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Synthesis of Oligo-*closo*-dodecaborates by Hüisgen Click Reaction as Encapsulated Agents for Preparation of High-Boron-Content Liposomes for Neutron Capture Therapy

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Abstract

High-boron-content compounds **8**, **10**, and **13** were designed and synthesized as boron agents encapsulated in liposomes by the Hüisgen click cycloaddition of *closo*-dodecaborate-containing azides and alkynes. These compounds have relatively low cytotoxicies and their GI_{50} values for B16, CT26, and HeLa cells are higher than 0.14 mM, indicating toxicities similar to that of BSH. High-boron-content liposomes were prepared using **13**, which has the largest number of boron atoms in a molecule among the compounds synthesized. The final boron concentration in **13**-encapsulating liposomes reached 11.0 x 10^3 ppm with a B/P ratio of 5.1. A significantly high tumor boron accumulation (72.2 ppm) was observed in tumor-bearing mice 36 h after the injection of **13**-encapsulating liposomes at a dose of 15 mgB/kg body weight with the tumor/blood (T/B) ratio of approximately 14.

Keyword

Boron neutron capture therapy (BNCT), *closo*-dodecaborate, Hüisgen click

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cycloaddition, liposome, boron delivery system, colon 26 cells

1. Introduction

Boron neutron capture therapy (BNCT) is a cell-selective radiation therapy that uses a combination of boron-10 (^{10}B) and thermal neutron. Neutron capture reaction of ^{10}B generates high linier energy particles, α-particle and Li nuclei, that travel a short distance (approximately 5−9 μm) equivalent to a cell diameter.[1] Therefore, it would be possible to destroy tumor cells selectively without seriously damaging adjacent healthy tissues if compounds containing ^{10}B atoms were selectively accumulated in tumor cells.[2-4] In order to achieve tumor tissue-selective ^{10}B delivery, we have focused on the liposomal boron delivery system.[5-7] In this system, the enhanced permeability and retention (EPR) effect is essential for accumulation of liposomes in tumor tissues and the encapsulated drugs in liposomes are transported to various tumors regardless their contents.[8] Various ^{10}B-encapusulated liposomes have been developed,[9-15] however, high lipid doses are always required to deliver adequate ^{10}B atoms to tumor tissues because of the osmotic reason.[16] We recently reported high boron content liposomes.[17-21] Sodium mercaptoundecahydrododecaborate (Na_2BSH)-encapsulating 10% distearoyl boron lipid (DSBL) liposomes have high boron content with a boron/phosphorous concentration (B/P) ratio of 2.6 that enables us to prepare liposome solution with 5.0 x 10^3 ppm ^{10}B concentration.[20] Furthermore, the use of spermidinium as a counter cation of BSH dramatically increased the encapsulation efficiency of BSH into liposomes and the final ^{10}B concentration of the liposome solution reached to approximately 1.4 x 10^4 ppm with the B/P ratio of 3.4.[21]

Recently, we have developed a method of Hüisgen click cycloaddition of various organic azides and *closo*-dodecaborate-containing alkynes.[22, 23] In this study, we designed and synthesized high-boron-content molecules as boron agents encapsulated in liposomes by the Hüisgen click cycloaddition of both *closo*-dodecaborate-containing azides and alkynes. We succeeded in the preparation of high-boron-content liposomes to reduce the osmotic pressure in their inner core and achieved an efficient system of

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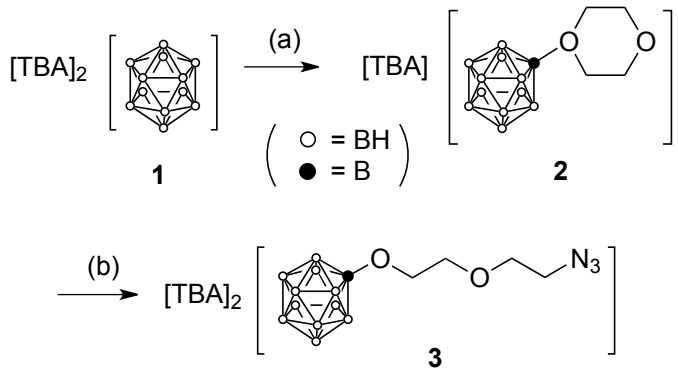
boron delivery to the tumor for BNCT.

2. Results and discussion

2.1. Chemistry

We first synthesized *closo*-dodecaborate azide **3** from the *closo*-dodecaborate tetrabutylammonium (TBA) form **1** using the ring opening reaction of the 1,4-dioxane-conjugated *closo*-dodecaborate according to the literature procedures reported by Bregadze and coworkers (Scheme 1).[24] The *closo*-Dodecaborate tetrabutylammonium form **1** was treated with $NaBF_4$ and HCl in 1,4-dioxane and the resulting 1,4-dioxane-conjugated *closo*-dodecaborane **2** underwent the ring opening reaction with TBA azide in CH_2Cl_2 to give the *closo*-dodecaborate azide **3** in 97% yield.

