluster nucleation [34].

3. Results

The properties of a critical gas cluster, and the probability that it will form in a lipid bilayer depend on two parameters: The degree of supersaturation $S$, and the lipid/cluster surface tension $\sigma$ (Eq. (3)). Using Eq. (2.b) for the surface tension acting on the nucleating bubble we minimize the free energy (Eq. (3)) to obtain the critical bubble size. In the limit where the bubble size is smaller than $h_0$, the monolayer thickness

$$r^* \approx \frac{3}{8} \left( 1 - \frac{\epsilon}{9\sqrt{2}} \right)$$  \hspace{1cm} (5.a)

where

$$\epsilon \equiv \frac{\ln S}{\sum_C \sum_i n_i \mu_i^2 / 2 \rho}$$  \hspace{1cm} (5.b)

represents the ratio between the chemical potential difference, which drives bubble formation, and the membrane effective perturbation energy, which opposes it. (In the derivation of Eq. (5) we used the fact that lipid molecules are largely incompressible, so $\nu \approx h_0 \Sigma_0$).

When $\epsilon$ is small, the driving force for bubble formation is weak, and a larger critical bubble is needed to overcome the surface tension penalty. As the driving force $\ln S$ increases, so does $\epsilon$, Therefore, the critical bubble size decreases. In fact, if the driving force is large enough, the critical bubble size may become zero or even negative, thereby indicating that in such a case any spontaneously-forming bubble would be stable.

The free energy of the critical bubble is

$$F(r^*) = \frac{9nB^{1/4}K^{1/4}h_0^{1/4}}{64\sqrt{2}} \left( 1 - \frac{\epsilon}{9\sqrt{2}} \right)^4$$  \hspace{1cm} (6.a)

and, therefore, the probability that such a bubble would spontaneously form is given by [34]

$$P(r^*) = \alpha \exp \left\{ -\frac{9nB^{1/4}K^{1/4}h_0^{1/4}}{64\sqrt{2}} \left( 1 - \frac{\epsilon}{9\sqrt{2}} \right)^4 \right\}$$  \hspace{1cm} (6.b)

where $\alpha$ is a rate constant that is expected to be independent of either bilayer parameters or gas concentration [34].

To test the model predictions, we need to evaluate the different parameters. We first consider the value of $\ln S$ that may apply here. As discussed in Section 2, $\ln S = \ln x$, where $\alpha$ is the partition coefficient between the gas phase and the media. In the case of lipid bilayers the gas first partitions into the aqueous solution and then into the lipid bilayer, so that, $\alpha = \alpha_{OG} \alpha_{GW}$ where $\alpha_{OG}$ is the partition coefficient between water and the surrounding gas, and $\alpha_{GW}$ is the partition coefficient between water and the lipid bilayer. The solubility of a gas such as N$_2$ in water, under atmospheric conditions and 25 °C is of order 0.15 and of O$_2$ it is of order 0.28 [37]. The partition coefficient of N$_2$ from water into lipid bilayers is of order 5, and for O$_2$ it is 2.5–3 [30,38]. Based on these numbers, $\ln S$ is of order (−0.5), too low to induce bubble nucleation (which requires that $\ln S$ be positive).

However, it has been shown that solutes are not uniformly distributed in the bilayer; the concentration of a hydrophobic molecule in the bilayer midplane may be twice that of the measured, average value [32]. In that region, then, $\ln S \approx 0.33$, a low value that, although favoring bubble nucleation, reflects the weak driving force for bubble formation in lipid bilayers.

Both membrane moduli, $B$ and $K$ have been found to depend on the bilayer thickness. Rawicz et al. [36] find that for saturated lipids, membrane moduli can be described by a polymer-chain model,
where $K = a_1 h_0^2/\Sigma_0$ and $B = a_2 h_0/\Sigma_0$ ($a_1, a_2$ are numerical constants). As a result, $\omega - \ln S/h_0$ and

$$P(r) = \omega \exp \left\{ - \frac{9\pi r h_0^2}{64\sqrt{2}\Sigma_0} \left( 1 - \frac{\ln S}{9\sqrt{2}a_0 h_0} \right)^4 \right\}$$

(7)

where $r = a_1^{1/4} a_2^{1/2}$ is a constant that depends on the particular lipid system properties.

