10.4　今後の展望

BNCTのためのホウ素キャリアの開発には，いわゆるナノモルレベルで薬理効果が要求される抗がん剤のようなドラッグデザインではなく，ミリモルレベルで投与できるのに十分な低毒性であり，なおかつ腫瘍細胞に集積することが必要とされる。BNCTにおいて1950年代に開発されたBSH，BPAという2剤以外には，まだ臨床応用されたホウ素薬剤は残念ながら登場してない。熱中性子源が原子炉から加速器に移行できれば都市部病院併設型加速器によるBNCTが可能となり，将来放射線療法の一般的治療法の1つになると考える。本稿で紹介したホウ素ナノキャリアは，大量のホウ素を腫瘍部位へ運ぶことが可能であるだけでなく，リガンドを導入することにより様々な癌種に対応した設計が可能であることから，BNCTの治療効果を高める上でも非常に有効なアプローチであると期待される。

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中性子捕捉がん治療のための次世代ホウ素デリバリーシステム

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1．はじめに

高齢化の進む我が国の死亡原因の第一位はがんであり、その年間死亡者数はおよそ 30 万人である。がん検診の普及、早期診断・早期治療、さらには初期治療としての手術・放射線・化学療法の進歩によって、ある程度治癒率の改善がみられるものの、化学療法では全身的な副作用との戦い、放射線治療では照射野内の正常組織損傷の問題が常に存在する。このような中で、化学療法と放射線療法の両方の原理を上手く利用したホウ素中性子捕捉療法（BNCT: boron neutron capture therapy）が注目されている。

熱中性子は人体には無害であるが、ホウ素 10 と反応することにより、リチウムとヘリウム（α線）を生じる。これらの粒子エネルギーは 2.79MeV とおよそ 1 つの細胞を破壊するのに十分なエネルギーである（式 1)。この核反応を利用しがんを殺傷するのが BNCT である。BNCT は、世界に先駆け日本で初めて臨床応用に成功を収めて以来、世界をリードしてきた分野であるが、原子炉からの熱・熱外中性子線を利用しているため、対応できる治療患者数および地域が限られている。現在、病院内設置可能な BNCT 用加速器の開発が日本をはじめ全世界で進められており、加速器から十分な熱中性子が得られるようになれば、都市型病院への併設が可能となることから BNCT は細胞選択的放射線療法として一般に普及することが期待される。

$$^{10}B + {}^{1}n \rightarrow {}^{7}Li + {}^{4}He + 2.79\ MeV \quad (1)$$

では、なぜホウ素分子なのか？中性子を原子核に照射した際に、中性子を捕捉する大きさ"中性子捕捉断面積"を主な元素について比較した（Table 1)。中性子捕捉断面積はバーン（1 barn = 10^{-24} cm^2）という単位で表される。^{135}Xe, ^{149}Sm, ^{151}Eu, ^{157}Gd などがきわめて大きい値を示している。^{10}B の中性子捕捉断面積は 3837 バーンとそれほど大きな値は示していないのに、中性子捕捉療法に有望であるのは主に次に挙げる 4 つの理由からである。(i) ^{10}B は非放射性で天然のホウ素に約 20%含まれるため入手容易である。(ii) 上で述べたように核反応の際の α 線の飛程が 1 個の細胞内に限られる。(iii) ホウ素の広範な化学反応性と安定性により種々の生物活性分子や生体関連物質への導入が可能である。(iv) 重金属のような高い毒性を示さない。一方、生体中の元素も中性子を捕捉して放射線を生じるが、その中性子捕捉断面積は ^{10}B よりも数桁小さな値なので（Table. 1）通常は無視できる。しかしながら水素と窒素は生体中に高濃度に存在するため、中性子の照射線量に大きく影響す

る。したがってこれらの影響を最小限にするためにも、腫瘍組織内の ^{10}B 濃度が 20～35μg/g、もしくは ^{10}B 原子が 10^9 個/細胞であれば、放射線量のおよそ 85%が ^{10}B の中性子捕捉反応から生じると計算されている [1]。最終的には照射できる中性子線量の上限は、水素と窒素が中性子を捕捉して出す放射線に周囲の正常組織がどれほど耐えられるかに依存する。このためにも ^{10}B ががん細胞に選択的に集積することが必要であり、実際に臨床上の立場から腫瘍組織内 ^{10}B 濃度が 30 μg/g 以上、^{10}B 濃度の腫瘍組織/血液および腫瘍組織/正常組織の比がいずれも 5 以上が望ましいとされている。

Table 1. Capture Cross Section Values of Various Nuclides for Thermal Neutrons

nuclide	cross section capture value[a]	nuclide	cross section capture value[a]
^{6}Li	942	H	0.332
^{10}B	3838	C	0.0037
^{113}Cd	20,000	N	1.75
^{135}Xe	2,720,000	O	<0.0002
^{149}Sm	41,500	P	0.19
^{151}Eu	59,002	S	0.52
^{157}Gd	240,000	Na	0.536
^{174}Hf	400	K	2.07

[a] Cross section capture values in barns.

2．ホウ素デリバリーシステム

近年、ホウ素のがん組織への有効な送達法としてドラッグデリバリーシステムの利用が注目されている [2]。リポソーム DDS を用いたホウ素デリバリーの方法として、大きく２つの戦略に分けられる（Figure 1）。一つは、ホウ素薬剤をリポソーム内に封入する方法である。この方法は、一般的なリポソームを用いた DDS を応用するものであり、BSH などのホウ素化合物を封入する [3-5]。もう一つの方法として、我々はホウ素をリポソーム膜に埋め込む方法を考えた。この方法では、リポソーム内にさらに抗がん剤などの薬剤を封入することができるため、化学療法との複合治療が期待できる。いずれの場合も、リポソーム膜を PEG 化することで EPR (enhanced permeability and retension) 効果を高めたり、さまざまな分子をリポソーム膜に結合させることにより、能動的にターゲティングできるような機能を持たせることが可能となってきた。

