特異的な感染機構の中心的役割を担っている。このような肝臓選択性は，他のキャリアーがEPR効果によって癌細胞に物質を送達する受動的標的化とは異なり，HBVが本来備えている能動的標的化機構であり，BNCも同機構を引き継いでいる。さらに，BNCには，HBVのようにウイルスのゲノムや遺伝子複製に必要な酵素などが含まれていないため，安全である。実際，BNCは遺伝子組み換え酵母を用いて大量生産される[20]が，この粒子は既に全世界で20年以上も臨床で使用されてきたB型肝炎ワクチンと同じ構造であるので，BNCを臨床応用する際の毒性や安全性に対する懸念を著しく低減できる。

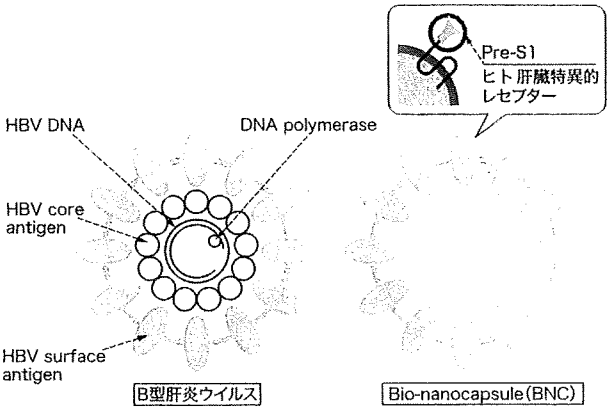

図2　B型肝炎ウイルスと同表面抗原粒子(HBsAg；BNCに相当)

❷ バイオナノカプセル内部への物質封入

実際にBNC内部に物質を封入するのは，当初はエレクトロポレーションを用いて物質をBNC内部に封入していたが[21]，BNCを医薬品として開発する場合に大量生産に不向きであることと，封入効率が一定でないことから，現在では一度リポソーム内部に物質を封入してBNCの膜融合活性を利用して融合することによりBNC内部に効率的に物質を封入している(リポソーム融合法)[22]。また，リポソームに封入できるものであればすべてBNC内部に移すことができるので，遺伝子，薬剤，蛋白質，siRNAなどの送達にBNCは使用できる。特に，従来不可能であった直径100 nmのポリスチレンビーズや数十kbpの発現ベクターをBNC内部に封入して，生体内でのピンポイントデリバリーができるようになった。

緑色蛍光蛋白質(GFP)の発現遺伝子を封入したBNCを，ヒト肝臓癌由来細胞株と他組織由来細胞株の培養液中に添加すると，ヒト肝臓癌由来細胞株のみにGFP由来の蛍光を観察することができた[21]。従来のカチオン性リポソームを使用する遺伝子導入試薬と比較すると，単位DNA当たりのGFP発現量は100倍以上であった。これは，BNCがリポソームとは異なりendocytosisに依存して細胞内に送達されるだけでなく，HBV由来の感染機構を保持していることを示している。

❸ バイオナノカプセルの再標的化

BNCは主にpre-S1領域によりヒト肝臓細胞を認識する。同領域をさまざまな生体認識分子に置換することで，他の細胞や組織を標的にすることが可能である。生体認識分子としては，抗体，糖鎖，サイトカイン，同レセプターおよびボーミングペプチド(生体内で組織特異性を持つ短鎖ペプチド)などさまざまなものが考えられ，その臨床応用に向けて研究が進んでいる(**図3**)。なかでも抗体は多彩な分子を認識することができるので，BNCの再標的化用の生体認識分子としては最適である。われわれは*Staphylococcus aureus*由来のprotein A由来のIgG Fc結合ドメインであるZドメインを2量体にしたZZドメインをpre-S1領域と交換した，ZZドメイン提示型BNCの遺伝子組み換え酵母による大量生産に成功した。このZZ提示型BNCは，抗体と共存させると，自動的に抗体提示型BNCとなる(**図3**)。

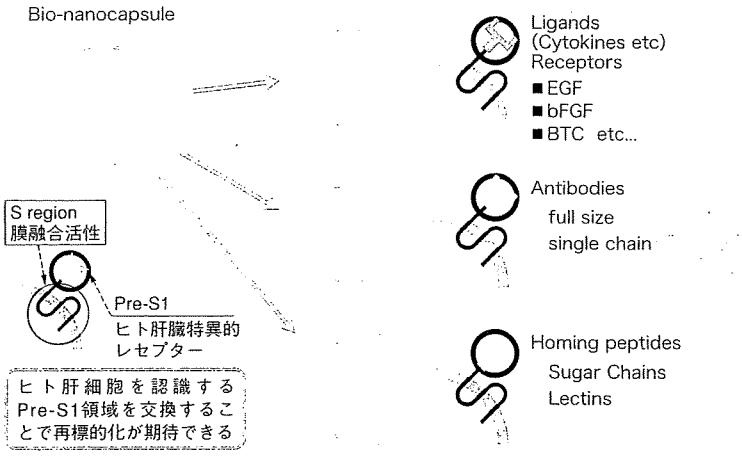

図3

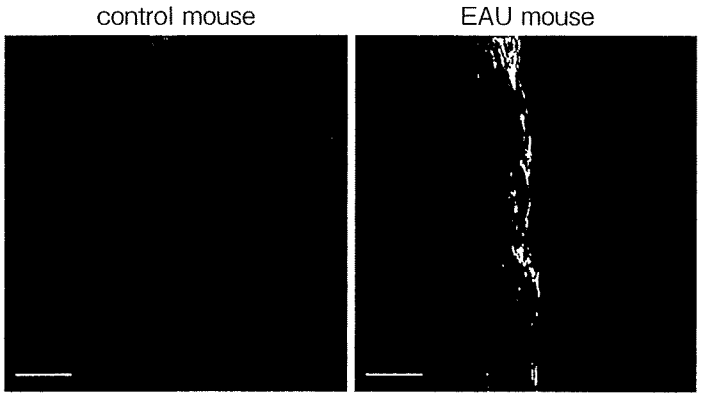

図4　EAUマウス網膜でのP-selectinの発現
FITC：P-selectin，Evans blue：血管内腔

❹ ぶどう膜炎治療へのバイオナノカプセルの応用

再標的化したBNCをヒトぶどう膜炎の動物モデルに応用することにより生体内でピンポイントに遺伝子や物質をデリバリーすることに成功した。

1) 実験的自己免疫性ぶどう膜炎(EAU)とP-selectinの発現

EAUはヒトぶどう膜炎の動物モデルであり，その病態・治療に関する研究はヒトぶどう膜炎の病態解明や新しい治療法の開発に貢献してきた。免疫抑制薬のシクロスポリンもEAUにおいてその有効性が確認された後，ヒトぶどう膜炎でも使用されるようになり，現在ではベーチェット病をはじめとする自己免疫性ぶどう膜炎における炎症抑制の有効な治療薬としての立場が確立されている。このように，EAUを使った治療実験は，ヒトぶどう膜炎の治療への応用を期待させる。