In Fig. 2 we plot the predicted values for $r^*$ and the (reduced) probability of critical bubble formation, as a function of monolayer thickness $h_0$, based on Eqs. (6) and (7). All values used for the different parameters are based on experimental measurements for PC bilayers [36]. We see that, as may be expected, the critical bubble size increases linearly with monolayer (namely, 1/2 bilayer) thickness. As a result, the probability of a critical bubble forming in a membrane decreases exponentially with $h_0$.

Fig. 2 suggests that increasing the bilayer thickness (everything else being equal) would decrease the probability of stable bubble nucleation in bilayers; Therefore, if bubble nucleation is the mechanism by which LFUS disrupt bilayers, the sensitivity of membranes to LFUS should decrease exponentially with bilayer thickness.

We have recently measured the LFUS-induced leakage of encapsulants from lipid vesicles [13]. In Fig. 3 we plot the fraction of encapsulant released from vesicles, $f$, after the application of LFUS for a period of 180 s; in systems where bubbles are more likely to nucleate, we expect that the fraction of release would be higher, and vice versa.

Fig. 3(a) depicts the release from binary mixtures of DPPC and DOPC. Previous studies have shown that the composition of DPPC/DOPC bilayers does not affect their thickness [39]. Our model (Eq. (7)) therefore predicts that for these systems the probability of bubble formation, and fraction of release, should be insensitive to bilayer composition. This is based on the assumption that $\ln S$ (which is equal to $\ln(\alpha)$, as discussed in Section 2.2) is insensitive to the lipid composition: Since the partition coefficient $\alpha$ depends on the differences in chemistry between the media, differences between different lipid tails are weak and therefore not expected to affect $\ln S$.

Fig. 3(a) shows that, for the system studied, the fraction of release for these mixtures is indeed insensitive to the bilayer composition. This result is somewhat unexpected: DOPC/DPPC bilayers are known to transition from a liquid-disordered to a liquid-ordered phase through a coexistence region, as a function of composition [39]. Thus, it may have been expected that the rate of release would depend on the composition as well [Small 2011]. However, our model predicts that the dominant parameter is the bilayer thickness. Thus, for this system $h_0$ remains virtually constant, bubble formation -- and thus release under LFUS -- should be as well.

Cholesterol is known to affect the thickness of lipid bilayers; Alwarawrah et al. [40] found that the thickness of DOPC/cholesterol mixtures increases with the cholesterol mole fraction up to approximately 0.3, after which it decreases slightly. In Fig. 3(b) we plot the fraction released from DOPC/cholesterol bilayers (with less than 20% DPPC), as a function of the bilayer thickness [13]. As in Fig. 3(a), we assume that $\ln S$ is constant.

We also plot the predictions of Eq. (7) using $\beta = 24$, as measured by Rawicz et al. [36]. We see that there is good agreement between the measured release and the one predicted based on our model: The fraction of release decreases with increasing bilayer thickness, with a slope that agrees with the model predictions for the probability of stable bubble formation.

4. Discussion and conclusions

LFUS has been shown to induce transient pores in lipid bilayers [9,17–19]. Since US is hardly absorbed by the membrane below the solid ordered–liquid disordered transition temperature [20,21], lipid packing disruption is unlikely to arise from direct interactions between the lipids and US. However, the indirect mechanism causing bilayer disruption and (ultimately) pore formation has not been clearly identified to date.

In this paper we suggest that preferential partitioning of gas from the aqueous media into the lipid bilayer can lead to the formation of stable microbubbles. These would expand and contract under applied LFUS, leading to disruption in lipid organization and, therefore, to leakage. To test this hypothesis, we derived a model for the formation of stable bubbles (namely, bubbles larger than the critical size) in lipid bilayers. The model accounts for the ‘surface tension’ between the bubble and the lipid tail environment, which arises from local deformation of the lipid packing.

