Available online at www.sciencedirect.com
SCIENCE DIRECT
International Journal of Pharmaceutics 320 (2006) 96–103
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Biodistribution and tumor-accumulation of gadolinium (Gd) encapsulated in long-circulating liposomes in tumor-bearing mice for potential neutron capture therapy

Uyen M. Le, Zhengrong Cui *

Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, 231 Pharmacy Building, Corvallis, OR 97331, United States

Received 20 January 2006; received in revised form 22 March 2006; accepted 17 April 2006
Available online 19 May 2006

Abstract

To deliver and maintain a sufficient amount of Gd into tumors is required for a successful Gd neutron capture therapy (Gd-NCT), but it has been proven to be rather challenging to achieve. Previously, we have reported a Gd-encapsulated liposome formulation that has the potential to overcome this challenge. In the present study, we sought to systemically evaluate the biodistribution and the tumor-accumulation of the Gd in model tumor-bearing mice. The Gd-encapsulated liposomes were injected into mice pre-grafted with two different model tumors. The Gd content in the tumors and other organs were determined at various time after the injection. A sufficient amount of Gd was readily delivered into those two different model tumors. Increasing the dose of Gd by injecting the Gd-encapsulated liposomes multiple times tended to increase the uptake of the Gd by the tumors. Finally, the uptake of Gd by tumors was inversely correlated with the size of the tumors. The Gd-encapsulated liposomes hold great potentials as a Gd delivery system for NCT of small- and medium-size tumors. Alternative strategies may have to be adopted in order to use NCT to treat large, advanced solid tumors, although for which, Gd-NCT might be advantageous over boron-NCT.

Keywords: Tumor size; Tumor type; NCT; Distribution kinetics

1. Introduction

NCT is a cancer therapeutic modality with promising potentials. In NCT, stable, non-radioactive nuclides are delivered into the target tumors. Upon irradiation by thermal or epithermal neutrons, the nuclides then produce localized cytotoxic radiations (Barth and Soloway, 1994; Carlsson et al., 2002). Earlier studies were mainly focused on using boron-10 (^{10}B) as the nuclide for the treatment of melanoma and brain glioma (Barth et al., 2005). Gd neutron capture therapy (Gd-NCT) is a new NCT approach using the γ-rays and auger electrons emitted from the ^{157}Gd (n,γ) ^{158}Gd reaction to kill tumor cells (Shih and Brugger, 1992). Gd-NCT is generally considered to be advantageous over B-NCT due to the 66 times larger thermal neutron capture cross-section of the Gd nuclide (Martin et al., 1989) and the longer range (>100 μm) of the γ-rays released by the Gd after a neutron irradiation (Brugger and Shih, 1989). The γ-rays are expected to kill tumor cells even when the Gd is outside the tumor cells, which eliminates the requirement for the delivery of the Gd into tumor cells (De Stasio et al., 2001; Hofmann et al., 1999). In addition, because many Gd compounds, such as the Gd-DTPA (diethylenetriaminepentaacetic acid), are used clinically as a contrast agent in magnetic resonance imaging (MRI) (Caravan et al., 1999), using Gd as the nuclide provides an opportunity to integrate MRI diagnosis with NCT.

One of the key criteria for the Gd-NCT to be successful is to deliver and maintain a sufficient amount of Gd into tumors during the neutron irradiation (Shikata et al., 2002). The optimal Gd concentration in tumors for Gd-NCT was estimated to be 50–200 μg/g wet tumor tissues (Shih and Brugger, 1992), which had proven to be rather challenging to achieve, if the Gd compounds are to be injected systemically. Thus, many Gd delivery systems, such as calcium carbonate microparticles (Miyamoto et al., 1997), lecithin microcapsules (Jono et al., 1999), lipid emulsions (Dierling et al., 2006; Miyamoto et al., 1999), gadopentetic acid–chitosan complex nanoparticles (Tokumitsu et al., 1999),

* Corresponding author. Tel.: +1 541 737 3255; fax: +1 541 737 3999.
E-mail address: Zhengrong.cui@oregonstate.edu (Z. Cui).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.04.009

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), had been prepared to enhance the delivery and the retention of Gd in tumors. Unfortunately, only a couple of those systems were able to deliver the required amount of Gd into tumors when they were intravenously (i.v.) injected into tumor-bearing murine models (Miyamoto et al., 1999). In order to deliver a sufficient amount of Gd into tumors, several of these Gd-systems were directly injected into tumors (Akine et al., 1992; Hofmann et al., 1999; Khokhlov et al., 1995; Matsumura et al., 2003; Tokumitsu et al., 1999, 2000). However, direct intratumor injection is not preferred for tumors that may not be easily located.

Ideally, Gd compounds or delivery systems should be intravenously injected to allow the Gd to spontaneously accumulate into tumors. To achieve this, we have developed a Gd-DTPA-encapsulated, PEGylated liposome formulation that encapsulated as high as 6.8 mg of Gd/mL of liposomes (Le and Cui, 2006). Due to the PEGylation and the high content of cholesterol in the lipid composition, the liposomes exhibited a prolonged blood circulation time (i.e., the $t_{1/2}$ in mouse blood was >24 h), which was expected to lead to repeated passages of the Gd-encapsulated liposomes through the tumor microvascular bed, and thus, a greater efficiency of extravasations per unit volume of convective transport time (Gabizon and Papahadjopoulos, 1988; Gabizon, 2001). In addition, because the Gd-DTPA was complexed with a cationic polymer, poly-L-lysine (pLL), prior to being encapsulated into the liposomes, the "leakage" of Gd from the liposomes was found to be very limited (Le and Cui, 2006). The pLL could be readily replaced with a USP material protamine sulfate to decrease any potential toxicity from pLL. More importantly, in a preliminary study, we have found that a tumor Gd concentration of more than 100 μg/g of wet tumor tissues was readily achieved when the Gd-encapsulated liposomes were injected (i.v.) into mice pre-established with a model tumor. These findings warrant further exploration of this Gd-encapsulated liposome formulation for Gd-NCT.

