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A Thermo-Sensitive Polymeric Gel Containing a Gadolinium (Gd) Compound Encapsulated into Liposomes Significantly Extended the Retention of the Gd in Tumors

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Gadolinium neutron capture therapy (Gd-NCT) is a promising approach to fight cancer. One key factor for the success of Gd-NCT is to deliver and maintain a sufficient amount of Gd inside tumors. A large amount of Gd can be readily introduced into tumors by direct intratumor injection. However, an innovative approach is needed to maintain the Gd in the tumors. We encapsulated a Gd compound into a liposome formulation and then dispersed the liposomes into a thermo-sensitive polymeric gel. In murine tumor models, we showed that this liposome-in-thermo-sensitive gel system significantly extended the retention of the Gd compound in tumors. This similar concept may be applied to prolong the retention of other cytotoxic chemicals in tumors, and thus, improve their anti-tumor efficacy.

Keywords neutron capture therapy; redistribution; release; local delivery

INTRODUCTION

Gadolinium neutron capture therapy (Gd-NCT) is a promising tumor therapeutic modality (Akine et al., 1993; Khokhlov et al., 1995). During a Gd-NCT, a stable and non-radioactive Gd compound is delivered into tumors. Upon irradiation by thermal or epithermal neutrons, the Gd emits cytotoxic γ-rays and auger electrons (Shih & Brugger, 1992). Gd-NCT is generally considered to be advantageous over boron-NCT due to the larger thermal neutron capture cross-section of the ^{157}Gd (255,000 barns, 66 times larger than that of the ^{10}B) and the longer range (> 100 μm) γ-rays generated after a neutron irradiation (Barth & Soloway, 1994; Brugger & Shih, 1989). Thus, in Gd-NCT, the short range auger electrons with high linear energy transfer will extensively destroy DNA in tumor cells, and the long range γ-rays are expected to kill tumor cells even when the Gd is outside of the tumor cells, eliminating the requirement for the intracellular delivery of the Gd (De Stasio et al., 2001; Hofmann et al., 1999).

One of the key factors for the success of Gd-NCT is its ability to deliver and maintain a sufficient amount of Gd in tumors (50–200 μg of Gd/g wet tumor tissues) during the neutron irradiation (Shih & Brugger, 1992). Although it is quite easy to deliver a high concentration of Gd in tumors by direct intratumor (i.t.) injection (Hofmann et al., 1999; Khokhlov et al., 1995), it is rather challenging to maintain the Gd in tumors in order to complete the NCT (Jono et al., 1999; Khokhlov et al., 1995; Tokumitsu, Ichikawa, & Fukumori, 1999). This is largely due to the rapid diffusion of the Gd compound out of the tumors after the injection. Therefore, there is a need for a delivery system to prevent or slow down the diffusion of the Gd compounds out of the tumors. To address this need, we proposed to encapsulate a Gd compound into liposomes and then disperse the Gd-encapsulated liposomes into a thermo-sensitive polymeric gel.

We hypothesized that the liposomes would slow down the diffusion of the Gd and that the polymeric gel would slow down the diffusion of the liposomes, and thus, ultimately prolong the retention of the injected Gd compound in the tumors. This is based on data in the literature and from our own studies. Previously, we have developed a Gd-DTPA (diethylenetriamine pentaacetic acid gadolinium (III)) carrier by encapsulating the Gd-DTPA into liposomes (Le & Cui, 2006b). The Gd-DTPA was complexed with a cationic polymer, poly L-lysine (pLL), to prevent or slow down the diffusion of the Gd-DTPA out of the liposomes. Data from our in vitro release study clearly showed that the encapsulation of the Gd-DTPA into the liposomes significantly slowed down the diffusion of the Gd-DTPA. Only less than 5% of the Gd-DTPA diffused out of the liposomes within 24 h (Le & Cui, 2006b). Similarly, it was reported that when certain tumor chemotherapy agents (i.e., doxorubicin or cisplatin) were dosed intratumorally, they stayed in the tumors much longer when given as a liposome formulation than when as a free drug (Harasym, Cullis, & Bally, 1997; Hwang, Lee, Hua, & Fang, 2007). Also, it was

