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Biochimica et Biophysica Acta 1665 (2004) 134–141

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Acoustically active liposomes for drug encapsulation and ultrasound-triggered release

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Received 2 March 2004; received in revised form 7 June 2004; accepted 16 July 2004
Available online 12 August 2004

Abstract

Acoustically active liposomes (AAL), previously developed as ultrasound contrast agents, contain small amounts of air. These AAL have potential to carry pharmaceutics and their acoustic activity could enable them to respond to ultrasound stimulation by releasing their contents. Since liposomes can entrap many kinds of drugs, if such entrapment did not affect their echogenicity, then the release of contents could potentially be controlled by ultrasound stimulation. The aim of this research was to investigate the capacity of acoustically active liposomes for hydrophilic molecule encapsulation and to determine their sensitivity to ultrasound-triggered release. Liposomes, composed of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and cholesterol, were made acoustically active by hydrating a lipid film, sonication, freezing in the presence of mannitol, lyophilization, and rehydration. As a test molecule, calcein was added in the hydration step. The procedure for generating acoustically active liposomes was compatible with an encapsulation efficiency of 15% or more. The presence of mannitol during freeze-drying was essential not only for generation of acoustic activity but also for efficient encapsulation. Ultrasound-triggered release was achieved by applying 1 MHz ultrasound at 2 W/cm^2 for 10 s. The inclusion of 4% diheptanolyphosphatidylcholine (DHPC) increased the sensitivity of liposomes to ultrasound stimulation and resulted in very efficient stimulated release of contents (1/3 released in 10 s, 2/3 released in six such applications). Release of contents was highly correlated with the loss of air induced either by ultrasound or rapid pressure reduction. These encapsulation and triggered release techniques are highly efficient, and hence may be applicable to drug delivery.

Keywords: Liposome; Ultrasound; Contrast agent; Mannitol; Drug delivery; Controlled release

1. Introduction

Liposomes have been under development as non-toxic, biodegradable and non-immunogenic drug delivery vehicles ever since shortly after their discovery by Bangham et al. [1]. They are suitable for delivering both hydrophilic and lipophilic drugs. When drugs are incorporated into liposomes, their pharmacokinetics are markedly changed, lowering systemic toxicity and reducing premature degradation or inactivation [2]. Furthermore, liposomes conjugated with an antibody can deliver drugs to specific tissues or cells, increasing the local concentration of drugs while decreasing the systemic side effects [3].

Ideally, liposomes should be able to carry an appropriate drug dose, remain stable in circulation [4], and then release their drug contents such that the local concentration is high enough to mediate an effective therapeutic effect at the target site. To deliver a high local dose, triggerable release would be desirable. Although a number of methodologies for both drug encapsulation and for prolonging the circulation of liposomes have been developed over past 20 years [5,6], few procedures are available for triggered drug release.

Strategies for triggering drug release from liposomes have been proposed, including chemical approaches [7], biological approaches [8], and methods based on physical phenomena such as electric fields [9], magnetic fields [10], temperature [11], visible light [12,13], pH [14], and ultrasound [15]. Ultrasound is of special interest in controlled release applications because ultrasound is non-invasive and

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doi:10.1016/j.bbamem.2004.07.003

yet is able to penetrate into the interior of the body. In addition, ultrasound can be focused on targeted sites and moreover has been shown to increase the permeability of blood–tissue barriers and cell membranes [16].

To achieve a satisfactory response to ultrasound stimulation, an ultrasound-sensitive vehicle is needed. Previous research led to development of acoustically active liposomes with an average size of 800 nm that can be used as contrast agents for ultrasound image enhancement [17]. Subsequently, research revealed that these liposomes encapsulated air, which is responsible for their acoustic activity [18]. Such liposomes are potential drug carriers for a variety of drugs, genes, therapeutic proteins and diagnostic agents. Because they contain air, these liposomes have the potential to respond to ultrasound stress by releasing their contents. In the present research, a fluorescent dye, calcein, was used as a drug "stand-in" and successfully encapsulated into acoustically active liposomes by freeze-drying in the presence of mannitol. These liposomes were shown to exhibit a low threshold for aqueous contents release upon application of ultrasound.

