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Lipid-membrane-incorporated arylboronate esters as agents for boron neutron capture therapy

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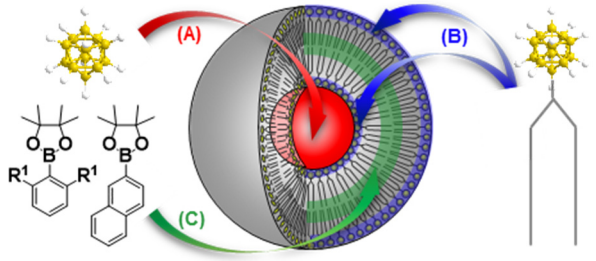
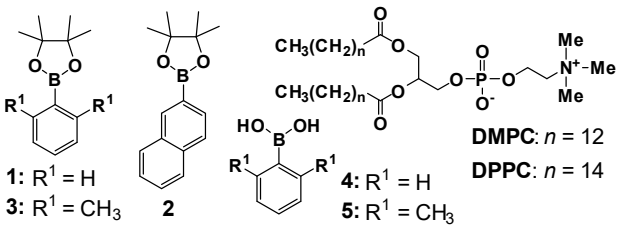
Abstract Arylboronate esters bearing methyl groups in both of their *ortho* positions were stably incorporated into lipid membranes at high concentrations without undergoing hydrolysis to the corresponding boronic acids. This method could be used in combination with previous methods to increase the maximum ratio of boron atoms in liposomal boron carriers.

Boron neutron capture therapy (BNCT) is based on the nuclear capture and fission reactions that occur when a non-radioactive ^{10}B atom is irradiated with low-energy thermal neutrons, resulting in the formation of excited $^{11}B^*$. This excited material subsequently decomposes to yield high energy alpha particles (^4He) and ^7Li nuclei via a linear energy transfer process.[1,2] Both of these particles can only travel very short distances (5–14 μm), and therefore only exhibit damaging effects within the immediate vicinity of the reaction, leading to highly selective, single-cell damaging events. The development of efficient boron carrier systems capable of delivering sufficient concentrations of boron to tumour cells to achieve effective BNCT is therefore highly desirable. Liposomal boron carriers have been studied extensively as efficient boron delivery systems in BNCT.[3–12] Although lipids generally exhibit low toxicity, high lipid doses can lead to liver toxicity as a result of saturating the lipid uptake mechanisms. To reduce the total lipid dose associated with the liposomal delivery systems used in BNCT, there is an urgent need for the development of liposomes with higher boron contents [*i.e.*, a higher boron/phosphorous concentration (B/P) ratio]. Two strategies have been investigated to date for the preparation of liposomal boron systems, including (i) the introduction of water-soluble boron compounds to liposomes via an internal water phase (Scheme 1A);[3–5] and (ii) the use of amphiphilic boron compounds embedded in a liposomal bilayer (Scheme 1B).[6–8] Recent developments in this area have made it possible to reduce the total lipid dose used in liposomal delivery systems based on a combination of these two methods; however, these systems are only capable of a B/P value of 3.6, which is unsatisfactory for BNCT.[9–12] According to the latter of the two methods described above, the boron atoms would exist on the surface of the lipid membranes, where they would behave in the same way as the hydrophilic head groups of the lipids. To the best of our knowledge, however, there have been no reports in the literature pertaining to the attachment of boron atoms to the inside portion of hydrophobic lipid membranes (Scheme 1C). The development of a new method for the stable incorporation of boron-containing moieties in hydrophobic lipid membranes would therefore represent a valuable addition to the two methods described above. Furthermore, all three of these methods could be used in combination to develop highly efficient liposomal delivery systems for BNCT.



Scheme 1 Three methods for preparing liposomal boron carriers, including (A) the introduction of water-soluble boron compounds to an internal water phase; (B) embedding amphiphilic boron compounds in a liposomal bilayer; and (C) incorporating arylboronate esters into the hydrophilic portion of lipid membranes.



