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Dodecaborate lipid liposomes as new vehicles for boron delivery system of neutron capture therapy

Manabu Ueno [a], Hyun Seung Ban [a], Kei Nakai [b], Ryu Inomata [a], Yasufumi Kaneda [c], Akira Matsumura [b], Hiroyuki Nakamura [a,*]

[a] Department of Chemistry, Faculty of Science, Gakushuin University, Mejiro, Toshima-ku, Tokyo 171-8588, Japan
[b] Department of Neurosurgery, Institute of Clinical Medicine, University of Tsukuba, Tenno-dai, Tsukuba 305-8575, Japan
[c] Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan

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ABSTRACT

closo-Dodecaborate lipid liposomes were developed as new vehicles for boron delivery system (BDS) of neutron capture therapy. The current approach is unique because the liposome shell itself possesses cytocidal potential in combination with neutron irradiation. The liposomes composed of *closo*-dodecaborate lipids DSBL and DPBL displayed high cytotoxicity with thermal neutron irradiation. The *closo*-dodecaborate lipid liposomes were taken up into the cytoplasm by endocytosis without degradation of the liposomes. Boron concentration of 22.7 ppm in tumor was achieved by injection with DSBL-25% PEG liposomes at 20 mg B/kg. Promising BNCT effects were observed in the mice injected with DSBL-25% PEG liposomes: the tumor growth was significantly suppressed after thermal neutron irradiation (1.8×10^{12} neutrons/cm^2).

1. Introduction

The cytotoxic effect of boron neutron capture therapy (BNCT) is due to the nuclear reaction of two essentially nontoxic species, boron-10 and thermal neutrons (Eq. 1):[1]

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

The resulting α-particle and Li nuclei are high linear energy transfer (LET) particles that exert the cytotoxic effect. The fact that LET particles travel a short range (approximately 10 μm) limits radiation-induced damage to cells containing boron-10. Therefore, the high accumulation and selective delivery of boron-10 into tumor tissue are the most important requirements to achieve efficient BNCT of cancers.[2–5] The amount of boron-10 necessary to realize fatal tumor cell damage is 20–35 μg/g tumor tissue.[6] At the same time, boron concentration in surrounding normal tissues and blood should be kept low to minimize damage to those tissues. Although mercaptoundecahydrododecaborate (BSH; $Na_2B_{12}H_{11}SH$)[7,8] and L-*p*-boronophenylalanine (L-BPA)[9,10] have been utilized for BNCT, the development of new boron-10 carriers that deliver an adequate concentration of boron-10 atoms to a tumor is still an important task to achieve effective cancer therapy.[11–13]

Recently much attention has been focused on the liposomal boron delivery system (BDS). Liposomes are efficient drug delivery vehicles because they can transport their contents to the interior of various tumors in a manner that is essentially independent of their contents. Therefore, boron compounds-encapsulated liposomes are attractive vehicles to deliver adequate quantities of boron to the tumor cells for BNCT. Various boron compounds-encapsulated BDSs have been developed including passive targeting liposomes[14–16] and/or active targeting liposomes by conjugating tumor specific ligands, such as mAb,[17–19] folate,[20] epidermal growth factor,[21] and transferrin (TF).[22,23] However, in order to deliver therapeutic quantities of boron to the tumor cells, concentrated aqueous solutions of polyhedral borane salts must be encapsulated. This causes osmotic problem of the liposome production.

In contrast, the development of lipophilic boron compounds embedded within the liposome bilayer is an attractive means to increase the overall incorporation efficiency of boron-containing species, as well as to raise the gross boron content of the liposome in the formation. Selective boron delivery to tumors by lipophilic species incorporated in the membranes of unilamellar liposomes was first demonstrated by Hawthorne and co-workers.[24,25] We previously reported the first synthesis of *nido*-carborane lipid (**1**) having a double-tailed moiety conjugated with *nido*-carborane as a hydrophilic moiety and its vesicle formation from **1** (Fig. 1).[26] Furthermore, we investigated the possibility of actively targeting boron liposomes to solid tumor by conjugating TF to the surface of the liposomes. Boron concentration of 22 μg ^{10}B/g tumor was observed

* Corresponding author. Tel.: +81 3 3986 0221; fax: +81 3 5992 1029.
E-mail address: hiroyuki.nakamura@gakushuin.ac.jp (H. Nakamura).