2. Materials and methods

Egg phosphatidylcholine (EggPC), dipalmitoyphosphatidylethanolamine (DPPE), dipalmitoyl phosphatidylglycerol (DPPG), diheptanolyphosphatidylcholine (DHPC) and cholesterol (CH) were purchased from Avanti (Alabaster, AL) and stored at −20 °C in chloroform. Calcein(2′,7′-[(bis[carboxymethyl]-amino)methyl]-fluorescein) and cobalt chloride ($CoCl_2$) were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of calcein (80 mM) were made by quickly dissolving solid calcein at pH 9.0 and then adjusting the pH to 7.5. Triton X-100 and 3-morpholinepropanesulfonic acid (MOPS) were also from Sigma. The ultrasound device was a Sonitron1000, from RichMar (Inola, OK).

2.1. Preparation of calcein-containing acoustically active liposomes

Liposomes were composed of EggPC/DPPE/DPPG/CH at a molar ratio of 69:8:8:15. Lipids (5 mg total weight) were mixed in chloroform in a glass vial. The solvent was removed by evaporation under argon in a 50 °C water bath with constant rotation until a thin film of lipids was formed on the vial wall. The resulting lipid film was then placed under high vacuum (<100 torr) for 2–4 h for complete removal of solvent. The dried lipid film was hydrated with 0.1 mM calcein in the desired concentrations of mannitol. The resultant liposomes (10 mg/ml final concentration) were sonicated for 5 min in a water bath and frozen at a −70 °C in a low-temperature freezer. Frozen liposomes were then lyophilized as previously described [17]. The lyophilized dry cake was either stored in 4 °C or resuspended at a concentration of 10 mg/ml in 10 mM MOPS buffer (pH 7.4) and used immediately.

2.2. Determination of encapsulation efficiency

Encapsulation efficiency and ultrasound-triggered release of acoustically active liposomes were determined fluorometrically using a previously developed method [19]. Briefly, 25 μl of resuspended liposomes (10 mg lipid/ml) were diluted to 500 μl with 50 mM MOPS buffer containing 110 mM NaCl. $CoCl_2$ was used to quench the fluorescence of external calcein so that the residual fluorescence represented the entrapped calcein. Then the liposomes were lysed with detergent to determine the background fluorescence at zero encapsulated volume. Thus, fluorescence intensity was measured before (F_b) and after (F_a) addition of 5 μl of 10 mM $CoCl_2$, and again, after addition of 25 μl 10% Triton X-100 (F_{totq}) at 490 nm Ex, 520 nm Em. The percentage of entrapment was calculated as:

$$\%\ \text{encapsulation} = \left(F_a - F_{totq}\right)/\left(F_b - F_{totq}\right) \times 100 \qquad (1)$$

2.3. Measurement of echogenicity

Liposomes, resuspended in PBS, were diluted to a concentration of 25 μg/ml for ultrasound imaging in 12×16 mm glass vials. Imaging was performed with a 20-MHz high-frequency intravascular ultrasound image catheter as described previously [17]. Briefly, images were recorded onto videotape, subsequently digitalized and computer analyzed in terms of a mean gray scale value (MGSV) of the entire image, white indicating complete reflection, and black, zero reflection.

2.4. Ultrasound-triggered release

Ultrasound-triggered release experiments were performed in a chamber (Fig. 1) consisting of a Plexiglas cylinder connecting the ultrasound probe at one end with a water-filled chamber at the other end. The apparatus was inclined at about 15° to the horizontal to reduce reflection. The water-filled tube was separated from the sample by a thin vinyl rubber diaphragm and, at the far end, an air interface provided for off-axis reflection. A 2×2 mm hole in the top of the chamber allowed for introduction of the liposome dispersion (400 μl).

After preliminary exploration of conditions, 1 MHz, 2 W/cm^2, 100% duty cycle for 10 s was chosen for subsequent experiments.

The release of calcein was measured using a modification of the encapsulation measurement procedure. Briefly, 100 μl of calcein-containing, acoustically active liposomes (10 mg lipid/ml), were diluted to 500 μl with 50 mM MOPS buffer containing 110 mM NaCl (to maintain isosmolality with liposome contents of 320 mM mannitol and 0.1 mM calcein.

