In Vitro Evaluation of Polymerized Liposomes as an Oral Drug Delivery System

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The physical characteristics of polymerized liposomes for potential use as an oral drug delivery system were examined *in vitro*. The trap efficiency in monomeric liposomes composed of 1,2-di (2,4-octadecadienoyl) phosphatidylcholine was increased from 3% for original multilamellar vesicles to 35% for freeze-thaw treated liposomes. Polymerized liposomes with azobis (isobutyronitrile) and azobis (2-amidinopropane) hydrochloride as radical initiators showed complete stability against solubilization by Triton X-100, a detergent chosen to mimic bile salts. Release rates of ^{14}C-BSA and ^{14}C-sucrose in media simulating the gastro-intestinal fluids was 50% less than from regular liposomes composed of hydrogenated egg phosphatidylcholine mixed with cholesterol (molar ratio 1:1), which can be regarded as one of the most stable types of regular liposomes. It was estimated that, when administered orally, polymerized liposomes can reach the intestine while maintaining their vesicle structure and keeping at least 75% of their original content.

KEY WORDS: oral drug delivery; polymerized liposomes; Peyer's patches; stability in G-I fluids.

INTRODUCTION

The oral route is the most convenient way to administer drugs and vaccines. However, this route is not applied for many substances which are unstable and poorly absorbed through the gastro-intestinal (G-I) tract (1,2). In such cases, the alternative route for administration is generally injection. Many efforts have been devoted to achieve absorption enhancement of complex drugs such as peptides from the G-I tract. Those include using peptidase inhibitors (3), surface active agents (4), molecular modification to hydrophobic prodrugs (5), and hybridization of a drug with a ligand that can promote active transport through the intestine (6).

A variety of polymeric microparticles have previously been explored as oral delivery systems for complex molecules (*e.g.*, peptides, vaccines) because of the ability of these particles to be taken up by Peyer's patches (PP). PP are a part of gut associated lymphoid tissue and are naturally responsible for the uptake of macromolecular antigens from the intestinal lumen to the underlying lymphatic system for the acquisition of mucosal immunity (7). Therefore, particulate systems which protect drug molecules by entrapment and are taken up by PP into the lymph circulation, may be a promising concept.

A number of studies dealing with the intestinal uptake of polymeric microparticles revealed that particles with more hydrophobic surfaces and with a diameter smaller than 5~10 μm tend to be taken up better (8,9). It occurred to us from such findings that liposomes, which consist of phospho-"lipid", may therefore be a promising particulate system for oral drug delivery to PP. Liposomes have several characteristics which may render them desirable as such an oral drug delivery vehicle including their ability to encapsulate hydrophilic and hydrophobic substances separately and their small size and corresponding narrow size distribution. In addition, it is possible to prepare liposomes without the aqueous phase being exposed to organic solvent (10) (aqueous solutes, especially peptides and proteins, often lose their activity when exposed to organic solvents). However, most liposomes are not stable to acids, bile salts or enzymes to which they are exposed during gastrointestinal exposure (11,12) and therefore little attention has been given to conventional liposomes as oral drug carriers.

Polymerized liposomes (13,14) were therefore considered as potential carriers for oral drug delivery. Polymerized liposomes are different from regular liposomes in that the phospholipid molecules comprising the bilayers are linked together by covalent bonds. Polymerized liposomes thus retain many of the liposome's original features mentioned above. Most studies of polymerized liposomes have dealt with liposomes of very small size and a low capacity to include substances in their aqueous space (15,16). To our knowledge, there have been no previous reports of polymerized liposomes as an oral drug carrier. In this article, as the first step to examine the potential applicability of polymerized liposomes to oral drug delivery, the stability of polymerized liposomes *in vitro* was examined.

