Supporting Information

Spermidinium *closo*-Dodecaborate-Encapsulating Liposomes as Efficient Boron Delivery Vehicles for Neutron Capture Therapy

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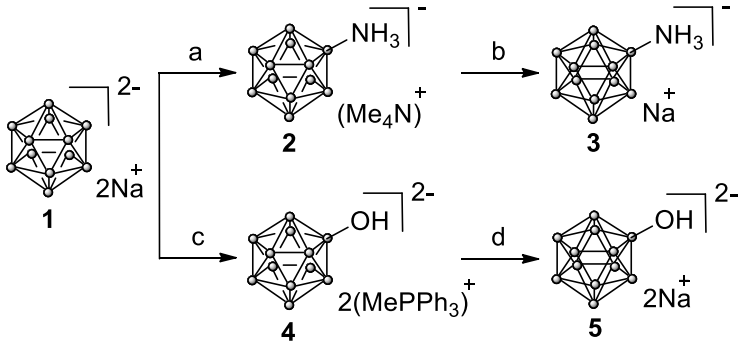
Materials and Methods

2.1. Chemicals

DSPC (MC-8080) and DSPE-PEG (SUNBRIGHT DSPE-020CN) were purchased from Nippon Oil and Fats (Tokyo, Japan). Cholesterol was purchased from Kanto Chemical (Tokyo, Japan). $(Et_3NH)_2[B_{12}H_{12}]$ and $Na_2[B_{12}H_{11}SH]$ (Na_2BSH) were purchased from Katchem Ltd. (Prague, Czech Republic). $Na_2[^{10}B_{12}H_{12}]$ and $Na_2[^{10}B_{12}H_{11}SH]$ ($Na_2{}^{10}BSH$) were kindly supplied by Stella Chemifa Co. (Osaka, Japan). *Closo*-dodecaborates, $Na[B_{12}H_{11}NH_3]$, $Na_2[B_{12}H_{11}OH]$, and $Na[^{10}B_{12}H_{11}NH_3]$ were synthesized from $(Et_3NH)_2[B_{12}H_{12}]$ and $Na_2[^{10}B_{12}H_{12}]$ according the literature procedures[1, 2] and transformed into their sodium forms by an ion-exchange resin (Amberlite IR-120). All other chemicals were of the highest grade commercially available. Spermidinium chloride solution was prepared from spermidine and aqueous 1N HCl solution (1:3 molar ratio).



Scheme S1. Synthesis of $Na[B_{12}H_{11}NH_3]$ and $Na_2[B_{12}H_{11}OH]$. Reagents and conditions: a. H_3NO_4S, Me_4NCl, H_2O, 80%. b and d. Ion exchange with Amberlite IR120 (Na^+ form), **3**: 95%, **5**: 85%. c. H_2SO_4, $CaCO_3$, $MePPh_3Br$, H_2O, 50%.

2.2. Cytotoxicity of sodium closo-dodecaborates (GI_{50})

Each sodium *closo*-dodecarborate (Na_2BSH, $Na[B_{12}H_{11}NH_3]$, $Na_2[B_{12}H_{11}OH]$ and $Na_2[B_{12}H_{12}]$) was dissolved in 1 mL of Phosphate buffered saline (PBS), and the solution was diluted with RPM1640 (1% penicillin and streptomycin, 10% fetal bovine serum (FBS)). In 96 well plates, mouse colon 26 cells (5×10^3 cells/well) were cultured with the medium containing *closo*-dodecaborates at each concentration (0.1, 0.3, 1, 3, 10 and 30 mM) and incubated for 72 h at 37°C in a CO_2 incubator. The ratios of viable cells were determined by MTT assay. Compound concentration required to inhibit cell growth by 50% (GI_{50}) was

determined from semi-logarithmic dose-response plots, and results represent the mean ± standard deviation (SD) of triplicate experiments.

