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Nanoparticles for human liver-specific drug and gene delivery systems: *in vitro* and *in vivo* advances

Takeshi Kasuya & Shun'ichi Kuroda[†]
Osaka University, Institute of Scientific and Industrial Research, Department of
Structural Molecular Biology, Ibaraki, 567 – 0047, Osaka, Japan

A wide variety of nanoparticles (NPs) that can deliver incorporated therapeutic materials such as compounds, proteins, genes and siRNAs to the human liver have been developed to treat liver-related diseases. This review describes NP-based drug and gene delivery systems such as liposomes (including lipoplex), polymer micelles, polymers (including polyplex) and viral vectors. It focuses upon the modification of these NPs to enhance liver specificity or delivery efficiency *in vitro* and *in vivo*. We discuss recent advances in drug and gene delivery systems specific to the human liver utilizing bio-nanocapsules comprising hepatitis B virus (HBV) envelope L protein, which has a pivotal role in HBV infection. These NP-based medicines may offer novel strategies for the treatment of liver-related diseases and contribute to the development of nanomedicines targeting other tissues.

1. Introduction

The liver is an essential organ because it metabolizes various waste products. It is protected by a major immune defense system called the reticulo-endothelial system (RES); macrophage-like Küppfer cells from the RES capture micro- and nano-scaled materials from the bloodstream in the liver [1,2]. Liver dysfunction can cause hepatitis, cirrhosis, hepatocellular carcinoma (HCC), hyperlipemia, hyperuricemia, type II diabetes and infarction. Drug delivery systems (DDS) and gene delivery systems (GDS) based on microparticles and nanoparticles (NPs) are considered promising candidates for treating these liver diseases. NPs (i.e., conventional anionic liposomes) were first used for DDS in the mouse liver for metal poisoning in 1973 [3]. The surface of NPs were not modified to enable liver specificity (hepatophilicity), but NPs passively accumulated in the liver due to the RES and exhibited acceptable therapeutic effects. Recent studies have highlighted two major problems that must be solved in order to develop conventional NPs as liver-specific 'nanomedicines': i) NPs are incorporated not by target hepatocytes, but presumably by Küppfer cells, leading to mistargeting of therapeutic materials at the cellular level in the liver; and ii) NPs are poorly directed to the cytoplasm yet actively directed to endosomes and lysosomes by the endocytosis pathway, which also leads to mistargeting at the intracellular level and may attenuate the effects of therapeutic materials. NPs have recently been modified to efficiently deliver incorporated drugs and genes to the cytoplasm of hepatocytes by overcoming these problems.

In this review, we discuss conventional NP-based DDS and GDS from the viewpoint of human liver-specific targeting at the cellular and intracellular levels. We introduce recent advances in bio-nanocapsule (BNC) technology [4], which could deliver drugs and genes actively to the cytoplasm of human hepatocytes by utilizing the infection mechanism of the hepatitis B virus (HBV).

2. Indications for liver-specific DDS- and GDS-based nanomedicines

The worldwide incidence of liver-related diseases is high. These include hepatitis, cirrhosis and HCC caused by chronic infection by HBV and hepatitis C virus (HBC), alcohol abuse and adiposis [5]; hyperlipemia by a genetic defect of low-density lipoprotein (LDL) receptors [6]; hemophilia by a genetic defect of blood clotting Factor XIII or IX [7]; intra-hepatic cholestasis by a genetic defect of bile transporters; and Wilson's disease (accumulation of copper ions) by a genetic defect of ATPase. Several drugs are available for treatment of these diseases, but liver-specific DDS and GDS may enhance efficacy and reduce the side effects of these drugs. Liver-specific GDS would facilitate 'protein supplementation therapy' for various diseases even if they were not related to the liver because the latter is thought to synthesize the therapeutic proteins ectopically *in vivo*.

3. Overview of conventional NP carriers for DDS and GDS

Many NPs have been developed as DDS and GDS carriers to maximize the therapeutic effects and minimize the side effects of incorporated therapeutic materials (**Figure 1**). The following properties (see also **Table 1**) should be optimized for the *in vivo* use of conventional NPs as DDS and GDS carriers.

i) *Acceptability to versatile payloads*: The NP carrier should incorporate various therapeutic materials (compounds, proteins, genes, siRNAs) in a similar way. This facilitates the development of a general purpose carrier and concurrent administration of mixed drugs.

ii) *Low or no toxicity*: The NP carrier should avoid the incorporation and use of potentially dangerous materials (e.g., viral genomes).

iii) *Low immunogenicity*: The NP carrier should neither induce anaphylaxes nor elicit neutralizing antibodies, even after long-term administration.

iv) *Stealth*: A NP carrier injected systemically should escape from the RES efficiently. Capture by the liver RES may contribute to liver-specific targeting but target cells are not hepatocytes but Küppfer cells, leading to mistargeting at the cellular level in the liver.

v) *Active targeting*: The NP carrier should recognize and attach target cells and tissues by a sensor molecule

displayed on its surface. NP carriers of about 100 nm in size are likely to accumulate spontaneously in tumors after systemic injection, but this passive targeting mechanism based on the enhanced permeability and retention (EPR) effect [8] is excluded from these criteria.

vi) *Appropriate size*: The size of NP carriers should be about 40 – 150 nm in diameter. Too small NPs (< 40 nm) and too large NPs (> 150 nm) are non-specifically removed from blood circulation by the function of kidney and RES in the liver, respectively [9]. NPs of about 40 – 150 nm could be used to target both tumors utilizing the EPR effect [8] and hepatocytes bypassing through the fenestrae in liver endothelial cells [10].

vii) *Appropriate surface charge*: The surface charge of NPs is known to affect severely the stability and biodistribution of systemically administrated NPs. For ideal delivery, the surface of NPs should be optimized so as not to be entrapped by unexpected tissues. For example, one positively charged NP (i.e., polyplex of polyethyleneimine and DNA) was efficiently accumulated in the lung after systemic administration [11].

viii) *Efficient cell-penetrating activity*: The NP carrier should possess cell-penetrating activity for active and rapid intrusion across the plasma membrane or the endosomal membrane of target cells and tissues because many therapeutic materials (particularly genes and siRNAs) function intracellularly.

ix) *Mechanism of intracellular targeting*: The NP carrier in target cells should bring and release payloads to the intracellular destination precisely (e.g., genes and siRNAs should be released in the nucleus and cytoplasm, respectively, not in endosomes).

x) *High productivity*: The NP carrier should be synthesized levelling large amounts easily and safely. Any infection risks to manufacture and environment should be avoided.

