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Development of High Boron Content Liposomes and Their Promising Antitumor Effect for Neutron Capture Therapy of Cancers

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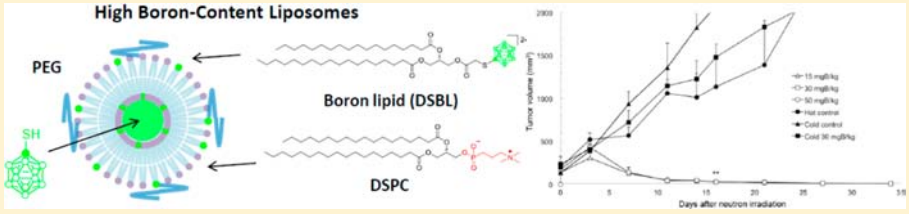
Supporting Information



ABSTRACT: Mercaptoundecahydrododecaborate (BSH)-encapsulating 10% distearoyl boron lipid (DSBL) liposomes were developed as a boron delivery vehicle for neutron capture therapy. The current approach is unique because the liposome shell itself possesses cytocidal potential in addition to its encapsulated agents. BSH-encapsulating 10% DSBL liposomes have high boron content (B/P ratio: 2.6) that enables us to prepare liposome solution with 5000 ppm boron concentration. BSH-encapsulating 10% DSBL liposomes displayed excellent boron delivery efficacy to tumor: boron concentrations reached 174, 93, and 32 ppm at doses of 50, 30, and 15 mg B/kg, respectively. Magnescope was also encapsulated in the 10% DSBL liposomes and the real-time biodistribution of the Magnescope-encapsulating DSBL liposomes was measured in a living body using MRI. Significant antitumor effect was observed in mice injected with BSH-encapsulating 10% DSBL liposomes even at the dose of 15 mg B/kg; the tumor completely disappeared three weeks after thermal neutron irradiation ($(1.5-1.8) \times 10^{12}$ neutrons/cm^2). The current results enabled us to reduce the total dose of liposomes to less than one-fifth compared with that of the BSH-encapsulating liposomes without reducing the efficacy of boron neutron capture therapy (BNCT).

INTRODUCTION

Boron neutron capture therapy (BNCT) functions as a double targeting therapy for cancer. Its therapeutic effect is realized by neutron beam irradiation and a boron delivery system (BDS). BNCT uses the nuclear reaction of two species, boron-10 (^{10}B) and thermal neutrons (eq 1).[1]

$$^{10}\mathrm{B} + {}^{1}\mathrm{n} \rightarrow {}^{4}\mathrm{He}(\alpha) + {}^{7}\mathrm{Li} + 2.4\ \mathrm{MeV} \quad (1)$$

Although the low-energy thermal neutrons (0.025 eV) are employed, the resulting α-particle and Li nuclei are high linear energy transfer (LET) particles that travel a short distance (approximately 5−9 μm) to destroy cells containing ^{10}B. If ^{10}B atoms were selectively delivered to intracellular regions of tumor tissue, it would be possible to kill tumor cells selectively without seriously damaging adjacent healthy tissues.[2−5] Although ^{10}B has a high cross section of 3838 barns for the capture of thermal neutrons, nuclides in living tissue also have certain capture cross sections; i.e., the capture cross sections of hydrogen and sodium are 0.332 and 0.43 barn, respectively. Therefore, 10^9 ^{10}B atoms per unit cell or 20−35 μg ^{10}B/g tumor tissue is necessary to fatally damage tumor cells[6] avoiding side effects to normal tissues in BNCT.

So far, two boron compounds have been utilized for BNCT. Mercaptoundecahydrododecaborate (BSH; $Na_2B_{12}H_{11}SH$) is an anionic boron cluster that is used for the treatment of malignant brain tumors[7,8] and L-*p*-boronophenylalanine (L-BPA) is a tyrosine mimic that is used to treat skin cancers.[9,10] ^{18}F-BPA was developed for positron emission tomography (PET) and is now an indispensable tool for the estimation of boron distribution in patients with tumor/normal tissue and tumor/blood ratios to determine the proper neutron doses before BNCT.[11] In the past decade, BNCT was utilized for various cancers, including head and neck cancers,[12,13] malignant meningeal tumors,[14] and hepatocellular carcinoma.[15] However, there is still an urgent

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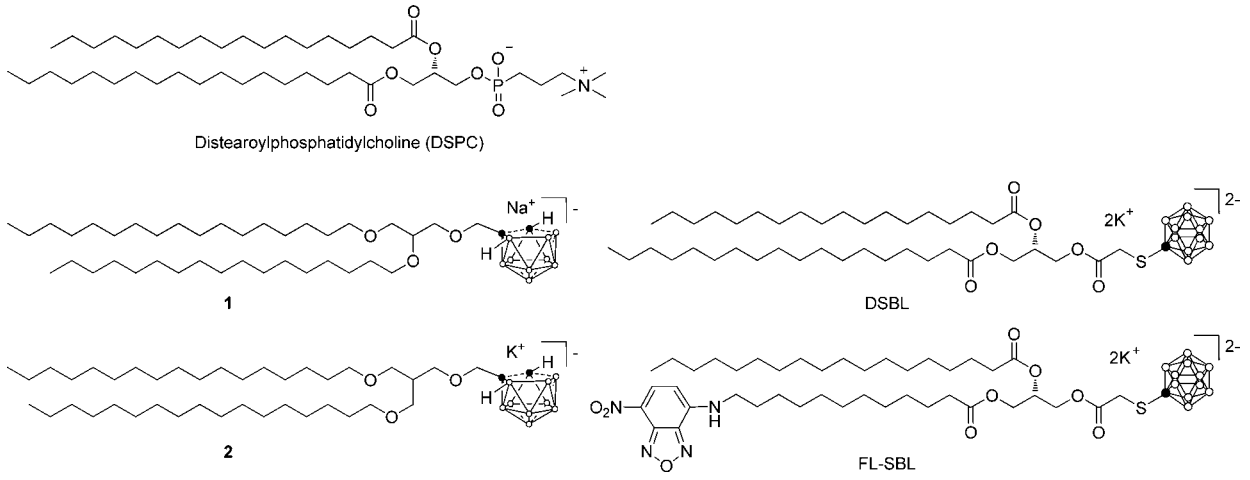

Figure 1. Structures of distearoylphosphatidylcholine (DSPC) and boron ion cluster lipids (**1**–**4**).

need to develop new ^{10}B carriers that deliver a sufficient concentration of ^{10}B atoms to a tumor to realize effective BNCT for a wide variety of cancer treatments.