Table S1. Growth inhibition of *closo*-dodecaborates toward colon 26 cells

closo-dodecaborate	GI_{50} (mM)	(ppm B)
Na_2BSH	2.1 ± 0.2	252
$Na[B_{12}H_{11}NH_3]$	32.9 ± 0.6	3,948
$Na_2[B_{12}H_{11}OH]$	7.7 ± 0.6	924
$Na_2[B_{12}H_{12}]$	5.1 ± 0.8	612

2.3. Preparation of liposomes encapsulated with various ammonium salts of closo-dodecaborates

An aqueous solution of various ammonium chloride salts (1,4-diaminobutane, spermidine, and spermine) of *closo*-dodecaborates were prepared by adding sodium *closo*-dodecaborates to a mixture of each amine and aqueous 1N HCl solution (1:2, 1:3, 1:4 molar ratio, respectively). Liposomes encapsulated with these ammonium *closo*-dodecaborates were prepared from DSPC, cholesterol and DSPE-PEG (1:1:0.11, molar ratio) by the reverse-phase evaporation (REV) method.[3] A mixture of DSPC (158 mg), cholesterol (77.3 mg), and DSPE-PEG-20CN (63.8 mg) were dissolved in 5 mL of a chloroform/ diisopropylether mixture (1 : 1, v/v) in a round-bottom flask. An aqueous solution of protonated ammonium forms (1,4-diaminobutane, spermidine, and spermine) of *closo*-dodecaborates (125 mM, 5 mL) was added to the lipid solution to form a w/o emulsion. The emulsion was sonicated for 3 min, and then, the organic solvent was removed under vacuum in a rotary evaporator at 55 °C for 30 min. to obtain a suspension of liposomes. The liposomes obtained were subjected to extrusion 10 times through a polycarbonate membrane of 100-nm pore size (Whatman, 110605, FILTER, 0.1 UM, 25 MM, Gentaur Molecular Products, Belgium), using an extruder device (LIPEX^{TM} Extruder, Northern Lipids, Canada) thermostated at 55°C. Purification was accomplished by ultracentrifuging at 200,000 g for 60 min at 4°C (himac cp 80 wx, Hitachi Koki, Japan), and the pellets obtained were resuspended in saline. Particle size and zeta potential of these liposomes were measured with an electrophoretic light scattering

spectrophotometer (Nano-ZS, Sysmex, Japan). B/P (boron concentration / phosphorus concentration) ratio was calculated from data obtained by the simultaneous measurement of boron and phosphorus concentrations by inductively coupled plasma atomic emission spectroscopy (ICP-AES, HORIBA, Japan).

Table S2. Physical characteristics of liposomes containing *closo*-dodecaborates associated with sodium and various ammonium cations[a]

Entry	Boron cluster	Particle size (nm)	Zeta potential (mV)[b]	Boron conc. (ppm)[b,c]	Phosphorus conc. (ppm)[b]	B/P ratio (%)	Liposome yield (%)[d]
1	Na_2BSH	102±0.2	−27.3±13.4	3,438 ±2.0	2864±18.3	1.2	58.2
2	$Na[B_{12}H_{11}NH_3]$	105±1.0	−24.9±16.6	4,072 ±22.8	1835±38.5	2.2	44.3
3	$Na_2[B_{12}H_{11}OH]$	110±1.2	−29.9±85.5	2,635 ±184.2	1600±99.0	1.5	38.7
4	$Na_2[B_{12}H_{12}]$	98±0.7	−25.8±11.9	3,133 ±10.3	1932±13.3	1.6	46.7
5	$(n\text{-}C_3H_7NH_3)_2BSH$	110±0.7	−23.6±19.1	2,874 ±47.7	2224.8±15.8	1.3	53.8
6	$(H_3NC_4H_8NH_3)BSH$	116±0.7	−25.4±18.3	4,711 ±17.4	1833.3±43.4	2.6	44.3
7	spd-BSH	99±0.2	−14.7±14.1	13,867 ±185.8	4046.0±18.3	3.4	97.9
8	(sperminium)BSH	101.2±1.7	−5.4±11.5	9,759 ±139.6	3558.8±44.5	2.7	86.5
9	spd-$[B_{12}H_{11}NH_3]$	100±0.2	−10.7±21.7	13,970 ±216.5	3943.0±43.4	3.5	95.4

[a]In all case, liposomes were prepared from DSPC, cholesterol, and DSPE-PEG2000 (1: 1: 0.11, molar ratio) by the REV method. Liposome diameter was approximately 100 nm as measured with an extrusion apparatus. [b]Data are expressed as means ± standard deviation (SD). [c]Boron and phosphorus concentrations were calculated for 1.5 mL of liposome solution. [d]Liposome yields were calculated from the phosphorus concentration of liposome solution based on the total phospholipids used in preparation.

