Preparation and Characterization of Echogenic Liposome as an Ultrasound Contrast Agent: Size-Dependency and Stabilizing Effect of Cholesterol on the Echogenicity of Gas-Entrapping Liposome

Atsuomi KIMURA, Atsuko SAKAI, Sei-ichi TSUKISHIRO, Shintaro BEPPU, and Hideaki FUJIWARA*

School of Allied Health Sciences, Faculty of Medicine, Osaka University, 1–7 Yamadaoka, Suita, Osaka 565–0871, Japan. Received March 6, 1998; accepted June 15, 1998

The liposome entrapping CO_2 gas inside the vesicle, which is called the echogenic liposome, has been made and characterized *in vitro* as an ultrasound contrast agent. The small unilamellar vesicle (SUV), large unilamellar vesicle (LUV) and multilamellar vesicle (MLV) as echogenic liposomes were compared in their echogenic efficiency and stability, and the effect of size and acoustic property was tested. The acoustic reflectivity increased with the increase in size of the vesicle, largest for the gas filled MLV among the three liposome suspensions. The acoustic reflectivity obtained with the echogenic MLV was larger than that of the gas bubbles enclosed within a surfactant mixture.

A half-lifetime of 39 min was observed for the MLV prepared from egg-yolk phosphatidylcholine liposomes. The duration of reflectivity was prolonged drastically to a half-lifetime of 866 min by incorporating cholesterol into the MLV, although the echogenicity was decreased by such incorporation. The stabilizing effect of cholesterol for the ordinary liposomal membrane was thus ascertained in the present case of the gas-entrapping liposome. Our findings encourage the future development of improved gas-entrapping liposomes for the clinical trials of ultrasound contrast agents.

Key words echogenic liposome; ultrasound contrast agent; acoustic reflectivity

Ultrasound imaging is prominent among several diagnostic imaging techniques such as those based on nuclear medicine or X-ray, in that it does not expose the patient to the detriment of any harmful side effects. Many advances have been made in recent years in ultrasound technology,[1] but it still includes some drawbacks requiring resolution for higher sensitivity and wider applicability. The ability of ultrasound imaging to detect diseases in a diagnostic site such as heart, spleen or liver depends on the difference in acoustic properties between those organs and the medium surrounding them. To promote the potential of ultrasound diagnostics, the development of novel contrast agents has been sought in recent years; this is useful for enhancement of the acoustic difference between target organs and the surrounding medium.

Ultrasound contrast agents are expected to improve the resolution of diagnostic images, and, in fact, different kinds of contrast agents have been developed and tested for cardiac imaging, blood-pool enhancement, tissue characterization, and improvement in the detection of lesions in organs such as liver and spleen.[1] As an example of these contrast agents, the gas bubble agent is recently gaining interest. The bubble is stabilized by special chemicals such as albumin,[2] carbohydrate microspheres,[3,4] perfluorocarbons[5,6] and liposomes[7,8]. To date, albumin-coated bubbles and gas-filled microspheres have been used in clinical trials and some encouraging results obtained. However, these have limitations as ultrasound contrast agents with regard to their ability to satisfy a variety of clinical use demands, and more advanced agents have to be developed such as those to prolong stability *in vivo* and to enable cardiac imaging.

Among the contrast agents mentioned above, the liposome which entraps gas inside a vesicle (echogenic liposome) is attractive. Liposome is a microscopic vesicle consisting of a phospholipid bilayer surrounding an aqueous solution. The lipid vesicle is formed spontaneously when phospholipid is hydrated in aqueous medium. Because of its entrapping ability, liposome has been considered for use as a drug carrying vesicle.[9] The liposome which entraps gas, however, differs from the classical one as confirmed by electron microscopy[10] in that the internal space is completely filled with sparse gas, differing from the outside dense fluid; also, it has a good property as acoustic reflector. For this reason, it has recently stirred great expectation as a novel type of contrast agent in ultrasound diagnostics.