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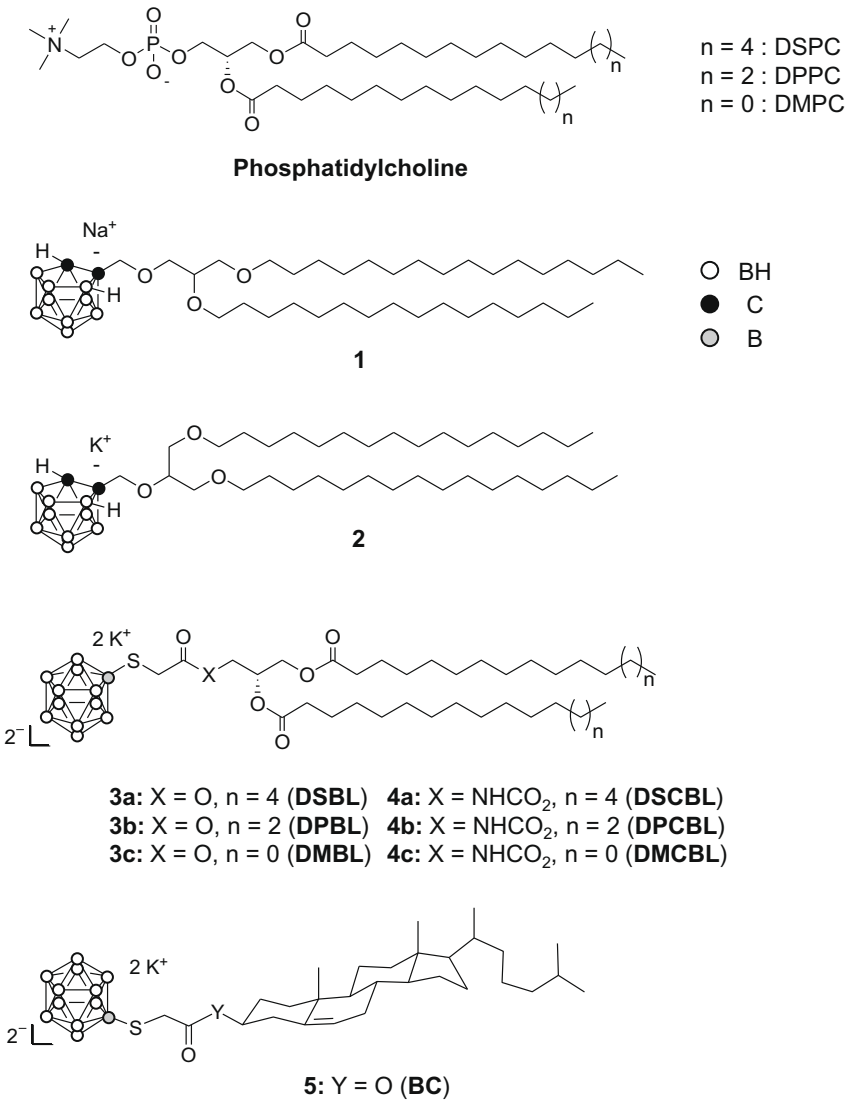

Figure 1. Structures of phosphatidylcholine, boron lipids (**1**–**4**).

in mice injected with the boron liposomes at 7.2 mg ^{10}B/kg body weight. However, the injection of a higher boron concentration (14 mg ^{10}B/kg body weight) resulted in acute toxicity to the mice.[27] Hawthorne and co-workers also recently reported similar acute toxicity in mice injected with *nido*-carborane lipid (**2**).[28] We surmised that this high toxicity may be caused by the *nido*-carborane structure, although the mechanism of *nido*-carborane cytotoxicity has not been studied in detail.

In order to overcome this drawback, we focused on BSH as an alternative hydrophilic boron cluster for boron lipids. BSH is a water-soluble divalent '*closo*-type' anion cluster that has significantly low toxicity. Because of this property, BSH has been utilized for BNCT. We were the first to synthesize *closo*-dodecaborate lipids (**3** and **4**) that possess the $B_{12}H_{11}S$ moiety as the hydrophilic function and have similar chirality to natural phospholipids, such as DSPC, in their lipophilic tails (Fig. 1).[29,30] We also focused on cholesterol, which is also one of the important contents for liposome formation, and synthesized *closo*-dodecaborate-conjugated cholesterols.[31] As we surmised, the liposomes prepared from the current *closo*-dodecaborate lipids and cholesterols did not show acute toxicity at 20 mg ^{10}B/kg body weight in healthy mice.[32] Recently, various boron compounds embedded within the liposome bilayer have been reported for BDS,[24,28,33–35] however their BNCT effects have not been reported yet. We found the significant BNCT effects of the mice treated with the current *closo*-dodecaborate lipid liposomes after neutron irradiation.[32] In this paper, we provide a full account of our BDS studies using *closo*-dodecaborate lipid liposomes.