We recently demonstrated that small hydrophobic π-conjugated molecules such as fullerenes, porphyrin, azobenzene and pyrenes could be incorporated into liposomes without using a chemical modification process to provide the necessary affinity for the lipid membranes.[13–20] These lipid membrane-incorporated guest molecules

(LMIG) were found to be stable in aqueous solution for up to 1 month. Based on the stability of these systems, it was envisaged that hydrophobic arylboronate esters could be used not to destabilise liposomes and we consequently evaluated the possibility of using this process as a third method for the preparation of liposomal boron systems (Scheme 1C).

We selected the pinacolato boronate ester group as a suitable boronate ester for this study, because of the known stability this group toward hydrolysis in water. Lipid-membrane-incorporated compounds **1**–**3** (LMI**1**–**3**), consisting of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), were prepared using a previously published premixing method by dissolving lipids and compounds **1**–**3** in chloroform followed by concentration and extraction with water (Scheme S1).[20,21] The solubilities of **1**–**3** in the liposomes were determined based on the absorbance characteristics of [**1**–**3**]/[DMPC] at 218, 279 and 275 nm, respectively, after subtracting the scattering derived from the DMPC liposomes (Fig. 1). The dashed lines in the insets of Fig. 1 show the extrapolated absorbance values corresponding to 100% of the guests being dissolved in water.

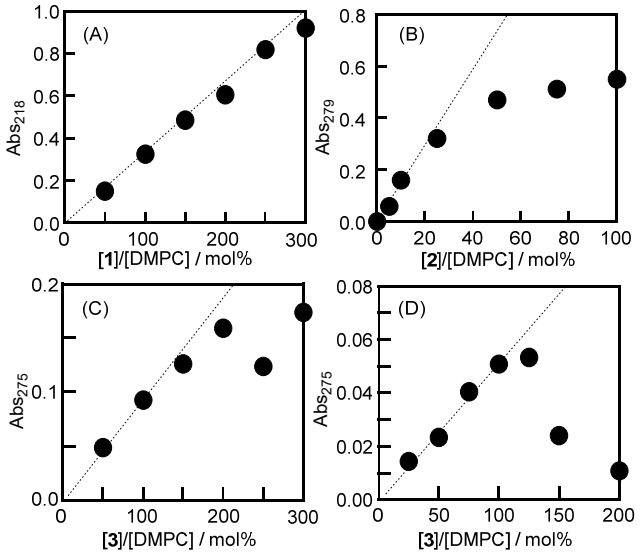


Fig. 1 Absorbance values (Abs) at λ_{max} versus [**1**, **2** or **3**]/[DMPC or DPPC] in (A) LMI**1** (DMPC), (B) LMI**2** (DMPC), (C) LMI**3** (DMPC) and (D) LMI**3** (DPPC). The λ_{max} values for LMI**1**–**3** were 218, 279 and 278 nm, respectively. All of the absorption spectra were obtained by subtracting the light scattering signals of the DMPC liposomes and were measured at 25 °C (1 mm cell, D_2O).

We examined the possibility of incorporating 4,4,5,5-tetramethyl-2-phenyl-1,3,2-dioxaborolane (**1**) into liposomes. This compound was selected as a model system because it represents the simplest possible π structure bearing a boronate ester. The absorbance of LMI**1** until [**1**]/[DMPC] = 300 mol% lay on the extrapolated line and was very similar to that of bare **1** in hexane (Figs. S1A, S1B and 1A). Consequently, **1** in LMI**1** was expected to exist in an isolated (*i.e.*, disaggregated) state. This result suggested that the lipid affinity of **1** was very high. We subsequently investigated the effect of the aryl moiety on the affinity toward the lipid membranes by preparing lipid-membrane-incorporated 4,4,5,5-tetramethyl-2-(naphthalen-2-yl)-1,3,2-dioxaborolane (**2**) (LMI**2**) bearing a naphthyl moiety. In the range of [**2**]/[DMPC] = 0–25 mol%, LMI**2** exhibited sharp absorption in the range of 250–400 nm. Furthermore, a plot showing λ_{max} (279 nm) versus [**2**]/[DMPC] for LMI**2** gave a straight line (Figs. S1C and 1B), which indicated that **2** existed in an isolated state within the lipid membrane. However, the absorbance of LMI**2** reached saturation above 50 mol% and the maximum ratio of [**2**]/[DMPC] was determined to be 25 mol%, which was much lower than that of [**1**]/[DMPC], indicating that the introduction of a naphthyl group led to a decrease in the affinity of the boronate ester for the lipid membranes.