In the present study, we sought: (i) to define the distribution and tumor uptake kinetics of the Gd encapsulated into the liposomes in model tumor-bearing mice, (ii) to evaluate the effect of multiple dosing and the type of tumors on the distribution and tumor uptake of Gd and (iii) to identify the relationship between the size of tumors and the amount of Gd that can accumulate in the tumors. The results from this study have clinically relevant implications.

2. Materials and methods

2.1. Materials

Soy hydrogenated phosphatidylcholine (Soy HPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (PEG 2000) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Cholesterol (Chol), Gd-DTPA, pLL (MW 5600), Sephadex-G75 and phosphate-buffered saline (PBS, pH 7.4) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cellulose dialysis membranes (MWC 50,000) were from Spectrum Chemicals & Laboratory Products (New Brunswick, NJ, USA). TC-1 cells were from Dr. T.C. Wu at the Johns Hopkins University. TC-1 cells were C57BL/6 mouse lung endothelial cells transformed with the HPV 16 E6 and E7 oncogenes and an activated H-ras (Lin et al., 1996). The 24JK tumor cell line, a tumor cell line derived from the MCA102 fibrosarcoma generated from C57BL/6 mice, was generated by Dr. P. Hwu in the National Cancer Institute (Hwu et al., 1995). Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL of penicillin (Invitrogen) and 100 μg/mL of streptomycin (Invitrogen).

2.2. Methods

2.2.1. Preparation of Gd-DTPA-encapsulated liposomes

Gd-encapsulated liposomes were prepared by the thin film hydration method with subsequent freeze–thaw as previously described (Le and Cui, 2006). Briefly, a thin film of soy HPC:Chol:PEG 2000 (50:35:5, molar ratio) was formed in the bottom of a glass tube by chloroform evaporation. The lipid thin film was suspended in an aqueous solution with Gd-DTPA/pLL complexes (1:0.25, w/w) by vigorous mixing at room temperature. The suspension was frozen and thawed for six cycles and sonicated for 15 min. The concentrations of HPC, Chol and PEG 2000 were 33, 12.73 and 12.15 mg/mL, respectively. One hundred nanometer range liposomes were prepared by extruding the suspension 11 times through 400 and 100 nm polycarbonate membranes sequentially (Avanti Polar Lipids). Free Gd-DTPA was removed by dialyzing against 0.9% NaCl solution through a cellulose dialysis membrane (MWC 50,000) for 15 h. The amount of Gd encapsulated in the liposomes was measured using an inductively coupled plasma optical emission spectrometer (ICP-OES, Teledyne Leeman Labs, Hudson, NH, USA) at 342.247 nm. The Gd content in the Gd-encapsulated liposomes was estimated to be 6.8 ± 0.3 mg pure Gd/mL. The encapsulation efficiency (%) of the Gd-DTPA in liposomes was $25.7 \pm 1.4\%$. The Gd-DTPA solution used to hydrate the lipids was 10% (w/v).

2.2.2. Biodistribution and tumor uptake study

Female C57BL/6 mice, 6–8 weeks old, were purchased from the Simenson Lab (Gilroy, CA, USA). All experiments were completed following the National Institutes of Health guidelines for care and use of laboratory animals. To establish tumors in mice, TC-1 cells (5×10^5) were subcutaneously (s.c.) injected in the flank of mice on day 0. On days 10–15, mice were injected (i.v.) with the Gd-encapsulated liposomes via the tail vein. The volume of the liposomes was adjusted to 200 μL/mouse (~20 μg Gd/g of body weight). Mice were euthanized at predetermined time points. Their tumor, blood, liver, spleen, heart, lung and kidney were collected, weighed, desiccated at 60 °C for 12 h, 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 content in the samples was then determined using ICP-OES. To estimate the total amount of Gd in the blood, the total blood volume of a

mouse was assumed to be 7.5% (v/w) of the mouse total body weight (Davies and Morris, 1993).

In the multiple dosing experiment, tumor-bearing mice were injected (i.v.) three times with the Gd-encapsulated liposomes at 0, 8 and 14 h, each time with a dose of 20 μg Gd/g of body weight. Twelve hours after the last injection, mice were euthanized with CO_2. The organs were harvested and Gd concentrations in them were determined as mentioned above.

To evaluate the biodistribution and tumor uptake of Gd by a different tumor, a similar experiment was completed in mice pre-grafted with 24JK tumors. Briefly, mice were seeded with 24JK cells (5×10^5) on day 0 in their flank. On day 15, mice were injected (i.v.) with a single dose of the Gd-encapsulated liposomes. About 12–13 h later, mice were euthanized; tumor and other organs were collected and processed as mentioned above.

2.2.3. 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, ANOVA followed by pairwise comparisons with Fisher's protected least significant difference (PLSD) procedure was used. Linear regressions were completed using the S-plus 7.0 software from the Insightful Corporation (Seattle, WA, USA). A p value of ≤ 0.05 (two-tail) was considered to be statistically significant.

3. Results and discussions

Gd-NCT is a promising therapeutic modality for solid tumors. One of the key factors for the success of Gd-NCT is to deliver and maintain a sufficient amount of Gd in tumor tissues during neutron irradiation (Shikata et al., 2002). We have previously reported a Gd-encapsulated liposome formulation, which: (i) had a blood half-life of more than 24 h in mice, (ii) was encapsulated with 6.8 mg of Gd/mL of liposomes and (iii) readily led to an average tumor Gd concentration of more than 100 μg Gd/g of wet tumors when intravenously injected into mice pre-grafted with model tumors (Le and Cui, 2006). These findings were encouraging because it was estimated that the optimal Gd concentration in tumors for Gd-NCT should be 50–200 μg/g tumor tissues (Shih and Brugger, 1992). In the present study, we systemically evaluated the biodistribution and tumor uptake of Gd in mice pre-established with two different model tumors and investigated the effect of tumor size on the tumor uptake of Gd encapsulated into the liposomes.

