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Effects of frequency and power of ultrasound on the size reduction of liposome

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ABSTRACT

The solutions of liposome made of L-α-dilauroyl phosphatidylcholine are sonicated at various powers and frequencies (43–480 kHz), and the resultant change in the size of liposome is measured by the dynamic light scattering method. The ultrasonic power dissipated into the solution is determined by the calorimetric method in order to compare the effects of ultrasound of different frequencies. The faster reduction of the mean size of liposome is achieved at the lower frequency. Comparing at the same frequency and total energy, short-time irradiation of strong ultrasound is more efficient than long-time irradiation of weak ultrasound. These results indicate that the small number of cavitation events with stronger physical disturbance on liposome can reduce the size of the liposome more efficiently than the large number of cavitation events with weaker disturbance.

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1. Introduction

Sonication is one of the popular methods to prepare liposome from the aqueous dispersion of phospholipids, which has been used from the beginning of the study of liposome (Huang, 1969). The unilamellar liposome whose diameter is smaller than 100 nm is obtained in a relatively easy way by sonication. There have been many studies on the size distribution and lamellarity of liposome after sonication, because they are important quantities for the applications of liposome (Zasadzinski, 1986; Maulucci et al., 2005; Pereira-Lachataignerais et al., 2006; Woodbury et al., 2006; Kim et al., 2007; Richardson et al., 2007). The reduction of the size of liposome by sonication is ascribed to the physical effects of acoustic cavitation, although there have been some debates on how the cavitation bubble breaks liposome (Finer et al., 1971; Lasic, 1988; Woodbury et al., 2006; Richardson et al., 2007).

Frequency and power are two of the important parameters of ultrasound. The information on how these parameters affect the size of liposome can open a way to control the size of liposome by tuning these parameters. Although there were some studies on the effect of ultrasonic power (Pereira-Lachataignerais et al., 2006; Richardson et al., 2007), however, no systematic study was performed on the effect of ultrasonic frequency so far as we know.

Frequency is a critical parameter that controls the behavior of cavitation. It has been revealed that the efficiency of sonochemical reaction is strongly dependent on frequency (Koda et al., 2003). We therefore expect that frequency also affects the formation process of liposome. In the preparation of liposome solution, however, one usually uses commercially available horn-type homogenizers or ultrasonic cleaning baths, which operate at several tens of kilohertz. In this work, we extend the frequency range from 45 to 480 kHz by utilizing the home-made ultrasonic reactor.

The evaluation of ultrasonic power is essential to study the effects of the power and frequency of ultrasound. In this work, we employ the calorimetric method to evaluate the ultrasonic power dissipated into solution. In the calorimetric method, the temperature rise of the solution during the sonication is monitored, and the thermal energy stored in solution is calculated. The ultrasonic power is then evaluated from the initial slope of the increase in temperature. Although the calorimetric method suffers from problems such as the heat production of transducer and the coupling between a thermocouple and viscous flow of the solution (Morris et al., 2008), it is a conventional way to measure the ultrasonic power in the field of sonochemistry (Contamine et al., 1995; Kimura et al., 1996) because it is easily applicable to various types of sonoreactors, and we apply it to the liposome solution for the first time to our best knowledge.

2. Experimental

2.1. Sample

The lipid we use in this work is L-α-dilauroyl phosphatidylcholine (DLPC, Nichiyu), which is used as received. We have chosen DLPC for the following two reasons. The first one is to avoid the oxidation of lipid molecules (Hauser, 1971). The transient radicals like •OH are formed by ultrasonic cavitation, which lead to various sonochemical reactions. Such a chemical effect of ultrasound is strong around 100–500 kHz (Koda et al., 2003), which is the fre-

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quency range we shall use in this work. Since the unsaturated parts of alkyl chain are subject to oxidation (Niki, 2001), we employed DLPC that has saturated alkyl chains. Second, the gel-liquid crystal phase transition temperature of the DLPC bilayer membrane is about 7 °C (Cherney et al., 2003), which is far lower than the temperature in the sample preparation and ultrasound irradiation. We can therefore consider that the lipid membrane is always in its fluid phase during experiment.