^{1}H NMR analysis was conducted to confirm the formation of LMI**1** (Fig. 2). Several peaks were observed at δ_H = 7.17–7.61 and 7.76 ppm in the ^{1}H NMR spectrum of LMI**1** (red and blue circles in Fig. 2B). If all of the available **1** was incorporated into the lipid membranes, then there would be no peaks corresponding to **1** in the spectrum because of the extreme broadening of the signals after its incorporation into the liposomes (Fig. 2B, [**1**]/[DMPC] = 100 mol%). The peaks observed in the spectrum were attributed to phenylboronic acid (**4**), which was formed by the hydrolysis of **1** (based on the peak observed at δ_H = 1.20 ppm, which was assigned to pinacol, Fig. 2B and 2C, green circles). Compound **1** was completely converted to compound **4** after one week at ambient temperature, based on the disappearance of the peak corresponding to the methyl protons of **1** at δ_H = 1.33 ppm (Fig. 2C, green circles). Although the ^{1}H NMR spectrum of compound **1** in the absence of liposomes contained several sharp peaks (Fig. 2A, blue circles), these peaks broadened considerably in the presence of liposomes (Fig. 2B and 2C, blue circles). There are two possible explanations for this behaviour, including (i) comparable exchange rates for the complexation-decomplexation of **4** with the liposomes on the ^{1}H NMR time scale; and (ii) the incorporation of **4** into small lipid aggregates. We evaluated both of these possibilities by measuring the ^{1}H NMR spectra of a mixture of DMPC liposomes containing high concentrations of **4**. The separation of the peaks between free **4** and **4** complexed with the liposomes did not occur as shown in Fig. 2D (blue circles). Furthermore, because the peak intensities of the small aggregates were very small in the range of 0.8–1.4 ppm compared with those of **4** (Fig. 2D, black circles), we therefore disproved that most of the molecules of **4** were incorporated in small lipid aggregates, as indicated in explanation (ii). These results therefore supported explanation (i), suggesting that the deprotection of **1** resulted in the formation of **4**, which was released from the liposomes to exist in an equilibrium between the bulk water and the liposomes (Scheme 2B).

To avoid the hydrolysis of the pinacolato boronate ester in **1**, we prepared lipid-membrane-incorporated 2-(2,6-dimethylphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**3**) (LMI**3**) bearing a methyl group at both of the *ortho* positions of the phenyl ring.[22] As shown in Figs. S1D and 1C, the absorbance values of LMI**3** were consistent with the extrapolated lines for concentrations of less than 150 mol%, but deviated from the lines at a concentration of 200 mol%. Given that no precipitation was observed in any of these samples of LMI**3**, this deviation was attributed to free compound **3**, which was not incorporated into the liposomes at concentration greater than 150 mol%. This material was subsequently removed by filtration through the membranes (pore size 50 nm) during the extrusion process. Compound **3** dissolved in water in the presence of the DMPC liposomes at a concentration of [**3**]/[DMPC] = 150 mol%, making it possible to confirm the formation of LMI**3** by ^{1}H NMR analysis at 100 mol% (Fig. 3B). The ^{1}H NMR spectrum of LMI**3** did not contain any peaks belonging to 2,6-dimethylphenylboronic acid (**5**) or the pinacol formed by the hydrolysis of **3** (Fig. 3A and 3B, blue circles). This result clearly demonstrated that **3** was not being hydrolysed in water. However, because broad peaks were observed in the range of 0.8–1.4 ppm for LMI**3**, compound **3** was adjudged to have destabilised the DMPC liposomes to a much greater extent than compound **1** (Fig. 3B, black circles). If the DMPC liposomes remained as large hollow particles with a diameter greater than 70 nm, then we wouldn't expect to see any broad peaks in the range of 0.8–1.4 ppm because of the

extreme broadening of the signals.[20] These results therefore suggest that some of the DMPC lipids formed small self-aggregates with **3** (Scheme 2C). Although we were unable to accurately calculate the peak intensities of DMPC because of considerable peak broadening,

