Magnetically-Responsive Polymerized Liposomes as Potential Oral Delivery Vehicles

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INTRODUCTION

Recently, nano- or microparticles have been used as a means to protect complex molecules such as peptides or proteins (e.g. vaccines) from the harsh gastrointestinal environment and targeting them to the Peyer's patches (1). In our laboratory, the potential of using polymerized liposomes as such delivery vehicles has been under investigation (2,3). When polymerized, liposomes have also been shown to remain stable in animal gastrointestinal tracts (3).

Successful application of vehicles such as polymerized liposomes for oral delivery is, to a large extent, dependent on the uptake of these particles by the Peyer's patches following oral administration (1). The epithelium overlying the Peyer's patches contain specialized "sampling" cells, which can take up a small percentage of the orally administered particles and transport them into the blood circulation (1,4). Previous work with polymerized liposomes confirmed that a small amount of orally-administered polymerized liposomes can be taken up by mouse Peyer's patches (3). However, the delivery efficiency is relatively low (2).

One factor that may, at least partially, be responsible for the less than optimal delivery efficiency observed for oral delivery vehicles is the difficulty for the vehicles to remain in the intestine near the Peyer's patches for an extended period of time, before they are swept away by intestinal contents and intestinal movement. In other words, if it were possible to localize these vehicles in the intestine for a longer period of time, it might increase their delivery efficiency.

In the literature, different approaches have been examined to attempt to slow down the intestinal transit of orally delivered drug carriers. For instance, mucoadhesive polymers which can adhere to mucus layer in the intestine have been widely studied (5,6). Here, the transit time of these polymeric carriers is determined by the physiological turn-over time of the mucus layer. This turn-over time is, in most cases, only slightly longer than the normal intestinal transit time.

In 1978, Widder *et al.* encapsulated Fe_3O_4 particles into albumin microspheres together with a chemotherapeutic agent. They injected the microspheres intravenously and then localized them to a specified *in vivo* target site using an external magnetic field (7). Since then, various applications have been proposed for injectable magnetically-responsive particles (8,9).

It occurred to us that we may be able to use magnetically-responsive polymerized liposomes as potential oral delivery vehicles. In this study, we incorporated Fe_3O_4 particles into polymerized liposomes so that the liposomes became magnetically responsive. After the liposomes were orally administered to mice, a magnetic field was created external to the mouse abdomen around the mouse intestine area to retain these magnetically-responsive liposomes within the intestine. In this way, we hope to study the effect of intestinal transit on liposome delivery efficiency.

EXPERIMENTAL METHODS

Preparation of Magnetically-Responsive Polymerized Liposomes

Liposomes were formed from 1,2-Di(2,4-octadecadienoyl)-sn-glycerol-3-phosphorylcholine (DODPC, Kawahara Yuka Co.) using thin-film hydration method. 1,2-Dipalmitoyl-L-3-phosphatidyl[N-methyl-^{3}H]choline ([^{3}H]DPPC, Amersham Life Science, Inc.) (1mCi/mL) was added as a lipid marker. The lipid film was hydrated with phosphate buffer saline solution (PBS, pH 7.4) containing 0.3% colloidal Fe_3O_4 particles (aqueous-based ferrofluids EMG 807, Ferrofluids Corp., NH) at 30°C. The liposomes formed were freeze-thawed and extruded through stacks of polycarbonate membrane filters of 100 nm pore size (Poretics, CA). Redox initiators $Na_2S_2O_5$ and $K_2S_2O_8$ (10 mol% of DODPC, $Na_2S_2O_5$: $K_2S_2O_8$ = 1:1) were added to the suspension and the liposomes were polymerized at room temperature overnight under inert gas.

The liposomes obtained were purified from the unencapsulated Fe_3O_4 particles on a Sephacryl-1000SF column (1.5cm×12cm, Pharmacia) with PBS as eluting buffer. The final lipid concentration was adjusted to ~ 10 mg/mL. The Fe_3O_4 particles that were not encapsulated in the liposomes were also eluted from the column and collected for further analysis.

