Boron Containing Macromolecules and Nanovehicles as Delivery Agents for Neutron Capture Therapy†

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Abstract: Boron neutron capture therapy (BNCT) is based on the nuclear capture and fission reactions that occur when non-radioactive boron-10 is irradiated with low energy thermal neutrons to yield high linear energy transfer (LET) alpha particles (^{4}He) and recoiling lithium -7(^{7}Li) nuclei. For BNCT to be successful, a sufficient number of ^{10}B atoms (~ 10^9 atoms/cell) must be selectively delivered to the tumor and enough thermal neutrons must be absorbed by them to sustain a lethal ^{10}B(n, _) ^{7}Li capture reaction. BNCT primarily has been used to treat patients with brain tumors, and more recently those with head and neck cancer. Two low molecular weight (LMW) boron delivery agents currently are being used clinically, sodium borocaptate and boronophenylalanine. However, a variety of high molecular weight (HMW) agents consisting of macromolecules and nanovehicles have been developed. This review will focus on the latter which include: monoclonal antibodies, dendrimers, liposomes, dextrans, polylysine, avidin, folic acid, epidermal and vascular endothelial growth factors (EGF and VEGF). Procedures for introducing boron atoms into these HMW agents and their chemical properties will be discussed. *In vivo* studies on their biodistribution will be described, and the efficacy of a subset of them, which have been used for BNCT of tumors in experimental animals, will be discussed. Since brain tumors currently are the primary candidates for treatment by BNCT, delivery of these HMW agents across the blood-brain barrier presents a special challenge. Various routes of administration will be discussed including receptor-facilitated transcytosis following intravenous administration, direct intratumoral injection and convection enhanced delivery by which a pump is used to apply a pressure gradient to establish bulk flow of the HMW agent during interstitial infusion. Finally, we will conclude with a discussion relating to issues that must be addressed if these HMW agents are to be used clinically.

Key Words: Boron neutron capture therapy, high molecular weight delivery agents and nanovehicles.

1. INTRODUCTION

After decades of intensive research, high grade gliomas, and specifically glioblastoma multiforme (GBM), are still extremely resistant to all current forms of therapy including surgery, chemotherapy, radiotherapy, immunotherapy and gene therapy [1-5]. The five year survival rate of patients diagnosed with GBM in the United States is less than a few percent [6, 7] despite aggressive treatment using combinations of therapeutic modalities. This is due to the infiltration of malignant cells beyond the margins of resection and their spread into both gray and white matter by the time of surgical resection [8, 9]. High grade gliomas are histologically complex and heterogeneous in their cellular composition. Recent molecular genetic studies of gliomas have shown how complex the development of these tumors is [10]. supratentorial gliomas must be regarded as a whole brain disease [14]. The inability of chemo- and radiotherapy to cure patients with high grade gliomas is due to their failure Glioma cells and their neoplastic precursors have biologic properties that allow them to evade a tumor associated host immune response [11], and biochemical properties that allow them to invade the unique extracellular environment of the brain [12, 13]. Consequently, high grade to eradicate microinvasive tumor cells within the brain. In order to successfully treat these tumors, therefore, strategies must be developed that can selectively target malignant cells with little or no effect on normal cells and tissues adjacent to the tumor.

Boron neutron capture therapy (BNCT) is based on the nuclear capture and fission reactions that occur when non-radioactive boron-10 is irradiated with low energy thermal neutrons to yield high linear energy transfer (LET) alpha particles (^{4}He) and recoiling lithium -7 (^{7}Li) nuclei. For BNCT to be successful, a sufficient number of ^{10}B atoms (~10^9 atoms/cell) must be delivered selectively to the tumor and enough thermal neutrons must be absorbed by them to sustain a lethal ^{10}B(n, α) ^{7}Li capture reaction. The destructive effects of these high energy particles are limited to boron containing cells. BNCT primarily has been used to treat high grade gliomas [15, 16], and either cutaneous primaries [17] or cerebral metastases of melanoma [18]. More recently, it also has been used to treat patients with head and neck [19, 20] and metastatic liver cancer [21, 22]. BNCT is a biologically rather than physically targeted type of radiation treatment. If sufficient amounts of ^{10}B and thermal neutrons can be delivered to the target volume, the potential exists to destroy tumor cells dispersed in the normal tissue parenchyma. Readers interested in more in depth coverage of other topics

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related to BNCT are referred to several recent reviews [23-25] and monographs [15, 24]. This review will focus on boron containing macromolecules and nanovehicles as boron delivery agents.

2. GENERAL REQUIREMENTS FOR BORON DELIVERY AGENTS

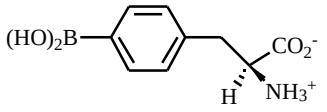
A successful boron delivery agent should have: 1. no or minimal systemic toxicity with rapid clearance from blood and normal tissues; 2. high tumor (~20 μg ^{10}B/g) and low normal tissue uptake; 3. high tumor:brain (T:Br) and tumor:blood (T:Bl) concentration ratios (> 3-4:1); 4. persistence in the tumor for a sufficient period of time to carry out BNCT. At this time *no* single boron delivery agent fulfills all of these criteria. However, as a result of new synthetic techniques and increased knowledge of the biological and biochemical requirements for an effective agent, a number of promising new boron agents has emerged and some of these are the subject of other papers in this special issue of the journal. The major challenge in their development has been the requirement for specific tumor targeting in order to achieve boron concentrations sufficient to deliver therapeutic doses of radiation to the tumor with minimal normal tissue toxicity. The selective destruction of GBM cells in the presence of normal cells represents an even greater challenge compared to malignancies at other anatomic sites.

3. LOW MOLECULAR WEIGHT DELIVERY AGENTS

In the 1950s and early 1960s clinical trials of BNCT were carried out using boric acid and some of its derivatives as delivery agents. These simple chemical compounds had poor tumor retention, attained low T:Br ratios and were non-selective [26, 27]. Among the hundreds of low-molecular weight boron-containing compounds that were synthesized, two appeared to be promising. One, based on arylboronic acids [28], was (*L*)-4-dihydroxy-borylphenylalanine, referred to as boronophenylalanine or BPA (Fig. **1**, **1**). The second, a polyhedral borane anion, was sodium mercaptoundecahydro-*closo*-dodecaborate [29], more commonly known as sodium borocaptate or BSH (**2**). These two compounds persisted longer in animal tumors compared with related molecules, attained T:Br and T:Bl boron ratios >1 and had low toxicity. ^{10}B enriched BPA, complexed with fructose to improve its water solubility, and BSH, have been used clinically for BNCT of brain, as well as extracranial tumors. Although their selective accumulation in tumors is not ideal, the safety of these two drugs following i.v. administration has been well established [30, 31].

4. HIGH MOLECULAR WEIGHT BORON DELIVERY AGENTS

High molecular weight (HMW) delivery agents usually contain a stable boron group or cluster linked *via* a hydrolytically stable bond to a tumor-targeting moiety, such as monoclonal antibodies (mAbs) or low molecular weight receptor targeting ligands. Examples of these include epidermal growth factor (EGF) or the mAb cetuximab (IMC-C225) to target the EGF receptor or its mutant isoform EGFRvIII, which are over-expressed in a variety of malignant tumors including gliomas, and squamous cell carcinomas of the head and neck [32]. Agents that are to be administered systemically should be water soluble, but lipophilicity is important in order to cross the blood-brain barrier (BBB) and diffuse within the brain and the tumor. There should be a favorable differential in boron concentrations between tumor and normal brain, thereby enhancing their tumor specificity. Their amphiphilic character is not as crucial for LMW agents that target specific biological transport systems and/or are incorporated into nanovehicles such as liposomes. Molecular weight also is an important factor, since it determines the rate of diffusion both within the brain and the tumor. Detailed reviews of the state-of-the-art of compound development for BNCT have been published [33, 34]. In this review, we will focus on boron containing macromolecules and liposomes as delivery agents for BNCT, and how they can be most effectively administered.



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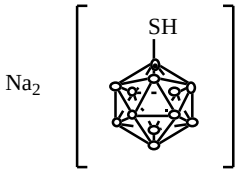

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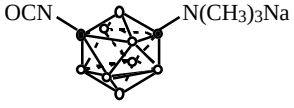

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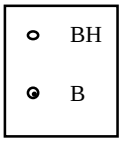


Fig. (1). Structures of two compounds used clinically for BNCT, dihydroxyborylphenylalanine or boronophenylalanine (BPA, **1**), and disodium undecahydro-closo-dodecaborte or sodium borocaptate (BSH, **2**) and the isocyanato polyhedral borane (**3**), which has been used to heavily boronate dendrimers.

