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Dodecaborate cluster lipids with variable headgroups for boron neutron capture therapy: Synthesis, physical–chemical properties and toxicity

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ABSTRACT

We have prepared two new boron-containing lipids with potential use in boron neutron capture therapy of tumors. These lipids consist of a diethanolamine frame with two myristoyl chains bonded as esters, and a butylene or ethyleneoxyethylene unit linking the doubly negatively charged dodecaborate cluster to the amino function of the frame, obtained by nucleophilic attack of the amino on the tetrahydrofurane and dioxane derivatives, respectively, of *closo*-dodecaborate. The latter cluster lipid can form liposomes at 25 °C whereas the former lipid at this temperature assembles into bilayer disks. Both lipids form stable liposomes when mixed with suitable helper lipids. The thermotropic behavior was found to be different for the two lipids, with the butylene lipid showing sharp melting transitions at surprisingly high temperatures. Toxicity *in vitro* and *in vivo* varies greatly, with the butylene derivative being more toxic than the ethyleneoxyethylene derivative.

1. Introduction

Boron neutron capture therapy (BNCT) is a radiation therapy for cancer treatment. The short ranged charged particles which are generated in the nuclear reaction between the nontoxic species ^{10}B and thermal neutrons have a great biological effect within the cancer cell. For successful treatment a high amount of boron (approx. 20–30 μg of boron-10 per gram of tumor) and a sufficiently selective accumulation is necessary. Several strategies have been investigated for the boron transfer system, e.g., antibodies [1], nucleic acid precursors [2] and porphyrin derivatives [3].

Liposomes show selective localization in tumors [4] and might be useful vehicles for boron transfer. In general it is possible to encapsulate low-molecular boron substances in the aqueous core of the liposomes or to incorporate boron-containing lipids in the liposomal membrane. The encapsulation method has been described in the literature [5], but this procedure holds some disadvantages. These include a sometimes low encapsulation efficiency, and leakage upon storage or in contact with serum. Further, it was recently shown that charged boron cluster compounds can profoundly affect the structure of liposomes [6]. These problems can be avoided by incorporating boron-containing lipids directly into liposomal membranes.

Only a few boron lipids are described in the literature. Lemmen et al. [7] reported a carborane-containing ether lipid which showed neither tumor selectivity nor retention in tumor tissue. Nakamura et al. [8] described a nido-carborane cluster lipid with a double tailed moiety which was tolerated well in mice [9]. A very similar lipid had been published later by Li et al. [10] but it turned out to be very toxic in mice.

The first dodecaborate ether lipid was described by Lee et al. [11]. Based on mercapto-undecahydro-*closo*-dodecaborate (BSH) different boron lipids have been synthezised. BSH is clinically used for BNCT and has a low toxicity. The boron lipids are expected to show a similar low toxicity. In 2007 Nakamura et al. [12] published a *closo*-dodecaborate-containing lipid. The boron lipids form liposomes in the presence of helper lipids. Recently we described two novel dodecaborate lipids [13]. Both lipids could be formulated into liposomes by use of the helper lipids DSPC (distearoylphosphatidylcholine) and cholesterol. One of the lipids, B-6-14, was shown to form liposomes also in the absence of helper lipids. The lipids were found to be nontoxic *in vitro*.

The existing dodecaborate lipids have a double negative charge. In this study we focused on lipids carrying only a single negative net charge; furthermore, we investigated the effect of different linkers between the dodecaborate cluster head group and the lipid

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backbone. These lipids differ from the lipid B-6-14 previously described by us [13] with respect to the net charge, the linker between the cluster and the hydrophobic moiety.

2. Experimental

2.1. General

A Bruker Esquire spectrometer was used for electrospray mass spectrometry. For boron-containing compounds the peak with the highest mass is declared. To identify the charge of the compound isotope satellite peaks are used. NMR spectra were recorded on a Bruker DPX 200 spectrometer. IR spectra were collected on a BioRad FTS 155 using KBr pellet. Melting points were measured on a Büchi 512 melting point apparatus.

3. Chemistry

3.1. 1-Tetramethylene-(3-oxa)-oxonium-closo-undecahydrododecaborate (-1), tetrabutylammonium salt (1a)

The convenient method recently described was used, in which *closo*-dodecaborate was reacted with hydrogen chloride in 1,4-dioxane in the presence of $NaBF_4$ [14].