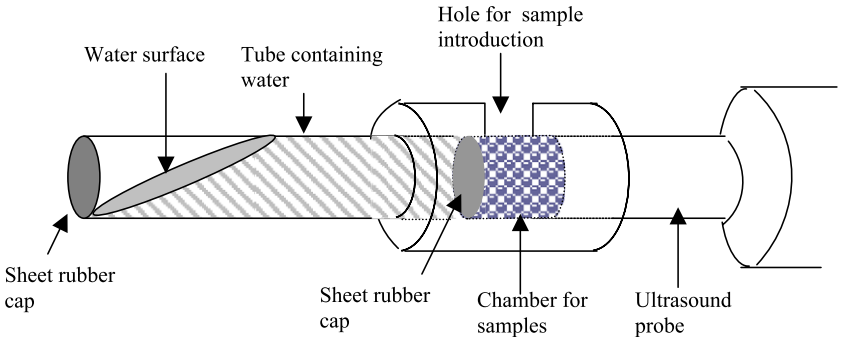

Fig. 1. Experimental apparatus for ultrasound-triggered release.

The fluorescence intensity (F_{in}) of the suspension was measured after addition of 5 μl of 40 mM $CoCl_2$. Subsequently, ultrasound was applied for 10 s. The resulting fluorescence intensity ($F_{ultrasound}$) was measured. Finally, 25 μl of 10% Triton X-100 was added and the fluorescence intensity (F_{totq}) was measured again. The amount of release was calculated from:

$$[F_{in} - F_{ultrasound})/[F_{in} - F_{totq})] \times 100 = \%\ \text{release} \qquad (2)$$

The release of air was measured by comparing the amount or air entrapped in liposomes before and after ultrasound stimulation. The method for determining entrapped air has been described previously [18].

2.5. Vacuum-induced release

Calcein-containing acoustically active liposomes (500 μl and 2 mg/ml), prepared by freeze-drying in the presence of 320 mM mannitol, were placed in a 50 ml polypropylene disposable syringe. A two-way Luer-lock stopcock was attached to the syringe and was closed. Two different rates of pressure change were produced by withdrawing the plunger to 50 ml volume in either 2 s or 5 min. The vacuum was held for 10 s and the procedure was repeated twice. The percentage of released calcein was measured in a way similar to that described above for ultrasound application.

2.6. Statistical analysis

Data are presented as mean±S.D. Comparisons between groups were made by ANOVA with significance taken to be $P<0.05$.

3. Results

3.1. Encapsulation efficiency and echogenicity

An ultrasound-sensitive liposome drug delivery system requires liposomes capable of encapsulating drugs as well as echogenicity that is not reduced by the encapsulation procedure. Thus, we began by measuring the encapsulation ability of acoustically active liposomes prepared using standard procedures. We also measured acoustic reflectivity (echogenicity) after entrapment and expressed it as the MGSV. Calcein was used as a marker for determining the encapsulation efficiency.

As shown in Fig. 2, in the presence of mannitol, calcein was efficiently incorporated into liposomes and high echogenicity was preserved. Entrapment depended on the concentration of mannitol, with higher mannitol concentrations yielding higher entrapment. The highest entrapment (14%) was obtained at a mannitol concentration of 0.4 M. An interesting finding was that the mannitol had a similar positive effect on echogenicity as on entrapment efficiency in the concentration range 0.1–0.4 M. At a mannitol concentration of 0.8 M, small amounts of mannitol crystals were observed and concomitantly, entrapment efficiency decreased, although echogenicity continued to increase.

3.2. Effects of mannitol and freeze-thawing on encapsulation efficiency

Since the encapsulation efficiency was highly correlated with the concentration of mannitol during freeze-drying, we

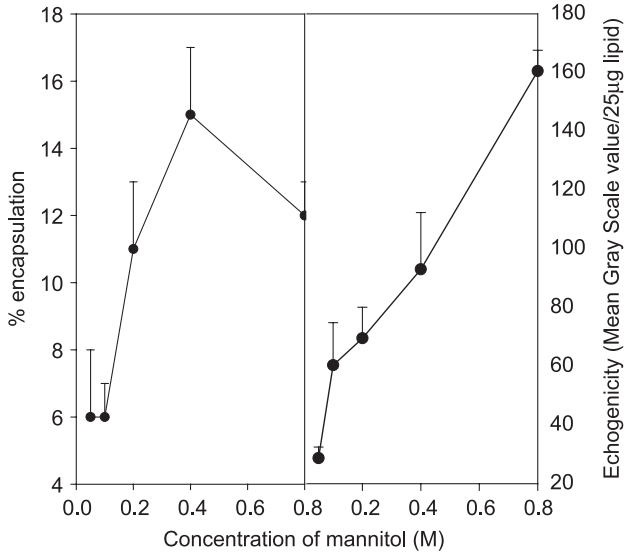

Fig. 2. Encapsulation efficiency and echogenicity of acoustically active liposomes (EggPC/DPPE/DPPG/CH 69:8:8:15) made by freeze-drying in the presence of different concentrations of mannitol. Mean±S.D. $n=6$.

