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Construction of arginine-rich peptide displaying bionanocapsules

Takuya Shishido [a], Daisaku Yonezawa [b], Kiyokazu Iwata [b], Tsutomu Tanaka [c], Chiaki Ogino [d], Hideki Fukuda [c], Akihiko Kondo [d,*]

[a] Department of Molecular Science and Material Engineering, Graduate School of Science and Technology, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan
[b] Department of Chemical Science and Engineering, Graduate School of Science and Technology, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan
[c] Organization of Advanced Science and Technology, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan
[d] Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

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ABSTRACT

Bionanocapsule (BNC) is a hollow nanoparticle composed of L-protein of the hepatitis B virus surface antigen. BNC can deliver genes or drugs into specific human hepatocytes, but delivery is limited to hepatocytes. In this study, we attempted to alter the specificity of BNCs by genetically introducing cell-penetrating peptides (CPPs), such as arginine-rich peptides, into BNCs. The CPP-fused BNC was efficiently internalized into various cell lines in a short period without significant cytotoxicity. These results show that CPP-BNC could be applied as an efficient carrier for gene and drug delivery.

Efficient intracellular delivery of bioactive molecules, such as genes, proteins and synthetic molecules, to the desired cells and tissues is of significant importance in fields varying from fundamental research to medical applications. In most cases, the use of genes or intact therapeutic proteins has been limited by their inability to cross the plasma membrane. Cell-penetrating peptides (CPPs) are very useful for efficient macromolecule delivery into cells[1]; however, direct conjugation of CPP to target molecules sometimes leads to a loss of function. Thus, liposomes displaying CPP (CPP-LIP) on their surface have been established as carriers to encapsulate and deliver genes and drugs.[2] Although liposomes can package therapeutic genes and drugs efficiently, they are not stable enough for *in vivo* use.[3] Therefore, it is important to develop carriers that can efficiently deliver macromolecules inside cells.

Bionanocapsules (BNCs) are hollow particles that are composed of L-protein derived from the hepatitis B virus with a lipid bilayer derived from yeast endoplasmic reticulum (ER) membrane.[4] BNCs are attractive carriers for human hepatocyte-specific drug delivery because several bioactive molecules, such as genes, chemical compounds and proteins, can be efficiently introduced into human hepatocytes, for which BNC has a specific affinity.[5] The advantages of BNC-mediated bioactive molecule delivery are safety, high stability and high transfection efficiency, allowing BNC to be used as a carrier both *in vitro* and *in vivo*.[5a] In the targeting of other types of cells, approaches to engineer BNC specificity have recently been developed. The PreS region, which has specific affinity for human hepatocytes,[6] was genetically eliminated from the L-protein region. Then the ZZ domain of protein A, which has affinity for several antibodies, was inserted. BNCs displaying the ZZ domain (ZZ-BNC) were prepared using yeast as the host, and antibody-displaying BNCs were demonstrated.[7]

However, antibody-mediated delivery using ZZ-BNC limits the types of targeted cells; a suitable antibody that will correspond to desired cells is needed. Here, in order to expand the utility of BNC, we developed a cell-penetrating peptide displaying BNCs (CPP-BNCs) as carriers for the intracellular delivery of bioactive molecules. We employed three types of arginine-rich CPPs: octaarginine (R8; RRRRRRRR),[8] Tat derived from the human immunodeficiency virus I (HIV-1) transactivator protein (Tat; GRKKR RQRRRPPQ)[9] and antennapedia derived from *Drosophila melanogaster* Antennapedia-homeodomain protein (Antp; RQIKIWFQN RRMKWKK).[10] These CPPs were genetically fused to L-protein and CPP-BNCs were successfully prepared using yeast as the host. Using fluorescence-labeled CPP-BNCs, we also demonstrated that CPP-BNCs could efficiently be introduced into various types of cells without cytotoxicity. The engineered BNCs in this study are promising carriers for the efficient delivery of bioactive molecules to various cells.

Abbreviations: BNC, Bionanocapsule; CPP, cell-penetrating peptides; LIP, liposome.

* Corresponding author. Tel./fax: +81 78 803 6196.
E-mail address: akondo@kobe-u.ac.jp (A. Kondo).