2.4. Effect of spermidinium cations on encapsulation of closo-dodecaborates in liposomes

The spd cation concentration dependent osmotic pressures with/without $[BSH]^{2-}$ in solutions were measured by the osmometer (Advanced™ Osmometer Model 3D3). The osmotic

pressures of the solutions were dependent on the total concentrations of both spd cation and $[BSH]^{2-}$ (data not shown). For instance, the osmotic pressures of Na_2BSH solution (125 mM) and spd chloride solution (125 mM) were 305 and 355 mOsm/kg H_2O, respectively, whereas that of a mixed solution of Na_2BSH and spd chloride (125 mM of each) was 592 mOsm/kg H_2O. Next, the viscosity of spd-$[B_{12}H_{12}]$ solution at various concentrations was measured by the viscometer (DV-1 + Viscometer, Brookfield, USA). The viscosities of water and saline (approximately 125 mM NaCl solution) were 1.23 and 1.28, respectively, whereas those of $Na_2[B_{12}H_{12}]$, spd cation, and spd-$[B_{12}H_{12}]$ solutions at 125 and 250 mM ranged from 1.23 to 1.29. The results indicate that the spd cation of $[B_{12}H_{12}]^{2-}$ does not affect the viscosity of internal aqueous solution of liposomes, although the osmotic pressure of the internal aqueous solution of liposomes is strongly dependent on the concentration of the contents.

Table S3. Viscosities of sodium and spermidinium cations of $[B_{12}H_{12}]^{2-}$ solution

Sample	Viscosity (cp)
water	1.23
$Na_2[B_{12}H_{12}]$ (125 mM)	1.27 - 1.29
$Na_2[B_{12}H_{12}]$ (250 mM)	1.23
Spermidine (125 mM)	1.23
Spermidine (250 mM)	1.23 - 1.25
spd-$[B_{12}H_{12}]$ (125 mM)	1.23 - 1.24
spd-$[B_{12}H_{12}]$ (250 mM)	1.22 - 1.24
NaCl solution (125 mM)	1.28

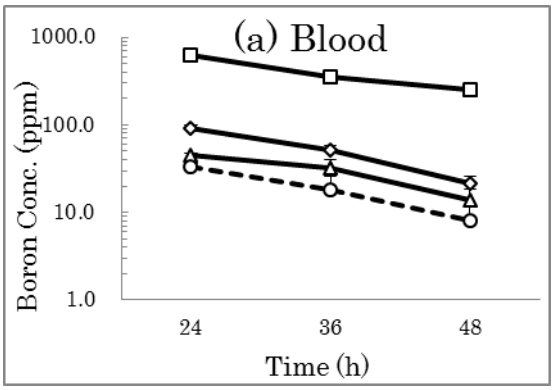
2.5. Transmission electron microscopy analysis

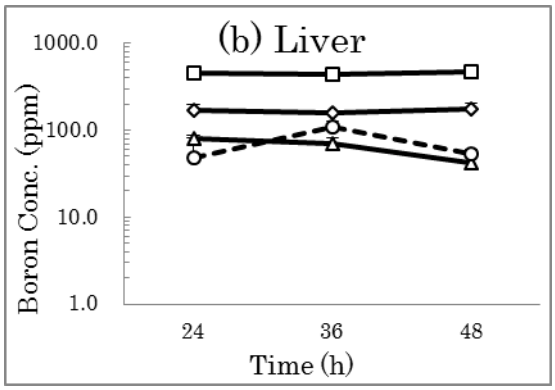
Vitrified specimens were prepared by placing 3 μL of the studied sample suspension on a 400 mesh copper grid with a holey carbon support. Each sample was blotted to a thin film and immediately plunged into liquid ethane in the Leica CPC cryoworkstation. The grids were viewed on a JEOL 2011 transmission electron microscope operating at an accelerating voltage of 200 kV. The microscope was equipped with a Gatan cryoholder and the samples were maintained at -177ºC during imaging. Electron micrographs were recorded with the Digital Micrograph software package under low electron dose conditions, to minimize electron bean radiation. Images were recorded on a Gatan 794 MSC 600HP cooled charge-coupled device