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shown that when mice were injected (i.t.) with anticancer chemicals in injectable gels or in solutions, sustained drug retention was observed only in tumors injected with the gels (Okino et al., 2003; Ruel-Gariepy et al., 2004; Smith et al., 1995). Finally, there were multiple previous reports of the preparation of liposomal gel formulations (liposomes in gel) (Alamelu & Rao, 1991; Bochot et al., 1998; Boulmedarat et al., 2005; Dai et al., 2006; DiTizio et al., 2000; Dragicevic-Curic et al., 2005; Glavas-Dodov et al., 2003; Glavas-Dodov et al., 2002; Gong et al., 2006; Langer et al., 2006; Mourtas et al., 2007; Ning et al., 2005; Paavola, Kilpelainen, Yliruusi, & Rosenberg, 2000; Pavelic, Skalko-Basnet, Filipovic-Grcic et al., 2005; Pavelic, Skalko-Basnet, & Jalsenjak, 2004; Pavelic, Skalko-Basnet, & Jalsenjak, 2005; Pavelic, Skalko-Basnet, & Schubert, 2001; Ruel-Gariepy et al., 2002; Takagi, Shimizu, & Yotsuyanagi, 1996; Weiner et al., 1985). Generally, it was shown that the incorporation of small molecules into the liposomal gel formulations reduced the release or diffusion of the molecules. However, successful in vivo applications of those liposomal gels were rare, if any, especially for local intratumor delivery.

To test this hypothesis, we chose to use the commercially available BD Matrigel™ Matrix (Matrigel). It is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma (Kleinman et al., 1982). Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, entactin, and nidogen (Kleinman et al., 1982). Below 22ºC, the Matrigel is in a liquid state. Above 22ºC, it rapidly transforms to a semi-solid matrix gel, resembling the mammalian cellular basement membrane. Its rapid gelation to form a semi-solid gel above 22ºC makes it ideal in which to disperse our Gd-DTPA-encapsulated liposomes. It was expected that this liposome-in-Matrigel formulation would rapidly gel upon injection into tumors, and thus, prevent or slow down the diffusion of the liposomes and the Gd-DTPA out of the tumors. In the present study, we have shown that this thermo-sensitive Matrigel with Gd-encapsulated liposomes dispersed inside significantly enhanced the retention of the Gd in tumors established in mouse models. A similar liposomal gel system may be used to intratumorally deliver other cytotoxic chemotherapy agents to improve the resultant anti-tumor effect.

MATERIALS

Soy hydrogenated phosphatidylcholine (Soy HPC), 1,2-distearoyl-Sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (PEG 2000), and polycarbonate membranes were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Cholesterol (Chol), Gd-DTPA, phosphate buffered saline (PBS, pH 7.4), chloroform, and poly-L-lysine (pLL) (MW 5,600) were purchased from Sigma-Aldrich (St. Louis, MO). Nitric acid was from Fischer Scientific, Inc. (Fair Lawn, NJ). Cellulose dialysis tubes (MWC 10,000) and cellulose dialysis membranes (MWC 50,000) were from Spectrum Laboratories, Inc. (New Brunswick, NJ). BD Matrigel™ matrix (Matrigel) was purchased from BD Biosciences (San Jose, CA). The PC-3 cells were from the ATCC (Manassas, VA). They were a human prostate cancer cell line initiated from a bone metastasis of a grade IV prostatic adenocarcinoma. The cells were grown in F-12K medium (ATCC) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). TC-1 cells were engineered by Dr. T. C. Wu's group at the Johns Hopkins University by transforming the primary lung cells from C57BL/6 mice with the human papillomavirus (HPV) type 16 E6 and E7 oncogenes and an activated H-*ras* (Lin et al., 1996). They were grown in RPMI1640 medium supplemented with 10% FBS, 100 U/mL of penicillin (Sigma), and 100 μg/mL of streptomycin (Sigma). EL4/PSA cells were kindly provided by Dr. Pavel Pisa in the Karolinska Hospital/Institute (Stockholm). This is a clonally-derived cell line stably transfected with the plasmid pCDNA3-PSA to express human PSA. The cells were grown in RPMI1640 medium in the presence of a selective antibiotic, G418 (500 μg/mL, Invitrogen).