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The expression plasmids for CPP-BNC were constructed as described below. The plasmid pGLDLIIP39-RcT contained HBV envelope L gene.[4] The gene encoding the deletion mutants L (Δ50–159) was prepared using a Quikchange Site-Directed Mutagenesis Kit (Stratagene Co., La Jolla, CA, USA) with pGLDLIIP39-RcT as a template and the following primers: 5′-GGTAGGAGCGGGCGGCC GCCCTGCACCGAAC-3′ and 5′-GTTCGGTGCAGGGCGGCCGCCCG CTCCTACC-3′. The resultant plasmid was named pGLDLd50. Three genes, encoding either R8, Tat or Antp, were prepared by annealing the following synthetic oligonucleotides: R8 (5′-GGGGGCGGCCG CAGACGCAGACGCAGAAGACGCGGCCGCGGG-3′ and 5′-CCCGCGGC CGCGTCTTCTGCGTCTGCTTCTGCGGCCGCCCCC-3′), Tat (5′-GGGGG CCGCTATGGTCGTAAGAAACGTCGCCAACGTCGCCGAAGCGGCCGC-3′ and 5′-CCCGCGGCCGCTTCGGCGACGTTGGCGACGTTTCTTACGACCA TAGCGGCC-3′) and Antp (5′-GGGGGCGGCCGCCGTCAAATCAAGAT TTGGTTCCAAAACCGTCGTATGAAGTGGAAGAAGGGCGGCCGCGGG-3′ and 5′-CCCGCGGCCGCCCTTCTTCCACTTCATACGACGGTTTTGGAACC AAATCTTGATTTGACGGCGGCCGCCCCC-3′), respectively. The annealed fragments were digested with NotI and were inserted into the NotI site of pGLDLd50, with the insertion orientation confirmed by DNA sequencing. The resultant plasmids were named pGLDLd50-R8, pGLDLd50-Tat and pGLDLd50-Antp. These plasmids were introduced into *Saccharomyces cerevisiae* AH22R$^-$ (**a** *leu2 his4 can1 cir*$^+$ *pho80*) using the spheroplast method, as described previously.[4]

The three CPP-displaying BNCs were overexpressed in *S. cerevisiae* AH22R$^-$ carrying the plasmids pGLDLd50-R8, pGLDLd50-Tat and pGLDLd50-Antp, respectively. The yeast cells were disrupted with glass beads and the CPP-BNC was purified by precipitation using polyethylene glycol 6000 (PEG6000), CsCl isopycnic ultracentrifugation and sucrose density gradient ultracentrifugation, according to the method described previously.[4] After purification, the protein concentration was determined using a *DC* protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Purified CPP-BNCs and wild-type BNC were analyzed using Western blotting with anti-S protein antibody as the primary antibody and an alkaline phosphatase-conjugated anti-mouse IgG antibody (Promega Corp., Madison, WI, USA) as the secondary antibody. Alkaline phosphate activity was chemiluminescently detected using CDP-*Star* Detection Reagent (GE Healthcare Bio-Sciences Corp., NJ, USA). The sizes of CPP-BNCs were determined by dynamic light scattering (DLS) using a Zetasizer Nano Particle Size Analyzer (Malvern Instruments Ltd, Worcestershire, UK), according to the manufacture's suggested procedure.

Purified CPP-BNCs were reacted with Alexa Fluor 488 Succinimidyl Esters (invitrogen) (2.6 mol equiv) in PBS for 1 h at room temperature. The mixture then was dialyzed against PBS overnight to remove free Alexa Fluor 488.

HeLa cells (human cervical carcinoma) and A431 cells (human epithelial carcinoma) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). Chinese hamster ovary (CHO) cells were cultured in DMEM/F-12 supplemented with 10% (v/v) FBS. These cells were maintained at 37 °C in 5% CO_2.

Approximately 2×10^4 cells of HeLa cells, A431 cells and CHO cells were seeded in 35 mm glass-bottom dishes. After incubation for 24 h, cells were treated with either CPP-BNCs or WT-BNC in serum-free medium at 37 °C for 1–3 h. The final concentrations of CPP-BNC were 0.1, 0.5, and 1.0 μM, respectively. The cells were then washed 3 times with PBS and observed by confocal microscopy (LSM 5 PASCAL, Carl Zeiss, Oberkochen, Germany).

Cytotoxicity of CPP-BNC was assayed using Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan), which contains WST-8 as coloring reagent. HeLa cells were seeded at 1×10^4 cells per well in 96-well plates and cultured overnight. The cells were washed 3 times with serum-free medium and treated with R8-BNC, Tat-BNC, Antp-BNC and WT-BNC in serum-free medium (final concentration: 1.0 μM) for 1 h. After washed 3 times and replaced with fresh medium with 10% FBS, CCK-8 was added 10 μl per well, and the cells were incubated for 2 h at 37 °C. The absorbance of culture medium was read at 450 nm using a Wallac 1420 Multilabel Counter (Perkin-Elmer, MA, USA). As well, after the treatment of each BNC, the cells were cultured for additional 24 h and WST-8 assay was carried out. In addition, cytotoxicity of R8-BNC was assayed about A431 and CHO cells in the similar method.