2. Materials and methods

2.1. Chemicals

DSPC (MC-8080) and DSPE-PEG (SUNBRIGHT DSPE-020CN) were purchased from Nippon Oil and Fats (Japan). Cholesterol (Chol) was purchased from Kanto Chemical (Japan). $Na_2{}^{10}B_{12}H_{11}SH$ was kindly supplied by Stella Chemifa (Japan). DSBL (**3a**), DPBL (**3b**), DMBL (**3c**), DSCBL (**4a**), DPCBL (**4b**), DMCBL (**4c**), and BC (**5**) were synthesized as previously described[29–31] and transformed into sodium forms by an ion-exchange resin (Amberlite IR-120). PKH Linker Kit (MINI67-1KT) was purchased from Sigma (USA). All other chemicals were of the highest grade commercially available.

2.2. Preparation and composition of boronated liposomes

Boronated liposomes and PEG boronated liposomes were prepared from boron lipids, DSPC, Chol (X:1 − X:1, molar ratio, $0 < X < 1$) and boron lipids, DSPC, Chol, DSPE-PEG (X:1 − X:1: 0.11, molar ratio, $0 < X < 1$), respectively. These boronated liposomes were prepared according to the reverse-phase evaporation (REV) method.[36] 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, 0.1UM, 25MM, Gentaur Molecular Products, Belgium), using an extruder device (LIPEX™ Extruder, Northern Lipids, Canada) thermostated 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 or PBS. Particle size distribution of the boronated liposomes was measured with an electrophoretic light scattering spectrophotometer (Nano-ZS, Sysmex, Japan). The compositions of boron lipids 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).

2.3. Transmission electron microscopy analysis

An aliquot of the sample solution was applied to electron microscope carbon-coated grids covered with parlodion backing. The excess of the solution was blotted with filter paper. Grids were immediately negatively stained with 2.5% uranyl acetate for 10 min. The grids were examined in a Transmission Electron Microscope (JEM 2000FX) at an accelerating voltage of 200 kV.

2.4. Cell culture and neutron irradiation

The mouse colorectal carcinoma cell line, colon 26, was maintained at 37 °C under 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, HyClone, USA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Invitrogen, USA). For subsequent experiments, the cells were seeded at a density of 5×10^3 cells/well in a 96-well plate (Greiner, Germany) and incubated at 37 °C for 20 h. Neutron irradiation was carried out in the Japan Research Reactor No. 4 (JRR4) of Japan Atomic Energy Agency.

Method A (no wash): The cells were incubated for 30 min in the presence of various concentrations of boron lipid-25% liposomes prepared from DSPC, ^{10}B-enriched boron lipids, and Chol (0.75:0.25:1, molar ratio) in medium. The cells were irradiated with thermal neutrons in the JRR4 for 30 min ($3.8–5.0 \times 10^{11}$ neutrons/cm^2). After irradiation, the cells were washed with PBS and incubated for 3 days in fresh medium. Cell viability was determined by the MTT assay.

Method B (wash): The cells were incubated for 30 min in the presence of various concentrations of boron lipid-25% liposomes (^{10}B-enriched) in medium. After the medium was exchanged with a fresh one, the cells were incubated for another 30 min and then irradiated with thermal neutrons in the JRR4 for 30 min ($3.8–5.0 \times 10^{11}$ neutrons/cm^2). After irradiation, the cells were washed with PBS and incubated for 3 days in fresh medium. Cell viability was determined by the MTT assay.

2.5. In vitro fluorescence imaging

PKH67-labeled boronated liposomes were prepared according to the conventional cell membrane labeling method (Sigma, PKH67 Green Fluorescent Cell Linker Kit). Briefly, pellets of boronated liposomes were dissolved in 250 μL of Diluent C, and then the boronated liposome solution was added dropwise into 1 μL of PKH67 dye stock solution. The mixture was maintained at 20 °C for 5 min and free PKH67 was removed by ultracentrifugation at 200,000*g* for 60 min at 4 °C. The obtained PKH67-labeled boronated liposomes were resuspended in PBS.