EAUの発症機序であるが，EAUを誘導可能な網膜特異抗原を免疫後，所属リンパ節で活性化された抗原特異的T細胞が眼内に侵入し，炎症性サイトカインを放出し，マクロファージや好中球が活性化・浸潤し，炎症を起こすと考えられている。眼内の炎症は，汎ぶどう膜炎として現れ，抗原免疫後10～14日で結膜充血，

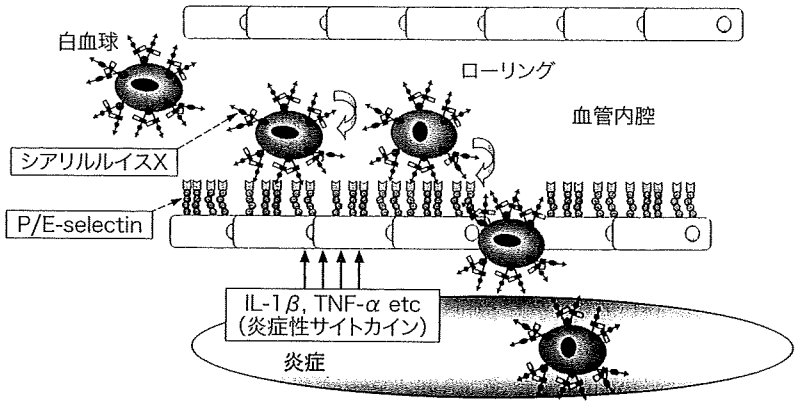

図5　ぶどう膜炎の網膜血管浸潤のメカニズム

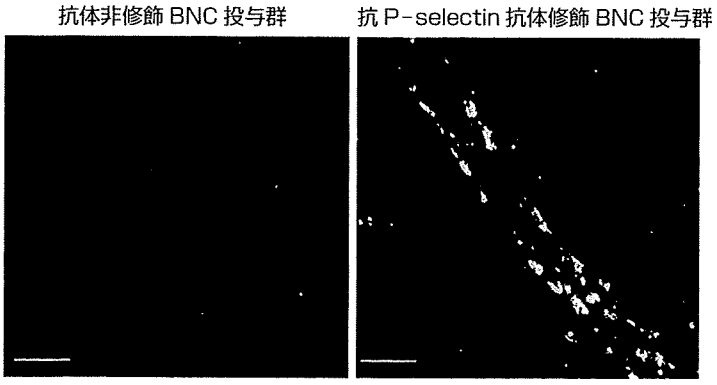

図6　GFP 遺伝子の発現
green：GFP，Evans blue：EUA マウスの血管内腔

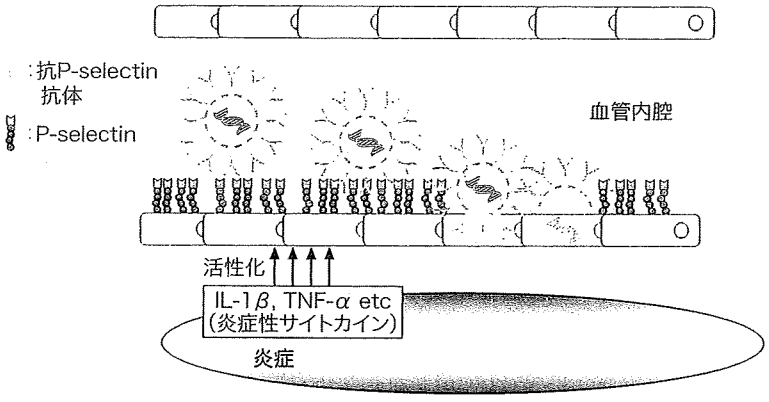

図7　抗 P-selectin 抗体結合 ZZ 提示型 BNC

前房内炎症，網膜血管炎の所見が出現し，数日で炎症はピークに達し，その後，徐々に自然消退することが知られている。また EAU の網膜静脈の血管内皮には P-selectin（**図4**）や E-selectin が発現しており，好中球は表面にはリガンドであるシアリルルイス X があり，それを

介して血管内皮と接触しローリングを行うようになる(**図5**)。

2) 抗体提示型バイオナノカプセルを用いた炎症部位の能動的標的化

リポソーム融合法により蛍光物質を封入したZZ提示型BNCと炎症部位に発現している抗P-selecitin抗体を，室温で30分間反応させたのち，炎症ピーク時のEAUマウスの尾静脈から投与すると，炎症部位の細胞に蛍光物質が取り込まれていた。さらにGFPを封入した抗P-selectin抗体結合ZZ提示型BNCをEAUマウスに投与した場合も，同様に炎症部位の細胞に蛍光発現を認めた(**図6**)。これらの結果から抗体提示型バイオナノカプセルを用いることにより薬剤，遺伝子を能動的に標的化できることが*in vivo*で示された(**図7**)(2008年日本眼炎症学会にて報告)。

3) バイオナノカプセルの臨床応用に際しての課題

ここまでBNCのDDS/GDS技術について述べたが，臨床応用に向けて解決すべき問題も多い。BNCはHBsAgのL蛋白質を基本としているため，ヒト免疫系により排除される可能性がある。特に，B型肝炎ワクチン接種者などの抗HBV抗体を持っているヒトにおいては，免疫反応から逃れるステルス化BNCが必要である。われわれは既にB型肝炎ワクチン接種者の体内で増殖するHBVエスケープ変異体の研究成果に基づき，いくつかのアミノ酸置換を加えた改変型BNCを開発し，その低抗原性ならびに高濃度の中和抗体存在下における感染性の保持を確認しており，長期的な治療におけるBNCの使用を可能にする研究も進めている。また，実際BNCにどのような薬剤や遺伝子を内包し，どれだけの量を投与すれば，ぶどう膜炎を長期間にわたって効果的に安全にコントロールできるかを検討していく必要がある。

おわりに

今日，多様なキャリアーの開発が世界中で進み，それに伴いDDS/GDSの進歩が目覚ましい。しかし，その中で*in vivo*で使用できるものは一部であり，*in vivo*で静脈注射によりピンポイントデリバリーに成功したものはBNCを含めごく一部である。BNCをはじめとする能動的標的化を可能とするキャリアーを利用した治療がぶどう膜炎をはじめとする慢性眼疾患の治療の突破口となることを期待したい。