Characterization of Magnetically-Responsive Polymerized Liposomes

The percentage of the Fe_3O_4 particles that was incorporated into the liposomes was determined using atomic absorption spectroscopy. The unencapsulated Fe_3O_4 particles collected from the Sephacryl-1000SF column were first hydrolyzed in concentrated nitric acid and concentrated hydrochloric acid according to a standard procedure (10). The hydrolysate was then analyzed at 248.3 nm wavelength using a Perkin-Elmer 403 Flame type atomic absorption spectrometer (Perkin Elmer, IL) to obtained its iron concentration (10). This information was then used to estimate the amount of unencapsulated Fe_3O_4.

The stability of these Fe_3O_4-containing polymerized liposomes against detergent dissolution was tested with Quasi-elastic Laser light Scattering (QELS) (Model BI-90, Brookheaven) using TritonX-100 as described before (2,3). Briefly, a change in scattered light intensity was recorded after addition of TritonX-100 to a diluted liposome suspension (lipid concentration ~ 1 mg/mL). Both polymerized as well as unpolymerized liposomes were examined.

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In Vitro Magnetic Responsiveness

An *in vitro* flow apparatus (Figure 1) similar to the one described by Senyei *et al.* (11) was used to examine the magnetic responsiveness of the Fe_3O_4-containing liposomes under flow conditions. A syringe pump (P), (Model M361, Orion Reserach, MA) was used to generate controlled flowrate with PBS in a silicone tubing (T), (1/16″ i.d., Biorad) (Figure 1). The tubing diameter was selected based on that of the mouse small intestine (12). A constant flowrate of PBS at 0.3 mL/min was used.

After the flowrate became constant, a 100 μL bolus of the Fe_3O_4-containing polymerized liposomes (S) was introduced into the flow stream through a three-way valve (V) (Biorad, CA). Fractions were then continuously collected from the end of the tubing (E) at one-minute intervals. This process was repeated with the silicone tubing securely positioned next to a rectangular-shaped neodymium permanent magnet (M; 2″ × 2″ × 0.5″, surface field strength 2500 Gauss), (Magnet Sales, CA). At all times, the magnetic field generated by the magnet was perpendicular to the flow stream. The amount of liposomes eluted in each fraction collected was quantified by analyzing the ^3H activity in the fraction using a Liquid Scintillation Counter (Tri-Carb 2000CA, Packard Instrument). The total amount of liposomes introduced into the flow stream was determined by measuring the ^3H activity in 100 μL of the liposome suspension.

In Vivo Absorption Study

Two groups of four mice each (female Balb/C of 19–21 grams, Charles River Lab) were used to study the *in vivo* uptake of Fe_3O_4-containing polymerized liposomes. All mice were fasted for at least 12 hours before the experiment but allowed free access to water. Each mouse was gavaged with 200 μL of Fe_3O_4-containing liposomes using a 24 gauge ball-tipped gavage needle (Harvard Apparatus, MA). Food was restored immediately after liposome administration. Forty minutes post administration, mice from groups 1 were restrained using acrylic mouse restrainers (Harvard Apparatus, MA) and each of the mice was placed on top of a neodymium permanent magnet identical to the one used above. The mice were positioned so that the magnetic field was localized around the intestinal area. The mice from group 2 were restrained but were not exposed to the magnetic field. Two hours post administration, all mice were anaesthetized with ether inhalation and blood was collected as described elsewhere (13). The mice were then sacrificed with carbon dioxide. Incisions were made in the abdomen (13) and the small intestines were removed first. Spleen, mesentery, liver, kidney, heart, and lung were collected. The excised intestines were then cut open along their mesenteric edges and the lumen unfolded with the mucosal surfaces facing upwards. The lumenal sides of the small intestines were thoroughly rinsed with PBS containing 1 mM dithiothreitol to clear away any residual lumenal contents. Peyer's patches were visually identified with their unique appearance as white opaque nodules and then excised. A patch-free intestinal segment of ~ 2 cm in length was cleaned and excised as well.

All tissue samples were patted dry with surgical gauze before their weights were recorded individually. Samples obtained were treated with the tissue solubilizer Soluene-350® (Packard Instruments, CT) and counted for ^3H activity using the Liquid Scintillation Counter. Total activity administered to each mouse was quantified by counting 200 μL of the original liposome suspension.

