Formation and Mechanical Stability of Phospholipid Vesicles

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Lamellar structure of phospholipid vesicles, obtained by short time ultrasonic radiation, was studied by using negative stain electron microscopy. It is expected that the number of lamellae are randomly distributed over a possible region which is determined by size of the fragments torn by the radiation. However, a more restricted distribution in the number of lamellae was observed, e. g., there were no larger unilamellar vesicles and no vesicles with a larger number of lamellae. The result could be explained in terms of the interbilayer interaction and the bending elastic energy considering the high curvature effect of bilayers. This suggested that sonicated vesicles were formed in order to take a mechanically stable lamellar structure.

Key words : Electron microscopy, Phospholipid, Sonicated vesicle, Lamellar structure, Curvature elasticity, Interbilayer interaction

Introduction

Since phospholipid vesicles were first prepared by Bangham et al.¹⁾, the unilamellar and multilamellar vesicles have been used in biology and medicine as model systems of biological membranes and drug carriers. The method of vesicle preparation has also been improved²⁻⁶⁾. In this situation, it is worth to study the nature of the stability of phospholipid vesicles. Multilamellar structures containing vesicles are easily obtained by dispersing bilayer-forming lipids in water, but they are unsuitable for model membranes because of their heterogeneity in size and of structural complication. However, sonication tears these multilamellar structures into small pieces and the torn fragments close to form well defined vesicles. These vesicles have been widely used as a model membrane system. Small unilamellar vesicles (SUV) are formed by prolonged ultrasonic radiation and

gel filtration chromatography²⁾. They are very homogeneous in size³⁾. Short time sonication produces small unilamellar vesicles and also multilamellar vesicles with a wide distribution in size⁷⁾. The size distribution of multilamellar vesicles varies with time and power level of the sonication⁷⁾ because of varying size of the torn fragments. Although the size of the vesicle and the number of the lamellae are limited by the size of the torn fragment, a question arises whether the number of lamellae are distributed at random within the possible geometrical restriction or not.

We have previously demonstrated that the lamellar structure of vesicles can be seen by negative stain electron microscopy without considerable break of vesicles, where the stain penetrates through the vesicle by incubation above the phase transition temperature⁸⁾. In this work, using this stain method, we measured the size and the number of lamellae of sonicated vesicles of phosphati-

Fig. 1 Negative stain electron micrograph of DPPC unilamellar and multilamellar vesicles obtained by short time sonication.

The vesicles were stained by incubation with phospho-tungstate solution (2%, pH 6.8) at a temperature above the phase transition.

dylcholine. We also tried to explain the observed results in terms of the interbilayer interaction and the bending elastic energy of bilayers.

Materials and Methods

DL- α - dipalmitoylphosphatidylcholine (DPPC), which was more than 99% pure, was purchased from Sigma Chemical Co. and used without further purification. The negative stain used was potassium phosphotungstic acid (Nissin EM Co.). The dry lipid was dispersed in distilled water without any salt at a concentration of 30 mg/ml. The dispersion was incubated for about 1 hour at a temperature (about 60°C) well above the phase transition temperature (42°C). The dispersion was sonicated at about 50°C with a tip-type ultrasonic apparatus (Nippon Seiki, US-150). The sonication time was varied at same power level of the sonicator. In order to obtain penetrated vesicles with the negative stain, the vesicle solution was mixed at about 60°C with an equivalent volume of the 2% stain solution adjusted to pH 6.8 and the mixture was incubated at 60°C for 1 hour. A drop of the mixture was applied onto a collodion membrane-coated grid and at once excess solution was drain-

ed off with a piece of filter paper. These procedures were carried out at room temperature. After drying, the grid was examined in an electron microscope (HITACHI, HU-12 A), operated at 95 KV.

In estimating the vesicle size from the micrographs, the observed sizes were multiplied by a factor of $1/\sqrt{2}$ because of a collapse effect of spherical vesicles during the drying procedure⁹⁾. For slightly deformed vesicles, the average values were adopted to vesicle size. The number of lamellae was measured by use of a magnifying glass.

