Preparation and Characterization of Dextran Magnetite-Incorporated Thermosensitive Liposomes: An on-line Flow System for Quantifying Magnetic Responsiveness

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Purpose. Dextran magnetite (DM)-incorporated thermosensitive liposomes, namely thermosensitive magnetoliposomes (TMs), were prepared and characterized in order to investigate their possibility for magnetic drug targeting. ***Methods.*** TMs containing calcein were prepared at various DM concentrations by reverse-phase evaporation of dipalmitoylphosphatidylcholine (DPPC). They were evaluated for their physicochemical properties including size, DM capture, magnetite distribution within liposomes, and temperature-dependent calcein release. Moreover, a novel on-line flow apparatus with a sample injector, a coil of tubing placed in an electromagnet, and a fluorescence detector was developed for quantifying the magnetic responsiveness of TMs. This device allowed us a real-time measurement of percentage holding of TMs by magnetic field. ***Results.*** Due to water-soluble property of DM, higher contents of magnetite up to 490 mg per mmol DPPC were successfully incorporated into the liposomes with DM than with conventional magnetite (Fe_3O_4). Thermosensitivity and lipid integrity of TMs were not influenced by inclusion of DM. Using the on-line flow system, percentage holding of TMs by magnetic field was shown to vary with several factors; it increases as the magnetic field strength increases, the fluid flow rate decreases, the magnetite content increases, and the liposome concentration increases. Typically, at 490 mg incorporated magnetite per mmol DPPC, 0.5 ml/min-fluid flow rate, and high magnetic field strength ($\geq$10 kiloGauss), approximately 100% of TMs were found to be held. ***Conclusions.*** The TMs were suggested to be useful in future cancer treatment by magnetic targeting combined with drug release in response to hyperthermia.

KEY WORDS: thermosensitive magnetoliposomes; drug targeting; cancer chemotherapy.

INTRODUCTION

There is considerable current interest in drug delivery improvement for increasing therapeutic efficacy and reducing systemic side effects. One of the major approaches is drug targeting to diseased organs or tissues by using magnetic field. Several types of magnetically responsive drug carriers have been proposed including albumin microspheres (1-2), chitosan microspheres (3), granules (4), and liposomes (5-8). Magnetoliposomes have been studied by several research groups. Kiwada *et al.* (5) reported a feasibility of magnetic liposomes as a targeting device for drugs. Cuyper and Joniau (6) presented generation and biophysical characterization of magnetoliposomes. Ishii *et al.* (7) described preparation, physicochemical properties of magnetoliposomes, and their possible use as a targeting carrier. More recently, Babincova (8) showed leakage of magnetoliposomes due to microwave irradiation.

The use of thermosensitive liposomes in conjunction with local hyperthermia has been attempted for cancer treatment (9-12). Besides earlier methods of heating (e.g., in a warm bath or with warmed perfusate), electromagnetic heating is now being investigated, especially for the local hyperthermia of deeper body structures. Dextran magnetite (DM) has been reported to be a new agent for selective heating by electromagnetic induction (13).

The above studies have led us to consider the combination of magnetoliposomes and thermosensitive liposomes, that is, the use of "thermosensitive magnetoliposomes" (TMs) for magnetic targeting associated with drug release in response to local hyperthermia. Therefore, the present research was designed to establish the condition of thermosensitive magnetoliposome (TM) preparation with an optimum magnetite content from the viewpoint of effective magnetic targeting. For this purpose, we have developed a novel on-line flow system for quantifying their magnetic responsiveness.

MATERIALS AND METHODS

Materials

DM was a gift from Meito Sangyo (Nagoya, Japan). Dipalmitoylphosphatidylcholine (DPPC) was kindly supplied by Nihon Yushi (Tokyo, Japan). Calcein (3,3′-Bis[N,N-di(carboxymethyl)-aminomethyl]-fluorescein), TES (N-tris[hydroxymethyl]methyl-2-amino-ethanesulfonic acid), and cobalt dichloride were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Polyethylene glycol *p*-*t*-octylphenol (Triton X-100) was supplied by Nacalai Tesque (Kyoto, Japan). All other reagents used were commercially available and of analytical grade.

The specifications of DM are as follows: a hematite impurity was under a detection limit according to the results of X-ray diffraction; an average molecular weight of dextran in DM is 4,000; a dextran/magnetite ratio is 0.43; its average core size is 8 nm ranging between 5 to 10 nm; and its coercivity, magnetic susceptibility, saturation magnetization, and T_2 relaxivity are 240 A/m, 0.3 $(gFe)^{-1}$, 0.1 $Wb/m^2 \cdot gFe$, and 220 $liter \cdot mmol^{-1} \cdot sec^{-1}$, respectively.

