J. Biochem. 2009;146(6)867–874
doi:10.1093/jb/mvp134

Biotinylated Bionanocapsules for Displaying Diverse Ligands Toward Cell-specific Delivery

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Received June 9, 2009; accepted August 10, 2009; published online August 25, 2009

Bionanocapsule (BNC) is hollow nanoparticle composed of the L-protein of the hepatitis B virus surface antigen. BNC allows targeted delivery of either genes or drugs only to hepatocytes, but not to other cell types. In this study, we attempted to alter the specificity of BNC by insertion of biotin-acceptor peptide (BAP), which is efficiently biotinylated using biotin ligase BirA from *Escherichia coli*. Using streptavidin as a linker, biotinylated BNC could be display various biotinylated ligands that are otherwise difficult to fuse with BNC, such as antibodies, synthetic peptides and functional molecules. BAP-fused BNC was efficiently biotinylated and effectively displayed streptavidin. Furthermore, we demonstrated that biotinylated BNC was internalized into targeted cells *via* biotinylated Nanobody displayed on the BNC surface. Biotinylated BNC permit display of diverse ligands, and thus have potential as a versatile carrier for drug delivery to a variety of target cells.

Key words: bionanocapsule, biotin-acceptor peptide, biotin ligase, drug delivery, nanobody.

Abbreviations: BNC, bionanocapsule; BAP, biotin-acceptor peptide: StAv, streptavidin; EGFR, epidermal growth factor receptor.

For medical and biological applications, efficient delivery system of bioactive molecules, such as genes, proteins or synthetic molecules, to target cells and tissues have been significantly important (*1*). Various kinds of nano-sized carriers to deliver bioactive molecules have been developed, including liposomes, micelles and polymers (*2*). However, these carriers have several limitations as targeted delivery systems: (i) poor control of biomacromolecules release from the carrier, (ii) difficulties of production with scaling-up method and (iii) carrier's instability in the blood (*3*). Therefore, the development of efficient carrier that can deliver macromolecules into target cells and tissues remains as an important goal.

Our group has developed a bionanocapsule (BNC) that is composed of the L-protein derived from the hepatitis B virus (HBV) surface antigen (HBsAg) and a lipid bilayer derived from yeast endoplasmic reticulum membrane (*4*). BNC is an attractive carrier for targeted drug delivery because of high stability in blood, high delivery efficiency and high capacity of bioactive molecules (*4*). Thus, BNC have been applied *in vitro* and *in vivo* as carriers of bioactive molecules, such as genes, chemical compounds and proteins, for specific delivery to human hepatocytes due to the hepatocyte specificity of PreS region of BNC (*5–8*). One of the drawbacks of BNC is that the use of BNC is limited for only hepatocytes. To overcome the limitation, the cell targeting specificity of BNC has been engineered. One example is ZZ domain of protein A from *Staphtlococcus aureus*, which has binding affinity for several antibodies, and the ZZ displaying BNC was constructed (*9*). Using this engineered BNC, anti-EGFR antibody was displayed on the BNC and the antibody-mediated protein delivery to EGFR-overexpressing cells was well demonstrated (*9*).

Here, to expand the versatility of ligand-display BNC, we proposed a versatile targeted delivery system that is based on biotinylated bionanocapsules (Bio-BNC). This BNC will accept biotin molecule by introduction of the biotin-acceptor peptide (BAP; GLNDIFEAQKIEWHE) (*10*) to the surface exposure region of BNC. A lysine side-chain of BAP is efficiently biotinylated by the *Escherichia coli* enzyme BirA (*11*). Various biotinylated ligands can be conjugated to Bio-BNC using high-affinity of biotin–streptavidin interaction ($K_d = 10^{-13}$ M) (*12*). For alternating of BNC specificity, we employed variable domains of a *camelid* heavy chain-only antibody as a model ligand (*13*). Biotinylated anti-EGFR *camelid* heavy chain-only antibody was displayed on the surface of Bio-BNC. Using this engineered BNC, we demonstrated that BNC-mediated target cell-specific delivery.