Colon 26 cells were maintained at 37 °C under 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. For subsequent experiments, the cells were seeded at a density of 5×10^4 cells in a 35 mm diameter dish (Greiner) and incubated at 37 °C for 20 h. The cells were incubated at 37 °C or 4 °C in the presence of NaN_3 (1 mM)[37] for another 3 h in the presence of PKH67-labeled boronated liposomes in medium. To prevent pH changes under CO_2-free condition, Leibovitz's L-15 medium (Invitrogen, USA) were used. After incubation, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, and coverslipped using Vectashield mounting medium (Vector Lavoratories, USA) for further analysis under a fluorescent confocal microscope (FV100D IX81, OLYMPUS, Japan).

2.6. Biodistribution of boronated liposomes in mice

Tumor-bearing mice (female, 5–6 weeks old, 16–20 g, Sankyo Labo Service, Japan) were prepared by injecting subcutaneously (s.c.) a suspension (2.5×10^6 cells/mouse) of colon 26 cells directly into the right thigh. The mice were kept on a regular chow diet and water and maintained under 12 h light/dark cycle in an ambient atmosphere. Biodistribution experiments were performed when the tumor diameter was 7–9 mm. The tumor-bearing mice were injected via the tail vein with 200 μL of BSH (6000 ppm B) in 0.9% NaCl solution or DSBL-25% and -50% PEG liposomes (2000 ppm B). 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 were excised, washed with 0.9% NaCl solution, and weighed. The excised tissues were digested with 2 mL of concd 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.

2.7. BNCT for tumor-bearing mice

Liposomes (DSBL-25%) were prepared from ^{10}B-enriched DSBL (**3a**), DSPC, Chol, and DSPE-PEG (0.25:0.75:1:0.11, molar ratio) and injected into colon 26 tumor bearing mice (female, 6–7 weeks old, 16–20 g) via the tail vein at a dose of 20 mg ^{10}B/kg (2000 ppm of ^{10}B concentration; 200 μL of boronated liposome solution). The mice were anesthetized with isoflurane (Forane, Abbott, Japan) and placed in an acrylic mouse holder 24 h after iv injection. The mice were irradiated in the JRR4 for 30 min at a rate of 1.8×10^{12} neutrons/cm^2. The BNCT effects 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.

3. Results and discussion

3.1. Characterization of boronated liposomes

The boronated liposomes were prepared from DSBL (**3a**), DSPC, Chol (X:1 − X:1, molar ratio, X <1) and DSBL, DSPC, Chol, DSPE-PEG (X: 1 − X:1:0.11, molar ratio, X <1) by the REV method and particle sizes were measured with an electrophoretic light scattering spectrophotometer. As shown in Table 1, particle sizes (diameter) were distributed in the range of 95–105 nm. The boronated liposomes from each boron lipid (**3b**–**c** and **4a**–**c**) also displayed similar particle size distribution (data not shown). DSBL/DSPC lipid ratios in the liposomes were calculated from the concentrations of boron and phosphorus determined by ICP-AES. The mixing ratio of DSBL to DSPC (X value) in the preparation of the liposomes is plotted on the abscissa and the DSBL/DSPC ratio in the liposomes obtained is plotted on the ordinate, as shown in Figure 2. It was revealed that the DSBL/DSPC ratio in the liposomal membrane is proportional to the mixing ratio in the preparation. The formation of DSBL-25% liposomes was analyzed under a transmission electron microscope by the negative staining method after extrusion through a 100 nm filter. As shown in Figure 3, boronated liposomes were formed as unilamellar particles measuring 100 nm in diameter.