Liposomes are efficient drug delivery vehicles because they can transport their contents to various tumors in a manner that is essentially independent of their contents. The accumulation of liposomes in tumor tissues caused by the enhanced permeability and retention (EPR) effect[16,17] is based on the abnormal architectures of newly formed tumoral blood vessels, that lose endothelial vessel cells without tight junctions.[18] Therefore, liposomes are considered attractive carriers for boron delivery to tumors.[19] Various boron-encapsulating BDSs have been reported, including passive targeting liposomes[20−22] and/or active targeting liposomes in conjunction with tumor-specific ligands, such as monoclonal antibodies,[23−25] folate,[26,27] epidermal growth factor,[28] and transferrin (TF).[29] TF-conjugated BSH-encapsulating liposomes injected at the dose of 35 mg B/kg highly accumulated in colon 26 tumor bearing mice (35.5 μg B/g), and the tumor growth was completely suppressed after thermal neutron irradiation. In this case, the concentration of boron used in the preparation of liposomes was limited due to osmotic reasons. The boron/phosphorus (B/P) ratio in the prepared liposomes was approximately 1.2, revealing that ca. 743 mg/kg of distearoylphosphatidylcholine (DSPC) is necessary to administer a boron dose of 35 mg B/kg. This total liposome dose is quite high for clinical application. An important character of an approved liposomal formulation (doxil) encapsulating an anticancer drug doxorubicin is the very high concentration of the encapsulated drug.[30] Conventionally, a preinjection of liposome at a high dose was done for an achievement of targeting through saturation of liver's scavenging capacity.[31] Such a high liposome dose may cause possible liver toxicity, since the liver's normal scavenging function is impared. Therefore, the low lipid dose obtained in doxil is believed to contribute to lowering the possible liver toxicity as well as to obtain unchanged pharmacokinetic behaviors in repeated injections, since lipid uptake saturation in liver is common with high lipid doses. A low lipid dose can prevent the liver toxicity that results from the saturating amount of lipid uptake and can achieve the unchanged pharmacokinetic behaviors that are important for the targeting therapies. In order to decrease the total liposome dose, the development of liposomes with higher boron content and therefore higher B/P ratio is necessary for practical use in BDSs.

In this study, we focused on lipophilic boron compounds embedded in a liposome bilayer. This strategy is an attractive means to increase the overall incorporation efficiency of boron-containing species, as well as to raise the gross boron content of liposomes. Selective boron delivery to tumors by lipophilic species incorporated in the membrane of unilamellar liposomes was first demonstrated by Hawthorne and co-workers.[32] They prepared liposomes from DSPC, cholesterol, K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$], and the polyhedral borane species, Na_3[*ae*-$B_{20}H_{17}NH_3$], and achieved the boron concentration of 48 μg B/g tumor in mice administered the dose of 18 mg B/kg. We reported the first synthesis of *nido*-carborane lipid having a double-tailed moiety conjugated with *nido*-carborane as a hydrophilic moiety and the vesicle formation of self-assembling *nido*-carborane lipids.[19,33] Furthermore, we prepared TF-conjugated boron liposomes from the *nido*-carborane lipid to actively target a solid tumor. Although the boron concentration of 22 μg B/g tumor was observed in mice injected with the actively targeting boron liposomes at 7.2 mg B/kg body weight, the injection of a higher boron concentration (14 mg B/kg body weight) resulted in acute toxicity to the mice.[34] Similar acute toxicity was also noted in the liposomes prepared from *nido*-carborane lipid (**2**) by Hawthorne and co-workers.[35] On the basis of these observations, we focused on BSH as an alternative hydrophilic boron cluster for boron lipids and developed *closo*-dodecaborate lipids that possess the $B_{12}H_{11}S$ moiety as the hydrophilic function and have similar chirality to natural phospholipids, such as distearoylphosphatidyl boron lipid (DSBL), in their lipophilic tails (Figure 1).[36,37] Remarkably, the liposomes prepared from these *closo*-dodecaborate lipids did not exhibit acute toxicity even at the dose of 30 mg B/kg and were excreted readily via the renal pathway by healthy mice. The DSBL liposomes were taken up into the cytoplasm by endocytosis without degradation of the liposomes. The boron concentration of 23 μg B/g tumor was achieved by injection of DSBL liposomes at the dose of 20 mg B/kg. Although promising BNCT effects were found in mice injected with DSBL liposomes and tumor growth was significantly suppressed one week after neutron irradiation, complete disappearance of the tumor was not observed and tumor regrowth was noted after two weeks.[38] Our *in vivo* imaging study revealed that the fluorescent boron lipid (FL-SBL)-labeled DSPC liposomes were delivered to tumor tissue but not distributed to hypoxic regions.[39] Tumor hypoxia suppresses the responsiveness of cancer cells to

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chemotherapy and promotes cancer progression, resulting in poor prognosis.[40] Therefore, the delivery of drugs to tissues located far from blood vessels is a critical issue for successful drug targeting in drug delivery systems. In this regard, the delivery of boron to the hypoxic regions is also an important issue for the efficient BNCT of cancers.[41] In this study, we try to resolve this delivery issue by releasing a low-molecular-weight boron compound from the liposome carrier at tumor tissues, since low-molecular-weight compounds can move away from the blood vessel through diffusion much more easily than liposomes. On the other hand, an appropriate drug release rate from the carrier in the tumor tissues can minimize the released drug's washing out from the tumor tissues into the blood. Although drug release rates of boron compounds in the tumor tissues are not measured in this paper, appropriate drug release rates in the tumor tissues are expected to be obtained because of successful achievements of considerably stable circulation in blood, a large amount of accumulation in tumor, and high antitumor activity.

