DEPARTMENT OF ENERGY – OFFICE OF SCIENCE – RESEARCH GRANT
OFFICE OF BIOLOGICAL AND ENVIRONMENTAL RESEARCH, MEDICAL SCIENCES DIVISION

FINAL REPORT

PROJECT TITLE: Boron-Containing Compounds for Liposome-Mediated Tumor Localization and Application to Neutron Capture Therapy

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OBJECTIVE

The principal objective of this project was to develop new boron-rich compounds and delivery methods to alleviate a persistent obstacle to boron neutron capture therapy: the need for selective and substantial boron-rich drug targeting to tumor tissues. This requirement is addressed through the development of boron-rich liposomal and related nanoparticles as boron delivery vehicles.

APPROACH

Medical application of boron neutron capture therapy (BNCT) has been significantly hindered by the slow development of boron drug-targeting methodologies for the selective delivery of high boron concentration sto malignant cells. We have successfully sought to fill this need by creating liposomes suitable as *in vivo* boron delivery vehicles for BNCT. Delivery of therapeutic quantities of boron to tumors in murine models has been achieved with small unilamellar boron-rich liposomes. Subsequently, attempts have been made to improve delivery efficiency of liposomes encapsulating boron-containing water-soluble species into their hollow core by incorporating lipophilic boron compounds as addenda to the liposome bilayer, incorporating boron compounds as structural components of the bilayer (which however, poses the risk of sacrificing some stability), and combinations thereof. Regardless of the method, approximately 90% of the total liposome mass remains therapeutically inactive and comprised of the vehicle's construction materials, while less than 5% is boron for neutron targeting.

Following this laboratory's intensive study, the observed tumor specificity of certain liposomes has been attributed to their diminutive size of these liposomes (30-150 nm), which enables these small vesicles to pass through the porous, immature vasculature of rapidly growing tumor tissue. We surmised that any amphiphilic nanoparticle of suitable size could possess some tumor selectivity. Consequently, the discovery of a very boron-rich nanoparticle delivery agent with biodistribution performance similar to unilamellar liposomes became one of our goals. Closomers, a

new class of polyhedral borane derivatives, attracted us as an alternative BNCT drug-delivery system.

We specifically envisioned dodeca (nido-carboranyl)-substituted closomers as possibly having a great potential role in BNCT drug delivery. They could function as extraordinarily boron-rich BNCT drugs since they are amphiphilic unimolecular nanoparticles presenting several advantages: tunable size through functionalization and branching, spherical shape due to the icosahedral B_{12}^{2-} core, promising water solubility resulting from degradation of all pendant closo-carborane groups to their hydrophilic nido anion substituents, and efficient boron delivery owing to the presence of 120 boron atoms which gives rise to a boron content as high as 40% by weight.

Keeping the new objective in mind, we have focused on the design, synthesis and evaluation of new and very boron-rich closomer species. Additionally, progress has also been made toward the evaluation of a newly synthesized boron-rich lipid as a substitute for DSPC in bilayer construction, and the boron content of the resulting liposomes has been greatly enhanced.

Related research involving the synthesis and self-assembly of carborane-containing amphiphiles has been systematically studied. Combined hydrophobic and hydrophilic properties of the single-chain amphiphiles allow their spontaneous self-assembly to form rods under a variety of variable conditions, such as concentration in the bilayer, carborane cage structure, chain-length, counterion identity, solvents, methods of preparation, and the ionic charge. On the other hand, the number of attached chains affects the self-assembly process. Particles having totally different shapes have been observed for dual-chain amphiphiles.

More than 32 publications resulted from this project during the duration of the grant and 4 other publications are in preparation. This Final Report for DOE Award number DE-FG03-95ER61975 summarizes results obtained during the entire funding period. The following published articles and annual project reports provide detailed results of this project.

1. Emil M. Georgiev, Kenneth Shelly, Debra A. Feakes, Jeremy Kuniyoshi, Solomon Romano, and M. Frederick Hawthorne, “Synthesis of Amine Derivatives of the Polyhedral Borane Anion $[B_{20}H_{18}]^{4-}$,” *Inorg. Chem.*, *35*, 5412 (**1996**).