METHODS

Preparation of Gd-DTPA-Encapsulated Liposomes (Gd-Liposome or Gd-LP)

Gd-liposomes were prepared by the thin film hydration method with subsequent repeated freezing-and-thawing (Le & Cui, 2006b). Briefly, a thin film of soy HPC:Chol:PEG 2000 (50:35:5, molar ratio) was formed in the bottom of a glass tube. The lipid thin film was suspended with an aqueous solution of Gd-DTPA, complexed with pLL (1:0.25, w/w). The suspension was frozen-and-thawed for 6 cycles, sonicated for 15 min, and then extruded 11 times sequentially through a 1,000 nm and a 400 nm-polycarbonate membrane. Free unencapsulated 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-DTPA encapsulated into the liposomes was determined using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Teledyne Leeman Prodigy, Teledyne Technologies Company, New Hampshire) at 342.247 nm.

Dispersing Gd-DTPA or Gd-Liposomes into Matrigel

Matrigel was thawed on ice overnight and then gently pipetted using pre-cooled pipette tips to ensure homogeneity. Pure Gd-DTPA solution or Gd-liposome suspension was mixed with the Matrigel at a volume ratio of 1:2 to prepare the Gd in gel formulation (Gd-Gel) or the Gd-liposomes in gel formulation (Gd-LP-Gel), respectively. The entire process was completed on ice to avoid gelling, and the final products were kept on ice prior to further use. At this ratio, the mixture rapidly gelled when placed into a 37ºC water bath (data not shown).

In Vitro Release of Gadolinium

Pure Gd-DTPA, Gd-Gel, Gd-LP, or Gd-LP-Gel, all containing ~574 μg of Gd-DTPA, was placed into a 1 mL cellulose ester dialysis tube. The tube was placed into a tube containing 12 mL of PBS (10 mM, pH 7.4) and incubated in a 37°C shaker incubator (VWR International). At predetermined time points, the dialysis tube was taken out and re-placed into another tube containing 12 mL of fresh PBS. The amount of Gd in the PBS was determined using ICP-OES.

Animal Studies

Female C57BL/6 mice (6–8 weeks) and male Nu/Nu mice (6 weeks) were from the Charles River Laboratories (Wilmington, MA). To establish tumors in nude mice, PC-3 cells in suspension were mixed (1:1 ratio, v/v) with the Matrigel, and a 0.1 mL of the mixture (1×10^6 cells) was subcutaneously (s.c.) injected in both the left and the right sides in the mouse flank on day 0. On day 27, mice (n = 3, with a total of 5–6 tumors) were intratumorally (i.t.) injected with a single dose of pure Gd-DTPA, Gd-Gel, Gd-LP, or Gd-LP-Gel. Mice were euthanized 10 min or 4 h after the injection. Tumors were collected, weighed, desiccated at 60°C for 12 h, and incinerated with nitric acid (6.6 N) at 60°C for 15 h (Le & Cui, 2006b). The concentration of the Gd in the samples was determined using ICP-OES.

To establish tumors in C57BL/6 mice, TC-1 cells or EL4/PSA cells (5×10^5) were s.c. injected in the flank of mice on day 0 (one tumor per mouse). In the case of TC-1 cells, on day 14, mice (n = 3–4) were i.t. injected with a single dose of pure Gd-DTPA, Gd-Gel, Gd-LP, or Gd-LP-Gel, and euthanized 4 h after the injection. Tumor, liver, spleen, and blood in the mice were collected to quantify the content of the Gd in them. The total blood volume of a mouse was assumed to be 7.5% (v/w) of its total body weight (Le & Cui, 2006a). In the case of the EL4/PSA tumors, on day 25, mice (n = 3–5) were i.t. injected with a single dose of the Gd-Gel or the Gd-LP-Gel, and the Gd content in the tumors was measured 4 h after the injection.