sought to determine whether the mannitol effect occurred during the freezing stage or during the sublimation stage of freeze-drying. "Empty" liposomes were made by a procedure analogous to that described for calcein-containing liposomes except that the calcein was omitted. Calcein (0.1 mM) was then added to the external phase. Subsequently, freeze-thawing or freeze-drying was performed.

As shown in Fig. 3, no detectable calcein was encapsulated prior to the freeze-thawing or freeze-drying steps. Also, freeze-drying in the presence of trehalose, a well-known cryoprotectant, prevented encapsulation of calcein by liposomes. On the other hand, in the presence of mannitol, both freeze-thawing and freeze-drying caused the entrance of calcein into liposomes, indicating that calcein was entrapped when liposomes were frozen in the presence of mannitol.

Since freezing in the presence of mannitol was responsible for calcein encapsulation, we tested entrapment efficiency as the function of the number of freeze-thaw cycles. As shown in Fig. 4, one freeze-thaw cycle in the presence of mannitol lead to the capture by liposomes of about 15% of the external calcein solution. The extent of entrapment stabilized at about 20% after three to four freeze-thawing cycles.

3.3. Vacuum-induced release of contents from acoustically active liposomes

Given the acoustic activity of calcein-containing liposomes, we next determined if reduced air pressure could cause release of the entrapped calcein. Fast and slow pressure changes were tested. A short-chain lipid, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC, Avanti), was added into the liposome preparation at a 4% (w/w) ratio, to test its effect on ultrasound sensitivity.

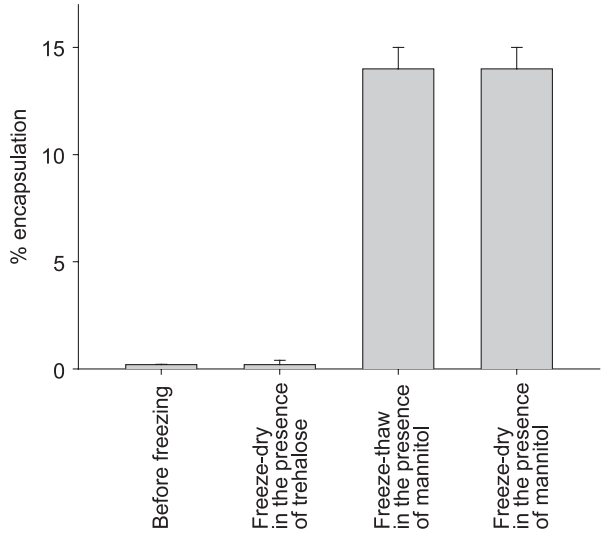

Fig. 3. Effect of freeze-drying and mannitol on the entrapment of calcein into liposomes. Liposomes were composed of EggPC/DPPE/DPPG/CH at a molar ratio of 69:8:8:15. Mean±S.D., n=3.

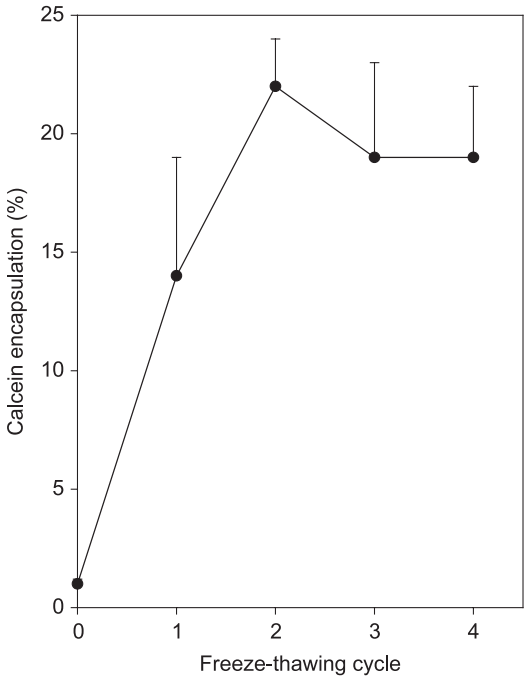

Fig. 4. Effect of number of freeze-thawing cycles on the calcein entrapment efficiency by liposomes (EggPC/DPPE/DPPG/CH at a molar ratio of 69:8:8:15). Mean±S.D.; n=3.