3.2. In vitro cytotoxicity of boronated liposomes after neutron irradiation

We next examined the effect of boronated liposomes (^{10}B-enriched) on colon 26 cells at various ^{10}B concentrations with thermal neutron irradiation. Cell viability with or without thermal neutron irradiation was measured by the MTT assay. In the case of method A (no wash), thermal neutron irradiation of cells was carried out in the presence of boronated liposomes prepared from DSBL (**3a**), DPBL (**3b**), DMBL (**3c**), DSCBL (**4a**), DPCBL (**4b**), and DMCBL (**4c**), BC (**5**) in the cell medium. BSH and $B(OH)_3$ were used as controls. As shown in Figure 4A, except DSCBL liposome, all boronated liposomes as well as BSH and $B(OH)_3$ controls showed no cytotoxicity when not irradiated with thermal neutrons. After thermal neutron irradiation, however, significant cell damage was observed in a boron-dose-dependent manner in all cases. Figure 4B shows BNCT effects on cells irradiated with thermal neutrons for 30 min after the cell medium was replaced with a boron-free one. Compared with liposomes composed of DMCBL and BC and boron compounds $B(OH)_3$ and BSH that showed no cytotoxicity, liposomes composed of DSBL, DPBL, DMBL, DSCBL, and DPCBL showed boron-dose-dependent cytotoxicity, and DSBL and DPBL were found to be potential boron lipids for liposomal boron vehicles. The results indicate that the boronated liposomes were taken up by the tumor cells and remained there for certain periods, whereas $B(OH)_3$ and BSH were readily washed out from the cells. In the current experiments, a slight difference in thermal neutron dose ranging from 3.8 to 5.0×10^{11} neutrons/cm^2 was observed on the 96-well plates. However, the difference did not affect cell viability remarkably.

Table 1
Particle size, polydispersity index, and zeta potential of DSBL liposomes

DSBL X value	Particle size[a] (nm)	Polydispersity index[a]	Zeta potential (mV)
0	94.8 ± 0.62	0.050 ± 0.003	−2.4
0.05	100.5 ± 0.17	0.033 ± 0.011	−42.8
0.1	95.6 ± 1.01	0.022 ± 0.011	−45.7
0.15	99.0 ± 0.20	0.029 ± 0.010	−42.5
0.25	102.0 ± 0.44	0.048 ± 0.005	−45.8
0.5	104.3 ± 0.61	0.034 ± 0.014	−46.7

[a] Data are expressed as means ± sem.

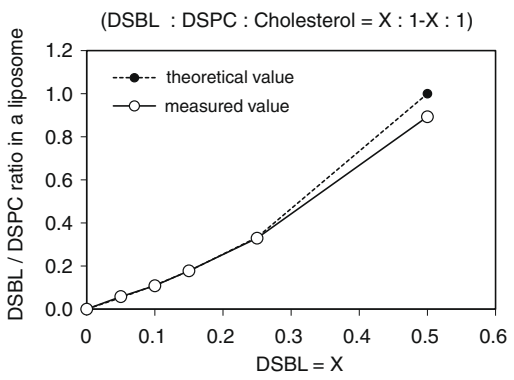

Figure 2. Composition of DSBL and DSPC in boronated liposome membranes. Boronated liposomes were prepared from DSBL, DSPC, and Chol (molar ratio is X:1 − X:1).

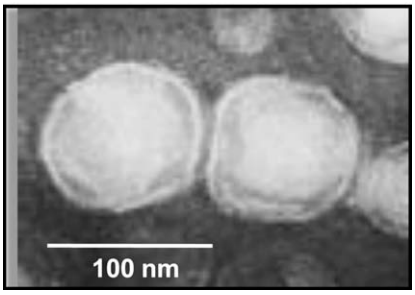

Figure 3. Transmission electron microscopy analysis of 25% DSBL liposomes.

3.3. Uptake of fluorescence-labeled boronated liposomes by endocytosis

Colon 26 cells were treated with DSBL-25% liposomes dyed green with PKH67. After 3 h incubation with the liposomes, the cells were washed with PBS and the PKH67-labeled DSBL liposomes were detected with a fluorescent confocal microscope. Figure 5A shows intracellular localization of PKH67-labeled DSBL liposomes at 37 °C. To confirm that the translocation of liposome is mediated by endocytosis, effects of endocytosis inhibitor sodium azide at low temperature.[37] As shown in Figure 5B, PKH67-labeled DSBL liposomes were localized on plasma membrane in the presence of sodium azide at 4 °C. These results indicate that DSBL liposomes were taken up into the cytoplasm by endocytosis without degradation of the liposomes.