3.1 Liposomes
Liposomes, single- or multi-lamellar nano-scaled hollow structures composed of a lipid bilayer, were discovered by Bangham *et al.* in 1964 [12]. Many innovations in the development of liposome-based DDS and GDS carriers have been made because liposomes can potentially incorporate hydrophilic drugs inside and hydrophobic drugs between the lipid bilayer (**Figure 1**, panel A) [13]. Several liposomal drugs have been commercialized for the treatment of cancers and fungal infection (e.g., Doxil® (Johnson & Johnson, USA [14]), Myocet® (Sopherion Therapeutics, USA [15]), DaunoXome® (Gilead Sciences, USA [16]), Ambisome® (Gilead Sciences, USA [17])). Liposomes can be produced simply with phospholipids, which are components of cellular membranes. Liposomes by itself show low toxicity and antigenicity in humans. Size, surface charges (ζ-potentials), stability in blood and the internal environment
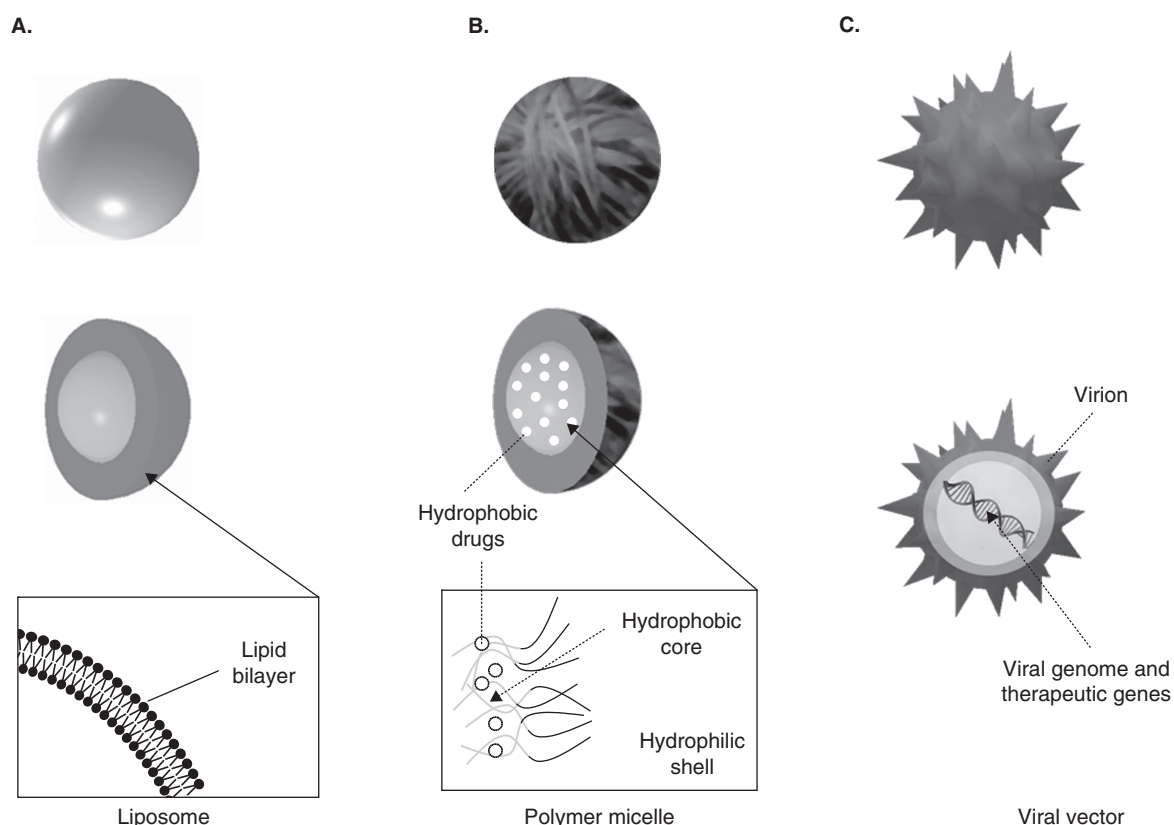
Figure 1. Conventional nanoparticles (NPs) for DDS and GDS. A. Liposomes composed of a lipid bilayer can incorporate hydrophilic drugs inside and hydrophobic drugs between lipid layers. **B.** Amphiphilic polymers spontaneously form a core–shell structure consisting of a hydrophobic core and hydrophilic shells, and can incorporate various hydrophobic drugs inside the hydrophobic cores. **C.** Viral vectors can deliver therapeutic genes (DNA or RNA) along with their genome at high efficiency owing to their infection mechanism.

Table 1. Comparison of NP-based DDS and GDS carriers.

Properties	Liposomes	Polymer micelles	Viral vectors	Bio-nanocapsules‡
GDS use	Yes	Yes	Yes	Yes
Controlled release	Yes	Yes	No	Possible
Toxicity	Low	Low	Medium–High*	Low
Antigenicity	Low	Low–Medium	Medium–High*	Low–Medium
Stealth	Low/High (with PEG)*	Low/High (with PEG)*	Low–Medium	Medium/High (with PEG)
Active targeting	No*	No*	No*	Yes (human liver)
Retargeting to other tissues	Possible	Possible	Difficult*	Possible
Cell-penetrating activity	No (applicable)*	No (applicable)*	Yes	Yes
Intracellular targeting	No (dependent on)	No (dependent on)	Yes	No (dependent on)
		Property of payload	Property of payload	Property of payload
Productivity	High	High	Low*	Medium-High

*Critical disadvantage.
‡In the *sensu strico* meaning, that is HBV envelope L particles.

are controllable by optimizing lipid formulation and surface treatment; a wide range of materials can therefore be stably incorporated into liposomes. For instance, liposomes composed of cationic phospholipids could incorporate nucleic acids (lipoplex formation) and deliver them to target cells *in vitro* [18]. For *in vivo* delivery, stealth (see above) can be added to liposomes by displaying the polyethylene glycol (PEG) moiety [19]. Recently, the systemic administration of lipoplex formation has been shown to induce innate immune responses and cytokine production [20-22], which would be reduced by PEGylation. Liposomes should be further modified to harbor an active targeting mechanism, efficient cell-penetrating activity and an intracellular targeting mechanism for development as general purpose carriers for *in vivo* DDS and GDS.