The results of these experiments are shown in Fig. 5A. A rapid reduction in pressure caused a 2-fold higher release than did slower pressure change. Liposomes containing 4% DHPC exhibited a 3.5-fold higher sensitivity to ultrasound stimulation than did those lacking the short-chain lipid.

Inclusion of DHPC in acoustically active liposomes increased their sensitivity to ultrasound, an effect that, in principle, could be due to (a) increased encapsulation of calcein, (b) increased air content or, (c) increased susceptibility to calcein release upon ultrasound treatment. To investigate the possible effect of DHPC on air entrapment and calcein encapsulation, echogenicity as well as calcein encapsulation were determined for both standard and DHPC-containing liposomes. As shown in Fig. 5B, incorporating 4% DHPC into acoustically active liposomes did not affect their echogenicity. Furthermore, entrapment of calcein into DHPC-containing liposomes did not change their echogenicity. Finally, as shown in Fig. 5C, the encapsulation efficiency was not affected by DHPC incorporation. It thus appears that DHPC confers increased sensitivity to ultrasound-triggered contents release.

3.4. Ultrasound-stimulated release of calcein from acoustically active liposomes

Although calcein-containing acoustically active liposomes were found to be pressure-sensitive in the very low frequency regime, the more important question was whether they would be sensitive to higher frequencies, in particular whether ultrasound could trigger the release of encapsulated