MATERIALS AND METHODS

Materials

The polymerizable phospholipid, 1,2-di (2,4-octadecadienoyl) phosphatidylcholine (DODPC) was purchased from Nippon Oil & Fats Co. Ltd. A thin-layer chromatography (Merck, Silica gel) developed in 65:35:5 chloroform/methanol/water (v/v) resulted a single spot at Rf=0.3. Azobis (isobutyronitrile) (AIBN) and azobis(2-amidinopropane) hydrochloride (AAPD) purchased from Polyscience (Warrington, PA) were used as polymerization initiators. AIBN and AAPD are purified by duplicate recrystallization in methanol and in water, respectively. The non-polymerizable phospholipid, hydrogenated egg phosphatidylcholine (Avanti polar lipids, Inc.; Alabaster AL), was used without further purification to prepare regular liposomes to be compared with polymerized liposomes.

^{14}C-labeled sucrose and ^{14}C-labeled bovine serum albumin (BSA), as model compounds to be entrapped in liposomes, and Triton X-100 were purchased from Sigma (St. Louis, MO). Taurocholate Na (Sigma; St. Louis MO) and phospholipase A_2 (Boehringer Mannheim; Indianapolis, IN)

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were used as received, for release studies. Other chemicals were analytical grade.

Preparation of Monomeric Liposomes

The preparation procedure was based on Ohno *et al.* (17) with some modifications. 200 mg of DODPC and 8.4 mg of AIBN were dissolved in a mixture of 2 ml of chloroform and 20 ml of diethyl ether. Solvents were evaporated at 30°C in a rotated round flask connected to an aspirator. To the dried thin film was added aqueous solution (2.5 ml) containing AAPD (13.9 mg) and ^{14}C-sucrose (0.1 μCi/ml) or ^{14}C-BSA (0.1 μCi/ml). The concentration of BSA in 0.1 μCi/ml ^{14}C-BSA solution was as low as 3.3 μg/ml. In order to minimize adsorption of ^{14}C-BSA on glass vessels during the preparation process, 1 mg/ml of non-labeled BSA was added to ^{14}C-BSA solution. Both AIBN and AAPD are 20 molar % to DODPC. The film was hydrated by gentle shaking under nitrogen gas at 40°C. The multilamellar-vesicle suspension thus prepared was treated by three cycles of freeze-thaw treatment. Freezing and thawing were performed respectively in liquid nitrogen and in a 40°C water bath. Probe sonication (Vibra Cell-250; Sonics & Materials Inc.) of 20 W was conducted at room temperature for 10 seconds to reduce the size of liposomes below about 10 μm diameter. As reference samples in release studies, regular liposomes composed of hydrogenated egg phosphatidylcholine and cholesterol (molar ratio 1:1) were prepared in a similar manner to DODPC liposomes, except that 1) 133 mg of hydrogenated egg phosphatidylcholine and 67 mg of cholesterol were dissolved in 20 ml of chloroform, 2) neither AIBN nor AAPD was added, 3) dried thin film was hydrated at 60°C, and 4) thawing in F-T treatment was done at 60°C.

Characterization of Liposomes

The size distribution of DODPC liposomes was measured by Coulter Counter Model ZB (Coulter Electronics Inc., Hialeah, FL) equipped with an aperture tube with a 50 μm hole. A channelizer was attached to the Coulter Counter to process raw data to obtain size distribution profiles. To measure the trap efficiency, liposomes were separated from unentrapped model compound by repeated centrifugation at 8000rpm (Sorvall RC-5B, DuPont) and replacement of buffer. To the centrifuged liposome pellet was added 1% Triton X-100 solution and sonication (40 W) was conducted at room temperature for 1 min. to extract the model compound from liposomes. Radioactivity was measured by liquid scintillation counting (Tri-carb 4530; Packard Instruments, Downers Grove, IL) after appropriate dilution. Freeze fracture electron micrographs were obtained to evaluate the inside structure of liposomes. As a cryoprotectant, 30% glycerol was used (18), and the fracturing and replication was done using a Balzers type 301 freeze fracture apparatus. Platinum replicas with carbon shadow were observed with a transmission electron microscope (EM-301, Phillips).