リポソーム膜内にホウ素を導入したホウ素リポソームの最初の報告は、Hawthorne らによって開発された一本鎖ホウ素イオンクラスター脂質 1（Figure 2）を用いたものであった [6]。この化合物は炭素鎖 16 の脂溶性部位と水溶性の nido 型カルボラン部位からなる両親媒性分子である。彼らは、DSPC、コレステロール、nido 型カルボラン脂質 1 からリポソームを調製した。EMT6 細胞を移植

したマウスを用いて生体内ホウ素分布を調べたところ、投与ホウ素濃度 6 mg/kg では腫瘍内ホウ素濃度が投与後 6 時間で 22 ppm、その後 16～30 時間はおよそ 34 ppm で一定であった。48 時間後には 25 ppm に低下したものの T/N 比は 8.4 であった。

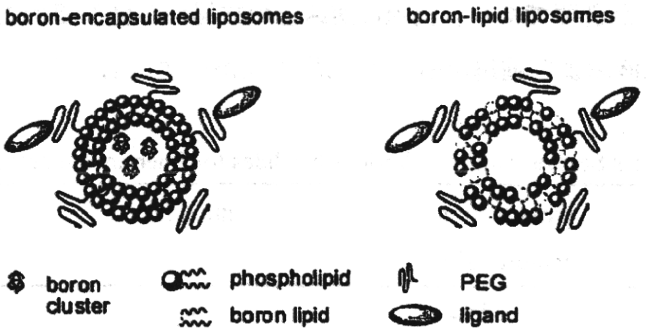

Figure 1. Boron-encapsulated liposomes and boron-lipid liposomes.

一方、我々はリポソーム膜へ効率良くかつ安定に生成するためには、二分子膜を形成しているリン脂質のように脂溶性部位が二本鎖であれば良いと考えた。そこで、二本鎖ホウ素イオンクラスター脂質 2 を設計した [7]。電子顕微鏡で確認したところ、合成したイオン性ホウ素クラスター脂質 2 から 150～200 nm の大きさのベシクルが形成していることが分かった。これは世界で初めてのホウ素脂質ベシクルである。このホウ素イオンクラスター脂質 2 と DSPC、コレステロールを用いて、能動的ターゲティングを指向した TF 結合型ホウ素クラスターリポソームを合成し、坦癌マウスを用いた体内分布ならびに中性子捕捉治療を行った [8]。左足に Colon 26 細胞を移植した BALB/c マウス（生後 6 週間、16～18 g）にトランスフェリン修飾型ホウ素クラスターリポソームをホウ素濃度で 7.2 mg/kg 投与したマウスでは、72 時間後、筋肉・心臓・脳ではホウ素蓄積はほとんど見られなかった。肺・血液ではおよそ 10 ppm、脾臓・肝臓では非常に高いホウ素蓄積が見られた。腫瘍内ホウ素蓄積量を見てみると 7.2 mgB/kg 投与した場合では 22 ppm、14.4 mgB/kg 投与の場合では 40 ppm であった。最近、Hawthorne らも同様な二本鎖ホウ素イオンクラスター脂質 3 を開発している [9]。

このように、二本鎖ホウ素イオンクラスター脂質は安定なホウ素リポソームを形成し、腫瘍へも効率よく集積することが分かった。しかしながら、ホウ素濃度で 14.4 mg/kg 投与した場合に急性毒性が一部のマウスに見られたことから、我々はより低毒性なホウ素脂質の開発を目指し次世代ホウ素イオンクラスター脂質 4 および 5 を設計した [10]。この脂質は、脂溶性部位に生体リン脂質 (Phosphatidylcholines) と同じ立体構造を有しており、リンカー部位にエステル基(脂質 4)またはカルバメート基(脂質 5)を有し、BSH と S を介して結合している。これらのホウ素脂質から調整したリポソームは、正常マウスに対しホウ素濃度で 20 mg/kg では急性毒性は見られなかった [11]。

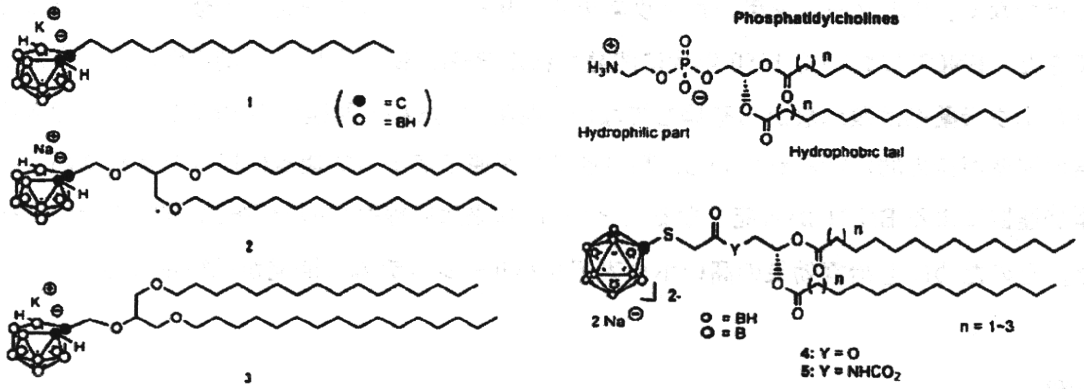

Figure. 2. Structures of *nido*-carborane lipids 1-3 and closo-dodecaborate lipids 4 and 5

3．BNCT 効果

ホウ素脂質 4 (n = 16)、DSPC、DSPE-PEG2000、コレステロール（それぞれ 0.25:0.75:0.1:1.0）から調整したホウ素リポソームを用いて上記と同様に Colon 26 細胞を移植した BALB/c マウスに尾静脈より投与し (20 mgB/kg) ホウ素分布を調べたところ、投与 24 時間後に腫瘍内ホウ素濃度が 22 ppm であった (Figure 3)。そこで、投与 24 時間後に中性子照射を行い腫瘍の経時的変化を調べたところ、Figure 4 に示すようにホウ素リポソームを投与したマウスでは、中性子照射 1 週間後には腫瘍の委縮が見られ増殖抑制が見られた。