We find that the size of the critical bubble is sensitive to the degree of supersaturation and to membrane moduli: the unperturbed thickness $h_0$, the area compression modulus $B$ and the bending modulus $K$. However, as shown by Rawicz et al. [36], both $B$ and $K$ can be related to the bilayer thickness. Thus, the dominant parameters are the supersaturation and the bilayer thickness.

The critical bubble size is set by a balance between the degree of supersaturation and the membrane ‘stiffness’, or resistance to local
packing deformations. As a result, increasing the bilayer thickness – which increases the modulus – increases the critical bubble size in a nearly linear manner (see Fig. 2a). The probability of critical bubble formation is set by the free energy of the critical sized bubble, and therefore decreases sharply with increasing bilayer thickness (Fig. 2b).

To the best of our knowledge there are no direct investigations of bubble formation in lipid bilayers; however, we propose that – if bubbles nucleate in a given lipid bilayer – the sensitivity of that membrane to LFUS would depend on the probability of bubble formation: Bilayers that are more sensitive to US would release the encapsulant more rapidly than those that are more resistant to US. If the release is indeed associated with the presence of stable gas bubbles, the more sensitive membranes should be those that are more likely to develop stable bubbles, namely, whose thickness is lower.

In Fig. 3 we plot, the fraction of release from lipid vesicles that have been subjected to LFUS for a period of 180 s [13]. Membranes that are more sensitive to LFUS would show a higher value of f, and vice versa. We first examine mixtures of DOPC/DPPC, where previous publications show that the bilayer thickness is insensitive to composition [39]. As a result, our model would predict that the sensitivity of the vesicles to LFUS would be independent of composition, as indeed observed (see Fig. 3a). In contrast, cholesterol is well known to affect the packing – and thus thickness – of bilayers. The experimentally measured release from DOPC/cholesterol bilayers (with low amounts of DPPC) is in both qualitative and quantitative agreement with the prediction of the model, namely, that the release is sensitive to the thickness, a function of cholesterol content. (Note that the model–predicted probability of bubble formation was calculated with no free parameters).

Both DOPC/DPPC and DOPC/cholesterol mixtures display different phases, and phase coexistence, depending on the composition [39]. It may be expected that the correlation used here between K, B and h₀ would break down when the phase changes, or when there is phase coexistence. Yet, although this may explain some of the deviations found, the general trend predicted by Eq. (7) is consistent with the available data. Thus (for a given supersaturation), the dominant parameter determining the probability of bubble formation in lipid membranes is the bilayer thickness.

Determining the degree of supersaturation for a given system is complex; the solubility of gases in aqueous media under typical laboratory conditions is well known and understood [37]. However, less is known regarding the partition coefficient between water and lipid bilayers [30,31], and, in particular, the effect – if any – of bilayer properties on the partition coefficient. Furthermore, the distribution of solubilized small molecules in bilayers is non-uniform, so that their concentration in the midplane is higher than in other regions [32]. In our analysis here we used a value for lnS that is consistent with available data, but more detailed analysis is required. Furthermore, the correlation between bubble formation and LFUS-induced release presented here neglects other potential phenomena (such as membrane healing) which may affect the observed release rate. It should be noted that the model, which assumes that the release is induced by the interactions between bubbles and LFUS, is not valid for high frequency or high intensity US where heating effects are known to dominate [5–8].

In conclusion, we present a model for the nucleation of stable gas bubbles in lipid bilayers. The model predicts that the probability of bubble nucleation is highly sensitive to the bilayer thickness, and largely insensitive to the bilayer phase. The probability of stable bubble formation is shown to correlate with the sensitivity of lipid bilayers to LFUS, suggesting that membrane disruption may be due to the interactions of embedded bubbles with the US field.

Acknowledgments

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References