In this paper, we developed high boron content liposomes by incorporating boron into both the interior aqueous core and the membrane of liposomes. Indeed, this strategy yielded significant antitumor effect on tumor-bearing mice after neutron irradiation, as well as a reduction of the total liposome dose, revealing that the current boronated liposome is one of the most promising candidates for practical use in BDSs for BNCT, although various boronated liposomes have been reported recently.[35,42−45]

MATERIALS AND METHODS

Chemicals. DSPC (MC-8080) and DSPE-PEG (Sunbright DSPE-020CN) were purchased from Nippon Oil and Fats (Tokyo, Japan). Cholesterol (Chol) was purchased from Kanto Chemical (Tokyo, Japan). ^{10}B-enriched BSH and *S*-cyanoethyl-protected ^{10}B-enriched BSH were purchased from Stella Pharma Co. (Osaka, Japan). Boron lipid (DSBL) was synthesized according to the previously described procedures with modification.[36,37] Synthetic route and detailed procedures are described in Scheme S1 in the Supporting Information. Meglumine gadoterate (Magnescope) was purchased from TERUMO Co. (Tokyo, Japan). All other chemicals were of the highest grade commercially available.

Preparation of DSBL Liposomes. DSBL liposomes were prepared from DSBL/DSPC/Chol/DSPE-PEG (X:1 − X:1:0.11, molar ratio, $0 < X < 0.25$). These boronated liposomes were prepared according to the reverse-phase evaporation (REV) method.[46] Total lipids of 200 mg were dissolved in 6 mL of chloroform/diisopropyl ether mixture (1:1, v/v) and 3 mL of distilled water was added to form a w/o emulsion. The emulsion was sonicated for 3 min, and then, the organic solvents were removed under reduced pressure in a rotary evaporator at 60 °C for 30 min to obtain a suspension of liposomes. The liposomes obtained were subjected to extrusion 10 times through a polycarbonate membrane filter of 100 nm pore size (Whatman, 110605, filter, 0.1 μm, 25 mm, Gentaur Molecular Products, Belgium), using an extruder device (LIPEX Extruder, Northern Lipids, Canada) thermostatted at 60 °C. Purification was accomplished by ultracentrifugation (himac cp 80 wx, Hitachi Koki, Japan) at 200 000 *g* for 60 min at 4 °C, and the pellets obtained were resuspended in 0.9% NaCl solution.

BSH-encapsulated DSPC liposomes and DSBL liposomes were prepared from DSPC/Chol/DSPE-PEG (1:1:0.11, molar ratio) and DSBL/DSPC/Chol/DSPE-PEG (0.1:0.9:1:0.11, molar ratio), respectively. These two liposomes were prepared according to the REV method mentioned above. Three milliliters of 125 mM BSH aqueous solution was used instead of distilled water in the protocol of the REV method.[29] Particle size distribution of the boronated liposomes was measured with an electrophoretic light scattering spectrophotometer (Nano-ZS, Sysmex, Japan). The compositions of DSBL and DSPC in liposomes were calculated from data obtained by the simultaneous measurement of boron and phosphorus concentrations by inductively coupled plasma atomic emission spectroscopy (ICP-AES, HORIBA, Japan). The emission wavelengths of 249.773 and 213.618 nm were used for determination of boron and phosphorus concentrations, respectively. Standard samples for boron and phosphorus solutions at 0.1, 1.0, and 10 ppm were prepared by diluting the boron standard solution (1000 ppm, Wako Pure Chemical Industries, Ltd.) and the phosphorus atomic absorption standard solution (1000 ppm, Sigma-Aldrich Inc.), respectively. The emission wavelengths of boron and phosphorus were calibrated using these standard samples, and then each concentration of samples was determined based on these calibration curves.

Preparation of Magnescope-Encapsulated DSBL Liposomes. Magnescope-encapsulated DSBL liposomes were prepared from DSBL/DSPC/Chol/DSPE-PEG (0.1:0.9:1:0.11, molar ratio) and Magnescope solution (0.5 M of meglumine gadoterate). The final gadolinium concentration of the resulting liposome solution was 2400 ppm (15.3 mM) determined by ICP-AES.

Biodistribution of DSBL Liposomes in Mice. Tumor-bearing mice (Balb/c, female, 5−6 weeks old) were prepared by injecting subcutaneously (s.c.) a suspension (2.5×10^6 cells/mouse) of colon 26 cells directly into the left thigh. Tumor-bearing mice or healthy mice were kept on a regular chow diet and water and maintained under 12 h light/dark cycle in an ambient atmosphere. The mice ($n = 5$) were injected via the tail vein with 200 μL of DSBL liposome solution. At selected time intervals after administration, the mice were lightly anesthetized and blood samples were collected from the retro-orbital sinus. The mice were then sacrificed by cervical dislocation and dissected. Liver, spleen, kidney, heart, brain, lung, muscle, and tumor (in the case of tumor-bearing mice) were excised, washed with 0.9% NaCl solution, and weighed. The excised tissues were digested with 2 mL of conc. HNO_3 (ultratrace analysis grade, Wako, Japan) at 90 °C for 1−3 h, and then the digested samples were diluted with distilled water. After filtering through a hydrophobic filter (13JP050AN, ADVANTEC, Japan), boron concentration was measured by ICP-AES. The % of injected dose/g in blood was calculated by assuming the total normalized blood volume of 7% of the body weight.[47]