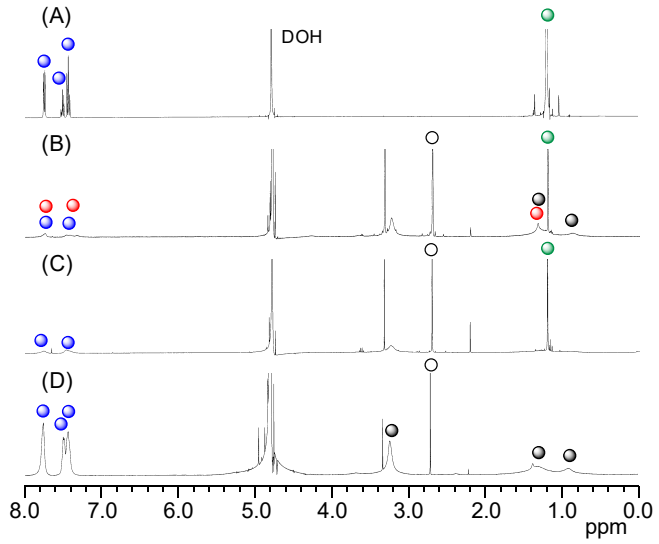


Fig. 2 ^{1}H NMR spectra (400 MHz, D_2O, 25 °C) of (A) a mixture of **4** and pinacol ([**4**] = [pinacol] = 13.0 mM), LMI**1** (B) immediately and (C) one week after its preparation ([**1**]/[DMPC] = 100.0 mol%, [DMSO] = 0.4 mM), and (D) a mixture of liposome and **4** [**4**]/[DMPC] = 300 mol%. ([DMPC] = 2.0 mM, [DMSO] = 0.4 mM). (●: **1**, ●: **4**, ●: pinacol, ●: DMPC lipid in the small aggregates and ○: DMSO).

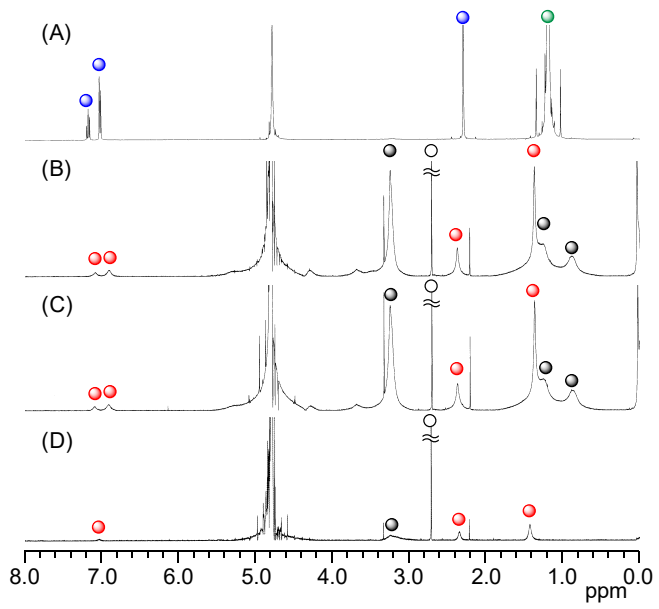
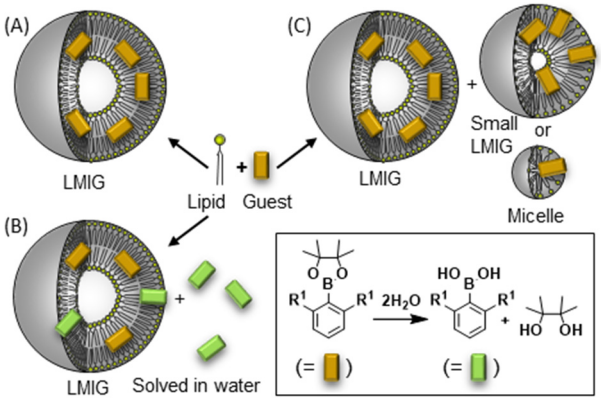