We previously reported that the PreS region, which has specificity for human hepatocytes in the L-protein, could be replaced by the ZZ domain, and the fusion protein would be expressed in the yeast and displayed on the surface of BNC.[7] In this study, we genetically substituted the PreS region with CPPs (Fig. 1) and prepared CPP-displaying BNCs. Figure 2A shows that R8-BNC, Tat-BNC and Antp-BNC were expressed in the yeast cells, as confirmed by western blotting. The yield of CPP-BNCs, obtained from the recombi-

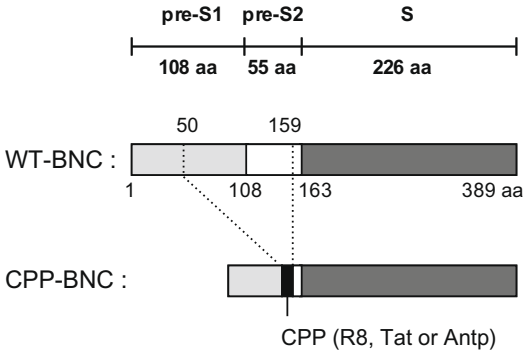

Figure 1. Schematic representation of CPP-BNCs. The hepatocyte-specific region corresponding to amino acids 50–159 of the L-protein were replaced by CPP as R8, Tat or Antp.

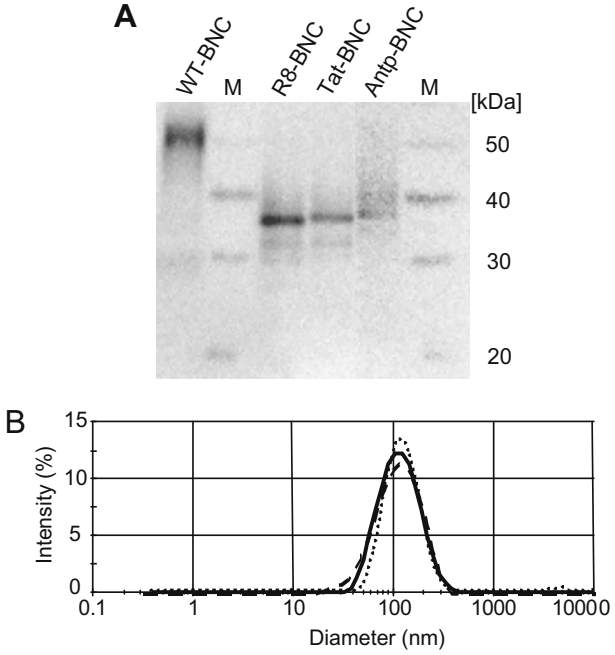

Figure 2. Characterization of CPP-BNCs. (A) Western blot analysis of CPP-BNCs. The purified CPP-BNCs were analyzed using an anti-S protein antibody. (B) DLS analysis of CPP-BNCs. The purified CPP-BNC analyzed by dynamic light scattering. R8-BNC (solid line), Tat-BNC (dotted line) and Antp-BNC (dashed line).

nant yeast cells grown in 1 L of the culture medium, were 1.92 mg, 1.57 mg and 0.50 mg, which was less than half that of the wild-type BNC (WT-BNC).[11] To confirm CPP-BNC particle formation, each size of the purified CPP-BNCs was analyzed using dynamic light scattering (Fig. 2B). The diameters of R8-BNC, Tat-BNC and Antp-BNC were about 107 nm, 110 nm and 106 nm, respectively, which is almost the same as that of WT-BNC,[11] suggesting that the CPP-BNCs formed nano-scale particles.