Statistical Analysis

The student t-test assuming equal variances was used if two groups were 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 procedure was used. A p value of ≤ 0.05 (two-tail) was considered to be statistically significant.

RESULTS AND DISCUSSIONS

In Vitro Release of Gd-DTPA

To maintain the Gd-DTPA inside tumors after it is injected directly into the tumors, the release of the Gd-DTPA from the Gd carrier needs to be controlled or prevented first. As shown in Figure 1, when placed into a dialysis tube, all free Gd-DTPA rapidly diffused out of the tube within 30 min. The incorporation of Gd-DTPA into the Matrigel significantly slowed down the diffusion of the Gd-DTPA as compared to the free Gd-DTPA. However, up to 20% of the Gd-DTPA still diffused out the gel within 24 h (Figure 1), indicating that the incorporation of the Gd-DTPA into the Matrigel alone may not be sufficient to prevent the diffusion of the Gd-DTPA out of tumors when directly injected into tumors. As expected, the release of the Gd-DTPA from the liposomes (Gd-LP) was very limited (Le & Cui, 2006b), less than 3% within 24 h. Dispersing the Gd-liposomes into the Matrigel did not further slow down the diffusion of the Gd (Figure 1). Apparently, the diffusion of the Gd-DTPA out of the liposomes was the rate-limiting step. Our previous data have shown that the complexation of the Gd-DTPA with the cationic polymer, pLL (MW 5600), helped to prevent or slow down the diffusion of the Gd-DTPA out of the liposomes (Le & Cui, 2006b). Although the in vitro release rate of the Gd-DTPA was not further decreased when the Gd-liposomes were dispersed into the Matrigel, it did not necessarily suggest that the Matrigel would be irrelevant in preventing the outflow of the Gd from tumors in vivo. Liposomes cannot diffuse out of the dialysis tube used in the present in vitro release study, but can directly diffuse out tumors in vivo. Therefore, the Matrigel may slow down the diffusion of the liposomes, and thus, the outflow of the Gd-DTPA in vivo.

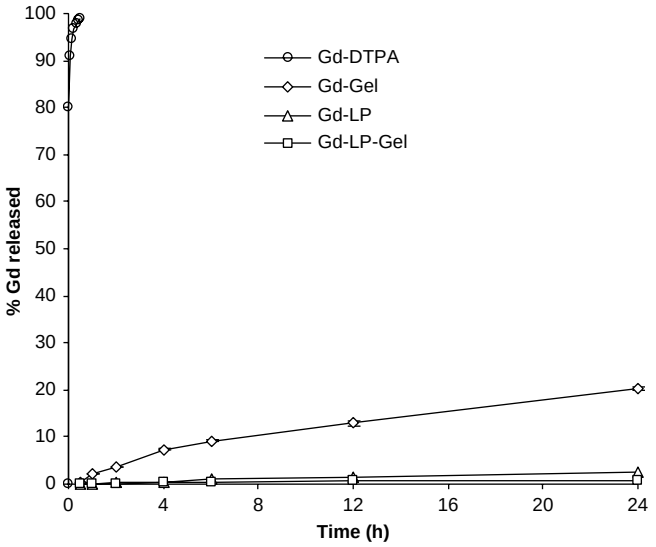

FIGURE 1. In vitro release of Gd-DTPA. Pure Gd-DTPA, Gd-Gel, Gd-LP, or Gd-LP-Gel was placed into a dialysis tube. The tube was placed into PBS, and the amount of Gd released as a function of time was monitored. Data shown are mean ± SD (n = 3).

The Retention of the Gd-DTPA in PC-3 Prostate Tumors in Nude Mice

To evaluate the ability of the Gd-LP-Gel to prolong the retention of the Gd inside tumors, a single dose of Gd-LP-Gel containing 60 μg of Gd was injected i.t. into the PC-3 prostate

tumors established in nude mice. Ten min after the injection, a Gd concentration as high as 3,794 ± 490 (μg per gram of tumor per mg of Gd injected) was recovered from the tumors (Figure 2). This corresponded to 64.5% of the injected Gd dose. As controls, tumors also were injected with the free Gd-DTPA, the Gd-Gel, or the Gd-liposome. After 10 min, a significant amount of the Gd also was recovered from all the tumors, but much less than from tumors injected with the Gd-LP-Gel (Figure 2).