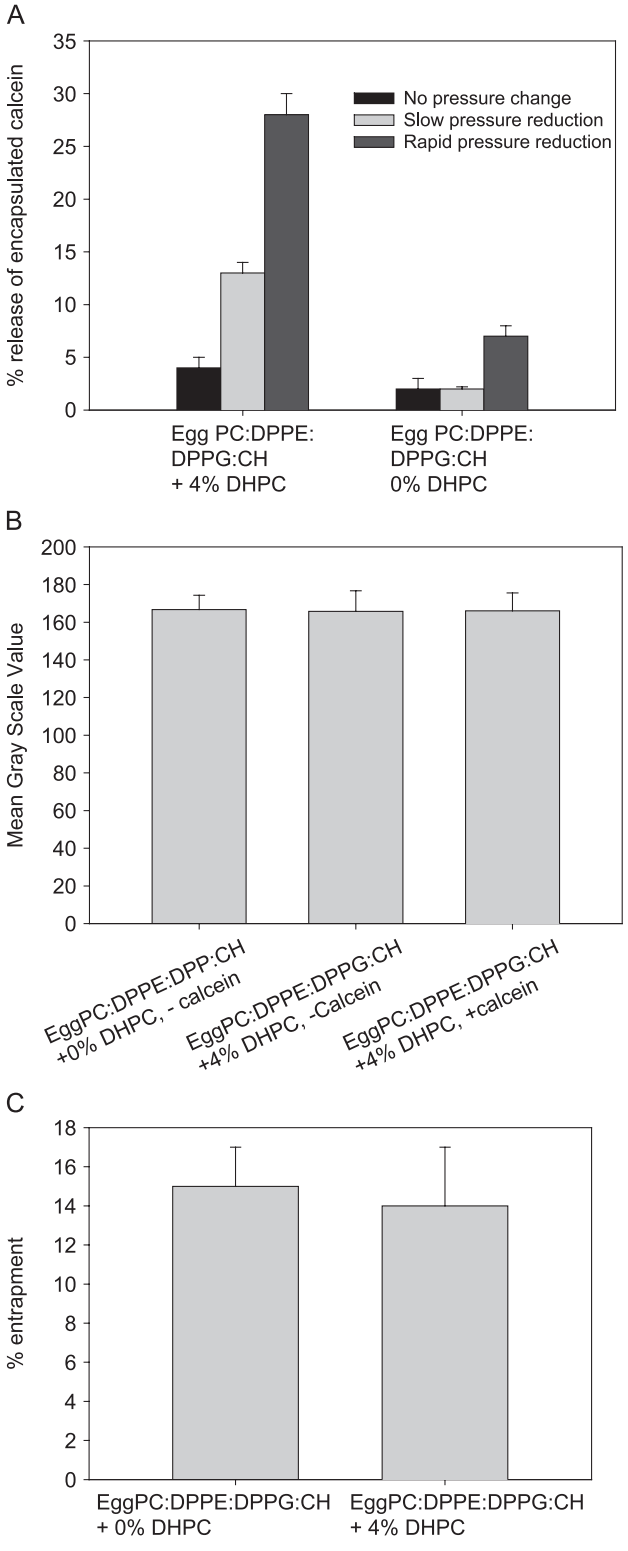

Fig. 5. (A) Effect of rapid and slow air pressure reduction on calcein release from acoustically active liposomes composed of EggPC/DPPE/DPPG/CH at a molar ratio of 69:8:8:15 with or without inclusion 4% DHPC. Mean±S.D. n=4. (B) Effect of incorporating DHPC or entrapping calcein into acoustically active liposomes on echogenicity. Mean±S.D. n=3. (C) Effect of incorporating DHPC on calcein entrapment into acoustically active liposomes. Mean±S.D. n=6.

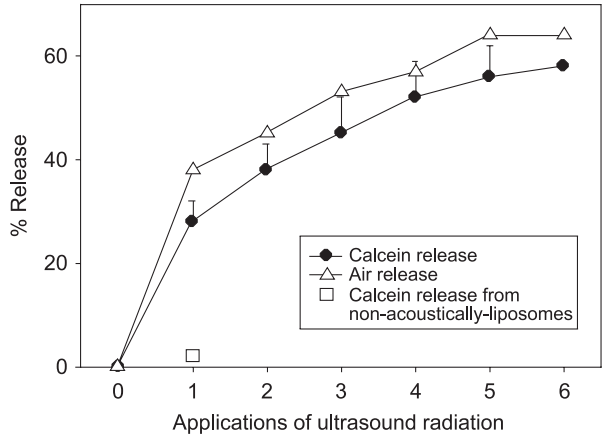

Fig. 6. Ultrasound-triggered release of calcein and air from acoustically active liposomes composed of EggPC/DPPE/DPPG/CH at a molar ratio of 69:8:8:15 including 4% DHPC. Mean±S.D., n=6.

calcein. We therefore subjected calcein-containing acoustically active liposomes to ultrasound at a variety of intensities and duty cycles. Ultrasound at 1 MHz, 2 W/cm^2, 100% duty cycle for 10 s, caused the highest release and was used in subsequent experiments. As shown in Fig. 6 (•), the first application of ultrasound triggered the release of 32% of the entrapped calcein; subsequent applications caused additional release but with diminishing effectiveness. Six such applications led to the release of a total of 62% of the initial contents. Of significance is that non-acoustically active liposomes were essentially unresponsive to ultrasound at this frequency and intensity. As shown by the open square in Fig. 6 (□), ultrasound application led to the release of only 2–3% of the calcein contained within those liposomes.

Also shown in Fig. 6 (△) are the results of a parallel experiment in which the amount of air remaining in the liposomal dispersion was measured. As may be seen, the release of calcein by ultrasound was highly correlated with the release of air. Although the time course of air loss was very similar to that of calcein release, in neither case was complete release achieved under the conditions used.

4. Discussion

Acoustically active liposomes, previously developed as contrast agents for ultrasound image enhancement, were found to efficiently entrap an internal volume marker while retaining their echogenicity. Such liposomes also efficiently released their contents upon ultrasound stimulation.