Fig. 3 ^{1}H NMR spectra (400 MHz, D_2O, 25 °C) of (A) a mixture of **5** and pinacol ([**5**] = [pinacol] = 11.0 mM), LMI**3** consisting of DMPC (B) immediately and (C) 1 week after its preparation ([**3**]/[DMPC] = 100 mol%, [DMPC] = 2.0 mM and [DMSO] = 0.4 mM), and (D) LMI**3** consisting of DPPC ([**3**]/[DPPC] = 100 mol%, [DPPC] = 2.0 mM and [DMSO] = 0.4 mM) (●: **3**, ●: **5**, ●: pinacol, ●: DMPC or DPPC lipid in the small aggregates and ○: DMSO).

the percentage of DMPC in the small aggregates was estimated to be approximately 20% of all the DMPC. The leakage percentage of **3** was determined to be 14% based on the peak intensities of **3** relative to DMSO, which was adopted as an internal standard (Fig. 3B, [DMSO] = 0.4 mM). Fig. 3C shows that the molecules of **3** in the liposomes were stable for one week at ambient temperature, as evidenced by the lack of any peaks corresponding to 2,6-dimethylphenylboronic acid (**5**) or pinacol. It is suggested that the reason for the stability of **3** is that the methyl groups in both of the *ortho* positions of the phenyl ring and in pinacol protect the boronate ester against attack by water (Fig. S4).

To inhibit the formation of the small aggregates, we prepared LMI**3** consisting of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), which has been reported to form much more stable liposomes than DMPC. Fig. 3D shows that the intensities of the broadened peaks observed by ^{1}H NMR in the range of 0.8–1.4 ppm dramatically decreased in the presence of DPPC compared with those observed in the in presence of DMPC (Fig. 3B). The broad peaks at 1.4, 2.4 and 7.0 ppm were assigned to molecules of **3** encapsulated in the small partially formed aggregates (Fig. 3D, red mark, Scheme 2C). The leakage percentage of **3** was determined to be 4% based on the peak intensities of **3** relative to DMSO. The absorbance values of LMI**3** consisting of DPPC reached saturation levels above 125 mol% and the maximum ratio of [**3**]/[DPPC] (100 mol%) was found to be much lower than that of [**3**]/[DMPC] (Figs. S1E and 1D).



Scheme 2 Schematic illustration of (A) stable LMIG [category (i)], (B) the dissolution of the guest molecules released from LMIG in water [category (ii)] and (C) the formation of small aggregates between the guest molecules released from LMIG and the lipids [category (iii)].

In summary, although compound **1** was effectively incorporated into liposomes in water, its pinacolato boronate ester moiety was partially hydrolysed to give phenylboronic acid (**4**), which was released from the lipid membrane. The hydrolysis of the boronate ester was inhibited using **3**, which was prepared as the 2,6-dimethylated analogue of **1**. Compound **3** was found to be stable in the liposomes for one week at ambient temperature. However, some of the molecules of **3** formed small aggregates such as micelles or very small liposomes with the lipids. The formation of these small aggregates was appreciably inhibited using DPPC. The B/P value is approximately 1.0 ([**3**]/[DPPC] = 100 mol%) and is not particularly high. However, it is envisaged that this method could be used in combination with two previously published methods to increase the maximum ratio of boron atoms in liposomal boron carriers, as shown in Scheme 1. There is therefore an urgent need to identify suitable derivatives of **3** that would be capable of forming stable liposomes at a high concentration.

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Notes and references

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