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MATERIALS AND METHODS

Plasmid Construction—The expression plasmid for BAP-BNC was constructed as described below. The plasmid pGLDLIIP39-RcT contains the gene for the HBV envelope L-protein (*5*). A fragment encoding the GLD promoter was amplified using pGLDLIIP39-RcT as a template. The primers utilized were 5′-GGGAGAT CTGCGAGCTTACCAGTTCTCAC-3′ and 5′-GGGGCGGC CGCGGATCCCCGCGGTGTTTTTATACTCGACCTCG-3′. The amplified fragment was digested with *Bgl*II/*Not*I and ligated into *Bam*HI/*Not*I sites of pGLDLd50 (*14*). The resultant plasmid was named pGLDsLd (1–159). Subsequently, a fragment encoding a region of the L gene (amino acids from 1 to 32), which had been fused to chicken-lysozyme signal peptide at the N-terminus and BAP at the C-terminus, was amplified in pGLDLIIP39-RcT using the following primers: 5′-GGGCCGCGGATGA GATCTTTGTTGATCTT-3′ and 5′-GGGGCGGCCGCCTT CGTGCCATTCGATCTTTTGAGCTTCGAAGATGTCGTT CAAACCGCGACCACCCCAATCTGGATTGTTTGAGT-3′. The amplified fragment was digested with *Sac*II/*Not*I and ligated into pGLDsLd (1–159). The resultant plasmid was named pGLDsLd33-BAP.

Next, the expression plasmid for BirA was constructed. The gene encoding BirA was fused to FLAG-tag at the C-terminus and then amplified in the *E. coli* strain NovaBlue {*endA1 hsdR17* (rk12–/mk12+) *supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proA+B+lacIqZ.M15*::Tn*10* (TcR)]} (Novagen, Darmstadt, Germany) using direct colony PCR with the following primers: 5′-GGGAGATC TATGAAGGATAACACCGTGCC-3′ and 5′-GGGGTCGAC TTACTTATCGTCGTCATCCTTGTAATCGAATTCTTTTT CTGCACTACGCAGGG-3′. The amplified fragment was digested with *Bgl*II/*Sal*I and ligated into pET32a (Novagen). The resultant plasmid was named pET32-BirA.

The expression plasmid for la1-BAP was constructed as follows. The gene encoding anti-EGFR *camelid* heavy chain-only antibody (*13*) containing plasmid was fully synthesized (Exigen, Co. Ltd, Tokyo, Japan) and the plasmid was named pUC-la1. The gene encoding the Trx-tag thioredoxin protein was fused to a His-tag at the C terminus and then amplified in pET32a using the following primers: 5′-GGGTCTAGAAATAATTTTGT TTA-3′ and 5′-TGCAGCTGCACCTGGGCCATAGATCTA GAACCGCGTGGCACCAGAC-3′. The fragment encoding the la1 gene was amplified using the plasmid pUC-la1 as a template with the following primers: 5′-AGATCTATGG CCCAGGTGCAGCTGCA-3′ and 5′-GGGAAGCTTGTCGA CGGATCCACCACCAGAACCACCACCAGAACCACCAC CCGAGCTCACGGTCACCTGGG-3′. The amplified fragments were mixed together and PCR was carried out using the following primers: 5′-GGGTCTAGAAATAATT TTGTTTA-3′ and 5′- GGGAAGCTTGTCGACGGATCCA CCACCAGAACCACCACCAGAACCACCACCCGAGCTC ACGGTCACCTGGG-3′. The amplified fragment encoding both the Trx-tag and la1 fusion protein was digested with *Xba*I/*Hind*III and ligated into pET32a. The resultant plasmid was named pET-Trx-la1. Subsequently, a fragment of the BAP gene was prepared by annealing the following synthetic oligonucleotides, 5′-GGGGGATCCGG TCTGAACGACATCTTCGAAGCTCAGAAAATCGAATGG CACGAATAATAGGTCGACCCC-3′ and 5′-GGGGTCGAC CTATTATTCGTGCCATTCGATTTTCTGAGCTTCGAAG ATGTCGTTCAGACCGGATCCCCC-3′. The annealed fragment was digested with *Bam*HI/*Sal*I and inserted into pET-Trx-la1. The resultant plasmid was designated pET-Trx-la1-BAP.

Expression and Purification of the Recombinant Protein—BAP-BNC was prepared as described previously (*5*). Briefly, the plasmid pGLDsLd33-BAP was introduced into *Saccharomyces cerevisiae* AH22R- (**a** *leu2 his4 can1 cir+pho80*) using the spheroplast method. The yeast cells overexpressing BAP-BNC were disrupted with glass beads and BAP-BNC was purified by precipitation with polyethylene glycol 6000 (PEG6000), CsCl isopycnic ultracentrifugation and sucrose density gradient ultracentrifugation. After purification, the protein concentration was determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

The plasmid pET-Trx-la1-BAP or pET32-BirA was transformed into *E. coli* BL21 (DE3) (Novagen). The cells were grown in LB medium containing 100 μg/ml ampicillin until the optical density at 600 nm (OD 600 nm) reached 0.8. At this time, expression of the protein was induced by addition of IPTG (final concentration: 0.3 mM). After an additional 16 h of culture at 25°C, the cells were harvested by centrifugation. The cell pellets were resuspended in 50 mM phosphate, 300 mM NaCl (pH 7.0) and lysed by sonication. Each of expressed protein were purified from the soluble fraction of the lysate using TALON metal affinity resins (Clontech, CA, USA) according to the manufacturer's protocol, and then was dialysed against 50 mM Tris–HCl and 150 mM NaCl (pH 8.0) at 4°C.

