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Long-circulating gadolinium-encapsulated liposomes for potential application in tumor neutron capture therapy

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Abstract

Gadolinium neutron capture therapy (Gd-NCT) is a promising cancer therapy modality. One of the key factors for a successful Gd-NCT is to deliver and maintain a sufficient amount of Gd in tumor tissues during neutron irradiation. We proposed to prepare a Gd delivery system by complexing a Gd-containing compound, diethylenetriaminepentaacetic acid (Gd-DTPA), with a polycationic peptide, poly-L-lysine (pLL), and then encapsulate the complexed Gd-DTPA into PEGylated liposomes. Complexation of Gd-DTPA with pLL not only enhanced the encapsulation efficiency of Gd-DTPA in liposomes, but also significantly limited the release of Gd-DTPA from the liposomes. A Gd-DTPA-encapsulated liposome formulation that contained 6.8 ± 0.3 mg/mL of pure encapsulated Gd was prepared. The blood half-life of the Gd encapsulated into the liposome formulation was estimated to be about 24 h in healthy tumor-free mice. About 12 h after the Gd-encapsulated liposomes were intravenously injected into mice with pre-established model tumors, the Gd content in the tumors reached an average of 159 μg/g of wet tumor tissue. This Gd-DTPA encapsulated liposome may be used to deliver Gd into solid tumors for NCT and tumor imaging.

Keywords: Complexation; Release; Biodistribution; Tumor uptake

1. Introduction

Neutron capture therapy (NCT) is a promising cancer therapeutic approach. In NCT, stable, non-radioactive nuclides are delivered to target tumors. The nuclides then produce localized cytotoxic radiations upon irradiation by thermal or epithermal neutrons (Barth and Soloway, 1994; Carlsson et al., 2002). Earlier studies were mainly focused on using boron-10 (^{10}B) as a nuclide for the treatment of melanoma and brain glioma. Gadolinium neutron-capture therapy (Gd-NCT) is a potential cancer therapy using the γ-rays and auger electrons emitted from the ^{157}Gd (n,γ) ^{158}Gd reaction to kill tumor cells. Gd-NCT is generally considered to be advantageous over B-NCT due to the 66 times larger thermal neutron capture cross section (Martin et al., 1989) and the long range (>100 μm) of γ-rays released by the Gd after neutron irradiation (Brugger and Shih, 1989).

One of the key factors for the success in Gd-NCT is to deliver and maintain a sufficient amount of Gd in tumor tissues during the neutron irradiation (Shikata et al., 2002). The optimal ^{157}Gd concentration in tumors for Gd-NCT was reported to be 50–200 μg/g tumor tissues (Shih and Brugger, 1992). Many drug delivery systems, such as calcium carbonate microparticles (Miyamoto et al., 1997), lecithin microcapsules (Jono et al., 1999), lipid emulsions (Miyamoto et al., 1999), gadopentetic acid–chitosan complexed nanoparticles (Tokumitsu et al., 1999), chitosan nanoparticles (Shikata et al., 2002), and lipid or emulsifying wax-based solid nanoparticles (Oyewumi and Mumper, 2003; Oyewumi et al., 2004; Watanabe et al., 2002), have been prepared to enhance the delivery and retention of Gd in tumors. In order to deliver a sufficient amount of Gd into tumors, several of these Gd-systems were directly injected into tumors. However, direct intratumor injection is not practical for tumors that may not be easily located and injected.

Ideally, Gd should be injected intravenously (i.v.), and then, allowed to accumulate into tumors. To achieve this, an appropriate delivery system is needed. An optimal Gd-delivery system should at first contain a large amount of Gd so that more Gd could be delivered into tumors. Some Gd-delivery systems that

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carried a high concentration of Gd were prepared in earlier studies. These included the Gd–chitosan nanoparticles (430 nm) containing 9.3% (w/w) of Gd (Tokumitsu et al., 1999), the distearylamide gadopentetic acid microcapsules (106–149 μm) containing 5.13% (w/w) of Gd (Jono et al., 1999), the gadolinium hexanedione (GdH) nanoparticles containing 2.5 mg/mL of GdH (Oyewumi and Mumper, 2002), and the Gd-incorporated lipid-nanoemulsions (100 nm) containing 3 mg/mL of Gd (Watanabe et al., 2002). Secondly, the release of Gd from the delivery system should be slow and limited, which will allow the Gd to remain inside the delivery system prior to reaching tumors. A slow and limited release should also slow down the diffusion of the Gd out of the tumors before and during the neutron irradiation. Another key requirement for an ideal Gd delivery system is that its particle size should be less than 150 nm in order to efficiently target tumors (Desai et al., 1997; Hobbs et al., 1998; Wu et al., 1993). Small size particles (about 100 nm) were reported to be ideal to avoid the uptake by liver macrophages and the reticuloendothelial system (RES) (Harashima et al., 1995; Litzinger et al., 1994) and to deliver molecules to specific tissues of interest (Kreuter, 1995). Finally, having a prolonged circulation time in blood is also critical because it has been shown that there was a strong correlation between the residence time of a drug delivery system in the blood and its uptake by implanted tumors in mice (Gabizon and Papahadjopoulos, 1988). It was reasoned that drug delivery systems have extravasations in tumors due to the passive convective transport through the leaky endothelium of tumors (Gabizon, 2001). A longer blood circulation time is associated with repeated passages of a high concentration of the delivery system through the tumor microvascular bed, and thus, a greater efficiency of extravasations per unit volume of convective transport. Coating of particles with polyethylene glycol (PEG) has been shown to have a pronounced effect on the distribution the particles in that it can lead to a prolongation of the circulation time of the particles in blood, a decrease in uptake of the particles by liver and spleen, and a corresponding increased accumulation of the particles in implanted tumors (Gabizon et al., 1990). It was believed that PEG reduced the coating (opsonisation) of delivery systems by plasma protein, and thus, enabled them to escape recognitions by liver and spleen (Allen, 1994).