Results and Discussion

Vesicle size and the number of lamellae

When the lipid dispersion was sonicated for a prolonged time (above 3 minutes), the dispersion became translucent indicating that almost all the vesicles were small unilamellar vesicles. Although the dispersion was stained and left in the gel phase at the application onto the grid, the electron micrograph showed that most of the vesicles were small unilamellar vesicles. This indicates that redistribution of vesicles induced by aggregation and staining is negligible. It may be considered that the electron microscopic observation reflects the vesicle distribution in the dispersion without the stain.

Fig. 1 shows a typical micrograph of sonicated vesicles with a short time period (one minute). Structures of unilamellar and also multilamellar vesicles can be seen without considerable break. This is in contrast to the observation of large multilamellar structures formed by aggregation and fusion in the case of staining at temperatures in the gel phase⁹⁾. The size of unilamellar vesicles was estimated to range from 13 nm to 34 nm in diameter (the average value of 19 nm) which is consistent with reported values¹⁰⁾. The bilayer repeat periods of the multilamellae were measured as about 6 nm in average which is also in good agreement with the X-ray data¹¹⁾.

In Fig. 2, numbers of lamellae are plotted as a

Fig. 2 Plots of the number of lamellae against the estimated outmost radius for vesicles observed in electron micrographs (open circles). Area with open circles bounded by solid lines indicates the possible region of the number of lamellae determined geometrically.

function of the outmost radius for the sonicated vesicles with the same time period. Possible limits of the number of lamellae are also shown as a region bounded by solid lines. The limiting numbers of lamellae were determined geometrically as follows. Here, we refer to the midsectional area of a bilayer spherical shell defined as the area of the center interface of the bilayer membrane. Then the total midsectional area of bilayers for a vesicle with n lamellae is written as

$$S = 4\pi \sum_{j=1}^n \{r + d(j-1)\}^2 \quad (1)$$

where r is the center radius of the inmost bilayer and d the bilayer repeat period. The outmost radius of the vesicle R_0 is related to r as $R_0 = (n-1)d + (d_1/2) + r$, where d_1 is the thickness of the bilayer. The limiting numbers of lamellae were obtained as a function of the outmost radius by calculating Eq. (1) for various values of the total midsectional area, S . In this calculation, values of $d_1 = 3.5$ nm and $d = 6.0$ nm were used. It is seen

that the observed numbers of lamellae are distributed to a lesser extent in comparison with the limiting numbers.

It is clearer that no larger unilamellar vesicles are seen. The restricted distribution in the number of lamellae suggests that vesicles are formed in favor of an energetically stable state in the lamellar structure within the limits. It should be noted that the stable state does not mean a thermodynamical equilibrium state but a quasi-stable one because of occurrence of vesicle aggregation.

Elastic energy and interbilayer interaction

In order to look for the stable lamellar structure of vesicles, we consider elasticity of the lamellar membranes and the interbilayer interaction. When torn fragments of lipid close to form vesicles, the accompanying curvature of the lamellae requires the bending elastic energy because membranes are forced to deform from their own preferable curvature. The lamellae of the vesicle are considered to be stabilized by the interbilayer interaction energy exceeding the elastic energy. Since vesicles were formed at temperatures above the phase transition, each bilayer membrane is thought to be a fluid membrane at the formation. Therefore the energy of the shear deformation can be neglected. A stretch of bilayers is also disregarded. The bending elastic energy of a vesicle with n lamellae is expressed by the sum of each bilayer contribution as

$$U_{el} = \sum_{j=1}^n g_j S_j \quad (2)$$

where S_j is the midsectional area of the j bilayer and g_j the bending elastic energy per unit area. Assuming the concentric structure of the lamellae, g_j is written for the j -th bilayer with radius of r_j as

$$g_j = \frac{1}{2} \kappa \left(\frac{2}{r_j} - c_0 \right)^2 \quad (3)$$

where κ is the bending elastic modulus of the bilayer membrane and c_0 is the spontaneous curva-

Fig. 3 Calculation of $U/(8\pi\kappa S)$ without high curvature effect ($c_0 = 0$) as a function of the number of lamellae for various values of $A/(8\pi\kappa) = 1 \times 10^{13} \text{ m}^{-2}$ (1), $2 \times 10^{13} \text{ m}^{-2}$ (2) and $3 \times 10^{13} \text{ m}^{-2}$ (3) with different values of (a) $S = 0.04 \mu\text{m}^2$ and (b) $S = 0.4 \mu\text{m}^2$. In the calculation, a value of $d = 6 \text{ nm}$ was used.