3.1. The biodistribution and tumor uptake of Gd in TC-1 tumor-bearing mice

In order to identify the time it takes for the content of Gd in tumors to reach the maximum, the kinetics of the Gd accumulation in tumor tissues was evaluated. The TC-1 tumor in C57BL/6 mice is a murine model of human cervical cancer (Lin et al., 1996). TC-1 cells grow rapidly in mice. Subcutaneously injected TC-1 cells (5×10^5 per mouse) generally kill the host mice in about 25–30 days, if left untreated (Cui and Huang, 2005). Thus, the TC-1 model tumors were used to evaluate the biodistribution and tumor uptake of Gd, not necessarily indicating to use the Gd-encapsulated liposomes to treat cervical cancers in the future, although it could be a possibility. As shown in Fig. 1A, the Gd encapsulated into the liposomes quickly reached tumor tissues and resulted in a pure Gd content of 33.9 ± 4.6 μg/g tumor tissues 3 h after the injection. The concentration of Gd in tumors then increased gradually and reached a maximal average value of 158.9 ± 48.7 μg/g tumor tissues 12 h after the injection (Fig. 1A). When measured 24 h after the injection, the concentration of Gd that remained in tumors was 103.1 ± 23.5 μg Gd/g wet tumor tissues, although this value was not significantly different from that in the 12 h time point ($p = 0.45$, two-tail) due to the large variations in the Gd content in tumors. Thus, in future studies, at least 12 h should be given to allow a sufficient amount of the i.v. injected liposome-encapsulated Gd to accumulate into tumor tissues. Finally, it should be pointed out that the amount of Gd in tumors achieved in this study not only surpassed the estimated concentration of Gd required for a successful NCT (Shih and Brugger, 1992), but also represent one of the highest Gd contents reported in tumor tissues when a Gd compound or delivery system was i.v. injected into a tumor-bearing animal model. Other high tumor Gd concentrations previously reported included the 101 μg Gd/g wet tumor tissues when a high Gd-nanoemulsion formulation (high Gd-nanoLE) was injected (i.v.) twice into tumor-bearing hamsters (Shikata et al., 2002) and the 107 μg Gd/g wet tumor tissues when an emulsion containing a distearylamide-Gd-DTPA was intraperitoneally injected into Greene's melanoma-bearing hamsters at a dose of 2.0 mL (6.0 mg Gd) per hamster (Miyamoto et al., 1999). 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; commercially available Gd-enriched compounds may contain as high as 99.5% of ^{157}Gd.

The biodistribution of the Gd encapsulated into liposomes in other organs of the tumor-bearing mice was also evaluated. Similar to the $t_{1/2}$ value previously reported in tumor-free Balb/C mice (Le and Cui, 2006), the $t_{1/2}$ of the liposome-encapsulated Gd in the blood of the TC-1 tumor-bearing mice was more than 24 h (Fig. 1B), suggesting that mouse species and their health condition did not significantly influence the behavior of the Gd-encapsulated liposomes in the blood. The content of Gd in the liver and spleen increased gradually and reached 24.1 ± 5.0 and 8.9 ± 1.9% (both are mean ± S.E.M.), respectively, of the total injected Gd, 24 h after the injection (Fig. 1B). The contents of Gd in other organs, including heart, lung and kidney, were all gradually decreased as a function of time, as shown in Fig. 1C.

For comparison, the contents of Gd in the tumor, blood, liver and spleen were also measured when free Gd-DTPA was injected (i.v.) into TC-1 tumor-bearing mice. As expected, the free Gd-DTPA was quickly cleared from the blood. Only 0.14 ± 0.01% of the total injected Gd-DTPA were remaining in the blood circulation 12 h after the injection. The Gd taken up by the tumors when the free Gd-DTPA in solution was injected was over 260-fold lower than that when the Gd encapsulated into liposomes was injected (Fig. 2). These findings clearly demonstrated the advantage of delivering the Gd-DTPA using the liposomes.