Our strategy is to utilize liposomes as a delivery system to deliver Gd into tumors. Liposomes have been studied for decades as a drug delivery system. They theoretically fulfill the key requirements to retain, target, and release drugs and have been evaluated clinically for drug delivery in a variety of diseases (Cagnoni, 2002; Muggia and Hamilton, 2001; Sparano and Winer, 2001). The unique characteristic of liposomes lies in their membrane structure composed of double phospholipid layers similar to biological membranes. They can encapsulate a large variety of both hydrophilic and hydrophobic compounds without the use of surfactants or other emulsifiers and can be introduced into the body without triggering immune rejection reactions. In fact, there are liposomally delivered drugs currently available on the market (e.g., daunoxome and doxil®) (Massing and Fuxius, 2000). Gd-DTPA, a nontoxic, hydrophilic, and stable chemical commonly used as a magnetic resonance imaging (MRI) diagnostic agent, which can potentially be useful for assessing tumors or metastases (Unger et al., 1990), was used as the source of Gd. The utilization of this MRI diagnostic agent promises the potential of coordinating MRI diagnosis with Gd-NCT using a single functional Gd compound in the future.

Our objective in this study was to design a long-circulating, small (100 nm) liposome preparation that encapsulates a high concentration, but slowly releasing, Gd. Moreover, the biodistributions of the encapsulated Gd in both healthy and model tumor-bearing mice were also evaluated.

2. Materials and methods

2.1. Materials

Magnevist® was from Berlex Laboratories (Wayre, NJ). Soy phosphatidylcholine (Soy PC), soy hydrogenated phosphatidylcholine (Soy HPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (PEG 2000) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Cholesterol (Chol), diethylenetriaminepentaacetic acid (Gd-DTPA), poly-L-lysine (pLL, MW 5600), Sephadex-G75, and phosphate buffered saline (PBS, pH 7.4) were purchased from Sigma–Aldrich (St. Louis, MO). Cellulose dialysis tubes (MWC 10,000 and 50,000) were from Spectrum Chemicals & Laboratory Products (New Brunswick, NJ).

2.2. Methods

2.2.1. Preparation of liposomes

Liposomes were prepared by the thin film hydration method followed by six cycles of freeze–thaw. Briefly, a thin film of phospholipids and cholesterol with PEG 2000 (10% molar ratio of the phospholipid) was formed in the bottom of a glass tube by chloroform evaporation. The lipid thin film was suspended in a Gd-DTPA aqueous solution or the aqueous solution of Gd-DTPA–pLL complex (Gd-DTPA:pLL = 1:0.25, w/w) by vigorous mixing at room temperature. For some preparations, the liposome was frozen and thawed for six cycles, sonicated for 5–6 min. One hundred nanometer-range liposomes were prepared by extruding the preparation 11 times sequentially through 1000, 400, and then 100 nm-polycarbonate membranes (Avanti Polar Lipids). Unencapsulated Gd-DTPA was removed by gel permeation chromatography using a Sephadex G-75 column or by dialyzing against 0.9% NaCl solution through a cellulose dialysis membrane (MWC 50,000) for at least 15 h.

2.2.2. Gel permeation chromatography (GPC)

The unencapsulated Gd-DTPA was removed by GPC. About 100 μL of liposomes were eluted with water through a Sephadex-G75 column (6 mm × 30 cm). Each elution fraction of 1 mL was collected, and the amount of Gd in each elution fraction was measured using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Teledyne Leeman Labs, Hudson, NH) at 342.247 nm. Based on the turbidity (OD655) of each elution fraction, liposomes were determined to be mainly in the third 1 mL fraction, which was the most turbid fraction.

The encapsulation efficiency of Gd in liposomes was calculated as following: encapsulation efficiency (%) = [Gd encapsulated/(Gd encapsulated + Gd unencapsulated)] × 100.

2.2.3. Dialysis

Gd-encapsulated liposomes were dialyzed against 4 L of physiological NaCl solution (0.9%, w/v) through a cellulose dialysis membrane (MWC 50,000) at room temperature for at least 15 h.

2.2.4. In vitro release of Gd-DTPA from liposomes

GPC-purified, Gd-DTPA-encapsulated liposomes (100 μg of pure Gd) were dispersed into PBS (10 mM, pH 7.4) and placed into a 1 mL cellulose ester dialysis tube. The tube was then placed into 12 mL of PBS (10 mM, pH 7.4) and incubated in a 37 ± 2 °C shaker incubator. At predetermined time points, the dialysis tube was taken out and re-placed into another 12 mL of fresh medium. The amount of Gd in the release medium was determined using ICP-OES.

