PULSATORY LIPOSOMES – A POSSIBLE BIOTECHNOLOGICAL DEVICE FOR CONTROLLED DRUG DELIVERY. II. THE PORE APPEARANCE[#]

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Abstract. In this paper we analyse an important event in the life cycle of the pulsatory liposome: the membrane rupture for pore opening. An unilamellar lipid liposome filled with an aqueous solution of an impermeant solute was introduced into a hypotonic aqueous medium. Due to transbilayer osmotic imbalance, water flows inside the liposome and swells it up to a critical size, when the upper elasticity limit of the bilayer is reached. In this state the membrane suddenly ruptures and a transbilayer pore appears. A part of the intracellular material leaks out through this pore and the liposome membrane relaxes and, finally, recovers. The pore appearance in spherical bilayers is a more intricate process compared to plane lipid bilayers. Here we have studied the influence of membrane stretching on the pore appearance. We have also calculated the energetic barrier for a pore opening and closing. The stability of the liposome with a pore on it was analyzed.

Key words: Stretched vesicle, pulsatory vesicle, energetic barriers, pore opening, pore closing.

INTRODUCTION

The passage of small molecules or nucleic acids encoding genetic information through a cellular membrane is a clue problem in drug delivery. Transbilayer pore formation is a way to increase the permeability of lipid bilayers. Pores can appear due to structural and dynamic properties of lipid bilayers [4–6]. These pores are commonly named stochastic pores [4, 6]. Thickness fluctuations caused by thermal motion of lipid molecules superpose on local variations in bilayer thickness which already exist due to selective association of phospholipids,

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[3, 7] creating favourable conditions for pore appearance [9]. The fluctuations in polar head groups density on the bilayer surface following thermal motion produce local superficial defects. The neighbouring lipid molecules rotate their polar heads such as to cover the low density regions to avoid the contact between hydrophobic chains and water. There are a lot of theoretical papers regarding pore formation, especially in plane lipid bilayers [4–6, 9–14].

The theoretical approach of stochastic transbilayer pore formation in lipid vesicles is more difficult because of their shape fluctuations. Pores may be formed as a result of membrane expansion. In this case, membrane tension changes with vesicle expansion, which is not the case for black lipid membranes, where membrane tension is constant due to the Langmuir-Blodgett plateau, providing a reservoir of lipid molecules.

On the other hand, there are many experimental methods to increase membrane permeability by favouring pore appearance: a) raising the osmotic pressure inside a vesicle; b) electroporation due to a voltage drop across the bilayer; c) irradiation of a vesicle; d) temperature jumps; e) plating on porous or patterned surfaces [1, 2, 14].

In this paper we performed a theoretical study on pore formation across a vesicle membrane which is tensed by the osmotic pressure. We should not forget that opening of a pore is an important event in the duty cycle of a pulsatory liposome.

MATERIALS AND METHODS

At high pressure, the thermal undulations of lipid membrane can be ignored, and the membrane energy is due to elastic stretching.

Let us take into account a vesicle in the unstretched state. This is an equilibrium state, characterized by $\sigma = 0$.

According to Hooke's law, the membrane elastic energy is:

$$W = E\frac{(\Delta S)^2}{2S_0} \tag{1}$$

where E is the two-dimensional stretching modulus and $\Delta S = 4\pi\left(R^2 - R_0^2\right)$. R_0 is the radius of the vesicle in the equilibrium state.

If the vesicle bilayer is stretched beyond its elastic limit, a pore is nucleated.

The pore appearance will lower the expansion surface ΔS, and consequently will decrease the elastic membrane energy, but it will contribute an additional energy due to the monolayers bending such that the lipid molecules head groups to cover the edge of the pore.

We consider a pore of area A is formed in a membrane initially expanded by ΔS. The elastic energy of such a membrane with a pore is:

$$W\left(R,r\right)=E\frac{\left(\Delta S-A\right)^2}{2S_0}+2\sqrt{\pi A}\,\gamma=E\frac{\left[4\pi\left(R^2-R_0^2\right)-\pi r^2\right]^2}{8\pi R_0^2}+2\pi r\gamma \qquad (2)$$

where r is the pore radius and γ is the pore line tension.