3.2 Polymer micelles

When amphiphilic block copolymers are suspended in aqueous solution containing hydrophobic compounds (e.g., anticancer drugs), they spontaneously assemble into nano-scaled plugged structures (polymer micelles) [23] comprising a hydrophobic core containing hydrophobic compounds and a hydrophilic shell (**Figure 1**, panel B). Polymer micelles using biodegradable copolymers were recently used to deliver hydrophobic anticancer drugs to tumors specifically by the EPR effect *in vivo*, and polymer micelles achieved controlled release of drugs in tumors longer than conventional liposomes [24,25]. Cationic polymer micelles such as polyethyleneimine and poly-L-lysine were also utilized for condensation (polyplex formation) and *in vitro* delivery of not only DNAs, [26,27] but also siRNAs [28]. The properties of polymer micelles are similar to those of liposomes (**Table 1**), so some polymer micelles-based nanomedicines have been developed as anticancer drugs harboring the *in vivo* tumor targeting utilizing EPR effect. These agents are currently in clinical trials in Japan, Europe and the USA.

3.3 Viral vectors

Various species of viruses have been developed as gene vectors but adenovirus [29], adeno-associated virus [30] and retrovirus [31] are used for gene therapy. These viruses (**Figure 1**, panel C) can efficiently attach to the surface of various mammalian cells and deliver viral genome into the nuclei or cytoplasm of target cells, thereby accomplishing highly efficient infection to transduce genetic information [32,33]. Recombinant viruses harboring an expression unit of therapeutic proteins or short-hairpin RNAs (shRNAs; for repressing harmful gene expressions) have therefore been utilized for gene therapy. When injected systemically, these viruses tended to accumulate in the liver by the action of the RES, but serious side effects were occasionally observed in patients (presumably by mistargeting of these vectors) [34,35]. Overexpression of some shRNAs in mice livers by the adeno-associated virus was found to kill the mice by oversaturation of endogenous

small RNA pathways [36]. Conventional viral vectors harboring efficient cell-penetrating activity and an intracellular targeting mechanism (see above) are considered to be promising GDS carriers, although they have certain disadvantages: i) unacceptability as DDS carriers; ii) toxicity derived from the viral genome; iii) high immunogenicity; iv) less stealth; v) fewer active targeting mechanisms; and vi) poor productivity. Viral vectors must jettison viral genetic compartments and diminish these disadvantages.

4. Obstacles to the development of liver-specific NP-based medicines

Most NPs used for DDS and GDS are 50 – 150 nm in diameter. When they are injected (i.v.) into normal animals without surface modifications, most NPs spontaneously and immediately (half-life [$T_{1/2}$] in the bloodstream is usually < 15 min) accumulate in the liver by the action of the RES, and the remainder settle in RES-rich tissues (e.g., spleen, kidney, lung). The destination of NPs in the liver is not target hepatocytes but Küppfer cells, which decompose therapeutic materials by macrophagocytosis, so this passive liver-targeting strategy is not appropriate for NP-based delivery of therapeutic materials. The NP surface must therefore be modified with a PEG moiety (PEGylation) [14,37] to inhibit binding of plasma proteins (opsonization) to consequently reduce non-specific entrapment by macrophagocytosis. PEGylation has become the general strategy for i.v. administration of high-molecular-weight medicines (therapeutic proteins) to be stabilized in the bloodstream [14,38,39]. Some PEGylated proteins were reported to lose targeting activity by PEG-derived steric hindrance. These proteins must substitute cleavable PEG for conventional PEG [40-42]. Repetitive administration of PEGylated materials was found to elicit anti-PEG IgM antibody, and induce accelerated blood clearance (ABC) [43,44]. These side effects of PEGylation led us to develop another strategy for adding stealth to NPs (see Section 8, below).

The second obstacle is that cellular uptake of NPs is often driven by an endocytosis pathway, as follows: i) cellular association of NPs; ii) internalization of NPs into endosomes; iii) exocytosis of some NPs by recycling endosomes; iv) endosomal escape of some NPs (rate-limiting step); v) degradation of some NPs in lysosomes; and vi) release of payloads into the cytoplasm. Some viral vectors possess strong endosomal escape functions to aid transfer of the viral genome from endosomes to nuclei, but most therapeutic materials delivered by conventional DDS are not actively directed to the cytoplasm [33]. To enhance the therapeutic effect of DDS and non-viral GDS, NPs are designed to escape the endosomal cascade by utilizing the pH gradient in endosomes (e.g., pH-dependent fusogenic lipids for liposomes [45], and polycationic polymers that rupture endosomal vesicles in response to low pH [46]).

5. *In vivo* targeting strategy for liver-specific NP-based medicines

Although SNALP (stable nucleic acid lipid particles) – cationic liposomes encapsulating siRNA without any targeting mechanism – showed great gene silencing effect in the liver [47], new liver-specific NP-based medicines should generally harbor an *in vivo* targeting mechanism. The sensor molecule on the surface of the NP recognizes the unique molecule of hepatocytes from the bloodstream, attaches to the surface of hepatocytes (not of other hepatic cells) and induces efficient cellular uptake of NPs.

A well-studied hepatic receptor is the asialoglycoprotein receptor (ASGPR) [48]. It is a type of lectin that recognizes sugar residues (e.g., galactose, lactose) and incorporates sugar-modified proteins into hepatocytes by the endocytosis pathway. Wu *et al.* [49] first demonstrated *in vivo* ASGPR-specific gene transfer in rat hepatocytes by using the DNA polyplex of asialomucoid (containing galactose residues)-conjugated poly-L-lysine. This glycoprotein-conjugated polyplex approach has been applied to various NP-based DDS and GDS carriers [50]. The liposome displaying asialofetuin possessing three N-linked glycans containing N-acetyllactosamine residues can deliver genes to mouse hepatocytes through intraportal injection *in vivo* [51]. The liposome displaying vitamin E-labeled asialofetuin can also deliver therapeutic genes to mouse hepatocytes through i.v. injection, and can show therapeutic effects on CCl_4-induced acute liver injury [52]. The siRNA dynamic polyconjugates labeled with *N*-acetylgalactosamine, designed to be degraded in acidic conditions (endosome), could efficiently deliver siRNA to the cytoplasm of mouse hepatocytes by systemic administration [53]. Polymer micelles can be similarly retargeted to mouse hepatocytes *in vivo* by conjugation with galactose [54]. It has recently been considered that low molecular weight glycans and glycolipids are appropriate for the retargeting of NPs because of their lower immunogenicity, as demonstrated in a spleen-specific liposome using mono-sialoganglioside GM1 [55].

The folate receptor would be useful for *in vivo* targeting of NPs to hepatocytes. It was shown that folate-conjugated PEGylated liposome accumulates in the mouse liver through i.v. injection because the receptor is highly expressed in the liver as well as in tumors [56,57]. Naturally occurring hepatophilic proteins and chylomicrons remnants [58] (e.g., LDL, high-density lipoprotein [HDL]) could be used for the retargeting of NPs to the liver, as demonstrated in the liver-specific DDS of antimalarial drugs [59]. When NP-based medicines require retargeting to other hepatic cells except hepatocytes, the mannosyl moiety and vitamin A moiety are actively recognized by Küppfer cells [60] and satellite cells [61], respectively, in animal models.

