Evaluation of the Hardness of Lipid Bilayer Membranes of Liposomes by the Ultrasound Attenuation Method

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Abstract: We attempted to evaluate the hardness of lipid bilayer membranes of liposomes by an ultrasound attenuation method and compared the results with those obtained by a representative fluorescence probe method using pyrene. We used multilamellar vesicles type of liposomes prepared by Bangham’s method. In the ultrasound attenuation method, the measurement of the ultrasonic attenuation constant of liposomes dispersions indicated that the hardness of the bilayer membranes of the liposomes increased with the increasing cholesterol concentration and the decreasing temperature. This tendency was identical with that found in the results obtained by the fluorescence probe method using pyrene. Therefore, we consider that the ultrasound attenuation method is useful for estimating the hardness of lipid bilayer membranes of multilamellar vesicles type of liposomes.

Key words: multilamellar vesicles type of liposome, hardness of lipid bilayer membrane, ultrasound attenuation method, ultrasonic attenuation constant, fluorescence probe method

1 Introduction

Liposomes are defined as structures composed of lipid bilayer membranes having an inner core of water phase. They contain water-insoluble compounds in the lipid bilayer membranes and water-soluble compounds in the aqueous inner core (1). Liposomes are widely used as a drug carrier, a model for biomembranes, etc., and have been studied pharmaceutically, biochemically, and physicochemically.

The mechanical properties of lipid bilayer membranes of liposomes are essential for their applications as a drug carrier (2). For example, the leakage of water-soluble compounds incorporated in the inner core of liposomes increases with the decrease in the hardness of the membranes (3). In general, the microfluidity, or the hardness, of bilayer membranes of liposomes can be evaluated by ESR spin probe (4) and fluorescence probe methods (5). These methods use chemicals, for example doxyl stearic acid as an ESR spin probe and pyrene as a fluorescence probe, which are incorporated in liposomes bilayer membranes.

We have investigated microbubbles coated with a surfactant monolayer shell as an ultrasound contrast agent (6). In a series of our studies on the microbubbles, we calculated the parameters of the mechanical properties of the shell of microbubbles, shell elasticity and shell friction parameters, using the data obtained in an
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ultrasound attenuation method (7). The ultrasound attenuation method can evaluate the hardness of the shell of the microbubbles through measurements of the ultrasonic attenuation constant of microbubble dispersions, and the method needs no chemicals in contrast to the ESR spin probe and the fluorescence probe methods. Thus, we expect that the ultrasound attenuation method will allow us to evaluate more readily the hardness of lipid bilayer membranes of liposomes.

In the present study, we prepared multilamellar vesicles type of liposomes using Bangham’s method and determined the hardness of liposomes bilayer membranes by the ultrasound attenuation method. In order to find the method useful for evaluating the hardness of liposomes bilayer membranes, moreover, we compared the results with these obtained by a representative fluorescence probe method using pyrene.

2 Materials and Methods

2.1 Materials

Dipalmitoylphosphatidylcholine (DPPC, 99.7 %) and cholesterol (99 %) were purchased from NOF Co., and SIGMA, respectively. They were used without further purification. Pyrene (98 %) was of Wako Pure Chemical Industries, Ltd., and was used after triple extraction with ethanol.

2.2 Preparation of Liposome Dispersions

We prepared multilamellar vesicles type of liposomes using Bangham’s method (5,8,9). Initially, DPPC and cholesterol were dissolved in chloroform in a test tube. The solvent was evaporated under N₂, and a thin lipid film was formed on the wall of the test tube. To remove the residual solvents completely, the test tube was allowed to stand in a desiccator under reduced pressure. Distilled water was then added into the test tube. The test tube was kept for 5 min at 60°C, and agitated on a mixer for a few minutes to swell the lipid film and to form aqueous liposomes dispersions. In order to obtain a more homogeneous size distribution of liposomes, the liposomes dispersion was extruded twice through a 10 µm of Millipore filter made of polycarbonate.

2.3 Measurement of the Ultrasonic Attenuation Constant of Liposomes Dispersions

Figure 1 shows the measuring system for the frequency characteristics of the ultrasonic attenuation constant in liposomes dispersions (7). Liposomes dispersions diluted with degassed water were poured into a cell with acoustic windows. Focused-type transducers (HARISONIC IS-0304-R, Staveley Sensors Inc., diameter: 0.25 inch) were used as a transmitting probe and a receiving probe, and the transmitted and received signals were processed with an ultrasonic analyzer (Model 5800, Panametrics). The frequency and the sound pressure of the ultrasound transmitted for measurement were 3.5 MHz and 10 kPa, respectively; the acoustic power of the ultrasound was less than 1 × 10⁻³ W. The resulting waveforms with and without liposomes dispersions were stored in a computer for further analysis. Using Fourier transform of these resulting waveforms, the frequency spectra of sound pressure were obtained. The frequency characteristics of the ultrasonic attenuation constant were then calculated comparing the frequency spectra of the sound pressure using the following equation (10):

\[ \alpha(f) = \frac{20}{d} \log \frac{p_w(f)}{p(f)} + a_s(f) \quad (1) \]

where \( \alpha(f) \) and \( p(f) \) correspond to the ultrasonic
attenuation constant and the sound pressure of frequency spectrum at a certain frequency, \( f \), respectively. Subscripts \( l \) and \( w \) represent the measurements in liposomes dispersion media and in degassed water media, respectively. Finally, \( d \) denotes the length of the cell between the acoustic windows.