The membrane surface tension is:

$$\sigma=\frac{\partial W}{\partial\left(\Delta S\right)} \qquad (3)$$

According to formula (3), and having in mind formula (1), the membrane tension for a stretched membrane without a pore is:

$$\sigma\left(R\right)=E\frac{R^2-R_0^2}{R_0^2} \qquad (4)$$

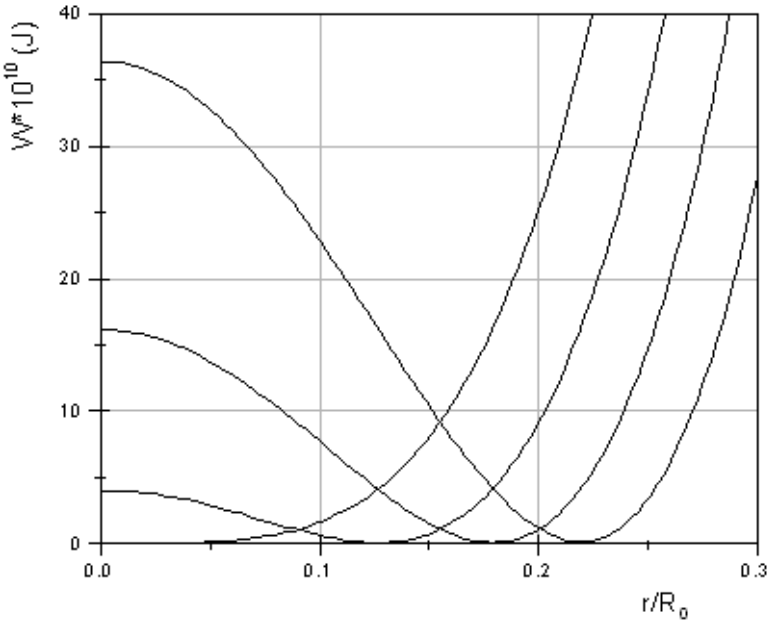

Fig. 1. The elastic energy of a vesicle of initial radius $R_0 = 20$ μm as a function of pore radius, for different stretching degrees. The curves correspond, from bottom to top (considering the part of each curve near the vertical axis), to: $R/R_0 = 1$; $R/R_0 = 1.002$; $R/R_0 = 1.004$; $R/R_0 = 1.006$. The two-dimensional stretching modulus was set to $E = 0.2$ N/m [1]. The linear tension of the pore edge was set to $\gamma = 8.1\times10^{-12}$ N [2]. One observes that the greater the membrane expansion, the deeper the minimum on the energy curve.

Similarly, using formula (3) and taking into account formula (2) for a stretched membrane with a pore, we can compute the membrane tension:

$$\sigma\left(R,r\right)=E\left[\frac{R^2-R_0^2}{R_0^2}-\frac{r^2}{4R_0^2}\right] \tag{5}$$

The result of the competition between the two terms of equation (2) determines two different behaviours of the vesicle which depend on the value of the membrane expansion, ΔS.

The dependence of the membrane energy for different values of ΔS is presented in Fig. 1.

Analysing Fig. 1, one observes that the increase of the membrane expansion determines the minimum of the energy curve to become deeper.

Depending on the size of expansion ΔS, we face two cases. If ΔS is:
-small, the membrane energy increases monotonically with the pore radius, and pore formation is unfavorable (curve 1);
-greater than a critical value, a minimum appears on the energy curve; this means the formation of a pore in membrane is possible (curves 2–4).

If a vesicle has the initial expansion ΔS when a pore is nucleated, the vesicle bilayer chooses its final state such as to minimize its energy.

From the condition of energy minimization as a function of pore radius given by relation (2), one finds:

$$\Delta S=\pi r^2+\frac{4\pi R_0^2\gamma}{E\,r} \tag{6}$$

In Fig. 2 we have plotted the dependence of ΔS on pore radius ($r > 0$), at the point where the vesicle energy reaches a minimum.