5. DENDRIMER RELATED DELIVERY AGENTS

5.1. Properties of Dendrimers

Dendrimers are synthetic polymers with a well-defined globular structure. As shown in Fig. **2**, they are composed of a core molecule, repeat units that have three or more functionalities, and reactive surface groups [35, 36]. Two techniques have been used to synthesize these macromolecules: divergent growth outwards from the core [37], or convergent growth from the terminal groups inwards towards the core[36, 38]. Regular and repeated branching at each monomer group gives rise to a symmetric structure and pattern to the entire globular dendrimers. Dendrimers are an attractive platform for macromolecular imaging and gene delivery because of their low cytotoxicity and their multiple types of reactive terminal groups [36, 39-44].

5.2. Boronated Dendrimers Linked to Monoclonal Antibodies

5.2.1. Boron Clusters Directly Linked to mAb

Monoclonal antibodies have been attractive targeting agents for delivering radionuclides [45], drugs [46-50], toxins [51] and boron to tumors [52-55]. Prior to the introduction of dendrimers as boron carriers, boron compounds were directly attached to mAbs [53, 54]. It has been calculated that ~ 10^9 ^{10}B atoms per cell (~ 20μg/g tumor) must be delivered in order to kill tumor cells [55, 56]. Based on the assumption of 10^6 antigenic receptor sites per cell, ~ 50-100 boron cage structures of carboranes, or polyhedral borane anions and their derivatives must be linked to each mAb molecule to deliver the required amount of boron for NCT. The attachment of such a large number of boron cages to a mAb may result in precipitation of the bioconjugate or a loss of its immunological activity. Solubility can be improved by inserting a water-soluble gluconamide group into the protein-binding boron cage compounds, thereby enhancing their water solubility [57]. This modification makes it possible to incorporate up to 1100 boron atoms into a human gamma globulin (HGG) molecule without any precipitation. Other approaches to enhance solubility include the use of negatively charged carboranes [58] or polyhedral borane anions [59], as well as the insertion of carbohydrate groups [60, 61]. A major limitation of using an agent containing a single boron cage is that a large number of sites must be modified in order to deliver 10^3 boron atoms per molecule of antibody and this can reduce its immunoreactivity activity. Alam *et al*. showed that attachment of an average of 1300 boron atoms to mAb 17-1A, which is directed against human colorectal carcinoma cells, resulted in a 90% loss of its immunoreactivity [62].

5.2.2. Attachment of Boronated Dendrimers to mAb

Dendrimers are one of the most attractive polymers that have been used as boron carriers due to their well-defined structure and large number of reactive terminal groups. Depending on the antigen site density, ~ 1000 boron atoms need to be attached per molecule of dendrimer and subsequently linked to the mAb. In our first study, second- and fourth-generation polyamido amino (PAMAM or "starburst") dendrimers, which have 12 and 48 reactive terminal amino groups, respectively, were reacted with the water soluble isocyanato polyhedral borane [$Na(CH_3)_3NB_{10}H_8NCO$] (**3**) [63, 64]. The boronated dendrimer then was linked to the mAb IB16-6, which is directed against the murine B16 melanoma, by means of two heterobifunctional linkers, m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS) and N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) [63, 65]. However, following intravenous (i.v.) administration, large amounts of the bioconjugate accumulated in the liver and spleen and it was concluded that random conjugation of boronated dendrimers to a mAb could alter its binding affinity and biodistribution. To minimize the loss of mAb reactivity, a 5th generation PAMAM dendrimers was boronated with the same polyhedral borane anion, and more recently it was site-specifically linked to the anti-EGFR mAb

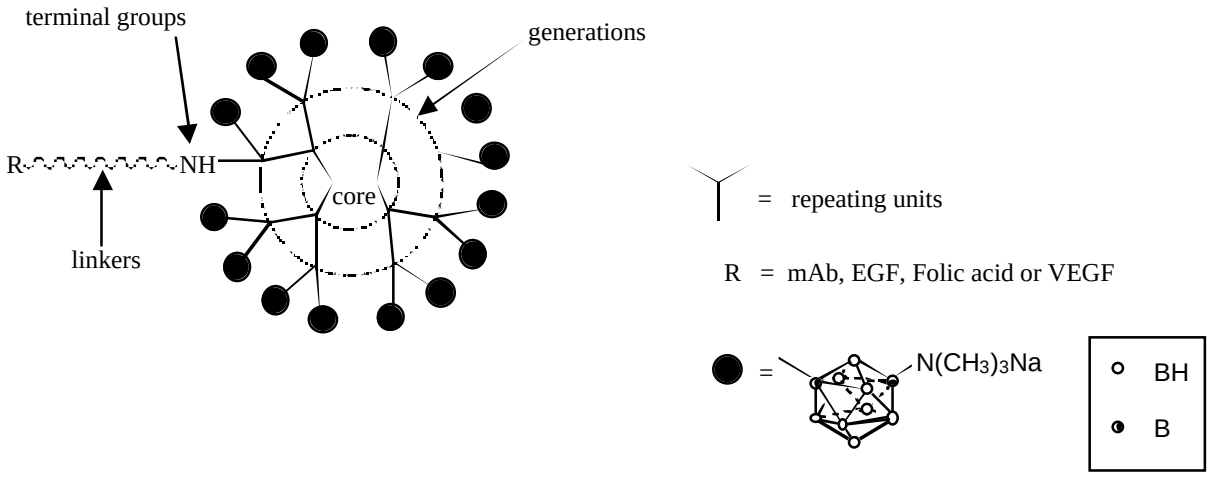


Fig. (2). Structure of a boronated PAMAM dendrimer that has been linked to targeting moieties. PAMAM dendrimers consist of a core, repeating units and reactive terminal groups. Each successfully higher generation of PAMAM dendrimer has an incremental number of terminal groups. Boronated dendrimers have been prepared by reacting them with water soluble isocyanato polyhedral boranes and subsequently attaching them to targeting moieties by means of heterobifunctional linkers.

cutuximab (or IMC-C225) or the EGFRvIII specific mAb L8A4 (Fig. **2**). Cetuximab was linked *via* glycosidic moieties in the Fc region by means of two heterobifunctional reagents SPDP and N-(k-maleimidoundecanoic acid) hydrazide (KMUH) [66, 67]. The resulting bioconjugate, designated C225-G5-B_{1100}, contained ~1100 boron atoms per molecule of cetuximab and retained its aqueous solubility in 10% DMSO and its *in vitro* and *in vivo* immunoreactivity. As determined by a competitive binding assay, there was a <1 log unit decrease in affinity for EGFR (+) glioma cell line F98 $_{EGFR}$, compared to that of unmodified cetuximab [66]. *In vivo* biodistribution studies, carried out 24 h after intratumoral (i.t) administration of the bioconjugate demonstrated that 92.3 μg/g of boron was retained in rats bearing F98 $_{EGFR}$ gliomas compared to 36.5 μg/g in EGFR (-) F98 parental tumors and 6.7 μg/g in normal brain [67].

5.3. Boronated Dendrimers Delivered by Receptor Ligands

5.3.1. Epidermal Growth Factors (EGF)

Due to its increased expression in a variety of tumors including high grade gliomas and its low or undetectable expression in normal brain, EGFR is an attractive target for cancer therapy [68-70]. As described above, targeting of EGFR has been carried out using either mAbs or alternatively, as described in this section, EGF, which is a single-chain, 53-mer heat and acid stable polypeptide. It binds to a transmembrane glycoprotein with tyrosine kinase activity, which triggers dimerization and internalization [71, 72]. Since the EGF boron bioconjugates have a much smaller MW than mAb conjugates, they should be capable of more rapid and effective tumor targeting than has been observed with mAbs [67, 73].