Polymerization of Liposomes

The monomeric DODPC liposome suspension without removal of unentrapped model compound was kept at 60°C for 5 hours with nitrogen gas sealing. After completion of polymerization, the model compound free from liposomes was removed by repeated centrifugation and replacement of buffer. The resultant polymerized liposomes were suspended in phosphate buffer. In a separate set of experiments to follow polymerization progress, monomeric DODPC liposomes were prepared in the same manner, but with pure water as the aqueous phase and kept at 60°C. At appropriate intervals, aliquots of the suspension were sampled and absorbance at 255 nm, which detects conjugated double bonds in DODPC molecules, was measured spectrophotometrically after appropriate dilution. The percentage of absorbance at 255 nm at each time point to the initial absorbance was regarded as polymerization progress %, neglecting a slight turbidity. The stability of the polymerized liposomes was estimated by the procedure of Ohno *et al.* (19). That is, the intensity of laser light scattering was measured (Dynamic light scattering apparatus BI-90; Brookheaven Instrument) with the stepwise addition of Triton X-100 at room temperature. The wavelength of the Argon-ion laser was 488 nm and the scattering angle was 90°.

Release Studies

60 mg of Polymerized (DODPC) or regular (hydrogenated egg phosphatidylcholine + cholesterol) liposomes containing ^{14}C-sucrose or ^{14}C-BSA of about 20 pCi were suspended in 10 ml of release medium and shaken slowly (100 strokes/min.) at 37°C under sealing with nitrogen gas. Release studies were done in triplicate for each sample and for each release medium. At appropriate intervals, the suspension was centrifuged to separate the supernatant. 1.5 ml of the supernatant was mixed with 10 ml of scintillation fluor (Aquasol 2; New England Nuclear) and radioactivity was measured using liquid scintillation counting(Tri-carb 4530; Packard Instruments, Downers Grove, IL). Fresh medium was added to the liposome pellet and the release study was continued after dispersion by vortexing.

The medium for the release study was designed to simulate gastrointestinal fluids. Because the gastric fluid has a pH around 2, one set of release media contained 0.01N hydrochloric acid and was adjusted to be isotonic using sodium chloride (model gastric medium). However, the composition of the intestinal fluid and concentrations of its components are not completely defined. Constituents of the intestinal fluid which may influence the stability of liposomes are bile salts and phospholipases. Sodium taurocholate has the highest capacity to solubilize lecithin among bile salts (20). Bile salt concentration in the intestinal lumen is 10mmol/liter (21), which corresponds to about 5 mg/ml. Phospholipase A_2 (PLA_2) concentration in pancreatic juice was measured as 1.5 to 16 U/ml (22). Calcium ion is needed to activate PLA_2 and the concentration should be between 1 and 10 mM (23,24). Consequently, a second set of release media in this study simulating intestinal fluid was chosen: 10 mg/ml sodium taurocholate, 5 U/ml PLA_2, and 3 mM calcium chloride in pH 7.4 phosphate buffer isotonic saline (model intestinal medium). When the model compound was ^{14}C-BSA, a release study in the model gastric medium was not done, because of degradation of BSA. Preliminary experiments (data not shown) showed that ^{14}C-BSA did not adsorb on glass vessels during release studies using the model intestinal medium.

RESULTS

Freeze-Thaw Treatment and Trap Efficiency

Multi-lamellar-vesicles (MLV) of DODPC prepared by the hydration of the dried lipid film showed as low as a 3.0 ± 0.6% trap efficiency for both ^{14}C-sucrose and ^{14}C-BSA. The trap efficiency is the ratio of aqueous solute entrapped in the liposomes divided by the total amount of the solute used. In this case, the radioactivity measured from liposome after removal of the model compound free from liposomes was 1.6 (±0.3) × 10^4 dpm, while the total radioactivity used in each preparation of liposomes was 0.25 μCi, which corresponds to 5.5 × 10^5 dpm. However, three cycles of freeze-thaw (F-T) treatment increased the trap efficiency up to 31 ± 4% for both ^{14}C-sucrose and ^{14}C-BSA, when the phospholipid concentration in the aqueous suspension was 80 mg/ml. This result was obtained from the fact that the radioactivity derived from liposomes was 1.7 (±0.2) × 10^5. Since the trap efficiency depends on the lipid concentration, the entrapped volume of the aqueous phase in unit moles of the lipid was calculated, assuming that the concentration of the model compound in the intra-liposome aqueous phase and in the inter-liposome aqueous phase was the same after F-T treatment (25). The trap volume reached 3.1 liter/mole. This is in the same order of magnitude as reported by Mayer *et al.*, that is 5.27 liter/mole when the lipid concentration was 100mg/ml (25).