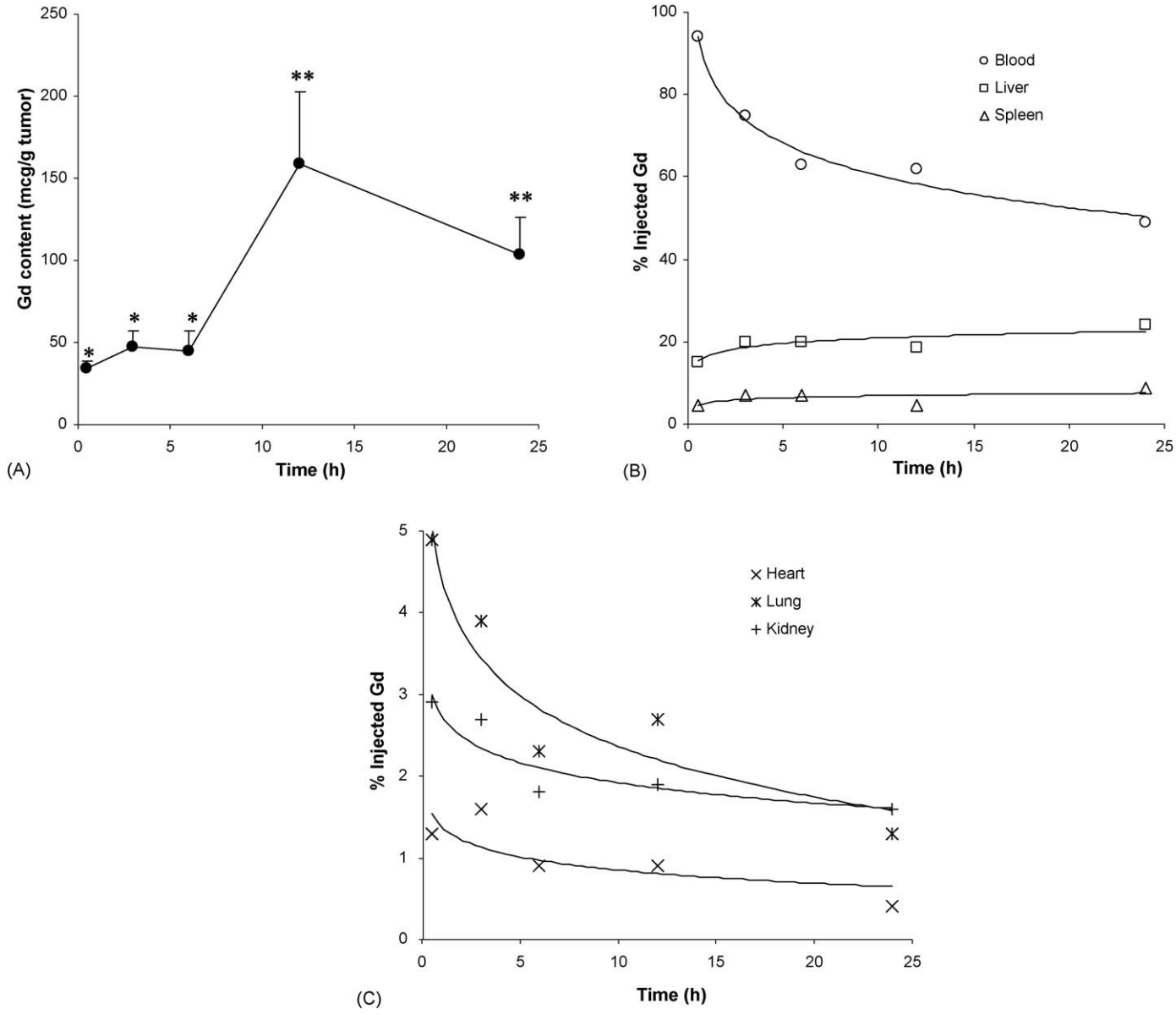


Fig. 1. The biodistribution of Gd encapsulated into the liposomes in TC-1 tumor-bearing mice. (A) The content of Gd in tumor tissues (μg Gd/g of tumors). ANOVA analysis revealed that there were significant differences among the values at the five different time points ($p = 0.02$). Asterisk (*) indicates that the values at 0.5, 3 and 6 h were not different from one another ($p = 0.66$, AVONA) and (**) indicates that the values at 12 and 24 h were comparable ($p = 0.29$, t-test). Data shown are mean ± S.E.M. ($n = 4$–7). (B and C) The percentage of the total injected Gd that was recovered in the blood and other organs.

Shown in Fig. 3 were the tumor-to-normal tissue ratios (T/N) of the Gd contents. Except in the spleen, the T/N ratios of the Gd content in blood and other organs (i.e., liver, heart, lung and kidney) were all above one, 12 h after the injection (Fig. 3A). Similar to previous reports about the splenic liposome uptake (Gabizon, 2001; Harrington et al., 2001), the liposome-encapsulated Gd was more concentrated in the spleen than in other organs that were examined. Thus, more modifications may have to be introduced into the Gd-liposome formulation to further reduce its uptake by the spleen. Otherwise, the spleen will have to be properly avoided when neutrons are to be applied in a NCT. In addition, the T/N ratios of the Gd in the tumor tissues over that in the rest of the non-tumor tissues (i.e., total body weight less tumor weight) were significantly higher than 1 in all the time points evaluated (i.e., ranged from 154 to 783) (Fig. 3B). These findings are important because they indicated a preferred accumulation of the Gd encapsulated into the liposomes in tumor tissues, which is expected to allow a selective targeting of neutrons to tumor tissues.

3.2. The biodistribution and tumor uptake of Gd in tumor-bearing mice after three injections of Gd-encapsulated liposomes

A single injection of our Gd-encapsulated liposomes had resulted in a sufficiently high concentration of Gd in tumors in the mouse model, although, as mentioned earlier, the Gd has to be ^{157}Gd-enriched. Thus, we evaluated the feasibility of further

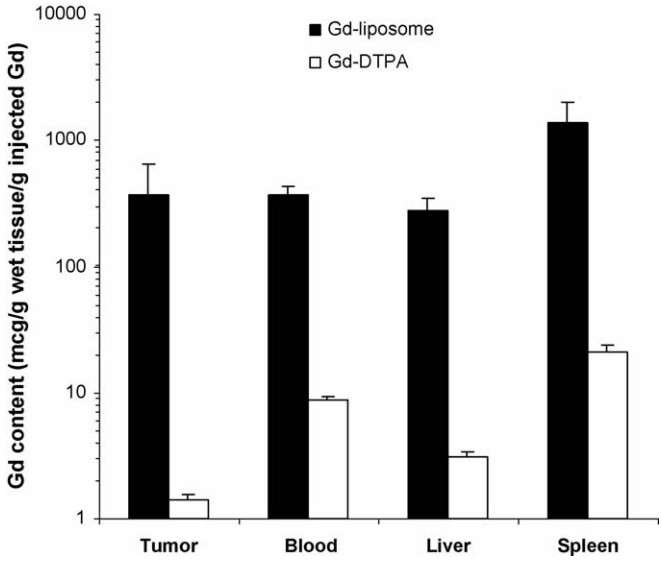


Fig. 2. The distribution of free Gd-DTPA and Gd-DTPA encapsulated into liposomes in TC-1 tumor-bearing mice. Data reported (mean ± S.D.) were the content of Gd normalized by the weight of the tissues and the total Gd injected (μg Gd/g of tissues/g of Gd injected). Statistical analyses (*t*-test) revealed that the values between the Gd-liposome and free Gd-DTPA were different from each other in all the organs tested (*p* values were 0.03, ≪0.05, ≪0.05 and 0.002 for tumor, blood, liver and spleen, respectively).

enhancing the amount of Gd that can be taken up by the tumors by dosing more Gd-encapsulated liposomes via multiple injections. The distribution and tumor uptake of Gd in TC-1 tumor-bearing mice after they were injected (i.v.) three times with the Gd encapsulated into the liposome were determined and compared to that after a single injection. The triple injections did not significantly change the relative distribution of Gd in all organs and tissues examined, except the tumor (Table 1). The percentage of the Gd injected that accumulated into tumors after the triple injections was more than doubled, when compared to that after a single injection (from 1.9 ± 0.4 to 3.9 ± 0.6%, $p = 0.007$). In fact, the actual amount of Gd accumulated into the tumors after the triple injections was 45.1 ± 13.0 μg, which was about 5.3-fold higher than that after the single injection (8.5 ± 4.8 μg). These data clearly demonstrated that increasing the dose of the Gd by multiple injections can further increase the uptake of the Gd incorporated into the liposomes by tumors, although it needs to be pointed out that the final Gd concentration accumulated in tumors after the triple injections was not significantly different from that after the single injection (158.9 ± 43.7 μg Gd/g tumor versus 233.9 ± 81.2 μg Gd/g tumor). This might be attributed to the relatively larger size of tumors in mice injected three times. Tending to be significantly different ($p = 0.08$, two-tail), the average weight of tumors in mice who were injected three times (360 ± 340 mg) was 3.75-fold larger than that in mice who were injected only once (96 ± 86 mg). As we will discuss in details later in Section 3.4, the tumor size could have a significant effect on the final concentration of Gd accumulated in the tumors.