3.2. 1-Tetramethyleneoxonium-closo-undecahydrododecaborate (-1), tetrabutylammonium salt (1b)

The method as for **1a** was used, but replacing hydrogen chloride with *p*-toluenesulfonic acid.

3.3. 4-(N,N-bis(2-hydroxyethyl)-N-ethoxy-ammonium)-ethoxy-undecahydro-closo-dodecaborate (-1), cesium salt (2a)

Compound **1a** (0.5 g, 1.05 mmol) and diethanolamine (2 equiv., 0.22 g, 2.1 mmol) were dissolved in dry acetonitrile and then heated to 82 °C for 4 days. The solvent was evaporated and the residue dissolved in methanol. After addition of cesium fluoride (1.5 equiv., 0.24 g, 1.58 mmol) which was dissolved in methanol a white precipitate was formed and filtrated. The product was dried in oil pump vacuum. Yield 0.465 g (95%), m.p. above 245 °C. MS (ESI, acetonitrile, *m*/*z*) negative 332.1 [A^--H_2], 355.8 [$A^--H^++Na^+$], 466 [$A^--H^++Cs^+$]; positive 132.8 [Cs^+], 467.2 [$A^-+Cs^++H^+$], 600.1 [$A^-+2\ Cs^+$]. NMR measurements were not very easy for **2a**, due to the formation of micelles.

^{1}H NMR (200 MHz, ([D_3] CD_3CN, 25 °C, TMS): δ = 3.3 (m, 6H, –O–CH_2–CH_2–O–CH_2–), δ = 3.63 (m, 4H, –CH_2-O–), δ = 3.86 (m, 6H, –N^+–(CH_2)$_3$), δ = 4.19 (m, 1H, –NH^+–). ^{13}C NMR (200 MHz, [D_6] DMSO, 25°C, TMS): δ = 52.84, 55,76, 67.67, 71.34. ^{11}B NMR (200 MHz, [D_6] DMSO, 25 °C): −22.9 (1B), −17.91 (5B), −16.65 (5B), 7.01 (1B). IR (KBr): ν = 3404 (–O–H), 2869 (C–H), 2484 (B–H), 1623 (–N–H), 1460 (C–H), 1163 (–C–N–), 1066 (–C–O–C–; –B–O–C–).

3.4. 4-(N,N-bis(2-hydroxyethyl)ammonium)-butoxy-undecahydro-closo-dodecaborate (-1), cesium salt (2b)

Similar to **2a**. Yield 0.45 g (94%), m.p. above 245 °C. MS (ESI, acetonitrile, *m*/*z*) negative 318.0 [A^-]; positive 132.9 [Cs^+]. Also for **2b**, NMR measurements were not very, due to the formation of micelles.

^{1}H NMR (200 MHz, [D2] D2O, 25 °C, TMS): δ = 1.41–1.79 (m, 6 H, –O–CH_2–CH_2 –CH_2–), 3.20–3.36 (m, 6 H, –N^+–(CH_2)$_3$), 3.48 (m, 1H, –NH^+–), 3.88 (m, 4H, –CH_2–O–). ^{13}C NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 24.59, 30.85, 55.24, 56.56, 58.50, 69.00. ^{11}B NMR (200 MHz, [D_6] DMSO, 25 °C): −22.52 (1B), −17.74–(−16.83) (10B), 6.60 (1B).

IR (KBr): ν = 3404 (–O–H), 2940 (C–H), 2865 (C–H), 2482 (B–H), 1631 (–NH–), 1464 (C–H), 1150 (–C–N–), 1059 (–B–O–C–).

3.5. 4-(N,N-bis(2-myristoyloxyethyl)-N-ethoxy-ammonium)-ethoxy-undecahydro-closo-dodecaborate (-1), cesium salt (B-Dioxan-14)