***In Vivo* Real-Time Tracking of Magnescope-Encapsulated DSBL Liposomes in Tumor-Bearing Mice Using MRI.** Tumor-bearing mice (Balb/c, female, 5−6 weeks old) were prepared by injecting subcutaneously (s.c.) a suspension (2.5×10^6 cells/mouse) of colon 26 cells directly into the back. The mice were kept on a regular chow diet and water and maintained under 12 h light/dark cycle in an ambient atmosphere. MRI experiments were performed when the tumor diameter was 7 to 9 mm. The tumor-bearing mice were injected via the tail vein with 200 μL of Magnescope-encapsulated DSBL liposome solution (2400 ppm; 15.3 mM gadolinium concentration). At selected time intervals after administration, the mice were lightly anesthetized and the contrast enhanced MR images by the liposomes were measured. MR images were taken with a Varian NMR system (9.4 T) equipped with a MR probe. Parameters of T1-weighted gradient echo protocol were TR/TE = 8.0/4.5, flip

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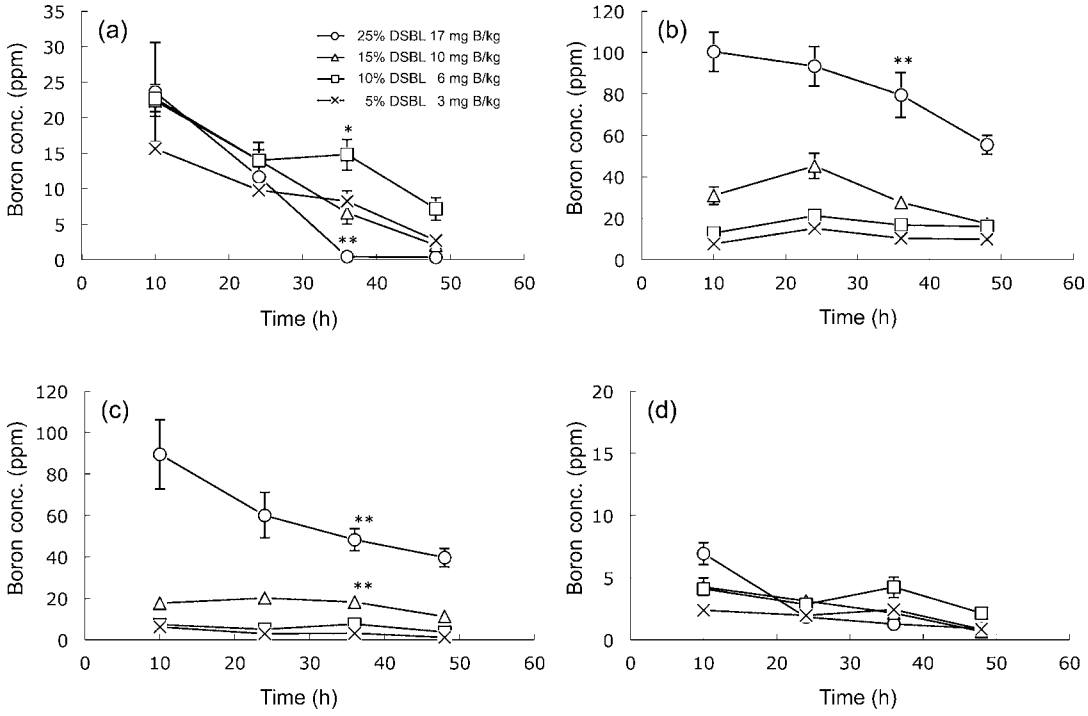

Figure 2. Time courses of boron concentrations in blood (a), liver (b), spleen (c), and kidney (d) of mice injected with DSBL liposomes containing 5%, 10%, 15%, and 25% DSBL (3, 6, 10, and 17 mg B/kg, respectively) via the tail vein. At selected time intervals after administration, blood samples were collected and digested with 2 mL of conc. HNO_3 at 90 °C for 1−3 h. Boron concentration of each tissue sample was determined by ICP-AES. Data are expressed as means ± SD (n = 5). Statistical significance: *P < 0.05 and **P < 0.01, compared with 5% DSBL (3 mg B/kg) 36 h after administration.

angle = 30°, field of view of 45 × 45 mm^2, a matrix size of 192 × 192, and thickness of 2 mm. For normalized signal intensity relative to the T1-weighted images, the tumor area was selected as a region of interest (ROI). The signal intensity of the ROI was compared with the intensity of a stock solution of 0.1 mM gadolinium ion in agarose gel.

Antitumor Effect on the Tumor-Bearing Mice Treated with Boronated Liposomes and Subsequent Neutron Irradiation. BSH-encapsulated DSBL-10% liposomes, which were prepared from ^{10}B-enriched DSBL, DSPC, Chol, and DSPE-PEG (0.1:0.9:1:0.11, molar ratio) and 125 mM BSH aqueous solution, were injected into colon 26 tumor bearing mice (female, 6−7 weeks old, 16−20 g, 5 mice in each group) via the tail vein at doses of 15 and 30 mg ^{10}B/kg (1500 and 3000 ppm of ^{10}B concentration; 200 μL of boronated liposome solution). The mice were placed in an acrylic mouse holder 36 h after i.v. injection. The mice were irradiated in the Kyoto University Reactor (KUR) for 50 min (1 MW) at a rate of (1.5−1.8) × 10^{12} neutrons/cm^2. The antitumor effects of BNCT were evaluated on the basis of the changes in tumor volume of the mice. Mortality was monitored daily and tumor volume was measured at intervals of a few days. To determine tumor volume, two perpendicular diameters of the tumor were measured with a slide caliper and calculation was carried out using the formula 0.5 ($A \times B^2$), where A and B are the longest and shortest dimensions of the tumor in millimeters, respectively. All protocols were approved by the Institutional Animal Care and Use Committee of Gakushuin University.

Statistical Analysis. The statistical significance of the results was analyzed using the Student's t test for unpaired observations and Dunnett's test for multiple comparisons.