Quite recently, the site-directed echogenic liposome has been developed and applied to the imaging of thrombi *in vitro*.[11,12] Such a targetable contrast agent will provide high concentration at the diagnostic site, and is expected to be utilized for not only diagnostics but also therapy. Given this potential, there is urgency for the basic study of echogenic liposome to promote development of such a liposome with versatility in medicine.

A physicochemical study was performed here to make and characterize the echogenic liposome, and the effect of the addition of cholesterol which is known to stabilize the liposomal membrane was investigated to achieve higher stability and efficiency of echogenicity.

Experimental

Materials Cholesterol was purchased from WAKO Pure Chemical Industry, Ltd. and recrystallized from methanol. Egg-yolk lecithin, glycerol monolaurate, cholesterol acetate and glycerol tripalmitate were purchased from Nakalai Tesque and used without further purification. 18-Crown-6 and cholesterol benzoate were purchased from Tokyo Kasei Organic Chemicals and used without further purification. Other chemicals were of analytical grade and used as purchased.

Preparation of Multilamellar Vesicle (MLV) Two grams of egg-yolk phosphatidylcholine was deposited on the inside wall of a round-bottom flask by removing the chloroform solvent by rotary evaporation. The evaporation was continued for ca. 6 h at 30 °C to assure complete removal of the solvent. Then a 50 ml solution of 150 mM NaCl and 100 mM K_2CO_3 was added to the film. By shaking it vigorously, a MLV suspension was obtained. The MLV containing a 30% cholesterol by molar ratio was prepared as needed (to measure the echogenicity of cholesterol-containing liposome).

Preparation of Small Unilamellar Vesicle (SUV) The MLV solution

* To whom correspondence should be addressed.

prepared as above was homogenized with the pressure of 15000 psi using an M-110 EH Microfluidizer from Microfluidics. The passage through the Microfluidizer was repeated *ca.* 10 times, and a translucent SUV solution was obtained.

Preparation of Large Unilamellar Vesicle (LUV) The LUV was prepared by the reverse-phase evaporation method developed by Szoka *et al.*[13] In brief, a 100 mg egg yolk phosphatidylcholine was dissolved in 6 ml of diethyl ether distilled immediately prior to use. Then, a 2 ml solution of 150 mM NaCl and 100 mM K_2CO_3 was added to the lipid/ether mixture. The resulting two-phase suspension was sonicated at 0 °C in a bath-type sonicator for ca.30 min to form a homogeneous suspension. The phase of ethyl ether was slowly removed by rotary evaporation at room temperature, leaving a translucent aqueous suspension of vesicles.

Preparation of Echogenic Liposome[7] An HCl solution was added to the vesicle suspension after the addition of NaCl solution to dilute external K_2CO_3 with NaCl so that the resulting solution of the vesicle suspension reached the following condition: 8.7 mM egg yolk lecithin; 150 mM NaCl and 100 mM K_2CO_3 inside the vesicle; 6.7 mM K_2CO_3, 290 mM NaCl and 75 mM HCl outside the vesicle. In this way, a preliminary solution was prepared. Afterwards, 0.2 mM of ionophore crown ether (18-crown-6) was added to the vesicle solution immediately prior to the acoustic analysis, to promote the exchange of external H^+ with internal K^+ and to generate CO_2 bubbles inside the vesicle.

Preparation of Echogenic Surfactant Mixture[14] A surfactant mixture was prepared by admixing glycerol monolaurate, cholesterol benzoate, cholesterol, cholesterol acetate and glycerol tripalmitate in a weight ratio of 3 : 1 : 1 : 1 : 1, respectively, to obtain a dry powdery surfactant mixture. A saturated solution of this mixture was formed by dissolving 0.1 g of the surfactant mixture into 100 ml of water. The resultant solution was shaken vigorously to obtain gas microbubbles.