Compound **2a** (0.3 g, 0.643 mmol) was suspended in dry acetonitrile and a 60% suspension of NaH (3.5 equiv., 0.09 g, 2.25 mmol) was added under N_2 atmosphere. The mixture was heated to 70 °C and stirred for 2 h. After cooling, myristoyl chloride (3 equiv., 0.476 g, 1.93 mmol) was added and stirred for 24 h. A precipitate was removed by filtration, followed by evaporation of the solvent. The residue was dissolved in hot methanol. The product **3a** precipitated upon cooling, was filtrated and dried in oil pump vacuum. Yield 0.228 g (40%) MS (ESI, acetonitrile, *m*/*z*) negative 752.6 [$A^-−H_2$], 776.6 [$A^-−H^++Na^+$], 792.6 [$A^-−H^++K^+$], 886.5 [$A^-−H^++Cs^+$]; positive 132.9 [Cs^+], 596.6 [$A^-−B_{12}H_{11}O$]. $^{1H\ NMR}$ (200 MHz, [D_1] $CDCl_3$, 25 °C, TMS): 0.89 (m, 6H, –CH_3), 1.26 (m, 44 H, –(CH_2)$_{11}$–CH_3), 1.59 (m, 6H, B–O–CH_2–, –CH_2–C=O), 2.36 (m, 4H, –CH_2–O–C=O), 3.61–3.95 (m, 7H, –CH_2–O–CH_2–, –CH_2–N^+–), 4.57 (m, 4H, –NH^+–, –CH_2–N^+–). ^{13}C NMR (200 MHz, ([D_1] $CDCl_3$, 25 °C, TMS): δ = 14.78, 23.76, 25.87, 30.75, 33.03, 34.96, 52.96, 53.89, 64.19, 70.51, 70.96, 174.19. ^{11}B NMR (200 MHz, ([D_6] DMSO, 25 °C): −21.85 (1B), −17.00 (10B), 6.49 (1B). IR (KBr): ν = 2920 (C–H), 2851 (C–H), 2482 (B–H), 1741 (–C=O), 1645 (–NH–), 1469 (C–H), 1170 (–C–N–), 1028 (–C–O–C–; –B–O–C–).

3.6. 4-(N,N-bis(2-myristoyloxyethyl)ammonium)-butoxy-undecahydro-closo-dodecaborate (-1), cesium salt (B-THF-14)

Prepared as described for **B-Dioxan-14.** Yield 0.196 g (35%) MS (ESI, acetonitrile, *m*/*z*) negative 737.7 [A^-]; positive 132.9 [Cs^+], 580.6 [$A^-−B_{12}H_{11}O$], 1004 [A^-+2Cs^+].

1H NMR (200 MHz, [D_6] DMSO, 25 °C, TMS): δ = 0.86 (m, 6H, –CH_3), 1.24 (m, 48H, –(CH_2)$_{11}$–CH_3, –CH_2–CH_2–), 1.48 (m, 6H, B–O–CH_2–, –CH_2–C=O), 2.18 (t, 4H, –CH_2–O–C=O), 2.80–3.80 (m, 6H, –CH_2–N^+–), 4.40 (m, 1H, –NH^+–). ^{13}C NMR (200 MHz, ([D_1] $CDCl_3$, 25 °C, TMS): 14.78, 23.79, 25.98, 30.58, 33.03, 34.54, 53.35, 55.23, 59.92, 69.97, 72.67, 175.57. ^{11}B NMR (200 MHz, [D_6] DMSO, 25 °C): −23.11 (1B), −18.15 to −16.62 (10B), 6.87 (1B). IR (KBr): ν = 2917 (C–H), 2850 (C–H), 2483 (B–H), 1850–1705 (–C=O), 1468 (C–H), 1306 (–C–N–), 1172 (–C–N–), 1056 (–B–O–C–).

4. Preparation of liposome samples

The boron-containing lipids were either used in pure form or mixed with equal molar amounts of DPPC and cholesterol. The lipid, or lipid mixture (DPPC/cholesterol/boron lipid (1:1:1)), was dissolved in chloroform and dried to a thin lipid film in a round-bottom flask. Then the lipid film was hydrated and dispersed by vortexing in 10 mM HEPES buffer saline, pH 7.4 (150 mM NaCl, 10 mM HEPES). The resulting suspension was frozen and thawed in 10 cycles followed by extrusion (21 times) through a polycarbonate membrane with a pore diameter of 100 nm (Avestin, Mannheim, Germany) at a temperature of 64 °C.

Final lipid concentration was 10 mM. Lipid content was measured by the Stewart assay [15], using appropriate standard curves for the individual lipids.

For DSC measurements, the pure lipids were dispersed from a lipid film (obtained as described above) by hydration with 10 mM HEPES 100 mM NaCl, pH 7.4, through ten freeze–thaw cycles and stored at 4 °C prior to measurement.