RESULTS

Time-Dependent Boron Distribution in Blood, Liver, Spleen, and Kidney. DSBL liposomes were prepared with various DSBL ratios (X = 0.05, 0.1, 0.15, and 0.25) by the REV method.[46] Final boron concentrations of the liposome solutions prepared for injection were 300, 600, 1000, and 1700 ppm, respectively. Healthy mice were injected intravenously with 200 μL of these DSBL liposome solutions via the tail vein at the final doses of 3, 6, 10, and 17 mg B/kg (5 mice in each group), respectively. At selected time intervals after administration, blood, liver, spleen, and kidney were excised and digested with conc. HNO_3 at 90 °C. The time courses of boron concentrations in the tissue samples were determined by ICP-AES, and the results are shown in Figure 2. In the case of 25% DSBL liposomes, blood boron concentration was 23 ppm 10 h after injection, but was reduced to almost 0 ppm 36 h after injection (Figure 2a). High accumulation of liposomes was noted in liver (Figure 2b) and spleen (Figure 2c), revealing that 25% DSBL liposomes were rapidly removed from blood by the reticuloendothelial system. Both 15% and 10% DSBL liposomes displayed similar blood boron concentrations 10 and 24 h after injection; however, 10% DSBL liposomes showed longer circulation in blood. Indeed, in the case of 10% DSBL liposomes, high blood boron concentrations were observed 36 and 48 h after injection. Although a similar tendency was observed in the biodistribution of 5% DSBL liposomes, blood boron concentration was relatively low due to the low boron dose. High boron accumulation in liver and spleen was also observed when 15% DSBL liposomes were injected (Figure 2b,c). Taken together, 10% DSBL liposomes are considered the most suitable boron delivery vehicle. Significant accumulation of DSBL liposomes was not observed in kidney regardless of the dose of DSBL liposomes injected (Figure 2d).

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Boron/Phosphorus (B/P) Ratio and Particle Size of BSH-Encapsulating Liposomes, 10% DSBL Liposomes, and BSH-Encapsulating 10% DSBL Liposomes. BSH-encapsulating liposomes and 10% DSBL liposomes were prepared from DSPC, Chol, and DSPE-PEG (molar ratio 1:1:0.11) and DSBL, DSPC, Chol, and DSPE-PEG (molar ratio 0.1:0.9:1:0.11), respectively. As shown in Table 1, the final B/P ratios of BSH-encapsulating liposomes and 10% DSBL liposomes were 1.2 and 0.5, respectively, and the B/P ratio affected the final boron concentrations of liposome solution. Actually, we were able to obtain the BSH-encapsulating liposome solution and 10% DSBL liposome solution with boron concentrations of 3000 ppm and 1000 ppm, respectively. These boron concentrations were maximum obtained values for the aqueous solutions free from insoluble aggregates. Furthermore, BSH-encapsulating 10% DSBL liposomes were prepared from DSBL, DSPC, Chol, and DSPE-PEG (molar ratio 0.1:0.9:1:0.11), and 125 mM BSH solution and the B/P ratio reached 2.6 (Table 1), which enabled us to prepare the liposome solution with high boron concentration (5000 ppm).

Table 1. Boron/Phosphorus (B/P) Ratio and Particle Size of BSH-Encapsulating Liposomes, 10% DSBL Liposomes, and BSH-Encapsulating 10% DSBL Liposomes

liposomes	B/P ratio	diameter size distribution (nm)[a]
BSH-encapsulating liposomes	1.2	110 ± 0.71
10% DSBL liposomes	0.5	121 ± 1.22
BSH-encapsulating 10% DSBL liposomes	2.6	127 ± 0.71

[a]Data are expressed as means ± SD.

In Vivo Real-Time Tracking of Magnescope-Encapsulating DSBL Liposomes by MRI. As 10% DSBL liposomes displayed long circulation in blood and the encapsulation of BSH in the inner aqueous core produced a high boron content vehicle, we next performed the *in vivo* real-time tracking of 10% DSBL liposomes by MRI. Magnescope was chosen as the MRI contrast agent and Magnescope-encapsulating 10% DSBL liposomes were prepared from DSBL, DSPC, Chol, and DSPE-PEG (molar ratio 0.1:0.9:1:0.11) and Magnescope solution (0.5 M) by the REV method. Tumor-bearing mice were injected intravenously with 200 μL of Magnescope-encapsulating 10% DSBL liposome solution via the tail vein at the final dose of 24 mg (0.153 mmol) Gd/kg. At selected time intervals after the injection, MRI measurement was performed. T_1-weighted gradient echo axial imaging was performed as well using the NMR system at 9.4 T. Signal intensities of the tumor area were compared before and after injection of the Magnescope-encapsulating 10% DSBL liposomes. As shown in Figure 3, the signal intensity of the tumor area was substantially increased 24 h after injection (Figure 3b,e) and the highest signal intensity was observed 36 h after injection (Figure 3c,f).

Biodistribution of BSH-Encapsulating 10% DSBL Liposomes in Tumor-Bearing Mice. Tumor-bearing mice were prepared by injecting subcutaneously (s.c.) a suspension (2.5×10^6 cells/mouse) of colon 26 cells directly into the left thighs. Biodistribution experiments were performed when the tumor diameter was 7 to 9 mm. BSH-encapsulating 10% DSBL liposomes were injected into colon 26 tumor bearing mice at doses of 50, 30, and 15 mg B/kg via the tail veins (5 mice in each group), and both distribution and tumor accumulation of boron were measured by ICP-AES. Figure 4a shows the time courses of boron blood clearance after injection of BSH-encapsulating 10% DSBL liposomes. Twenty-four hours after injection of 50, 30, and 15 mg B/kg, boron concentrations of 334, 180, and 47 ppm were observed in blood, respectively, and those concentrations decreased gradually in a time-dependent manner. Figure 4b–d shows the time courses of boron concentrations in liver, kidney, and spleen, respectively. Time-dependent accumulation of boron was observed in liver, and boron concentrations of 279 and 178 ppm were detected 72 h after injection of 50 and 30 mg B/kg, respectively. When 15 mg B/kg was injected, boron concentration reached a maximum 36 h after injection and decreased gradually thereafter (Figure 4b). Compared to the moderate boron accumulation in kidney (Figure 4c), BSH-encapsulating 10% DSBL liposomes were highly accumulated in spleen 24 h after injection, and the boron concentrations were decreased gradually in a manner similar to blood clearance (Figure 4d). In contrast, significant boron accumulation in tumor was observed 36 h after injection; boron concentrations reached 174 and 93 ppm at doses of 50 and 30 mg B/kg, respectively. Even at the low boron dose of 15 mg B/kg, boron concentrations of 32 and 29 ppm were observed in tumor 24 and 36 h after injection, respectively. These boron concentrations in tumor are sufficiently high to induce an efficient BNCT effect on cancer.