In order to evaluate the feasibility of using the dialysis tube to measure the release of Gd-DTPA, the diffusion of pure Gd-DTPA through the membrane of the tube in PBS (10 mM, pH 7.4) was also measured.

Moreover, the release of Gd from liposomes in PBS buffer (10 mM, pH 7.4) containing fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) was also measured using the same procedure as mentioned above, except that the PBS medium was replaced by FBS in PBS (FBS/PBS, 10%, v/v).

2.2.5. Biodistribution

The biodistribution of Gd-DTPA-encapsulated liposomes was carried out in 6–8-week-old female Balb/C mice or model tumor-bearing C57BL/6 mice (Charles River Laboratories, Wilmington, MA). All experiments were completed following the National Institutes of Health guidelines for care and use of laboratory animals. To evaluate the distribution of Gd encapsulated into liposomes in healthy, tumor-free mice, Balb/C mice were injected (i.v.) with a dose of liposomes equivalent to 20 mg Gd per kg body weight, and euthanized 6, 10 or 24 h later after the injection. The blood, liver, spleen, heart, lung, and kidney were collected, desiccated at 60 °C overnight, and then incinerated with nitric acid (6.6N) at 60 °C for 15 h. The samples were filtrated through a 0.45 μm filter. Gd concentration was determined using ICP-OES. To estimate the concentration of Gd in blood, the total blood volume of a mouse was assumed to be 7.5% (v/w) of its total body weight (Davies and Morris, 1993; Mosqueira et al., 2001).

The distribution of Gd in tumor-bearing mice was evaluated similarly. The tumor cells used were the TC-1 tumor cell line, which was engineered by Dr. T.C. Wu at the Johns Hopkins University (Baltimore, MD) from C57BL/6 mouse lung endothelial cells. Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 100 U/mL of penicillin (Sigma), and 100 μg/mL of streptomycin (Sigma) and cultured at 37 °C in a humidified incubator containing 5% CO_2. Mice ($n = 7$) were subcutaneously implanted with TC-1 cells (5×10^5/mouse) on day 0. The tumors reached around 4–7 mm in diameter in about 10 days. On day 10, the mice were injected via the tail vein with Gd-DTPA encapsulated in one of the liposome formulations (formulation VI). The total Gd amount injected with 414 μg pure Gd/mouse (∼20 mg Gd/kg body weight). As a control, TC-1 tumor-bearing mice ($n = 4$) were also injected via the tail vein with Gd-DTPA dissolved in PBS (10 mM, pH 7.4). About 12 h later, mice were euthanized; organs and tissues were harvested; Gd content in them was measured.

2.2.6. Statistical analysis

The Student's *t*-test assuming equal variances was used if two groups were to be compared. If more than two groups were involved, the one-way analysis of variance (ANOVA), followed by pair-wise comparisons with Fisher's protected least significant difference (PLSD) procedure, was used. A p value of ≤ 0.05 (two-tail) was considered to be statistically significant.

3. Results and discussions

3.1. In vitro release of Gd-DTPA from liposomes

In order to deposit a large amount of Gd into tumors, the encapsulated Gd should not significantly leak out from the liposomes. In addition, a slow and limited release may keep the Gd that are delivered into tumors remaining inside the tumors prior to the neutron irradiation (Hobbs et al., 1998). Due to the high water solubility of Gd-DTPA, we expect that the release of Gd-DTPA from liposomes will be very rapid and extensive. It has been reported that the covalent conjugate of Gd-DTPA and pLL substantially slowed down the blood clearance of Gd-DTPA (Vexler et al., 1994). Moreover, Gd-DTPA and pLL conjugate has been used to enhance tissue signals in MRI (Bock et al., 1997; Curtet et al., 1998; Su et al., 1998; Uzgiris, 2004). However, the effect of complexing pLL with Gd-DTPA on the release of Gd-DTPA from liposomes has not been evaluated. We hypothesized that complexation of Gd-DTPA with pLL will decrease the release of Gd-DTPA from liposomes because the Gd-DTPA/pLL complex has a relatively larger molecular size than Gd-DTPA alone. To test this hypothesis, four liposome formulations (Soy PC:Chol = 3:2, molar ratio) with or without PEG 2000 (10%, m/m) coating were prepared using the thin film hydration method without multiple freeze-thaw cycles. The liposomes were: (I) liposome encapsulated with Gd-DTPA (LP-Gd-DTPA); (II) liposome encapsulated with Gd-DTPA complexed with pLL (LP-Gd-DTPA–pLL); (III) PEG-coated liposome encapsulated with Gd-DTPA (PEG-LP-Gd-DTPA); and (IV) PEG-coated liposome encapsulated with Gd-DTPA complexed with pLL (PEG-LP-Gd-DTPA–pLL).

We have found that complexation of Gd-DTPA with pLL enhanced the encapsulation efficiency of the Gd-DTPA in liposomes and that a Gd-DTPA:pLL ratio of 1:0.25 (w/w) led to the highest Gd-DTPA encapsulation efficiency. Further increasing the amount of pLL did not lead to a significantly higher Gd-DTPA encapsulation. Thus, this ratio was chosen to complex Gd-DTPA with pLL in further studies.