Scheme 1. Synthesis of *closo*-dodecaborate azide **3**[24]



Reagents and conditions: (a) $NaBF_4$ (5 equiv.), 4N HCl, 1,4-dioxane, 87%; (b) Bu_4NN_3, CH_2Cl_2, 97%.

We examined the Hüisgen click cycloaddition of the monopropagylated *closo*-dodecaborate (Scheme 2). Mercaptoundecahydrododecaborate (BSH) tetramethylammonium (TMA) form **4** underwent the Michael addition reaction with malononitrile to protect the mercapto function with a cyanoethyl group.[25] The protected thio-*closo*-dodecaborate **5**[25] reacted with propargyl bromide to give sulfonium ion **6**,[22]

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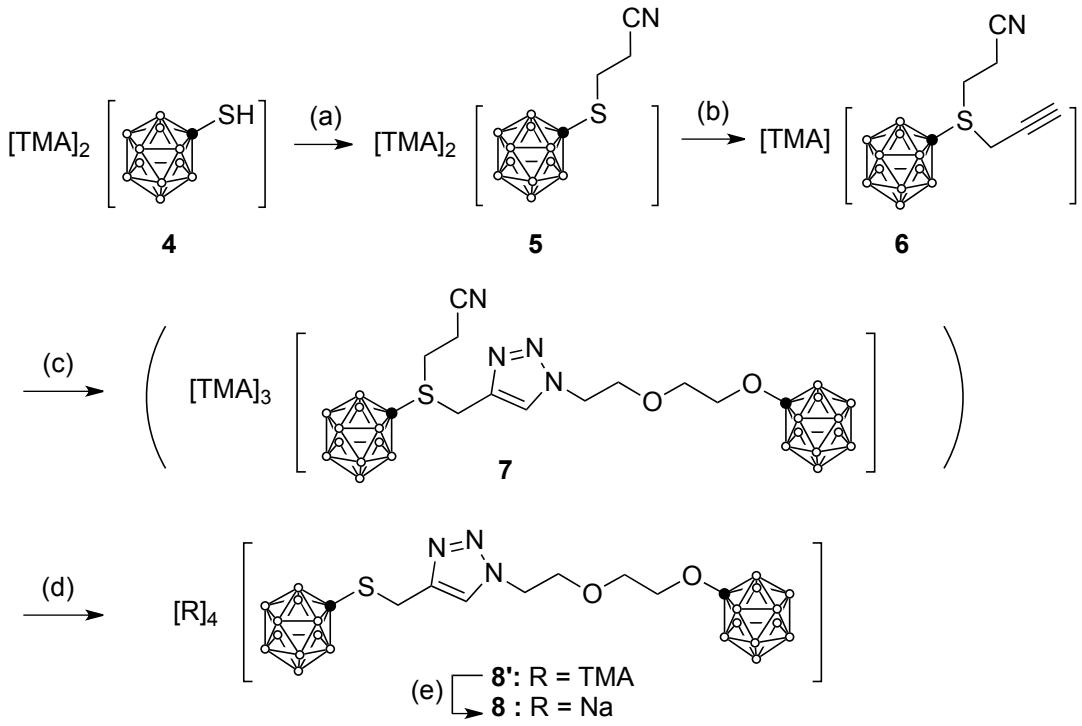
which underwent the Hüisgen click cycloaddition with *closo*-dodecaborate azide **3** in the presence of $Cu(OAc)_2$ and sodium ascorbate in CH_3CN to give the corresponding triazole **7**.[22,26] A cyanoethyl group was removed by TMAOH as a base to give bis-*closo*-dodecaborate **8'** as a TMA form, which was readily converted to the sodium form **8** by treatment with the amberlite Na^+ form.

Scheme 2. Synthesis of bis-*closo*-dodecaborate **8**



Reagents and conditions: (a) NaOH, acrylonitrile, CH_3CN/H_2O (4/1), 97%; (b) propargyl bromide, CH_3CN/H_2O (4/1), 72%; (c) i. **3**, $Cu(OAc)_2$, sodium ascorbate, CH_3CN; ii. TMACl, CH_3OH; (d) TMAOH, CH_3CN, 63% in three steps; (e) amberlite Na^+ form, CH_3CN/H_2O (4/1), 97%.