The reaction conditions for biotinylation were as follows: 5.0 μM BAP-BNC or la1-BAP, 200 μM biotin, 260 μM BirA, 4.0 mM ATP and 1.0 mM $MgCl_2$. The reaction mixture was incubated at room temperature for 45 min, and then dialysed against PBS overnight to remove free biotin.

The biotin-to-protein molar ratio of the biotinylated proteins was determined using the HABA Biotin Quantitation Kit (AnaSpec, Inc. CA, USA). After biotinylated proteins were dialysed against distilled water overnight, the HABA assay was performed according to the manufacturer's protocol.

Western Blot Analysis—Purified BAP-BNC and biotinylated BAP-BNC (Bio-BNC) were analysed by western blotting analysis using either alkaline phosphatase (AP)-conjugated streptavidin (Invitrogen, CA, USA) or the anti-HBsAg antibody from an IMx enzyme immunoassay (EIA) kit (Abbott Laboratories, Abbott Park, IL, USA) as the primary antibody, and an AP-conjugated anti-mouse IgG antibody (Promega Corp, Madison, WI, USA) as the secondary antibody. The blots were developed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Promega Corp). For analysis of glycosylation, BAP-BNC were treated with Peptide: *N*-glycosidase F (PNGaseF) (New England Biolabs Inc., Ipswich, MA, USA) at 37°C for 1 h. The PNGaseF-treated samples were also analysed by western blotting. la1-BAP and biotinylated la1-BAP (la1-Bio) were analysed using AP-conjugated streptavidin as described above.

Dynamic Light Scattering Analysis—BAP-BNC size was determined by dynamic light scattering (DLS) using a Zetasizer Nano Particle Size Analyzer (Malvern Instruments Ltd, Worcestershire, UK), according to the manufacturer's protocol. In addition, the size of the Bio-BNC, Bio-BNC conjugated with 0.5 mol equivalent of streptavidin, and la1–streptavidin–BNC complexes (la1-Bio : streptavidin : Bio-BNC protein = 1.0 : 0.5 : 1.0 mol equiv.) was determined using DLS.

Enzyme-linked Immunosorbent Assay—The interaction between Bio-BNC and streptavidin was confirmed using a sandwich enzyme-linked immunosorbent assay (ELISA). A monoclonal anti-HBsAg antibody (Acris Antibodies, Hiddenhausen, Germany), which had been dissolved in coating buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH 9.6), was added to 96-well plates (Corning, Inc., Corning, NY, USA). The antibody was allowed to bind the plates overnight at 4°C (final concentration: 0.1 μg/well). After washing with PBS containing 0.05% Tween 20 (polyoxyethylenesorbitan monolaurate), StartingBlock™ Blocking Buffer (Pierce Biotechnology, Inc., IL, USA) was added to each well to block non-specific binding and the plate was incubated for 1 h at 37°C. After washing once more, Bio-BNC was added to each well (10.0 μg/well) and the plate was incubated for 2 h at room temperature. As negative controls, BAP-BNC without BirA treatment or the mixture of BAP-BNC and biotin was also added to each well. After additional washing, horseradish peroxidase (HRP)-conjugated streptavidin (Pierce Biotechnology, Inc.) diluted with PBS was added (0.01 μg/well) and the plate was incubated for an additional 1 h at room temperature. After a final washing, substrate solution containing 2,2-azino-di(3-ethylbenzthiazoline) sulfonic acid (ABTS; Zymed Laboratories, San Francisco, CA, USA) and H_2O_2 was added to the plate, and the reaction was allowed to proceed in the dark at room temperature. The absorbance of each well was measured at 405 nm using a Wallac 1420 Multilabel Counter (Perkin Elmer, MA, USA).

Cell Cultures—A431 (human squamous carcinoma) cells were obtained from Riken BioResource Center (Tsukuba, Japan). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in 5% CO_2.

Flow Cytometry—Purified Bio-BNC was reacted with Alexa Fluor 488 succinimidyl esters (Invitrogen) as previously described (*14*). Alexa-labeled Bio-BNC was mixed with 0.5-mol equivalent of streptavidin (Nakalai Tesque, Kyoto, Japan) (StAv-BNC). After 30 min incubation, 1.0 nmol of BNC were conjugated with 0, 0.25, 0.5, 1.0 and 10.0 nmol of la1-Bios for 15 min and la1-displaying BNC (la1-BNC) formed. Approximately 2×10^5 A431 cells were seeded in 12-well plates and cultivated for 24 h. After washing with serum-free medium, Alexa-labeled la1-BNC (final concentration: 1.0 μM) were added to the cells, which were then cultured for 3 h. After the cells were washed twice with serum-free medium, the forward scatter (FSC), side scatter (SSC) and green fluorescence of the cells were measured using a BD FACSCanto II (Becton, Dickinson and Company, NJ, USA). Based on FSC versus SSC characteristics, live cells were isolated from dead cells and cellular debris. The fluorescent intensity of la1-BNC-transfected cells was determined by subtracting the background fluorescence of the untreated cells from the fluorescence of the total cell population.