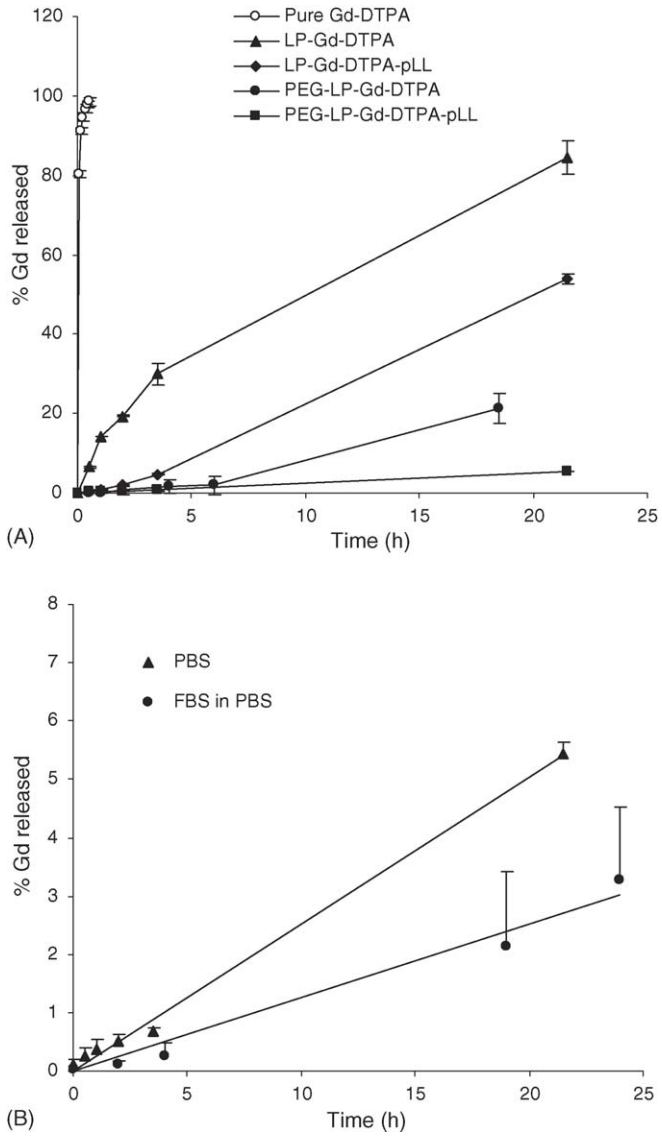

Fig. 1. The release of Gd-DTPA from liposomes. (A). The diffusion of Gd-DTPA through the dialysis membrane and the release of Gd-DTPA from liposome formulation I through IV were determined at 37 °C in PBS medium. Pure Gd-DTPA solution or GPC-purified, Gd-DTPA-encapsulated liposomes, each containing 100 μg of pure Gd, was placed into a cellulose ester dialysis tube (MWC 10,000). The dialysis tube was placed into 12 mL of PBS (pH 7.4, 10 mM). The amount of Gd released was determined using ICP-OES at 342.247 nm. Data shown are mean ± S.D. ($n = 3$). The liposomes were comprised of soy PC and Chol at a molar ratio of 3:2. Formulation I: LP-Gd-DTPA: liposome encapsulated with Gd-DTPA; formulation II: LP-Gd-DTPA-pLL: liposome encapsulated with Gd-DTPA-pLL; formulation III: PEG-LP-Gd-DTPA: PEG-coated-liposome encapsulated with Gd-DTPA; formulation IV: PEG-LP-Gd-DTPA-pLL: PEG-coated liposome encapsulated with Gd-DTPA-pLL. (B). The release of Gd-DTPA from liposome formulation IV in PBS or FBS/PBS (10%, v/v) medium.

Data from Fig. 1A clearly supported our hypothesis. The release of Gd-DTPA from formulation I (LP-Gd-DTPA) was very fast, with approximately 30% being released within 4 h. Complexation of Gd-DTPA with pLL (formulation II) significantly slowed down the release. Only about 5% of Gd-DTPA was released within the same period of time (4 h). Interestingly, coating of PEG on the surface of the liposomes also helped to reduce the release of Gd-DTPA from the liposomes (Fig. 1A, formulation III). It is possible that the saturated phospholipids (DSPE), to which the PEG molecule was attached, helped to enhance the rigidity of liposome membrane, and thus, decreased the leakage of the Gd-DTPA from the liposomes. The release of Gd-DTPA from formulation IV was the slowest. Only less than 4% of Gd-DTPA was released within 22 h, demonstrated the existence of a synergistic effect by complexing Gd-DTPA with pLL and coating the liposomes with PEG 2000.

In order to simulate the release of Gd-DTPA from liposomes in a more biologically relevant medium, the release of the Gd-DTPA was also evaluated in FBS/PBS (10%, v/v). Because formulation IV had the lowest release rate, the release of Gd-DTPA from it was measured. The release of Gd-DTPA was slightly slower in the FBS/PBS medium than in the PBS (Fig. 1B), promising a slow and limited release in vivo.