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CHAPTER EIGHT

BIO-NANOCAPSULE–LIPOSOME CONJUGATES FOR *IN VIVO* PINPOINT DRUG AND GENE DELIVERY

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Abstract

A bio-nanocapsule (BNC) is an ~50-nm hepatitis B virus (HBV) subviral particle comprising HBV envelope L proteins and a lipid bilayer, and is synthesized in recombinant *Saccharomyces cerevisiae*. When BNCs are administered intravenously in a mouse xenograft model, they can accumulate specifically in human liver-derived tissues and enter cells efficiently by the HBV-derived human liver-specific infection machinery, localized at the outer-membrane pre-S region of the L protein. BNC specificity for the human liver can be altered to other tissues by substituting the pre-S region using targeting molecules (e.g., antibodies, lectins, cytokines). BNCs can spontaneously form complexes with liposomes (LPs) by the membrane fusogenic activity of the pre-S region. LPs containing various therapeutic materials (e.g., chemicals, proteins, DNA, RNA) can therefore be covered with BNCs to form an ~150-nm BNC–LP conjugate. BNC–LP conjugates injected intravenously can deliver incorporated materials to target tissues specifically and efficiently by utilizing the HBV-derived infection machinery. The stability of BNC–LP conjugates in the blood circulation is similar to that of PEGylated LPs. In this chapter, we describe the preparation and *in vivo* application of BNC–LP conjugates, and the potential of BNC–LP conjugates as *in vivo* pinpoint drug delivery systems.

1. Introduction

Liposomes (LPs) are one of the most promising drug delivery system (DDS) carriers for genes and drugs, and several LP-based medicines have entered clinical use (Lorusso *et al.*, 2007; Markman, 2006; Richardson, 2006; Rosenthal *et al.*, 2002). Most LP-based medicines are nanoscaled. They are used for the delivery of anticancer drugs to carcinomatous lesions by taking advantage of the enhanced permeation and retention effect of solid tumors (Maeda *et al.*, 2000). The blood vasculature in tumors is leaky, because it possesses larger sized pores than the blood vasculature in healthy tissues. A nanomedicine of diameter ~100 nm can therefore readily seep out from the blood circulation and accumulate in tumors ("passive targeting"). The major obstacle for passive targeting is the immune system in the liver and spleen, that is, the reticuloendothelial system (RES) in which macrophages (including Kupffer cells) capture opsonized micro- and nanoscaled compounds (Moghimi *et al.*, 2001). To escape the RES, recent LPs have been modified with polyethylene glycol (PEG) (i.e., PEGylated LPs),

thereby enhancing their stability in the blood circulation by avoiding LPs from binding to serum proteins ("opsonization") (Lasic and Martin, 1995; Moghimi and Szebeni, 2003). PEGylated LPs containing anticancer drugs could dramatically reduce adverse side effects, but several reports have noted that the LP encapsulation of anticancer drugs contribute only to the reduction of side effects, not to the enhancement of therapeutic effects (Hong *et al.*, 1999; Parr *et al.*, 1997). This indicates that modifying LPs for the delivery of drugs to actively target *foci* ("active targeting") is required.

After attachment onto the cell surface, LPs are usually incorporated within target cells through an endocytotic cascade. Most of the drugs in LPs are degraded in late endosome and lysosome without exhibiting sufficient therapeutic effects. In contrast to LPs, several viral vectors (e.g., adenovirus, adeno-associated virus, lentivirus, retrovirus) are widely used in gene delivery. This is because recombinant viral vectors can efficiently pass across the plasma membrane and deliver their genomic information to the cell nucleus as well as escaping from the endocytotic cascade. These viral vectors may have unexpected severe side effects originating from the viral genome (Marshall, 2002; Savulescu, 2001), but the efficiency of gene delivery is much higher than that of lipoplexes (LP–DNA complexes) (Hama *et al.*, 2006). Taken together, the next generation of LP-based nanomedicines must have the following features: (1) viral genome-free structure; (2) active targeting machinery; (3) escape machinery from the RES; (4) escape machinery from the endosomal degradation pathway; and (5) intracellular targeting machinery.

2. First-Generation Bio-Nanocapsules

In the process of developing a hepatitis B virus (HBV) vaccine, we succeeded in overexpressing HBV subviral L particles in *Saccharomyces cerevisiae* (Kuroda *et al.*, 1992). The L particle is an ~50-nm hollow capsule comprising HBV envelope L proteins and a lipid bilayer (Yamada *et al.*, 2001). In 2003, we unexpectedly found that the L particles could incorporate genes and drugs by electroporation, and deliver the incorporated materials specifically and efficiently to cell lines (*in vitro*) and tumors (*in vivo*) derived from the human liver (Yamada *et al.*, 2003). We therefore designated the HBV L particle as a "bio-nanocapsule" (BNC) (Yu *et al.*, 2005). The hepatophilic and highly efficient delivery properties of BNCs are considered to be based on the infection mechanism of HBV. BNCs and HBV display the N-terminal half of the outer-membrane pre-S region of L protein (Fig. 8.1), which is postulated to have a pivotal role in the human liver-specific attachment of HBV (Kasuya *et al.*, 2008a,b). We succeeded in the continuous expression (>1 month) of the human blood clotting factor

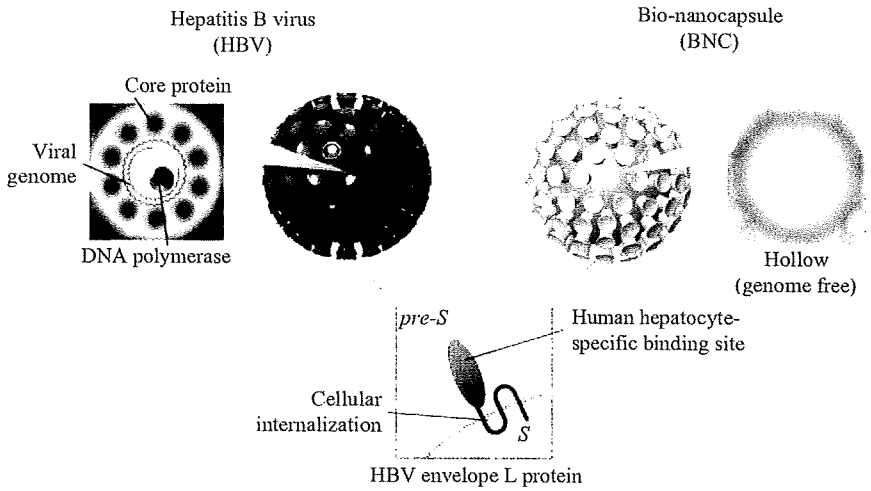

Figure 8.1 HBV and BNC (schematic).