Methods

Preparation of TMs. TMs were prepared by reverse-

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phase evaporation (14, 15) at various concentrations of DM. Briefly, DPPC was dissolved in isopropyl ether and chloroform mixture (1:1, v/v). Then, it was emulsified with DM suspensions containing 10 μ*M* calcein by 5-min sonication at 40°C in a bath sonicator (Sonorex Super RK 156 BH, 150/300 W, 35 kHz, Bandelin Electronic, Berlin, Germany). The organic solvent was evaporated in a rotary evaporator at 42°C and low pressure (260-400 mmHg). TMs were separated from nonencapsulated DM and calcein by centrifugation (960 × g) at 4°C for 20 min and TMs were resuspended in 20 m*M* TES buffer solution containing 0.84% NaCl (pH 7.0). This step was repeated 3 times. The concentration of DPPC was adjusted to 20 m*M*. The average diameter of TMs was measured using a laser particle size analyzing system (Photal LPA-3000/3100, Otsuka Electronics, Osaka, Japan). The entrapment of calcein in the liposomes was 40%, and the concentration of calcein in the total volume of the liposome suspension can be calculated as 4 μ*M*. For this research, calcein was encapsulated to characterize the thermosensitivity and magnetic responsiveness of TMs.

Determination of Phosphorus and Magnetite. Total phosphorus was determined based on the method of Bartlett (16). Magnetite was determined colorimetrically according to the method described by Kiwada *et al.* (5). Total phosphorus and magnetite content of each TM suspension were measured before and after centrifugation for isolation of nonencapsulated DM. Then, magnetite content in a unit of mg per mmol DPPC and percentage capture of DM were calculated.

Transmission Electron Microscopy. All observations were made on an electron microscope (JEM-200 CX, JEOL, Tokyo, Japan). Samples were not stained because magnetite can be visualized in the electron microscope by virtue of its electron density.

Differential Scanning Calorimetry. TMs containing 48.1 and 490 mg magnetite per mmol DPPC were analyzed with a differential scanning calorimeter (SSC/560, Daini Seikosha, Tokyo, Japan) from 20 to 70°C at the scanning rate of 0.5°C/min.

Temperature-Dependent Release of Calcein from TMs. Ten μl of the calcein-entrapped TMs containing 48.1 mg magnetite per mmol DPPC were added to 1 ml of preheated (at 22.8, 30.2, 35.4, 40.1, 42.0, and 45.0°C) 20 m*M* TES buffer solution containing 0.84% NaCl and incubated for 5 min. Immediately after the incubation, the samples were ice-cooled and kept at 0.5°C. According to the method of Oku *et al.* (17), the amount of calcein trapped in liposomes was determined fluorometrically by using a spectrofluorometer (Model RF-5000, Shimadzu, Tokyo, Japan) set at 490 nm for excitation and 520 nm for emission. Fifty μl of the samples were diluted to 2550 μl (V_1) with TES buffer solution containing 0.84% NaCl and the fluorescence of the samples was measured before (F_1) and after (F_2) the addition of 50 μl of 100 m*M* $CoCl_2$ solution (V_2, 2600 μl). Subsequently, 50 μl of 10% Triton X-100 solution was added (V_3, 2650 μl) and the fluorescence was measured again (F_3). The percentage release of calcein from TMs was calculated as:

$$\% \text{ Release} = \left(1 - \frac{E_i}{E_r}\right) \times 100 \quad (1)$$

where E is expressed as:

$$E = \frac{F_2/V_2 - F_3/V_3}{F_1/V_1 - F_3/V_3} \quad (2)$$

The subscripts i and r refer to the sample incubated at indicated temperature and at 22.8°C (when no calcein release is supposed), respectively.

Holding Experiments. An *in vitro* on-line flow system fabricated for quantifying the magnetic responsiveness of TMs is illustrated in Fig. 1. The system consisted of a solvent delivery pump (Model 6000A, Waters, Milford, MA, USA), a polyethylene tubing (i.d.; 1.00 mm, o.d.; 1.50 mm), a sample injector (Model U6K, Waters), an electromagnet (Model MCD-1B, JASCO, Tokyo, Japan) connected with a power supply (Model 7020A, JASCO), a scanning fluores-

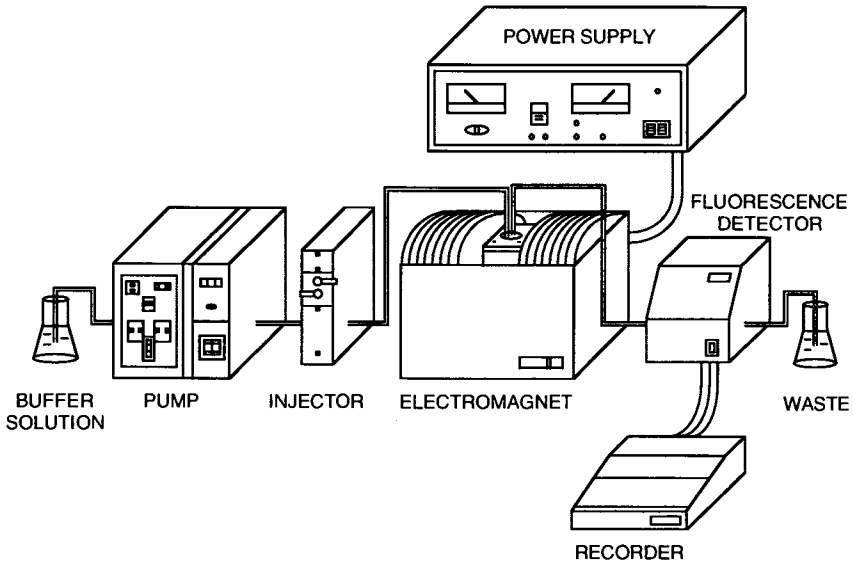

Fig. 1. Diagram of an on-line flow system for measuring magnetic responsiveness of thermosensitive magnetoliposomes.