To evaluate the trap ratio increase, freeze-fracture electron micrographs of the liposomes before and after F-T treatment were observed. Figure 1(a),(b) are examples showing a dramatic structural change inside the liposomes. That is, the original MLV (Fig. 1(a)) has many concentric lamellae and, as a result, a small volume of the intra-liposome aqueous space. However, F-T treatment changed the inside structure of liposomes, as shown in Figure 1(b), where small liposomes are included in a large one and there are large aqueous spaces between them. Morphologies inside of MLVs and F-T treated liposomes that were observed in other freeze-fracture electron micrographs (not shown) were similar to the typical examples in Figure 1(a) and Figure 1(b), respectively.

In addition, the size distribution before and after F-T treatment was measured (Fig. 2). The height of each bar shown as "total volume of liposomes" on the ordinate of Figure 2 represents the sum of apparent volume of all the liposomes that belong to each size range shown on the abscissa, included in 1 ml of the liposome suspension. The apparent volume of a liposome means a volume that includes bilayers of the liposome and intra-liposome aqueous spaces. The total apparent volume of liposomes in the range from 1 to 9 μm increased by the F-T treatment. The increase in the sum of all the volumes from 1 to 9 μm was 3.0 times.

Polymerization of DODPC liposomes was done without removal of unentrapped model compounds. Trap efficiency after polymerization was similar to that of pre-polymerization.

Polymerization Progress

DODPC liposomes that were prepared with pure water as the aqueous phase instead of the model compound solutions were used to follow polymerization progress, because of the UV absorbance of the model compound. Polymerization progress was followed by measuring spectrophotometric absorbance at 255 nm. The percentage decrease of absorbance at each time point from the initial absorbance was designated as polymerization progress %. Polymerizations using AIBN or AAPD individually or together were performed at 60°C. With only AIBN as an initiator, some conditions allowed good polymerization progress. For example, when pure chloroform was used to dissolve lipid, which would be dried via a rotary evaporator to form the thin film, polymerization progress % terminated between 0 and 50%, unreproducibly. Mixing of diethyl ether with chloroform gave better results. Nevertheless, even using the mixture of diethyl ether and chloroform, polymerization was not initi-