3.4. Biodistribution of boronated liposomes in mice

Figure 6A and B show the time courses of boron concentrations in various tissues of tumor-bearing mice after injecting DSBL-25% PEG liposomes (20 mg B/kg) and BSH (60 mg B/kg). In the case of BSH, boron concentration in blood was 62.7 ppm 1 h after injection but dropped to 15.7 and 0.48 ppm 3 and 10 h after injection, respectively. Boron concentration in the other tissues decreased along with the disappearance of BSH in blood due to high renal clearance. Boron concentration of 37.8 ppm was observed in tumor with a tumor/blood ratio of ~0.6 at 1 h after injection. In contrast,

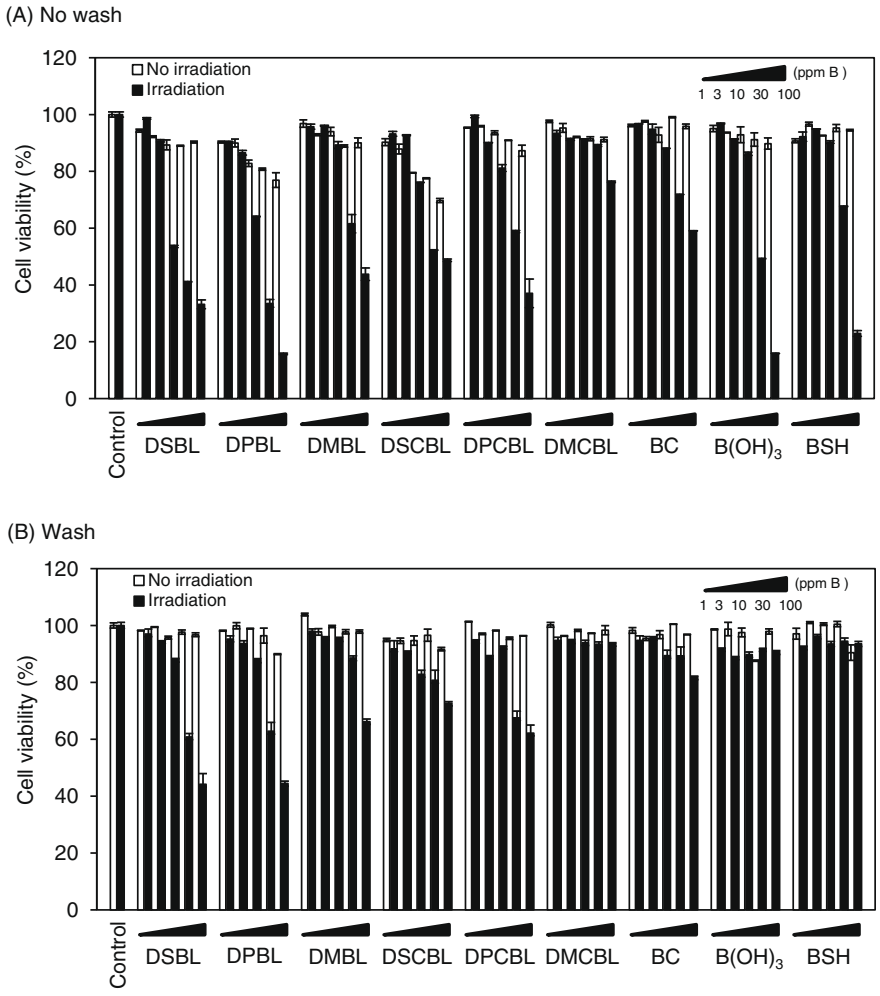

Figure 4. Effects of boronated liposomes on colon 26 cell viability after thermal neutron irradiation or no irradiation. All boronated liposomes were composed of 25% boron lipids or boron cholesterols. (A) Colon 26 cells were incubated in a 96-well microplate at 37 °C in 5% CO_2 in air for 30 min in the presence of boronated liposomes. The cells were irradiated with thermal neutrons for 30 min (no wash). Three days after irradiation, cell viability was determined by the MTT assay. (B) Colon 26 cells were incubated in a 96-well microplate at 37 °C in 5% CO_2 in air for 30 min in the presence of boronated liposomes. The cells were irradiated with thermal neutrons for 30 min after medium exchange (wash). Three days after irradiation, cell viability was determined by the MTT assay. Data are expressed as means ± sem (n = 3).