These retargeting strategies have been applied only on non-viral NPs (mainly liposomes), facilitating the *in vivo* active liver-specific targeting of NPs. Viral NPs intrinsically possess efficient cell-penetrating activity (**Table 1**) but it is difficult for them to synchronize additional targeting activity with endogenous cell-penetrating activity. Nevertheless, promising liver-specific NPs should accomplish precise *in vivo* targeting and high transfection efficiency regardless of their origins.

6. Development of BNC for liver-specific DDS and GDS

6.1 What is a BNC?

The term bio-nanocapsule (BNC) was first used by our research group in 2003 [4,62]. *Sensu lato* means 'a nano-scaled capsule consisting of proteins produced by biotechnological techniques'. We preliminarily propose that the BNC family can be classified into at least three groups based on protein content.

6.1.1 BNCs exclusively containing proteins

Some viral capsid proteins (e.g., HBV core proteins [63]) are known to assemble into nanocapsules (20 – 30 nm in diameter) spontaneously by protein–protein interactions of capsid monomers without other components. These capsules possess a rigid and inflexible structure, and the internal volume is smaller than that of other NPs. Viral capsid proteins can show strong immunogenicity. These BNCs are therefore considered inappropriate for use as DDS and GDS carriers.

6.1.2 BNCs containing < 50% (w/w) proteins and > 50% (w/w) phospholipids

The hemagglutinating virus of Japan (HVJ; also known as 'Sendai virus') envelope (HVJ-E) vector consists of ~ 70% phospholipids and ~ 30% transmembrane protein complex (HN and F proteins indispensable for HVJ infection) and is a size-flexible nanocapsule. HVJ-E vector can be prepared from purified HVJ by ultraviolet (UV) treatment, which eliminates the viral RNA genome [64]. The vector possesses HVJ-derived strong membrane fusion activity, so it can incorporate various materials (drugs, genes) by liposome fusion and deliver them to various cells efficiently in a non-specific manner. Confirming complete removal of viral genome from HVJ-E preparations is difficult. Strong immunogenicity is another disadvantage of this vector. Retargeting the vector to specific cells and tissues *in vivo* has not been achieved. These problems should be solved in order to expand the possibilities of this promising vector in clinical medicine.

6.1.3 BNCs containing > 50% (w/w) proteins and < 50% (w/w) phospholipids

HBV is a 42-nm enveloped DNA virus (**Figure 2**) that specifically infects the human liver and causes severe diseases (e.g., hepatitis, cirrhosis and cancer). To prevent blood-mediated transmission of HBV, the envelope S
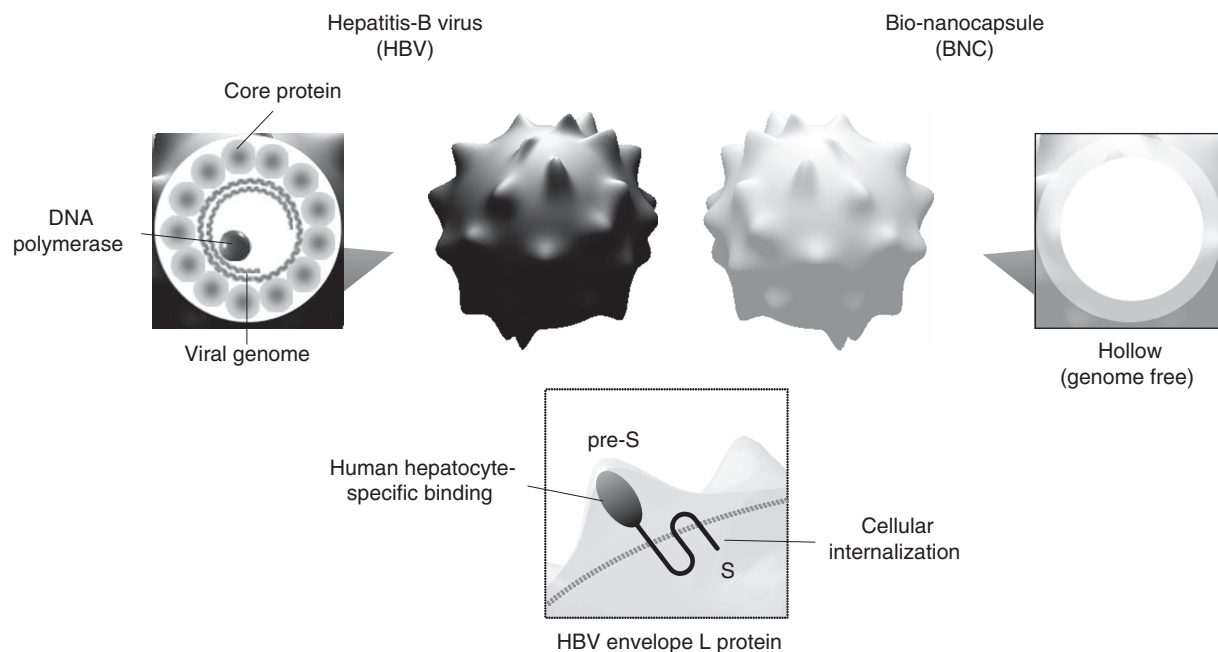
Figure 2. Comparison of hepatitis B virus (HBV) and bio-nanocapsule (BNC). HBV is an encapsulated DNA virus consisting of envelope protein, core protein, DNA polymerase and viral DNA genome. BNC is a hollow subviral particle consisting of HBV envelope L proteins embedded in a lipid bilayer, which is synthesized by recombinant yeast cells. The N-terminal L protein is a pre-S region which is displayed on the surface of BNC and HBV and functions as a human hepatocyte-specific binding site. The C-terminal-half of L protein is an S protein, which is a transmembrane protein and is required for particle formation and cellular internalization.

protein (226 amino acid residues containing three transmembrane segments) has been synthesized as about 20-nm S particles in the endoplasmic reticulum of eukaryotic cells (i.e., Chinese hamster ovary cells, *Saccharomyces cerevisiae*) and utilized as an immunogen of hepatitis B vaccine for the last three decades. In 1992, aiming to add new antigens to the conventional hepatitis B vaccine, we initially succeeded in overexpression of HBV envelope L protein (pre-S region [163 amino acid residues] + S protein) as about 70-nm hollow-particle form in *Saccharomyces cerevisiae* (**Figure 2**) [65]. Physicochemical analyzes showed that L particles consist of > 90% L proteins embedded in < 10% phospholipids (lipid bilayer); particle structure is very stable against heat and chemicals, and particle diameter varies from 50 – 500 nm in response to physical conditions [66]. The high content of protein would strengthen the structure of L particles, and the phospholipids may enhance the size flexibility of L particles by interfering with the interactions between L proteins in the lipid bilayer [67]. Several lines of evidence indicated that the pre-S region and S protein have pivotal roles in the mechanism of HBV infection, that is the human hepatocyte-specific receptor [68] and cellular internalization [69], respectively. The HBV envelope particles produced by *Saccharomyces cerevisiae* (e.g., TGP-943) are completely free from the HBV genome, and have been commercialized as hepatitis B vaccine for humans [70]. This led our research group to designate the HBV envelope L particle as *sensu stricto* bio-nanocapsule (BNC) and

examine if BNC incorporates various materials (drugs, genes) and delivers them to human liver-related tissues *in vivo*.