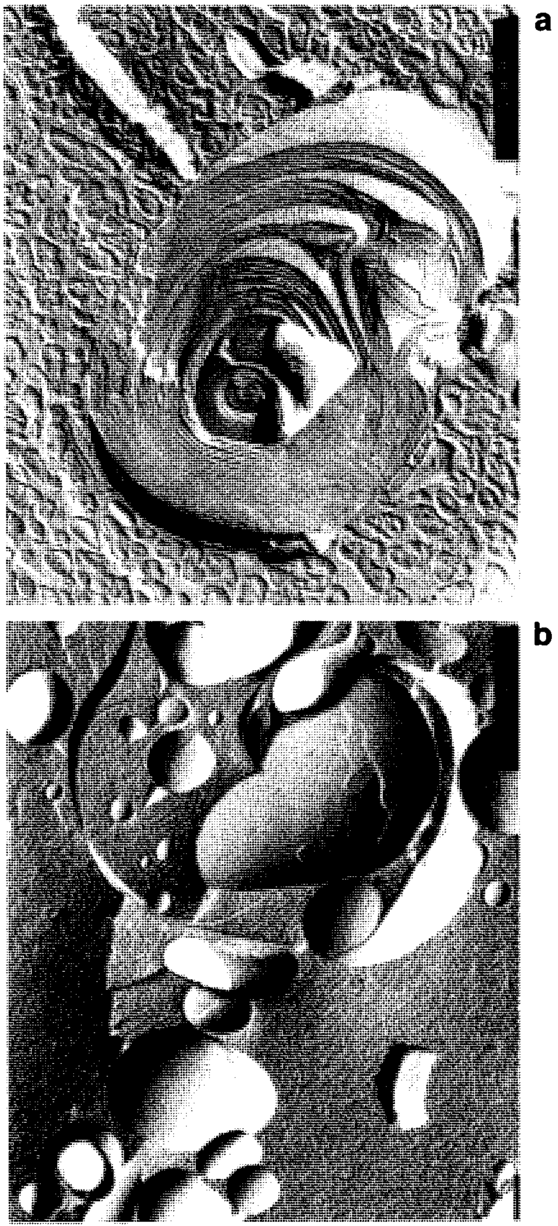

Fig. 1. Inside structure of liposomes before (a) and after (b) freeze-thaw treatment observed with freeze fracture electron microscope.

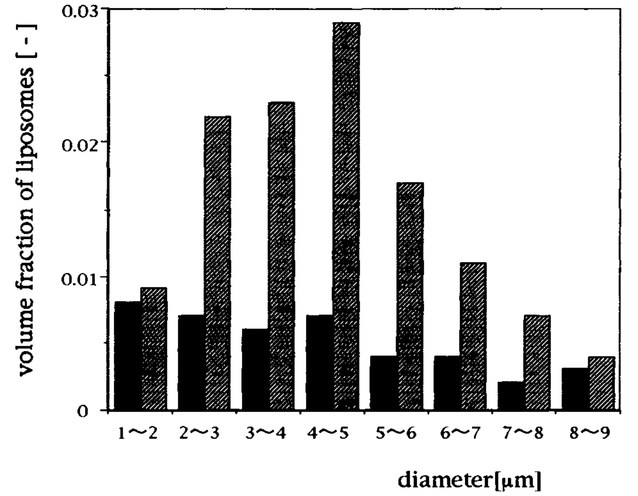

Fig. 2. Size distribution of liposomes before and after freeze-thaw (F&T) treatment. (Ordinate represents the total of external volume of liposomes that belong to each size range shown in abcissa, in 1 mL of the liposome suspension) ■, before F&T; ▨, after F&T.

ated when the solvents were completely removed by high vacuum drying. The profiles of polymerization progress % are shown in Figure 3. The profile with AIBN as the initiator was obtained when the mixture of diethyl ether and chloroform was used to dissolve the lipid and AIBN, and the evaporation of the solvents were performed via a rotary evaporator connected to an aspirator but not via high vacuum. The polymerization progress % with AAPD terminated 45 ± 5% reproducibly under all conditions, regardless of the lipid solvent or the evaporation process of the solvent. The polymerization progress % terminated around 50% using either AIBN or AAPD, and reached almost 100% with both. From Figure 3, about 5 hours is enough to complete the polymerization with radical initiators at 60°C.

Stability of Polymerized Liposomes

The principal driving force to maintain the bilayer and vesicle structure of liposomes is the hydrophobic interaction and Van der Waals force between each phospholipid molecule. Therefore, strong surface active agents including bile salts in the intestine could possibly break liposomes by extraction and solubilization of phospholipids. Because the purpose of preparing polymerized liposomes instead of regular ones is to get higher stability in the G-I tract, the stability of polymerized liposomes against surface active agents was examined. Evaluation was performed by measuring the intensity of laser light scattering from the liposome suspension with stepwise addition of Triton X-100. As shown in Figure 4, addition of Triton X-100 to the pre-polymerized liposome suspension gave a sudden decrease in intensity, suggesting complete solubilization. In contrast, polymerized liposomes, especially those prepared with both initiators, showed little change in intensity by the addition of Triton. These results suggest the high stability of polymerized liposomes against breakage which may be induced by surface active agents in the G-I tract, such as bile salts.