The procedure used to conjugate EGF to a boronated dendrimer was slightly different from that used to boronate mAbs. A fourth-generation PAMAM dendrimer was reacted with the isocyanato polyhedral borane $Na(CH_3)_3NB_{10}H_8$ NCO. Next, reactive thiol groups were introduced into the boronated dendrimer using SPDP, and EGF was derivatized with m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sMBS). The reaction of thiol groups of the derivatized, boronated dendrimer with maleimide groups produced stable BSD-EGF bioconjugates, which contained ~ 960 atoms of boron per molecule of EGF [74]. The BSD-EGF initially was bound to the cell surface membrane and then was endocytosed, which resulted in accumulation of boron in lysosomes [74]. Subsequently, *in vitro* and *in vivo* studies were carried out to evaluate the potential efficacy of the bioconjugate as a boron delivery agent for BNCT [73]. As will be described in more detail later on in this review, therapy studies demonstrated that $F98_{EGFR}$ glioma bearing rats that received either boronated EGF or mAb by either direct i.t. injection or convection enhanced delivery into the brain had a longer mean survival time than animals bearing F98 parental tumors [75-77].

5.3.2. Folate Receptor Targeting Agents

Folate receptor (FR) is overexpressed on a variety of human cancers, including those originating in ovary, lung, breast, endometrium and kidney [78-80]. Folic acid (FA) is a vitamin that is transported into cells *via* FR mediated endocytosis. It has been well documented that the attachment of FA *via* its γ-carboxylic function to other molecules does not alter endocytosis by FR-expressing cells [81]. FR targeting has been used successfully to deliver protein toxins, chemotherapeutic, radio-imaging, therapeutic and MRI contrast agents [82], liposomes[83], gene transfer vectors [84], antisense oligonucleotides [85], ribozymes and immunotherapeutic agents to FR-positive cancers [86]. In order to deliver boron compounds, FA was conjugated to heavily boronated 3rd generation PAMAM dendrimers containing polyethylene glycol (PEG) [87]. PEG was introduced into the bioconjugate to reduce its uptake by the reticuloendothelial system (RES), and more specifically, the liver and spleen. It was observed that folate linked to 3rd generation PAMAM dendrimers containing 12-15 decaborate clusters and 1-1.5 PEG_{2000} units had the lowest hepatic uptake in C57Bl/6 mice (7.2-7.7% injected dose [I.D.] /g liver). *In vitro* studies using FR (+) KB cells demonstrated receptor-dependent uptake of the bioconjugate. Biodistribution studies with this conjugate, carried out in C57Bl/6 mice bearing subcutaneous(s.c.) implants of the FR (+) murine sarcoma 24JK-FBP, demonstrated selective tumor uptake (6.0% ID/g tumor), but there was high hepatic (38.8% ID/g) and renal (62.8% ID/g) uptake [87].

5.3.3. Vascular Endothelial Growth Factor (VEGF)

There is preclinical and clinical evidence indicating that angiogenesis plays a major role in the growth and dissemination of malignant tumors [88, 89]. Inhibition of angiogenesis has yielded promising results in a number of experimental animal tumor models [90, 91]. The most important molecular targets have been vascular endothelial growth factor (VEGF) and its receptor (VEGFR) [92, 93]. We recently have constructed a human $VEGF_{121}$ isoform fused to a novel 15-aa cysteine-containing tag designed for site-specific conjugation of therapeutic and diagnostic agents [94]. A boronated 5th generation PAMAM dendrimer (BD), tagged with a near-infrared (IR) fluorescent dye Cy5, was conjugated using the heterobifunctional reagent sulfo-LC-SPDP to BD/Cy5 *via* reactive SH-groups generated in the VEGF fusion protein by mild dithiothreitol (DTT) treatment. The bioconjugate, designated VEGF-BD/Cy5, contained 1050-1100 boron atoms per dimeric VEGF molecule. VEGF-BD/Cy5 retained the *in vitro* functional activity of VEGF $_{121}$, which was similar to that of native VEGF. Tagging the bioconjugate with Cy5 dye permitted near-IR fluorescence imaging of its *in vitro* and *in vivo* uptake. *In vitro* uptake was determined by incubating VEGF-BD/Cy5 with VEGFR-2 overexpressing PAE/KDR cells and *in vivo* uptake was evaluated in mice bearing s.c. tumor implants. *In vitro*, the bioconjugate localized in the perinuclear region and *in vivo* it primarily localized in regions of active angiogenesis. Furthermore, depletion of VEGFR-2 overexpressing cells from the tumor vasculature with VEGF-toxin fusion protein significantly decreased bioconjugate uptake, indicating that these cells were the primary targets of VEGF-BD/Cy5. These studies demonstrated that VEGF-BD/Cy5 potentially could be used as a diagnostic agent [94]. Further studies are planned to evaluate its therapeutic efficacy using the F98 rat glioma model, which we have used extensively to evaluate both low and HMW boron delivery agents [95].

5.4. Other Boronated Dendrimers

An alternative method to deliver boron compounds by means of dendrimers is to incorporate carborane cages within the dendrimer (Fig. **3**). Matthew *et al*. have reported that 4, 8 or 16 carboranes could be inserted into an aliphatic polyester dendrimer by means of a highly effective synthon, a bifunctional carborane derivative with an acid group and a benzyl ether protected alcohol [96]. The procedure employed a divergent synthesis with high yield. The resulting polyhydroxylated dendrimer was water soluble with a minimum ratio of eight hydroxyl groups per carborane cage. Carborane containing dendrimers potentially could be used as boron delivery agents for BNCT, since it is possible to control the number of carborane moieties and overall solubility.

6. LIPOSOMES AS BORON DELIVERY AGENTS

6.1. Overview of Liposomes

Liposomes are biodegradable, non-toxic vesicles that have been used to deliver both hydrophilic and hydrophobic agents (Fig. **4**) [97]. Both classical and PEGylated ("stealth") liposomes can increase the amounts of anticancer drugs that can be delivered to solid tumors by passive targeting. Rapidly growing solid tumors have increased permeability to nanoparticles due to increased capillary pore size. These can range from 100 to 800 nm compared to 60-80 nm for those in normal tissues, which are impermeable to liposomes. In addition, tumors lack efficient lymphatic drainage, and consequently, clearance of extravasated liposomes is slow [98]. Modification of the liposomal surface by PEGylation or attachment of antibodies or receptor ligands, will improve their selective targeting and increase their circulation time [98].

6.2. Liposomal Encapsulation of Sodium Borocaptate and Boronophenylalanine

Liposomes have been extensively evaluated as nanovehicles for the delivery of boron compounds for NCT [99, 100]. *In vitro* and *in vivo* studies have demonstrated that they can effectively and selectively deliver large quantities of boron to tumors and that the compounds delivered by liposomes have longer tumor retention time. BPA is an amino acid analogue that is preferentially taken up by cells with increased metabolic activity, such as tumor cells of varying

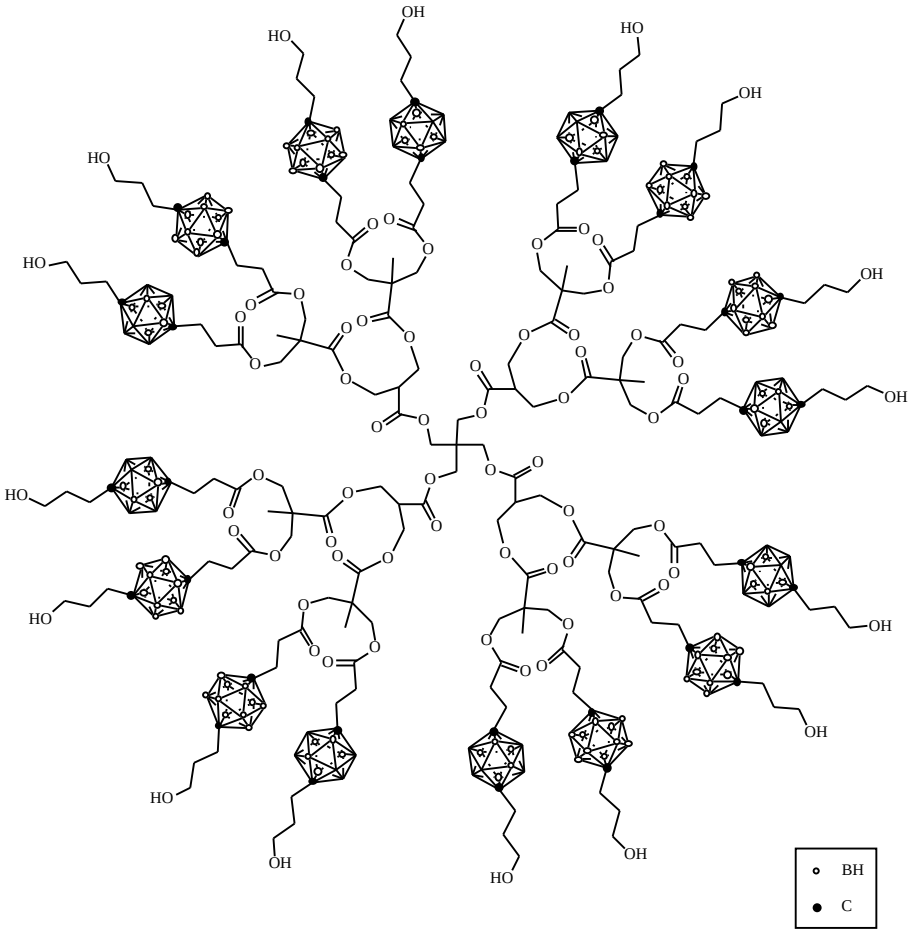


Fig. (3). Structure of a carborane-containing aliphatic polyester dendrimer. Carborane cages were incorporated into the interior of the dendrimer structure and the peripheral hydrophilic groups improved water solubility and were available for modification.