On this graph we can see that there is a critical value of ΔS, marked with $\Delta\overline{S}$, where the energy has the lowest value. This critical value of vesicle expansion, $\Delta\overline{S}$, corresponds to a transmembrane pore with the smallest radius, denoted by $\overline{r}$.

Starting from the function $\Delta S(r)$, we can easily find the critical values of both vesicle expansion and pore radius:

$$\overline{r}=\sqrt[3]{\frac{2\gamma R_0^2}{E}} \tag{7}$$

$$\Delta\overline{S}=3\pi\sqrt[3]{\left(\frac{2\gamma R_0^2}{E}\right)^2} \tag{8}$$

Between the lowest value of membrane expansion for which transmembrane pore formation is possible and the area of this pore there is a surprisingly simple relation:

$$\Delta\overline{S} = 3\,\overline{A} \tag{9}$$

or an equivalent relation between their radii:

$$\overline{r}^{\,2} = \frac{4}{3}\left(\overline{R}^{\,2} - R_0^2\right) \tag{10}$$

We must retain from relation (7) that the critical pore radius depends on the initial size, R_0, of the lipid vesicle.

In order for a pore to appear on a membrane stretched with ΔS, the membrane must overcome an energy barrier. We define the energy barrier for pore formation and denote it by W_{bo}, as the difference between the maximum energy W of the membrane bearing a pore and the membrane energy at point $r = 0$, when the pore is absent.

The energy barrier for pore opening is equal to:

$$W_{\mathrm{bo}}\left(r, R, R_0\right) = W\left(R, r\right) - W\left(R, 0\right) = \frac{E\pi}{8R_0^2}\left[r^4 - 8\,r^2\left(R^2 - R_0^2\right)\right] + 2\pi\gamma\, r \tag{11}$$

The initial energy barrier for pore formation corresponding to the membrane critical expansion, $\Delta\overline{S}$, is equal to:

$$\overline{W}_{\mathrm{bo}} = W\left(\overline{R}, \overline{r}\right) - W\left(\overline{R}, 0\right) = \frac{3\pi}{4}\sqrt[3]{\frac{2\gamma^4 R_0^2}{E}} \tag{12}$$

Also, we define the energy barrier for pore closure as the difference between the maximum and minimum values of energy function.

We denote the energy barrier for pore closure by ΔW_{bc}. It is equal to:

$$W_{\mathrm{bc}} = \max W\left(R, r > 0\right) - \min W\left(R, r > 0\right) \tag{13}$$

Thus, in order to determine the energy barrier for pore closure, we must calculate the minimum and maximum values of the energy function $W(R,r)$. In other words we must solve the equation:

$$r^3 - a\;\; r + b = 0 \tag{14}$$

The right member of equation (14) is the first derivative of $W(R,r)$ as a function of pore radius, r.

The coefficients a and b are:

$$a = 4\left(f^2 - 1\right)R_0^2 = \frac{\left(f^2 - 1\right)S_0}{\pi} = \frac{\Delta S}{\pi} \tag{15}$$

$$b = \frac{4R_0^2}{E} \tag{16}$$

We have denoted the surface expansion coefficient by f. Thus:

$$f = \frac{S}{S_0} = \frac{R^2}{R_0^2} \tag{17}$$

We can easily see that if the following condition:

$$a > \frac{3}{2}\sqrt[3]{2b^2} \tag{18}$$

is accomplished, equation (14) has three real solutions. We denote by r_1 and r_2 the positive solutions of equation (14) for which the energy function has a maximum and a minimum value, respectively.