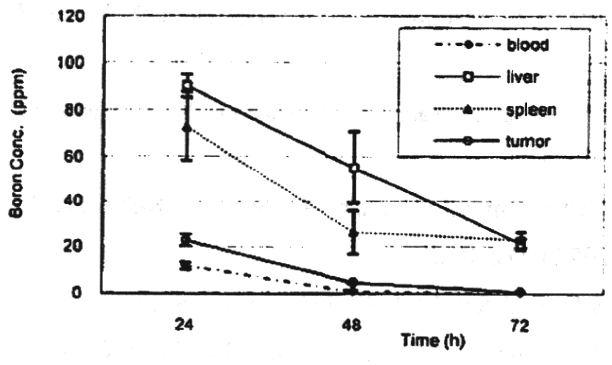

Figure 2. Time course of biodistribution of boron liposomes prepared from 4c in tumor-bearing mice.

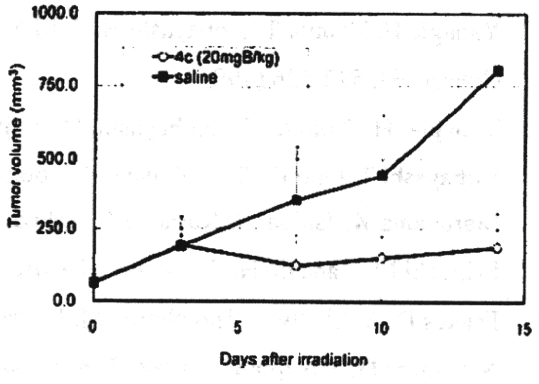

Figure 3. Tumor growth curve of mice bearing colon 26 tumors after injection of 20mg ^{10}B/kg of boronliposomes, thermal neutron irradiation with 0.9-1.4 x 10^{12} n/cm^2.

4．おわりに

BNCT のためのホウ素キャリアーの開発には、いわゆるナノモルレベルで薬理効果が要求される抗がん剤のようなドラッグデザインではなく、ミリモルレベルで投与できるのに十分な低毒性であり、なおかつ腫瘍細胞に集積することが必要とされる。そのために、低毒性小分子ホウ素化合物の

開発だけでなく、リポソームを用いたホウ素デリバリーシステムの開発が十数年前から盛んに研究されてきた。BNCT において 1950 年代に開発された BSH、BPA という２剤以外には、まだ臨床応用されたホウ素薬剤は残念ながら登場してない。現在、核燃料の問題から BNCT に適応できる小型加速器の開発が精力的に行われている。熱中性子源が原子炉から加速器に移行できれば都市部病院併設型加速器による BNCT が可能となることから、将来放射線療法の一般的治療法の一つになるであろう。そのためにも治療効果の高いホウ素デリバリーシステムの開発が期待される。

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Development of Boron Delivery System for Neutron Capture Therapy of Cancer

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Boron neutron capture therapy (BNCT) is a binary cancer treatment based on the nuclear reaction of two essentially nontoxic species, ^{10}B and thermal neutrons. The neutron capture reaction by ^{10}B produces an α-particle and a lithium-7 ion bearing approximately 2.4 MeV, and these high linear energy transfer particles afford precise cell killing. Therefore, the marked accumulation and selective delivery of boron into tumor tissues are the most important requirement to achieve an effective BNCT of cancers. We focused on a liposomal boron delivery system. Accumulation of boron in the liposomal bilayer is highly potent, because drugs can be encapsulated into the vacant inner cell of a liposome. Furthermore, functionalization of liposomes is possible by combination of lipid contents. Therefore, boron and drugs may be simultaneously delivered to tumor tissues for both BNCT and chemotherapy. We recently developed the *nido*-carborane lipid, which has a double-tailed moiety conjugated with *nido*-carborane as a hydrophilic function. The longer survival of tumor-bearing mice injected with the boronated liposomes was observed after BNCT. However, significant toxicity was also observed in the mice injected at higher boron concentrations. In this paper, we report synthesis of *closo*-dodecaborate containing boron lipids. Our design of the boron lipids is based on biomimetic composition of phosphatidylcholines. Mercaptoundecahydrododecaborate ($B_{12}H_{11}SH^{2-}$, BSH) was chosen as an alternative hydrophilic function of boron lipids. BSH is a water-soluble divalent anion cluster and significantly lowered toxicity, and has thus been utilized for clinical treatment of BNCT. We prepared the boronated liposomes from diacylphosphatidylcholines and boron lipids and examined their BNCT effect using tumor bearing mice. Suppression of tumor growth was observed in the mice injected with the boronated liposomes two weeks after neutron irradiation.

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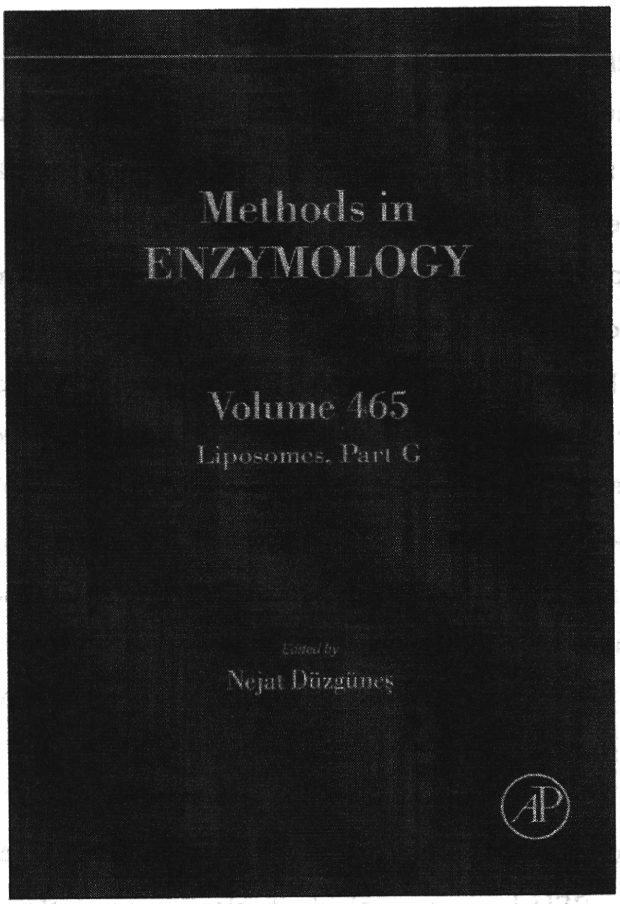