4.1. Cryotransmission electron microscopy (cryo-TEM)

A small amount (~1 μl) of the liposome suspension (final lipid concentration 5 mM) was transferred to a polymer-coated copper grid and shock-frozen from 25 °C by injection into liquid ethane. The vitrified sample was examined in Zeiss EM 902 A electron microscopes, operating at an accelerating voltage of 80 keV in filtered bright field image mode at $\Delta E = 0$ eV. The stage temperature was kept below 108 K, and images were recorded at defocus settings between 1 and 3 μm. Images were recorded by a slow scan charge-coupled device (SSCCD) camera using the minimal dose focusing device. To assess the reproducibility of the results several images were recorded in different areas of the specimen.

4.2. Differential scanning calorimetry measurements

Differential scanning calorimetry (DSC) measurements were carried out on a VP-DSC microcalorimeter from Microcal (Northhampton, MA), using the pure boron lipid (final lipid concentration 5 mM). The samples were degassed under vacuum prior to the measurement. For the up- and downscans a scan rate of 60 °C/h and a filtering period of 2 s were used. From each scan a buffer background scan was subtracted. For data analysis, the software package ORIGIN (Microcal) was used. The buffer system for this measurement was 10 mM HEPES with 100 mM sodium chloride.

4.3. Cytotoxicity assay

The cell line V79 (lung fibroblasts of Chinese hamster) was used and cultivated with Hamś F10 medium and 10% newborn calf serum at 37 °C and 5% CO_2. 11.000 cells per well were seeded into 96-well plates and grown for 24 h. Then the cells were incubated with different concentrations of boron-containing liposomes for 24 h. Liposomal formulation of DPPC/cholesterol/boron lipid (1:1:1) was used. The cell survival was determined with the WST-1 test system. The supernatant was removed and 100 μl of a WST-1 stock solution (1:4 diluted with PBS and further 1:10 with medium) per well were filled. After 4–6 h the absorbance was measured at 450 nm.

The IC_{50} values were obtained by fitting a sigmoidal curve with the following equation:

$$f = \frac{a}{(1 + e^{-(x-IC_{50})/b})}$$

in which f corresponds to the percentage survival of cells, a corresponds to the highest point of response, x to the concentration of the tested substance, b to the slope of the response curve, and IC_{50} to the concentration of the tested substance that provokes 50% cell death.

4.4. Animal experiments

Animal experiments were carried out with Balb/c mice bred specifically for scientific purposes, following the legally required permission by the government of the Free Hanseatic City of Bremen. Animals were housed in cages with ample access to water and food.

Liposomes (100 μl) were injected into the tail vein of female Balb/c mice. The animals were observed for acute toxicity for 24 h.

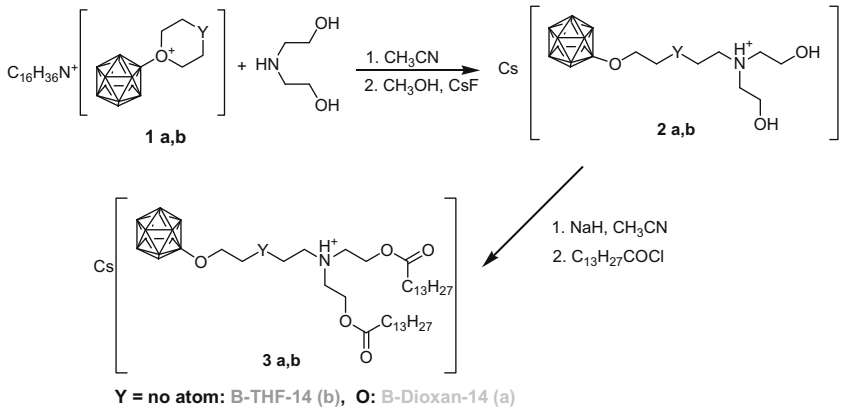
5. Results and discussion

In this work, two new lipids were synthesized which differ from the lipid B-6-14 previously described by us [13] by carrying only one negative net charge. The lipids consist of a double-tailed moiety and a dodecaborate cluster as head group, connected with different spacers. The synthesis requires the connection between a derivative of the $B_{12}H_{12}^{2-}$ cluster and a lipid backbone. We used nucleophilic ring opening reactions between the tetrahydrofurane (THF) and dioxane derivatives of the cluster and the secondary amine diethanolamine (Scheme 1).

Semioshkin et al. [16] recently reported reactions of oxonium derivatives with amines, but under different conditions and not applied to boron lipids. The lipid moiety prepared here is similar to that of the recently published lipids B-6-14 and B-6-16 [13].