Since the Matrigel is a thermo-sensitive gel, which very rapidly transforms to a semi-solid matrix gel at above 22ºC, we speculated that when the Gd-LP-Gel was injected into tumors in vivo, the Matrigel might have immediately gelated into a semi-solid state, and thus, prevented the significant outflow of the Gd-liposomes from the tumors. However, in the absence of the Matrigel, a significant fraction of the Gd-liposomes injected into the tumors might have been forced out of the tumors because of the increased intratumor pressure generated by the injection. Thus, it was likely that the Matrigel has prevented or decreased the outflow of the liposomes immediately after the injection. If what was injected was not forced out of the tumors immediately after the injection, the liposomes alone were sufficient to prevent or slow down the diffusion of the Gd-DTPA out of the tumors. For example, 4 h after the injection, the Gd-DTPA content in tumors injected with the Gd-liposomes did not significantly decrease, regardless of the presence or absence of the Matrigel (Figure 2). However, when the Gd-DTPA was not encapsulated into the liposomes, almost all of the Gd-DTPA injected into the tumors diffused out of the tumors within those 4 h, regardless of whether it was incorporated into the Matrigel or not (Figure 2). Apparently, in order to maintain a larger fraction of the injected Gd-DTPA inside tumors, it was the best to combine the encapsulation of the Gd-DTPA into liposomes and the dispersion of the liposomes into the thermo-sensitive Matrigel.

The Retention of the Gd-DTPA in TC-1 and EL4/PSA Tumors in C57BL/6 Mice

To further confirm the ability of the Gd-LP-Gel to retain the Gd-DTPA in tumors, two other tumor models, TC-1 and EL4/PSA in C57BL/6 mice, were used. Again, as shown in Figures 3A and 4,

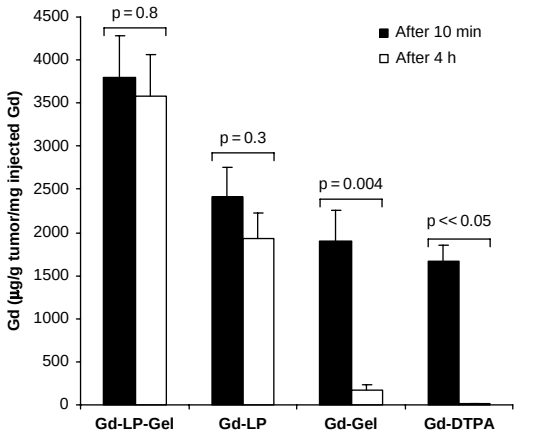

FIGURE 2. The retention of the Gd in PC-3 tumors. PC-3 cells were s.c. injected into mice to create tumors. Twenty-seven days later, Gd-LP-Gel, Gd-LP, Gd-Gel, or free Gd-DTPA was injected into the tumors. Ten min or 4 h later, the tumors were collected and weighed, and the content of Gd inside them was determined. Ten min after the injection, the value of the Gd-LP-Gel was significantly higher than that of the others (p = 0.003). Four h after the injection, the values of all four treatments were significantly different from one another ($p << 0.05$).