### 2.4 Measurement of the Hardness of Lipid Bilayer Membranes Using a Fluorescence Probe Method with Pyrene

Liposomes dispersion containing \( 6.0 \times 10^{-3} \) mol/l of pyrene was prepared by the same method as described. The fluorescence spectra from pyrene in liposomes dispersions were measured with a fluorescence spectrophotometer (V-530, JASCO Co.). The excitation wavelength was 335 nm and the emission wavelength was in the range from 350 to 650 nm. Pyrene monomer generally forms excimer by face-to-face collision above a certain concentration of pyrene. Fluorescence peaks at 395 and 473 nm are assigned to pyrene monomer and its excimer, respectively. The excimer/monomer ratio of emission intensity (IE/IM) indicates the microfluidity in the surroundings of pyrene. The larger \( \text{IE}/\text{IM} \) of pyrene in lipid bilayer membranes, the higher the microfluidity, or the lower the hardness of the membranes (5,11).

### 3 Results and Discussion

**Figure 2** shows the experimentally obtained frequency characteristics of the ultrasonic attenuation constant of liposomes dispersion at 20 \( ^\circ \)C. The molar ratio of cholesterol against DPPC in the liposomes bilayer membranes was 3:7. The peak at the maximum attenuation constant (arrow in Fig. 2) is closely related to the hardness of liposomes bilayer membranes; an increment in the hardness of liposomes bilayer membranes causes the peak at the maximum attenuation constant to shift to higher frequency.

In general, when multilamellar vesicles type of liposomes is exposed to 25-60 W of ultrasound, the liposomes are transformed into unilamellar vesicles type of liposomes (12). As described in Materials and Methods, however, the acoustic power of the ultrasound transmitted in the ultrasound attenuation method was considerably weak (less than \( 1 \times 10^{-3} \) W). Thus, it was implied that the transmitted ultrasound could not influence or break the structures of the liposomes. In fact, we observed no change in the structures of the liposomes under a microscope after exposing the liposomes to the ultrasound in the method.

First, we determined changes in the hardness of liposomes bilayer membranes with addition of cholesterol using the ultrasound attenuation method. Cholesterol is well known as an important material that depresses the permeation of incorporated compounds through the membranes because it increases the hardness of the membranes (13). **Figure 3** shows changes in the frequency of the peak at the maximum attenuation constant with the concentration of cholesterol at 20 \( ^\circ \)C. Here, the horizontal line represents the molar ratio of cholesterol against DPPC. The results on the hardness evaluated by the fluorescence probe method using pyrene are also given at **Fig. 3**. The addition of cholesterol raised the frequency at the maximum attenuation constant and made the lipid bilayer membranes of liposomes harder. By comparison the ratio IE/IM decreased with the increasing concentration of cholesterol and the microfluidity of bilayer membranes diminished, which indicates that the hardness of the membranes rises with increasing the concentration of cholesterol.

The dependence of the hardness of lipid bilayer membranes of liposomes on the temperature was also evaluated by the ultrasound attenuation method. **Figure 4** shows the results on the hardness of lipid bilayer membranes obtained by the ultrasound attenuation method and by the fluorescence probe method using pyrene. The molar ratio of cholesterol against DPPC in
The frequency at the maximum attenuation constant became higher. The values of $I_E/I_M$ decreased by the decreasing temperature. The results obtained by both methods indicate that the hardness of liposome bilayer membranes is raised by the decreasing temperature, which is consistent with the temperature dependency of the properties of liposome bilayer membranes (9).

These findings described above confirm that the ultrasound attenuation method allows us to evaluate the hardness of lipid bilayer membranes of multilamellar vesicles type of liposomes as does the fluorescence probe method using pyrene. The method is based on the difference in the ultrasonic attenuation between liposomes dispersions and water media. In this study, the multilamellar vesicles type of liposomes were used as a sample, and the ultrasonic attenuation of the liposomes dispersions would be caused by the multilamellar membranes of the liposomes. As the lipid bilayer membranes of liposomes become thinner, or unilamellar, the ultrasonic attenuation in the liposomes dispersions is closer to that in water media because the volume fraction of inner water phase in the liposomes becomes larger, which results in no difference in the ultrasonic attenuation between the liposomes dispersion and water media. It is suggested, therefore, that it is difficult to measure the hardness of the membranes of unilamellar vesicles type of liposomes in the ultrasound attenuation method.

We can conclude that the ultrasound attenuation method is useful for evaluating the hardness of bilayer membranes of multilamellar vesicles type of liposomes. In ESR spin probe and fluorescence probe methods for measuring hardness of the liposomes bilayer membranes, chemicals are used as a probe and are incorporated in the membranes. On the other hand, the ultrasound attenuation method needs no chemicals to evaluate the hardness of the membranes. Therefore, we consider that the ultrasound attenuation method is more readily for evaluating the hardness of lipid bilayer membranes of natural liposomes without chemicals.

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References

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