For the ring opening reactions the THF- or dioxane derivative of the cluster reacts with 2 equiv. diethanolamine followed by an esterification at the hydroxyl groups with the acid chloride (Scheme 1) The first reaction step proceeds with a yield of 94–95% and the esterification, with 35–40%. Both new dodecaborate cluster lipids contain the same lipid backbone and the same head groups, but varying linkers between them.

The phase transition temperature of the pure B-THF-14 lipid was determined with differential scanning calorimetric measurements (DSC) (Fig. 1) A sharp peak at 48.9 °C and a small pre-transition peak at 46 °C is observed. These temperatures are not comparable to other lipids containing the same hydrophobic part. Dimyristoylphosphatidylcholine (DMPC) with the same alkyl chain length has a transition temperature of 24.3 °C. The dodecaborate lipid B-6-14 with two myristoyl chains (recently described by us [13]) on the same diethanolamine frame (differing only in the linker to the cluster) does not exhibit a pre-transition peak and the main transition is at 18.8 °C, in the same temperature region as DMPC.



Scheme 1. Synthesis of boron lipids.

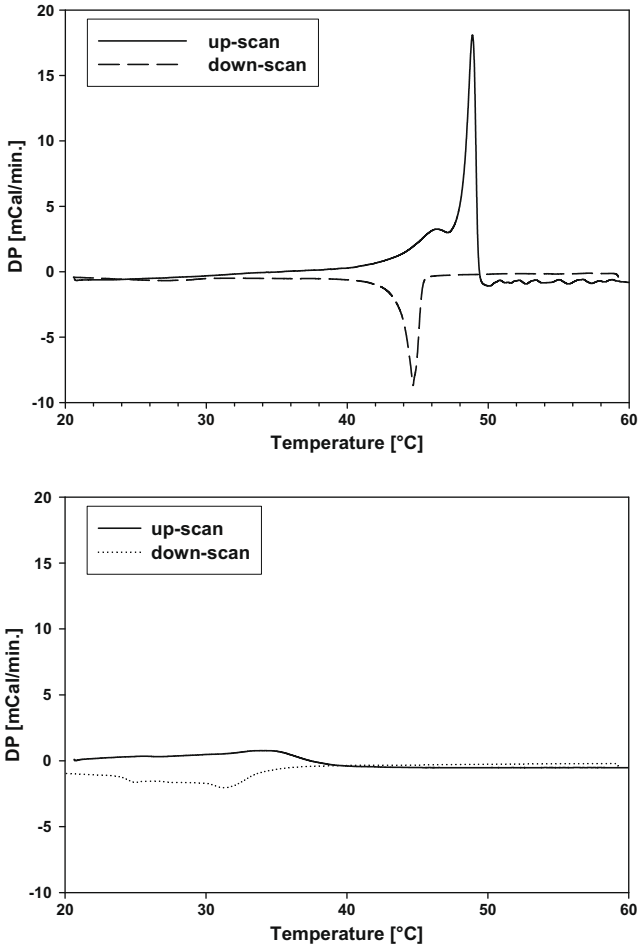


Fig. 1. DSC of pure films of B-THF-14 (above) and B-Dioxan-14 (below) (the first up-scan and the first down-scan are shown). Lipid concentration 5 mM.

The temperature difference between the transition peaks in the up- and down scan (approx. 4°C) is remarkable; such strong hysteresis effect has not been described for other lipids.

For B-Dioxan-14 a phase transition temperature could not be identified. A very broad transition between 20 and 40 °C with minimal endothermic demand is observed. Broad transitions are normally found for lipid species that are not able to form ordered, gel-phase, structures.

The transition temperature depends on both the nature of the headgroup and on the alkyl chain length. Thus DMPC with choline as headgroup has a transition at 24 °C whereas the transition temperature of DMPE with an ethanolamine headgroup lays at 57 °C [17]. B-THF-14 and B-Dioxan-14 share the same polar headgroup, i.e., the dodecaborate cluster. The transition temperature is well known to increase with longer alkyl chain length e.g., DMPC (dimyristoyl-phosphatidylcholine) (24 °C) to DSPC (distearoyl-phosphatidylcholine) (54 °C). The phase transition temperatures are different, however, for B-THF-14 and B-Dioxan-14 despite the fact that they have the same fatty acid chains. The DSC data from pure B-THF-14 and B-Dioxan-14 thus indicate that the phase transition depends also on the linker between the cluster head group and the lipid backbone.