IX gene in a mouse xenograft model by a single intravenous injection of an electroporated BNC (Yamada *et al.*, 2003). We also succeeded in obtaining a significant reduction in the size of human liver-derived tumors in a mouse xenograft model by a single intravenous injection of an electroporated BNC containing the herpes simplex virus thymidine kinase (HSV-tk) gene and sequential treatment with gancyclovir (an HSV-tk-dependent inhibitor of DNA polymerases) (Iwasaki *et al.*, 2007). Engineered BNCs, of which the human liver-specific attachment site of the pre-S region is changed to other targeting molecules (epidermal growth factor (EGF), anti-EGF receptor (EGFR) monoclonal antibody), have been shown to accumulate in nonhuman liver tissues *in vitro* and *in vivo* (Tsutsui *et al.*, 2007; Yamada *et al.*, 2003). This strongly suggests that BNCs are promising *in vivo* pinpoint DDS carriers targeting not only human liver tissue but also other tissues.

3. Second-Generation BNCs

Electroporation was used to transiently induce micropores across the lipid bilayer of BNCs. It was found that BNCs stored for long periods tended to show lower incorporation efficiency than freshly prepared BNCs. The intra- and intermolecular disulfide bonds of L proteins (14 Cys residues in three transmembrane segments of the L protein) were postulated to gradually form in a time-dependent manner, which would make BNCs

resistant to electroporation. We substituted each Cys residue with Ala or Ser by genetic modification, and identified at least eight Cys residues of the L protein unnecessary for BNC formation (Nagaoka *et al.*, 2007). The BNC harboring the eight Cys → Ala or Cys → Ser mutations showed good incorporation efficiency in an electroporation method. This method was not suitable for introducing large materials into a BNC (e.g., >20-kbp plasmid for gene therapy; >10-nm fluorescence-labeled polystyrene beads for bioimaging) and adopting the electroporation method for the good manufacturing practice (GMP)-based production of BNC-based nanomedicines would be very difficult. We recently found that the N-terminal half of the L protein possesses membrane fusogenic activity (Matsuzaki *et al.*, unpublished data). BNCs spontaneously form an ~150-nm rigid complex with LPs (BNC–LP conjugate) in which multiple BNCs are embedded on the surface of LPs (Fig. 8.2) (Jung *et al.*, 2008). This property of BNC allows incorporation of various therapeutic materials into BNC–LP conjugates as follows. First, various materials (even 40-kbp plasmid, 100-nm fluorescent polystyrene beads) are constantly incorporated into LPs by conventional methods. Second, LPs are covered with BNCs harboring tissue specificity and high infectivity by the fusogenic activity of BNC. Third, BNC–LP conjugates can deliver various materials incorporated specifically and efficiently into human liver-derived tumors in a mouse xenograft model

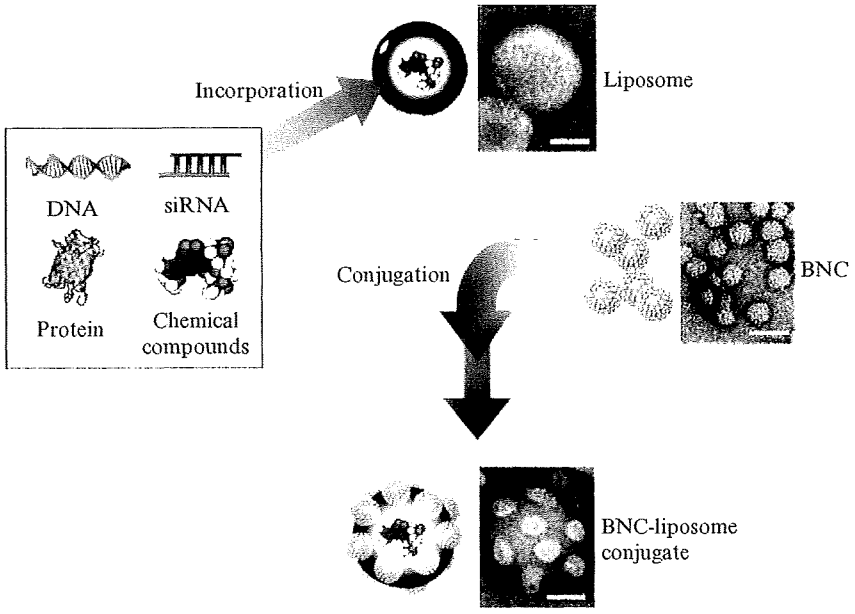

Figure 8.2 Preparation of BNC–LP conjugates (bars: 50 nm).

through intravenous injection (Jung *et al.*, 2008). Additionally, this method can be expanded to GMP-based mass production more readily than electroporation. We therefore designated the BNC–LP conjugate as a "second-generation BNC" (Kasuya and Kuroda, 2009).

With respect to the attachment of BNC–LP conjugates to human liver cells, the N-terminal half of the pre-S region has as a specific receptor for the human liver, which was demonstrated in HBV and BNCs. The intracellular drug release from BNC–LP conjugates may be mediated by the cellular internalization activity (another fusogenic activity) of the C-terminal half of the pre-S region and S region (see Fig. 8.1), as demonstrated in HBV (Glebe and Urban, 2007). In fact, an engineered BNC lacking the N-terminal half of the pre-S region can form a BNC–LP conjugate and deliver incorporated materials to target cells (Kasuya *et al.*, 2008c). BNC–LP conjugates therefore possess the advantages of LPs and viral vectors, namely the use of versatile materials (as with LP) and the HBV-derived infection and active targeting machinery. These results indicate that BNC–LP conjugates are more promising than BNC *per se* as *in vivo* pinpoint DDS carriers.

4. Retargeting of BNC–LP Conjugates

Due to the narrow tropism of HBV (because of the function of the N-terminal half of the pre-S region), systemically injected BNCs accumulate specifically in human liver-derived tissues *in vivo*. For expanding the indications of BNC-based nanomedicines, it is important to establish the methodology for retargeting BNC from human liver to nonliver tissues by substitution of the pre-S region by other targeting molecules (e.g., cytokines, growth factors, receptors, antibodies, glycans, lectins, aptamers). First, the pre-S (3–77) region is replaced with EGF by genetic engineering. The EGF-displaying BNC lost the specificity to human liver cells and obtained new specificity to EGFR-overexpressing A431 cells *in vitro* (Yamada *et al.*, 2003). This approach needs a time-consuming step for constructing the expression system and sometimes fails to achieve the high productivity of BNCs in yeast cells. Next, a large part (50–159) of the pre-S region is replaced with the *Staphylococcus aureus* protein A-derived IgG Fc-binding ZZ domain. Beyond expectation, the ZZ domain-displaying BNC (ZZ–BNC) is efficiently synthesized in yeast cells. After ZZ–BNC is mixed with anti-EGFR IgG, the mixture is injected intracranially to a glioma-transplanted mouse *orthograft* model. The anti-EGFR IgG-displaying BNC accumulated in the EGFR-overexpressing transplanted glioma in the mouse corpus striatum (Kurata *et al.*, 2008; Tsutsui *et al.*, 2007), indicating that the antibody-displaying ZZ–BNC is applicable for