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CHAPTER TEN

LIPOSOMAL BORON DELIVERY FOR NEUTRON CAPTURE THERAPY

Hiroyuki Nakamura

Contents

Abstract

Tumor cell destruction in boron neutron capture therapy (BNCT) is due to the nuclear reaction between ^{10}B and thermal neutrons. The thermal neutrons have an energy of 0.025 eV, clearly below the threshold energy required to ionize

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tissue components. However, neutron capture by ^{10}B produces lithium ion and helium (α-particles), which are high linear energy transfer (LET) particles, and dissipate their kinetic energy before traveling one cell diameter (5–9 μm) in biological tissues, ensuring their potential for precise cell killing. BNCT has been applied clinically for the treatment of malignant brain tumors, malignant melanoma, head and neck cancer, and hepatoma using two boron compounds: sodium borocaptate ($Na_2{}^{10}B_{12}H_{11}SH$; $Na_2{}^{10}BSH$) and L-*p*-boronophenylalanine (L-^{10}BPA). These low molecular weight compounds are cleared easily from the cancer cells and blood. Therefore, high accumulation and selective delivery of boron compounds into tumor tissues are most important to achieve effective BNCT and to avoid damage of adjacent healthy cells. Much attention has been focused on the liposomal drug delivery system (DDS) as an attractive, intelligent technology of targeting and controlled release of ^{10}B compounds. Two approaches have been investigated for incorporation of ^{10}B into liposomes: (1) encapsulation of ^{10}B compounds into liposomes and (2) incorporation of ^{10}B-conjugated lipids into the liposomal bilayer. Our laboratory has developed boron ion cluster lipids for application of the latter approach. In this chapter, our boron lipid liposome approaches as well as recent developments of the liposomal boron delivery system are summarized.

1. INTRODUCTION

Boron neutron capture therapy (BNCT) was first proposed as a binary approach to cancer treatment (Locher, 1936). The cell-killing effect of BNCT is due to a nuclear reaction of two essentially nontoxic species, boron-10 (^{10}B) and thermal neutrons (Eq. (10.1)).

$$^{10}B + {}^{1}n \rightarrow {}^{4}He(\alpha) + {}^{7}Li + 2.4\,MeV \qquad (10.1)$$

The resulting α-particles and Li nuclei are high linear energy transfer (LET) particles and dissipate their kinetic energy before traveling one cell diameter (5–9 μm) in biological tissues ensuring their potential for precise cell killing. Their destructive effect is highly observed in boron-loaded tissues. Therefore, high accumulation and selective delivery of boron-10 into the tumor tissue are the most important requirements to achieve efficient neutron capture therapy of cancer (Barth *et al.*, 1990; Hawthorne, 1993; Soloway *et al.*, 1998). The amounts of boron-10 required to obtain fatal tumor cell damage has been calculated to be more than 20–30 μg/g of tumor tissue (Barth *et al.*, 1992). At the same time, the boron concentration in the surrounding normal tissues and blood should be kept low to minimize damage to the normal tissues. BNCT has been applied clinically for the treatment of patients with malignant brain tumors and malignant melanoma, using sodium mercaptoundecahydrododecaborate ($Na_2{}^{10}B_{12}H_{11}SH$; $Na_2{}^{10}BSH$) (Nakagawa and Hatanaka, 1997;

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Soloway *et al.*, 1967) and L-*p*-boronophenylalanine (L-^{10}BPA) (Mishima *et al.*, 1989; Synder *et al.*, 1958), respectively. Furthermore, positron emission tomography (PET) using ^{18}F-BPA has been developed (Imahori *et al.*, 1998). The structures of boron compounds which have been utilized for clinical treatment of BNCT are shown in Fig. 10.1. Since the achievement of ^{18}F-BPA PET imaging, we have been able to predict tumor/blood and tumor/normal tissue ratios of L-^{10}BPA before neutron irradiation. This PET technology also displayed selective accumulation of ^{18}F-BPA in various tumors. Thus, BNCT has been applied for various cancers including head and neck cancer, lung cancer, hepatoma, chest wall cancer, and mesothelioma (Aihata *et al.*, 2006; Kato *et al.*, 2004; Suzuki *et al.*, 2007). Number of cases for treatment of cancers with BNCT at KUR (Kyoto University Reactor) is summarized in Fig. 10.2. Although the number of

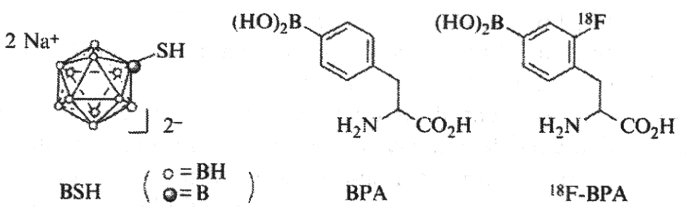

Figure 10.1 Structures of boron compounds utilized for clinical treatment of BNCT.