In this release study, we have placed the Gd-DTPA-encapsulated liposomes in a cellulose membrane dialysis tube to prevent the liposomes from entering the release medium. Data in Fig. 1A also showed that the cellulose dialysis membrane was feasible for studying the release of Gd-DTPA from the liposomes because the rate for the diffusion of pure Gd-DTPA through the dialysis membrane was much greater than that for the release of Gd-DTPA from any of liposome formulations. Pure Gd-DTPA has a molecular weight of 547.58. It diffused through the dialysis membrane (MWC 10,000 Da) very rapidly. In fact, nearly all of the free Gd-DTPA (99%) was diffused out of the dialysis membrane within 30 min.

3.2. Optimization of the Gd-encapsulated liposome formulation

A key factor for the success of Gd-NCT is to develop a delivery system that can efficiently deliver and retain a sufficient amount of Gd into tumors prior to the neutron irradiation. Although the release of Gd-DTPA for formulation IV was slow and limited, in a preliminary biodistribution study, we found that this formulation IV (Soy PC:Chol:PEG = 60:40:6, molar ratio) was quickly cleared from the blood when injected (i.v.) into mice. Only about 14% of injected dose was recovered in the blood 4 h after the injection. It was believed that the low level of Chol and the use of Soy PC, an unsaturated phospholipid with a low transition temperature (less than 37 °C), were responsible for the short blood circulation time. In order to prolong the circulation time of Gd in blood, saturated phospholipids with a high transition temperature, such as Soy HPC and DSPC, were combined with a higher concentration of Chol to increase the rigidity of the membrane of the liposomes. Three different liposome formulations were prepared with PC, Chol, and PEG 2000 at a molar ratio of 50:35:5 and an aqueous solution of Gd-DTPA (10%, w/v) complexed with pLL (Gd-DTPA:pLL = 1:0.25, w/w). They were formulation V, liposomes prepared with Soy PC as phospholipid (LP-Soy PC); formulation VI, liposomes prepared with Soy HPC (LP-Soy HPC); and formulation VII, liposomes prepared with DSPC (LP-DSPC).

Fig. 2 shows the GPC profiles of these three liposome formulations. The encapsulation efficiencies of Gd-DTPA in formulations V, VI, and VII were presented in Table 1. Formulation VI

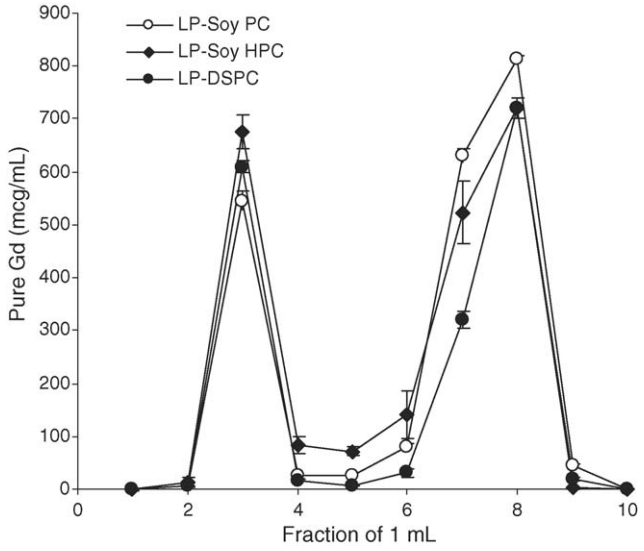

Fig. 2. The separation of the unencapsulated Gd-DTPA from Gd-DTPA-encapsulated liposomes by gel permeation. PEGylated liposomes prepared with different PCs (soy PC, Soy HPC, and DSPC) and PEG 2000 (PC:Chol:PEG 2000 = 50:35:5, molar ratio, Gd-DTPA:pLL = 1:0.25, w/w) were applied into a sephadex G75 column (diameter = 0.6 cm, length = 30 cm) and eluted with de-ionized water. The concentration of Gd in each elution fraction of 1 mL was measured using ICP-OES at 342.247 nm. Data reported are mean ± S.D. ($n = 3$). Formulation V: LP-Soy PC: liposome prepared with Soy PC as phospholipid; formulation VI: LP-Soy HPC: liposome prepared with Soy HPC as phospholipid; formulation VII: LP-DSPC: liposome prepared with DSPC as phospholipid.