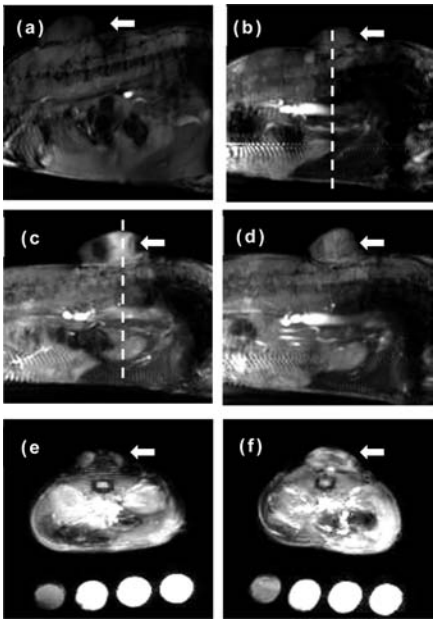

Figure 3. Gadolinium-enhanced MRI contrast images of tumor-bearing mice before injection (a) or 24 h (b), 36 h (c), and 48 h (d) after injection with Magnescope-encapsulating 10% DSBL liposomes. The mouse head is directed to the right and the tail, to the left. Parts (e) and (f) are the cross-section images taken at the point marked by dashed lines in (b) and (c), respectively. Tumor is transplanted to the back of mice and its location is indicated by a white arrow. Each sample was injected into tumor-bearing mice via the tail vein at the dose of 24 mg (0.153 mmol) Gd/kg.

BNCT Effect of BSH-Encapsulating 10% DSBL Liposomes in Tumor-Bearing Mice. The antitumor effect of BSH-encapsulating 10% DSBL liposomes in conjunction with thermal neutron irradiation was examined in colon 26 tumor bearing mice at KUR. As the highest boron concentration in tumor was observed 36 h after injecting BSH-encapsulating 10% DSBL liposomes, thermal neutron irradiation of the tumor-transplanted left thighs of mice was carried out 36 h after

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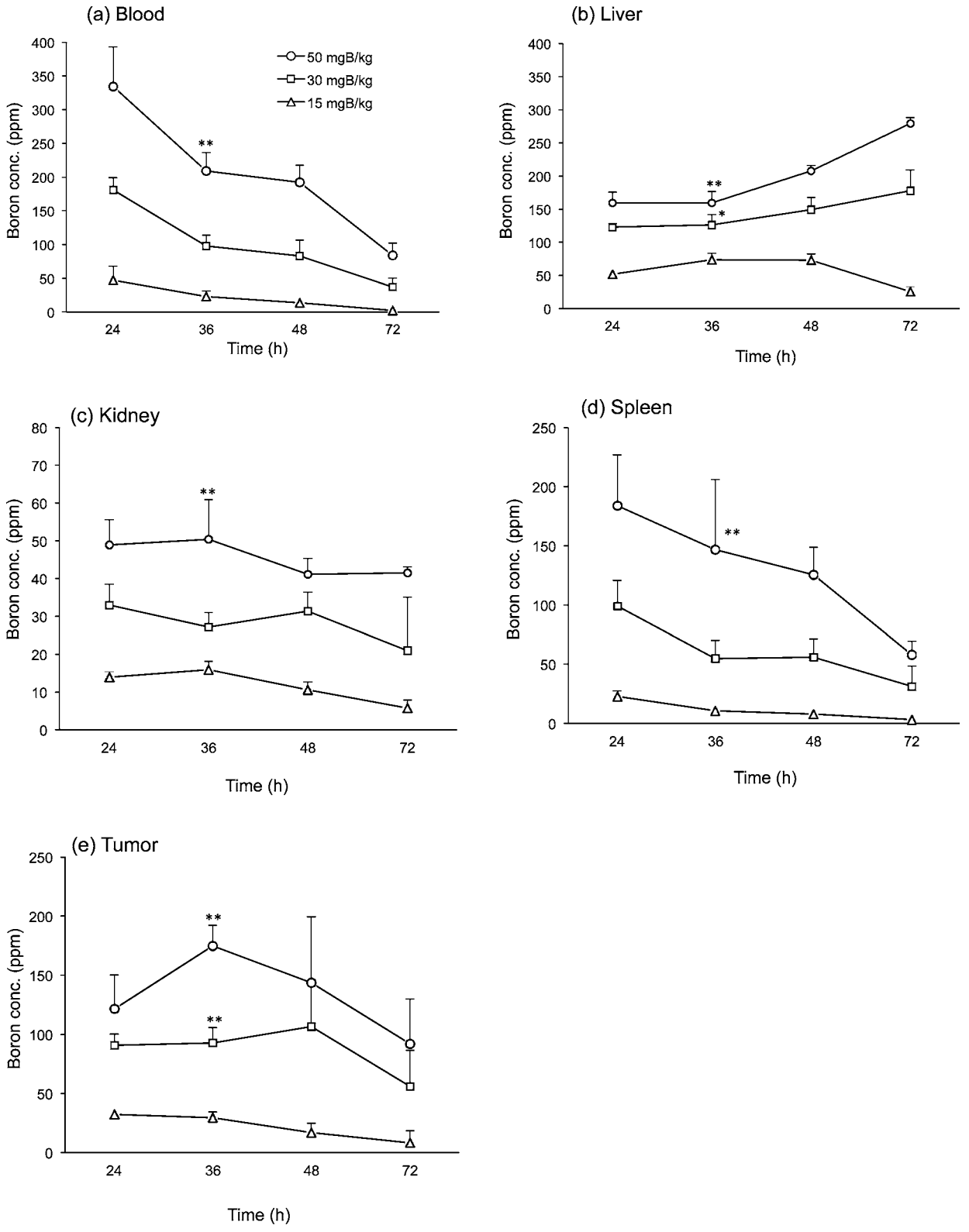