6.2 First generation BNC

In 2003, we succeeded in incorporating genes (green fluorescent gene [GFP]-expression vector) and a drug (calcein) into BNC by applying electroporation and delivering these payloads to the human HCC-derived cell lines NuE and Huh7, and also to the primary human hepatocyte efficiently *in vitro* (cultured cells) [4]. Next, since the tropism of HBV is restricted to the liver cells of humans and chimpanzees, the human HCC-derived tumors on the back of mouse xenograft model have routinely used as a substituent of human liver tissue. The same BNC preparations injected intravenously could deliver genes and drugs to NuE- and Huh7-derived tumors specifically in a mouse xenograft model (*in vivo*) without a detectable level of mistargeting to control human colon cancer cell line WiDr-derived tumor and major normal tissues (**Figure 3**). Recently, we established transgenic rats whose livers express a putative HBV receptor (SCCA1, squamous cell carcinoma antigen-1), and injected the same BNC preparations in its tail vein [71]. Immunohistochemical analyses of livers demonstrated that BNC was incorporated by albumin-expressing hepatocytes preferentially, not by Küppfer cells, suggesting that BNC could target hepatocytes in human liver. BNC could transduce the gene expression of therapeutic levels of human clotting Factor IX (for hemophilia B [4])
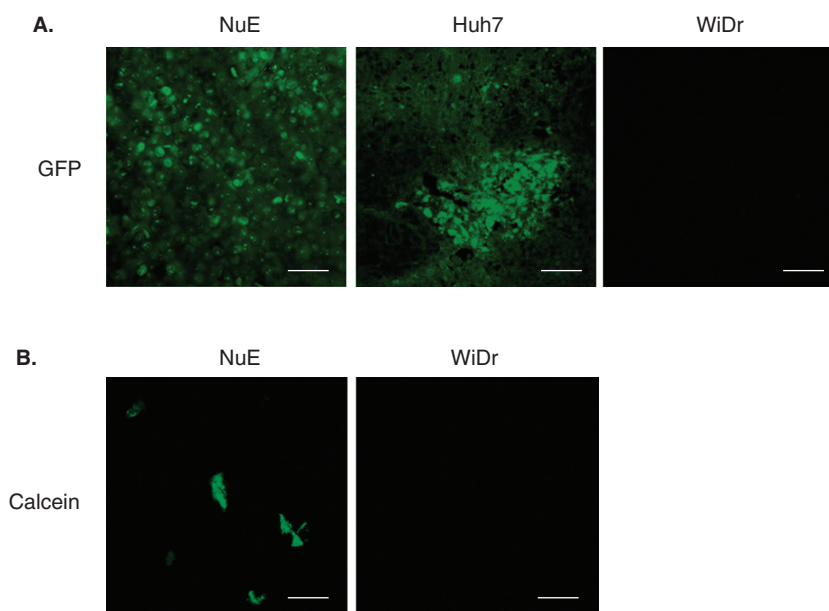
Figure 3. *In vivo* delivery of genes and drug to human liver-derived tumors in a mouse xenograft model using first-generation BNCs. A. BNC containing the GFP-expression plasmid was delivered not to the control tumor (WiDr) but to human liver-derived tumors (NuE and Huh7). **B.** BNC containing the fluorescent chemical compound calcein was also delivered to the human liver-derived NuE tumor. Bars: 100 μm.

and herpes simplex virus type-1 thymidine kinase (for transplanted NuE-derived tumor [72]) in animal models. These data indicated for the first time that yeast-derived HBV envelope L particle, so far known as an immunogen of hepatitis B vaccine, is a promising and efficient NP carrier for human liver-specific *in vivo* pinpoint DDS and GDS.

6.3 Membrane fusion activity of BNC

As described in section 4, new NP-based DDS and GDS carriers should be incorporated into cells not by endocytosis and deliver payloads directly to the cytoplasm. For example, HVJ-E vector could deliver payloads directly to the cytoplasm owing to HVJ-derived membrane fusion activity [64]. It was also demonstrated that liposomes displaying Arg-rich cell-penetrating peptide are efficiently incorporated into the cytoplasm by endocytosis followed by membrane fusion [73]. Because BNC has been considered to transfect human liver cells in a manner similar to HBV [4], of which the transfection mechanism is still controversial (endocytosis or membrane fusion), we examined if BNC possesses membrane fusion activity using a lipid mixing assay using doubly fluorescence-labeled liposomes [74]. Under conditions in which HVJ and PEG showed strong activity, we found that BNC by itself showed the same level of membrane fusion activity (Matsuzaki *et al.*, manuscript submitted). Deletion analyses of L protein revealed that the domain responsible for this activity is not involved in the C-terminal-half of the pre-S region, as reported previously [75]. This result strongly suggested that BNC (presumably also HBV) infects human

liver cells in a membrane-fusion manner, but not excluding the possibility of endocytosis in the infection process of HBV and BNC.