boron concentration of 22.7 ppm in tumor with a tumor/blood ratio of ~2 was observed 24 h after administration of DSBL-25% PEG liposomes, and boron concentration gradually decreased thereafter. High boron concentration was observed also in liver and spleen. The high boron concentration in spleen may be due to the instability of the DSBL-25% PEG liposomes present in blood. In general drug delivery systems, the high accumulation of drug-encapsulating or -attaching nanoparticles in other tissues, such as liver and spleen, sometimes induces side effects due to the cytotoxicity of the accumulated drugs. Current boron lipid liposomes displayed significantly low toxicity and were readily eliminated from the tissues within three weeks after injection. Therefore, it is considered that the high accumulation of boron in liver and spleen observed in Figure 6A would not have serious side effects unless thermal neutron irradiation is carried out on these tissues. In this regard, *BNCT is a double-targeting therapy that involves boron delivery to and neutron irradiation of cancers.*

3.5. BNCT effect of boronated liposomes on tumor-bearing mice

The cytotoxicity of DSBL-25% PEG liposomes was examined by irradiating colon 26 tumor bearing mice with thermal neutrons. When colon 26 cells transplanted into the left thighs of mice showed logarithmic growth, the mice were given saline as control and DSBL-25% PEG liposomes at a dose of 20 mg ^{10}B/kg. As the highest boron concentration in tumor (22.7 ppm at a dose of 20 mg B/kg) was observed 24 h after injecting DSBL-25% PEG liposomes, thermal neutron irradiation of the tumor-transplanted left thighs of mice was carried out 24 h after the injection while shielding bodies with the acrylic mouse holder. As shown in Figure 7, tumor volume in mice treated with DSBL-25% PEG liposomes was significantly inhibited after thermal neutron irradiation. The tumor volumes were ~20% of those of control mice two weeks after the neutron irradiation.

4. Conclusion

We newly prepared the *closo*-dodecaborate lipids containing liposomes as BDS vehicles for neutron capture therapy. The current approach is unique because *the liposome shell itself possesses cytocidal potential in combination with neutron irradiation*. The boronated liposomes composed of DSBL or DPBL, in particular, displayed high cytotoxicity with thermal neutron irradiation in colon 26 cells. The efficient in vitro BNCT effects were due to the uptake of the boro-

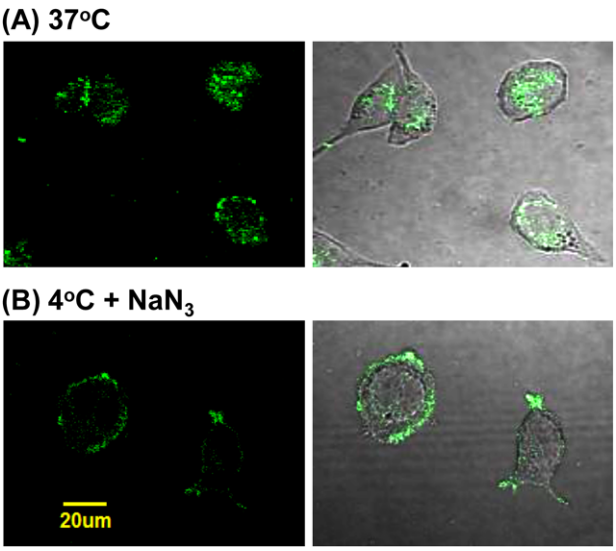

Figure 5. Intracellular localization of PKH-labeled boronated liposomes. (A) PKH67-labeled DSBL-25% liposomes were incubated at 37 °C for 3 h in colon 26 cells, and visualized under a fluorescent confocal microscope. (B) The cells were incubated at 4 °C for 3 h with PKH67-labeled DSBL-25% liposomes in the presence of NaN_3 (1 mM).

nated liposomes in the cytoplasm by endocytosis, which was observed in the fluorescence experiments using PHK67-labeled DSBL-25% liposomes. The previously developed boronated liposomes composed of *nido*-carborane lipid **1** showed acute toxicity within one day at a dose of 14 mg B/kg,[27] however the boronated liposomes described in this paper did not show acute toxicity toward healthy mice at a dose of 20 mg B/kg. Furthermore, the boronated liposomes composed of *closo*-dodecaborate lipids were readily eliminated from the body, whereas BSH-conjugated cholesterols BC and BBC were not eliminated and remained in tissues even after three weeks. Boron concentration of 22.7 ppm in tumor was achieved by injection with DSBL-25% PEG liposomes at 20 mg B/kg in tumor-bearing mice. As described, 20–35 ppm boron concentrations are required to realize fatal tumor cell damage, therefore the concentration observed by injection with DSBL-25% PEG liposomes in tumor-bearing mice is expected to result in the fatal tumor cell damage with BNCT. In fact, significant suppression of tumor growth was observed after thermal neutron irradiation. This is the first promising BNCT of tumor-bearing mice with the boron lipid liposomes, although various boron compounds embedded within the liposome bilayer have been reported. As the internal aqueous core of the examined boronated liposomes is still vacant, drugs, including boron compounds, can be encapsulated in it. In this regard, boron-10 and drugs may be simultaneously delivered