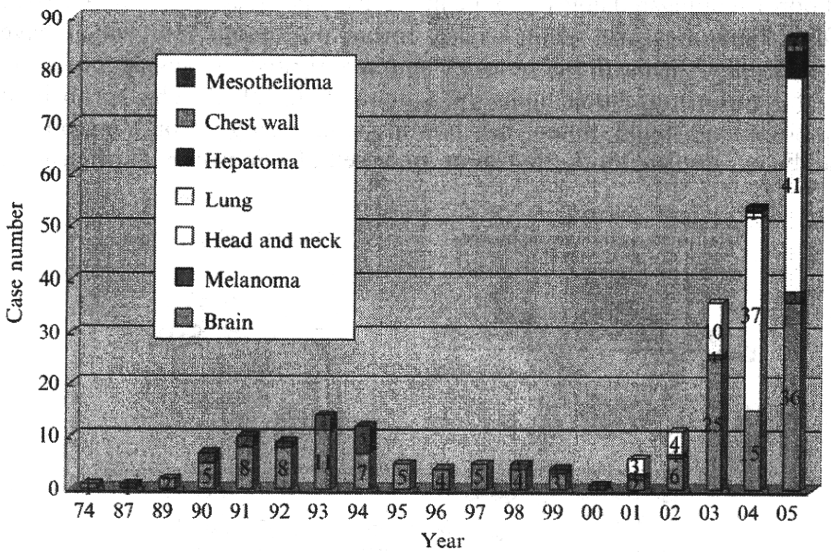

Figure 10.2 Number of cases for treatment of cancers with BNCT at KUR.

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cases is increasing, development of new ^{10}B-carriers that deliver an adequate concentration of ^{10}B atoms to tumors is still an important requirement for effective and extensive cancer therapy in BNCT.

Recent promising approaches that meet the requirement entail the use of small boron molecules (Cai *et al.*, 1997; Gedda *et al.*, 1997; Kelly *et al.*, 1994; Nakamura *et al.*, 1997; Yamamoto and Nakamura, 1993; Yamamoto *et al.*, 1995), such as porphyrins (Alam *et al.*, 1989; Kahl and Li, 1996; Kahl *et al.*, 1990; Miura *et al.*, 1996; Murakami *et al.*, 1993; Woodburn *et al.*, 1993), nucleosides (Rong and Soloway, 1994; Schinazi and Prusoff, 1985; Sood *et al.*, 1989; Yamamoto *et al.*, 1992), and amino acids (Kahl and Kasar, 1996; Kirihata *et al.*, 1995; Malan and Morin, 1996; Nakamura *et al.*, 1998, 2000; Srivastava *et al.*, 1997; Takagaki *et al.*, 1996), and boron-conjugated biological complexes, such as monoclonal antibodies (Alam *et al.*, 1985; Goldenberg *et al.*, 1984; Pak *et al.*, 1995), epidermal growth factors (Capala *et al.*, 1996; Gedda *et al.*, 1996; Yang *et al.*, 1997), carborane oligomers (Cai *et al.*, 1997; Fulcrand-El Kattan *et al.*, 1994; Nakanishi *et al.*, 1999; Sood *et al.*, 1990), micells (Wei *et al.*, 2003), and dendrimers (Backer *et al.*, 2005; Shukla *et al.*, 2003; Wu *et al.*, 2004).

Liposomes are efficient drug delivery vehicles, because encapsulated drugs can be delivered selectively to tumors. Therefore, liposomal boron delivery system, in this context, is also considered to be potent for BNCT due to the possibility to carry a large amount of ^{10}B compound. Two approaches have been investigated for liposomes as boron delivery vehicles: (1) encapsulation of boron compounds into liposomes and (2) incorporation of boron-conjugated lipids into the liposomal bilayer, as shown in Fig. 10.3. Our laboratories first synthesized a boron ion cluster lipid which have double alkyl chains in the molecule and investigated the vesicle formation of the boron ion cluster lipids (Nakamura *et al.*, 2004). The boron lipid liposomes are highly potent because drugs, including boron compounds, can be encapsulated into the vacant inner cell of a liposome. Furthermore,

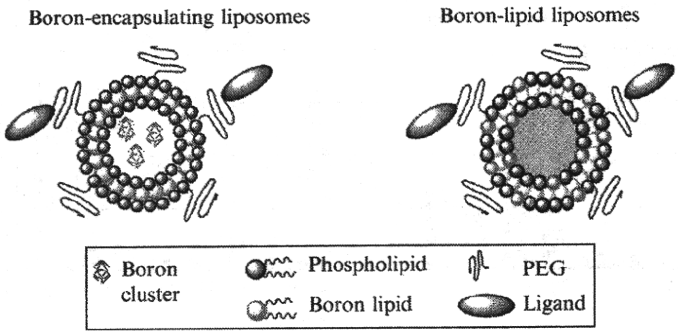

Figure 10.3 Boron-encapsulating liposomes and boron-lipid liposomes.

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functionalization of liposomes is possible by combination of lipid contents. Therefore, ^{10}B and drugs may be simultaneously delivered to tumor and this will lead to the combination therapy of both BNCT and chemotherapy for cancers. In this chapter, liposomal boron delivery systems of both boron-encapsulation and boron-lipid liposome approaches are described.

2. Boron-Encapsulation Approach

Liposomal boron delivery system was first reported by Yanagië *et al.* (1989, 1991). They investigated a BSH-encapsulated liposome which was conjugated with a monoclonal antibody specific for carcinoembryonic antigen (CEA). A new murine monoclonal antibody (2C-8) was prepared by immunizing mice *i.p.* with CEA producing human pancreatic cancer cell line, AsPC-1. SDS–PAGE and Western blot analysis showed that 2C-8 monoclonal antibody recognized CEA. This anti-CEA monoclonal antibody was conjugated with large multilamellar liposomes incorporated ^{10}B compound ($Cs_2{}^{10}BSH$). The liposome was prepared from egg yolk phosphatidylcholine, cholesterol, and dipalmitoylphosphatidylethanolamine (1/1/0.05), and $Cs_2{}^{10}BSH$ was encapsulated. The liposomes were treated with dithiothreitol and suspended in the *N*-hydroxysuccinimidyl-3-(2-pyridyldithio)propionate-treated antibody solution for conjugation. This immunoliposome was shown to bind selectively to human pancreatic carcinoma cells (AsPC-1) bearing CEA on their surface and inhibit tumor cell growth on thermal neutron irradiation (5×10^{12} neutrons/cm^2) *in vitro*. Furthermore, the theraputic effects of locally injected BSH-encapsulated immunoliposome on AsPC-1 xenografts in nude mice were evaluated. After intratumoral injection of the immunoliposomes, boron concentrations in tumor tissue and blood were 49.6 ± 6.6 and 0.30 ± 0.08 ppm, respectively. Tumor growth of mice with intratumoral injection of BSH-encapsulated immunoliposomes was suppressed with thermal neutron irradiation (2×10^{12} neutrons/cm^2) *in vivo*. Histopathologically, hyalinization and necrosis were found in the immunoliposome-treated tumors (Yanagië *et al.*, 1997).