ture which allows for an asymmetry of the membrane¹²⁾. The total bending elastic energy of the vesicle is given as

$$U_{el} = 2\pi\kappa \sum_{j=1}^n (2 - c_0 r_j)^2 \quad (4)$$

When all of the lamellae are symmetric membranes, i. e., $c_0 = 0$, the total energy is reduced to $8\pi\kappa n$, which is independent of the vesicle size.

The interbilayer interaction energy of a multilamellar vesicle can be written as

$$U_{int} = -AS' \quad (5)$$

where A is the attractive interaction energy per unit area and S' the total midsectional area of the interbilayers. S' is given by

$$S' = 4\pi \sum_{j=1}^{n-1} \left\{ r + d \left(j - \frac{1}{2} \right) \right\}^2 \quad (6)$$

Using the total midsectional area of bilayers S , the interbilayer interaction energy is rewritten as

$$U_{int} = -A \left[2\pi r \{ r + d(n-1) \} - S \left(1 - \frac{3}{2n} \right) \right] \quad (7)$$

It is noted the interbilayer interaction energy is zero for unilamellar vesicles.

Since electrostatic forces are disregarded for the neutral membranes as used here, the interbilayer interaction is considered to arise from van der Waals interaction as an attractive force¹³⁾. Values of the Hamaker constant were reported to be in a range of $2.3\text{--}8.2 \times 10^{-21} \text{ J}$ for phospholipid/water systems^{14~17)}. Using X ray data of $d_1 = 3.5 \text{ nm}$ and the thickness of the water layer between two bilayers d_w of 2.5 nm for DPPC/water system above the phase transition¹¹⁾, the van der Waals interaction energy per unit area is $0.7\text{--}2.4 \times 10^{-5} \text{ J/m}^2$. Reported values of the bending elastic modulus κ are widely ranged from $0.4 \times 10^{-19} \text{ J}$ to $2 \times 10^{-19} \text{ J}$ for bilayers of phosphatidylcholine^{18~23)}. Using the above values of the van der Waals interaction energy and the bending elastic modulus κ , the ratio $A/(8\pi\kappa)$ ranges from $1 \times 10^{12} \text{ m}^{-2}$ to $3 \times 10^{13} \text{ m}^{-2}$.

Next we have calculated the total energy of the vesicle, $U = U_{el} + U_{int}$ for various values of the ratio $A/(8\pi\kappa)$ and of the total area of bilayers S . Fig. 3 shows the examples of the calculation of $U/(8\pi\kappa S)$ as a function of the number of lamellae for the case of symmetrical bilayers, i. e., $c_0 = 0$. It demonstrates that, for a smaller value of S (Fig. 3a), the total energy increases with an increase in the number of lamellae indicating that the bending elastic energy cannot be overcome by the interbilayer attractive interaction because of large curvatures for respective bilayers. This implies that only the unilamellar vesicle is stable, which is in disagreement with the observed result. For large S (Fig. 3b), the total energy decreases indicating that there is a possibility of the formation of stable multilamellar vesicles. However, the number of lamellae of the stable vesicles was limited to a small range even though a large value of $A/$

Fig. 4 Calculation of $U/(8\pi\kappa S)$ with high curvature effect as a function of the number of lamellae for various values of $A/(8\pi\kappa) = 1 \times 10^{13} \text{ m}^{-2}$ (1), $2 \times 10^{13} \text{ m}^{-2}$ (2) and $3 \times 10^{13} \text{ m}^{-2}$ (3) with different values of (a) $S = 0.04 \mu\text{m}^2$ and (b) $S = 0.4 \mu\text{m}^2$. These values are the same as the calculation shown in Fig. 3. The curvature effect was taken for bilayers of radii below 50 nm.

$(8\pi\kappa)$ was used. This also disagrees with the observed result. The discrepancy is considered to arise from an over estimation of the bending elastic energy of the vesicle layers with small radii.