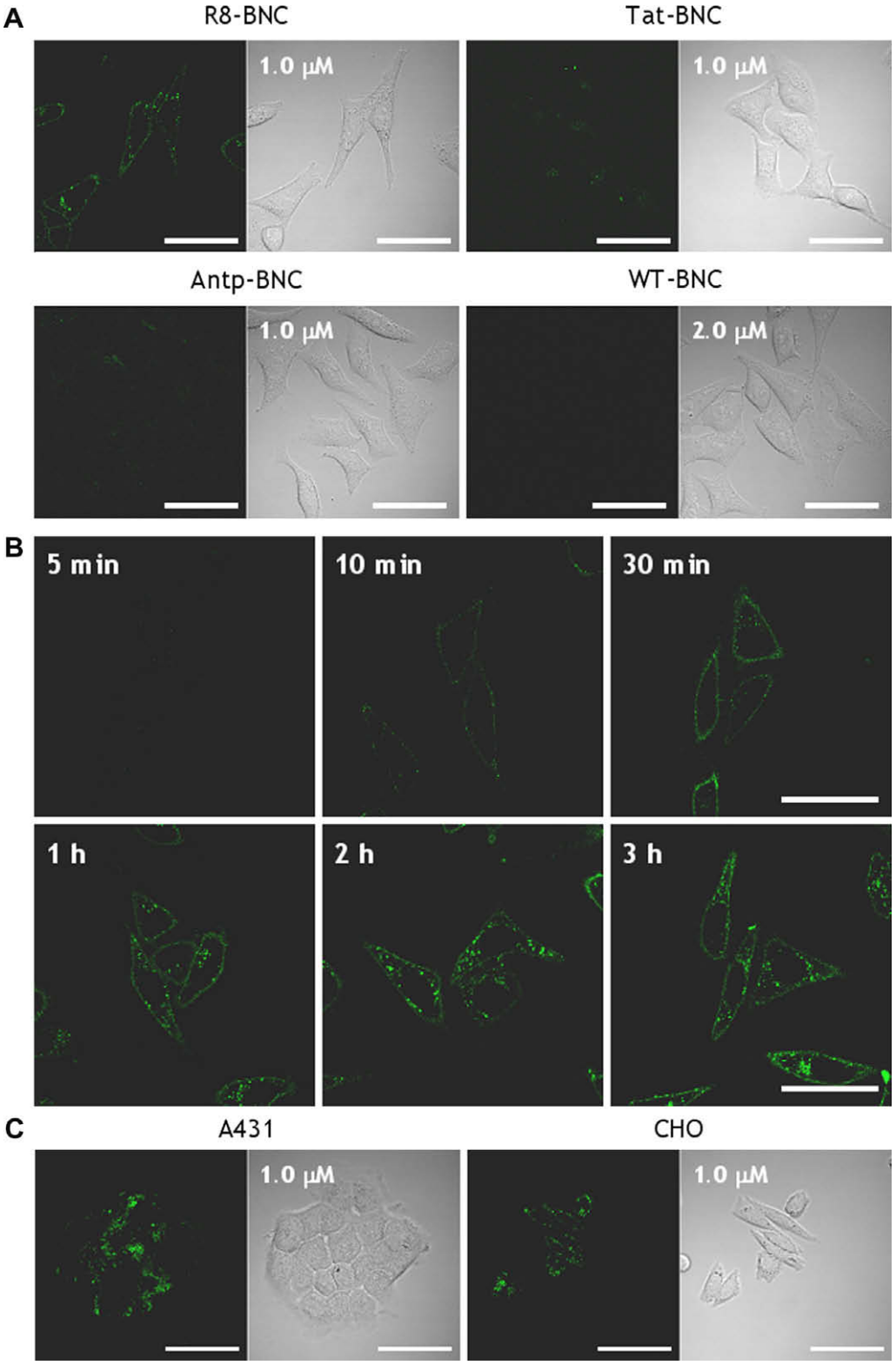

Figure 3. Transduction of CPP-BNC into Hela, A431 and CHO cells. (A) HeLa cells were incubated with indicated concentrations of Alexa488-labeled R8-BNC, Tat-BNC, Antp-BNC or WT-BNC for 1 h in serum-free medium. (B) The time course of internalization of R8-BNC. HeLa cells were incubated with 1.0 μM Alexa-labeled R8-BNC for up to 3 h in serum-free medium. (C) Internalization of R8-BNC into other cells. A431 and CHO cells were incubated with 1.0 μM Alexa-labeled R8-BNC for 1 h in serum-free medium. After incubation, cells were washed 3 times with PBS, then were observed using confocal microscopy. Scale bar, 50 μm.