Sample solutions are prepared by the extrusion method, which is a popular method to prepare liposome of intermediate size (Szoka et al., 1980; Endruschat and Henschke, 2000). The powder of DLPC is first dispersed into distilled water, and the dispersion is mechanically stirred by a magnetic stirrer for several hours. The molar concentration of DLPC in the dispersion is 5.0×10^{-5} mol/dm^3. The dispersion is then extruded 10 times through the polycarbonate membrane filter of 400 nm poe size (Advantec) in order to improve the reproductivity of the initial size of the aggregate. The extrusion is performed by hand with a plastic syringe equipped with a membrane filter. The mean diameter of the initial aggregate is about 300 nm, which is determined by the dynamic light scattering (DLS) method. It is confirmed by liquid chromatography that the loss of DLPC during the extrusion and sonication processes is small.

2.2. Sonication

The frequencies of the ultrasound used in this experiment are 43, 133, and 480 kHz. Two different ultrasonic baths are made for this experiment.

Fig. 1(a) shows the ultrasonic bath used for 480 kHz experiment. The body of the bath made of brass is cylinder shape, and the ultrasonic transducer is equipped at the bottom of the cylinder. The inner diameter of the cylinder is 50 mm. The transducer (Honda Electronics) is made of PZT ceramics and its shape is the circular plate whose diameter and thickness are 30 and 5 mm, respectively. The transducer is pasted onto the stainless steel plate of 0.1 mm thickness, which is fixed to the body of the cell. The ultrasonic transducer is driven by the power amplifier (HSA4014, NF), whose signal is seeded by the synthesizer (FS-1131, TOA Electronics). The sample solution is held within the cylinder-shape cell made of quartz glass, whose inner diameter is 30 mm. The volume of the sample solution is 35 cm^3. The thickness of the bottom of the quartz cell is 0.5 mm. The quartz cell is fixed to the body of the bath through the cap at the top of the body. The distance between the top of the transducer and the bottom of the quartz cell is 30 mm. The space between the transducer and the quartz cell is filled with the distilled water. The ultrasound is transferred from the transducer to the sample solution indirectly through distilled water, which is degassed before use in order to avoid the creation of bubbles during experiment.

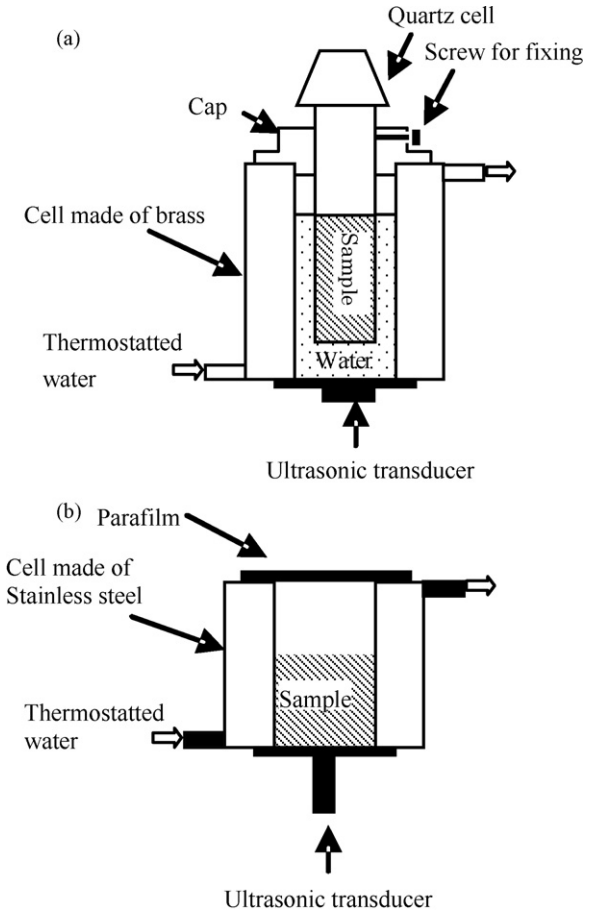

Fig. 1. The illustrations of the ultrasonic baths used for (a) high-frequency (480 kHz) and (b) low-frequency (43 and 133 kHz) experiments.