Hawthorne and coworkers succeeded in the preparation of boron-encapsulating liposomes with mean diameters of 70 nm or less from distearoylphosphatidylcholine (DSPC) and cholesterol in 1992. The hydrolytically stable borane anions $B_{10}H_{10}{}^{2-}$, $B_{12}H_{11}SH^{2-}$, $B_{20}H_{17}OH^{4-}$, $B_{20}H_{19}{}^{3-}$, and the normal form and photoisomer of $B_{20}H_{18}{}^{2-}$ were encapsulated in liposomes as their soluble sodium salts. Although the boron compounds used do not exhibit an affinity for tumors and are normally rapidly cleared from the body, liposomes were observed to

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selectively deliver the borane anions to tumors. High tumor concentrations were achieved in the therapeutic range (>15 μg of boron per g of tumor) while maintaining high tumor-boron/blood-boron ratios (>3). The most favorable results were obtained with the two isomers of $B_{20}H_{18}^{2-}$. These boron compounds have the capability to react with intracellular components after they have been deposited within tumor cells by the liposome, thereby preventing the borane ion from being released into blood (Shelly *et al.*, 1992). Furthermore, an apical–equatorial (*ae*) isomer of the $B_{20}H_{17}NH_3^{3-}$ ion, $[1\text{-}(2'\text{-}B_{10}H_9)\text{-}2\text{-}NH_3B_{10}H_8]^{3-}$, which was produced from the reaction of the polyhedral borane ion $B_{20}H_{18}^{2-}$ with liquid ammonia, was encapsulated into liposomes prepared with 5% PEG-200-distearoyl phosphatidylethanolamine. The PEGylated liposomes exhibited a long circulation lifetime due to escape from reticuloendothelial system (RES), resulting in the continued accumulation of boron in the tumor over the entire 48-h experiment and reaching a maximum of 47 μg of boron per g of tumor (Feakes *et al.*, 1994).

Boron-containing folate receptor-targeted liposomes have been developed by Lee and coworkers (Pan *et al.*, 2002). Expression of the folate receptor (FR) is frequently is amplified among human tumors. Two highly ionized boron compounds, $Na_2[B_{12}H_{11}SH]$ and $Na_3(B_{20}H_{17}NH_3)$, were incorporated into liposomes by passive loading with encapsulation efficiencies of 6% and 15%, respectively. In addition, five weakly basic boronated polyamines investigated were incorporated into liposomes by a pH-gradient-driven remote-loading method with varying loading efficiencies. 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. The *in vitro* uptake of folate-conjugated boron-encapsulating liposomes was investigated using human KB squamous epithelial cancer cells, which have amplified FR expression. Higher cellular boron uptake (up to 1584 μg per 10^9 cells) was observed with FR-targeted liposomes than with nontargeted control liposomes (up to 154 μg per 10^9 cells), irrespective of the chemical form of the boron and the method used for liposomal preparation.

Kullberg and coworkers investigated EGF-conjugated PEGylated liposome delivery vehicle, containing water-soluble boronated phenanthridine, WSP1, or water-soluble boronated acridine, WSA1, for EGFR targeting. In the case of WSA1, a ligand-dependent uptake was obtained and the boron uptake was as good as if free WSA1 was given. No ligand-dependent boron uptake was seen for WSP1-containing liposomes. Thus, WSA1 is a candidate for further studies. Approximately 10^5 boron atoms were in each liposome. A critical assessment indicates that after optimization up to 10^6 boron atoms can be loaded. *In vitro* boron uptake by glioma cells (6.29 ± 1.07 μg/g cells) was observed with WSA1-encapsulated EGF-conjugated PEGylated liposomes (Kullberg *et al.*, 2003).

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Cetuximab-conjugated liposome was also investigated as an althernative immunoliposome for targeting of EGFR(+) glioma cells. Lee and coworkers developed cetuximab-immunoliposomes via a cholesterol-based membrane anchor, maleimido-PEG-cholesterol (Mal-PEG-Chol), to incorporate cetuximab into liposomes. BSH-encapsulated cetuximab-immunoliposomes were evaluated for targeted delivery to human EGFR gene transfected $F98_{EGFR}$ glioma cells. Much greater (approximately eightfold) cellular uptake of boron was obtained using cetuximab-immunoliposomes in EGFR(+) $F98_{EGFR}$ compared with nontargeted human IgG-immunoliposomes (Pan *et al.*, 2007).