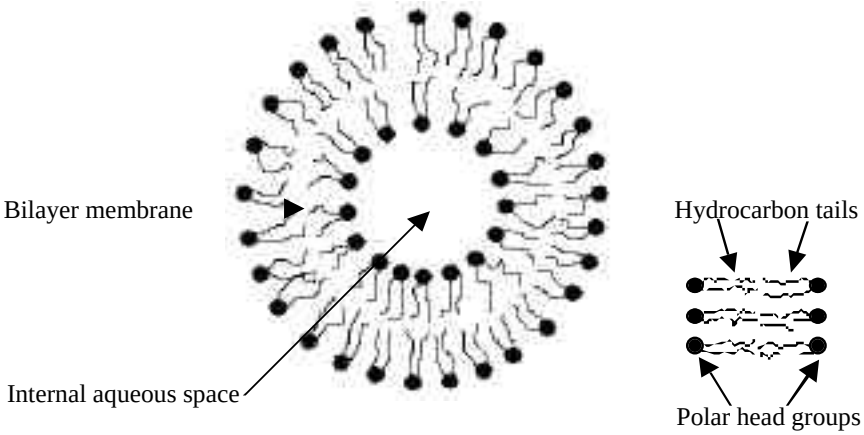


Fig. (4). Schematic diagram of the structure of a liposome, which has an aqueous core and a lipid bilayer membrane. The latter is composed of polar head groups and hydrocarbon tails.

histopathologic types including melanomas[31, 101], gliomas [102] and squamous cell carcinomas [103, 104]. Because of its low aqueous solubility, BPA has been used as a fructose complex, which has permitted it to be administered i.v. rather than orally [105, 106]. Following i.v. administration of BPA, which had been incorporated into conventional liposomes, there was rapid elimination by the reticuloendothelial system (RES) with very low blood and liver boron concentrations at 3 hr. In contrast, if BPA was incorporated into liposomes composed of DSPE-PEG, therapeutically useful tumor boron concentrations (> 20 μg/g) were seen at 3 and at 6 hr indicating that PEG-liposomes had evaded the RES [107]. In addition, BPA has been incorporated into the lipid bilayer of liposomes, composed of either the positively charged lipid 1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP) or the zwitterionic lipid, 1, 2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE) [108]. Cationic liposomes have been widely used as carriers of biomolecules that specifically target the cell nucleus [109], which would be advantageous for BNCT. Another clinically used drug, sodium borocaptate (BSH), has been incorporated into liposomes composed of DPPC/Chol in a 1:1 molar ratio with or without PEG stabilization [110]. The average diameter of liposomes containing BSH was in the range of 100-110 nm. Both types of liposomes resulted in a significant improvement in their circulation time compared to that of free BSH. At 24h following i.v. injection of PEG-liposomes, 19% of the injected dose of boron was in the blood compared to 7% following formulation of BSH in conventional liposomes. The mean percent uptake by the liver and spleen was not significantly different for the two types of liposomes. However, the blood:RES ratios were higher for PEG-liposomes at all time points indicating that a higher fraction of the injected dose of BSH was still in the blood. Ji *et al*. have reported that there were no significant differences in the *in vitro* uptake by 9L gliosarcoma cells of free BSH *versus* a liposomal formulation after 16 h incubation. However, cellular persistence was increased at 12 h and 24 h for BSH-loaded liposomes [111]. BSH also has been incorporated into transferrin (TF) conjugated PEG liposomes (TF-PEG liposomes) [112], which then were taken up by cells *via* transferrin receptor-mediated endocytosis. Intravenous administration of this formulation increased boron retention at the tumor site compared with PEG liposomes, bare liposomes or free BSH and suppressed tumor growth following BNCT. These results suggested that TF targeted liposomes might be useful as an intracellular targeting vehicle.

6.2.2. Liposomal Encapsulation of Other Carboranes

Polyhedral boranes[34] and carboranes [113, 114] are another class of boron compounds that have been used for NCT. They contain multiple boron atoms per molecule, are resistant to metabolic degradation, and are lipophilic, thereby permitting easier penetration of the tumor cell membrane [113]. In addition to BSH, carboranylpropylamine (CPA, Fig. **5**, **4**) [115] has been incorporated into conventional and PEGylated liposomes by active loading, using a transmembrane pH gradient [115, 116]. Although as many as 13, 000 molecules of CPA were loaded into liposomes having a mean average diameter of 100 nm, there was *in vitro* toxicity to both the glioblastoma cell line SK-MG-1 and normal human peripheral blood lymphocytes. Borane anions, such as $B_{10}H_{10}^{2-}$, $B_{12}H_{11}SH^{2-}$, $B_{20}H_{17}OH^{4-}$ and $B_{20}H_{19}^{3-}$, and the normal form and photoisomer of $B_{20}H_{18}^{2-}$, also have been encapsulated into small unilamellar vesicles with mean diameters of 70 nm or less [117-120]. These were composed of the pure synthetic phospholipids, distearoyl phosphatidylcholine and cholesterol [117]. Although encapsulation efficiencies were only 2-3%, following i.v. injection these liposomes were selectively delivered to the tumors in mice bearing the EMT6 mammary carcinoma and had attained boron concentrations of >15 μg boron/g, with a T:Bl ratio of > 3. Two isomers of $B_{20}H_{18}^{2-}$ attained boron concentrations of 13.6 and 13.9μg/g and T:Bl ratios of 3.3 and 12, respectively, at 48 h following administration. High boron retention and prolongation of their circulation time was observed due to the interaction with intracellular components after it had been released from liposomes within tumor cells [117].

In order to examine the effect of charge and substitution on the retention of boranes, two isomers $[B_{20}H_{17}NH_3]^{3-}$ and $[1\text{-}(2'\text{-}B_{10}H_9)\text{-}2\text{-}NH_3B_{10}H_8]^{3-}$ (**5**) were prepared from the polyhedral borane anion $[n\text{-}B_{20}H_{18}]^{2-}$ (**6**) [118, 119]. The

sodium salts of these two isomers had been encapsulated within small unilamellar liposomes, composed of distearoyl phosphatidylcholine/ cholesterol at a 1:1 ratio. Both isomers of $[B_{20}H_{17}NH_3]^{3-}$ had excellent tumor uptake and selectivity in EMT 6 tumor bearing mice, even at very low injected doses, and this resulted in peak tumor boron concentrations of 30-40 μg B/g and a T:Bl ratio of ~ 5. Due to low boron retention of liposomal $Na_3[B_{20}H_{19}]$ and $Na_4[e^2\text{-}B_{20}H_{17}OH]$ and rapid clearance of liposomal $[2\text{-}NH_3B_{10}H_9]^-$, the enhanced retention of liposomal $Na_3[ae\text{-}B_{20}H_{17}NH_3]$ was not due to the anionic charge or substitution in the borane cage. Rather, it could be attributed to their facile intracellular oxidation to an extremely reactive NH^{3-} substituted $[n\text{-}B_{20}H_{18}]^{2-}$ electrophilic anion, $[B_{20}H_{17}NH_3]^-$. Another anion $[ae\text{-}B_{20}H_{17}NH_3]^{3-}$ also was encapsulated into liposomes prepared with 5% PEG-2000-distearoyl phosphatidylethanolamine as a constituent of the membrane. These liposomes had longer *in vivo* circulation times, which resulted in continued accumulation of boron in the tumor over the entire 48 h time period, and reached a maximum concentration of 47 μg B/g tumor.