In a vesicle bilayer with large curvature, the ratio of the outside surface area S_0 to the inside one S_1 increases abruptly with increase in the curvature as expressed geometrically by

$$\frac{S_0}{S_1} = \left(\frac{2r + d_1}{2r - d_1} \right)^2 \quad (8)$$

For example, when $d_1 = 3.5 \text{ nm}$, the outside surface area is larger than the inside one by 15% for $r = 50 \text{ nm}$ and by 103% for $r = 10 \text{ nm}$. The difference in the surface area raises an asymmetry of the bilayer which leads that $c_0 \neq 0$. Furthermore, in the bilayer with a large curvature, the lateral area available per lipid molecule is not uniform from the surface to the center of the bilayer. This

disordered arrangement in the molecular packing¹⁰⁾ leads a smaller modulus of the bending elasticity than that of bilayers with a small curvature. In other words, the deformation with a large curvature is not only elastic but plastic. It is difficult to determine practically both values of the spontaneous curvature c_0 and the bending elastic modulus κ for such a large curvature bilayer. Previously, one of the present authors has analyzed the stability of small unilamellar vesicles (SUV) where the vesicle is stable when the bilayer has its own preferable radius of curvature due to the asymmetry of the bilayer. The bending elastic energy has been estimated to be reduced by a factor of a significantly small value of 2.5×10^{-3} ²⁴⁾. It is difficult to distinguish between the contribution of the spontaneous curvature c_0 to the elastic energy and that of κ . Then we calculated the total energy on the assumptions that the spontaneous curvature varies with the radius r as $c_0 = 1/2 r$ and that the bending modulus also varies as r/r_c for bilayers with smaller radii than an appropriate radius of r_c . Fig. 4. shows the examples of the calculated results for the case of $r_c = 50 \text{ nm}$. It demonstrates that there are more stable multilamellar vesicles than unilamellar vesicles even for the smaller value of S , which is in contrast to the results without the high curvature effect as shown in Fig. 3. Next we looked for a stable region of the number of lamellae from the calculation of $U/(8\pi\kappa S)$ for vesicles with the curvature effect using the parameters of $A/(8\pi\kappa) = 3 \times 10^{13} \text{ m}^{-2}$, $d_1 = 3.5 \text{ nm}$, $d = 6 \text{ nm}$ and $r_c = 50 \text{ nm}$. When we took the numbers of lamellae for which the $U/(8\pi\kappa S)$ values range from the minimum value up to $4 \mu\text{m}^{-2}$, as shown in Fig. 5, a region of the number of lamellae was obtained, which was consistent with the observed result. The value of $4 \mu\text{m}^{-2}$ in the depth of $U/(8\pi\kappa S)$ represents the energy of the bilayer membrane per unit area U/S is $2400k_B T \mu\text{m}^{-2}$ for $\kappa = 1 \times 10^{-19} \text{ J}$. For comparison, this figure also shows a region for vesicles without the curvature effect, which was obtained by use of the same parameters. It is

Fig. 5 Stable numbers of lamellae as a function of the outmost radius for vesicles with high curvature effect (the region bounded by solid lines) and without the curvature effect (the region bounded by dotted lines and uniamellar vesicles with radii below 50 nm).

These regions were determined by taking the numbers of lamellae where the $U/(8\pi\kappa S)$ values are ranged from the minimum value up to $4\mu\text{m}^{-2}$. The parameter used are $A/(8\pi\kappa) = 3 \times 10^{13} \text{ m}^{-2}$, $d_1 = 3.5 \text{ nm}$ and $d = 6 \text{ nm}$. For comparison, the observed result is replotted and the geometrically limiting numbers of lamellae are shown as broken lines.

clear that the region without the curvature effect is widely different from the observed result. The consistency between the region with curvature effect and the observed result implies that the bilayer with large curvature has small bending elastic energy due to an asymmetry of the bilayer.

In conclusion, the electron microscopic observation showed that the number of lamellae of sonicated vesicles were more restricted than the possible geometrical restriction. The result was well explained in terms of the interbilayer interaction and the bending elastic energy considering the high curvature effect of bilayers. This indicates that the vesicles are formed in favor of a mechanically stable lamellar structure.

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