Maruyama and coworkers developed a new type of target-sensitive liposomes, in which transferrin-coupling pendant-type PEG liposomes were extravasated effectively into solid tumor tissue in colon 26 tumor-bearing mice, and internalized into tumor cells (Ishida *et al.*, 2001). Transferrin (TF) receptor-mediated endocytosis is a normal physiological process by which TF delivers iron to the cells and higher concentration of TF receptor has been observed on most tumor cells in comparison with normal cells. TF-PEG liposomes showed a prolonged residence time in the circulation and low RES uptake in tumor-bearing mice, resulting in enhanced extravasation of the liposomes into the solid tumor tissue. Once at the tumor site, TF-PEG liposomes were internalized into tumor cells by receptor-mediated endocytosis. TF-PEG liposomes were taken up into endosome-like intracellular vesicles. Therefore, the clearance of TF-PEG liposomes from tumor tissue is so impaired that they remain in the tumor interstitium for a long time. Thus, the potential of liposomes for selective delivery of therapeutic quantities of ^{10}B to tumors has been studied (Maruyama *et al.*, 2004). TF-PEG liposomes and PEG liposomes encapsulating $Na_2{}^{10}BSH$ were prepared and their tissue distributions in colon 26 tumor-bearing mice after *i.v.* injection were compared with those of bare liposomes and free $Na_2{}^{10}BSH$. When TF-PEG liposomes were injected at a dose of 35 mg ^{10}B/kg, a prolonged residence time in the circulation and low uptake by the RES were observed in colon 26 tumor-bearing mice, resulting in enhanced accumulation of ^{10}B into the solid tumor tissue (e.g., 35.5 μg of boron per g of tumor). TF-PEG liposomes maintained a high ^{10}B level in the tumor, with concentrations over 30 μg of boron per g of tumor for at least 72 h after injection. On the other hand, the plasma level of ^{10}B decreased, resulting in a tumor/plasma ratio of 6.0 at 72 h after injection. Administration of $Na_2{}^{10}BSH$ encapsulated in TF-PEG liposomes at a dose of 5 or 20 mg ^{10}B/kg and irradiation with 2×10^{12} neutrons/cm^2 for 37 min produced tumor growth suppression and improved long-term survival compared with PEG liposomes, bare liposomes, and free $Na_2{}^{10}BSH$. Masunaga and coworkers evaluated biodistribution of $Na_2{}^{10}BSH$- and $Na_2{}^{10}B_{10}H_{10}$-encapsulated TF-PEG liposomes in SCC VII tumor-bearing mice (Masunaga *et al.*, 2006). The time course of the

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change in the ^{10}B concentration in tumors loaded with both liposomes were similar except that ^{10}B concentrations were greater 24 h after the loading of $Na_2{}^{10}B_{10}H_{10}$ than $Na_2{}^{10}BSH$ in TF-PEG liposomes and ^{10}B concentration in tumors was 35.6 μg of boron per g of tumor with injection of $Na_2{}^{10}B_{10}H_{10}$-encapsulated TF-PEG liposomes (35 mg ^{10}B/kg).

3. Boron Lipid-Liposome Approach

Development of lipophilic boron compounds, embedded within the liposome bilayer, provides an attractive method to increase the overall efficiency of incorporation of boron-containing species, as well as raise the gross boron content of the liposomes in the formulation. Hawthorne and coworkers first introduced *nido*-carborane as hydrophilic moiety into the amphiphile and this single-tailed *nido*-carborane amphiphile was utilized for liposomal boron delivery using tumor-bearing mice (Feakes *et al.*, 1995; Watson-Clark *et al.*, 1998). They synthesized the *nido*-carborane amphiphile **1** (Fig. 10.4) and prepared boronated liposomes composed of DSPC, cholesterol, and **1** in the bilayer. After the injection of liposomal suspensions in BALB/c mice bearing EMT6 mammary adenocarcinomas, the time-course biodistribution of boron was examined. At the low injected doses normally used (5–10 mg ^{10}B/kg), peak tumor boron concentrations of

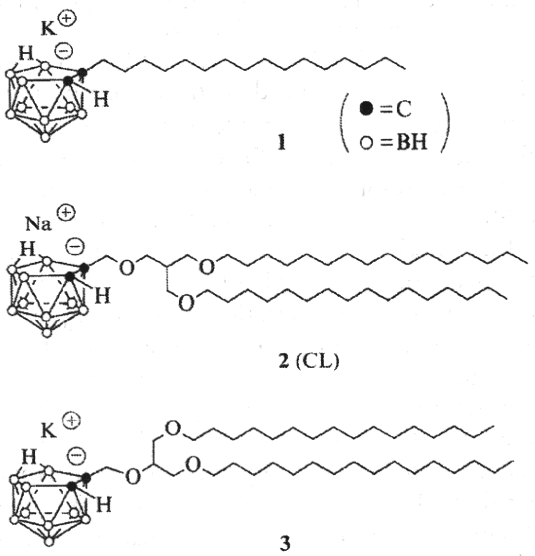

Figure 10.4 Structures of *nido*-carborane lipids.

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35 μg of boron per g of tumor and tumor/blood boron ratios of ~8 were achieved. These values are sufficiently high for the successful application of BNCT. The incorporation of both **1** and the hydrophilic species, $Na_3[1\text{-}(2'\text{-}B_{10}H_9)\text{-}2\text{-}NH_3B_{10}H_8]$, within the same liposomes demonstrated significantly enhanced biodistribution characteristics, exemplified by maximum tumor boron concentration of 50 μg of boron per g of tumor and tumor/blood boron ratio of 6.

Our laboratories designed the *nido*-carborane lipid **2** (CL), which has a double-tailed moiety conjugated with *nido*-carborane as a hydrophilic moiety (Nakamura *et al.*, 2004). This lipid has a symmetric carbon in the lipophilic alkyl chain. Analysis in a transmission electron microscope by negative staining with uranyl acetate showed a stable vesicle formation of CL. Furthermore, we focused on overexpression of TF receptor on most cell surfaces. Thus we investigated active targeting of the boron liposomes to solid tumor by functionalization of TF on the surface of their liposomes and achieved a boron concentration of 22 μg ^{10}B per g of tumor by the injection of the liposomes at 7.2 mg ^{10}B/kg body weight with longer survival rates of tumor-bearing mice after BNCT (Miyajima *et al.*, 2006). The detailed protocols for synthesis of the CL and *in vivo* BNCT effects of the CL-liposomes are described.

4. *NIDO*-CARBORANE LIPID LIPOSOMES

4.1. Synthesis of the *nido*-carborane lipid (CL)

Chemical synthesis of the *nido*-carborane lipid (CL) is shown in Scheme 10.1. Reaction of two equivalents of heptadecanol with 3-chloro-2-chloromethyl-1-propene using NaH as base gives the diether **4** in 93% yield and the hydroboration of **4** gives the corresponding alcohol **5** in 71% yield. The alcohol **5** is converted into the propargyl ether **6** in 48% yield by the treatment with propargyl bromide, and the decaborane coupling of **6** is carried out in the presence of acetonitrile in toluene under reflux condition to give the corresponding *ortho*-carborane **7** in 80% yield. The degradation of the carborane cage by the treatment with sodium methoxide in methanol affords the *nido*-carborane lipid **2** (CL) in 57% yield.