3.3. The uptake of Gd by 24JK tumors in mice

To evaluate the effect of tumor type on the tumor uptake of Gd, the biodistribution and tumor uptake of Gd in 24JK tumor-bearing mice were examined. The 24JK cells were a B6 sarcoma cell line and can grow into tumors when injected into C57BL/6 mice (Hwu et al., 1995), although the growth tended to be much slower than that of the TC-1 tumors. Again, an average Gd content of 58.8 ± 12.9 μg/g of wet tumor tissues was achieved 13 h after the Gd-encapsulated liposomes were injected (i.v.) into the 24JK tumor-bearing mice (Table 2). Thus, we expect that our Gd-encapsulated liposomes can be used to deliver a sufficient amount of Gd into solid tumors of other different origins for potential NCT, although it needs to be pointed out that, the differences in the extent of the vascularity in different tumors could have significant effects on the Gd that can accumulate in tumors because it had been shown that a tumor that is poorly vascularized tended to take less liposomes (Gillies et al., 1999; Harrington et al., 2000). As will be discussed in details in the following section, the extent of the vascularity of the 24JK tumors was quite different from that of the TC-1 tumors.

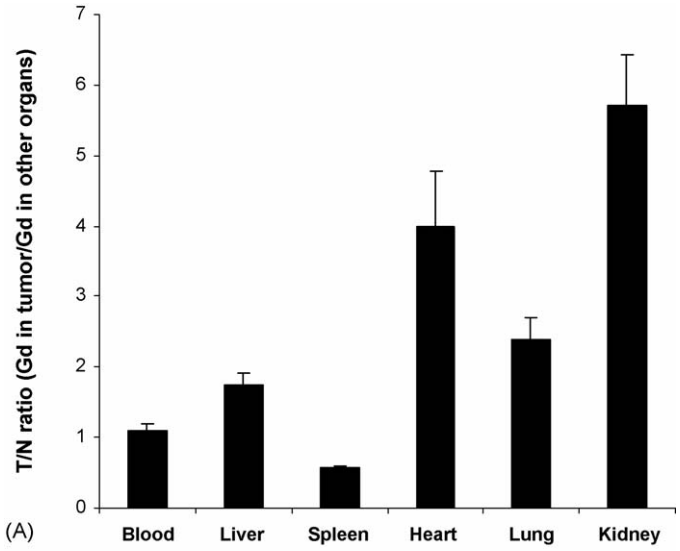
3.4. The effect of tumor size on the uptake of Gd encapsulated into the liposomes by tumors

Data from our preliminary studies have suggested that the size of the tumors tended to influence the uptake of Gd by tumors in vivo. A higher percentage of the total injected Gd tended to

Table 1
Comparison of the biodistribution and tumor uptake of Gd in TC-1 tumor-bearing mice when mice were injected once or three times with the Gd encapsulated into liposomes

	Single dose (414 μg/mouse)	Triple dose (1187 μg/mouse)
Gd concentration in tumors (μg Gd/g tumor)	158.9 ± 43.7 (34.7–365.6)	233.9 ± 81.2 (63.1–430.5)
Gd amount in tumor (μg)	8.5 ± 4.8	45.1 ± 13.0
Tumor (%injected)	1.9 ± 0.4	3.9 ± 0.6
Blood (%injected)	61.2 ± 5.9	60.6 ± 5.9
Liver (%injected)	24.4 ± 1.9	21.6 ± 4.6
Spleen (%injected)	4.4 ± 0.8	4.1 ± 1.7
Heart (%injected)	0.9 ± 0.1	1.1 ± 0.7
Lung (%injected)	2.5 ± 0.5	2.5 ± 0.4
Kidney (%injected)	1.8 ± 0.2	2.0 ± 0.2

TC-1 tumor-bearing C57BL/6 mice were injected (i.v.) with Gd-encapsulated liposomes either once or three times (8 and 6 h apart). Twelve hours later, tumors, blood and other organs were collected and the amount of Gd in them was determined. Data reported are mean ± S.D. ($n = 4$ or 7).



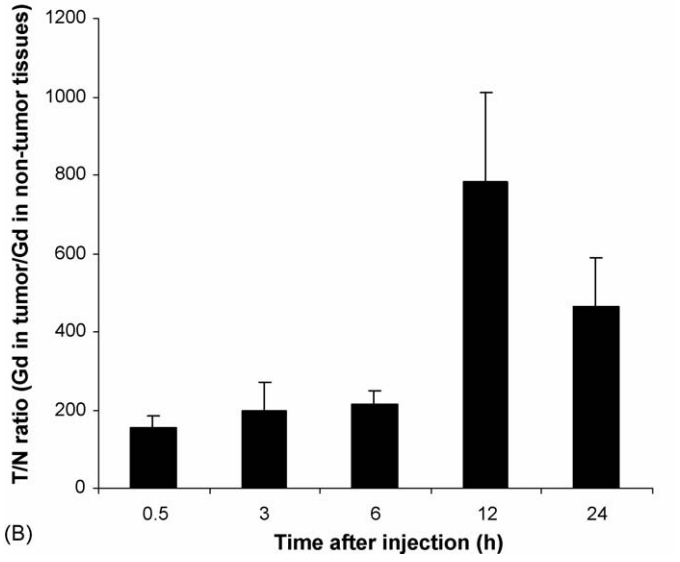


Fig. 3. The ratios of the concentration of Gd in tumors over that in other organs. Tumor-bearing C57BL/6 mice were injected via the tail vein with a single dose of the Gd-encapsulated liposomes (20 μg Gd/g of body weight). (A) Twelve hours after the injection, mice were euthanized to determine Gd distribution. The ratios shown in *Y*-axis were the concentration of Gd in tumors (μg Gd/g tissue) over that in other organs (μg Gd/g tissue) [i.e., T/N ratio, Gd_{tumor} (μg/g)/$Gd_{non\text{-}tumor\ tissues}$ (μg/g)]. Data shown were the mean from seven mice. (B) The T/N ratios of the concentration of Gd in tumors (μg Gd/g tumor) over that in the non-tumor tissues (i.e., body weight minus tumor weight) as a function of time (hours after the initial tumor cell injection). Data shown were mean ± S.E.M. (n = 3–7).