Figure 4. Time courses of boron concentrations in blood (a), liver (b), kidney (c), spleen (d), and tumor (e) of colon 26 tumor bearing mice. BSH-encapsulating 10% DSBL liposomes (200 μL) were injected at doses of 50, 30, and 15 mg B/kg via the tail vein. Data are expressed as means ± SD (n = 5). ○, 50 mg B/kg; □, 30 mg B/kg; Δ, 15 mg B/kg. Statistical significance: *P < 0.05 and **P < 0.01, compared with 15 mg B/kg of BSH-encapsulating DSBL liposomes 36 h after injection.

injection while shielding mouse bodies with an acrylic mouse holder (5 mice in each group). The tumor growth curves are shown in Figure 5. "Hot control (-●-)" and "Cold control (-▲-)" represent tumor volumes of mice with and without thermal neutron irradiation, respectively. At doses of 15, 30, and 50 mg ^{10}B/kg, tumor growth in mice treated with BSH-encapsulating 10% DSBL liposomes was significantly inhibited after thermal neutron irradiation, and the tumor disappeared

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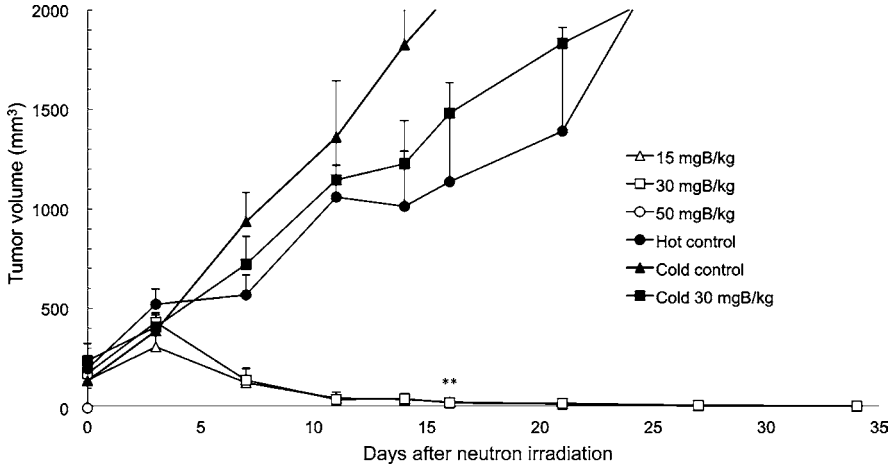

Figure 5. Tumor volumes in mice (Balb/c, female, 6 weeks old, 14−20 g) bearing colon 26 solid tumor after i.v. injection of BSH-encapsulating 10% DSBL liposomes (15 (-Δ-), 30 (-□-), 50 (-○-) mg B/kg) and thermal neutron irradiation for 50 min ($(1.5-1.8) \times 10^{12}$ neutrons/cm^2) 36 h after injection. "Hot control (-●-)" represents tumor volume of mice without injection of liposomes but with thermal neutron irradiation. "Cold control (-▲-)" represents tumor volume of mice without injection of liposomes and thermal neutron irradiation. "Cold 30 mgB/kg (-■-)" means tumor volume of mice injected with BSH-encapsulating 10% DSBL liposomes (30 mg B/kg) without thermal neutron irradiation. Data are expressed as means ± SD (n = 5). Statistical significance: $^{**}P < 0.01$, compared with hot control 16 days after neutron irradiation.

within three weeks even when the dose of 15 mg ^{10}B/kg was injected. However, the inhibition was not observed in mice injected with BSH-encapsulating 10% DSBL liposomes (30 mg ^{10}B/kg) without thermal neutron irradiation, or in hot and cold control mice.

■ DISCUSSION

The development of new ^{10}B carriers that deliver a sufficient amount of ^{10}B atoms to tumor is indispensable for the effective BNCT of cancers. To achieve successful treatment of cancer by BNCT, 20−35 μg ^{10}B/g tumor tissue is necessary. In this regard, liposomal BDS is efficient because liposomes can transport a large amount of ^{10}B atoms in a vehicle to a tumor even if the boron compounds have low affinity to the tumor.