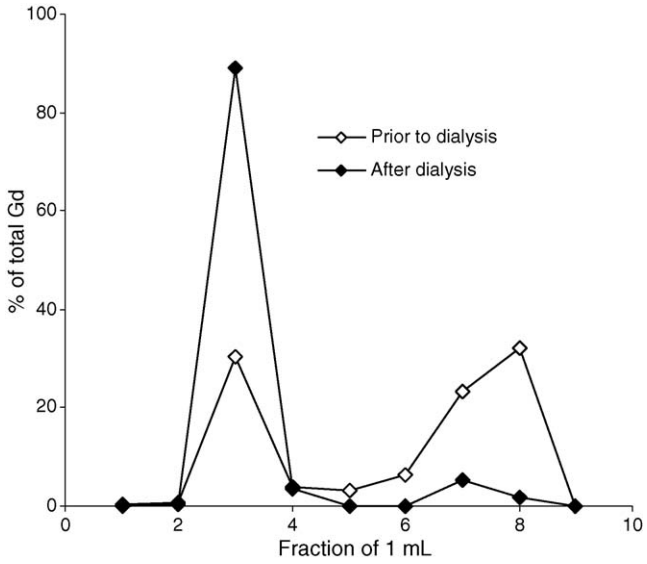

Fig. 3. The GPC profiles of Gd-DTPA-pLL-encapsulated liposomes prior to and after dialysis. One hundred microliters of Gd-DTPA-pLL-encapsulated liposomes (Soy HPC:Chol:PEG = 50:35:5, molar ratio; Gd-DTPA:pLL = 1:0.25, w/w) was applied into a sephadex G75 column (diameter = 0.6 cm, length = 30 cm) and eluted with de-ionized water. The Gd concentration in each 1 mL of elution fraction was measured using ICP-OES at 342.247 nm. Data shown were the percent of the total Gd that were applied into the column in every 1 mL fraction. Prior to dialysis: freshly prepared liposome formulation VI was applied directly to a GPC column. After dialysis: formulation VI was dialyzed against NaCl (0.9%, w/v) for 15 h at room temperature and then applied to the same GPC column.

had the highest encapsulation efficiency, and thus, the highest amount of Gd-DTPA encapsulated, with an estimated encapsulated Gd-DTPA concentration of 23.6 ± 1.1 mg/mL, which corresponded to 6.8 ± 0.3 mg/mL of pure Gd. Although there were reports of Gd-delivery systems with a higher Gd concentration in earlier studies (Jono et al., 1999; Tokumitsu et al., 1999), the previously reported systems were either too large (i.e., the 100-μm particles) (Jono et al., 1999) or may not be stable in a biological medium (i.e., chitosan–gadopentetic acid complex, 400 nm) (Tokumitsu et al., 1999), and thus, may only be suitable for direct intratumoral injection. Torchilin (2000) and Weissig et al. (2000) prepared a liposome encapsulated with 31% (w/w) of Gd for MRI (Torchilin, 2000; Weissig et al., 2000). However, the concentration was expressed in term of the weight of Gd over the weight of the Gd-liposomes, and the solubility of the Gd-liposomes was not reported. To our knowledge, the value of 6.8 ± 0.3 mg/mL represents a high amount of pure Gd that had been encapsulated into a Gd delivery system with small particle size (<150 nm) (Devoisselle et al., 1988; Grunder et al., 1998; McDannold et al., 2004; Oyewumi and Mumper, 2002; Tilcock et al., 1991; Unger et al., 1990; Watanabe et al., 2002). As predicted, the release of Gd-DTPA from these three formulations was also very slow and limited (Table 1). These liposomes, with small size, high concentration of Gd, and low Gd release rate, were expected to be a suitable delivery system for targeting Gd into tumors.

Table 1
The encapsulation efficiency and release of Gd-DTPA from liposome formulations V, VI, and VII

	Phospholipids		
	Soy PC	Soy HPC	DSPC
Encapsulation efficiency (%)	19.2 ± 0.6	25.7 ± 1.4[a]	21.0 ± 0.3
% Release	1.83 ± 0.57	1.13 ± 0.05	1.14 ± 0.15

The encapsulation efficiency of Gd-DTPA and the percent of Gd-DTPA released from liposomes (Phospholipid:Chol:PEG = 50:35:5, m/m/m, Gd-DTPA:pLL = 1:0.25, w/w) after 24 h of incubation at 37 °C in PBS were determined. Data reported are mean ± S.D. ($n = 3$).

[a] Indicates that the encapsulation efficiency for Soy HPC was significantly higher than those for Soy PC ($p = 0.002$) and DSPC ($p = 0.005$). The releases of Gd-DTPA from those three liposome formulations were comparable.

Although GPC efficiently removed free Gd-DTPA from liposomes, Gd-DTPA was significantly diluted after this step. Thus, the liposomes were dialyzed against physiological NaCl solution for at least 15 h to remove free Gd-DTPA. As shown in Fig. 3, almost all of the unencapsulated Gd-DTPA was removed using this dialysis procedure.

3.3. Biodistribution of Gd-DTPA-encapsulated liposomes in mice

The distribution of Gd in healthy, tumor-free mice was initially determined 6 h after i.v. injection. As shown in Fig. 4, increasing the ratio of Chol in the liposome (i.e. from Soy PC:Chol = 3:2 to 50:35, molar ratio) significantly enhanced the blood circulation time of Gd. For formulation V (Soy PC:Chol = 50:35), about 40% of the Gd injected was still in