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5. Fangbiao Li, Kenneth Shelly, Robert R. Kane, Carolyn B. Knobler and M. Frederick Hawthorne, "The Novel [*n*-$B_{20}H_{18}$]$^{2-}$ Induced Nucleophilic Ring-Opening of Tetrahydrofuran by Alkoxide Anions," *Angew. Chem.*, *Int. Ed., 35*, 2646 (**1996**).

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10. Kenneth Shelly, Emil M. Georgiev, Rachel Watson-Clark and M. Frederick Hawthorne, "Boron Delivery to Tumors for BNCT: Recent Murine Results with Liposomes," *Advances in Neutron Capture Therapy, Vol. II, Chemistry and Biology*, Edited by B. Larsson, et al., Elsevier: Amsterdam, 357 (**1997**).

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13. Fangbiao Li, Kenneth Shelly, Carolyn B. Knobler and M. Frederick Hawthorne, "Formation of a Novel Amidinium-bridged Polyhedral Borane Ion by Incorporation of Acetonitrile Solvent Molecule," *Angew. Chem. Int. Ed.* , *37 ,* 1865 (**1998**).

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19. Jason Thomas and M. Frederick Hawthorne, "Dodeca(carboranyl)-substituted Closomers: Toward Unimolecular Nanoparticles as Delivery Vehicles for BNCT," *Chem. Comm.***,** 1884 (**2001**).

20. George E. Laramore, K.J. Stelzer, R. Risler, J.L. Schwartz, J.J. Douglas, J.P. Einck, D.W. Nigg, C.A. Wemple, J.K. Hartwell, Y.D. Harker, Patrick R. Gavin and M. Frederick Hawthorne, "Neutron Capture Therapy (NCT) Enhancement of Fast Neutron Radiotherapy: Application to Non-Small Cell Lung Cancer," Proceedings of the Sixteenth International Conference on Application of Accelerators in Research and Industry, J.L. Duggan and I.L. Morgan, Editors. American Institute of Physics (**2001**).

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24. K.J. Stelzer, P. R. Gavin, R. Risler, H. Kippenes, M. F. Hawthorne, D.W. Nigg and G. E. Laramore, "Boron Neutron Capture-Enhanced Fast Neutron Therapy (BNC/FNT) for Non-Small Cell Lung Cancer in Canine Patients," *Frontiers in*

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30. M. Frederick Hawthorne and Mark W. Lee, "A Critical Assessment of Boron Target Compounds for Boron Neutron Capture Therapy", *J. Neuro-Oncol.*, 62, 33-45 (**2003**).

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32. M. Frederick Hawthorne, "New Discoveries at the Interface of Boron and Carbon Chemistries," *Pure Appl. Chem.*, *75*, 1157 (**2003**).

33. Michael J. Bayer and M. Frederick Hawthorne, "An Improved Method for the Synthesis of [*Closo*-$B_{12}(OH)_{12}$]$^{-2}$", *Inorg. Chem.* 43, 2018-2020 (**2004**).

Articles submitted or in preparation for submission

1. Ling Ma, Julie Orf, M. Frederick Hawthorne, "Closomers of High Boron Content: Synthesis, Characterization and Potential Application as Unimolecular Nanoparticle Delivery Vehicles for BNCT", *J. Am. Chem. Soc.* **2005**, to be submitted.
2. Ling Ma, Jiaxing Huang, M. Frederick Hawthorne, " Camouflaged Carborane Ampiphiles: Synthesis and Self-assembly", in preparation.
3. Tiejun Li, Satish S. Jalisatgi, Michel J. Bayer, A. Maderna, Saeed I. Khan, M. Frederick Hawthorne, " Organic Syntheses on an Aromatic Icosahedral Borane Surface: Closomer Structures with Twelvefold Functionality", *J. Am. Chem. Soc.* **2005**, to be submitted.
4. Tiejun Li, Julie Orf, M. Frederick Hawthorne, " Liposomes from Novel Carborane-containing Lipids for Boron Neutron Capture Therapy", *J. Am. Chem. Soc.* **2005**, to be submitted.

RESULTS

The results summarized below are from the latest renewal of this grant from the years 2001-2004.

A. Closomers

Closomers, based on the derivatives of icosahedral and aromatic [*closo*-$B_{12}(OH)_{12}]^{2-}$ ion are of special interest to us. Two types of closomers; ester- and ether-linked, have been designed and synthesized.