The two solutions accomplish the conditions:

$$0 < r_1 < \sqrt[3]{\frac{b}{2}} \tag{19}$$

$$r_2 > \sqrt[3]{\frac{b}{2}} \tag{20}$$

With these notations, the energy barrier for pore closure is:

$$\Delta W_{\mathrm{bc}} = W\left(r_1\right) - W\left(r_2\right) \tag{21}$$

After extensive and difficult calculations one obtains:

$$\Delta W_{\mathrm{bc}} = \frac{\sqrt{3}\pi}{6}\frac{a^2 E}{R_0^2}\cos\left(\frac{\pi}{6} + \frac{\alpha}{3}\right)\left[\frac{3}{2}\frac{b}{a}\sqrt{\frac{3}{a}} - \cos\left(\frac{\pi}{3} - \frac{\alpha}{3}\right)\right] \tag{22}$$

where:

$$\cos\alpha = -\frac{3}{2}\frac{b}{a}\sqrt{\frac{3}{a}} \tag{23}$$

RESULTS AND DISCUSSION

The theoretical results obtained above were applied for a unilamellar vesicle having a radius $R_0 = 20$ μm. Its elastic properties are described by two-dimensional stretching modulus $E = 0.2$ N/m [1]. The linear tension of the pore edge is $\gamma = 8.1\times10^{-12}$ N [2].

The energy of a vesicle membrane initially expanded by ΔS with a pore of radius r opened on it is given by equation (2). The pore formation decreases the energy corresponding to the elastic membrane tension, as described by the first term, but increases the energy due to linear tension of the pore edge described by the second term.

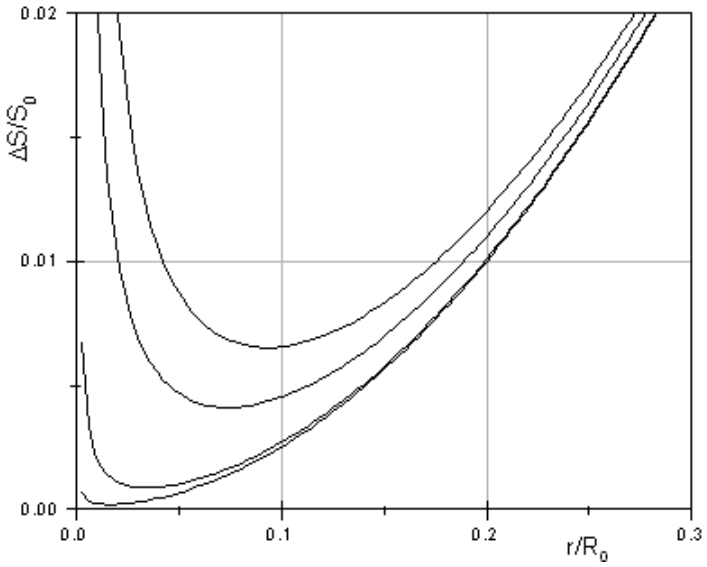

Fig. 2. The vesicle expansion ΔS for which the energy for the opening of a pore of radius r is minimized, as a function of pore radius. The curves have been plotted, from top to bottom, for vesicles with initial radius: $R_0 = 0.1$ μm; $R_0 = 0.2$ μm; $R_0 = 2$ μm; $R_0 = 20$ μm. The two-dimensional stretching modulus was set to $E = 0.2$ N/m [1]. The linear tension of the pore edge was set to $\gamma = 8.1\times10^{-12}$ N [2].

In Fig. 2 we can see that, for a vesicle of initial radius R_0, the appearance of a pore is possible only if the vesicle expansion is beyond a critical value, $\Delta\bar{S}$.

If $\Delta S > \Delta\bar{S}$, the pore radius may have values between a minimum value and a maximum value. This may be easily seen on each of graphs of Fig. 2.

If $\Delta S = \Delta\bar{S}$, the pore radius has a single value. This was named as a critical value. The critical values, $\bar{r}$ and $\Delta\bar{S}$, are given by formulae (7) and (8), respectively.

For the four vesicles used for drawing the curves in Fig. 2, the critical values are given in Table 1.