6.4 Second generation BNC

From the establishment of BNC technology, electroporation had been widely used to incorporate payloads into BNC in the laboratory. It was found that BNC stored for long periods tends to show lower incorporation efficiency than freshly prepared BNC. The intra- and inter-molecular disulfide bonds of L proteins (total 14 Cys residues in three transmembrane segments of L protein) were postulated to form gradually in a time-dependent manner, which would make BNC resistant to electroporation. We substituted each Cys residue with Ala or Ser by genetic modification, and identified at least eight Cys residues of L protein unnecessary for BNC formation [76]. The BNC harboring the eight Cys to Ala/Ser mutations showed good incorporation efficiency in an electroporation method. This method is not applicable for introducing large materials into a BNC (e.g., > 20-kbp plasmid for gene therapy; > 10-nm fluorescence-labeled polystyrene beads for bio-imaging), and it is virtually impossible to adopt the electroporation method for the good manufacture protocol (GMP)-based production of BNC-based nanomedicines. Based upon the findings that BNC possesses membrane fusion activity (see above), a new method has recently been developed for incorporating payloads into BNC by using liposome fusion (**Figure 4A**) [77]. Various materials are first incorporated into liposomes by conventional methods, and then mixed with
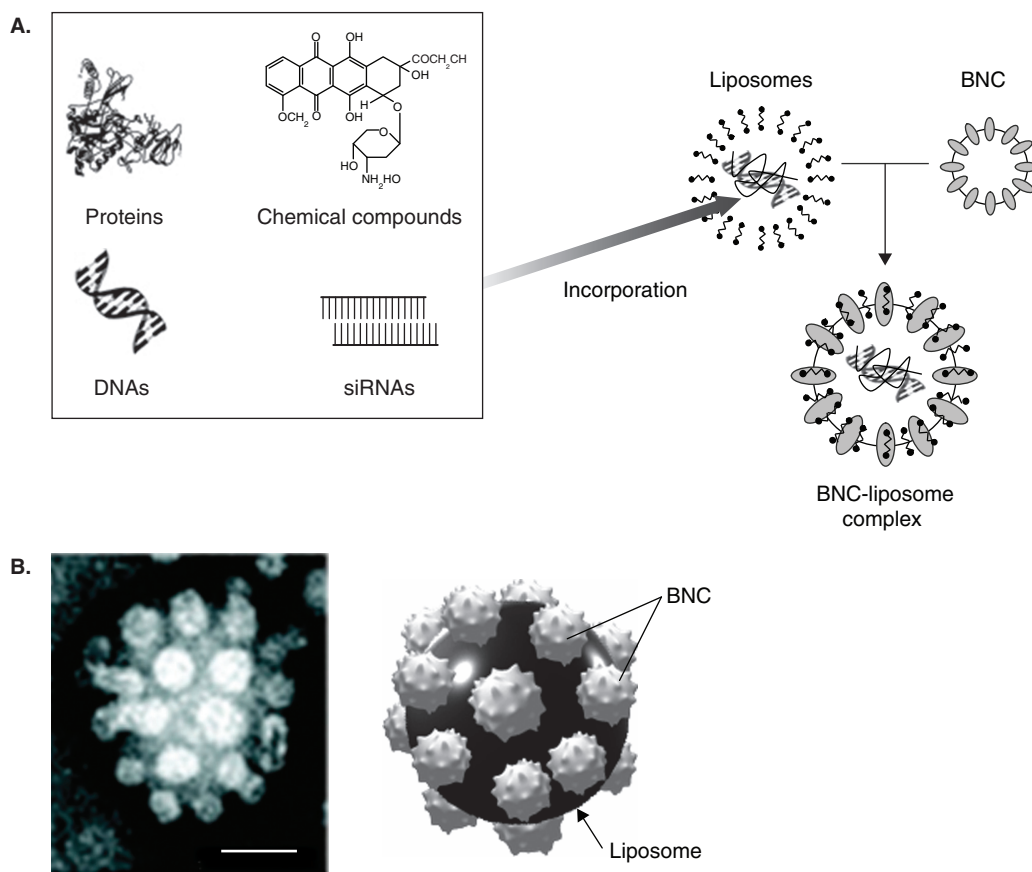
Figure 4. Second generation BNC (BNC–liposome complex). A. Preparation procedures for BNC–liposome (BNC–Lp) complex. **B.** Transmission electron microscopy (TEM) image and schema of BNC–Lp complex. Bars: 100 nm.

BNC to allow formation of BNC–liposome (BNC–Lp) complex (**Figure 4B**). The BNC–Lp complex containing 35-kbp GFP-expression vector showed good transfection efficiency only in human hepatocarcinoma cells HepG2 *in vitro* (**Figure 5**, upper panels), whereas neither conventional cationic liposomes nor electroporated BNC (first-generation BNC) could introduce the plasmid into any cells. The BNC–Lp complex containing 4.7-kbp GFP-expression plasmid injected intravenously was revealed to show higher expression of GFP specifically in the NuE-derived tumors of the mouse xenograft model (**Figure 5**, lower panels). The BNC–Lp complex succeeded in delivering fluorescence-labeled 50-nm polystyrene beads to the same tumors *in vivo* [77]. These results confirmed that the BNC–Lp complex functions better than electroporated BNC, whereas the size of the BNC–Lp complex (about 150 nm in diameter) is 1.5-fold larger than that of BNC (**Figure 4B**). The BNC–Lp complex may expand the possibility of BNC-based nanomedicines in clinical fields because GMP-based productions of liposomes and BNC (HB vaccine) are established in pharmaceutical companies worldwide.