Although initially employed for preparing acoustically active liposomes, the procedure of freeze-drying in the presence of mannitol has been found to be a superior method for encapsulation of a hydrophilic solute encapsulation. As shown here, an encapsulation efficiency of about 15% was obtained by this method; moreover, encapsulation efficiency could be significantly improved (to 20%) without

diminishing echogenicity by simply increasing the number of freeze-thaw cycles. As in the case of entrapment of air to generate echogenic liposomes, effective encapsulation of calcein was dependent on the formulation procedure, in particular on the presence of moderate concentrations of mannitol during the initial freezing. When calcein was added to the outside of "empty" liposomes, an encapsulation efficiency of about 15% was achieved after freeze-thawing or freeze-drying in the presence of mannitol, indicating that the liposome membrane opens and reseals during those operations. These data are consistent with the investigation of Janicki et al. [21], which showed that large liposomes lyophilized in the presence of mannitol tend to increase in both size and encapsulation efficacy. This behavior contrasts with that of trehalose, which is a well-known cryoprotectant that very effectively stabilizes liposomes during freeze-drying by forming a glassy shell around them [22]. Mannitol does not significantly reduce the tendency of water to form ice crystals and itself readily crystallizes in the cold. Such crystal formation evidently leads to membrane damage, including rupture and subsequent resealing of the participating bilayers [18]. Since vesicle rupture, which is necessary for efficient solute encapsulation, and encapsulation of air, which is necessary for ultrasound reflectivity, must both involve some damage or defect generation in the bilayer, it is perhaps not surprising that these two phenomena arise in the same procedure.

A priori, there are three possible kinds of particles: those which contain just calcein; those which contain just air; and those which contain both air and calcein. Based on our observation that: (i) most echogenic liposomes floated, and (ii) a rapid pressure drop caused a release of a considerable proportion of their contained calcein, we conclude that the bulk of the population falls into the third category, that is, air and calcein entrapped in the same particle. For those liposomes that are sensitive to ultrasound or pressure stress, they are vesicles that contain two compartments, the smaller of which contains air and is bounded by a monolayer, and the larger of which contains the aqueous phase. Because of energetic reasons, the air must face a hydrophobic surface (the surface tension of oil–air surfaces is invariably lower than that of water–air surfaces), the air pocket of echogenic liposomes must be bounded by the acyl chains of the lipids. In Fig. 7, the air is shown in between the two monolayers of the vesicle. Another configuration is also possible, namely a lipid monolayer-covered air bubble within the aqueous compartment of an otherwise conventional liposome.

The encapsulation properties of acoustically active liposomes shed some light on their structure. The amount of entrapped aqueous marker was reported here, but one can also calculate encapsulated volume from other information available. For this calculation, we assume that all liposomes have a diameter of about 1 μm (the measured mean diameter is 0.8 μm; however, there is considerable heterogeneity in the population so that the mean volume must be larger than that of a liposome with the mean diameter) [18]. Given an

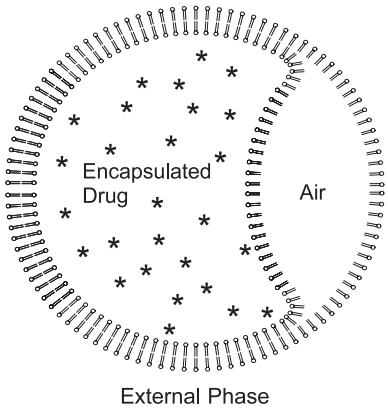

Fig. 7. Suggested structure of an acoustically active liposome, on aqueous contents of which may be released by ultrasound treatment. It is expected that considerable heterogeneity exists in the preparations such that the proportion of volume occupied by air and aqueous compartments would vary considerably. Furthermore, it is unlikely that all liposomes are unilamellar.

area per lipid molecule of 0.6 nm^2, there would be about 5×10^{11} bilayer vesicles in 10 mg liposome dispersion. At a diameter of 1 μm the volume of this population is calculated to be 200 μl. This is in very good agreement with the measured 15–20% encapsulation of marker. We previously found that 100 μl of air was entrapped in 10 mg liposomes [18]. Although clearly an approximation because we ignore heterogeneity, this analysis gives us a rough picture of an average echogenic liposome being about a micron in diameter and consisting of about 2/3 aqueous phase and 1/3 air.

Their structure predisposes acoustically active liposomes to be susceptible to rupture by pressure reduction and hence also by ultrasound irradiation. We presume it is the rarefaction phase of the sound wave that is responsible for content release, that is, when the negative ultrasound wave impinges upon the liposomes, the air pocket expands, stressing the monolayers bounding it as well as those in the adjacent bilayer (*surrounding* bilayer in the case of an isolated air bubble within the liposome). If the pressure drop is large enough, the stress will exceed the elastic limit of the weakest surface and at some point, either the bilayer or the monolayers must rend. When the integrity of the vesicle is lost, some or all of the contents (depending upon how long resealing takes) will be released.