Transduction of la1-BNC into A431 Cells—Alexa-labelled Bio-BNC was mixed with 0.5-mol equivalent of streptavidin for 30 min (StAv-BNC) and 1.0-mol equivalent of la1-Bio for 15 min to prepare la1-BNC. In addition, Alexa-labelled streptavidin was conjugated with la1-Bio (2.0 mol equiv.) for 15 min (la1-StAv). Approximately 5×10^4 A431 cells were seeded into a 35-mm glass-bottom dish and cultured for 24 h. After washing with serum-free medium, Alexa-labelled la1-BNC or la1-StAv was added to the A431 cells (final concentration: 1.0 μM). Bio-BNC or StAv-BNC was added to A431 cells as negative controls. After 1 or 3 h of incubation, the cells were washed three times with serum-free medium and observed with a 5-Pa laser-scanning confocal microscope (LSCM) (Carl Zeiss, Oberkochen, Germany). Fluorescent images were acquired using the 488-nm line of an Ar laser for excitation and a 505-nm band pass filter for emission. The specimens were viewed using a 63-fold magnification, oil immersion objective.

RESULTS AND DISCUSSION

Preparation of BNC Displaying BAP—Our strategy to alter specificity of BNC by using biotin–streptavidin interaction is illustrated in Fig. 1. First, each of BAP-fused BNC (BAP-BNC) and biotinylated ligand was prepared, respectively. Then BAP-BNC, streptavidin

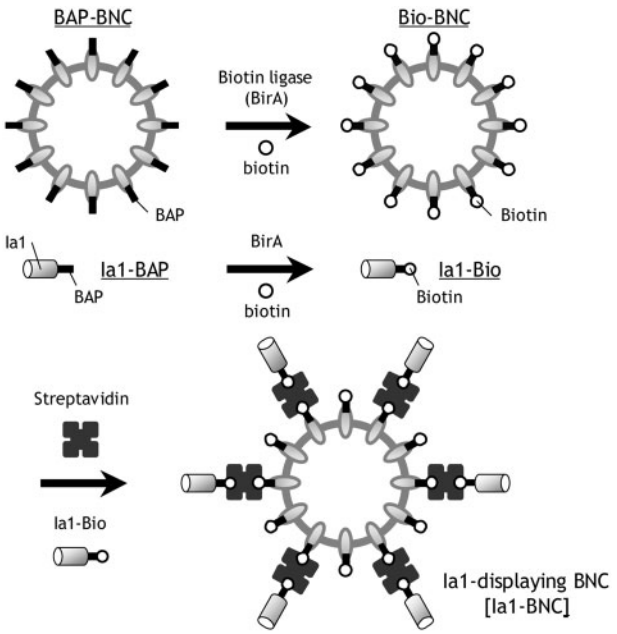

Fig. 1. **Schematic illustration of the construction of the la1-sptreptavidin–BNC complex.** BAP-BNC or la1-BAP were biotinylated by the biotin ligase BirA (Bio-BNC or la1-Bio). la1-Bio was displayed on the surface of Bio-BNC *via* streptavidin (la1-BNC).

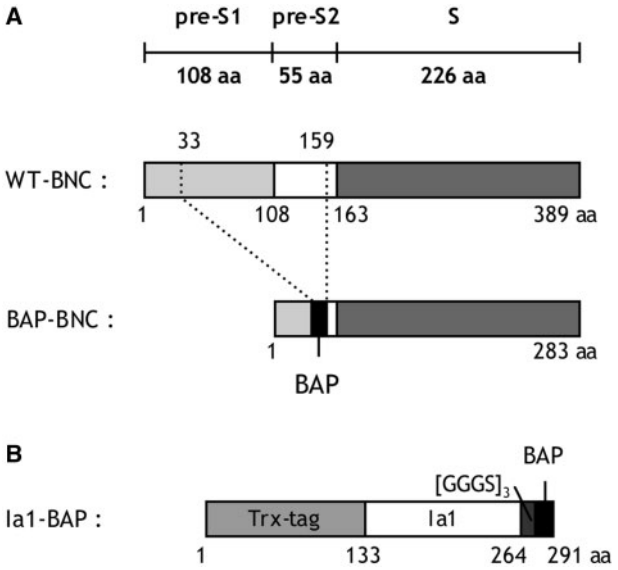

Fig. 2. **Schematic representation of both a BAP-BNC and a Ia1-BAP.** The hepatocyte-specific region corresponding to amino acids 33–159 of the L-protein was replaced with 15 aa of the biotin-acceptor peptide (BAP) (A). Ia1 was fused to BAP via the flexible linker ($(GGGS)_3$) at the C terminus (B).

