Spermidinium closo-dodecaborate-encapsulating liposomes as efficient boron delivery vehicles for neutron capture therapy†

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Boron neutron capture therapy (BNCT) has been attracting growing interest as one of the minimally invasive cancer therapies.1 Mercaptoundecahydrododecaborate (Na[B12H11SH]2) and L-boronophenylalanine (L-BPA) have been used in BNCT for many years. L-BPA, in particular, has been widely used for the treatment of not only melanoma but also brain tumor2 and head and neck cancer3 because it can be taken up selectively by tumor cells through an amino acid transporter.4 The accelerator-based BNCT is now undergoing phase I clinical study for the treatment of brain tumor and head and neck cancer patients in Japan.5,6

In recent years, liposomal 10B carriers have attracted attention as some of the efficient boron delivery systems in BNCT.7–10 Several efficient in vivo BNCTs have been reported. Yanagi and coworkers demonstrated the first antitumor effect of Na2BSH-encapsulating liposomes conjugated with a monoclonal antibody specific for the carcinoembryonic antigen.9a,b Marruyama and co-workers developed transferrin-conjugating Na3B12H11NH3 into the internal aqueous core and K[nido-7-CH3(CH2)15-7,8-C2B9H11] into the bilayer membrane to increase the boron content in liposomes.6b,13

We previously developed Na2BSH-encapsulating 10% distearoyl boron lipid (DSBL)8,13 liposomes that have high boron content with excellent boron delivery efficacy to tumors.14 In this communication, we studied the effects of the counter cations of boron clusters on liposome formation to develop high boron content liposomes for BNCT by overcoming osmotic pressure limitations.

We selected three closo-dodecaborates, Na[B12H12], Na[B12H11OH]2, and Na[B12H11NH3]2 in addition to Na2BSH (Fig. 1). We first tested the cytotoxicity of the closo-dodecaborates toward colon 26 cells. The closo-dodecaborates are relatively nontoxic and the GI50 values of Na[B12H12], Na2BSH, Na[B12H11NH3], and Na[B12H11OH] are 5.1, 2.1, 32.9, and 7.7 mM, respectively (Table S1 in the ESI†). Liposomes containing the closo-dodecaborates were prepared from DSPC, cholesterol, and DSPE-PEG2000 by the reverse phase evaporation method with sizes of approximately 100 nm in diameter. The results are summarized in Table 1. The final boron and phosphorus concentrations of liposome solution containing Na[B12H12] were 3438 ± 2.0 and 2864 ± 18.3 ppm, respectively, and the B/P ratio was 1.2 (entry 1). The higher B/P ratio indicates the higher boron content in liposomes. The liposome yield was 58% based on the total phospholipids used in the preparation. The B/P ratio of the liposome containing Na[B12H11NH3] was 2.2 (entry 2), which was slightly higher than those of liposomes containing Na2BSH, Na[B12H11OH], and Na[B12H11NH3] (1.2–1.6, entries 1, 3, and 4). In the case of Na[B12H11NH3], an ammonium ion group served as one of the two counter cations of the closo-dodecaborate bilayer have been studied.10–12 Hawthorne and coworkers developed liposomes incorporating Na1+[1-2(B10H9)-2-NH3B10H8] into the internal aqueous core and K[nido-7-CH3(CH2)15-7,8-C2B9H11] into the bilayer membrane to increase the boron content in liposomes.6b,13

Although they succeeded in completely suppressing tumor growth in mice after neutron irradiation, the concentration of inner 10B of liposomes was limited in preparation due to osmotic reasons. For this reason, boron lipids embedded within the liposome

**Table 1.** Summary of liposome formation and cytotoxicity.

<table>
<thead>
<tr>
<th>Liposome Type</th>
<th>Final Boron (ppm)</th>
<th>Final Phosphorus (ppm)</th>
<th>B/P Ratio</th>
<th>Cytotoxicity (GI50) (mM)</th>
<th>Counter Cation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na2BSH</td>
<td>3438 ± 2.0</td>
<td>2864 ± 18.3</td>
<td>1.2</td>
<td>5.1</td>
<td>Na2BSH</td>
</tr>
<tr>
<td>Na[B12H12]</td>
<td>2871 ± 19.4</td>
<td>2378 ± 10.2</td>
<td>1.2</td>
<td>2.1</td>
<td>Na[B12H12]</td>
</tr>
<tr>
<td>Na[B12H11OH]</td>
<td>3296 ± 16.5</td>
<td>2858 ± 17.8</td>
<td>1.2</td>
<td>32.9</td>
<td>Na[B12H11OH]</td>
</tr>
<tr>
<td>Na[B12H11NH3]</td>
<td>3559 ± 16.8</td>
<td>3064 ± 13.2</td>
<td>1.2</td>
<td>7.7</td>
<td>Na[B12H11NH3]</td>
</tr>
<tr>
<td>Na[B12H11NH]</td>
<td>3438 ± 2.0</td>
<td>2864 ± 18.3</td>
<td>1.2</td>
<td>5.1</td>
<td>Na[B12H11NH]</td>
</tr>
</tbody>
</table>