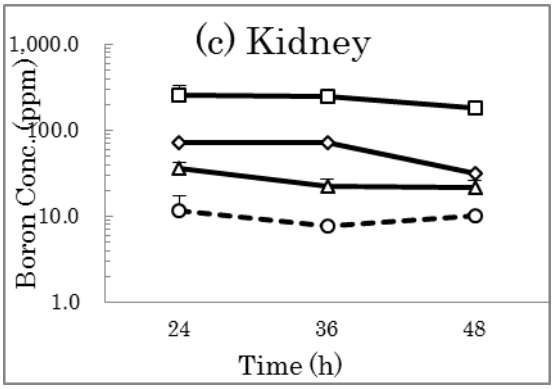
(CCD) camera.

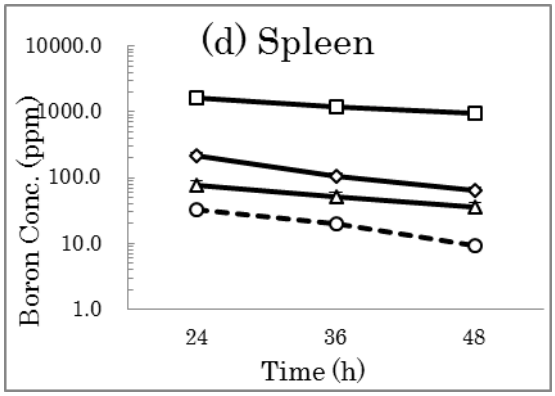
2.6. Biodistribution of spd-closo-dodecarborate encapsulating liposomes in tumor-bearing mice

Tumor-bearing mice (female, 5-6 weeks old, 16-20 g, Sankyo Labo Service Co., Japan) were prepared by injecting subcutaneously (s.c.) a suspension (1.0×10^6 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 to 9 mm. The mice were injected via the tail vein with 200 μL of spd- *closo*-dodecarborate encapsulating liposomes (spd-BSH and spd-[$B_{12}H_{11}NH_3$]) at 1,500, 3,000, and 10,000 ppm B concentrations and sodium *closo*-dodecarborate-encapsulated liposomes (Na_2BSH and $Na[B_{12}H_{11}NH_3]$) at 3,000 ppm B concentration. At selected time intervals (24, 36, 48, and 72 h) 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, kidney, spleen, and tumor were excised, washed with 0.9% NaCl solution, and weighed. The excised organs 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.









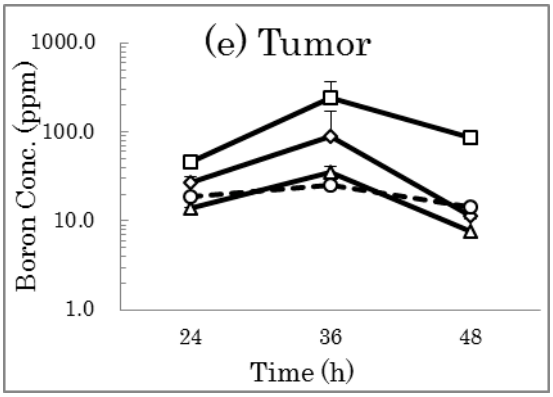


Fig. S1 Time courses of distribution of spd-[$B_{12}H_{11}NH_3$]- and Na[$B_{12}H_{11}NH_3$]-encapsulating liposomes (△, spd-[$B_{12}H_{11}NH_3$]: 15 mg[B]/kg; ◇, spd-[$B_{12}H_{11}NH_3$]: 30 mg[B]/kg; □, spd-[$B_{12}H_{11}NH_3$]: 100 mg[B]/kg; ○, Na[$B_{12}H_{11}NH_3$]: 15 mg[B]/kg). Each liposome was injected into tumor-bearing mice (Balb/c, female, six weeks old, 14-20 g) via the tail vein. Data are expressed as means ± SD (n = 5).