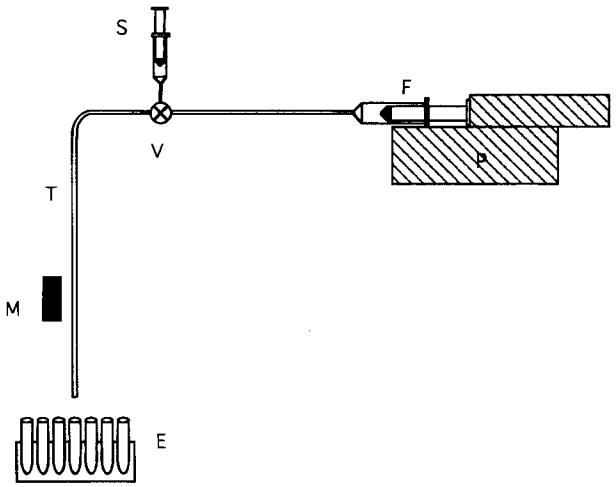

Fig. 1. Schematic representation of the *in vitro* flow apparatus for the examination of magnetic responsiveness of Fe_3O_4-containing liposomes. E: eluted fraction collector; M: neodymium magnet; P: syringe pump; S: sample injection port; T: silicone tubing; V: 3-way valve.

RESULTS AND DISCUSSION

The intestinal transit of liposomes is relatively fast in mice (~ 1–2 hours for small intestine) (3,12). During this time, liposomes are continuously pushed downward by intestinal flow as well as intestinal motions. As a result, most liposomes fail to have sufficient time to reach the Peyer's patches before they are swept further down. In order to slow down the liposome transit so as to retain the liposomes in the intestine for a prolonged period of time, magnetically responsive liposomes were prepared. These liposomes are localizable at desired areas when they are exposed to an external magnetic field.

The amount of Fe_3O_4 particles that were encapsulated inside the liposomes was about 30% of the total amount added. This was estimated from the iron concentration in the hydrolysis product of the unencapsulated Fe_3O_4 particles, which was determined using atomic absorption spectrometry.

The size distribution of Fe_3O_4-containing liposomes was determined using Quasi-elastic Laser light Scattering. The liposomes had an average diameter of about 100 nm. This was also confirmed by transmission electron microscopy (data not shown). The polymerized liposomes remained intact in the presence of the detergent Triton X-100 (Figure 2). On the other hand, the unpolymerized liposomes were completely dissolved by the addition of Triton X-100 (Figure 2). This indicates that incorporation of Fe_3O_4 particles in the liposomes does not interfere with the lipid polymerization process, and the polymerized liposomes are stable against detergent dissolution.

The magnetic-responsiveness of the liposomes was examined under flow conditions using the *in vitro* flow apparatus. The normal fluid flowrate in the mouse small intestine is only about 0.03 mL/min, as estimated from the small intestine transit time for fluid (12,14) and the small intestine length (12). When 0.03 mL/min was used in our flow apparatus, 100% retention of the liposomes were observed in the presence of the magnet

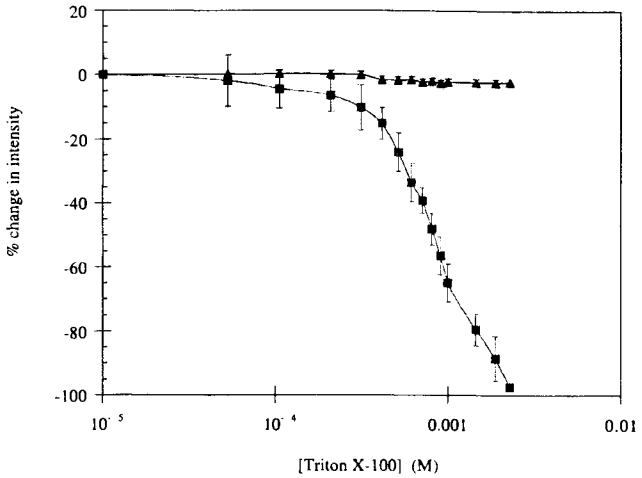

Fig. 2. *In vitro* stability of Fe_3O_4-containing liposomes against TritonX-100 dissolution, measured with Quasi-elastic Laser light Scattering (QELS) technique: comparison between unpolymerized (■) and polymerized (▲) liposomes.