**Fig. 1.** Structures of closo-dodecaborates used for encapsulation in liposomes.
We speculated that ammonium counter cations would affect the encapsulation of closo-dodecaborates in liposomes. Recently, Gabel and coworkers reported that Na2BSH induces aggregation and membrane rupture, increasing wall thickness of the liposome and triggering the release of liposome contents. Indeed, it is known that tetramethylammonium (TMA) salts of closo-dodecaborates are insoluble in water, and the ion-exchange from Na2BSH to (TMA)2BSH proceeds readily, whereas the ion-exchange from (TMA)2BSH to Na2BSH is not easy. We predicted that encapsulation as well as liposome yield would be increased if we could reduce this interaction in the preparation of closo-dodecaborate-encapsulating liposomes. Thus we prepared various ammonium salts of BSH and examined their encapsulation into dodecaborate-encapsulating liposomes. Indeed, it is known that tetramethylammonium (TMA) salts of closo-dodecaborates are insoluble in water, and the ion-exchange from Na2BSH to (TMA)2BSH proceeds readily, whereas the ion-exchange from (TMA)2BSH to Na2BSH is not easy. We predicted that encapsulation as well as liposome yield would be increased if we could reduce this interaction in the preparation of closo-dodecaborate-encapsulating liposomes. Thus we prepared various ammonium salts of BSH and examined their encapsulation into liposomes.

We next examined boron distribution of the spd-closo-dodecaborate-encapsulating liposomes in colon 26 tumor-bearing mice. The liposomes were injected at doses of 15, 30, and 100 mg [B] kg⁻¹ body weight via the tail veins. Na2BSH- and Na2[B12H11NH3]₂-encapsulating liposomes were also injected at a dose of 30 mg [B] kg⁻¹ as control experiments. The time courses of boron distribution in each organ are shown in Fig. 4. Blood boron concentrations of 460.7, 104.0, and 33.2 ppm were detected 24 h after injection of spd-BSH-encapsulating liposomes (100, 30, and 15 mg [B] kg⁻¹), respectively (Fig. 4a). Blood boron concentration in mice injected with 100 mg [B] kg⁻¹ of spd-BSH-encapsulating liposomes did not decrease notably during the 48 h period, whereas those in mice injected with 30 and 15 mg [B] kg⁻¹ of spd-BSH-encapsulating liposomes gradually decreased in a time-dependent manner. The time courses of boron concentrations in liver, kidneys, and spleen are shown in Fig. 4b–d, respectively. Boron concentrations of 528.5, 144.2, and 74.4 ppm in liver were observed 48 h after the injection of 100, 30, and 15 mg [B] kg⁻¹ of spd-BSH-encapsulating liposomes, respectively. In the meantime, maximum

![TEM images of Na2BSH-encapsulating liposomes (left) and spd-BSH-encapsulating liposomes (right). Scale bar represents 200 nm.](Image 14x290 to 26x354)

![Fig. 2 Effect of the amount of spd cation on spd-BSH encapsulation in liposomes. Boron/phosphorus (B/P) ratios of (spd)-closo-dodecaborate-encapsulating liposomes are shown in the vertical axis.](Image 14x290 to 26x354)
tumor boron concentrations of 202.7 and 82.4 ppm were achieved 36 h after injection at doses of 100 and 30 mg [B] kg⁻¹, respectively. Even at the low boron dose of 15 mg [B] kg⁻¹, the tumor boron concentration was 34.0 ppm at 36 h after injection (Fig. 4e). We also demonstrated the boron distribution of Na₂BSH-encapsulating liposomes in tumor-bearing mice for comparison. Although blood and liver boron concentrations after injection of Na₂BSH-encapsulating liposomes at a dose of 30 mg [B] kg⁻¹ were similar to those after injection of spd-BSH-encapsulating liposomes at a dose of 30 mg [B] kg⁻¹, kidney and spleen boron concentrations after injection of Na₂BSH-encapsulating liposomes were lower than those after injection of spd-BSH-encapsulating liposomes up to 48 h. The tumor boron concentration at 36 h after injection of Na₂BSH-encapsulating liposomes was 31.9 ppm, although the clearance of Na₂BSH-encapsulating liposomes was slow (Fig. 4e).

A similar tendency was observed in spd-[B₁₂H₁₁NH₃]-encapsulating liposomes (Fig. S1, ESI†). Blood, kidney, and spleen boron concentrations gradually decreased after injection. Maximum tumor boron concentrations of 242.2, 88.7, and 35.4 ppm were achieved 6 h after injection at doses of 100, 30, and 15 mg [B] kg⁻¹, respectively. Interestingly, significant tumor boron accumulation was also observed in the case of Na₂[B₁₂H₁₁NH₃]encapsulating liposomes.