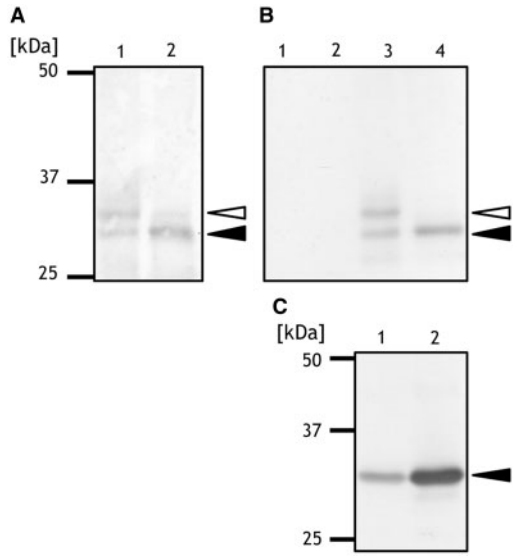

Fig. 3. **Characterization of BAP-BNC.** Western blot analysis of BAP-BNC. (A) The purified BAP-BNC was analysed using an anti-HBsAg antibody. Lane 1, BAP-BNC; 2, PNGaseF-treated BAP-BNC. (B) Biotinylated BAP-BNC (Bio-BNC) were analysed after treatment with streptavidin-AP. Lane 1, BAP-BNC; 2, PNGaseF-treated BAP-BNC; 3, Bio-BNC; 4, PNGaseF-treated Bio-BNC. Two bands corresponding to the unglycosylated (closed arrow) and glycosylated (open arrow) forms of BAP-BNC were detected. (C) Ia1-BAP and biotinylated Ia1-BAP (Ia1-Bio) were also analysed after treatment with streptavidin-AP. Lane 1, Ia1-BAP; 2, Ia1-Bio.

and biotinylated ligand were mixed together, and the ligand-displayed BNC *via* biotin–streptavidin interaction was constructed.

We replaced the pre-S region, which has specificity for human hepatocytes, with BAP *via* genetic manipulation (Fig. 2A) and prepared BAP-displaying BNC (BAP-BNC) using yeast as a host. The BAP-BNC yield obtained from the recombinant yeast cells was 1.35 mg/l, which was less than half of the yield of wild-type BNC (*15*). After ultracentrifugal purification, two major bands, which were corresponded to the glycosylated and unglycosylated forms of BAP-BNC, respectively, as previously reported (Fig. 3A, lane 1) (*15*). As expected, after treatment of BAP-BNC with PNGaseF, the upper band shifted to a molecular weight of 31.6 kDa, which is consistent with the molecular weight of the BAP-BNC protein (Fig. 3A, lane 2). It clearly showed that the successful production and purification of BAP-BNC. As shown in Fig. 4, the size of the purified BAP-BNC was analysed by DLS. The average diameter of the BAP-BNC was ~92.2 nm (Fig. 4A) and polydispersity index value (PDI) is 0.238, which is similar to that of WT-BNC (*15*), suggesting that the BAP-BNC particle was nano-sized particle.

Biotinylation of BAP-BNC—To ensure the labeling of BAP-BNC with biotin, BAP-BNC was incubated with biotin ligase BirA from *E. coli*, which catalyses the biotinylation of BAP. To improve the biotinylation rate and yield, an excess amount of biotine and BAP was appilied to modification reaction.

Biotinylated BAP-BNC (Bio-BNC) was analysed by western blotting using StAv-AP (Fig. 3B). Bio-BNC after BirA treatment showed two major bands (lane 3), while Bio-BNC treated with BirA followed by PNGaseF showed one band (lane 4). These results clearly showed that the efficient conjugation of biotin to the BAP-BNC using BirA was well achieved and both the glycosylated and non-glycosylated forms of BAP-BNC were biotinylated efficiently. The average diameter of Bio-BNC was about 94.2 nm and PDI is 0.248, which was nearly identical to that of BAP-BNC (Fig. 4B). Then, the ratio of biotinylation was measured using a HABA Biotin Quantification Kit and the biotin number conjugated to BNC was estimated to be 79.2 molecules/BNC. The biotinylation yield based on BAP concentration is 0.79/BAP, because BNC usually consists of about 110 molecules of L-protein.