Because the Hüisgen click cycloaddition proceeded smoothly between the ionic propargylated *closo*-dodecaborate and the ionic *closo*-dodecaborate azide, we next examined the double Hüisgen click cycloaddition (Scheme 3). *closo*-Dodecaborate **4** was treated with an excess amount of propargyl bromide to give bispropargylated

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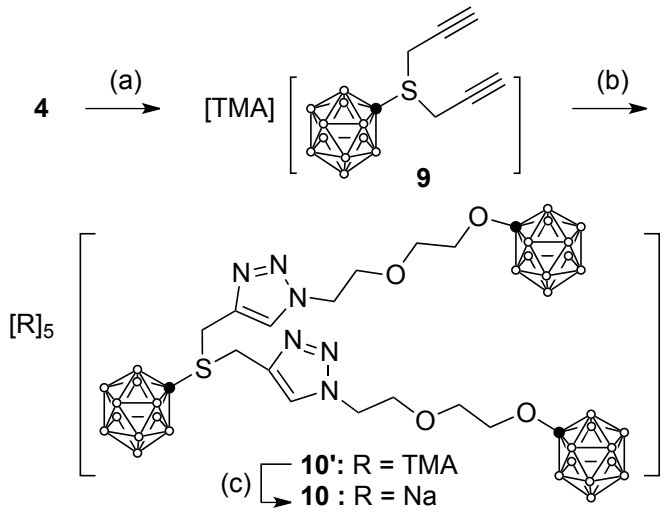
sulfonium **9**[22] in 87% yield. The Hüisgen click cycloaddition of **9** with *closo*-dodecaborate azide **3** proceeded smoothly in the presence of $Cu(OAc)_2$ and sodium ascorbate and the tris-*closo*-dodecaborate sodium form **10** was obtained after treatment with the amberlite Na^+ form.

Scheme 3. Synthesis of tris-*closo*-dodecaborate **10**



Reagents and conditions: (a) propargyl bromide (excess), CH_3CN/H_2O (4/1), 87%; (b) i. **3**, $Cu(OAc)_2$, sodium ascorbate, CH_3CN; ii. TMACl, CH_3OH, 63% in two steps; (c) amberlite Na^+ form, CH_3CN/H_2O (4/1), 80%.

Tetrakis-*closo*-dodecaborate penta sodium salt **13** was also synthesized from $TMA[B_{12}H_{11}NH_3]$ (**11**) similarly to the synthesis of **10**. Briefly, **11** was treated with excess amounts of propargyl bromide (10 equiv.) to afford **12**,[23] followed by Hüisgen click cycloaddition with *closo*-dodecaborate azide **3** to give the corresponding tetrakis-*closo*-dodecaborate **13'**. The sodium form **13** was obtained after treatment of **13'** with the amberlite Na^+ form.

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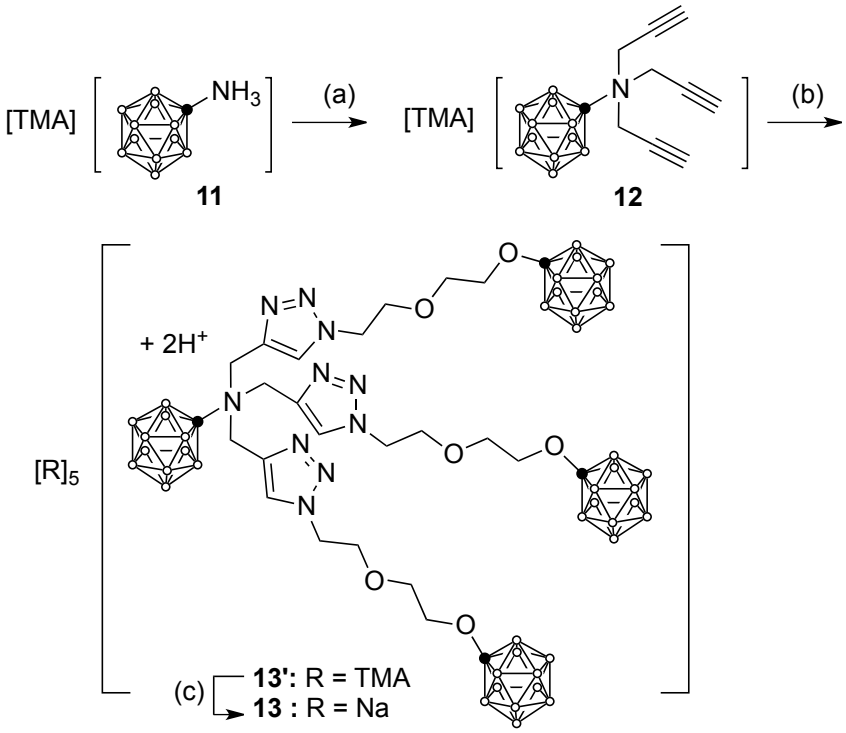
Scheme 4. Synthesis of tetrakis-*closo*-dodecaborate **13**



Reagents and conditions: (a) propargyl bromide (excess), KOH, CH_3CN/H_2O (4/1), 66%; (b) i. **3**, $Cu(OAc)_2$, sodium ascorbate, CH_3CN; ii. TMACl, CH_3OH, 85% in two steps; (c) amberlite Na^+ form, CH_3CN/H_2O (4/1), 80%.