Finally, we examined the antitumor effect of liposomes containing spd closo-dodecaborates in colon 26 tumor bearing mice exposed to thermal neutron irradiation. Thermal neutron irradiation of the tumor-transplanted left thighs of mice was carried out 36 h after injection. The tumor growth curves of mice are shown in Fig. 5 (and in Fig. S2, ESI†). “Hot control (●)” and “Cold control (×)” represent tumor volumes of mice injected with saline with and without thermal neutron irradiation, respectively. Tumor growth was significantly suppressed in mice treated with spd-[¹⁰BSH]- and spd-[¹⁰B₁₂H₁₁NH₃]-encapsulating liposomes at doses of 15, 30, and 100 mg [¹⁰B] kg⁻¹ and exposed to thermal neutron irradiation. The tumor completely disappeared within three weeks even when a dose of 15 mg [¹⁰B] kg⁻¹ was employed. Liposomes containing Na₄[¹⁰BSH] and Na₄[¹⁰B₁₂H₁₁NH₃] also inhibited tumor growth at a dose of 30 mg [¹⁰B] kg⁻¹, and the tumor was completely controlled three weeks after thermal neutron irradiation. Tumor growth was suppressed in mice treated with Na₂[¹⁰BSH] solution (100 mg [¹⁰B] kg⁻¹) during the two weeks after thermal neutron irradiation. However, the tumor started to grow thereafter (Fig. 5). In contrast, tumor growth was not suppressed in mice treated with Na₁⁰⁰[B₁₂H₄NH₃] solution (100 mg [¹⁰B] kg⁻¹) even after thermal neutron irradiation (Fig. S2, ESI†).

Fig. 4 Time courses of distribution of spd-BSH- and Na₂BSH-encapsulating liposomes (Δ, spd-BSH: 15 mg [B] kg⁻¹; ○, spd-BSH: 30 mg [B] kg⁻¹; ▲, spd-BSH: 100 mg [B] kg⁻¹; ▲, Na₂BSH: 15 mg [B] kg⁻¹). Each liposome was injected into tumor-bearing mice via the tail vein. Data are expressed as means ± SD (n = 5).

Fig. 5 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 × 10¹² neutrons per cm²) or not exposed to thermal neutron irradiation (cold). Irradiation was performed 36 h after injection of liposomes containing spd-[¹⁰BSH] (Δ, 15; ○, 30; ▲, 100 mg [¹⁰B] kg⁻¹) and Na₂[¹⁰BSH] (▲, 30 mg [¹⁰B] kg⁻¹), 1 h after injection of Na₂[¹⁰BSH] solution (▲, 100 mg [¹⁰B] kg⁻¹; ●, hot control; ×, cold control. **p < 0.01, compared with hot control.

Fig. 6 Survival curve of tumor-bearing mice after thermal neutron irradiation. The irradiation was performed 36 h after injection of closo-dodecaborates (●, spd-BSH: 30 mg [¹⁰B] kg⁻¹; ○, spd-BSH: 15 mg [¹⁰B] kg⁻¹; ▲, spd-[¹⁰B₁₂H₁₁NH₃]: 15 mg [¹⁰B] kg⁻¹ for 50 min (1.3–2.2 × 10¹² neutrons per cm²); ×, cold control; ●, hot control. Mice were sacrificed when their tumor volumes reached ~3000 mm³.

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was observed in the mice treated with spd-[10B]SH-encapsulating liposomes at a dose of 30 mg [10B] kg⁻¹; 100% of the mice survived up to 100 days after the thermal neutron irradiation.

We succeeded in the preparation of high boron content liposomes. The use of spd as a counter cation of closo-dodecaborates was essential to obtain the liposomes with high yields and high B/P ratios. All of the mice injected with 30 mg [10B] kg⁻¹ of spd-[10B]-encapsulating liposomes were completely cured while five of seven mice injected with 15 mg [10B] kg⁻¹ of spd-[10B]-SH-encapsulating liposomes were cured 100 days after thermal neutron irradiation. The results indicate that the total amount of phospholipids could be reduced to less than one-seventh of those used to prepare Na₂[10BSH]-encapsulating liposomes. 19 We believe that the spd-closo-dodecaborate-encapsulating liposomes are promising candidates for clinical use in BNCT.

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Notes and references

18 All protocols were approved by the Institutional Animal Care and Use Committee of Gakushuin University.
19 Conventionally, a preinjection of liposomes at a high dose was given for an achievement of targeting through saturation of liver's scavenging capacity (see; Y. Y. Kao and R. L. Juliano, Biochim. Biophys. Acta, 1981, 677, 453). Such a high liposome dose may cause possible liver toxicity, since the liver's normal scavenging function is impaired. Indeed, an important character of an approved liposomal formulation (doxil) encapsulating an anticancer drug doxorubicin is the very high concentration of the encapsulated drug (also see; P. G. Tardi, N. L. Boman and P. R. Cuill, J. Drug Targeting, 1996, 4, 129).