Size Measurement The mean particle size and size distribution of the vesicle were determined at room temperature by laser light diffraction using a Shimadzu SALD-2000 particle analyzer (Shimadzu) equipped with a semiconductor laser at an exciting wavelength of 680 nm. The mean particle size and size distribution were evaluated as volume weighted values.

Imaging and Videodensitometric Analysis of Echogenic Liposome "Brightness" About 50 ml of the echogenic liposome was transferred into a thin rubber condom (Okamoto). The suspension was stirred gently and left standing for a minute prior to recording the image of echogenic liposome. Imaging of the echogenic liposome was performed with a Toshiba SSH-140A clinical ultrasound scanner (Toshiba). Instrument settings for gain, zoom, dynamic range and output level were held constant at optimized values for all samples. Images were recorded on a VHS videotape. The relative echogenicity of the echogenic liposome was measured as the apparent brightness which was objectively assessed by computer-assisted videodensitometry and quantified as gray scale 256 values. All image processing and analyses were performed with a Tom Tec Color Cardiology work station. Measurement of time dependency of the echogenicity was made using different batches of the sample.

Results and Discussion

Comparison of Various Types of Echogenic Vesicles The ultrasound echogenicity (gray scale) of the prepared vesicles is summarized in Table 1, where the mean particle size (diameter) of the vesicles is also included. The echogenicity increased with the size of gas-entrapping liposome, and dependence of the apparent brightness on the size is illustrated in Fig. 1. Theoretically, it is known that the echogenicity of gas bubbles is expressed by the sixth-power dependence on their size in the first approximation,[1] in which the backscattered power received by the transducer is stated to be proportional to the sixth-power of the radius of gas bubbles as well as to the fourth-power of the ultrasonic frequency up to resonance. Since the gray scale is the logarithmic value of the backscattered power, it is difficult to straightforwardly discuss the apparent brightness in relation to the particle size of the echogenic liposome. However, clearly, the simple theoretical correlation between the particle size and the acoustic reflectivity was not observed in the present study (Fig. 1). That is, the apparent brightness of the echogenic MLV showed much higher dependence on particle size than the other echogenic liposomes. This was probably due to the extra effects of the bubble resonance phenomenon. When free gas bubbles are used for echogenicity experiments, resonance effects are reported to occur in the imaging band from 1 to 10 MHz for bubbles with a diameter on the order of 10 μm.[15] Therefore, in the present study, the resonant phenomenon can be observed for an echogenic MLV which has a diameter of 10.7 μm.

Table 1. A Comparison of the Reflectivity of Various Echogenic Liposomes

Microbubble	Diameter (μm)	Concentration, c (mg/ml)	Apparent brightness (gray scale)
SUV	0.8 (0.5)	6.7	65 (19)
LUV	6.8 (2.6)	6.7	97 (35)
MLV	10.7 (0.9)	6.7	213 (21)
MLV (egg PC/Chol.)[a]	18.0 (3.0)	6.7	134 (10)
Surfactant mixture suspension	5.0 (0.6)	1.0	129 (3)
Saline+CO_2	—	—	40 (6)

The standard deviation is given in the parenthesis. *a*) The liposome contains 70% egg-yolk phosphatidylcholine and 30% cholesterol by molar ratio.

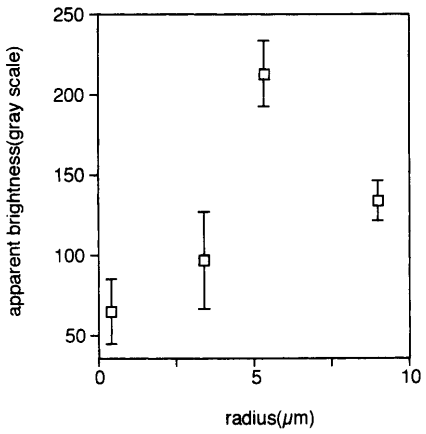

Fig. 1. Particle Size Dependency of the Reflectivity of Echogenic Liposome

Figure 2 shows the population distribution of optical particle size; the mean particle size is about 10 μm in diameter. This diameter of echogenic MLV is comparable to that reported by Unger *et al.*,[10] although it is about five times as large as the diameter of the classical MLV. Therefore, it seems that the diameter of liposome becomes larger than the original one when gas is entrapped inside the vesicle.