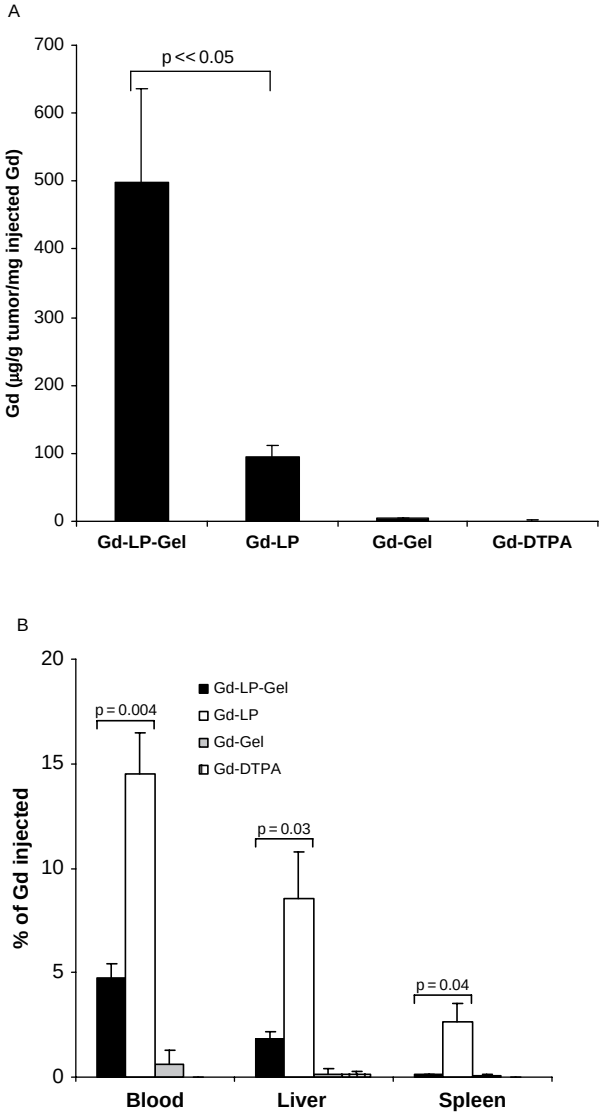

FIGURE 3. The retention of the Gd in TC-1 tumors. A single dose of Gd-LP-Gel, Gd-LP, Gd-Gel, or Gd-DTPA was injected into TC-1 tumors (120 μg of Gd/mouse). Four h later, mice were sacrificed. Their tumor, blood, liver, and spleen were harvested to quantify Gd content. A. Gd-DTPA recovered from tumors. Data shown are mean ± SEM. (n = 4–6). The levels of Gd in tumors of the four different groups were significantly different among one another ($p << 0.05$). B. The content of Gd in blood and other organs. The values of the Gd-LP and the Gd-LP-Gel were different from each other in the blood, liver, and spleen.

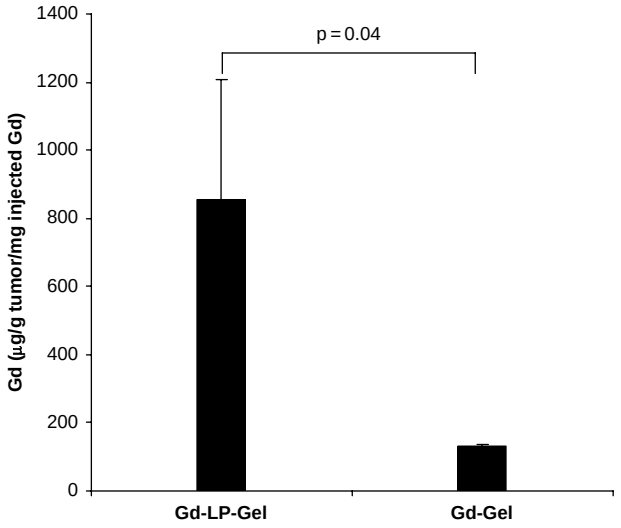

FIGURE 4. The retention of the Gd in EL4/PSA tumors. The Gd-LP-Gel or Gd-Gel was directly injected into EL4/PSA tumors. After 4 h, tumors were collected and weighed, and the Gd content was determined. Data shown are mean ± SEM (n = 3–5). The levels of Gd in tumors in the two groups were significantly different from each other (p = 0.04).