cence detector (Model 470, Waters), and a recorder (Chromatopac C-R6A, Shimadzu, Kyoto, Japan). A coil of 60 cm-length polyethylene tubing was positioned between the pole pieces of the electromagnet (Fig. 2).

The electromagnet was turned on at the selected magnetic field strength, which was measured by a Gauss meter (Model HGM-8200, ADS Co., Tokyo, Japan). Then, a flow of 20 m*M* TES buffer solution containing 0.84% NaCl was generated at the known rates between 0.5 and 8.0 ml/min. Thereafter, an aliquot (80 μl) of TM suspension (0.1 m*M* DPPC) was loaded into the injector with a microliter syringe and injected into the polyethylene tubing. The electromagnet was allowed to run until it was turned off at the predetermined time. Subsequently, the coil of polyethylene tubing was taken off from the magnetic field and vigorously agitated using a vortex mixer to make the held TMs detach from this coil. All the holding experiments were performed at room temperature.

The amount of TMs held by magnetic field was calculated as percentage holding:

$$\% \text{ Holding} = \frac{\text{Second peak area}}{\text{First peak area} + \text{Second peak area}} \times 100 \quad (3)$$

where the first peak of the elution pattern represents a fraction of TMs which was not held by magnetic field and the second peak indicates the other fraction which was held but later forced to detach from the coil of polyethylene tubing.

Considering that very high incorporation of DM may hinder efficient entrapment of drug molecules in TMs, the magnetic holding of TMs was also evaluated as the ratio of percentage holding and incorporated magnetite (mg/mmol DPPC), defined as "percentage holding relative to magnetite content", in order to find the smallest amount of DM that can still accomplish reasonably high efficiency of the magnetic holding.

Effect of the Concentration and Volume of Injected TM Suspension on Percentage Holding. TM suspensions containing 209 mg magnetite per mmol DPPC were injected at various concentrations (as m*M* DPPC) and volumes into the on-line flow system for measuring their percentage holding. The experiments were carried out at 1.0 ml/min-fluid flow rate and at 15 kiloGauss-magnetic field strength.

RESULTS

Preparation, Magnetite Content, and DM Capture of TMs

TMs with an average diameter of 1.16 ± 0.37 μm (mean ± SD, $N = 10$) were successfully prepared in the presence of calcein and DM, and used throughout the experiments. The concentration of DM did not influence the size of liposomes (result not shown). The content of incorporated magnetite varied from 10.3 to 490 mg per mmol DPPC when DM was used at 1.33 to 167 m*M* (as magnetite) for preparation. Figure 3 shows percentage capture of DM in TMs as a function of DM concentration used for TM preparation. As the DM concentration increased, the percentage capture of DM sharply decreased at the first phase of the curve, indicating that DM is adsorbed on liposomal bilayers. The slower decline in the percentage capture most likely represents entrapped DM in the internal phase of liposomes as well as adsorbed DM on the liposomal bilayers.

Transmission Electron Microscopy

As inspected from the electron micrographs (Fig. 4), a large number of TMs were completely filled with DM when they were prepared at high DM concentrations. In contrast, they were coated by or partially filled with DM when prepared at lower DM concentrations.

Differential Scanning Calorimetry

Figure 5 depicts the thermal curve with a sharp transition peak at 42°C for TMs at 48.1 mg magnetite per mmol DPPC. The same result was obtained at a higher DM concentration, i.e., 490 mg magnetite per mmol DPPC. This suggests that DM does not interrupt the lipid packing within the liposomal bilayers.

Temperature-Dependent Release of Calcein from TMs

Percentage release of calcein was the greatest at 42°C (Fig. 6), the phase transition temperature of DPPC. Thus, it

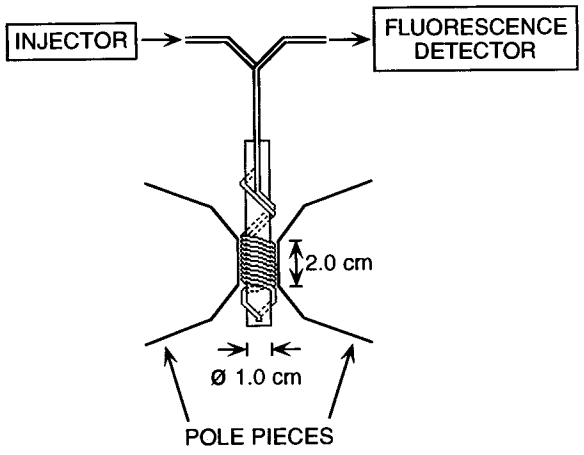

Fig. 2. Illustration of a tubular polyethylene coil placed between the pole pieces of an electromagnet.