The final products were characterized by ^1H, ^{13}C, and ^{11}B NMR spectroscopies and high-resolution time-of-flight mass spectroscopy (HR-TOF-MS) using their TMA form. In particular, HR-TOF-MS is one of the useful methods to identify current oligo anion cluster compounds because the HR-TOF mass spectra showed a unique property, namely, the isomer effects caused by the large number of boron atoms and their oligo anion characteristic. Figure 1 shows both the observed and calculated HR-TOF-MS peaks of compounds **8**, **10**, and **13**. Compound **8** consisting of two *closo*-dodecaborate clusters possessing four negative charges has a total of twenty-four boron atoms in a molecule. Each boron atom has an atomic weight of **10** (20%) or **11** (80%); therefore, the mass peaks of compound **8** exhibited unique isotope patterns with intervals of

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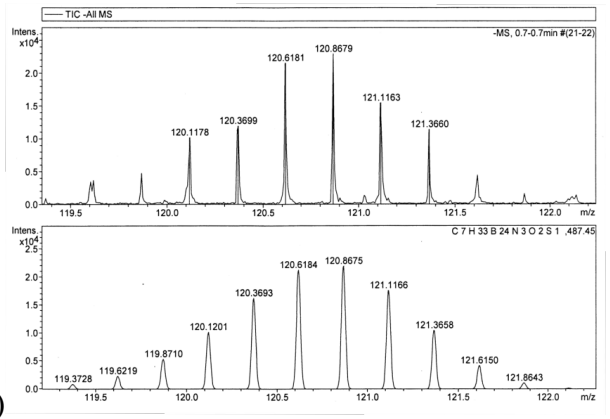
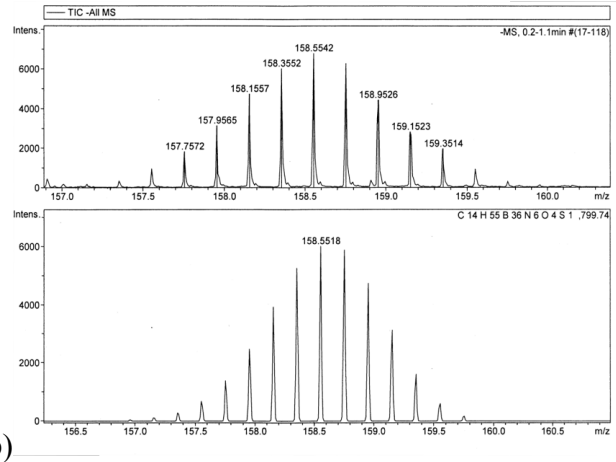
one-quarter of the average m/z, which were in good agreement with the calculated peaks, as shown in Figure 1a. Compound **10** consists of three *closo*-dodecaborate clusters and a sulfonium cation. Therefore compound **10** possesses five negative charges with a total of thirty-six boron atoms in a molecule. The observed mass peaks of compound **8** were in good agreement with the calculated peaks (Figure 1b). In the case of compound **13**, four *closo*-dodecaborate clusters with a total of forty-eight boron atoms are included in a molecule. The mass peaks were observed at intervals of one-fifth, not one-seventh, of the average m/z, probably due to an ammmonium cation and two protonation at imidazole moieties in a molecule (Figure 1c).



(a)



(b)

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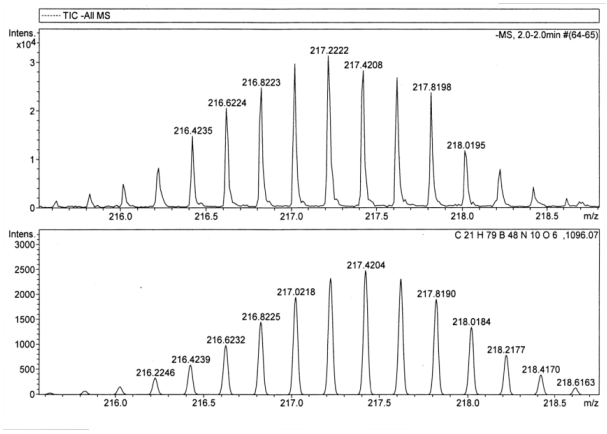

(c)

Figure 1. Observed (top panels) and calculated (bottom panels) HR-TOF-MS peaks of compounds **8** (a), **10** (b), and **13** (c).