in vivo use. In 2008, because the ZZ domain contains many Lys residues, ZZ–BNC was modified with *N*-hydroxysuccinimide (NHS)-biotin to display biotin molecules onto the BNC surface. The biotinylated ZZ–BNC can be used for displaying various biotinylated targeting molecules (see above) by using an avidin (e.g., streptavidin, neutravidin) as an adaptor. For instance, the *Phaseolus vulgaris* agglutinin-L_4 isolectin (L_4-PHA)-displaying BNC (PHA–BNC) has been shown to accumulate *in vivo* in highly metastatic malignant tumors which overexpress β1-6-branching *N*-acetylglucosamine (GlcNAc), a specific ligand of L_4-PHA (Kasuya *et al.*, 2008c). PHA–BNC–LP conjugate can deliver luciferase-expressing plasmid to the β1-6GlcNAc-overexpressing cells, showing that the lack of a large part of the pre-S region does not affect the formation of BNC–LP conjugates. We recently revealed that the fusogenic activity of BNC required for LP conjugation is delineated in the short sequence at the N-terminal of pre-S region, which remains in ZZ–BNC (Matsuzaki *et al.*, unpublished data). These data strongly suggested that the engineered BNCs (ZZ–BNC, biotinylated ZZ–BNC) and their LP conjugates can be used for lesion-specific pinpoint DDS carriers.

In this chapter, we describe methods to prepare BNC–LP conjugates containing DNA, and BNC–LP conjugates containing an anticancer drug (doxorubicin (DOX)), and the effects of these BNC–LP conjugates in *in vitro* and *in vivo* systems.

5. Overexpression of BNCs in *S. cerevisiae*

BNC is produced in *S. cerevisiae* AH22R$^-$ (*a leu2 his4 can1 cir$^+$ pho80*) strain (Kobayashi *et al.*, 1988). The yeast cells are transformed by the spheroplast method (Hinnen *et al.*, 1978) with the YEp plasmid pGLDLIIP39-RcT (Kuroda *et al.*, 1992), which encodes the N-terminal chicken lysozyme signal sequence-fused HBV envelope L protein (subtype *adr*) under the glyceraldehyde-3-phosphate dehydrogenase (GLD; also called TDH3) gene promoter. For production of ZZ–BNC, the DNA segment encoding the pre-S region (50–159) in the plasmid pGLDLIIP39-RcT is replaced with the DNA segment encoding the IgG Fc-binding ZZ domain derived from *S. aureus* protein A (Tsutsui *et al.*, 2007). The LEU2$^+$ yeast transformants are cultured in a synthetic selection medium 8S5N-P400 (Yamada *et al.*, 2001) at 30 °C for 7 days, harvested by centrifugation, and stored at −80 °C. The amount of BNC in yeast cells is estimated to be ~40% of total soluble proteins (Kuroda *et al.*, 1992).

5.1. Purification of BNCs by ultracentrifugation (≤10 mg protein per lot)

BNC and ZZ–BNC can be readily purified by ultracentrifugation owing to the overexpression of BNCs in yeast cells. The yeast cells (about 20 g wet weight) are disrupted with glass beads (0.5 mm in diameter, 175 ml) using a BEAD-BEATER (BioSpec Products, Bartlesville, OK, USA) in 160 ml of 0.1 *M* sodium phosphate buffer (pH 7.2) containing 7.5 *M* urea, 15 m*M* ethylenediaminetetraacetic acid (EDTA), 4 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.1 m*M* 4-amidinophenyl-methanesulfonyl fluoride (APMSF), and 0.1% (v/v) Tween 80. The crude extract obtained by centrifugation at 34,780×*g* at 4 °C for 30 min (about 6 g of protein) is mixed with PEG 6000 solution (15%, w/v at final concentration) to precipitate BNCs. The precipitants (about 3 g of protein) are subjected to CsCl isopycnic ultracentrifugation (10–40%, w/v) using an SW28 rotor (Beckman Coulter, Inc., Fullerton, CA, USA) at 24,000 rpm at room temperature for 15 h. Fractions containing BNCs are determined by sandwich enzyme-linked immunosorbent assay (ELISA) for BNC using an IMx HBsAg assay system (Abbott Laboratories, Abbott Park, IL, USA). Positive fractions are subjected twice to sucrose density gradient ultracentrifugation (10–50%, w/v) using an SW28 rotor at 24,000 rpm at room temperature for 15 h. Fractions containing BNCs are concentrated by ultrafiltration using an Amicon Ultra 100,000 NMWL (Millipore, Billerica, MA, USA) and subjected to a Sephacryl S-500 HR (GE Healthcare, Waukesha, WI, USA) gel-filtration column equilibrated with phosphate-buffered saline (PBS) containing 1 m*M* EDTA. The protein concentration of BNCs is measured by a bicinchoninic acid (BCA) assay kit (Sigma-Aldrich, St Louis, MO, USA) using bovine serum albumin (BSA) as a calibration standard. Approximately 10 mg (as a protein) of BNC is obtained from recombinant yeast cells grown in 2 l of culture medium (Yamada *et al.*, 2001).

5.2. Purification of BNCs using column chromatography

The ultracentrifugation step is rate-limiting, time-consuming, and produces a low yield, so obtaining >10 mg of BNC per lot in 1 week is difficult. Based on the heat stability of BNCs (Yamada *et al.*, 2001), a large amount of yeast crude extract can be processed immediately by heat treatment (Jung *et al.*, unpublished data). The crude extract of yeast cells is obtained by the method described earlier, and then dialyzed three times against PBS containing 1 m*M* EDTA at 4 °C for 2 h to remove urea. The crude extract (about 6 g of protein) is dispensed to 40-ml plastic tubes, incubated at 70 °C for 20 min, and then centrifuged at 34,780×*g* at 4 °C for 30 min to remove yeast-derived proteins. The supernatant (about 800 mg of protein) is subjected to a sulfate-cellulofine column (1.6 × 20 cm; Chisso Corp., Tokyo,

Japan) equilibrated with PBS containing 150 m*M* NaCl. After the stepwise elution of PBS containing 1 *M* NaCl, the fraction containing a BNC is concentrated by ultrafiltration using an Amicon Ultra 100,000 NMWL (about 120 mg of protein) and then subjected to a Sephacryl S-500 HR gel-filtration column (1.6 × 60 cm) equilibrated with PBS containing 1 m*M* EDTA. About 60 mg of the highly purified BNC is obtained from the yeast cells grown in 2 l of culture medium.

5.3. Purification of ZZ–BNC using column chromatography

Porcine IgG is precipitated from porcine serum (Sigma-Aldrich) with $(NH_4)_2SO_4$ (40% saturation), dissolved in PBS, and dialyzed against PBS at 4 °C for 48 h. The dialyzed solution is mixed with two-times volume of 60 m*M* acetate buffer (pH 4.8) and then mixed with *n*-caprylic acid to achieve a final concentration of 6.8% (v/v). After incubation at room temperature for 30 min with gentle stirring, the supernatant containing purified IgG is obtained by brief centrifugation. Purified porcine IgG is conjugated to an NHS-activated Sepharose 4B Fast Flow (GE Healthcare) column according to manufacturer's instructions. The IgG-conjugated Sepharose column is equilibrated with PBS.