4.2. Stability of the *nido*-carborane lipid vesicles

The stability of the boron cluster vesicles in fetal bovine serum (FBS), which can be considered as a model of blood, is examined using the vesicle solution. A boron cluster vesicle fraction is added to FBS (the volume ratio FBS:vesicle solution = 9:1) and the mixture is incubated at 37 °C with stirring. The fluorescence of the FBS solution is measured at 0–18 h.

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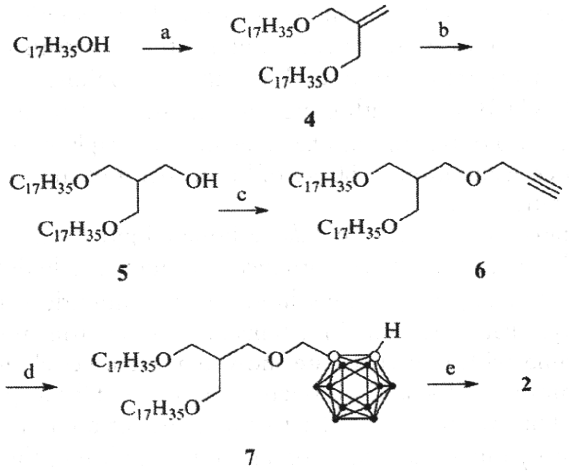

Scheme 10.1 Synthesis of the boron lipid 2. *Reagents*: (a) 1—NaH, THF, 2—$CH_2NC(CH_2Cl)_2$, 93%; (b) 1—$BH_3 \cdot Me_2S$, 2—H_2O_2, NaOH, 71%; (c) 1—NaH, THF, 2—propargyl bromide, 58%; (d) $B_{10}H_{14}$, CH_3CN, toluene, 80%; (e) NaOMe, MeOH, 57%.

No increase of the fluorescence intensity of the FBS solutions is observed during 18 h. Therefore, the calcein encapsulated into the boron cluster vesicle is not released. This result indicates that the boron cluster vesicle prepared from the *nido*-carborane lipid is quite stable in the FBS solution at 37 °C.

4.3. Incorporation of the *nido*-carborane lipid into liposomal membranes

The effect of the accumulation ratio of DSPC and CL on the liposome formation is examined under various mixing ratios. PEG liposomes are prepared from DSPC, CH, CL, and PEG-distearoylphosphatidylethanolamine (DSPE) (1:1:x:0.11, x = 0–1). The lipid concentration is estimated by phosphorus assay (Fiske and Subbarow, 1925). Boron content is determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Very interestingly, the ratio of DSPC and CL in the liposomes increased in proportion to the increase of the mixing ratio of CL to DSPC in the solution. Furthermore, it was observed that CL was incorporated into the liposome membranes with five times higher concentration than DSPC (Nakamura *et al.*, 2004) (Fig. 10.5).

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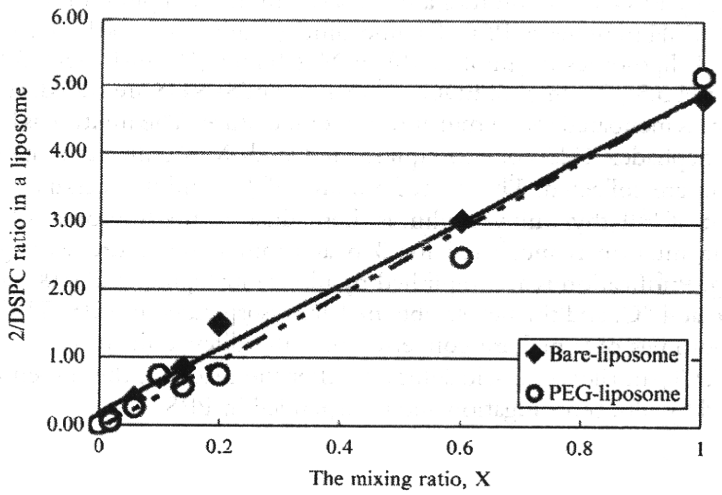

Figure 10.5 Incorporation of the *nido*-carborane lipid (CL) into liposomal membranes. The bare-liposome was prepared from DSPC, CH, and CL (the mixing ratio of 1:1:*x*; $x = 0$–1), and the PEG-liposome was prepared from DSPC, CH, CL, and PEG-DSPE (the mixing ratio of 1:1:*x*:0.11, $x = 0$–1).

5. Transferrin-Conjugated *nido*-Carborane Lipid Liposomes

5.1. Preparation of liposomes

TF(−)-PEG-CL liposomes are prepared from DSPC, CH, DSPE-PEG-OMe, DSPE-PEG-O-$(CH_2)_5CO_2H$, and CL (molar ratio 1:1:0.11:0.021:0.25) by the reverse-phase evaporation (REV) method. A mixture of DSPC, CH, DSPE-PEG-OMe, and CL are dissolved in chloroform/diisipropylether mixture (1:1, v/v) in a round-bottomed flask. The volume ratio of the aqueous phase to the organic phase is maintained at 1:2. The emulsion is sonicated for 1 min, and then the organic solvent is removed under vacuum in a rotary evaporator at 37 °C for 1 h to form a lipid gel. The gel obtained is subjected to extrusion through a polycarbonate membrane of 100 nm pore size, using an extruder device (Lipex Biomembrane, Canada) thermostated at 60 °C. Purification is accomplished by ultracentrifuging at 200,000×*g* for 20 min at 4 °C, and the pellets obtained are resuspended in PBS buffer. Liposome size is measured with an electrophoretic light scattering spectrophotometer (ELS-700, Otsuka Electronics, Tokyo).