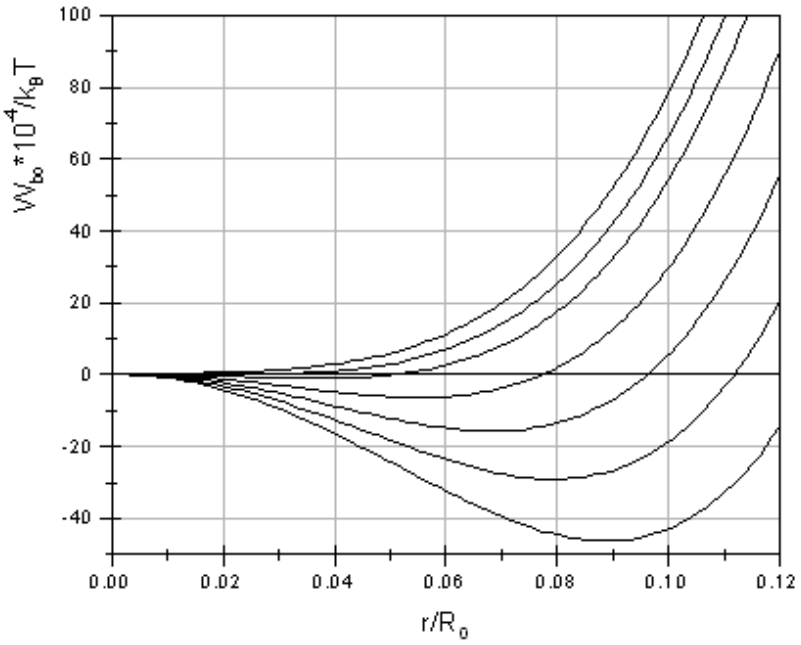

Fig. 3. The energy $W_{\mathrm{bo}}(r,R,R_0)$ necessary to open a pore of radius r in a lipid vesicle of radius $R_0 = 20$ μm, for different degrees of membrane stretching. The vesicle membrane stretching is described by the ratio R/R_0. The graphs, from top to bottom, were plotted for: $R/R_0 = 1$; $R/R_0 = 1.0001$; $R/R_0 = 1.0002$; $R/R_0 = 1.0004$; $R/R_0 = 1.0006$; $R/R_0 = 1.0008$; $R/R_0 = 1.0012$. The two-dimensional stretching modulus was set to $E = 0.2$ N/m [1]. The linear tension of the pore edge was set to $\gamma = 8.1\times10^{-12}$ N [2].

Let us consider a lipid vesicle in completely relaxed state ($\sigma_0 = 0$) of radius R_0, characterized by elasting stretching modulus E. The elastic energy gained by a membrane of initial surface $S_0 = 4\pi R_0^2$ following expansion with ΔS is given by formula (1). Let us suppose a pore of radius r is formed in the membrane initially expanded by ΔS. The energy of such a membrane with a pore is calculated using the formula (2).

Table 1

Critical values of the extended surface, $\Delta\overline{S}$, and of the pore radius, $\overline{r}$, for liposomes of various initial size

$R_0\times10^{-3}$ (nm)	$\Delta\overline{S}\times10^{-4}$ (nm^2)	$\overline{r}$ (nm)
20	63.3290762	317.480
2	2.939491	68.399
0.2	0.136440	14.736
0.1	0.054147	9.269