2.2. Cell Growth Inhibition

Table 1 shows the molecular weight (MW), boron weight (BW), and cell growth inhibition by the synthesized compounds. Cell growth inhibition was measured in B16 melanoma, CT26 colon cancer, and HeLa cell lines incubated at 37 ^{o}C for 72 h and expressed as the half maximal inhibitory concentration (GI_{50}) for cell growth. BW increased according to the number of boron clusters included in a molecule. BSH, which has been clinically used for the BNCT of brain tumors, did not significantly inhibit B16 cell growth at 1 mM concentration and its GI_{50} values for CT26 and HeLa cells were 0.30 and 0.58 mM, respectively. Compound **11** did not significantly inhibit the growth of all cell lines at 1 mM. Compound **8** having two boron clusters was found to have a low cytotoxicity similarly to BSH and **11**. Both compounds **10** and **13**, which have three and four boron clusters in each molecule, respectively, also showed relatively low cytotoxicities with GI_{50} values ranging from 0.14 to 0.22 mM.

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Table 1. Inhibition of growth of compounds toward B16 melanoma, colon 26, and HeLa cell lines[a] by synthesized compounds

Compd	MW (g/mol)	BW[b] (g/mol)	GI_{50} (mM)		
			B16	CT26	HeLa
4 (BSH)[c]	179.8	129.7	>1	0.30 ± 0.05	0.58± 0.02
11[c]	219.9	129.7	>1	>1	>1
8	574.8	259.4	>1	0.47± 0.05	>1
10	907.8	389.2	0.21± 0.03	0.22± 0.01	0.21± 0.02
13	1,245.6	518.9	0.14± 0.02	0.15± 0.01	0.16± 0.01

[a] Cells (5×10^3 cells per well of a 96-well plate) were incubated at 37 °C for 72 h in RPMI-1640 medium (100 μL) containing various concentrations of the synthesized compounds. After the incubation, cell viability was determined by MTT assay. The drug concentration required to reduce cell viability by 50% (IC_{50}) was determined from the semilogarithmic dose-response plots. [b] Boron weight in a molecule. [c] Sodium forms of *closo*-dodecaborates were used for cell growth inhibition assay.

2.3. Preparation and characterization of closo-dodecaborates-encapsulating liposomes.

Next oligo-*closo*-dodecaborate-encapsulating liposomes were prepared by the reverse phase evaporation (REV) method. In order to obtain high-boron-content liposomes, two compounds **10** and **13**, which have high BWs, were selected and used for the preparation of *closo*-dodecaborate-encapsulating liposomes. BSH was used as a control. Distearoylphosphatidylcholine (DSPC), cholesterol and distearoylphosphatidylethanolamine (DSPE)-PEG2000 (1:1:0.11, molar ratio) were dissolved in chloroform and isobutyl ether (1:1 v/v) and the synthesized *closo*-dodecaborates **10** and **13** dissolved in water (125 mM) were added. The mixture was sonicated to obtain an emersion and then organic solvents were removed under reduced pressure. The resulting liposomes were extruded 10 times through a polycarbonate membrane of 100 nm pore size using an extruder device and purified by ultracentrifugation at 200,000 x *g* for 60 min at 4 °C. The resulting liposome pellets

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were dissolved in a minimum amount of saline to make a final liposome solution. Table 2 shows a summary of the characteristics of the resulting oligo-*closo*-dodecaborates-encapsulating liposomes. The average particle sizes of the liposomes encapsulating BSH, **10**, and **13** were 110.7, 113.2, and 113.4 nm, respectively. Their zeta potentials were -25.8, -23.7, and -24.4 mV, respectively. These values are slightly positive compared with the zeta potential of the empty PEG-liposomes (-27.3 mV). The final boron concentration and the boron and phosphorus concentration (B/P) ratio of the liposome solutions increased with the BW of encapsulated compounds. The final boron concentration in BSH-encapsulating liposome solution was 3.5 x 10^3 ppm with a B/P ratio of 1.2, whereas that of compound **10**-encapsulating liposome solution was 9.0 x 10^3 ppm, indicating that the B/P ratio markedly increased to 3.9. Furthermore, in the case of compound **13**, which contains four *closo*-dodecaborates in a molecule (BW = 518.9), the final boron concentration in the liposome solution reached 11.0 x 10^3 ppm with the highest B/P ratio (5.1). These results indicate that the encapsulation of oligo-*closo*-dodecaborates is efficient for the preparation of high-boron-content liposomes under limited osmotic pressure.