Next, to confirm that Bio-BNC interacts with streptavidin, a sandwich ELISA was performed (Fig. 5). Bio-BNC was added to a 96-well plate that had been coated with anti-HBsAg antibody. As negative controls, BAP-BNC without BirA treatment, or the mixture of BAP-BNC and biotin were used. After washing, the streptavidin-HRP was added to each well for detection of BNC that interacted with streptavidin-HRP. Figure 5 shows the significant higher absorbance was detected for Bio-BNC. In contrast, the absorbance measured for both BAP-BNC without BirA treatment and the mixture BAP-BNC and biotin was negligible. These results suggest biotinylated BNC could be conjugated with streptavidin on its surface through the biotin. Further improvement to produce Bio-BNC is now undergoing such as co-expression of BAP-BNC and BirA in the yeast host cell.

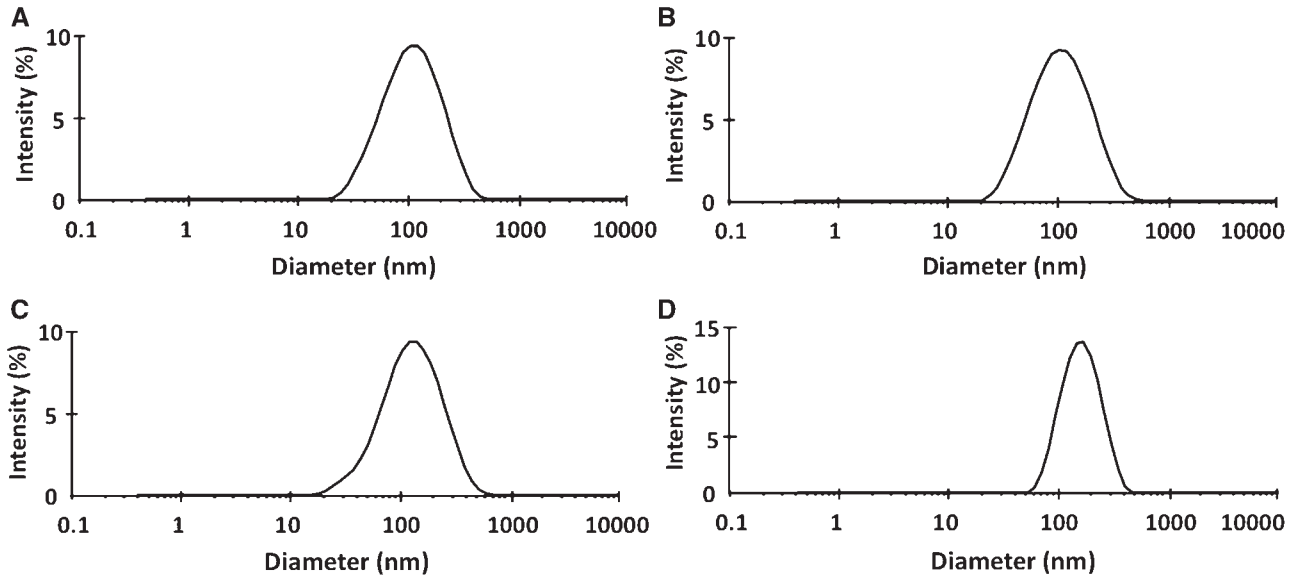

Fig. 4. **DLS analysis of BAP-BNC.** BAP-BNC (A), Bio-BNC (B), Bio-BNC conjugated with 0.5 mol equiv. of streptavidin (C) and la1-displaying BNC complexes (la1-Bio : streptavidin : Bio-BNC protein = 1.0 : 0.5 : 1.0 mol equiv.) (D).

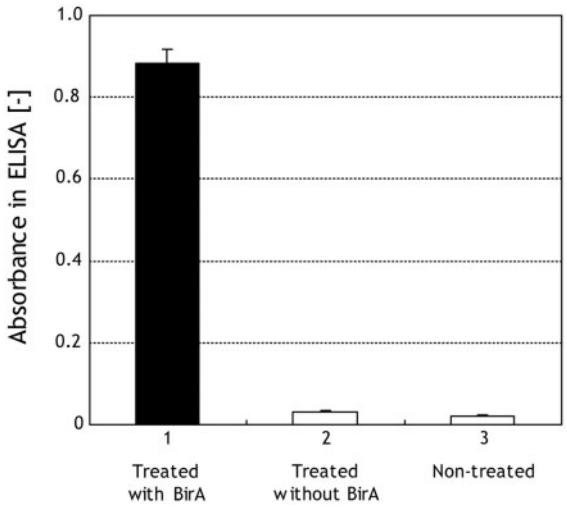

Fig. 5. **Sandwich ELISA of Bio-BNC using a monoclonal anti-HBsAg antibody and streptavidin-HRP.** (1) Biotinylated BAP-BNC treated with BirA. (2) Non-treated BAP-BNC mixed with only the biotin in the biotinylation buffer. (3) Non-treated BAP-BNC. A_{405} values are means ± SD of three independent experiments.