It was observed that slow pressure reduction was relatively ineffective. This may be because it leads to such a gradual loss of air that the critical stress for bilayer or monolayer rupture is not attained. On the other hand, if the air pocket expands more rapidly than air is lost by diffusion into the external aqueous phase, then the monolayer or bilayer will reach the lysis threshold, and most liposomes will release some contents. In any case, the openings must reseal, and this could well occur after all air, but not all contents, have been released. It was noted that short-chain lipid in liposomes increased their air sensitivity. Given the information in Fig. 5B and C, the actual air content and

calcein entrapment did not change by inclusion of DHPC, so it appears that the increased sensitivity of the DHPC-containing liposomes is not due to an increased entrapment of air or of calcein marker. Although it is well known that DHPC can destabilize bilayers by lowering their lysis tension, we suspect that, for low pressure-induced lytic events, the lowering of the lysis tension is not likely to be the critical factor. We believe this to be the case because, at the ultrasound intensities we used, the pressure amplitude variation should be large enough to rend normal bilayers, even in the absence of DHPC. More important with respect to complete contents release may be the lifetime of the opening of membrane in the liposomes. Since short-chain lipids stabilize broken edges of bilayers [20,23], the openings created by membrane stretch should remain open longer, allowing more contents to be lost.

Ultrasound has been used to trigger release from several different drug delivery preparations [24,25]. Rapoport et al. [24] reported that ultrasound at frequencies of 20 or 70 kHz and power 2 or 1 W/cm^2, gave rise to release of up to12% of a drug contained within polymeric micelles. Insonation of tissue after administration of such preparations successfully inhibited tumor proliferation without killing cells. Such a frequency is much lower than that used clinically (1–20 MHz), however, and at 1 MHz and 2 W/cm^2 only 3% release was obtained. At 7 W/cm^2, a 9% drug release was obtained but such power levels can cause substantial cell damage. As described in another report, Unger et al. [25] recently entrapped hydrophobic drugs into lipid shells of gas-filled microspheres by incorporating soybean oil in the shell. Sixty percent of the microspheres were lysed by application of 2.5 MHz ultrasound at 0.8 W/cm^2 for 30 min. In our experiments (Fig. 6), ultrasound at 1 MHz and 2 W/cm^2 (10 s) triggered an approx. 30% release of contents. Although they do not give complete release, echogenic liposomes nevertheless seem to constitute one of the most sensitive ultrasound-controlled release systems yet described.

For convenience of assay, calcein was used as hydrophilic drug stand-in. It registers the fraction of the aqueous phase entrapped and hence also provides a good measure of how much of a hydrophilic drug would be encapsulated by echogenic liposomes. Because liposomes also have the potential to entrap hydrophobic drugs and other bioactive molecular such as proteins, enzymes, oligonucleotides and DNAs, the possibility should not be overlooked that these could also be encapsulated into acoustically active liposomes and also be released upon ultrasound stimulation.

Liposomes can be conjugated with antibodies, peptides, etc., and ultrasound can be focused on essentially any specific site, so, such combinations of targeting procedures should allow for highly localized drug delivery [26,27]. A previous investigation of acoustically active liposomes for diagnostic imaging showed that intravenous injection of 2 mg anti-fibrinogen echogenic liposomes enhanced the image of left ventricular thrombus within 10 min [28]. Admittedly, however, drug delivery in vivo may require longer circulation times than does imaging, and may require measures to avoid uptake by the RES. Methods to reduce RES uptake include inclusion of polyethylene glycol-derivatized (PEG) lipids or increased lipid dose [29]. The former approach seems quite feasible with acoustically active liposomes because we have found that their echogenicity is not significantly changed by PEG inclusion (to be published). Thus, the strategy of ultrasound-controlled drug delivery with acoustically active liposomes may have many potential applications to specific clinical conditions such as cancer, thrombus, restenosis and angiogenesis.

Acknowledgements

This work was supported by an American Heart Association Fellowship 0225486Z to S. Huang and NIH grant GM52329. We thank David McPherson for use of the ultrasound imaging facilities and Christy Holland for advice on ultrasound irradiation procedures.

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