Release Studies

Release studies were done under conditions simulating the G-I environment. The release profiles of the model compounds, ^{14}C-labeled sucrose or ^{14}C-labeled BSA, from polymerized liposomes and from regular liposomes, as references, are shown in Figures 5 and 6. Regular liposomes are prepared from hydrogenated egg phosphatidylcholine and cholesterol (molar ratio 1:1). Because of the degradation of BSA, a release study of ^{14}C-BSA in the model gastric medium was not done. The release patterns of ^{14}C-sucrose from both polymerized and regular liposomes were usual, *i.e.*, fast in the initial stage and gradually slower. The released percentage from polymerized liposomes was less than half of the percentage from regular ones. Polymerized liposomes released 25% and 7% of their contents in the model intestinal medium and in the model gastric medium, respectively, in 2 days, while regular liposomes released 70% and 40% in the former and latter medium, respectively, in the same period. In the case of ^{14}C-BSA release, polymerized liposomes showed an irregular pattern, *i.e.*, initial burst up to 25% in 1

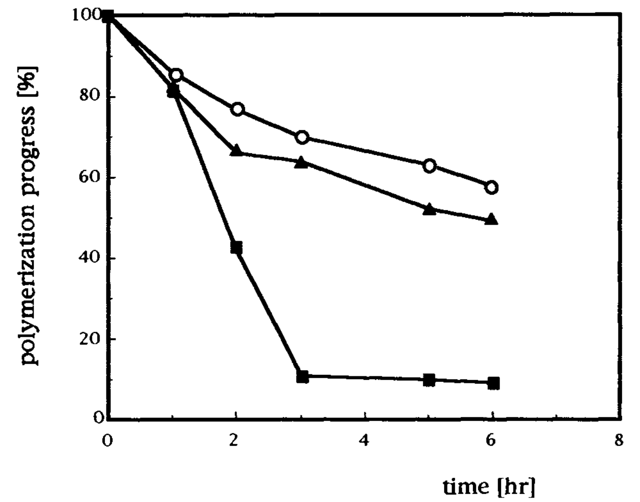

Fig. 3. Polymerization profiles of DODPC, that form liposome bilayers. radical initiator; ○ : AIBN, ▲ : AAPD, ■ : both.

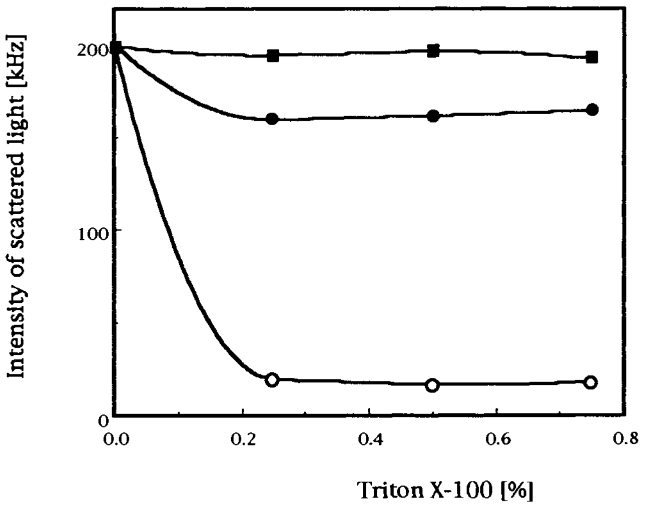

Fig. 4. Stability of polymerized liposomes against Triton X-100. ○, before polymerization; ●, polymerized by AIBN; ■, polymerized by both AIBN and AAPD.