To evaluate the transduction efficiency of these CPP-BNCs, HeLa cells were incubated with Alexa488-labeled R8-BNC, Tat-BNC, Antp-BNC or WT-BNC. HeLa cells were seeded and incubated with 0.1, 0.5 or 1.0 μM of Alexa488-labeled CPP-BNC. The cells were then washed 3 times with PBS and analyzed by confocal microscopy. Figure 3A shows that the fluorescence of 1.0 μM R8-BNC was localized around the cell membrane and partly inside. As a negative control, internalization of WT-BNC was hardly observed when the cells were incubated with 2.0 μM of Alexa488-labeled WT-BNC. In the case of Tat-BNC and Antp-BNC, the lower levels of internalized fluorescence were observed, which is similar to the previous reports that oligoarginine (R8-12) exerts high transduction efficiency against HeLa compared with Tat and Antp.[12] However, the transduction efficiency of CPP seems to depend on the combination of the cargo and the targeted cells. In the case of CPP-LIP, Antp-LIP showed higher transduction efficiency against airway cells than R8-LIP and TAT-LIP.[2c] In this study, R8-BNC was utilized in the following experiment as an appropriate carrier.

We then evaluated the time course of R8-BNC internalization. After HeLa cells were incubated with R8-BNC (1.0 μM), cells were washed and observed by confocal microscopy (Fig. 3B). R8-BNC appeared to localize in the cell membrane a mere 10 min after incubation, which was much faster than WT-BNC.[5a] Internalization of R8-BNC appeared to begin after incubation for 30 min and achieved efficient internalization after 1 h, which is consistent with R8-liposome.[2b] In the case of either CPP-conjugates or fusion proteins, the internalization was typically established within 5–30 min, depending on the cargos.[13] The reason the internalization of R8-BNC was slightly slower than R8-conjugates might derive from the size of the R8-BNC.[14] Nevertheless, this result shows that R8-BNC was able to efficiently internalize into the cell in a short time, compared with WT-BNC. As well, when HeLa was incubated with R8-BNC at 4 °C for 1 h, R8-BNC was frequently inhibited to internalize into cytoplasm, which shows that most of R8-BNC appeared to enter the cell *via* energy-dependent transport (data not shown).

To confirm that R8-BNC can internalize other cell lines, human epithelial carcinoma cell A431 and Chinese hamster ovary (CHO) cells were incubated with 1.0 μM of R8-BNC for 1 h in a serum-free medium and were observed by confocal microscopy (Fig. 3C). R8-BNC could internalize A431 cells as well as CHO cells efficiently. These results indicated that R8-BNC could be an efficient carrier of the gene and drug delivery into various cells, which differs from ZZ-BNC that limits the types of targeted cells without suitable antibody.

Finally, we examined the cytotoxicity of R8-BNC in order to confirm that the high transduction efficiency was related to cell viability. In the case of CPP-LIP, Antp-LIP, which showed the highest transduction efficiency, proved the most cytotoxic in airway cells.[2c] HeLa cells, A431 and CHO cells were treated with R8-BNC (1.0 μM) for 1 h in serum-free medium, WST-8 assay was carried out using Cell Counting Kit-8 (Fig. 4). As a result, R8-BNC showed no significant cytotoxicity, which is similar to the R8 peptide.[15] Additional 24 h incubation after R8-BNC treatment also observed no cytotoxicity. Moreover, Tat-BNC, Antp-BNC and WT-BNC also exhibited no cytotoxicity in HeLa cells (data not shown). Therefore, these results suggest that R8-BNC has both safety and high efficiency when applied as a carrier for gene and drug delivery.

In conclusion, we produced novel particles having cell-penetrating peptides on their surface. We also optimized the internalization of R8-BNC into various cells with no cytotoxicity. R8-BNC will be a powerful tool for bioactive molecule delivery to various kinds of cells.

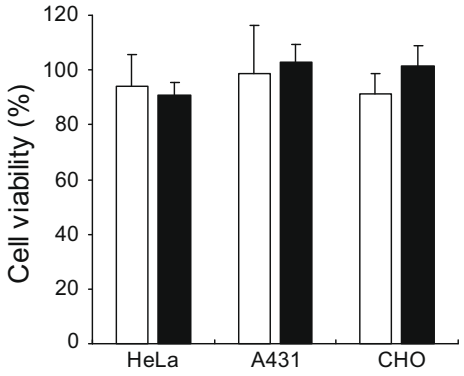

Figure 4. Cytotoxicity of R8-BNC in HeLa, A431 and CHO cells. These cells were incubated with R8-BNC for 1 h. The number of viable cells was counted subsequently (white) or after additional 24 culture in FBS containing medium (black), using CCK-8. Cell viability was defined as the ratio of the number of viable cells treated with CPP-BNC to the number of non-treated cells. The data presented is given as the mean and standard deviation of three independent experiments.

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