Fig. 1(b) illustrates the ultrasonic bath used for 43 and 133 kHz experiments. The shape of the bath is almost the same as that for the 480 kHz experiment. The inner diameter of the bath is 55 mm, slightly larger than that for the 480 kHz experiment. The largest difference is that the sample solution is directly in contact with the vibrating plate equipped with the ultrasonic transducer. The body of the bath is made of stainless steel in order to reduce the contamination of the sample due to the erosion of the body of the bath. The ultrasound is emitted from the Langevin transducer (Honda Electronics) at the bottom of the bath, which operates at both 43 and 133 kHz. The thickness of the vibrating plate is 2 mm. The power amplifier used to drive this transducer (1140LA-1331, ENI co.) is different from that used in the 480 kHz experiment, and the synthesizer is the same. The top of the bath is covered with parafilm during the experiment in order to minimize the loss of solution due to the evaporation and the ultrasonically induced atomization.

The temperature of the sample is controlled by circulating the water thermostated at 25 °C. The temperature rise after the sonication is lower than 10 °C.

In the 480 kHz experiments, the amplitude of the output current of the power amplifier is strongly dependent on the frequency. It can be attributed to the resonance due to the standing wave at the vibrating plate and the bottom of the quartz cell. Since the sound velocity of water decreases with increasing temperature, the resonance frequency changes during the sonication due to the fluctuation and drift of the temperature. The frequency of the synthesizer is always adjusted during the sonication so as to maximize the current amplitude displayed on the front panel of the power amplifier. The range of the frequency adjustment is 480 ± 10 kHz. Such frequency adjustment is not necessary in the 43 and 133 kHz experiments.

2.3. Determination of the ultrasonic power

The time dependence of the temperature of the solution under the irradiation of the strong ultrasound is determined by the balance between the power of ultrasound dissipated into the solution and the heat transfer from the solution to the surroundings. The evaluation of the latter is difficult in general. However, since the heat transfer is driven by the temperature gradient, its effect become negligible at the beginning of the sonication when the temperatures of the solution and surroundings are close to each other. Under such condition, the ultrasonic power, P_U, is given by

$$P_U = MC_p \frac{dT}{dt}, \quad (1)$$

where M and C_p stand for the mass and the isobaric heat capacity per unit mass, respectively, and dT/dt denotes the initial

slope of the time dependence of the temperature of the sample solution.

The calorimetric determination of the ultrasonic power is performed at the condition same as the irradiation of the ultrasound to the sample solution, except that the sample solution is replaced by distilled water. The temperature is measured by a *T*-type sheathed thermocouple. The output voltage of the thermocouple is detected by the data logger (NR-500NR TH08, Keyence), and stored in a computer for analysis. The error of the ultrasonic power is typically ±10%, which is determined by the repeated measurements.

2.4. Measurement of the size of liposome

The size and size distribution of liposome before and after the sonication are measured by DLS method. DLS is a popular way to analyze the size of colloid particles that probes the hydrodynamic diameter of the particle through the translational diffusion coefficient (Berne and Pecora, 1976; Okubo et al., 1996).

The quantity that is obtained in the DLS measurement is the time correlation function of the intensity (square of the electric field), which is mathematically converted into the time correlation function of the electric field, denoted as $g_1(t)$. The decay of $g_1(t)$ reflects the translational motion of the particles in solution.

When the colloid particle is spherical and its size distribution is monodisperse, the time dependence of $g_1(t)$ is given by

$$g_1(t) = \exp(-Dq^2 t), \tag{2}$$

where D and q are the translational diffusion coefficient and the scattering vector, respectively. The magnitude of the scattering vector, q, is defined as

$$q = \frac{4\pi n}{\lambda_0} \sin\left(\frac{\theta}{2}\right), \tag{3}$$

where n, λ_0, and θ stand for the refractive index of the solution, wavelength of the excitation light, and scattering angle, respectively. The translational diffusion coefficient, D, is related to the hydrodynamic diameter of the particle, d, through the Stokes–Einstein relationship as

$$D = \frac{k_B T}{3\pi\eta_0 d}, \tag{4}$$

where k_B, T, and η_0 denote the Boltzmann constant, absolute temperature, and shear viscosity of the solvent, respectively.