Table 2
The biodistribution and tumor uptake of Gd in 24JK tumor-bearing mice

	Gd content
Tumor (μg/g tissue)	58.8 ± 12.9
Tumor (%injected)	1.0 ± 0.6
Blood (%injected)	57.8 ± 4.1
Liver (%injected)	13.6 ± 1.3
Spleen (%injected)	6.8 ± 1.2
Heart (%injected)	1.1 ± 0.6
Lung (%injected)	1.2 ± 0.2
Kidney (%injected)	1.8 ± 0.4

C57BL/6 mice with 24JK tumors were injected (i.v.) with the Gd-encapsulated liposomes (Gd, 30 μg/g body weight in 0.2 mL) when the tumors were about 5–7 mm in diameter. Mice were euthanized 13 h later to determine Gd distribution. Data were reported as the percentages of the total injected dose (%injected dose). Data in tumors were also reported as μg of Gd/g of tumors. In only one mouse, the Gd content was below 50 μg/g tumor. Data reported are mean ± S.D. (n = 5). This experiment was repeated twice, and similar results were obtained.

be recovered in large tumors than in small ones. However, the Gd content reported as μg of Gd/g of wet tumor tissues tended to be smaller in larger tumors. These observations agreed well with previous reports showing that tumor size influenced the uptake of PEGylated liposomes by tumors in patients and in mice grafted with model tumors (Harrington et al., 2000, 2001). This prompted us to identify the relationship between TC-1 tumor size and the in vivo uptake of the Gd encapsulated into the liposomes by the tumors, whose size ranged from less than 1 mg to more than 2 g. As shown in Fig. 4A, there was a strong inverse correlation between the Gd concentration in the TC-1 tumors shown as μg of Gd in tumors/g of tumors/g of total Gd injected (μg Gd/g tumor/g Gd injected) and the tumor size (g), which can be described using the following equation ($p < 0.0001$): Gd [(μg)/g tumor/g Gd injected] = 29.746 × (tumor weight)$^{-0.7267}$. A similar equation can also be derived for the uptake of Gd by 24JK tumors: Gd = 32.132 × (tumor weight)$^{-0.4502}$ ($R^2 = 0.54$) (Fig. 4A). These equations indicated that increasing tumor size will lead to a decrease in the concentration of Gd that can be delivered into tumors. This relationship can be explained by the poor or heterogeneous vascularization of larger tumors (Acker et al., 1990; Gillies et al., 1999; Harrington et al., 2000; Sevick and Jain, 1989; Su et al., 1996). Thus, from the two equations shown for the TC-1 and the 24JK tumors, it is clear that the extents of the vascularity in these two tumors were different from each other. Also, for TC-1 tumors, the effect of tumor size on the Gd uptake tended to be more dramatic than that for 24JK tumors. This tumor size dependence of liposome-encapsulated Gd uptake is clinically relevant. Similar to the delivery of anti-tumor chemicals using long-circulating liposomes or other particulates, for small- and medium-size solid tumors, a sufficient amount of Gd is expected to be readily delivered into the tumors for a successful NCT. However, for those very small, non-vascularized or very large, poorly vascularized solid tumors, the advantage of using the liposome as a delivery system for Gd might be limited. Thus, alternative strategies might have to be applied to treat the very small, non-vascularized tumors and the very large, poorly vascularized and locally advanced tumors. Increasing the dose of the Gd-encapsulated liposomes by injecting more frequently and/or using more liposomes might increase the Gd uptake by large tumors as shown in Table 1. However, the extent to which this strategy of increasing dose can help might be limited. In chemotherapy, one of the strategies is to deliver multiple cycles of cytotoxic chemicals over a period of many weeks in an attempt to reduce the tumor mass and its interstitial pressure and increase tumor blood flow (Harrington et al., 2000). This strategy might be adopted in future Gd-NCT. Finally, the percentage of the total injected Gd that was recovered in the tumor tissues was also found to be closely correlated to the tumor weight (Fig. 4B, $p = 0.002$).