$[B_{20}H_{17}SH]^{4-}$(**7**), a thiol derivative of $[B_{20}H_{18}]^{4-}$, possesses a reactive thiol substituent and this can be oxidized into the more reactive $[B_{20}H_{17}SH]^{2-}$ anion. Both of these were considered to be essential for high tumor boron retention [120] and had been encapsulated into small, unilamellar liposomes. Biodistribution was determined after i.v. injection into BALB/c mice bearing EMT6 tumors. At low injected doses, tumor boron concentrations increased throughout the duration of the experiment, resulting in a maximum concentration of 47 μg B/g tumor at 48 h, which corresponded to 22.2% ID/g and a T:Bl ratio of 7.7. This was the most promising of the polyhedral borane anions that had been investigated for liposomal delivery. Although they were able to deliver adequate amounts of boron to tumor cells, their application to BNCT has been limited due to their low incorporation efficiency (~3%).

Lipophilic boron compounds incorporated into the lipid bilayer would be an alternative approach. Small unilamellar vesicles composed of 3:3:1 ratio of distearoylphosphatidylcholine, cholesterol and K[*nido*-7-$CH_3(CH_2)_{15}$-7, 8-$C_2B_9H_{11}$] (**8**) in the lipid bilayer and $Na_3[a2\text{-}B_{20}H_{17}NH_2CH_2CH_2NH_2]$ (**9**) in the aqueous core were produced as a delivery agents for NCT mediated synovectomy [121]. Biodistribution studies were carried out in Louvain rats that had a collagen-induced arthritis. The maximum synovial boron concentration was 29 μg/g tissue at 30 h and this had only decreased to 22 μg/g at 96 h following i.v. administration. The prolonged retention by synovium provided sufficient time for extensive clearance of boron from other tissues so that at 96 h the synovium:blood (Syn:Bl) ratio was 3.0. In order to accelerate blood clearance, serum stability of the liposomes was lowered by increasing the proportion of K[*nido*-7-$CH_3(CH_2)_{15}$-7, 8-$C_2B_9H_{11}$] embedded in the lipid bilayer. Liposomes were formulated with a 3:3:2 ratio DSPC:Ch:K[*nido*-7-$CH_3(CH_2)_{15}$-7, 8-$C_2B_9H_{11}$] in the lipid bilayer and $Na_3[a2\text{-}B_{20}H_{17}NH_2CH_2CH_2NH_2]$ was encapsulated in the aqueous core. The boron concentration in the synovium reached a maximum of 26 μg/g at 48 h with a Syn:Bl ratio of 2, following which it slowly decreased to 14 μg/g at 96 h at which time the Syn:Bl ratio was 7.5 [121]. Another method to deliver hydrophilic boron containing compounds would be to incorporate them into cholesterol in order to target tumor cells expressing amplified low density lipoprotein (LDL) receptors[122-124]. Glioma cells, which absorb more cholesterol, have been reported to take up more LDL than the corresponding normal tissue cells [125-127]. The cellular uptake of liposomal cholesteryl 1, 12-dicarba-closo-dodecaboranel-carboxylate (**10**) by two fast growing human glioma cell lines, SF-763 and SF-767 was mediated *via* the LDL receptor and was much higher than that of human neurons. The cellular boron concentration was ~10-11 times greater than that required for BNCT [128].

6.3. Boron Delivery by Targeted Liposomes

In order to improve the specificity of liposomally encapsulated drugs and to increase the amount of boron delivered, targeting moieties have been attached to the surface of liposomes. These could be any molecules that selectively recognized and bound to target antigens or receptors that were overexpressed on neoplastic cells or tumor associated neovasculature. These have included either intact mAb molecules or fragments, low molecular weight, naturally occurring or synthetic ligands such as peptides or receptor binding ligands such as EGF. To date, liposomes linked to mAbs or their fragments [129], EGF[130], folate [131] and transferin [112] have been the most extensively studied as targeting moieties.

6.3.1. Immunoliposomes

The murine anti-carcinoembryonic antigen (CEA) mAb 2C-8 has been conjugated to large multilamellar liposomes containing ^{10}B compounds[132, 133]. The maximum number of ^{10}B atoms attached per molecule of mAb was ~ 1.2×10^4. These immunoliposomes bound selectively to the human pancreatic carcinoma cell line, AsPC-1 that overexpressed CEA. Incubating the immunoliposomes with either MRKnu/nu-1 or AsPC-1 tumor cells, suppressed *in vitro* tumor cell growth following thermal neutron irradiation [134]. This was dependent upon the liposomal concentration of the ^{10}B-compound and on the number of molecules of mAb conjugated to the liposomes. Immunoliposomes containing either $(Et_4N)_2B_{10}H_{10}$ and linked to the mAb MGb 2, directed against human gastric cancer[135, 136] or water soluble boronated acridine (WSA, **11**) linked to Trastuzumab, directed against HER-2, have been prepared and evaluated *in vitro*[129]. There was specific binding and high uptake of these immunoliposomes, which delivered a sufficient amount of ^{10}B to produce a tumoricidal effect following thermal neutron irradiation.

6.3.2. Folate Receptor-Targeted Liposomes

A highly ionized boron compound, $Na_3B_{20}H_{17}NH_3$, was incorporated into liposomes by passive loading[131, 137, 138]. This showed high *in vitro* uptake by the FR expressing human cell line KB (American Type Culture Collection CCL 17), which originally was thought to be derived from a squamous cell carcinoma of the mouth, and subsequently was shown to be identical to HeLa cells, as determined by isoenyzyme markers, DNA fingerprinting and karyotypic analysis. KB tumor bearing mice that received either FR-targeted or non-targeted control liposomes had equivalent