When the colloid particles in solution have a size distribution, Eq. (2) is extended as follows:

$$g_1(t) = \int dD f(D) \exp(-Dq^2 t). \tag{5}$$

It should be noted here that the distribution of the diffusion coefficient in Eq. (5), denoted as $f(D)$, is weighted by the scattering cross section of the particle. A simple and straight-forward calculation shows that the average of the translational diffusion coefficient, $\langle D\rangle$, is related to the time-derivative of $g_1(t)$ as

$$\langle D\rangle \equiv \frac{\int dD[Df(D)]}{\int dD(D)f} = \frac{d}{dt}[\ln g_1(t)]\bigg|_{t=0}, \tag{6}$$

which can be converted into the mean diameter, $\langle d\rangle$, by Eq. (4) as

$$\langle D\rangle = \frac{k_B T}{3\pi\eta_0\langle d\rangle}, \tag{7}$$

Commercially available DLS equipment (DLS-7300NI, Otsuka electronics) is used in this work. The excitation light is 488.0 nm line of the argon ion laser, and the right-angle ($\theta = \pi/2$) scattering is collected. The size distribution of liposome is analyzed by the Marquardt method, which is implemented in the standard software provided by the manufacturer. In principle, the mean diameter can be obtained by the simple algebraic average of the size distribution. However, it does not work due to the large particle that is ascribed to the stainless steal powder, as will be shown in the next section. We therefore employ the mean diameter from Eq. (7). Since $\langle d\rangle$ defined in Eq. (7) is the harmonic mean of the diameter, it is little affected by the existence of the small fraction of large particles. It is experimentally confirmed that the size distributions before and after sonication do not change for several days, and we do not take special care to measure the size distribution after sonication as soon as possible.

3. Results and discussions

Fig. 2 shows $g_1(t)$ of the sample after various sonication times. The frequency and power of the ultrasound are 480 kHz and 8 W, respectively. The correlation function before sonication is also plotted for comparison. As is clearly seen, the decay of $g_1(t)$ becomes faster with increasing sonication time, which indicates the decrease in the size of aggregates. In addition, the curvature of $\ln g_1(t)$ also increases with the sonication time, suggesting that the size distribution becomes wider.

The correlation functions in Fig. 2 are converted into the size distribution in Fig. 3(a). The size of the aggregate before sonication shows unimodal distribution, whose center is about 300 nm. We can consider that the multilamellar liposome is formed by the extrusion process (Endruschat and Henschke, 2000). The distribution at the smaller size (~100 nm), which is considered to be unilamellar liposome, increases with the sonication time, and the bimodal distribution is clearly observed after the sonication for 3 h. Woodbury et al. (2006) reported that the size distribution of liposome during mild sonication become bimodal, whereas unimodal distribution is observed after strong sonication.

The size distributions after the sonication at the same frequency and increased power (18 W) are exhibited in Fig. 3(b). The decrease in the size of liposome becomes faster by increasing the power of ultrasound as is expected. The bimodal distribution is observed at the intermediate sonication time (1 h), and the unimodal distribution around 70 nm is obtained after 3 h.

Fig. 4 exhibits the size distribution after the sonication of 43 kHz and 8 W. It should first be noted that the sonication times shown in Fig. 4 are several tens of minutes, whereas those of 480 kHz are several hours. Since the small liposome of about 50 nm diameter is formed within 1 h, the effect of the 43 kHz ultrasound on the size-modification is larger than that of the 480 kHz one.