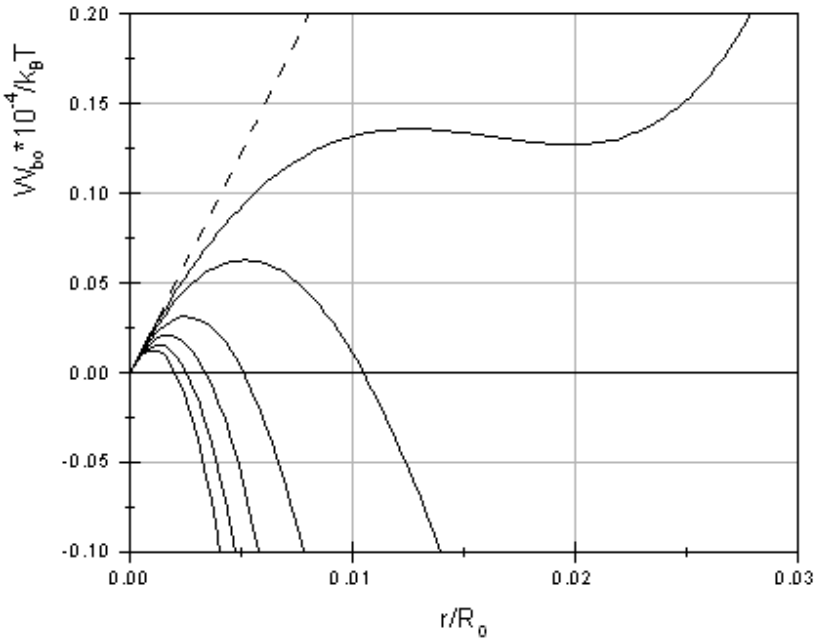

Fig. 4. A magnified view of the first part of the curves plotted in Fig. 3, in order to see the existence of a maximum value of the membrane energy change due to pore appearance.

The energy change of the membrane initially extended with ΔS due to the opening of a pore is given by formula (11).

One has to observe that the change in membrane energy due to appearance of a pore depends not only on the pore size, but also on both the initial size of untensed membrane (R_0) and the size of the tensed vesicle just before pore opening.

We have computed the change in membrane energy due to appearance of a pore for two liposomes of initial untensed size $R_0 = 2$ μm. Each liposome was supposed to exist in seven states of stretching characterized by ratio R/R_0 at the moment when the transbilayer pore appeared.

In Fig. 3 we have plotted the dependence of membrane energy change of a lipid vesicle of initial radius $R_0 = 20$ μm on pore radius for all the seven stretched states. For this lipid vesicle ($R_0 = 20$ μm), the membrane energy change has a minimum value for all stretched states, beginning with the stretched state characterized by $R/R_0 = 1.0004$. This is a very important property, because such vesicles with one pore on them reach a final stable state.

In Fig. 4 we have plotted a magnified view of the beginning part ($r/R_0 < 0.03$) of the curves in Fig. 3. On this graph we can notice that the stretched membrane energy change due to a pore opening also has a maximum value if $R/R_0 \geq 1.0001$. This means that, for pore opening, the system must overcome an energy barrier even if the membrane is in a tensed state.

Therefore, we can define an energy barrier for pore opening, as well as an energy barrier for pore closure. The values of these energy barriers were computed and were written in Table 2.

Table 2

The energy barriers for opening and closure of a transbilayer pore formed on vesicles of radius $R_0 = 20$ μm for different degrees of surface stretching expressed in $k_B T$ units

R/R_0	1.0001	1.0002	1.0004	1.0006	1.0008	1.0012
ΔE_{max}	1357.639	631.944	291.667	243.069	153.935	125.472
ΔE_{op}	1357.639	631.944	291.667	243.069	153.935	125.472
$\Delta E_{min} \times 10^{-4}$	0.1273	–0.9884	–6.3900	–15.7789	–29.1149	–46.4061
$\Delta E_{cls} \times 10^{-4}$	0.0085	1.0516	6.4192	15.8032	29.1303	46.4186

It is very interesting that a vesicle bearing a pore is more stable as it is more stretched. This property is very useful for biotechnology applications based on pulsatory liposomes, because the internal fluid must be leaked out in the external environment.

CONCLUSIONS

The appearance of a pore in spherical vesicles is different from the appearance of a pore in plane lipid bilayers. A lipid vesicle bearing an open pore may be stable. The pore may have a radius different from zero, but less than the maximum radius which the pore can reach for that vesicle.

We must emphasize that the existence of a stable pore depends on the initial size of the vesicle. Also, the existence of a stable pore depends on the membrane stretching at the moment of pore opening. In our case, for a vesicle with a radius of 20 μm, it is posible to have a stable pore (and an energy barrier for closure) if the membrane is stretched, but for a vesicle of 0.2 μm in radius the appearance of a stable pore is impossible.

The possibility of existence of a stable pore in the vesicle membrane is very important for applications of liposomes in biotechnology as vehicles for targeted and controlled delivery of special substances (drugs, genetic material, etc.) [8, 11, 12–16].

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