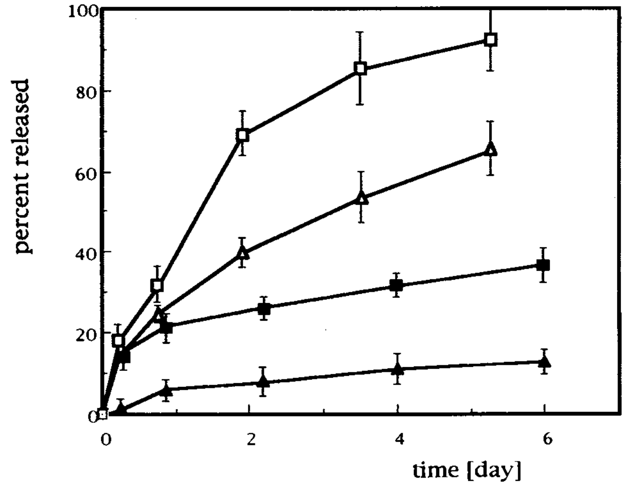

Fig. 5. Release profiles of ^{14}C-sucrose from polymerized* or regular** liposomes. (* by both initiators, ** hydrogenated egg PC + cholesterol) ■, polymerized liposomes in bile medium; ▲, polymerized liposomes in pH2 medium; □, regular liposomes in bile medium; △, regular liposomes in pH2 medium.

day and subsequent very slow release in the model intestinal medium. Even with the initial burst, the release from polymerized liposomes was slower than from regular ones (Fig. 6).

DISCUSSION

In preparing DODPC monomeric liposomes, the trap efficiency increased by F-T treatment. The inside structure of liposomes before treatment was very dense consisting of many concentric lamellae with narrow interstices (Fig. 1(a)) and the size distribution was broad from less than 1 μm to around 10 μm (Fig. 2). F-T treatment not only converted the structure of each liposome to a loose one containing large intra-liposome aqueous spaces (Fig. 1(b)), but also increased the total apparent volume of liposomes with diameters from 1 to 9 μm as shown in Fig. 2. Therefore, the increase of both intra-liposome aqueous volume in each liposome and the total apparent volume of all the liposomes contributed to increase the total trap efficiency. It is possible that there exists liposomes smaller than 1 μm diameter, the detection limit in size distribution measurements (Fig. 1). However, the contribution of such small liposomes to the total apparent volume and the trap efficiency would be very small, because volume is proportional to the third power of diameter.

After F-T treatment, polymerization of DODPC liposomes was done without removal of unentrapped model compound. The trap efficiency was not changed by polymerization. This suggests that the inside morphology of liposomes was unchanged during polymerization, and supports that the distribution of model compounds between intra- and inter-liposome aqueous phase was already equilibrated by F-T treatment.

Certain conditions are needed to achieve good polymerization when AIBN was used as the radical initiator as discussed in RESULTS. When pure chloroform was used and/or mixed solvents of chloroform with diethyl ether were completely removed, and polymerization progress % terminated between 0 and 50% unreproducibly, white spots were discerned on the dried lipid film before addition of the aqueous phase. Therefore, it was believed that AIBN recrystallized out of the lipid film to form the spots during the solvent evaporation process, and that the recrystallized AIBN was not available to initiate polymerization. On the other hand, Takeoka *et al.* (26) prepared small unilamellar vesicles (SUV) of DODPC by a similar procedure, except that they used pure chloroform to dissolve DODPC and AIBN, and they conducted intense sonication (60 W for 20 min) to make very small liposomes (ca. 30nm diameter). Afterward, polymerization of DODPC initiated by AIBN progressed to around 50% polymerization progress %. Therefore, it can be estimated that the intense sonication accelerated the recrystallized AIBN to dissolve in the aqueous phase, and to partition into the lipid layer. The re-partitioned AIBN was available to initiate polymerization. Consequently, the solvents, the evaporation rate, and the degree of dryness should be carefully determined to prevent recrystallizing of AIBN in the preparation process without intense sonication.

It was already known with SUV (17) that the conjugated double bond at the position 1 chain of each DODPC molecule is located deep in the hydrophobic region of the bilayer and only AIBN can initiate polymerization of this double bond. On the other hand, the conjugated double bond at the position 2 chain is located close to the aqueous phase and only AAPD, which dissolves in the aqueous phase, can initiate polymerization of this double bond. It is shown in this study that polymerization progress % terminated around 50% when one of the radical initiators was used (Fig. 3). These results suggest that AIBN or AAPD can initiate polymerization of one of the conjugated double bonds at the position 1 chain and at the position 2 chain of DODPC in the large liposomes as well as in SUV. These results are reasonable because of the lower curvature of the bilayers and closer packing of the phospholipid molecules in the large liposomes.