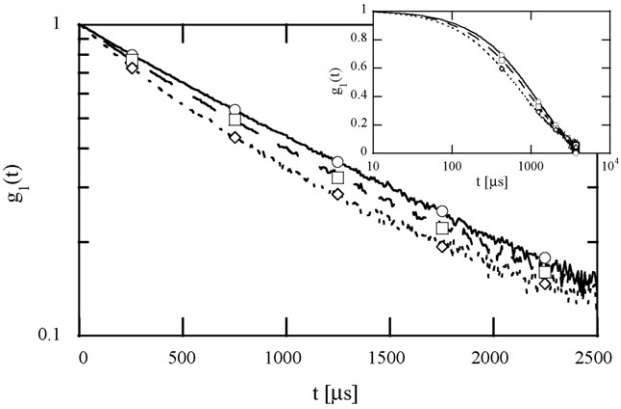

Fig. 2. The first-order time correlation functions of DLS measurements, $g_1(t)$, after the sonication at 480 kHz and 8 W are plotted. The solid, dashed and dotted curves denote the correlation function after the sonication of 0 h (before sonication), 1 h and 3 h, respectively. The *y*-axis is drawn in a log scale in the main panel, while the *x*-axis is in a log scale in the inset.

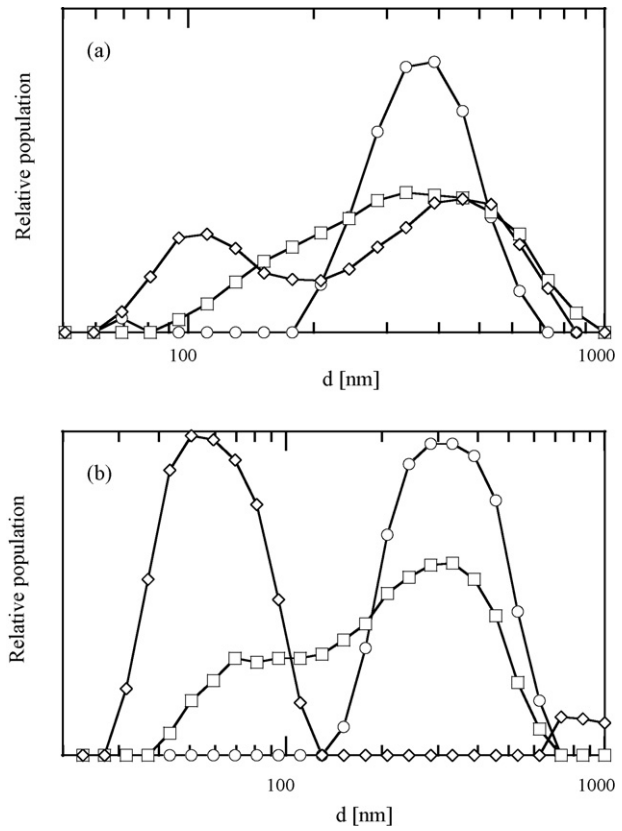

Fig. 3. The size distributions of liposome after sonication at 480 kHz are exhibited. The powers of the ultrasound are 8 and 18 W in (a) and (b), respectively. The sonication times are 0 h (circles), 1 h (squares), and 3 h (diamonds), respectively.

In addition to the distribution smaller than 1 μm that is ascribed to the DLPC liposome, the particles larger than 1 μm are found after the 30 min sonication. By naked eyes we find that the solutions after the 43 and 133 kHz sonication are contaminated with small black powders, which originate in the erosion of the body or the vibrating plate of the ultrasonic bath. We therefore assign the particles larger than 1 μm to the metal powders, not to the aggregates of lipids. A small distribution around 1 μm is also found after the irradiation of 480 kHz ultrasound (Fig. 3(b)). We consider it is due to the product of the sonochemical reaction of DLPC, because contamination by metal powers cannot occur due to the indirect irradiation condition. It is actually confirmed that further sonication at 480 kHz

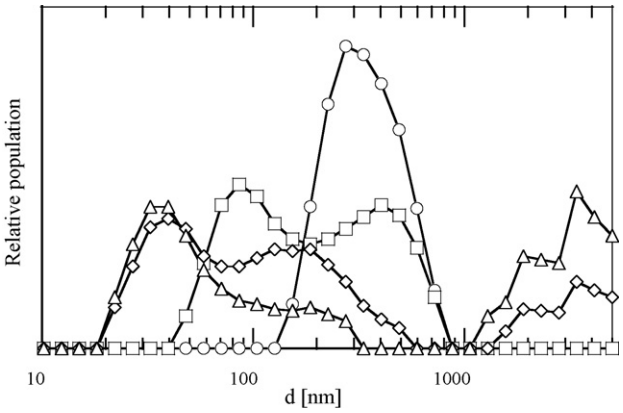

Fig. 4. The size distribution of liposome after the sonication at 43 kHz and 8 W are plotted. The sonication times are 0 min (circles), 10 min (squares), 30 min (diamonds), and 60 min (triangles), respectively.