the Gd-LP-Gel significantly extended the retention of the Gd in both tumors. The average content of the Gd in tumors in the C57BL/6 mice was lower than that in the nude mice (Figure 2) simply because the tumors in the C57BL/6 mice were significantly larger than in the nude mice (0.82 ± 0.64 vs. 0.13 ± 0.04, $p << 0.05$). For the TC-1 tumors in C57BL/6 mice, we also quantified the amount of the Gd recovered from the blood, spleen, and liver to examine the redistribution of the Gd-DTPA 4 h after the i.t. injection. The free Gd-DTPA was rapidly eliminated from the mice (Figure 3B), in agreement with what was observed when the free Gd-DTPA was injected intravenously (Le & Cui, 2006a). However, when the Gd-liposomes (Gd-LP and Gd-LP-Gel) were injected directly into tumors, a significant fraction of the injected Gd was recovered from the blood, liver, and spleen 4 h after the injection (Figure 3B), in agreement with our previous finding that the liposomes had a long half-life in blood (Le & Cui, 2006b). When the Gd-liposomes in Matrigel (Gd-LP-Gel) were injected into tumors, the Gd was also recovered from the blood, liver, and spleen 4 h after the injection, but significantly less than when the Gd-liposomes were injected into tumors in the absence of the Matrigel (Figure 3B). This is likely because the Matrigel limited the diffusion of the Gd-liposomes from the tumors. Therefore, a lower percent of the i.t. injected Gd-liposomes was diffused out the tumor and into the blood circulation for redistribution.

Previously, liposomes in polymeric hydrogels, such as the thermo-sensitive chitosan-based gel (Ruel-Gariepy et al., 2002) and the poloxamer-based gel (Fattal, De Rosa, & Bochot, 2004), have been developed, aimed at enhancing the retention of drugs when delivered locally (e.g., topical, vaginal, or intravitreal) (Alamelu & Rao, 1991; Bochot et al., 1998; Boulmedarat et al., 2005; Dai et al., 2006; DiTizio et al., 2000; Dragicevic-Curic et al., 2005; Glavas-Dodov et al., 2003; Glavas-Dodov et al., 2002; Gong et al., 2006; Langer et al., 2006; Mourtas et al., 2007; Ning et al., 2005; Paavola et al., 2000; Pavelic, Skalko-Basnet, Filipovic-Grcic et al., 2005; Pavelic, Skalko-Basnet, & Jalsenjak, 2004; Pavelic, Skalko-Basnet, & Jalsenjak, 2005; Pavelic, Skalko-Basnet, & Schubert, 2001; Ruel-Gariepy et al., 2002; Takagi et al., 1996; Weiner et al., 1985). However, most of the studies were limited to in vitro characterization and release studies, and there rarely were any data showing the in vivo performance of these systems. In one study, Ning et al. (2005) evaluated the efficacy of using a clotrimazole-in-liposomes-in-carbopol gel system to deliver the clotrimazole vaginally and found that the liposomal gel system was more effective in treating yeast in rat vagina, compared to a commercial clotrimazole ointment (Ning et al., 2005). However, the activity of the clotrimazole-in-liposomes without the carbopol gel was not reported, making it impossible to know whether the liposomal gel system had prolonged the retention of the clotrimazole locally. In another study, Fattal et al. (2004) tested the feasibility of intravitreal delivery of oligonucleotides using liposomes in a thermo-sensitive poloxmer gel. Unfortunately, it was found that the oligos were cleared from the vitreous humor significantly faster when they were in the liposomal gel system than when they were in the liposomes alone without the gel (Fattal et al., 2004). The authors attributed the short residence time of the oligos within the vitreous obtained with the liposomal gel system to the slightly increased release of the oligos from the liposomes in the presence of the polymers (poloxamer). In our study, the dispersion of the Gd-liposomes into the Matrigel did not affect the release of the Gd-DTPA from the liposomes (Figure 1), which might partially explain the prolonged retention of the Gd in the tumors when delivered intratumorally using the Gd-liposomes-in-Matrigel formulation.

We have shown that dispersing the Gd-DTPA-liposomes into a thermo-sensitive polymeric gel helped to prolong the retention of the Gd-DTPA in the tumors. This was confirmed in three different solid tumor models. Similar systems may be used in future Gd-NCT trials to improve the efficacy of NCT. Moreover, other cytotoxic chemotherapeutic agents also may be locally delivered into tumors using similar liposomes-in-thermo-sensitive gel systems to prolong the residence time of the chemotherapeutic agents in the tumors, and thus, to improve their anti-tumor efficacy and decrease their non-targeted toxicity.

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