Preparation of the crude extract of yeast and heat treatment are the same as the BNC protocol. The supernatant is subjected to the porcine IgG-conjugated Sepharose column (affinity chromatography) to capture ZZ–BNC specifically using the IgG–ZZ domain interaction. The column is washed extensively with 75 m*M* Tris–HCl (pH 7.2) containing 10 m*M* NaCl, and then ZZ–BNC is obtained by the stepwise elution of 10 m*M* Tris–HCl (pH 7.2) containing 3.5 *M* NaSCN, 500 m*M* NaCl, and 10 m*M* EDTA. Fractions containing ZZ–BNC are subjected to a Sephacryl S-500 HR gel-filtration column (1.6 × 60 cm) equilibrated with PBS containing 1 m*M* EDTA. About 30 mg of highly purified ZZ–BNC can be obtained from yeast cells grown in 2 l of culture medium.

6. Conjugation of BNCs with LPs

The LPs used for BNC conjugation (Fig. 8.2) can be prepared by conventional methods such as solvent evaporation (Bangham and Horne, 1964), ethanol injection (Batzri and Korn, 1973), and reverse-phase evaporation (Szoka and Papahadjopoulos, 1978). The lipid composition of LPs should be optimized for the materials to be incorporated, for example, cationic lipids for the preparation of DNA- or siRNA-containing LPs (i.e., lipoplexes; Chapter 14 of volume 465, Düzgüneş *et al.*, 2002; Li and Huang, 2006), and anionic/neutral lipids for chemical compounds

containing LPs. To our knowledge, all LPs that have been employed have resulted in the formation of BNC–LP conjugates.

6.1. Example 1.1: Preparation of BNC–LP conjugates containing DNA (BNC–lipoplex conjugates)

For preparation of a DNA–LP complex (lipoplex), we routinely use commercially available lyophilized cationic LPs containing *O,O'*-ditetradecanoyl-*N*-(α-trimethylammonioacetyl)diethanolamine chloride (DC-6-14; Kikuchi *et al.*, 1999) as the major cationic lipid, an essential component for the cellular uptake of DNA, for example, Coatsoame-EL-01-D (NOF, Tokyo, Japan) and LipoTrust series (Hokkaido System Science, Sapporo, Japan). In the case of Coatsome-EL-01-D, 1.5 mg of lyophilized LP (as lipids) is dissolved in 1 ml of 250 μg/ml luciferase (LUC) expression vector pGL3 (Promega, Madison, WI, USA) and incubated for 15 min to allow lipoplex formation. Aliquots of lipoplex (100 μg LP, 16.7 μg DNA) are mixed with freeze-dried BNC (100 μg as protein) and incubated at room temperature for 15 min. By changing the amount of LP used for 16.7 μg DNA, a series of lipoplex and BNC–lipoplex conjugates are prepared with an N/P ratio (molar ratio of nitrogen-atom content in cationic lipids to phosphorous-atom content in plasmid DNA) from 0.3 to 2.4. The *z*-averaged sizes and ζ-potentials of lipoplex and BNC–lipoplex conjugate are measured at 25 °C using a Zetasizer Nano-ZS (Malvern Instruments Ltd., Worcestershire, UK). When the content of cationic lipids in lipoplex is increased, *z*-averaged sizes and ζ-potentials are apt to increase to >1 μm and >0 mV, respectively, in accordance with the increase in N/P ratio (Fig. 8.3A). BNCs are found to keep the sizes of BNC–lipoplex conjugates to <200 nm and ζ-potentials negative.

6.2. Example 1.2: *In vitro* transfection with BNC–lipoplex conjugates

About 1×10^5 of human cells (hepatocarcinoma HepG2, colon carcinoma WiDr, cervical carcinoma HeLa) are seeded on a 24-well cell culture plate (Iwaki, Tokyo, Japan) and incubated for 24–48 h in a humidified atmosphere at 37 °C in 5% (v/v) CO_2. Aliquots of BNC–lipoplex conjugates containing pGL3 (N/P ratio = 1.4; 100 μg LP, 16.7 μg DNA, and 100 μg BNC (as protein)) are diluted with complete cell culture medium containing 10% (v/v) fetal bovine serum and antibiotics, and applied to each well with 1 μg/ml (as protein of BNC, 0.5 μg/well) at final concentration. The medium is exchanged after incubation for 3–6 h to avoid nonspecific cellular uptake, and then incubated for 48 h to allow LUC expression.

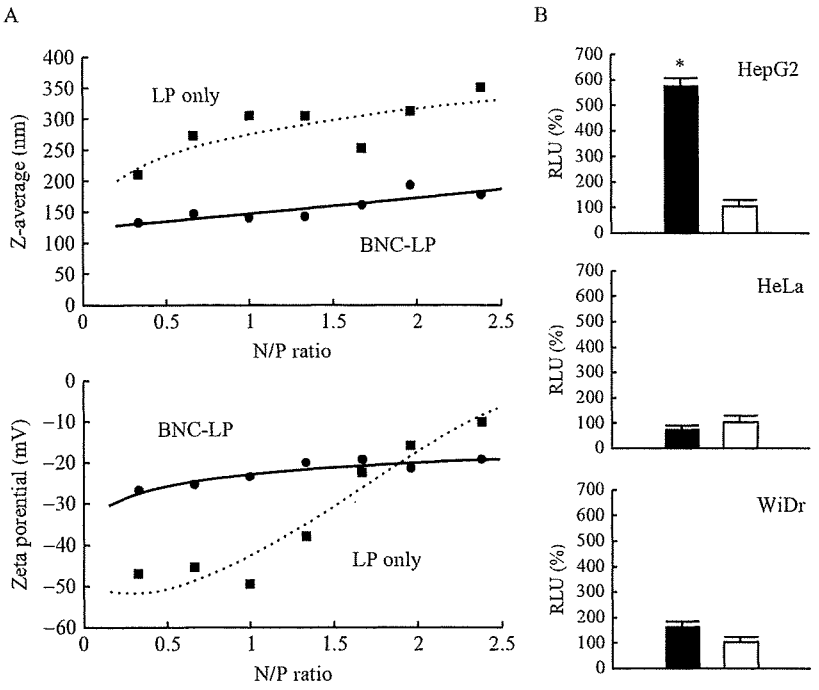

Figure 8.3 Effect of BNC conjugation on lipoplex (DNA-containing LPs). (A) z-averaged sizes (upper panel) and ζ-potentials (lower panel) of BNC–lipoplex conjugates (*circles* with *solid line*) and lipoplex (*squares* with *dashed line*) at the indicated N/P ratio. (B) *In vitro* transfection with LUC expression plasmid by BNC–lipoplex (*closed bars*) and lipoplex (*open bars*). LUC activities in cell lysates are measured 48 h after transfection. Mean ± S.D. ($n = 9$). $^*p < 0.05$.