Table 2. Characteristics of *closo*-dodecaborate-encapsulating liposomes

Encapsulated Compd.	Size (nm)	Zeta potential (mV)	Boron conc. (ppm)[a]	B/P ratio[b]
4 (BSH)	110.7 ± 0.7	-25.8 ± 15.6	3.5 x 10^3	1.2
10	113.2 ± 1.6	-23.7 ± 18.5	9.0 x 10^3	3.9
13	113.4 ± 1.0	-24.4 ± 16.0	11.0 x 10^3	5.1

[a] After purification by ultracentrifugation at 200,000 x *g* for 60 min at 4 °C, the resulting liposome pellets were dissolved in a minimum amount of saline, and the boron concentration in the final liposome solutions was determined by ICP-AES. [b] Boron-to-phosphorus concentration ratios of the final liposome solutions determined by ICP-AES.

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2.4. Biodistribution of the closo-dodecaborate 13-encapsulating liposomes in tumor-bearing mice.

Finally, the boron biodistribution of the *closo*-dodecaborate **13**-encapsulating liposomes was examined in colon 26 tumor-bearing mice. The liposomes were injected at doses of 15 mgB/kg body weight via the tail veins. BSH-encapsulating liposomes were also injected at the same dose as that used in control experiments. After 36 h, boron concentration in blood, tumor, liver, kidney, and spleen were measured by ICP-AES. The results are summarized in Table 3. The boron concentration in blood against **13**-encapsulating liposomes was lower than that against BSH-encapsulating liposomes, revealing that the half-life of **13**-encapsulating liposomes in blood was relatively shorter probably owing to their instability during their circulation in blood. Indeed, **13**-encapsulating liposomes were highly accumulated in the spleen (193.4 ppm). Interestingly, the boron concentration in the tumor against **13**-encapsulating liposomes was four times higher than that against BSH-encapsulated liposomes. In the case of BSH-encapsulating liposomes, the boron concentration in the tumor was similar to that in blood, indicating that BSH-encapsulating liposomes passively accumulated in the tumor tissue and that BSH was readily eliminated from cells. In contrast, the boron concentration in the tumor was much higher than that in blood, and the tumor/blood (T/B) ratio was approximately 14, indicating that compound **13** was not easily eliminated from cells probably owing to its large molecular size and electronically highly charged characteristic.

Table 3. Boron concentrations in various organs of tumor-bearing mice 36 h after injection of BSH- and *closo*-dodecaborate **13**-encapsulating liposomes[a]

Encapsulated Compd.	Blood	Tumor	Liver	Kidney	Spleen
4 (BSH)	19.1 ± 1.9	18.5 ± 3.5	80.1 ± 3.4	20.8 ± 0.5	32.3 ± 1.9

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13	5.2 ± 1.1	72.2 ± 11.5	60.5 ± 2.3	33.7 ± 2.2	193.4 ± 15.6

[a] Liposomes encapsulating each compound were injected into colon 26 tumor-bearing mice (n = 5) at a dose of 15 mgB/kg body weight via the tail vein. The boron concentration in the tumor and organs 36 h after the injection were measured by ICP-AES.

3. Conclusion

We succeeded in synthesis of high-boron-content molecules **8**, **10**, and **13** as boron agents encapsulated in liposomes by the Hüisgen click cycloaddition of *closo*-dodecaborate-containing azides and alkynes. These boron compounds showed relatively low cytotoxicities and the concentrations that cause 50% growth inhibition (GI_{50}) for B16 melanoma cells, CT26 colon cancer cells, and HeLa cells were higher than 0.14 mM, indicating toxicities similar to that of BSH. Using compound **13**, which has the largest number of boron atoms in a molecule with a boron weight (BW) of 518.9 among the compounds synthesized, we were able to prepare high-boron-content liposomes with a reduced osmotic pressure in their inner core. The final boron concentration in **13**-encapsulating liposome solution reached 11.0 x 10^3 ppm with a boron/phosphorous (B/P) ratio of 5.1. A significantly high tumor boron accumulation was observed in tumor-bearing mice 36 h after the injection of **13**-encapsulating liposomes at a dose of 15 mgB/kg body weight with a high tumor/blood (T/B) ratio. Although boron accumulation in the spleen was relatively high, the current approach is considered to be promising for the development of an efficient system of boron delivery to the tumor for boron neutron capture therapy (BNCT). Further study to reduce boron accumulation in the spleen is now ongoing in our laboratory.