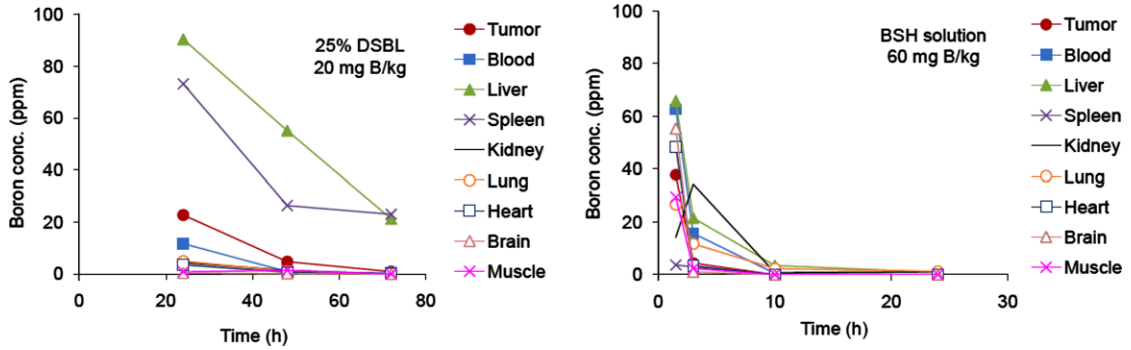

Figure 6. Time course of biodistribution of (A) DSBL-25% PEG liposomes (20 mg B/kg) and (B) BSH solution (60 mg B/kg). Each sample was injected into tumor-bearing mice (Balb/c, female, 6 weeks old, 14–20 g) via the tail vein.

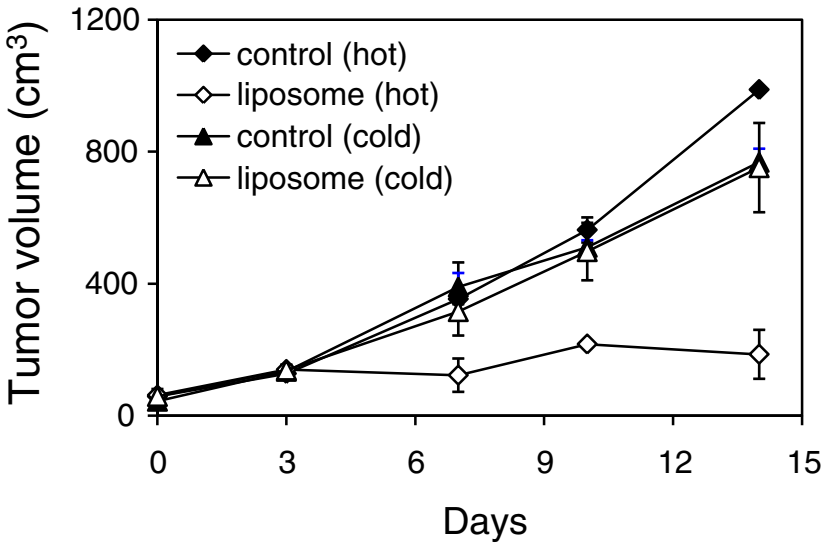

Figure 7. Tumor volume in mice (Balb/c, female, 6 weeks old, 14–20 g) bearing colon 26 solid tumor with thermal neutron irradiation (hot) for 30 min (1.8×10^{12} neutrons/cm^2) or without irradiation (cold). The irradiation was performed at 24 h after iv injection of DSBL-25% PEG liposomes (20 mg B/kg). Data are expressed as means ± sem ($n = 3$).

to a tumor, realizing combination therapy consisting of BNCT and chemotherapy. Investigation for further efficient BDS including active targeting to tumor by functionalization of the boronated liposomes are going on in our laboratory.

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