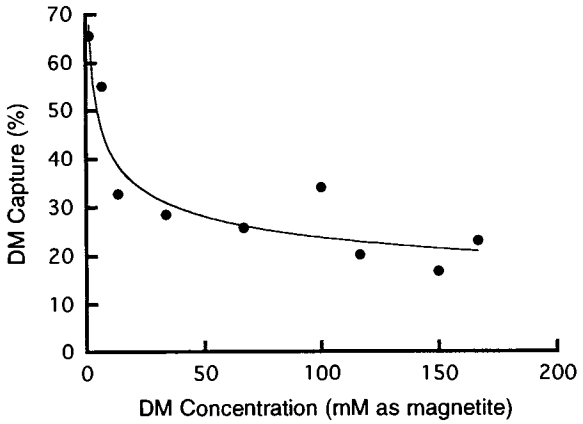

Fig. 3. Dextran magnetite (DM) capture of thermosensitive magnetoliposomes as a function of DM concentration used for their preparation.

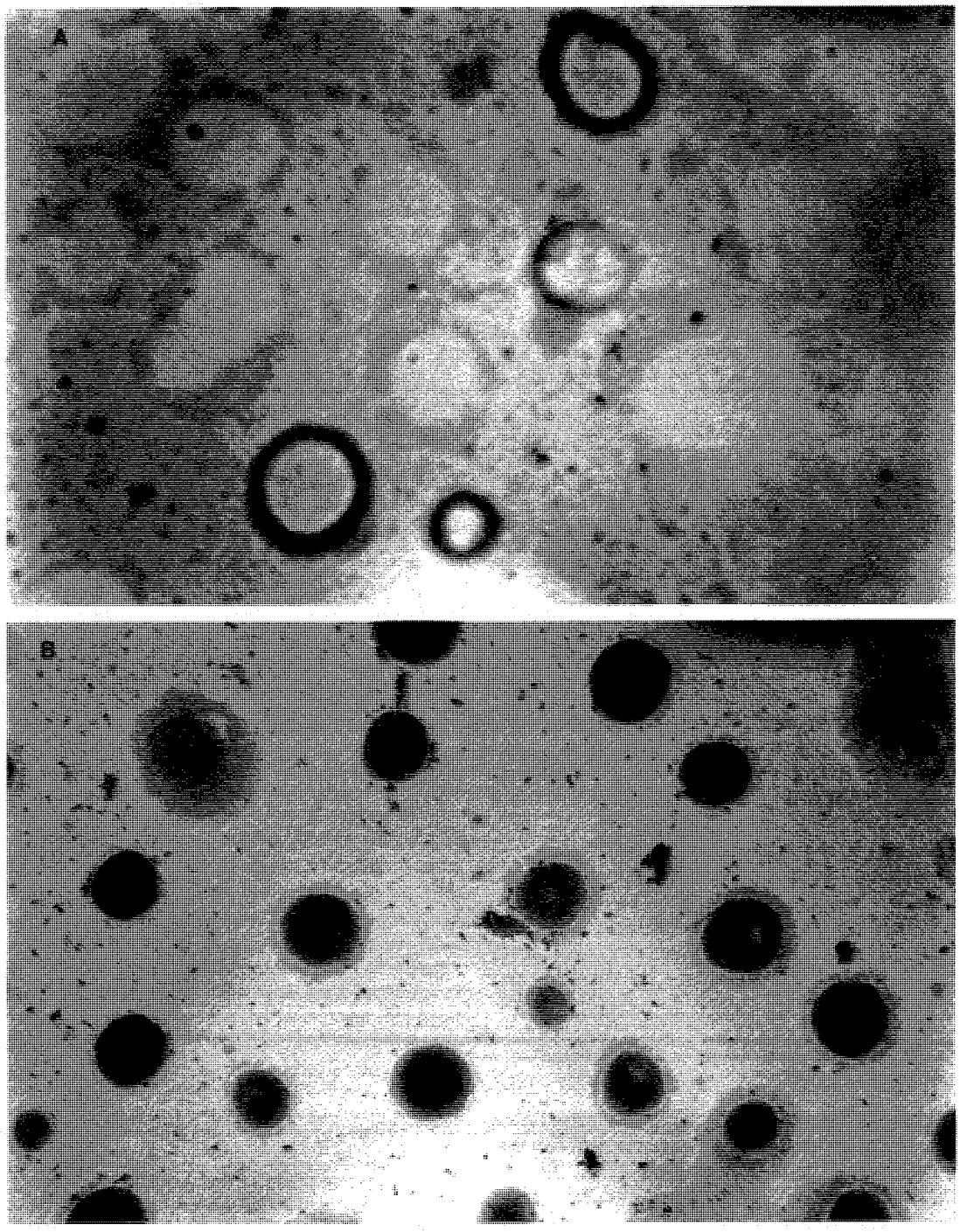

Fig. 4. Transmission electron micrographs of thermosensitive magnetoliposomes containing different amounts of magnetite: (A) 10.3; (B) 209; (C) 490 mg/mmol DPPC. 30,000×.

appears that DM does not qualitatively interfere with a temperature-dependent release characteristic of TMs.