B-Dioxan-14 displays no characteristic gel- to liquid crystalline phase transition and behaves more like a detergent in the DSC. We therefore suspect that the membrane packing is disturbed in this case, possibly due to the more hydrophilic and flexible nature of the linker.

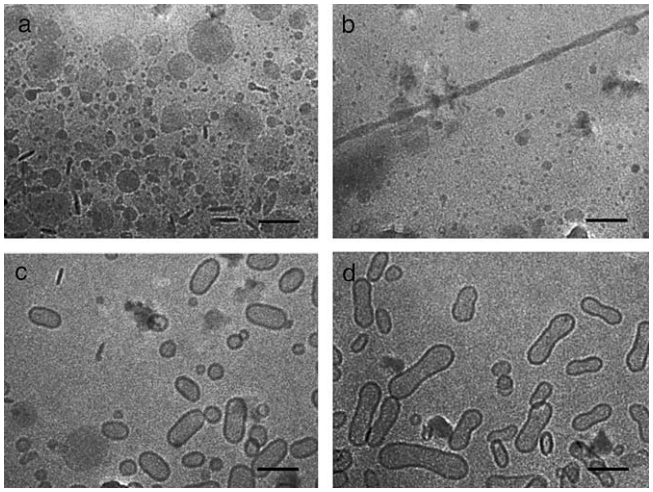


Fig. 2. Cryo-TEM pictures from pure boron lipids: (a) and (b) B-THF-14; (c) and (d) B-Dioxan-14. Scale bar 100 nm.

Cryo-TEM was used to investigate the structures formed from the pure boron lipids (Fig. 2).

B-THF-14 forms predominantly bilayer disks as can be seen in Fig. 2a. The structures are heterogeneous in size; some of them are smaller than 100 nm which is the diameter of the extrusion membrane pores. Long twisted bilayer bands are also found (Fig. 2b), but no closed vesicles could be observed. At the temperature of observation (25 °C), B-THF-14 is in the gel phase, which is less prone to allow the presence of liposomes. For comparison, samples prepared from the lipids B-6-14 and B-6-16 displayed very large bilayer sheets below the phase transition temperatures [13].

In contrast B-Dioxan-14 forms closed liposomes. Some spherical structures are observed, but most of the material is found in tubular peanut-shaped liposomes. The long axis of the tubular structures is very heterogeneous in length. Some of the tubular structures are larger than 200 nm. A few bilayer disks were found in coexistence with the liposomes.

The difference in morphology between the preparations of B-Dioxan-14 and B-THF-14 are striking. The only chemical difference between them is in the spacer linking the negatively charged cluster to the positively charged central nitrogen atom; that of B-Dioxan-14 contains an additional oxygen atom. This should increase the hydrophilicity, as well as the flexibility, of the spacer in comparison to that of B-THF-14. As the class of lipids is, however, new, a detailed molecular picture of the structure would require a molecular dynamics simulation.

Cryo-transmission electron microscopy was employed also to visualize the structure in samples where the boron-containing lipids had been mixed with helper lipids. For liposomal preparations, DPPC/cholesterol/boron-containing lipid in the molar ratio of 1:1:1 were used. DPPC is a commonly used structural component in the formulation of unilamellar liposomes. It consists of a zwitterionic choline-based headgroup and a double-tailed lipid moiety. The physical similarity between DPPC and the boron lipids makes a facile incorporation into the bilayer probable.

As shown in Fig. 3, most of the material forms unilamellar, closed vesicles which exhibit a tolerable size distribution. In the case of B-THF-14 a few multilamellar structures can be observed.

The cryo-TEM pictures demonstrate the possibility to prepare spherical and unilamellar liposomes from the present dodecaborate cluster lipids in the presence of helper lipids. By means of this liposome formulation a high amount of boron can be transferred to the cancer cells. For successful BNCT 10–30 ppm boron is required which corresponds to approx. $(1–3) \times 10^9$ boron atoms for an

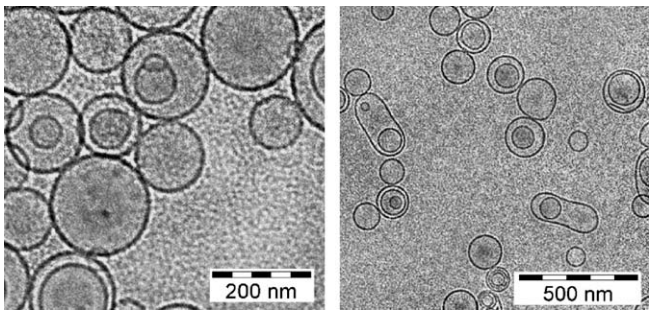


Fig. 3. Cryo-TEM pictures of DPPC/cholesterol/B-THF-14 liposomes (left) and DPPC/cholesterol/B-Dioxan-14 liposomes (right). Scale as indicated.