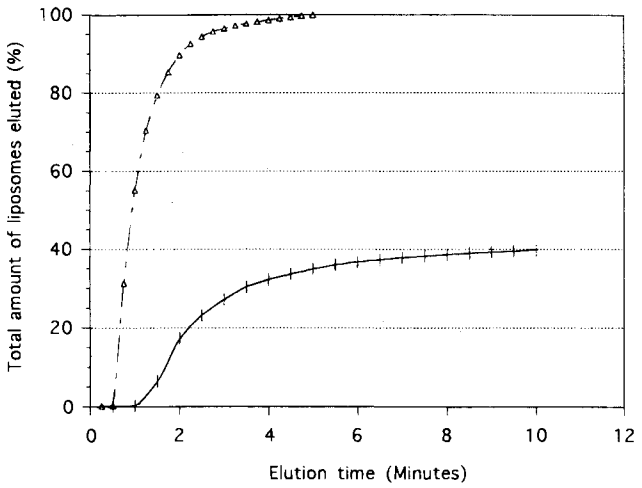

Fig. 3B. Magnetic responsiveness of liposomes: cumulative elution profiles from the *in vitro* flow apparatus with (△) or without (+) the effect of a magnetic field.

(data not shown). In other words, no elution of liposomes was observed. As a result, a flowrate of 0.3 mL/min was selected in this study to examine the responsiveness of these liposomes under extreme flow shear conditions.

^3H activity in each of the fractions collected from the *in vitro* flow apparatus was normalized against the total amount of liposomes introduced into the flow. The results were plotted versus elution time (Figure 3A). In the presence of the magnet, the majority of the liposomes were eluted at a later time as compared to the case when no magnet was used. The elution peak was observed at an elution time of two minutes as compared to one minute in the absence of the magnet (Figure 3A). This indicates that the Fe_3O_4-containing liposomes were indeed responsive to the magnetic field generated by the magnet.

The cumulative amount of liposomes eluted at any time point can also be obtained by taking the summation of all the liposomes that have already eluted by that particular time point.

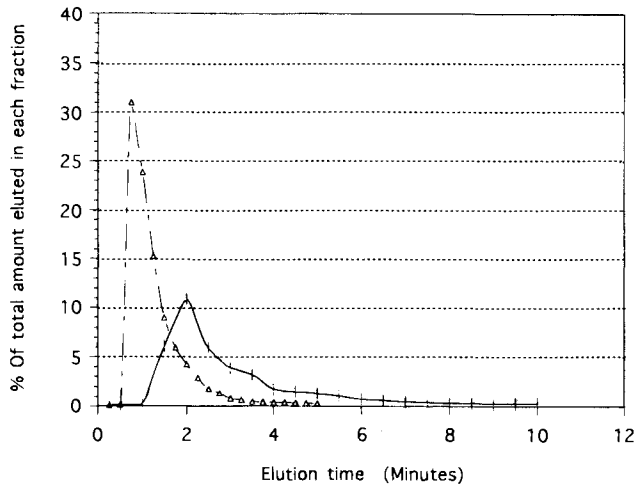

Fig. 3A. Magnetic responsiveness of Fe_3O_4-containing liposomes: elution profiles from the *in vitro* flow apparatus with (△) or without (+) the effect of a magnetic field.

The results were expressed in percentages and plotted in Figure 3B. By 5 minutes, 100% of the liposomes introduced were eluted from the tubing when the magnet was not used. In other words, no retention of liposomes in the tubing was observed when the magnet was absent. In the case when the magnet was used, the curve levels off at 10 minutes. By then only 40% of the total amount introduced were eluted from the tubing. The remaining 60% was retained within the tubing by the applied magnetic field.

In vivo absorption of the Fe_3O_4-containing liposomes was examined in mice. After liposome administration, the mice were given access to food and water for 40 minutes before they were restrained and exposed to the magnetic field. This was chosen based on the liposome gastric empty time in fed mice (3). In this way, when the external magnetic field was applied, the majority of the liposomes were localized within the intestine instead of the stomach.