2.7. BNCT effect on tumor-bearing mice treated with spd-closo-dodecarborate encapsulating liposomes.

Each spermidinium and sodium *closo*-dodecarborate encapsulating liposome was prepared from DSPC, cholesterol, and DSPE-PEG in a solution of ^{10}B-enriched *closo*-dodecaborates (spd-[^{10}BSH], spd-[$^{10}B_{12}H_{11}NH_3$], Na_2[^{10}BSH], or Na[$^{10}B_{12}H_{11}NH_3$]), by the REV method, and injected into colon 26 tumor-bearing mice (female, 5-6 weeks old, 16-20 g, Sankyo Labo Service, Japan) via the tail vein at a dose of 15, 30, 100 mg [^{10}B]/kg (1,500, 3,000, 10,000 ppm ^{10}B concentration; 200 μL of spd-*closo*-dodecarborate-encapsulated liposomes solution) and 30 mg [^{10}B]/kg (3,000 ppm ^{10}B concentration; 200 μL of sodium *closo*-dodecarborate

encapsulating liposome solution). The sodium *closo*-dodecarborate solutions at a dose of 30 mg [^{10}B]/kg (3,000 ppm of ^{10}B concentration; 200 μL) were used as a control experiments. The mice were anesthetized with isoflurane (Forane, Abbott, Japan) and placed in an acrylic mouse holder 36 h after i.v. injection. The mice were irradiated in the KUR atomic reactor for 50 min at a rate of 1.3-2.2 × 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. Mice were sacrificed when their tumor volumes reached ~3000 mm^3. All protocols were approved by the Institutional Animal Care and Use Committee of Gakushuin University.

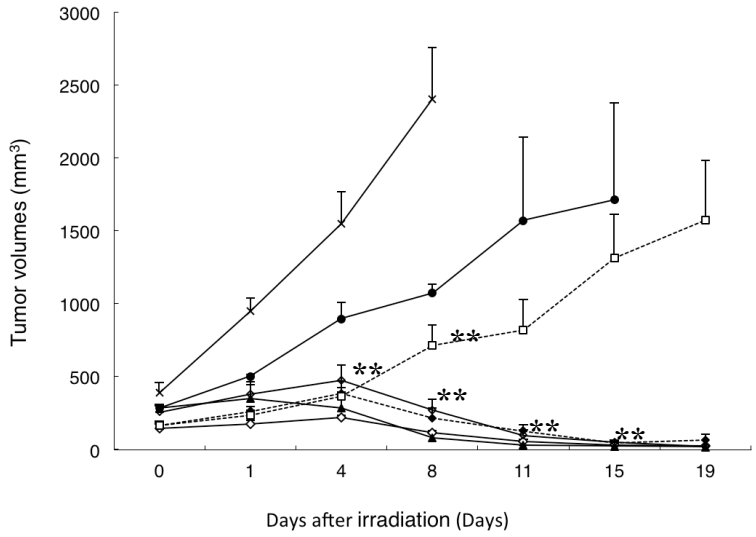


Fig. S2 Tumor volumes in mice (Balb/c, female, six weeks old, 14-20 g) bearing colon 26 solid tumor, exposed to thermal neutron irradiation (hot) for 50 min (1.3-2.2 × 10^{12} neutrons/cm^2) or not exposed to thermal neutron irradiation (cold). Irradiation was performed 36 h after injection of liposomes containing spd-[$^{10}B_{12}H_{11}NH_3$] (▲, 15; ◆, 30; ■,100 mg [^{10}B]/kg) and Na[$^{10}B_{12}H_{11}NH_3$] (◇, 30 mg [^{10}B]/kg), or 1 h after injection of Na[$^{10}B_{12}H_{11}NH_3$] solution (□, 100 mg [^{10}B]/kg). ●, hot control; ✳, cold control. Data are expressed as means ± SD (n = 7). Statistical significance: **P*<0.05 and ***P*<0.01, compared with irradiation control at each time point.

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.

References

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