6.5 Retargeting of BNC

The human liver-specific transfection mechanism of BNC and BNC–Lp complex is based on the HBV infection mechanism but it is valuable to describe the strategy for retargeting BNC and BNC–Lp complex to other cells and tissues. The recognition of human hepatocytes by HBV and BNC is owned by the N-terminal-half of the pre-S region [68] (**Figure 6A**). As a first example, by replacing the 3 – 77 amino acid residues of L protein with epidermal growth factor (EGF), we demonstrated the *in vitro* retargeting of BNC from human HCC NuE cells to EGF receptor (EGFR)-expressing A431 cells without decreasing transfection efficiency (**Figure 6B**) [4]. We then generated the BNC displaying the IgG Fc-binding ZZ-tag, derived from *Staphylococcus aureus* protein A, in a similar way. The ZZ-tag-displaying BNC (ZZ-BNC) was conjugated with anti-EGFR antibodies, labeled chemically with fluorescence, and injected into mouse brain harboring EGFR-expressing glioma tissues. It was shown that anti-EGFR antibody-displaying BNC accumulated efficiently in the glioma, which indicates that antibody can be used for *in vivo* retargeting of BNC [78]. More recently, we chemically
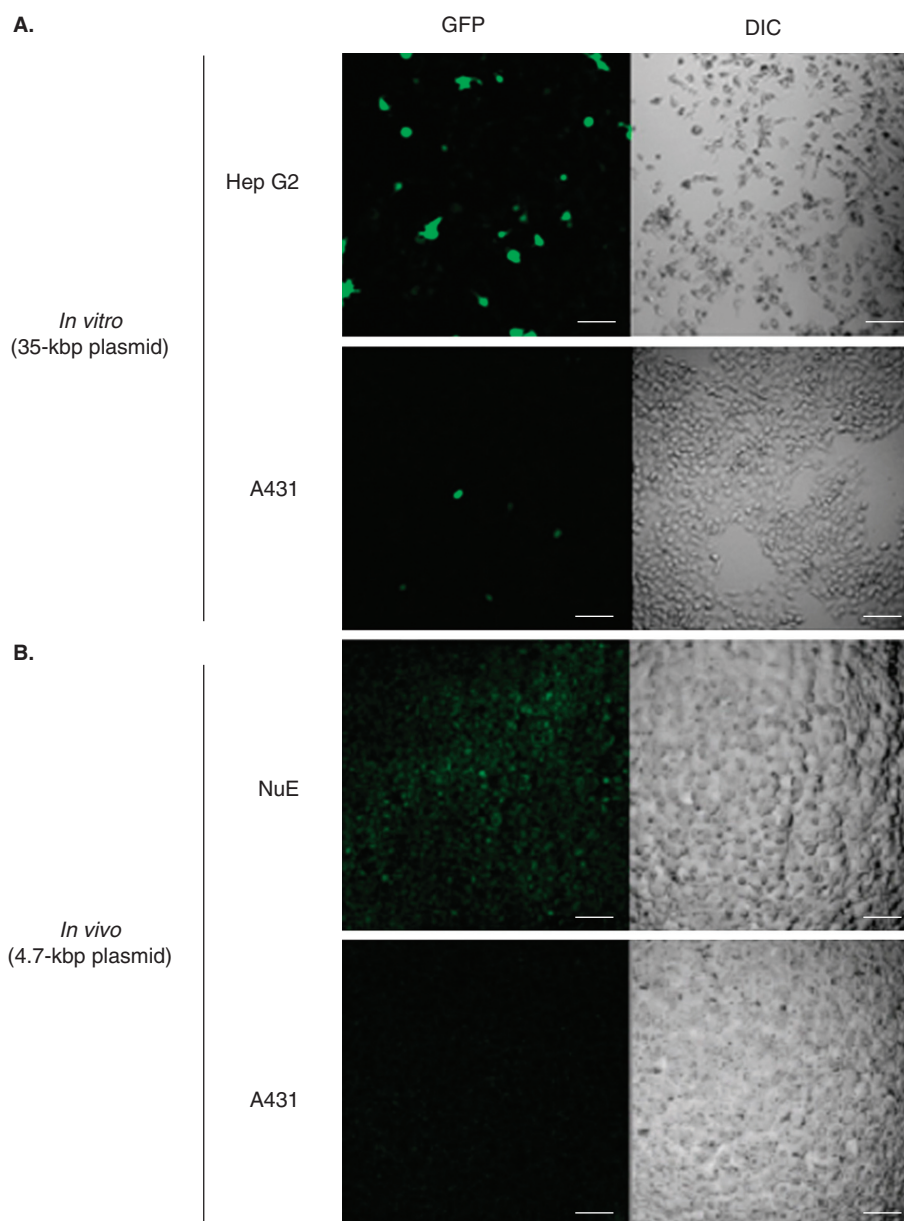
Figure 5. *In vitro* **and** *in vivo* **delivery of genes to human liver-derived cells using second-generation BNCs. A.** BNC–liposome (BNC–Lp) complex containing a 35-kbp GFP-expression plasmid was delivered *in vitro* not to control cells (A431) but to human liver-derived cells (HepG2). **B.** BNC-Lp complex containing the 4.7-kbp GFP-expression plasmid was delivered *in vivo* not to control tumors (A431) but to human liver-derived tumors (NuE) in a mouse xenograft model. Bars: 100 μm.

modified the free Lys residues of ZZ-BNC with *N*-hydroxysuccinimide (NHS)-biotin and then conjugated with avidin and biotin-labeled *Phaseolus vulgaris* agglutinin-L_4 isolectin (L_4-PHA). The L_4-PHA lectin-displaying BNC injected intravenously was shown to deliver drugs and genes to the malignant tumors overexpressing *N*-acetylglucosaminyltransferase-V (GnT-V) in a mouse xenograft model that synthesizes a ligand for L_4-PHA, β(1-6) branching *N*-acetylglucosamine (GlcNAc) (**Figure 6C**) [79].

Taken together, the BNC and BNC–Lp complex can be retargeted to non-hepatic cells and tissues by displaying various bio-recognition molecules (e.g., cytokines, receptors, antibodies, antigens, glycans, lectins, aptamers, homing peptides) without affecting the other advantages of BNC.

6.6 Immunologic considerations of BNC

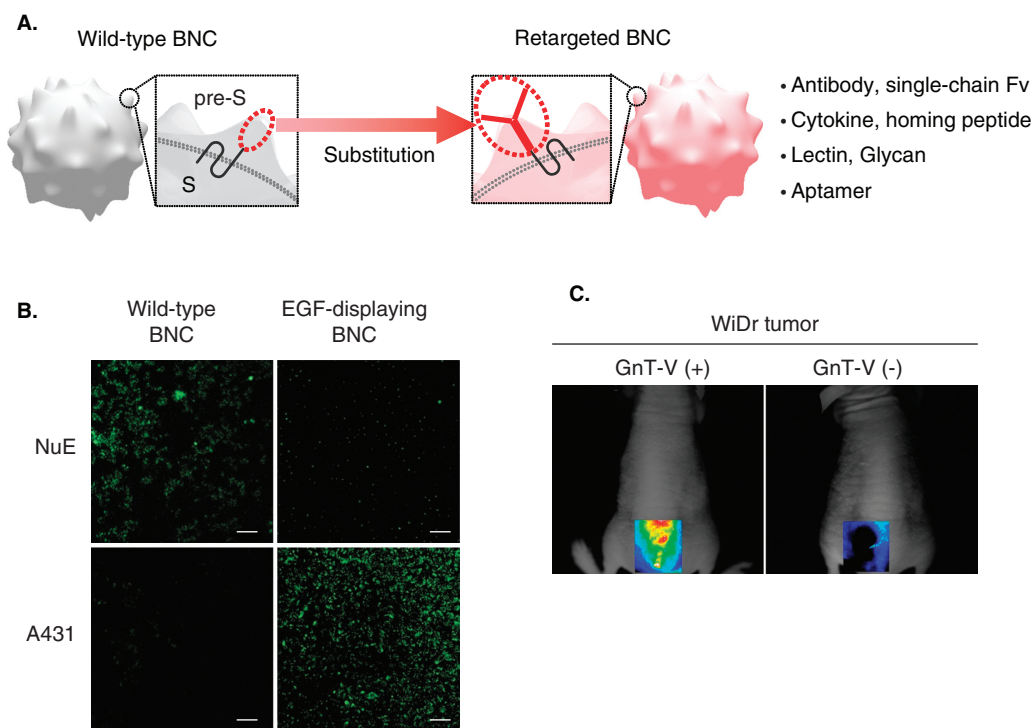As highlighted in *The Lancet* [80], BNC and BNC–Lp complex are considered to elicit unexpected immunologic