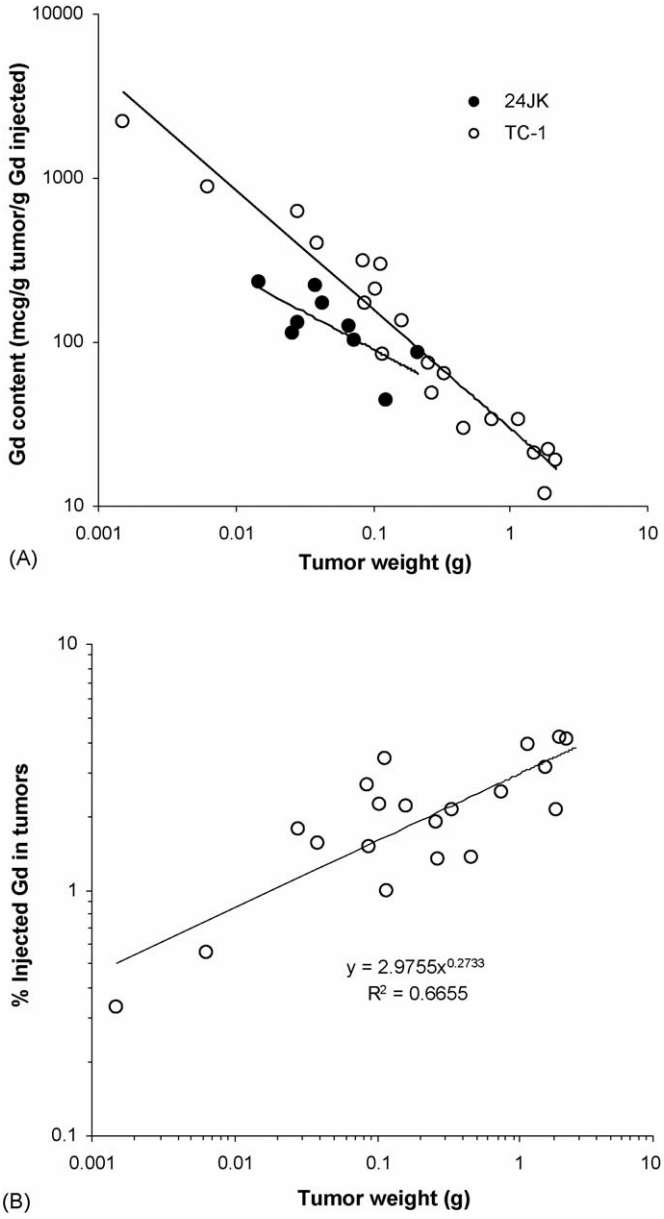


Fig. 4. The effect of tumor size on the uptake of Gd by tumors. TC-1 tumor-bearing C57BL/6 mice ($n = 20$) or 24JK tumor-bearing mice ($n = 9$) were i.v. injected with the Gd-encapsulated liposomes. Mice were sacrificed 12–13 h later. (A) The relationship between the final Gd concentration in tumors (TC-1, ◯; 24JK, ●, unit in μg Gd/g of wet tumor tissues/g of injected Gd) as a function of the tumor weight (g). A linear regression analysis using the S-plus software revealed p values of $\ll 0.05$. (B) The relationship between the percentage of the total injected Gd recovered in the TC-1 tumors (%ID) as a function of the tumor weight (g). A linear regression analysis revealed a p value of 0.002.

It needs to be pointed out that the relationships between tumor Gd uptake and tumor size might indicate another advantage of Gd-NCT over B-NCT because the long range γ-rays emitted from the Gd can kill cells without being taken up by the cells; while, in the case of B-NCT, the B has to be delivered inside tumor cells for the short range α-particles emitted by the boron to kill the tumor cells. Thus, Gd-NCT may have an improved efficiency in killing tumors in the less- or non-vascularized tissues in large tumors than B-NCT, although experiments have to be carried out to confirm it.

The composition of the liposome is similar to that of some commercial liposomally delivered anti-cancer drugs, such as Doxil® and daunoxome (Massing and Fuxius, 2000). Gd-DTPA is essentially an inert complex (Caravan et al., 1999). Although the pLL component in the Gd-encapsulated liposomes may be potentially toxic, it can be easily replaced by a USP material such as the protamine sulfate. Both pLL and protamine are polycations and can provide positively charged groups to complex with the Gd-DTPA. Thus, it is expected that this Gd-encapsulated liposome formulation will have a good safety profile.

In conclusion, we reported that a Gd concentration of above 50 μg/g of wet tumor tissues can be readily achieved by injecting (i.v.) our Gd-encapsulated liposomes into mice. It took about 12 h for the Gd concentration in tumors to reach its maximum. Finally, a strong inverse correlation between tumor size and the final amount of Gd that accumulated into the tumors was identified, suggesting that alternative strategies might have to be adopted for Gd-NCT to be effective in large, poorly vascularized, advanced solid tumors or very small and non-vascularized tumors.

References

Acker, J.C., Dewhist, M.W., Honore, G.M., Samulski, T.V., Tucker, J.A., Oleson, J.R., 1990. Blood perfusion measurements in human tumours: evaluation of laser Doppler methods. Int. J. Hyperthermia 6, 287–304.

Akine, Y., Tokita, N., Tokuuye, K., Satoh, M., Fukumori, Y., Tokumitsu, H., Kanamori, R., Kobayashi, T., Kanda, K., 1992. Neutron-capture therapy of murine ascites tumor with gadolinium-containing microcapsules. J. Cancer Res. Clin. Oncol. 119, 71–73.

Barth, R.F., Coderre, J.A., Vicente, M.G., Blue, T.E., 2005. Boron neutron capture therapy of cancer: current status and future prospects. Clin. Cancer Res. 11, 3987–4002.

Barth, R.F., Soloway, A.H., 1994. Perspective on boron neutron capture therapy. Int. J. Radiat. Oncol. Biol. Phys. 28, 1059–1060.

Brugger, R.M., Shih, J.A., 1989. Evaluation of gadolinium-157 as a neutron capture therapy agent. Strahlenther. Onkol. 165, 153–156.

Caravan, P., Ellison, J.J., McMurry, T.J., Lauffer, R.B., 1999. Gadolinium(III) chelates as MRI contrast agents: structure, dynamics, and applications. Chem. Rev. 99, 2293–2352.

Carlsson, J., Forssell-Aronsson, E., Glimelius, B., 2002. Radiation therapy through activation of stable nuclides. Acta Oncol. 41, 629–634.

Cui, Z., Huang, L., 2005. Liposome-polycation-DNA (LPD) particle as a carrier and adjuvant for protein-based vaccines: therapeutic effect against cervical cancer. Cancer Immunol. Immunother. 54, 1180–1190.

Davies, B., Morris, T., 1993. Physiological parameters in laboratory animals and humans. Pharm. Res. 10, 1093–1095.

De Stasio, G., Casalbore, P., Pallini, R., Gilbert, B., Sanita, F., Ciotti, M.T., Rosi, G., Festinesi, A., Laorcca, L.M., Rinelli, A., Perret, D., Mogk, D.W., Perfetti, P., Mehta, M.P., Mercanti, D., 2001. Gadolinium in human glioblastoma cells for gadolinium neutron capture therapy. Cancer Res. 61, 4272–4277.

Dierling, A.M., Sloat, B.R., Cui, Z., 2006. Gadolinium incorporated reconstituted chylomicron emulsion for potential application in tumor neutron capture therapy. Eur. J. Pharm. Biopharm. 62, 275–281.

Gabizon, A., Papahadjopoulos, D., 1988. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc. Natl. Acad. Sci. U.S.A. 85, 6949–6953.