When microparticles are administered orally, they will

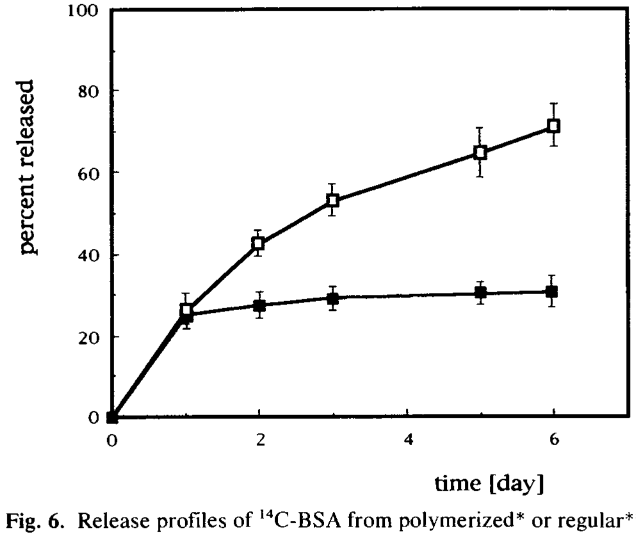

Fig. 6. Release profiles of ^{14}C-BSA from polymerized* or regular** liposomes in bile medium. (* by both initiators, ** hydrogenated egg PC + cholesterol) ■ : polymerized liposomes, □ : regular liposomes.

first reach the stomach where the environment is strongly acidic. In several hours (27), they transfer into the intestine where bile salts and enzymes including phospholipases are secreted in the neutral pH fluid. Transit time in the intestine is up to 24 hours (27). Therefore, polymerized liposomes have to be stable under physiologic conditions for up to 24 hours. However, in this preliminary study to examine the applicability of polymerized liposomes to oral drug delivery, stability of polymerized liposomes and of regular liposomes were compared. In future studies, the stability and other surface characteristics of polymerized liposomes, that may influence uptake efficiency and process after the uptake, should be examined. From such considerations, stability was evaluated in 1% Triton, which may be more surface-active than many physiological agents, and release profiles were observed for periods longer than 24 hours.

Polymerized liposomes show complete stability against solubilization even in nearly 1% Triton (Fig. 4). The results suggest that orally administered polymerized liposomes would potentially maintain their particulate structure in the G-I tract. The *in vitro* release rates from polymerized liposomes were slower than from regular liposomes composed of egg hydrogenated phosphatidylcholine and cholesterol(1:1 molar ratio) (Figs. 5 and 6). Both polymerized and regular liposomes were prepared under non-isotonic conditions and the *in vitro* release studies were performed under isotonic conditions. Although these conditions may accelerate release, it is obvious that the release rate from polymerized liposomes was relatively slower than that from regular ones. The regular liposomes of this composition can be regarded as one of the most stable types of monomeric liposomes because of the high phase transition temperature (approx. 45°C) of the phospholipid (28) and high cholesterol content (29). Therefore, the release rates of ^{14}C-BSA or ^{14}C-sucrose from polymerized liposomes are slower than any type of regular liposomes. The release percentage from polymerized liposomes in the simulated G-I fluid was at most 25% in the first 24 hours (Figs. 5 and 6). Considering the usual transit time of substances in the G-I tract, the release rate is slow enough to keep at least 75% of the original content in polymerized liposomes until they are transferred to the PP region. In addition, slow release characteristics from polymerized liposomes were observed not only for large molecules such as BSA, but for small ones such as sucrose.

The characteristics of polymerized liposomes as shown above suggest the potential applicability for oral drug delivery. Future studies will examine the toxicity of polymerized liposomes, uptake by Peyer's patches, cellular processing of polymerized liposomes *in vivo* and the characteristics of polymerized liposomes that maximize uptake by Peyer's patches.

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