The polyhedral ion precursor, [*closo*-$B_{12}H_{12}]^{2-}$, has been routinely hydroxylated by 30% H_2O_2 to form [*closo*-$B_{12}(OH)_{12}]^{2-}$ on a multi-gram scale in our laboratory. Recently, it has been scaled up to a fifteen-gram level by adding H_2O_2 in portions, and monitoring the reaction by ^{11}B NMR. This improved procedure affords yields of >95% and requires two to three weeks reaction time. Larger scale preparations appear to be feasible.

The synthesis of a boron-rich carborane-containing closomer, [*closo*-$B_{12}\{OCO(CH_2)_4Cb\}_{12}]^n$, where Cb is closo-C-methyl-ortho-carboranyl and n = 2- was described in our previous publication[1]. Cesium fluoride was used to deboronate the twelve closo-carborane pendant groups, and afford tetradecacesium salts of the nido-closomer, where Cb represents the nido-C-methyl carborane anion and n = 14-. The essentially spherical shape of these molecular structures resembling small amphiphilic micelles (unimolecular nanoparticles) in size and shape make these materials of interest for BNCT applications and such species have been synthesized for evaluation.

B. Unimolecular Nanoparticles for Boron Delivery

Closo-ether and Closo-ester-linked Closomers

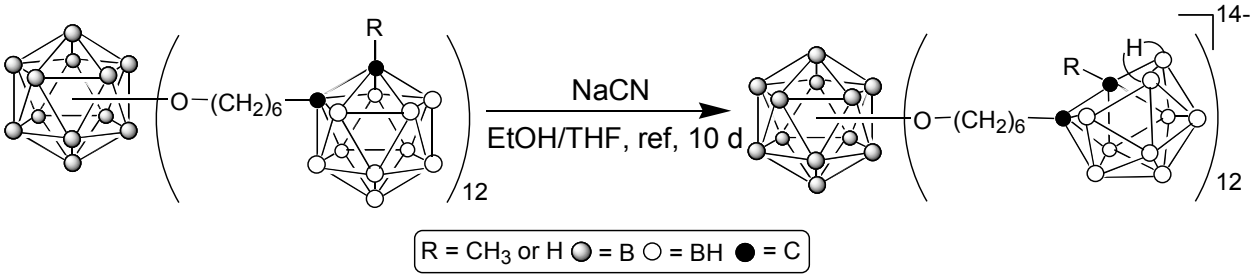
Three new closomers, one ester- and two ether-linked, were synthesized. The closomers are [*closo*-$B_{12}\{OCO(CH_2)_4Cb\}_{12}]^n$, where Cb is closo-C-ortho-carboranyl and n = 2-; [*closo*-$B_{12}\{O(CH_2)_6Cb\}_{12}]$, where Cb is closo-C-methyl-ortho-carboranyl, and closo-

C-ortho-carboranyl, respectively. The uncharged, 24-electron, stable hypercloso-, ether-linked closomers were obtained from the facile 2-eletron oxidation of the 26-electron [*closo*-B_{12}]$^{2-}$ scaffold. All three closomers were purified by column chromatography and characterized by ^{1}H, $^{13}C\{^{1}H\}$, ^{11}B NMR spectroscopy and HRMS.

Nido-ether-linked Closomers

Highly charged nido-closomer tetradeca anions derived from the corresponding closo-ether-linked closomers have been obtained via the deboronation of the closo-closomers by CsF. A mixture of THF and ethanol was employed as the reaction solvent with ether-linked closomers. The white products are the nido-closomers with the B_{12} central core reduced to its (-2) oxidation state. In the air, the latter turns pink or even purple because of partial air-oxidation to the corresponding paramagnetic nido-closomers having their central core in a (-1) oxidation state. The tetradecacesium salts of the nido-closomers which resulted from the CsF deboronation of the closo derivatives are not water-soluble. The extremely poor solubility in water prevented us from obtaining the corresponding sodium salts by ion exchange. Other deboronation reagents, such as KF and piperidine, have been investigated under variety of conditions, but no satisfactory results were obtained.

Our planned biodistribution and toxicity studies were hindererd by a lack of pure, water-soluble nido-closomers. This led us to a new carborane deboronation reagent which would also supply sodium ion. Sodium cyanide was examined and found to deboronate closo-carboranes to their nido derivatives. This reagent was then employed to obtain the sodium salt of nido-ether-linked closomers. The reaction was carried out in refluxing THF and ethanol (1:2). These nido-closomers were characterized by ^{1}H, $^{13}C\{^{1}H\}$, ^{11}B NMR spectroscopy and HRMS.