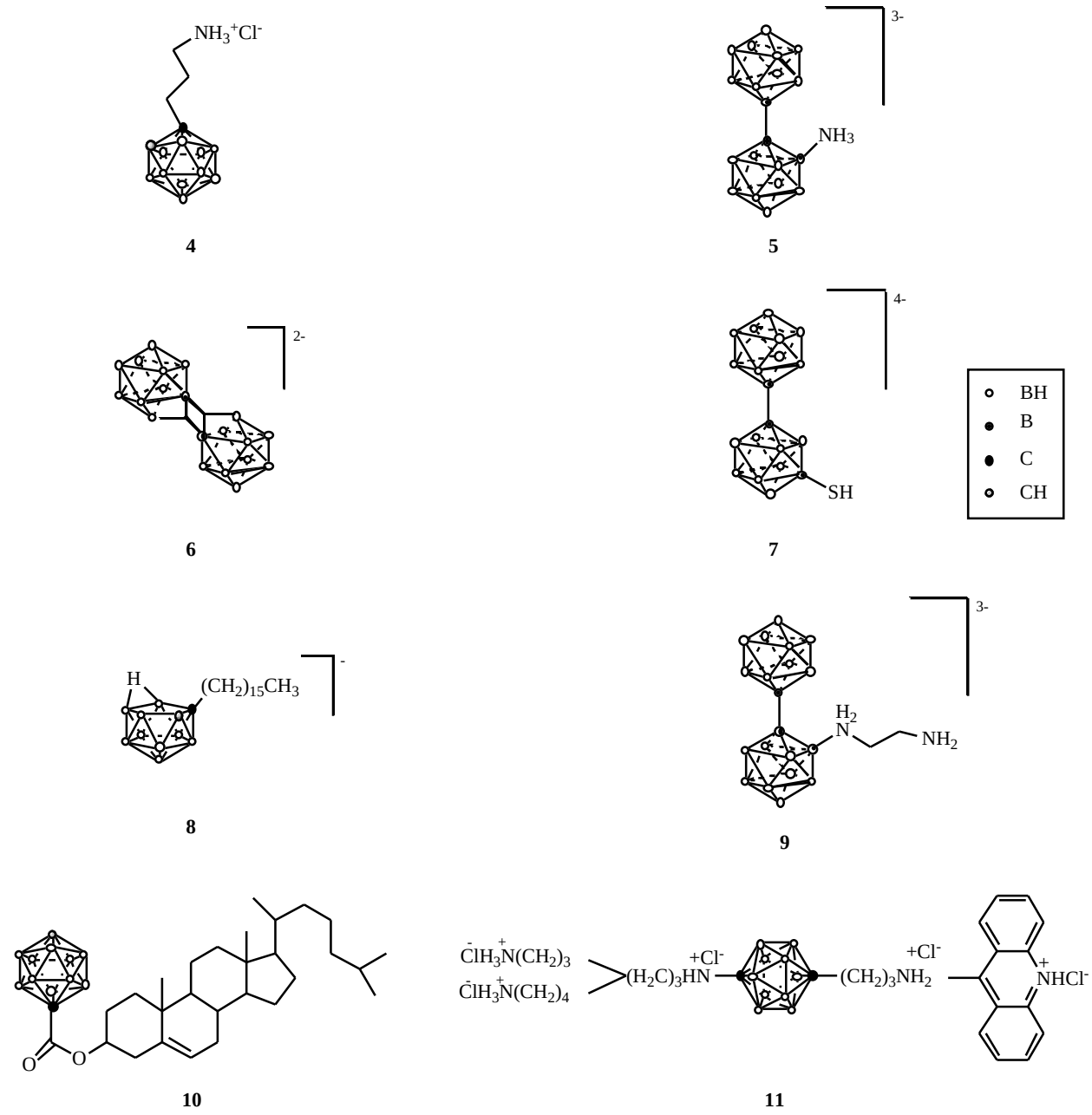


Fig. (5). Structures of hydrophilic and lipophilic boron containing compounds that have been incorporated into liposomes. Carboranylpropylamine (CPA, **4**), [1-(2'-$B_{10}H_9$)-2-$NH_3B_{10}H_8]^{3-}$ (**5**), [n-$B_{20}H_{18}]^{2-}$ (**6**), $[B_{20}H_{17}SH]^{4-}$(**7**), Na_3[*a*2-$B_{20}H_{17}NH_2CH_2CH_2NH_2$] (**9**) and boronated water soluble acridine (WSA, **11**) were encapsulated into the aqueous core. K[*nido*-7-$CH_3(CH_2)_{15}$-7, 8-$C_2B_9H_{11}$] (**8**) and cholesteryl 1, 12-dicarba-closo-dodecaboranel-carboxylate (**10**) were incorporated into the lipid bilayer of liposomes.

tumor boron values (~ 85 μg/g), which attained a maximum at 24 h, while the T:Bl ratio reached a maximum at 72 h. Additional studies were carried out with the lipophilic boron compound, K[*nido*-7-$CH_3(CH_2)_{15}$-7, 8-$C_2B_9H_{11}$. This was incorporated into large unilamellar vesicles, ~ 200 nm in diameter, which were composed of egg PC/chol/K[*nido*-7-$CH_3(CH_2)_{15}$-7, 8-$C_2B_9H_{11}$] at a 2:2:1, mol/mol ratio, and an additional 0.5 mol % of folate-PEG-DSPE or PEG-DSPE for the FR-targeted or nontargeted liposomal formulations [139]. The boron uptake by FR-overexpressing KB cells, treated with these targeted liposomes, was ~ 10 times greater compared with those treated with control liposomes. In addition, BSH and five weakly basic boronated polyamines were evaluated (Fig. **6**). Two of these were the spermidine derivatives N^5-(4-carboranylbutyl)spermidine 3HCl (**12**) and N^5-[4-(2-aminoethyl-o-carboranyl)butyl] spermidine 4HCl (**13**). Three were the spermine derivatives N^5-(4-o-carboranylbutyl) spermine 4HCl (**14**), N^5-[4-(2-aminoethyl-o-carboranyl)butyl]

spermine 5HCl (**15**), and N^5, N^{10}-bis(4-o-carboranylbutyl) spermine 4 HCl (**16**). These were incorporated into liposomes by a pH-gradient-driven remote-loading method with varying loading efficiencies, which were influenced by the specific trapping agent and the structure of the boron compound. Greater loading efficiencies were obtained with lower molecular weight boron derivatives, using ammonium sulfate as the trapping agent, compared to those obtained with sodium citrate.

6.3.3. EGFR Targeted Liposomes

Acridine is a water soluble (WS), DNA-intercalator. Its boronated derivative WSA was incorporated into liposomes composed of EGF-conjugated lipids. Their surface contained ~ 5 mol % PEG and 10-15 molecules of EGF, and 10^4 to 10^5 of WSA molecules were encapsulated. These liposomes had EGFR specific cellular binding to cultured human glioma cells [130, 140] and were internalized following specific binding to the receptor. Following internalization, WSA primarily was localized in the cytoplasm, and had high cellular retention with 80% of the boron remaining cell-associated after 48 h [141].

7. BORON DELIVERY BY DEXTRANS

Dextrans are glucose polymers that consist mainly of a linear α-1, 6-glucosidic linkage with some degree of branching *via* a 1, 3-linkage[142, 143]. Dextrans have been used extensively as drug and protein carriers to increase drug circulation time[144, 145]. In addition, native or chemically-modified dextrans have been used for passive targeting to tumors, the RES or active receptor-specific cellular targeting. To link boron compounds to dextrans [146], β-decachloro-o-carborane derivatives, in which one of the carbon atoms was substituted by $-CH_2CHOHCH_2-O-CH_2CH=CH_2$, were ep-