We have focused on lipophilic boron compounds embedded in a liposome bilayer as an alternative strategy for the liposomal BDS and developed boron cluster lipids, including DSBL. Our previous study revealed that 25% DSBL liposomes did not circulate in blood for a long time, resulting in poor EPR effect and low boron accumulation in tumor. In the current study, we optimized the DSBL contents in liposomes and found that 10% DSBL liposomes are the best choice to achieve the longest circulation in blood. The % injected dose/g in blood is calculated to be 16.4% and 17.3% at 24 and 36 h after injecting a dose of 6 mg B/kg, respectively, revealing that 10% DSBL liposomes have a long-circulation property. However, the B/P ratio of the resulting liposomes is low due to the low DSBL loading. Finally, we prepared liposomes with high boron content through the combination of DSBL embedment and BSH encapsulation in liposomes, and in this case, the B/P ratio reached 2.6. It was reported that BSH induces aggregation of phosphatidylcholine-containing liposomes.[48] Therefore, the anionic *closo*-dodecaborate moiety of DSBL may interact with the choline function in the liposomal membrane to prevent BSH-induced aggregation of liposomes, enhancing the encapsulation of BSH in 10% DSBL liposomes. We prepared both BSH-encapsulating liposomes and BSH-encapsulating 10% DSBL liposomes in a 125 mM concentration of BSH solution. On the basis of the observed boron concentration and volume of each liposome as shown in Table 1, the estimated concentrations of BSH in BSH-encapsulating liposomes and BSH-encapsulating 10% DSBL liposomes are calculated to be approximately 37 mM and 50 mM, respectively. These results also show the enhancement of the encapsulation of BSH in 10% DSBL liposomes. We compared the pharmacokinetics of the BSH-encapsulating liposomes and the BSH-encapsulating 10% DSBL liposomes in healthy organs at the same dose (11 mg B/kg). As shown in Figure S1 in the Supporting Information, the 10% DSBL liposomes show higher boron concentration in blood than the BSH-encapsulating liposomes, revealing that the BSH encapsulated in 10% DSBL liposomes tolerates well whereas the BSH encapsulated in DSPC liposomes leaks during blood circulation. Higher boron accumulation of BSH-encapsulating 10% DSBL liposomes than the BSH-encapsulating liposomes were also observed in other organs such as spleen and kidney probably due to the different boron concentrations in blood. This BSH-encapsulating 10% DSBL liposome preparation enabled us to inject high boron doses of up to 50 mg B/kg and showed excellent boron delivery efficacy to tumor: boron concentrations in tumor reached 174 and 93 ppm at doses of 50 and 30 mg B/kg, respectively. Surprisingly, 32 ppm boron concentration in tumor was observed 24 h after injecting a dose of 15 mg B/kg (Figure 4c). Although high boron accumulation was observed in liver, kidney, and spleen (Figure 4b−d), there would be no serious side effects unless thermal neutron irradiation was carried out on those tissues. In this regard, BNCT functions as a double-targeting therapy that involves boron delivery to and thermal neutron irradiation of tumors. Indeed, acute toxicity was not observed in mice treated with various doses of the BSH-encapsulating 10% DSBL liposomes and then subjected to thermal neutron irradiation. Tumor growth in mice treated with those liposomes was suppressed one week after thermal neutron irradiation and disappeared completely within three weeks.

We previously developed fluorescence-labeled boron lipid (FL-SBL) liposomes and examined their *in vivo* biodistribution in tumor-bearing mice. We found that the boron lipid liposomes are localized in tumor but not distributed in hypoxic regions.[39] In general, hypoxic cells are located at a distance from blood vessels (>150 μm) because of the low oxygen supply, and tumor hypoxia affects the responsiveness of cancer cells to chemotherapy and

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promotes cancer progression, resulting in poor prognosis.[40] Our previous study of the BNCT effect on tumor-bearing mice injected with DSBL liposomes revealed that tumor regrowth occurred two weeks after neutron irradiation.[38] Thus, BSH encapsulation is necessary to deliver boron to hypoxic regions, probably by means of the transcellular diffusion of the encapsulated boron agents.[41]

MRI study showed that the highest intensity was observed in tumor 36 h after injection of Magnescope-encapsulating 10% DSBL liposomes (Figure 3). A similar tendency was observed in the time-dependent boron distribution shown in Figure 4e, revealing that estimation of the real-time biodistribution of DSBL liposomes in a living body is possible by using MRI. It is known that Magnescope has a short half-life in blood after intravenous administration (approximately 20 min).[49] Therefore, the MRI contrast effect of Magnescope would not be observed several hours after injection if Magnescope leaked from the liposomes during circulation in blood. The fact that significant enhancement by the MRI contrast agent was noted even at 36 h after injection indicated that Magnescope was encapsulated in the liposomes and delivered to the tumor region. These results suggest that a combination of ^{10}B and ^{157}Gd neutron capture therapy would be possible for the treatment of cancer using Magnescope-encapsulating 10% DSBL liposomes.

In this study, we observed the efficient antitumor effect of the BSH-encapsulating 10% DSBL liposomes with neutron irradiation even at the dose of 15 mg ^{10}B/kg. The B/P ratio of the BSH-encapsulating 10% DSBL liposomes is 2.6, which is higher than those of the BSH-encapsulating liposomes (1.2) and the 10% DSBL liposomes (0.5). As we mentioned earlier, ca. 743 mg/kg of DSPC is necessary for injection of a boron dose of 35 mg ^{10}B/kg to achieve efficient BNCT in the case of BSH-encapsulating liposomes. This quite high liposome dose may cause lipid saturation in liver, resulting in liver toxicity. In the current study, efficient anticancer effect was observed in tumor-bearing mice injected with the dose of 15 mg ^{10}B/kg, revealing that the total amount of DSPC necessary for administration can be reduced to ca. 147 mg/kg, which is less than one-fifth of the amount of BSH-encapsulating liposomes.

CONCLUSION

We prepared BSH-encapsulating 10% DSBL liposomes as a boron delivery vehicle for neutron capture therapy. The current approach is unique because the liposome shell itself possesses cytocidal potential in addition to its encapsulated agents. The BSH-encapsulating 10% DSBL liposomes have high boron content with a B/P ratio of 2.6 and display excellent boron delivery efficacy to tumor, which enabled us to reduce the total dose of liposomes to less than one-fifth compared with that of the BSH-encapsulating liposomes without reducing the efficacy of BNCT. Therefore, we believe that the BSH-encapsulating 10% DSBL liposomes are a promising candidate for practical use in BNCT.

ASSOCIATED CONTENT

Supporting Information

Details on the synthesis of boron lipid, DSBL and *in vivo* biodistribution of the BSH-encapsulating liposomes and the BSH-encapsulating 10% DSBL liposomes in organs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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