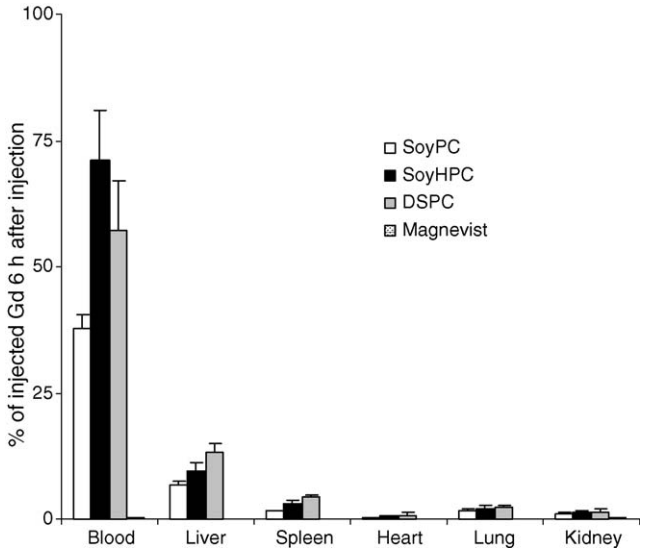

Fig. 4. The biodistribution of Gd-DTPA encapsulated into liposomes in healthy mice. Balb/C mice ($n = 5$) were injected via the tail vein with a single dose (20 mg of pure Gd/kg of body weight) of Magnevist® solution or Gd-DTPA-pLL-encapsulated liposomes. About 6 h after the injection, mice were sacrificed, and their blood, liver, spleen, heart, lung, and kidney were harvested. The concentration of Gd in them was determined using ICP-OES at 342.247 nm. Data shown are mean ± S.E.M. ($n = 5$). Formulation V: LP-Soy PC: liposome prepared with Soy PC as phospholipid; formulation VI: LP-Soy HPC: liposome prepared with Soy HPC as phospholipid; formulation VII: LP-DSPC: liposome prepared with DSPC as phospholipid. The concentrations of Gd in the blood for formulations VI and VII were comparable, but significantly higher than that for formulation V ($p = 0.006$ and $p = 0.04$, respectively). ANOVA analyses revealed that there were significant differences among the concentrations of Gd in the liver ($p < 0.0001$) and the spleen ($p < 0.00001$) of mice injected with formulations V, VI, and VII.

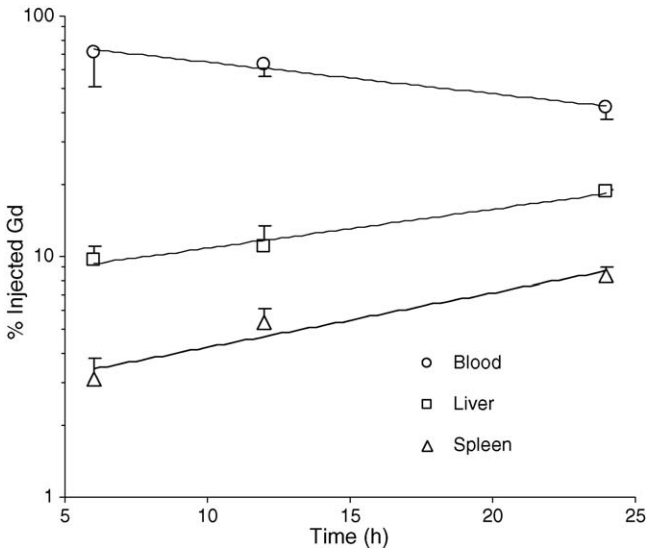

Fig. 5. The distribution kinetics of Gd encapsulated into liposome formulation VI in healthy mice. Balb/C mice were injected via the tail vein with a single dose (20 mg of pure Gd/kg of body weight) of liposome formulation VI. About 6, 12, and 24 h after the injection, mice ($n = 5$) were sacrificed, and their blood, liver, and spleen were harvested. The concentration of Gd in them was determined using ICP-OES. Data shown are mean ± S.E.M. ($n = 5$).

the blood 6 h after the injection, compared to only 14% for formulation IV (Soy PC:Chol = 3:2) 4 h after the injection (Fig. 4). Furthermore, liposomes prepared with saturated phospholipids with a high transition temperature, such as Soy HPC and DSPC, had a longer blood circulation time when compared to liposomes prepared with unsaturated Soy PC (Fig. 4). Also, it needs to be pointed out that, when pure Gd-DTPA alone (Magnevist) was dosed similarly, it was quickly cleared, with only a recovery rate of 0.58 ± 0.15% 6 h after the injection. Because the percent of Gd remained in the blood for formulation VI (LP-Soy HPC) tended to be slightly higher than that for formulation VII (LP-DSPC), and formulation VI encapsulated a higher amount of Gd, it was used to further evaluate its blood circulation and biodistribution in tumor-free and tumor-bearing mice.

Fig. 5 shows the percent of Gd recovered in the blood, liver, and spleen of tumor-free mice 6, 12 and 24 h after the i.v. injection of formulation VI. The percent of Gd remained in the blood was 71 ± 20%, 63 ± 7%, and 42 ± 5% (mean ± S.D.) of the total injected Gd dose 6, 10, and 24 h after the injection, respectively. The half-life of the Gd in the blood was calculated to be 24 h, higher than the 19 h blood half-life for a previously reported Gd-liposome formulation composed of the gadopentetate dimeglumine lipid and ditricosadiynoyl tricosadinynayl phosphatidylcholine in rats (Storrs et al., 1995). This value was also in sharp contrast to the blood $t_{1/2}$ of other previously reported Gd-liposome preparations, which ranged from 2 h (Bertini et al., 2004) to 4 h (Unger et al., 1990) to complete clearance within 12 h (Tilcock et al., 1989). Finally, the uptake of the Gd by the liver increased from 10% (at 6 h) to 19% (at 24 h) of the injected dose. Similarly, the uptake by the spleen was also increased from 3% (at 6 h) to 8% (at 24 h) of the injected dose, clearly showing the uptake by the RES was limited.