Scale-up of the synthesis of the nido species and further purification for biodistribution and toxicity purposes have been completed. The biodistribution and toxicity studies will soon be carried out by using tumor-bearing Balb C mice at Washington State University.

C. Multimolecular Nanoparticles and Self-assembly

Camouflaged Carborane Amphiphiles: Synthesis and Self-assembly

We previously reported the synthesis and self-assembly[2] of amphiphilic camouflaged carborane derivatives Cb*$(CH_2)_4NH_3^+$, where Cb* represents the

octamethyl-ortho-carborane cage. This preliminary study demonstrated that such species might have the potential to form self-organized supramolecular aggregates which resemble those available from similar C_{60} derivatives. This predicated further investigation of such amphyphilic species based upon hydrophobic, B-methylated carborane derivatives. A systematic study of the self-assembly process has been carried out to better understand and control this phenomenon having a variety of potential applications.

A series of polymethylated carborane-containing amphiphiles, **1-10**, with the general formula $\mathbf{Cb^*[(CH_2)_nNH_3^+]_m}$, where Cb* represents the B-methylated-carborane cage, **n =** 3, 4, 5, and 6; **m =** 1 or 2**,** were synthesized and characterized by NMR, and HRMS (Table 1). Amphiphiles **1** and **2** are isomers with Cb* = octamethyl-*ortho*- and octamethyl-*meta*-carborane cages, respectively, n = 3, and m = 1. Compounds **3** to **6** are amphiphiles which contain decamethyl-para-carborane cages with chain lengths comprised of from three to six $-CH_2-$ Thus, Cb* is the decamethyl-*para*-carborane cage and m = 1, n = 3, 4, 5, and 6, respectively. These amphiphiles were synthesized to study the relationship of chain-length to self-assembly and to explore the higher symmetry of the *para*-carborane derivative compared to that of the *ortho*- and *meta*-carborane analogues. Compound **7**, where Cb* is the octamethyl-*ortho*-carborane cage with m = 1 and n = 4, was initially studied and demonstrated self-assembly[2]. Compounds **8** and **9**, where Cb* is the octamethyl-*meta*-carborane cage with m = 2, n = 3 and 4 respectively, have two hydrophilic chains which allows the study of structure and hydrophilicity effects on self-assembly. Amphiphile **10**, $Cb^*(CH_2)_3COO^-$, where Cb* represents the octamethyl-*ortho*-carborane cage, is anionic.