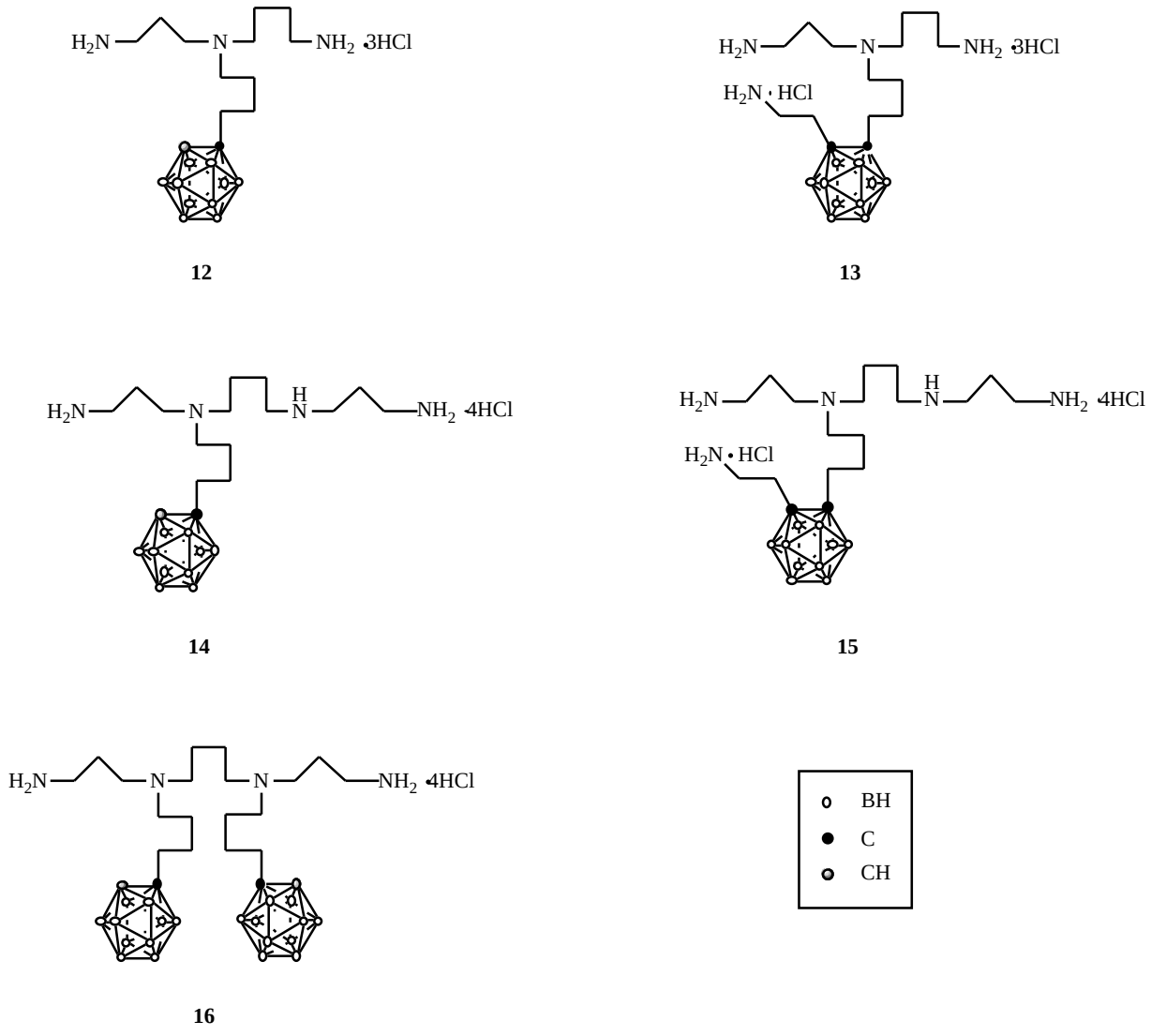


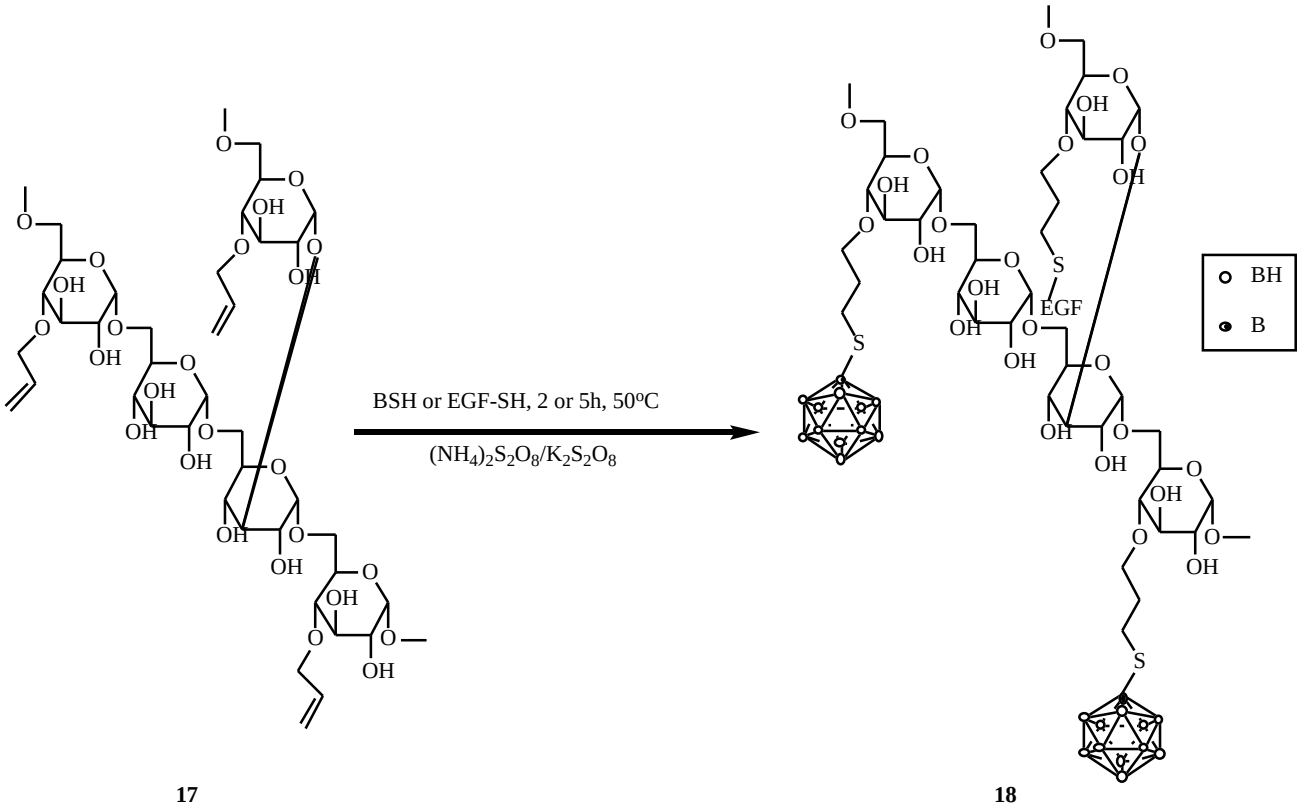
Fig. (6). Structures of five weakly basic boronated polyamines encapsulated in FR targeting liposomes. Two spermidine (**12**, **13**) and three spermine derivatives (**14**-**16**), which contain hydrophilic amine groups and lipophilic carboranyl cages had DNA-binding properties.

oxidized and then subsequently bound to dextran with a resulting boron content of 4.3% (w/w) [147]. The modified dextran then could be attached to tumor specific antibodies [147-150]. BSH was covalently coupled to dextran derivatives by two methods [151]. In the first method, dextran was activated with 1-cyano-4-(dimethylamino)pyridine (CDAP) and subsequently coupled with 2-aminoethyl pyridyl disulfide. Then, thiolated dextran was linked to BSH in a disulfide exchange reaction. A total of 10-20 boron cages were attached to each dextran chain. In the second method, dextran was derivatized to a multiallyl derivative (Fig. **7**, **17**), which was reacted with BSH in a free-radical-initiated addition reaction. Using this method, 100-125 boron cages could be attached per dextran chain, suggesting that this derivative might be a promising template for the development of other HMW delivery agents. In the second method, designed to target EGFR overexpressing cells, EGF and BSH were covalently linked to a 70 kDa dextran (**18**) [152-154]. Bioconjugates, having a small number of BSH molecules, attained maximum *in vitro* binding at 4 h with the human glioma cell line U-343 MGaC12:6. In contrast, there was a slow increase of binding over 24 h for those having a large number of BSH molecules. Although most of the bioconjugates were internalized, *in vitro* retention was low, as was *in vivo* uptake following i.v. injection into nude mice bearing s.c. implants of Chinese hamster ovary (CHO) cells transfected with the human gene encoding EGFR (designated CHO-EGFR). However, following i.t. injection, boron uptake was higher with CHO-EGFR(+) tumors compared to wildtype EGFR(-) CHO tumors [155].

8. OTHER MACROMOLECULES USED FOR DELIVERING BORON COMPOUNDS

Polylysine is another polymer having multiple reactive amino groups, that has been used as a platform for the delivery of boron compounds [53, 156]. The protein-binding polyhedral boron derivatives, isocyanatoundecahydro-*closo*-dodecaborate ($B_{12}H_{11}NCO^{2-}$), was linked to polylysine and subsequently to the anti-B16 melanoma mAb IB16-6 using two heterobifunctional linkers, SPDP and sulfo-MBS. The bioconjugate had an average of 2700 boron atoms per molecule and retained 58% of the immunoreactivity of the native antibody, as determined by a semiquantitative immunofluorescent assay or by ELISA. Other bioconjugates prepared by this method had >1000 boron atoms per molecule of antibody and retained 40-90% of the immunoreactivity of the native antibody [53]. Using another approach site-specific linkage of boronated polylysine to the carbohydrate moieties of anti-TSH antibody resulted in a bioconjugate that had a ~6 × 10^3 boron atoms with retention of its immunoreactivity [156].

A streptavidin/biotin system also has been developed to specifically deliver boron to tumors. Biotin was linked to a mAb and streptavidin was attached to the boron containing moiety. The indirect linking of boron to the mAb minimized



17 **18**

Fig. (7). Preparation of EGF-targeted, boronated dextrans. The bioconjugate was prepared by a free-radical-initiated addition reaction between multiallyl dextran derivatives and BSH or thiolated EGF at 50 °C using $K_2S_2O_8$ as a initiator.

loss of its immunoreactivity. BSH was attached to poly-(D-glutamate D-lysine) (poly-GL) *via* a heterobifunctional agent[157]. This boronated poly-GL then was activated by a carbodiimide reagent and in turn reacted with streptavidin. Another approach employed a streptavidin mutant that had 20 cysteine residues per molecule. BSH was conjugated *via* sulfhydryl-specific bifunctional reagents to incorporate ~ 230 boron atoms/molecule [158]. A closomer species with an icosahedral dodecaborate core and twelve pendant anionic *nido*-7, 8-carborane groups was developed as a new class of unimolecular nanovehicles for evaluation as a delivery agent for BNCT [159].