Holding Experiments

Figure 7 illustrates the elution patterns of TMs (490 mg magnetite/mmol DPPC) from the on-line flow system at various fluid flow rates and magnetic field strengths. Figure 8 reveals the percentage holding of TMs at various conditions of magnetite content, magnetic field strength, and fluid flow rate. Typically, at 490 mg magnetite per mmol DPPC, 0.5 ml/min-fluid flow rate, and high magnetic field strength (≥10 kiloGauss), approximately 100% of TMs were found to be held. It was demonstrated in Figs. 8A-E that the holding of TMs increases as the magnetic field strength increases, the fluid flow rate decreases, and the magnetite content increases. Almost none of TMs were able to be held at 8.0 ml/min. TMs containing 48.1 mg magnetite per mmol DPPC were only held in a small fraction even at a high magnetic

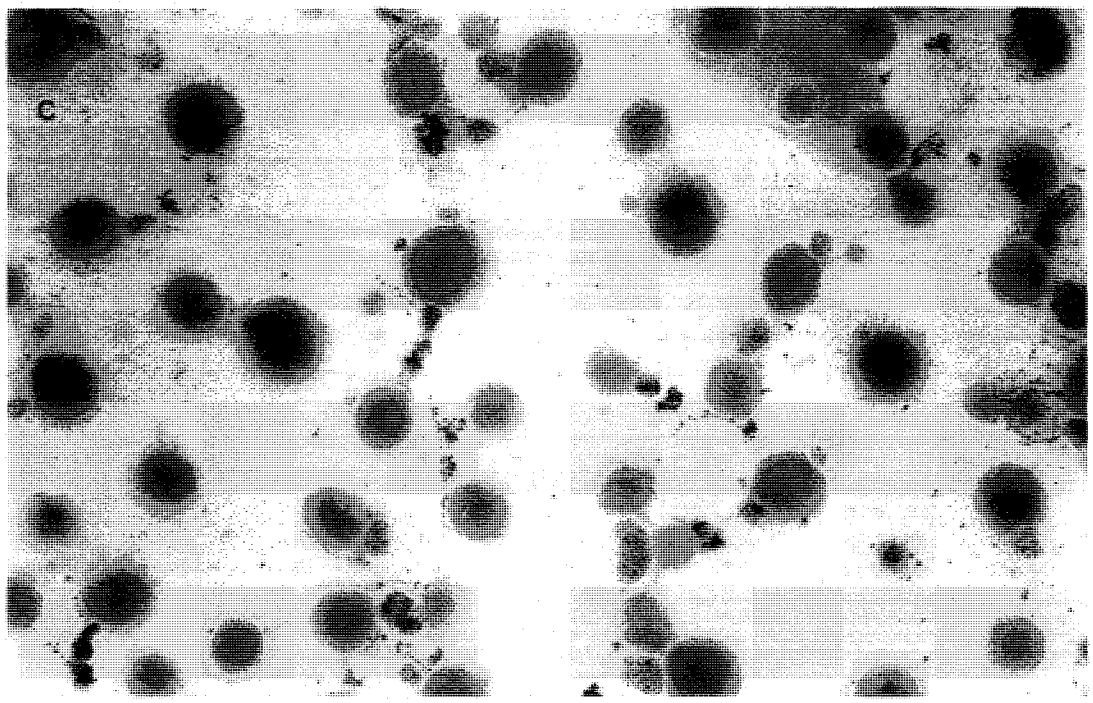

Fig. 4. Continued.

field strength (15 kiloGauss) and low fluid flow rate (0.5 ml/min). There was nearly no holding of TMs containing 10.3 mg magnetite per mmol DPPC. Figure 9 displays the correlation between percentage holding relative to magnetite content and magnetite content at various fluid flow rates and magnetic field strengths. At 0.5-2.0 ml/min and 5-15 kiloGauss, percentage holding relative to magnetite content was the highest when they contained 135 or 209 mg magnetite per mmol DPPC except at 5 kiloGauss and 2.0 ml/min. However, for TMs containing 490 mg magnetite per mmol DPPC, percentage holding relative to magnetite content was predominant at 4.0 ml/min and at 5, 10, and 15 kiloGauss and was also uppermost at 1 kiloGauss and at 0.5 and 1.0 ml/min.

Effect of the Concentration and Volume of Injected TM Suspension on Percentage Holding

As shown in Fig. 10, the holding of TMs was raised from 25 to 66% when the concentration of the injected TM suspension was elevated from 0.05 to 4.0 m*M* DPPC. In contrast, the percentage holdings of TMs at the injected suspension volumes of 40, 80, and 160 μl were determined to be 35.1 ± 3.0%, 35.3 ± 0.7%, and 34.4 ± 2.3% (mean ± SD, N = 3), respectively.