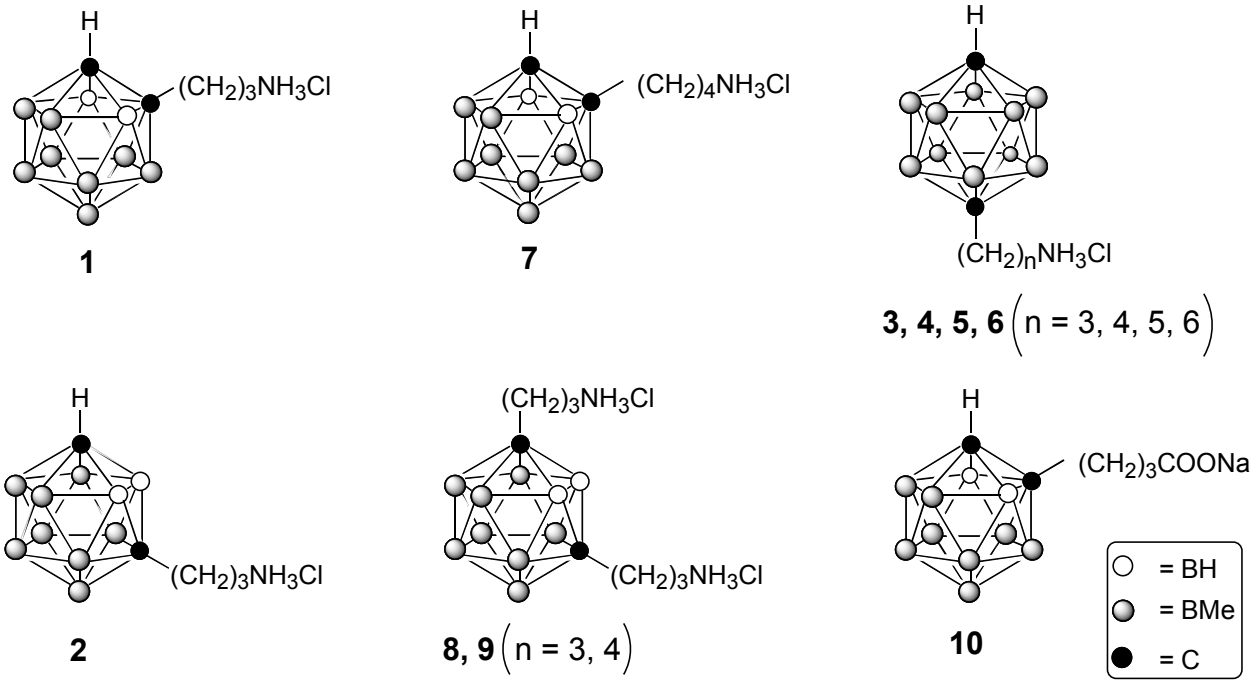


***Table 1**. Camouflaged Carborane Amphiphiles*

Compounds **1** to **6,** and **10** are soluble in methanol and ethanol, but insoluble in water. A few milligrams of these amphiphiles dispersed in water at a concentration of 4 to 5 mM and then ultrasonicated for five minutes provides a translucent suspension. One drop of the suspension was transferred to a TEM grid and air-dried. TEM images revealed that species **1** to **7** all formed one-dimensional rods having different characteristic dimensions. Amphiphiles **2** and **7** formed the longest, most uniform rods whose width and length averaged 300 nm and 70 μm, respectively. Compound **8** was used as a model for a concentration effect study. It was ultrasonicated in water at concentrations as low as 1.0 mM, and as high as 9.0 mM. Hair-like fibers formed at both concentrations, but the fibers formed at 9.0 mM are shorter. Strikingly, all of the self-assembled structures can be observed under an optical microscope, which provides a much simpler way to study these self-assembled structures. XRD, TG/DTA and FTIR have also been used to study these rod structures.

Dual-chain compounds **8** and **9** were synthesized to investigate their aggregation behavior. They both have greater solubility in water than that of **1** to **7**, as expected. Ultrasonicated suspensions were directly examined under an optical microscope, no fibers were found, but small spheres were observed. Feather-like aggregation was observed for compound **9** at a concentration of 8 mM. Detailed studies are still underway with the double-chained amphiphiles.

D. A Boron-rich Lipid for Liposome Bilayer Construction

Liposomes remain as the preferred tumor-selective boron carrier system for BNCT in research efforts have involved the incorporation of very boron-rich liposome construction modules in order to increase the boron content in the injectable vessicles. Typical unilamellar liposome formulations with encapsulated hydrophilic boron compounds contain a relatively small quantity of boron (less than 5% dry weight). Over 90% of the mass in these liposomes (not including the encapsulated water) is comprised of the material which makes up the phospholipid bilayer. A liposome bilayer incorporating, or constructed from, boron-rich lipophilic species (carboralipids) has the potential to greatly increase the boron content of the formulation and the quantity of boron which could be delivered by such liposome.

The sodium salt, $Na[C_2B_9H_{11}CH_2OCH\{CH_2O(CH_2)_{15}CH_3\}_2]$ (referred to as sodium DAC) is structurally comparable with distearoylphosphatidylcholine (DSPC), and it has been used to replace DSPC in conventional bilayer formulations also containing cholesterol. The sodium salt of DAC resulted from the NaOH deboronation of the corresponding closo compound.

Equal quantities of DAC and cholesterol were sonicated in PBS buffer. Stable unilamellar liposomes with an average diameter of 50 nm and 8% boron content by weight were produced. These vesicles possess excellent storage stability and they may be stored for a few weeks at room temperature. Toxicity studies with Balb C mice demonstrated that sodium DAC liposomes are too toxic for use. Consequently, the amount of DAC incorporated into the bilayer has been lowered by the addition of DSPC giving the ratio of DSPC:DAC:cholesterol 1:1:2. An injected dose having as little as 6

mg of B per Kg of tumor was examined, but toxicity remained an issue. Studies of these enhanced boron delivery systems will continue as resources allow.

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