4. Experimental

4.1. General methods

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^{1}H NMR and ^{13}C NMR spectra were measured with Jeol JNM-AL 300 (300 MHz) and Varian Unity-Inova 400 (400 MHz) spectrometers. ^{1}H NMR and ^{13}C NMR chemical shifts are expressed in parts per million (ppm, δ units), and coupling constants are expressed in hertz (Hz). IR spectra were measured with a Shimadzu FTIR-8200 A spectrometer. UV/Vis spectra were measured with a Shimadzu 2450 PC spectrophotometer over the 300–700 nm wavelength range. Elemental analyses were performed witha Perkin–Elmer 2400 automatic elemental analyzer. The fluorescence (excitation and emission) spectra were determined with a Jasco FP-6500 PC spectrophotometer. Electron spray ionization (ESI) mass spectra were recorded with a Shimadzu LCMS-2010 eV spectrometer. Analytical thin-layer chromatography (TLC) was performed on glass plates (Merck Kieselgel 60 F254, layer thickness 0.2 mm). Compounds containing boron clusters were visualized with the aid of $PdCl_2$ or $KMnO_4$. Column chromatography was performed on silica gel (Merck Kieselgel 70–230 mesh). All reactions were performed with shielding from light in dry solvents under nitrogen atmosphere using standard Schlenk techniques. DSPC and DSPE-PEG (SUNBRIGHT DSPE-020CN) were purchased from Nippon Oil and Fats (Tokyo, Japan). All chemicals were of analytical grade and were used without further purification. Compound **3** was synthesized from *closo*-dodecaborate **1** according to the literature procedures[24,26] and compounds **6**, **9** and **12** were synthesized from **4** or **11** according to our previously reported procedures.[22,25]

*4.2. Synthesis of bis-closo-dodecaborates (**8**)*

Compound **6**[22] (170 mg, 0.5 mmol) was dissolved in CH_3CN and $Cu(OAc)_2$ (27 mg, 0.2 mmol) and sodium ascorbate (52 mg, 0.3 mmol) were added. Then the azide **3**[26] (378 mg, 0.5 mmol) was added to the mixture and the reaction mixture was stirred at room temperature for 24 h. After insoluble materials were removed by filtration, the filtrate was evaporated under the reduced pressure. The residue was dissolved in

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methanol and a methanol solution of tetramethylammonium chloride (TMACl, 6 mol/L) was added to precipitate the compound **7**. The resulted precipitate was dissolved in CH_3CN and a CH_3CN solution of tetramethylammonium hydroxide (TMAOH, 10 mol/L, 0.2 mL) was added dropwise. The resulting precipitate was filtered and washed with methanol followed by ether. Purification by recrystallization from CH_3CN gave the product **8'** as a tetramethylammonium (TMA) salt in 63% yield; Mp. >200 ^oC; IR (KBr, cm^{-1}): 3599, 3028 ν (C-H), 2485 ν (B-H), 1635 ν (C=C), 1485, 1163, 1110 ν (C-H), 1288 ν (N=N), 1066 ν (B-B), 1027; ^1H NMR (400 MHz; CD_3CN): δ 7.73 (s, 1H), 4.44 (t, J = 5.2 Hz, 2H), 3.80 (t, J = 5.2 Hz, 2H), 3.65 (s, 2H), 3.49 (s, 4H), 2.20 (s, 48H); ^{13}C NMR (300 MHz; CD_3CN): δ 149.02, 122.87, 72.62, 68.96, 67.70, 55.35, 55.29, 55.25, 49.86, 27.06; 37.42; ^{11}B NMR (300 MHz; CD_3CN): δ 1.62, -19.11, -21.39, -22.80, -27.58; HRMS (ESI, negative) *m/z* calcd. for $C_7H_{33}B_{24}N_3O_2S_1$ $[M/4]^{4-}$: 120.8675, found: 120.8679. Compound **8'** was converted the corresponding sodium form **8** in 97% yield by treating with amberlite Na^+ form for 12 h in CH_3CN/H_2O.

*4.3. Synthesis of tris-closo-dodecaborates (**10**)*

This compound was synthesized from compound **9**[22] (211 mg, 0.7 mmol), $Cu(OAc)_2$ (36 mg, 0.2 mmol), sodium ascorbate (65 mg, 0.5 mmol), and the azide **3** (491 mg, 0.65 mmol) using the procedure described for **8** to give **10'** as a tetramethylammonium (TMA) salt in 89% yield; mp. >200 ^oC; IR (KBr, cm^{-1}): 3599, 3029, 2921 ν (C-H), 2480 ν (B-H), 1635 ν (C=C), 1489, 1164 ν (C-H), 1288 ν (N=N), 1053 ν (B-B), 1027; ^1H NMR (400 MHz; CD_3CN): δ 8.02 (s, 2H), 4.43 (m, 8H), 3.79 (m, 4H), 3.49 (s, 8H), 3.10 (s, 60H); ^{13}C NMR (400 MHz; CD_3CN): δ 138.4, 126.13, 72.60, 68.84, 67.89, 55.60, 55.56, 55.52, 50.26, 37.42; ^{11}B NMR (300 MHz; CD_3CN): δ 1.62, -19.11, -21.39, -22.80, -27.58; HRMS (ESI, negative) *m/z* calcd. for $C_{14}H_{55}B_{36}N_6O_4S_1$ $[M/5]^{5-}$: 158.5518, found: 158.5542. Compound **10'** was converted the corresponding sodium form **10** in 80% yield by treating with amberlite Na^+ form for 12 h in CH_3CN/H_2O.