9. DELIVERY OF BORON CONTAINING MACROMOLECULES TO BRAIN TUMORS

9.1. General Considerations

Drug delivery to brain tumors is dependent upon 1. the plasma concentration profile of the drug, which depends upon the amount and route of administration, 2. the ability of the agent to traverse the blood brain barrier (BBB), 3. blood flow within the tumor, and 4. the lipophilicity of the drug. In general, a high steady-state blood concentration will maximize brain uptake, while rapid clearance will reduce it, except in the case of intra-arterial (i.a.) drug administration. Although the i.v. route currently is being used clinically to administer both BSH and BPA, this may not be ideal for boron containing macromolecules and other strategies must be employed to improve their delivery.

9.2. Drug-transport Vectors

One approach to improve brain tumor uptake of boron compounds has been to conjugate them to a drug-transport vector by means of receptor specific transport systems [160, 161]. Proteins such as insulin, insulin-like growth factor (IGF), transferrin (TF) [162], and leptin can traverse the BBB. BSH encapsulated in TF-PEG-liposomes had a prolonged residence time in the circulation and low RES uptake in tumor-bearing mice, resulting in enhanced extravasation of the liposomes into the tumor and concomitant internalization by receptor-mediated endocytosis [163, 164]. Mice that received BSH containing TF-liposomes had a significant prolongation in survival time compared to those that received PEG-liposomes, bare liposomes and free BSH, thereby establishing *proof-of-principle* for transcytosis of a boron containing nanovehicle [112].

9.3. Direct Intracerebral Delivery

Studies carried out by us have clearly demonstrated that the i.v. route of administration is not suitable for delivery of boronated EGF or mAbs to glioma bearing rats [75, 165]. Intravenous injection of technetium-99m labeled EGF to rats bearing intracerebral implants of the C6 rat glioma, which had been genetically engineered to express the human EGFR gene, resulted in 0.14% ID localizing in the tumor. Intracarotid (i.c.) injection with or without BBB disruption increased the tumor uptake to 0.34 to 0.45% ID/g, but based even on the most optimistic assumptions the amount of boron that could be delivered to the tumor by i.v. injection, this would have been inadequate for BNCT [165]. Direct i.t. injection of boronated EGF (BSD-EGF), on the other hand, resulted in tumor boron concentrations of 22μg/g compared to 0.01 μg/g following i.v. injection and almost identical boron uptake values were obtained using the $F98_{EGFR}$ glioma model [77]. This was produced by transfecting F98 glioma cells with the gene encoding human EGFR. Based on our biodistribution results, therapy studies were initiated with the $F98_{EGFR}$ glioma in syngeneic Fischer rats. $F98_{EGFR}$ glioma bearing rats that received BSD-EGF i.t. had a mean survival time (MST) of 45 ± d compared to 33 ± 2 d in animals that had EGFR (-) wildtype F98 gliomas. Since it is unlikely that any single boron delivery agent will be able to targ*et al*l tumor cells, the combination of i.t. administration of BSD-EGF with i.v. injection of BPA was evaluated. This resulted in further increase in MST to 57 ± 8 d compared to 39 ± 2 d for i.v. BPA alone [73]. These data provide *proof-of-principle* for the idea of using a combination of low and HMW boron delivery agents.

9.4. Convection Enhanced Delivery

CED, by which therapeutic agents are directly infused into the brain, is an innovative method to increase their uptake and distribution [166-168]. Under normal physiological conditions, interstitial fluids move through the brain by both convection and diffusion. Diffusion of a drug in tissue depends upon its molecular weight, ionic charge and its concentration gradient within normal tissue and the tumor. The higher the molecular weight of the drug, the more positively charged the ionic species, the lower its concentration, then the slower its diffusion. For example, diffusion of antibody into a tumor requires 3 days to diffuse 1mm from the point of origin. Unlike diffusion, however, convection or "bulk" flows results from pressure gradient that is independent of the molecular weight of the substance. CED potentially can improve the targeting of both low and HMW molecules, as well as liposomes, to the CNS by applying a pressure gradient to establish bulk flow during interstitial infusion. The volume of distribution (V_d) is a linear function of the volume of the infusate (V_i). CED has been used to efficiently deliver drugs and HMW agents such as mAbs and toxin fusion proteins to brain tumors [168-170]. CED can provide more homogenous dispersion of the agent and at higher concentrations that otherwise would be attainable by i.v. injection [165]. For example, in our own studies, CED of ^{125}I labeled EGF to $F98_{EGFR}$ glioma bearing rats resulted in 47% ID/g of the bioconjugate localizing in the tumor compared to 10% ID/g in normal brain at 24 h following administration. The corresponding boron values were 22 and 2.9 - 4.9μg/g, respectively [76]. Based on these results, therapy studies were initiated. $F98_{EGFR}$ glioma bearing rats that received BD-EGF by CED had a MST of 53 ± 13 d compared to 40 ± 5 d for animals that received BPA i.v [73]. Similar studies have been carried out using either boronated cetuximab (IMC-C225) or the mAb L8A4 [171, 172], which is specifically directed against the tumor specific mutant isoform, EGFR vIII, and comparable results were obtained [173]. Direct intracerebral administration of these and other HMW agents by CED has opened up the possibility that they actually could be used clinically, since CED is being used to administer radiolabeled antibodies, toxin fusion proteins and gene vectors to patients with GBM. It is only a matter of time before

this approach also will be used to deliver both low and HMW boron containing agents for NCT.

10. CLINICAL CONSIDERATIONS AND CONCLUSIONS

In this review we have focused on HMW boron delivery agents and nanovehicles that potentially could be used clinically for targeting intra and extra-cranial tumors. Animal studies, carried out in glioma bearing rats, have demonstrated that boronated EGF and the mAb, cetuximab, both of which bind to EGFR, selectively targeted receptor (+) tumors following direct i.c. delivery. Furthermore, following BNCT, a significant increase in MST was observed, and this was further enhanced if BPA was administered in combination with the HMW agents. These studies provide *proof-of-principle* first for the potential utility of HMW agents, and second, the therapeutic gain associated with combining them with LMW boron delivery agents. A major question is whether any of these agents will ever be used clinically? There are a number of critical issues that must be addressed if BNCT is to ever become a useful modality for the treatment of cancer. *First*, large clinical trials, preferably randomized, must be carried out in order to convincingly demonstrate the efficacy of the two drugs that currently are being used, BPA and BSH. Once this has been established, then studies with HMW EGFR targeting agents could move forward. Both direct i.t. injection [174, 175] and CED [170, 176-179] have been used clinically to deliver mAbs and toxin fusion proteins to patients who have had surgical resection of their brain tumors. These studies provide a strong clinical rationale for the direct intracerabral delivery of HMW agents. Initially, the primary focus should be on determining the safety of administering them to patients prior to surgical resection of their brain tumors. Once this has been established, then biodistribution studies could be carried out in patients who were going to have surgical resection of their brain tumors. Tumor and normal tissues would be analyzed for their boron content, and if there was evidence of preferential tumor localization with boron concentrations in the range 10-20 μg/g and normal brain concentrations of < 5 μg/g, then therapy studies could be undertaken. Since there is considerable variability in EGFR expression in gliomas, it is highly unlikely that any single agent will be able to deliver the requisite amount of boron to all tumor cells, and that they would be used in combination with BPA/BSH. This general plan also would be applicable to the other HMW delivery agents and nanovehicles that have been discussed in this review. The joining together of chemistry and nanotechnology [180, 181] represents a major step forward for the development of effective boron delivery agents for NCT. Nanovehicles offer the possibility of tumor targeting with enhanced boron payloads. Potentially, this could solve the central problem of how to selectively deliver large number of boron atoms to individual cancer cells.

As can be seen from the preceding discussion, the development of HMW boron delivery agents must proceed in step with strategies to optimize their delivery and an appreciation as to how they would be used clinically. Intracerebral delivery has been used in clinically advanced settings, but nuclear reactors, which currently are the only source of neutrons for BNCT, would not be conducive to this. Therefore, the development of accelerator neutron sources, which could be easily sited in hospitals, is especially important. This also would facilitate the initiation of large scale clinical trials at selected centers that treat large numbers of patients with brain tumors and would permit evaluation of new boron delivery agents. In conclusion, as should be apparent from this review, there is a plethora of HMW boron delivery agents that have been designed and synthesized. The challenge is to move from experimental animal studies to clinical biodistribution studies, a step which has yet to be taken.

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