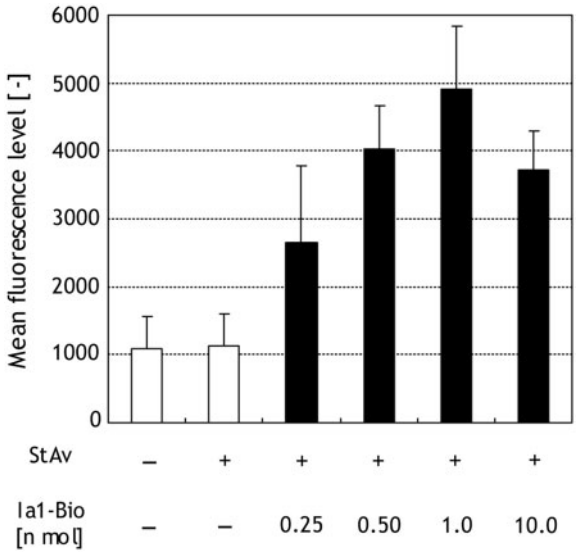

Fig. 6. **Quantitative analysis of the transfection efficiency of BNC conjugated with la1.** One nanomole of Alexa488-labelled Bio-BNC, which had been mixed with 0.5 mol equiv. of streptavidin (StAv), was conjugated with 0, 0.25, 0.5, 1.0 and 10.0 nmol of la1-Bio (la1-BNC). The Alexa-labelled la1-BNC was added to A431 cells and incubated for 3 h. After washing three times with serum-free medium, the cells were recovered and evaluated using FACS. Fluorescent intensity data are means ± SD of five independent experiments.

A DLS assay was performed to determine the diameter of the Bio-BNC after conjugation with streptavidin (Fig. 4C). The diameter of Bio-BNC conjugated with 0.5 mol equivalent of streptavidin was 114 nm and PDI is 0.357, which was about 21 nm larger than that of BAP-BNC. When Bio-BNC was mixed with more than 1.0 mol equivalent, the peak of aggregation was detected at around 4,000 nm (data not shown). This result confirms binding of streptavidin to the surface of Bio-BNC.

Preparation of Biotinylated Nanobody—Nanobody, which is derived from variable domains of *camelid* heavy chain-only antibody, was applied as a surface ligand for effective targeting of Bio-BNC. We employed la1, one kind of Nanobody, which has high specificity for human EGFR (*13*), as a model ligand and we genetically introduced BAP at the C-terminus of la1 *via* the $(GGGS)_3$ flexible linker (la1-BAP) (Fig. 2B). Thioredoxin–la1–BAP

fusion proteins were efficiently produced using *E. coli* as a host. The yield of la1-BAP was 22.5 mg/l. Purified la1-BAP was similarly biotinylated by BirA treatment and analysed by western blotting using StAv-AP (Fig. 3C). As shown Fig. 3, la1-BAP was efficiently biotinylated after BirA treatment. The la1-BAP was also slightly biotinylated, which was attributed to endogenous BirA in *E. coli*. From the HABA assay, 90% of la1-BAP was biotinylated, showing high biotinylated efficiency.

Internalization of Bio-BNC into Target Cells via Anti-EGFR Nanobody—Accumulation of BNC around the target cells is first step of the targeted bioactive molecule delivery. Therefore, we chose Human squamous carcinoma A431 cells as model cell, which express the large amount of EGFR (*16*), and optimized the accumulated condition of la1-displayed BNC around A431 cell. To form Bio-BNC conjugated with streptavidin (StAv-BNC), 1.0 nmol of Alexa-labelled Bio-BNC protein was incubated with 0.5 nmol of streptavidin for 30 min. Subsequently, StAv-BNC was incubated with la1-Bio (0, 0.25, 0.5, 1.0 and 10.0 nmol). After 15 min, la1-displayed StAv-BNC (la1-BNC) were incubated with A431 cell for 3 h, and then analysed by flow cytometry (Fig. 6). As the amount of la1-Bio was increased, the mean fluorescence intensity of la1-BNC treated A431 cell increased gradually. Mean fluorescence intensity was highest when 1.0 nmol of la1-Bio was used. The excess amount of la1-Bio is not appropriate because of