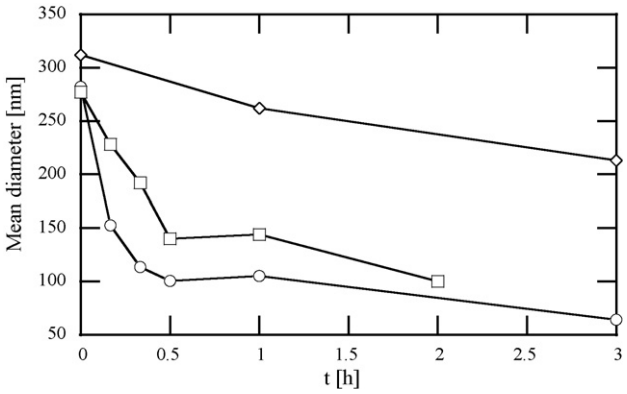

Fig. 5. The mean diameters of the liposome are plotted as the function of sonication time, *t*. The ultrasonic power is 8 W, and the frequencies of the ultrasound are 43 kHz (circles), 133 kHz (squares) and 480 kHz (diamonds), respectively.

(18 W and 5 h) leads to the formation of large aggregates and the loss of the concentration of DLPC. Although lipid aggregates may also contribute to the distribution around 1 μm, we believe its effect is small, because the harmonic mean diameter shown in Fig. 5 corresponds to the distribution below 1 μm.

The mean diameters are calculated from Eqs. (6) and (7), and plotted as the function of time in Fig. 5. The results of different ultrasonic frequencies are compared, keeping the ultrasonic power constant (8 W). The efficiency of reducing the size of liposome clearly increases with lowering the frequency. Since the breaking of the large liposome is considered to be induced by the physical effect of cavitation, the higher efficiency of the lower-frequency ultrasound is in harmony with a common sense in sonochemistry that the physical effect of cavitation is stronger at the lower frequency.

One may consider that the comparison at the constant power is not appropriate because the volume of the solution is different (50 cm^3 for 43 and 133 kHz, and 35 cm^3 for 480 kHz). We admit that it is better to compare the results at the same power *density*. Since the sample volume is larger at 43 and 133 kHz experiments (50 cm^3) than the 480 kHz one (35 cm^3), the power density is smaller. Therefore, the comparison at the constant energy density will make the difference between the results of low- and high-frequency experiments larger, and it does not affect the conclusion that the ultrasound of lower frequency is more efficient to reduce the size of liposome.

The effect of frequency on the size reduction of liposome can be related to that on the strength of cavitation caused by the difference in the bubble dynamics. The amplitude of the oscillation of a cavitation bubble is larger at lower frequency because the bubble experiences longer time of negative pressure to glow larger (Leighton, 1977; Brotchie et al., 2009). The impulsive shock wave and the microjet stream created by a bubble are thus stronger at the lower frequency. On the other hand, the number of cavitation bubble increases with increasing frequency due to the short wavelength of the ultrasound and the short lifetime of the cavity, which are considered as the reason for increasing sonochemical efficiency with increasing frequency (Koda et al., 2003). Therefore, a liposome feels the small number of strong impact under the low-frequency sonication, whereas the high-frequency ultrasound exerts the large number of weak impacts. Since the breakage of large liposome to make smaller ones involve plastic deformation, it is natural to consider that the small number of strong impact is favorable when the total energy is equal.

As is already shown in the comparison between Fig. 3(a) and (b), the size reduction of liposome is faster under the stronger ultrasound. The faster decrease in the liposome size with higher ultrasonic power is rather trivial in a sense, because breaking the

large liposome into smaller ones is an energy-consuming process. We consider that the comparison should be performed at the constant energy dissipated into the solution, rather than the constant sonication time.