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*4.4. Synthesis of tetrakis-closo-dodecaborates (**13**)*

This compound was synthesized from compound **12**[22] (240 mg, 0.7 mmol), $Cu(OAc)_2$ (50 mg, 0.3 mmol), sodium ascorbate (103 mg, 0.6 mmol), and the azide **3** (1.6 g, 2.1 mmol) using the procedure described for **8** to give **13'** as a tetramethylammonium (TMA) salt in 85% yield; mp. >200 ^oC; IR (KBr, cm^{-1}) 3601, 3028, 2923 ν (C-H), 2924 ν (N=N), 2481 ν (B-H), 1635 ν (C=C), 1485, 1288, 1163ν (C-H), 1415 ν (B-N), 1109 ν (C-N), 1052 ν (B-B), 949, 823, 720 ν (C-H); ^1H NMR (400 MHz; CD_3CN): δ 8.01 (s, 3H), 4.85 (s, 6H), 4.48 (t, J= 5.2 Hz), 3.80 (t, J= 5.2 Hz, 6H), 3.49 (s, 12H), 3.13 (s, 84H); ^{13}C NMR (400 MHz; CD_3CN): δ 139.46, 127.86, 72.54, 69.16, 67.59, 57.18, 55.32, 55.26, 55.24, 50.00; ^{11}B NMR (300 MHz; CD_3CN): δ 1.67, -21.42, -22.81, -27.64; HRMS (ESI, negative) *m/z* calcd. for $C_{21}H_{77}B_{48}N_{10}O_6$ $[M+2H/7]^{5-}$: 217.4204, found: 217.4208. Compound **13'** was converted the corresponding sodium form **13** in 91% yield by treating with amberlite Na^+ form for 12 h in CH_3CN/H_2O.

4.5. Cytotoxicity of closo-dodecaborates

B16 melanoma, colon 26, and HeLa cells were used for the cell growth inhibition assay. The cells (5×10^3 cells per well of a 96-well plate) were seeded in RPMI-1640 medium and pre-incubated at 37 ^oC for 24 h. Then the cell medium was replaced by the RPMI-1640 medium (100 μL) containing compounds at various concentrations. After incubation for 72 h, 3'-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) in PBS (5 mg/mL, 10 μL) was added to each well, and the cells were further incubated at 37 ^oC for 2 h. After removal of the medium, DMSO (100 mL) was added and the absorbance at 570 nm was determined with a microplate reader. The drug concentration required to reduce cell viability by 50% (IC_{50}) was determined from semilogarithmic dose-response plots.

New Journal of Chemistry Accepted Manuscript

4.6. Preparation of closo-dodecaborates-encapsulating liposomes

closo-Dodecaborates-encapsulating liposomes were prepared from DSPC, cholesterol and DSPE-PEG (1:1:0.11, molar ratio) by the reverse-phase evaporation (REV) method. Briefly, a mixture of DSPC (158 mg, 0.2 mmol), cholesterol (77.3 mg, 0.2 mmol), and DSPE-PEG (63.8 mg, 0.02 mmol) were dissolved in 5 mL of $CHCl_3$/diisopropyl ether mixture (1:1, v/v) in a round-bottom flask. Aqueous solutions of various *closo*-dodecaborates (125 mM) was added to the lipid solution to form an emulsion. The emulsion was sonicated for 3 min, and then, the organic solvent was removed under the reduced pressure using a rotary evaporator at 55 ^{o}C to obtain a suspension of liposomes. The resulting liposomes were subjected to extrusion 10 times through a polycarbonate membrane of 100 nm pore size (Whatman, 110605, FILTER, 0.1UM, 25MM, Gentaur Molecular Products, Belgium), using an extruder device (LIPEX^{TM} Extruder, Northern Lipids, Canada) thermostated at 55 ^{o}C. Purification was accomplished by ultracentrifugation at 200,000 x *g* for 60 min at 4 ^{o}C (Himac CP80WX, Hitachi Koki, Japan), and the pellets obtained were resuspended in saline (1.5 mL). Final boron concentrations of the resulting liposome solutions were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

4.7. Boron biodistribution in tumor-bearing 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 left 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 tumor-bearing mice were injected via the tail vein with 200 μL of the *closo*-dodecaborates encapsulating liposomes (1,500 and 3,000 ppm B). The mice were

New Journal of Chemistry Accepted Manuscript

anesthetized and blood samples were collected from the retro-orbital sinus 36 h after injection. 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 90ºC 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. All protocols were approved by the Institutional Animal Care and Use Committee of Gakushuin University.

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26. It is possible to contain some TBA cations derived from **3** in the counter cations of compound **7**.

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