average mammalian cell [18]. Justus et al. [13] calculated that a 100 nm liposome with an equimolar ratio of DSPC, cholesterol and boron lipid is capable of transferring approx. 6.5×10^5 boron atoms. Only half of that number of boron atoms (3.8×10^5) can be encapsulated in 100 nm liposomes at a dodecaborate cluster concentration of 0.1 M (assuming a volume per liposome of 5.2×10^6 nm^3).

The liposomes prepared here can most probably be tagged with tumor-seeking entities [19] and thereby a selective tumor accumulation should be possible.

In an *in vitro* experiment the toxicity of B-THF-14 and B-Dioxan-14 was determined. We found that B-THF-14 inhibits the cell growth by 50% at a concentration of 0.38 mM. B-Dioxan-14 is less toxic and has an IC_{50} value of 2 mM (Fig. 4).

The liposomal formulation with boron-containing lipid/DSPC/cholesterol (molar ratio 1:1:1) and 2 mol% DSPE-PEG_{2000} was tested for toxicity in mice (0.43 mg boron in 100 μl). The liposomes were injected intravenously into the tail vein of female Balb/c mice. For B-Dioxan-14 no acute toxicity was found but B-THF-14 was lethal after a few minutes. As a consequence, the choice of the linker is an important factor for toxicity. In this case, the introduction of an ether function in the hydrocarbon spacer leads to a decrease of toxicity.

We have previously found that the *in vitro* toxicity of the dodecaborate cluster lipids B-6-14 and B-6-16 [13] decreases with increasing alkyl chain length. The preparation of the lipids B-THF-16 and B-Dioxan-16 with palmitoyl instead of myristoyl chains is in progress. Possibly, the toxicity decreases similarly.

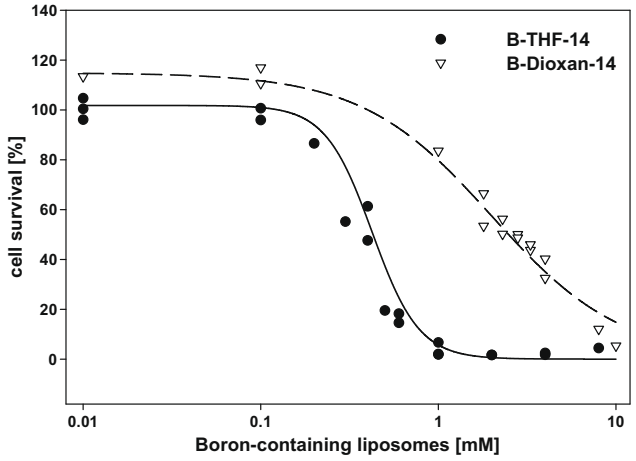


Fig. 4. Survival of V79 Chinese hamster cells exposed to B-THF-14 (circles) and B-Dioxan-14 (triangles), respectively. The solid and the dashed lines are the fitted curves from which the IC_{50} value was calculated.

We have to date no explanation for the high toxicity of B-THF-14. The mechanism by which the boron-containing lipids interact with the cells is not known in any detail. It is possible that the boron-containing liposomes are completely internalized into the cells but an exchange of boron-containing lipid between liposome and cell membrane likely also occur. In this case the membrane potential would decrease because of the negative lipid charge and a dysfunction of the membrane channels would be possible. Alternatively, the membrane fluidity could be changed by incorporation of the lipid into the membranes of cells (including blood cells).

In general the high boron carrying capacity make dodecaborate cluster lipids attractive agents as boron delivery systems for BNCT. B-THF-14 and B-Dioxan-14 form different, but small stable structures (100–200 nm when extruded through a 100-nm filter) when used in pure form. They represent the first dodecaborate-containing lipids which can form structures compatible with intravenous injection without helper lipids which are stable even upon storage at 4 °C. B-Dioxan-14 with its low toxicity might represent a suitable boron carrier for boron neutron capture therapy.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jorganchem.2008.12.044.

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