Figure 6. Retargeting of BNC to other cells and tissues. A. Strategy for retargeting of BNC. **B.** EGF-displaying BNC can deliver incorporated calcein to EGF receptor-overexpressing A431 cells, and wild-type (original) BNC to the NuE cells. Bars: 100 μm. **C.** Specific accumulation of L_4-PHA isolectin-displaying BNC to the *N*-acetylglucosaminyltransferase-V (GnT-V)-overexpressing WiDr tumor *in vivo*.

reactions in humans because they are similar to an immunogen of conventional hepatitis B vaccines. In particular, the intravenously injected BNC and BNC–Lp complex may be immediately neutralized by anti-HBV antibodies in HB vaccines. To overcome these problems, according to the amino acid substitutions naturally occurring in the HBV escape mutants which propagate in HB vaccinees [81], we introduced two amino acid substitutions (Glu-292 to Arg, Gly-308 to Arg) in L protein (Jung *et al.*, manuscript submitted). Original and mutated BNC (stealth BNC) did not show passive and active anaphylaxes. The ED_{50} value (minimum dose required for 50% seroconversion of anti-BNC antibodies in Balb/c mice [intraperitoneal injection]) of stealth BNC was < 10% that of original BNC. We then prepared a mouse xenograft model which received the i.v. injection of human anti-HBV IgG in advance and examined whether stealth BNC (electroporated BNC or BNC–Lp complex) could deliver drugs and genes to human liver-related tumors in the mouse xenograft model. Even in the mouse possessing > 500 mIU/ml anti-HBV antibody (> 50-fold of HBV protection level in human), these BNCs could deliver drugs and genes to target tumors without reduction of transfection efficiency. These results strongly suggested that stealth BNC will be a promising human liver-specific DDS and GDS carrier, regardless of the existence of anti-HBV antibodies in the blood.

7. Conclusions

It is plausible that NP-based DDS and GDS carriers accelerate development of novel therapeutic agents against not only liver-related diseases, but also other diseases. New NP-based carriers should fulfil the criteria listed in section 3. We consider BNC to be the most promising NP-based carrier among existing DDS and GDS carriers.

8. Expert opinion

We herein proposed eight criteria for NP-based DDS and GDS carriers and compared BNC with conventional carriers on the basis of these criteria (**Table 1**). Conventional liposomes and polymer micelles that do not fulfil two criteria (active targeting and cell-penetrating activity) have been used to deliver anticancer drugs to tumors in humans. Viral vectors that do not fulfil four criteria (toxicity, antigenicity, active targeting and productivity) have been used to transduce therapeutic genes through direct intra-tumor injections in humans. These situations strongly suggested that extensive optimizations of NP-based DDS and GDS carriers are not necessary for the treatment of serious diseases that may be incurable and which have no alternative therapy. It is essential to enhance the safety and efficacy of these carriers by optimizing all the properties listed in Section 3

to expand the use of NP-based DDS and GDS carriers in other diseases.

Current NP-based carriers have been developed by two independent research fields: material science and biologic science. The former produced non-viral carriers (liposomes and polymer micelles) and the latter produced viral carriers. As summarized in **Table 1**, each NP-based carrier does not necessarily fulfil all criteria. In the last decade, interdisciplinary study has generated a novel-type NP-based carrier, a hybrid of non-viral and viral carriers, to fulfil the criteria as far as possible. For example, the HVJ-E vector is a hybrid of HVJ virus and liposomes (Section 6.1.2), which is designed to add HVJ-derived membrane fusion activity to conventional liposomes. It is unfortunate that the HVJ-E vector should eliminate the following problems; remaining RNA genome, antigenicity and no targeting mechanism. As described in section 6.1.3, since yeast-derived HBV subviral particles (HBsAg particles) have been confirmed as an extremely safe bio-material by their long-term clinical use as an immunogen of recombinant hepatitis B vaccine, we have recently developed BNC technology, a hybrid of HBV subviral particles and liposomes. The carrier is considered to fulfil most of the criteria and harbors no serious problem. We are continuing the optimization of BNC as an ideal NP-based DDS and GDS carrier according to the strategies detailed below.

1. *Evaluation of PEG-like property of BNC*: PEGylation has been widely used for various NP-based carriers to enhance their stability in blood. In the course of developing PEGylation-less BNC–Lp complex containing doxorubicin (Dox), the mice intravenously injected with BNC-Lp (8 mg/kg body as Dox) or PEGylated Lp (8 mg/kg body as Dox) showed similar half-life ($T_{1/2}$) of Dox in the bloodstream (about 90 min). Because the surface of BNC is very similar to that of HBV, which circulates in the body and attaches to the human liver specifically, the modification of NPs with BNC would be an alternative method for PEGylation. We are currently determining the amount of BNC on the surface of NP that is most effective for escaping from RES-rich organs *in vivo*.

2. *Investigation of retargeting molecules for BNC*: Retargeting ability is a unique property of BNC. Although various molecules have been proposed to retarget NPs from *in vitro*

studies, a few molecules can function *in vivo* through systemic injection because the targets of these molecules are often inaccessible from the bloodstream. It is necessary to synchronize the retargeting molecule with the cell-penetrating moiety of BNC by changing the amounts of the molecules on the surface of BNC. Using BNC instead of PEGylation (see above) may reduce the steric hindrance of the surface of BNC, enhancing the activities of the retargeting molecule and the cell-penetrating moiety. We are currently investigating retargeting molecules for the *in vivo* use of BNC.

3. *Investigation of intracellular trafficking pathway for BNC*: Several parts of L protein have been reported to possess cell-penetrating activity, strongly suggesting that BNC and HBV are incorporated into cells by a membrane fusion mechanism. We are currently elucidating the intracellular fate of BNC and HBV in human liver cells by the combination of molecular cell biological and immunocytochemical techniques.

We believe these approaches will shed light on the function of BNC on a molecular basis, and facilitate the use of BNC-based nanomedicines in clinical fields.

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Declaration of interest

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Affiliation

Takeshi Kasuya PhD & Shun'ichi Kuroda[†] PhD
[†]Author for correspondence
Osaka University,
Institute of Scientific and Industrial Research,
Department of Structural Molecular Biology,
Ibaraki, 567-0047, Osaka, Japan
Tel: +81 6 6879 8462; Fax: +81 6 6879 8462;
E-mail: skuroda@sanken.osaka-u.ac.jp

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