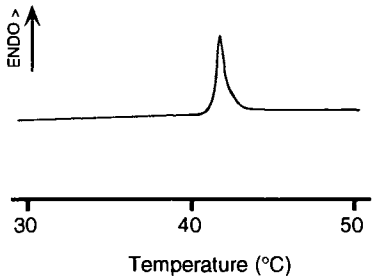

Fig. 5. DSC thermogram of thermosensitive magnetoliposomes at 48.1 mg magnetite/mmol DPPC.

DISCUSSION

This study attempted to establish the condition of TM preparation with an optimum magnetite content from the viewpoint of effective magnetic targeting. TMs containing different amounts of magnetite could be prepared by reverse-phase evaporation using dextran magnetite (DM). DM is a submicron complex consisting of magnetic iron oxide core surrounded by dextran chains (18). Since DM disperses uniformly in aqueous phase, it is likely that DM can be easily incorporated into liposomes. In contrast, conventional magnetite may not be a good candidate for this purpose because it is insoluble in both aqueous and organic solvents (15). Magnetoli-

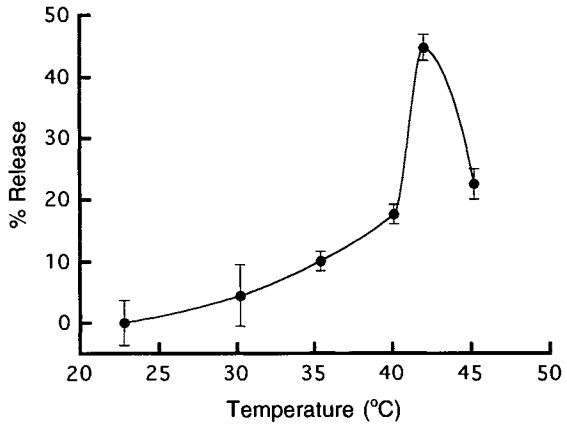

Fig. 6. Temperature dependence of calcein release from thermosensitive magnetoliposomes. Each point is the mean ± SD of three experiments.

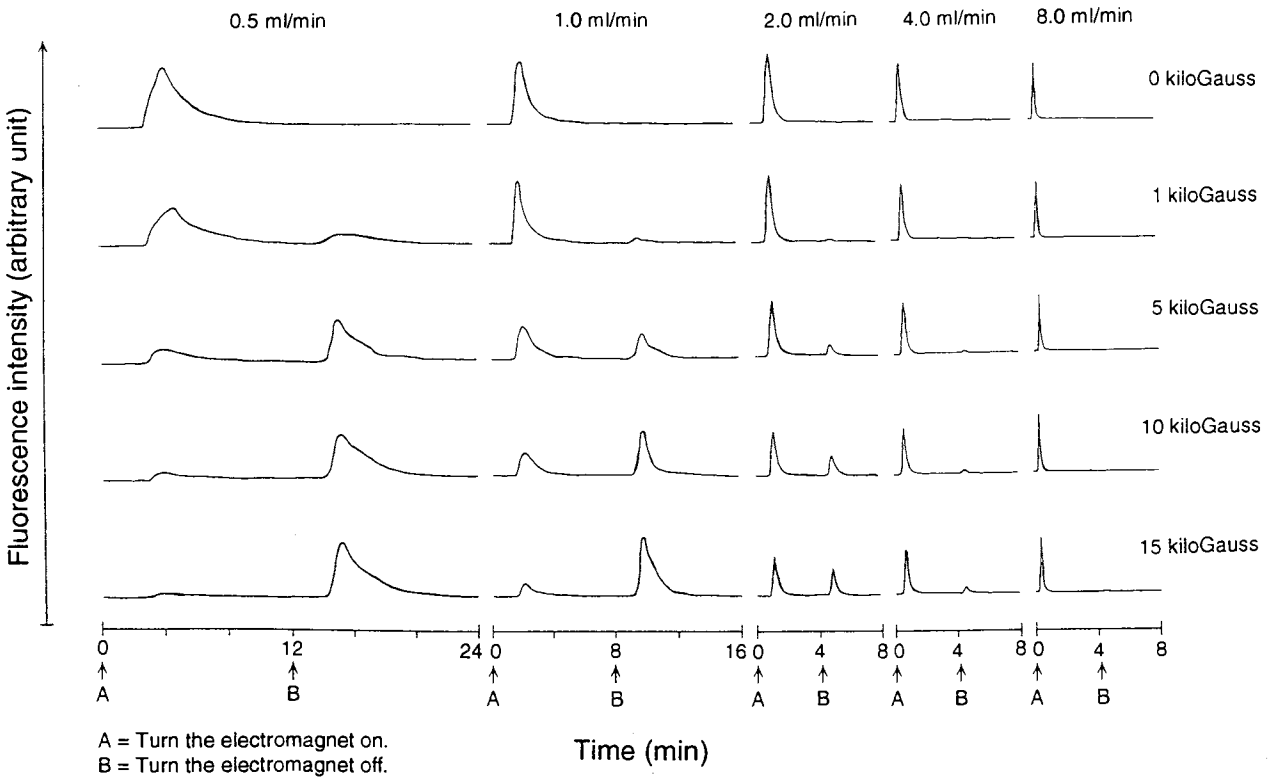

Fig. 7. Typical elution patterns of thermosensitive magnetoliposomes from the on-line flow system.

posomes prepared by using conventional magnetite (5) contained only 2.2-41 mg magnetite per mmol of lipid, resulting in a lower holding (4-48%) by magnetic field even at a low fluid flow rate (0.08-0.51 ml/min). On the contrary, our TMs could contain a high amount of magnetite (490 mg magnetite/mmol DPPC) resulting in a higher holding at its best (~100%).