With its long blood half-life in mice, we expected that a sufficient amount of Gd will be delivered into tumors using our formulation VI for Gd-NCT. As shown in Table 2, an average of 158.8 ± 115.6 μg of Gd per gram of tumor tissue was accu-

Table 2
Biodistribution of Gd encapsulated into liposome formulation VI in tumor tissues and other organs of model tumor-bearing mice

	Pure Gd content (injected dose, 414 μg Gd)
Tumor (μg/g tissue)[a]	158.8 ± 115.6 (34.7–365.6)
Blood (% injected Gd)	57.8 ± 11.6 (%)
Liver (μg/g)	121 ± 7.0
Spleen (μg/g)	577 ± 81.8
Heart (μg/g)	44.7 ± 3.1
Lung (μg/g)	74.2 ± 3.3
Kidney (μg/g)	31.6 ± 1.6

C57BL/6 mice ($n = 7$) were implanted with TC-1 tumor cells (5×10^5) on day 0. Ten days later, mice were injected (i.v.) with Gd encapsulated in liposome formulation VI (~20 mg Gd/Kg body weight). Mice were euthanized 12 h after the injection; their tumor, blood, liver, spleen, heart, lung, and kidney were harvested. Gd content in them was determined using ICP-OES. Data shown are mean ± S.D. ($n = 7$). The Gd content in the blood was reported as the % of total injected Gd that remained in the blood. The Gd contents in other tissues and organs were reported as the final pure Gd amount (μg) per gram of wet tissue.

[a] The range of Gd content in tumors was shown in parenthesis. Only one out of seven mice had a Gd content of less than 50 μg/g tumor tissue in its tumor.

mulated into the tumor tissues 12 h after the Gd-encapsulated liposome formulation VI was injected into tumor-bearing mice via the tail vein. Of the seven mice evaluated, only one had a Gd content of less than 50 μg/g tumor tissues (34.7 μg/g). The large variation in the tumor uptake may be caused by the variation in tumor size (Harrington et al., 2000). In contrast, the Gd recovered in tumors when a free Gd-DTPA solution was injected (i.v.) into similar TC-1 tumor-bearing mice was only 1.43 ± 0.14 μg of Gd/g of tumors, which was about 260-fold less than when the Gd-DTPA-encapsulated liposomes were injected. As mentioned earlier, the optimal Gd concentration in tumors for a successful Gd-NCT was estimated to be 50–200 μg/g tumor tissue (Shih and Brugger, 1992). More Gd is expected to be delivered to tumors if more formulation VI is dosed by multiple injections. The tumor uptake of Gd may also be enhanced by coupling appropriate monoclonal antibodies onto the surface of liposomes to further target tumors (Blanco et al., 2005; Desormeaux and Bergeron, 2005; Laginha et al., 2005). Therefore, it is believed that this Gd encapsulated liposome formulation VI can be used to deliver a sufficient amount of Gd in tumors for Gd-NCT. Of course, a ^{157}Gd-enriched Gd compound has to be used when performing NCT because natural Gd only contains 15.56% of ^{157}Gd. Finally, it needs to be mentioned that, in the tumor-bearing mice, the uptake of the Gd by the liver and the spleen after the Gd-encapsulated liposomes were injected tended to be high, although it was unlikely to be caused by the species of the mice or the tumor per se. Nevertheless, this RES uptake may be further decreased in future studies by decreasing the size of the liposomes, by increasing the content of the PEG 2000 in the liposomes, or by using PEG with a longer chain, such as the PEG 7000 (Drummond et al., 1999).

Another advantage for our Gd-liposome is its potential application in MRI. The MRI technique has been used to examine brain and other parts of the central nervous system, blood vessel, and tumors. In MRI, contrast agents increase the difference in the intensity of signals from tissues with and without them. Gd-DTPA-encapsulated liposomes had been shown to be effective for MRI due to its high relaxivity (Curtet et al., 1998; Torchilin, 2000; Weissig et al., 2000). Therefore, our Gd-DTPA-pLL-encapsulated liposome preparation has the potential for integrating MRI diagnosis and Gd-NCT, which is advantageous over some previously reported Gd-delivery systems utilizing hydrophobic Gd-compounds, such as distearylamide of gadopentetic acid (Jono et al., 1999), gadolinium hexanedione (Oyewumi and Mumper, 2002), and gadolinium acetylacetonate (Dierling et al., 2005), as the source of Gd.

In conclusion, we prepared a Gd-DTPA-encapsulated liposome formulation that had 6.8 ± 0.3 mg/mL of pure Gd encapsulated in the liposomes. The complexation of Gd-DTPA with pLL significantly slowed down the release of Gd-DTPA from the liposomes. This encapsulated Gd-DTPA had a half-life of about 24 h in mouse blood. Moreover, an average of 158.8 ± 115.6 of Gd per gram of tumor tissues was delivered into tumors 12 h after the Gd-encapsulated liposomes were injected (i.v.) into mice pre-implanted with model tumors. This Gd-encapsulated liposome formulation is expected to be promising for delivering Gd into tumors for future Gd-NCT and/or MRI.

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