In Fig. 6(a), the mean diameter of liposome after the 480 kHz sonication is plotted against the energy density, which is the sonication time multiplied by the ultrasonic power and divided by the mass of the solution. Compared at the same energy density, the smaller liposome is produced by the stronger ultrasound. Fig. 6(b) exhibits the results of 43 kHz sonication in the same way as Fig. 6(a). The short-time irradiation of strong ultrasound shows higher efficiency also in the case of 43 kHz ultrasound.

The higher efficiency of the stronger sonication can be interpreted similarly to the discussion on the effect of frequency. The stronger ultrasound exerts more intense impact on a liposome at the collapse of a bubble, while a liposome feels a large number of weak impacts under the long irradiation of weak ultrasound. Therefore, we can understand the higher efficiency of the strong ultrasound in terms of the strength of the impact on a liposome, as is the case of the effect of frequency.

According to Fig. 6, the mean sizes of the liposome finally obtained by the strongest sonication used in this work are 50–70 nm, irrespective of the frequency of the ultrasound. It suggests that the final size of liposome after sonication is determined by the stability of the liposome itself, not by the process how the small liposome is formed. It is interesting to see whether the final size of liposome depend on the initial state, and it can be tested experimentally with extrusion through membranes of different pore sizes. Our preliminary results suggest that the same final size is achieved by the sonication at 500 kHz to liposome of 100 nm diameter. However, more experimental studies will be required for conclusive discussions, which we shall left for future studies.

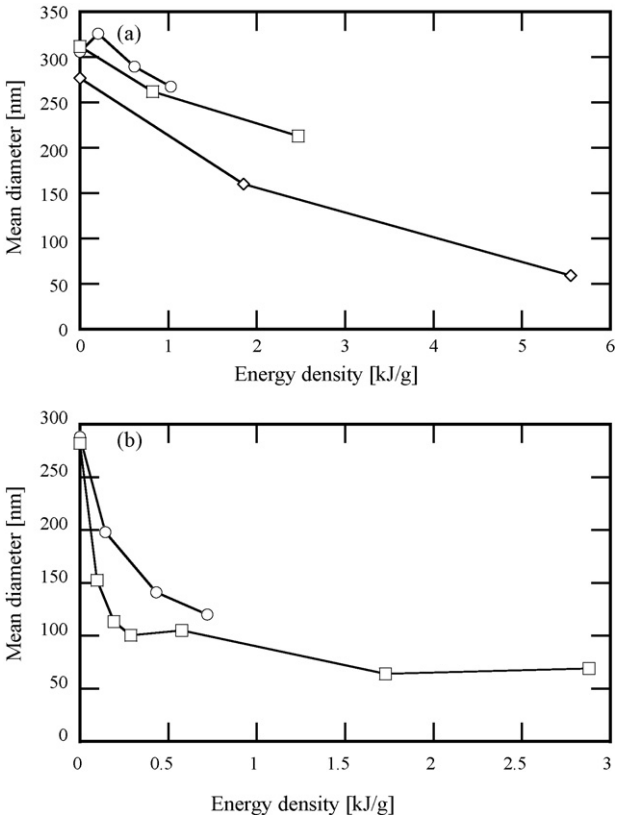

Fig. 6. The mean diameters after the sonication at the same frequency and different ultrasonic powers are compared as the function of the energy densities dissipated into the solution. (a) The results at 480 kHz. The ultrasonic powers are 2 W (circles), 8 W (squares), and 18 W (diamonds), respectively. A small initial increase in the 2 W experiment is ascribed to an experimental error. The results at 43 kHz are exhibited in (b). The ultrasonic powers are 2 W (circles) and 8 W (squares), respectively.

4. Summary

The solution of DLPC liposome of about 300 nm diameter is sonicated at various frequencies ranging from 43 to 480 kHz, and the subsequent change in the size of liposome is measured by DLS. The power of ultrasound dissipated into the solution is determined by the calorimetric method, and the efficiency of the ultrasound to reduce the size of liposome is evaluated.

The ultrasound of lower frequency can reduce the liposome size faster within the frequency range studied here. The short-time irradiation of the strong ultrasound is more efficient than the long-time weak sonication. These two results are understood in terms of the energy of an impact at the collapse of the bubble to break the liposome.

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