After lysis with passive lysis buffer (Luciferase Assay Kit, Promega), intracellular activities of LUC can be measured by a GloMax 20/20n Luminometer (Promega) using the LUC substrate luciferin. Human liver-derived HepG2 cells transfected with BNC–lipoplex conjugates containing pGL3 showed approximately five times higher LUC expression than those transfected with lipoplex containing pGL3, whereas human nonliver-derived HeLa cells and WiDr cells showed no significant differences in LUC expression between BNC–lipoplex conjugates and lipoplexes (Fig. 8.3B). These data indicated that the BNC–lipoplex conjugates deliver the LUC plasmid to human liver cells specifically *in vitro*. This prompted us to examine if BNC–lipoplex conjugates are more suitable than conventional lipoplexes for an *in vivo* pinpoint gene delivery system.

6.3. Example 1.3: *In vivo* transfection with BNC–lipoplex conjugates

Xenograft mouse models bearing tumors on their backs are generated using nude mice (5 weeks, male, BALB/c-nu/nu; CLEA Japan, Tokyo, Japan) under guidelines of the Institute of Scientific and Industrial Research, Osaka University, Japan. The cancer cell lines used are NuE and Huh-7 (human hepatocarcinoma), WiDr (human colon carcinoma), A431 (human epithelial carcinoma), PC12 (rat adrenal pheochromocytoma), and MDA-MB-435 (human breast carcinoma). About 1×10^6 cells are mixed with 50 μl of BD Matrigel Matrix HC (BD Biosciences, Bedford, MA, USA) and subcutaneously injected into the back of mice. After 2–4 weeks, mice possessing a tumor of appropriate diameter 1 cm can be obtained.

For the delivery of green fluorescent protein (GFP)-expression plasmid pEGFP-C1 (Clontech, Mountain View, CA, USA), BNC–lipoplex (N/P ratio = 1.4; 100 μg LP, 16.7 μg pEGFP-C1, and 100 μg BNC (as protein)) are intravenously injected to each tumor-bearing mouse. At 1 week after injection, mice are killed, and tumors and tissues (liver, lung, spleen, brain, heart, kidney) are isolated. Tissues and tumors are embedded in plastic resin or cryomold, cut into sections of 5 μm thickness, and observed using fluorescent microscopy or confocal laser scanning microscopy. GFP expression can be observed in only human liver-derived tumors, not in other tumors and tissues (Jung *et al.*, 2008).

6.4. Example 2.1: Preparation of BNC–LP conjugates containing DOX

Stock solutions of dipalmitoylphosphatidylcholine (DPPC, NOF), dipalmitoylphosphatidylethanolamine (DPPE, NOF), dipalmitoylphosphatidylglycerol sodium (DPPG-Na, NOF) are prepared in a chloroform/methanol (2:1, v/v) mixture at a concentration of 50 m*M*. A stock solution of cholesterol (Chol, NOF) is prepared in the same chloroform/methanol mixture at a concentration of 200 m*M*. All stock solutions are stored at −80 °C and prewarmed to 60 °C before use. Phospholipids and cholesterol (DPPC:DPPE:DPPG-Na:Chol = 15:15:30:40 molar ratio) are dissolved in the chloroform/methanol mixture in a round-bottomed flask, and then evaporated at 60 °C by utilizing a rotary evaporator to produce a thin hemispherical lipid film. The film is hydrated in buffer containing 10 m*M* HEPES (pH 4.0) and 120 m*M* $(NH_4)_2SO_4$ at 60 °C. The freeze–thaw cycle is repeated five times. The crude solution of LP is subjected to a Lipex extruder (Northern Lipids, Vancouver, BC, Canada) equipped with a polycarbonate filter (pore size, 200 nm) at 60 °C five times, followed by seven times with a filter (pore size, 50 nm). The z-average size of LP is

measured by dynamic light scattering at 25 °C using a Zetasizer Nano-ZS. The LPs are subjected to a Sephadex G-25 (GE Healthcare) gel-filtration column equilibrated with 10 m*M* HEPES buffer (pH 7.4) containing 100 m*M* NaCl and 3.4% (w/v) sucrose. Total lipid concentration is calculated from that of DPPC, which is determined by a Laboassay Phospholipid Kit (Wako, Osaka, Japan) utilizing choline oxidase and [*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline] (DAOS) (Takayama *et al.*, 1977). To introduce DOX (also called adriamycin; Wako) into LPs by a remote-loading method, 200 μl of 10 mg/ml DOX–HCl solution is added to 15 mg of LP solution (as total lipids, prewarmed at 60 °C), and then incubated for 20 min at 60 °C with gentle stirring. After free DOX is removed by a Sephadex G-25 gel-filtration column, LPs containing DOX (about 2 mg DOX–HCl per 15 mg total lipid) can be obtained.

Aliquots of LPs containing DOX (2 mg LP, 0.27 mg DOX–HCl) are gradually added to freeze-dried BNC (100 μg as protein) and incubated at room temperature for 15 min to form a BNC–LP conjugate containing DOX. The diameter of the conjugate is estimated to be ~140 nm by the dynamic laser scattering method.

6.5. Example 2.2: *In vitro* cytotoxic effects of BNC–LP conjugates containing DOX

About 5×10^3 cells (HepG2, Huh-7, MDA-MB-435) cultured on a 96-well plate for 24–48 h are incubated with BNC–LP conjugates containing DOX at appropriate concentration (1–100 μg/ml as DOX) for 6–24 h and then incubated for 48–72 h in fresh complete medium. Cellular viability can be measured by the formation of formazan from a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a tetrazole probe using a CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega). The strong cytotoxicity of BNC–LP conjugates containing DOX, comparable to that of DOX itself, can be observed in only human liver-derived HepG2 and Huh-7 cells, not in human nonliver-derived MDA-MB-435 cells (Fig. 8.4A). When comparing the cytotoxicity of BNC–LP conjugates with that of LP, BNC–LP conjugates showed strong cytotoxicity only toward HepG2 cells and Huh-7 cells, whereas LP did not show severe cytotoxicity to any cells. The half maximal inhibitory concentration (IC_{50}) of BNC–LP containing DOX for Huh-7 cells is 12 μg/ml, whereas that of LP containing DOX is 100 μg/ml. Doxil, a commercial PEGylated LP containing DOX (Markman, 2006), also showed a cytotoxicity curve similar to that of LP containing DOX. These data indicated that BNCs confer a human liver-specific active targeting function to LPs containing DOX.