Table 1 also compares the echogenicity of gas-entrapping liposomes with the saline solution including gas bubbles of carbon dioxide. The echogenicity of MLV was higher than that of the control gas-generated saline solution, but that of SUV or LUV suspension was comparable to that of the control solution (Table 1). Thus the importance of the multilamellar structure[10] is supported for the enhancement of the echogenicity and for greater stability of large gas-entrapping liposomes.

The echogenicity of gas-containing liposomes is compared with that of the surfactant mixture suspension in Table 1. We can say that we have obtained a gas-containing liposome which exhibits echogenicity superior to that of the surfactant

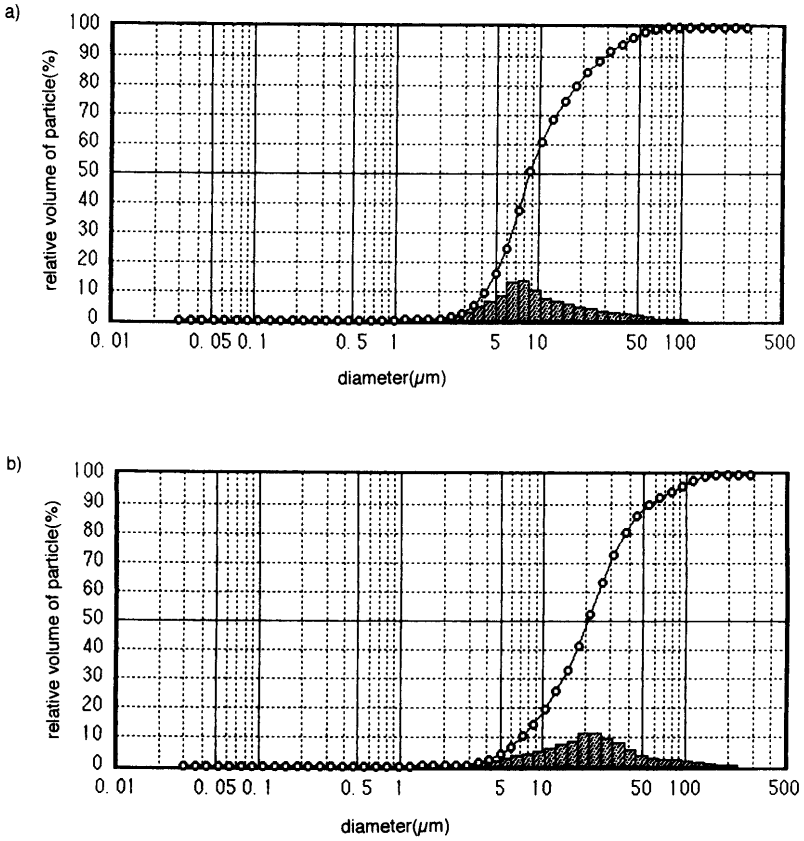

Fig. 2. Population Distribution of Optical Particle Size of a) the Echogenic MLV, b) the Echogenic MLV Containing 30% Cholesterol by Molar Ratio

mixture suspension. However, the concentration of the echogenic liposome is about seven times as high as that of the suspension, and the particle size of the surfactant mixture suspension is smaller than that of the echogenic liposome. Accordingly, improvement in the size- and concentration-dependency of the echogenicity of the gas-containing liposome is desirable for its future application as a targetable contrast agent.