DM may somehow reduce the release rate and/or extent of entrapped materials. However, the present results show that DM did not qualitatively interfere with a temperature-dependent release characteristic of TMs (Fig. 6), which is consistent with the fact that it did not interrupt the lipid packing within the liposomal bilayers (Fig. 5). We recently

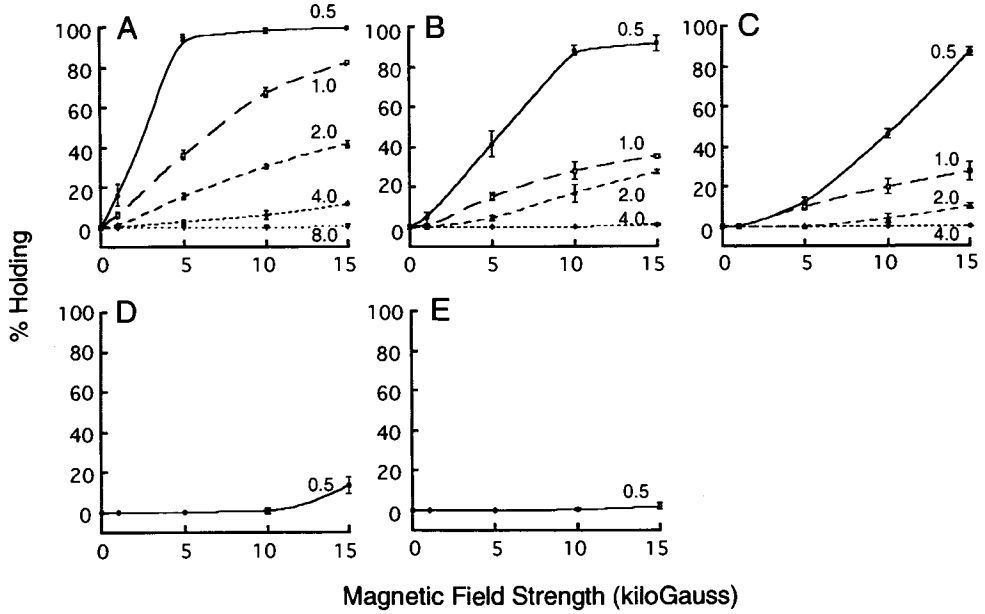

Fig. 8. Correlation between the percentage holding of thermosensitive magnetoliposomes and applied magnetic field strength at various fluid flow rates and at different amounts of incorporated magnetite: (A) 490; (B) 209; (C) 135; (D) 48.1; (E) 10.3 mg/mmol DPPC. The numbers accompanying the lines correspond to the fluid flow rates. Each point is the mean ± SD of three experiments.

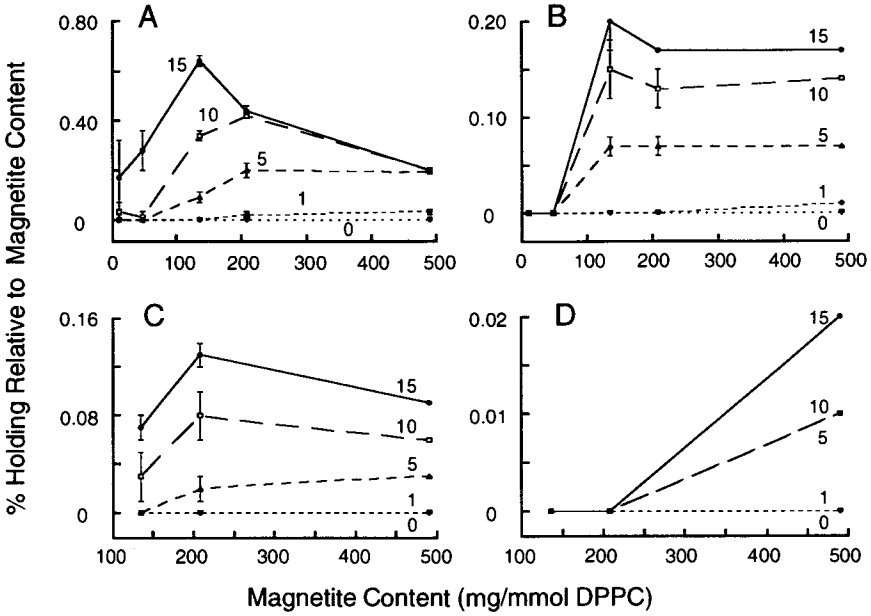

Fig. 9. Correlation between the percentage holding relative to magnetite content and magnetite content of thermosensitive magnetoliposomes at various magnetic field strengths and fluid flow rates: (A) 0.5; (B) 1.0; (C) 2.0; (D) 4.0 ml/min. The numbers accompanying the lines correspond to the strengths (kiloGauss) of magnetic field. Each point is the mean ± SD of three experiments.