Gabizon, A.A., 2001. Stealth liposomes and tumor targeting: one step further in the quest for the magic bullet. Clin. Cancer Res. 7, 223–225.

Gillies, R.J., Schornack, P.A., Secomb, T.W., Raghunand, N., 1999. Causes and effects of heterogeneous perfusion in tumors. Neoplasia 1, 197–207.

Harrington, K.J., Mohammadtaghi, S., Uster, P.S., Glass, D., Peters, A.M., Vile, R.G., Stewart, J.S., 2001. Effective targeting of solid tumors in patients with locally advanced cancers by radiolabeled pegylated liposomes. Clin. Cancer Res. 7, 243–254.

Harrington, K.J., Rowlinson-Busza, G., Syrigos, K.N., Abra, R.M., Uster, P.S., Peters, A.M., Stewart, J.S., 2000. Influence of tumour size on uptake of (111)ln-DTPA-labelled pegylated liposomes in a human tumour xenograft model. Br. J. Cancer 83, 684–688.

Hofmann, B., Fischer, C.O., Lawaczeck, R., Platzek, J., Semmler, W., 1999. Gadolinium neutron capture therapy (GdNCT) of melanoma cells and solid tumors with the magnetic resonance imaging contrast agent Gadobutrol. Invest. Radiol. 34, 126–133.

Hwu, P., Yang, J.C., Cowherd, R., Treisman, J., Shafer, G.E., Eshhar, Z., Rosenberg, S.A., 1995. In vivo antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. Cancer Res. 55, 3369–3373.

Jono, K., Ichikawa, H., Fujioka, K., Fukumori, Y., Akine, Y., Tokuuye, K., 1999. Preparation of lecithin microcapsules by a dilution method using the Wurster process for intraarterial administration in gadolinium neutron capture therapy. Chem. Pharm. Bull. 47, 54–63.

Khokhlov, V.F., Yashkin, P.N., Silin, D.I., Djorova, E.S., Lawaczeck, R., 1995. Neutron capture therapy with gadopentetate dimeglumine: experiments on tumor-bearing rats. Acad. Radiol. 2, 392–398.

Le, U.M., Cui, Z., 2006. Long-circulating gadolinium-encapsulated liposomes for potential application in tumor neutron capture therapy. Int. J. Pharm. 312, 105–112.

Lin, K.Y., Guarnieri, F.G., Staveley-O'Carroll, K.F., Levitsky, H.I., August, J.T., Pardoll, D.M., Wu, T.C., 1996. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res. 56, 21–26.

Martin, R.F., D'Cunha, G., Pardee, M., Allen, B.J., 1989. Induction of DNA double-strand breaks by 157Gd neutron capture. Pigment Cell Res. 2, 330–332.

Massing, U., Fuxius, S., 2000. Liposomal formulations of anticancer drugs: selectivity and effectiveness. Drug Resist. Updat. 3, 171–177.

Matsumura, A., Zhang, T., Yamamoto, T., Yoshida, F., Sakurai, Y., Shimojo, N., Nose, T., 2003. In vivo gadolinium neutron capture therapy using a potentially effective compound (Gd-BOPTA). Anticancer Res. 23, 2451–2456.

Miyamoto, M., Hirano, K., Ichikawa, H., Fukumori, Y., Akine, Y., Tokuuye, K., 1999. Biodistribution of gadolinium incorporated in lipid emulsions intraperitoneally administered for neutron-capture therapy with tumor-bearing hamsters. Biol. Pharm. Bull. 22, 1331–1340.

Miyamoto, M., Ichikawa, H., Fukumori, Y., Akine, Y., Tokuuye, K., 1997. Design and preparation of gadolinium-reservoir microcapsules for neutron-capture therapy by means of the Wurster process. Chem. Pharm. Bull. 45, 2043–2050.

Oyewumi, M.O., Mumper, R.J., 2003. Influence of formulation parameters on gadolinium entrapment and tumor cell uptake using folate-coated nanoparticles. Int. J. Pharm., 85–97.

Oyewumi, M.O., Yokel, R.A., Jay, M., Coakley, T., Mumper, R.J., 2004. Comparison of cell uptake, biodistribution and tumor retention of folate-coated and PEG-coated gadolinium nanoparticles in tumor-bearing mice. J. Control. Release 95, 613–626.

Sevick, E.M., Jain, R.K., 1989. Geometric resistance to blood flow in solid tumors perfused ex vivo: effects of tumor size and perfusion pressure. Cancer Res. 49, 3506–3512.

Shih, J.L., Brugger, R.M. (Eds.), 1992. Gadolinium as a neutron capture therapy agent. Med. Phys. 19, 733–744.

Shikata, F., Tokumitsu, H., Ichikawa, H., Fukumori, Y., 2002. In vitro cellular accumulation of gadolinium incorporated into chitosan nanoparticles designed for neutron-capture therapy of cancer. Eur. J. Pharm. Biopharm. 53, 57–63.

Su, M.Y., Wang, Z., Roth, G.M., Lao, X., Samoszuk, M.K., Nalcioglu, O., 1996. Pharmacokinetic changes induced by vasomodulators in kidneys, livers, muscles, and implanted tumors in rats as measured by dynamic Gd-DTPA-enhanced MRI. Magn. Reson. Med. 36, 868–877.

Tokumitsu, H., Hiratsuka, J., Sakurai, Y., Kobayashi, T., Ichikawa, H., Fukumori, Y., 2000. Gadolinium neutron-capture therapy using novel gadopentetic acid–chitosan complex nanoparticles: in vivo growth suppression of experimental melanoma solid tumor. Cancer Lett. 150, 177–182.

Tokumitsu, H., Ichikawa, H., Fukumori, Y., 1999. Chitosan–gadopentetic acid complex nanoparticles for gadolinium neutron-capture therapy of cancer: preparation by novel emulsion-droplet coalescence technique and characterization. Pharm. Res. 16, 1830–1835.

Watanabe, T., Ichikawa, H., Fukumori, Y., 2002. Tumor accumulation of gadolinium in lipid-nanoparticles intravenously injected for neutron-capture therapy of cancer. Eur. J. Pharm. Biopharm. 54, 119–124.