Effect of Cholesterol on Stability of the Echogenic Liposome The echogenicity of MLV which contains 30% cholesterol by molar ratio is listed in Table 1; it was not large and smaller than that of the echogenic MLV. This is due to the lack of resonance effects as stated above, since the diameter of the cholesterol-containing vesicle was much larger than that of the echogenic MLV (Fig. 2b). Therefore, for a rigorous comparison of these two types of gas-entrapping liposomes as the echogenic agent, it is necessary to prepare the more uniform vesicle suspension utilizing a technique such as extrusion.[16]

Figures 3a and b show the time dependency of echogenicity for the MLV and the MLV containing cholesterol, respectively. It is obvious that the duration of the echogeniciy increased drastically for the cholesterol-containing MLV. While the echogenicity of MLV demonstrated an exponential decay and had an easily estimated half-lifetime of 39 min, the echogenicity of cholesterol containing MLV continued for more than 300 min and the half-life time was extrapolated to be 866 minutes. This supports the enhanced stability of liposomal membrane when cholesterol is included. Unger[7] reported that the addition of 20% cholesterol by molar ratio had little effect on the echogenicity itself or on the stability of the echogenic liposome. On the other hand, it has been reported that the addition of 30% cholesterol by molar ratio into the membrane composition increases the acoustic reflectivity and the *in vitro* and *in vivo* stability of the echogenic liposome.[12,17] It is also obvious from the present study that the duration of the echogenicity increases drastically when 30% cholesterol by molar ratio is incorporated into the liposomal membrane. The integrity of liposomal membrane is known to depend on the content of cholesterol, and cholesterol of more than 20% by molar ratio must be uniformly mixed in the membrane composition.[18] Therefore, the integrity and rigidity of liposomal membrane determined by the content of cholesterol are considered important for the acoustic reflectivity and stability of the echogenic liposome as observed in this study.

Figure 3c shows the time dependency of the gas bubbles formed in the surfactant mixture for comparison. The cholesterol-incorporated echogenic liposome is seen to be comparable to the microbubbles formed by the surfactant mixture with regard to the duration of echogenicity. Since the stability and efficiency of the echogenic liposome can be improved further as mentioned above, the echogenic liposome is expected to be a useful contrast agent in clinical trials in the future.

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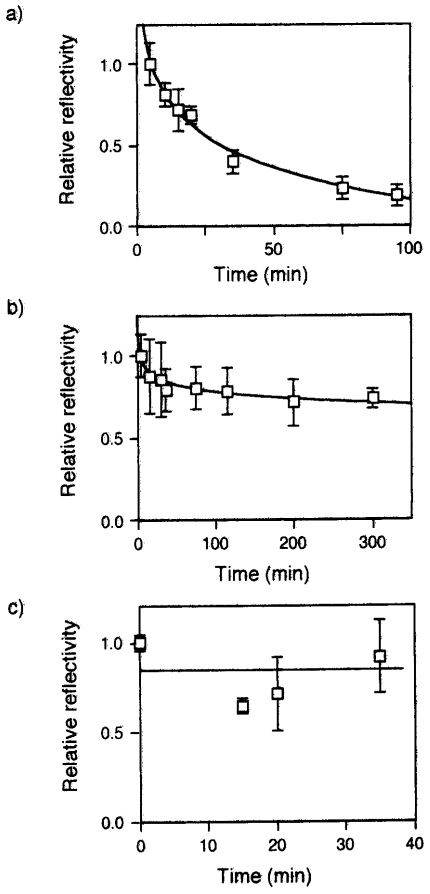

Fig. 3. Time Duration of the Echogenicity of a) the Echogenic MLV, b) the Echogenic MLV Containing 30% Cholesterol by Molar Ratio, c) the Surfactant Mixture Microbubbles

Graph shows the apparent brightness (gray scale) versus time. The line included was obtained from the non-linear least squares fitting (a, b) and the mean value of measured echogenicity (c).

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