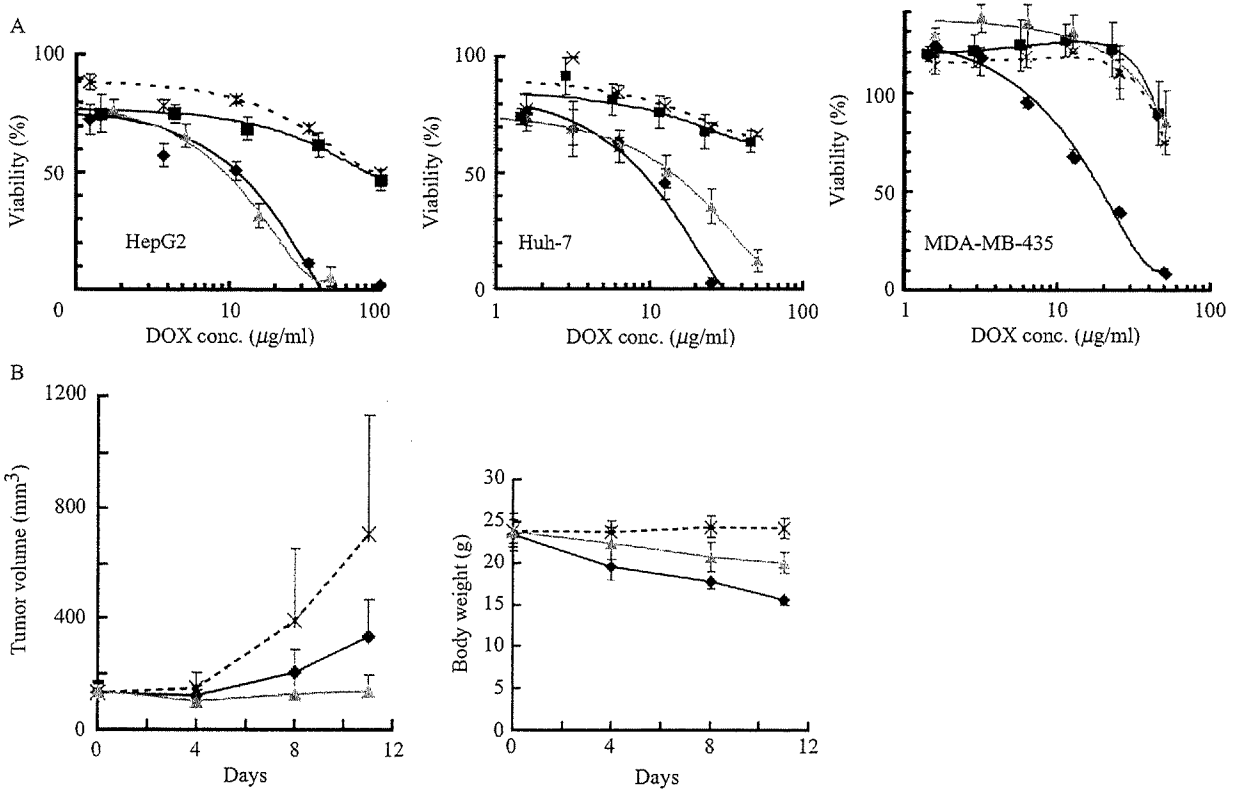

Figure 8.4 Effect of BNC conjugation on LP containing DOX. (A) *In vitro* cytotoxicity curves of BNC–LP conjugates. BNC–LP conjugates containing DOX (*triangles* with *gray lines*), LP containing DOX (*squares* with *solid lines*), DOX alone (*diamonds* with *solid lines*), and doxil (*crosses* with *dashed lines*). HepG2 cells (left panel), Huh-7 cells (center panel), and MDA-MB-435 cells (right panel). Mean ± SD ($n = 9$). (B) *In vivo* tumor suppression by BNC–LP conjugates. BNC–LP conjugates containing DOX (*triangles* with *gray lines*), DOX alone (*diamonds* with *solid lines*), and untreated control (*crosses* with *dashed lines*). Tumor volume (left panel) and body weight (right panel). Mean ± S.D. ($n = 9$).

6.6. Example 2.3: *In vivo* therapeutic effects of BNC–LP conjugates containing DOX

To the mouse xenograft model bearing Huh-7-derived tumors (about 1 cm in diameter), about 6 mg/kg (as DOX–HCl) of BNC–LP conjugates containing DOX is intravenously injected every 4 days under 10 ml/kg conditions. Largest (a) and smallest (b) superficial diameters of the tumor are measured every day, and tumor volume (V) is calculated as $V = ab^2/2$. BNC–LP conjugates containing DOX efficiently suppressed tumor growth, but identical amounts of DOX injected by LP only and injected alone failed to suppress it (Fig. 8.4B). As for toxicity, mice given about 8 mg/kg (as DOX–HCl) every 4 days by a BNC–LP conjugate showed less loss of body weight than those by DOX alone. A significant change in weight was not observed in tissues (liver, spleen, kidney, heart) 12 days after the first injection (data not shown). These data indicated that BNC–LP conjugates containing DOX possess not only active targeting machinery but also less toxicity.

When a mouse xenograft model bearing Huh-7-derived tumors received a single intravenous injection of BNC–LP conjugate containing DOX, LP containing DOX, doxil (PEGylated LP containing DOX), and DOX alone (8 mg/kg as DOX), blood is collected from the tail vein at 0, 30, 60, 120, and 180 min after the first injection. Plasma is collected by centrifugation at 3000 rpm at 4 °C, and DOX is extracted with a five volumes of the chloroform/methanol (4:1, v/v) mixture. After evaporation of solvent with nitrogen gas, the dried DOX fraction is subjected to high-performance liquid chromatography (HPLC) equipped with an octylsilanized gel column (Nucleosil 100 5C-18, Chemco Scientific Co. Ltd., Osaka, Japan) equilibrated with a 20 mM PBS/methanol mixture (25:75, v/v) containing 40 mM sodium heptane sulfonate. Flow rate is 1 ml/min, and DOX-derived fluorescence (excitation, 475 nm; emission, 554 nm) is determined using purified DOX as the internal standard. The initial concentration of DOX (0 min) in plasma is about 100 μg/ml in all mice (Fig. 8.5). The plasma concentration of DOX after 60 min in mice injected with a BNC–LP conjugate containing DOX is much higher than those with LP containing DOX and DOX alone, which is comparable to those with doxil. BNC conjugation, like PEGylation, enhances the robustness of LP against the RES.

7. Preparation of Antibody-Displaying BNC–LP Conjugates

ZZ–BNC–LP conjugates are prepared using ZZ–BNC instead of BNC by the method described earlier for *in vitro* and *in vivo* retargeting of BNC–LP conjugates according to antibody affinity. LP conjugation must