reported that dynamic stirring of liposomal suspension enhances the thermosensitive release of calcein (19). Therefore, although the release of calcein from TMs was measured only in a static condition in this study, its thermosensitive release should take place more easily in a dynamic condition. Thus, the TMs were suggested to be useful not only for drug targeting but also for drug release in response to hyperthermia, especially electromagnetic induced local hyperthermia (20-22). In addition, it has been found that DM has very low toxicity as represented by its LD_{50} (mouse, i.v. dose) of 2000 to 6000 mg/kg calculated as iron, in contrast to the LD_{50} of the conventional magnetite sols that is 300 to 600 mg/kg (18).

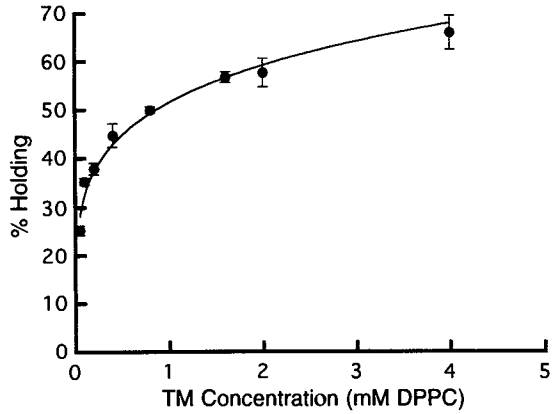

Fig. 10. Effect of the concentration of injected thermosensitive magnetoliposome (TM) suspension (209 mg magnetite/mmol DPPC) on percentage holding. Each point is the mean ± SD of three experiments.

As shown in Fig. 7, the width of the first peak in the elution pattern was not influenced by magnetic field strength but by fluid flow rate; the slower the fluid flow rate is, the longer is the time needed for applying magnetic field because it requires more time for the TMs to flow through the magnetic field-exposed area at low fluid flow rate. Thus, the time for applying magnetic field to obtain the highest percentage holding should be determined by fluid flow rate. A possibility of calcein leakage during the holding experiment could be excluded, because 1) complete holding was achieved in some cases, 2) the first peak of the elution pattern did not show any tailing, and 3) the baseline of the elution pattern did not drift.

As can be examined from Figs. 4 and 9, TMs containing medium DM concentration (135 and 209 mg magnetite per mmol DPPC) coated by and partially filled with DM can respond to magnetic field with the highest percentage holding relative to magnetite content at higher magnetic field and at the fluid flow rate of 0.5-2.0 ml/min or 1.1-4.2 cm/sec, which corresponds to mammalian blood flow in capillaries, venules, and some parts of arterioles (23). This observation agrees with the suggestion (1) that peripheral distribution of incorporated magnetite particles within a drug carrier (albumin microsphere) offers a higher magnetic moment. On the other hand, TMs that contain an insufficient amount of magnetite (10.3 mg magnetite per mmol DPPC) could not be efficiently held by magnetic field even though the magnetite particles also distribute peripherally within the liposomes. Thus, TM suspension containing a medium concentration of magnetite (i.e., approximately 140-210 mg magnetite/mmol DPPC) may be suitable for drug targeting using an external magnetic field.

Figure 10 clearly reveals that the concentration of the

injected TM suspension has a positive effect on magnetic targeting. The exact mechanism of this effect is not known, but one possible explanation is that when the concentration of the injected suspension was raised, a larger amount of TMs first attached onto the flow tube wall by magnetic field and aligned themselves along the magnetic field lines; this may induce the subsequent fractions to be more attracted to the chain of TMs. Moreover, this may also decrease the fluid flow rate near the tube wall causing the following TMs easily to be held. However, the effect of the concentration of the injected suspension on percentage holding became less at higher concentrations (>3m*M* DPPC, see Fig. 10), possibly due to the counteracting effect of shearing force on the fluid flow. On the other hand, the volume of the injected TM suspension had no influence on percentage holding presumably because the constant number of TMs per unit volume does not promote the chain formation of TMs along the magnetic field lines.

In conclusion, the TMs formulated in this study were shown to be efficiently held by magnetic field in an on-line flow system. It was clearly demonstrated that the magnetic responsiveness of TMs is complicated by many factors, such as fluid flow rate, strength of the applied magnetic field, magnetite content of TMs, distribution of magnetite particles within TMs, and concentration of injected TM suspension, which all together determine the holding efficiency of these TMs. Further study should be directed to *in situ*/*in vivo* application of TMs since we believe that they are useful in cancer treatment by magnetic targeting combined with drug release in response to electromagnetic induced local hyperthermia.

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