The amounts of radioactivity retained in all tissue samples were measured and divided by the total activity administered to obtain the percent-distribution of the radiolabel in each tissue. These percentages were then normalized with individual tissue sample weight to compensate for animal-to-animal variations.

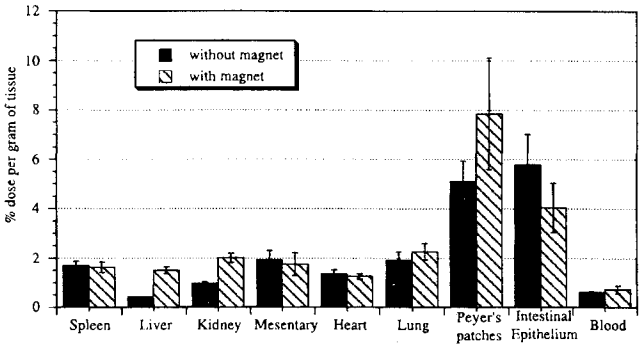

Fig. 4A. *In vivo* distribution of Fe_3O_4-containing polymerized liposomes in mouse tissues after a single dose oral administration. Each column represents the average of data collected from four mice. The errors presented were standard deviations calculated.

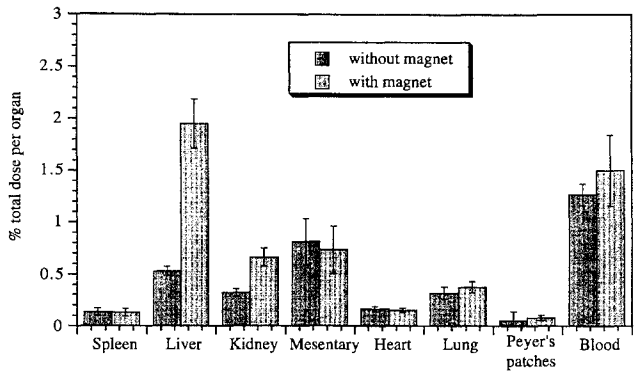

Fig. 4B. Retention of Fe_3O_4-containing polymerized liposomes in mouse tissues after a single dose oral administration. Each column represents the average of data collected from four mice. The errors presented were standard deviations calculated.

The normalized results are summarized in Figure 4A for all tissues examined. Compared to the animals that were not exposed to the magnetic field, a significantly higher level ($p < 0.001$, ANOVA test) of radioactivity was seen in organs such as liver and kidney when the animals were exposed to the magnetic field (Figure 4A).

If we take an average weight for each tissue as described previously (15), the amount of liposomes retained in each tissue can be estimated (Figure 4B). The tissue weights used for the estimation were calculated by averaging the tissue weight measurements obtained from all eight mice used. The summation of the numbers from all tissues shown in Figure 4B gave an estimation of the net delivery efficiency of the liposomes. When no magnet was used, 3.5 ± 0.6% of the total amount administered was absorbed. This is consistent with the bioavailability previously observed for polymerized liposomes that do not contain Fe_3O_4 particles (3,15). In comparison, an increased amount of the Fe_3O_4-containing liposomes (5.6 ± 1.0%) were absorbed when the mice were exposed to the external magnetic field. This latter value (5.6%) was significantly higher than 3.5% when the two numbers were examined using ANOVA test ($p < 0.05$). These results indicate that by localizing the liposomes in the intestine, we were able to increase the delivery efficiency for these liposomes.

The amount of liposome uptake *in vivo* was estimated here from the amount of radioactivity found in the tissues. This was justified by previous observations that polymerized liposomes remain intact in mouse gastrointestinal tract, and the radioactive membrane markers stay tightly associated with the liposomes (3). As a result, the amount of radioactivity detected in the tissues can effectively represent the amount of intact liposomes taken up from the gastrointestinal tract (3).

Finally, whether or not the percentage of liposomes absorbed can be increased beyond 5.6% by such strategies as including stronger magnetic particles into the liposomes, using a stronger external magnetic field, or other related strategies remains to be seen.

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