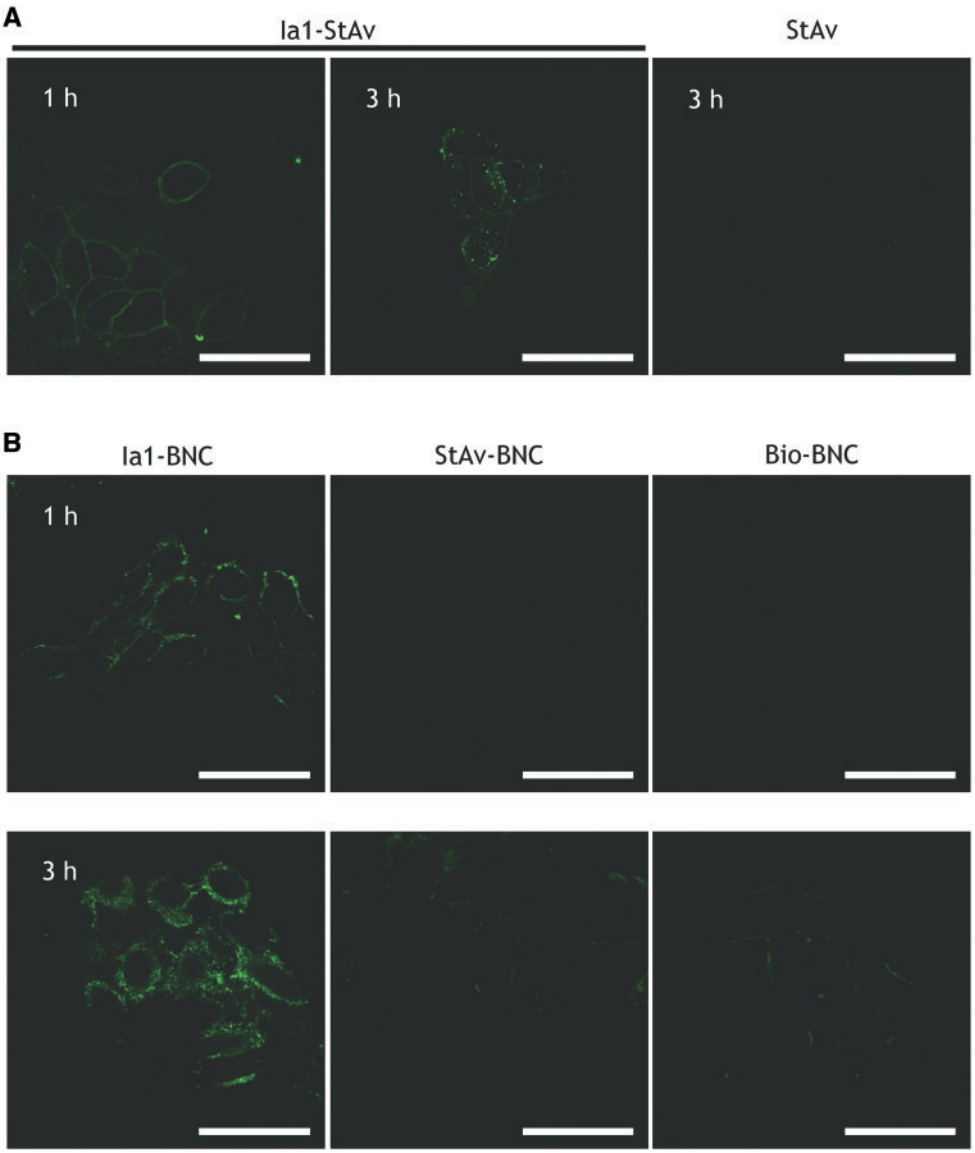

Fig. 7. **Internalization of Bio-BNC into A431 cells via an anti-EGFR Nanobody.** (A) One micromolar concentration of Alexa488-labelled streptavidin was conjugated with 2.0 μM of la1-Bio. As a negative control, A431 cells were incubated with 1.0 μM of Alexa488-labeled streptavidin. (B) 1.0 μM of Alexa488-labelled Bio-BNC, which displayed la1 and streptavidin (la1-BNC), were added and incubated for either 1 h or 3 h in serum-free medium. As negative controls, A431 cells were incubated with 1.0 μM of Bio-BNC or streptavidin-conjugated Bio-BNC (StAv-BNC). Scale bar: 50 μm.

competition for binding to the EGFR in A431 cell between free la1-Bio and la1-BNC. This result suggests that the optimal conjugation ratio of la1-Bio : streptavidin : Bio-BNC protein to form la1-displaying BNC is 1.0 : 0.5 : 1.0. Figure 4D shows that the diameter of la1-displayed StAv-BNC is 140 nm and PDI is 0.334.

We investigated the internalization property of la1-BNC by incubation with A431 cells (Fig. 7B). After 1 h of incubation, la1-BNC was detected at the cell surface, but they were not internalized. After 3h of incubation, la1-BNC was efficiently internalized into the cells, similar to the result reported previously for anti-EGFR-antibody-displaying ZZ-BNC-EGFP (*9*). As a negative control, StAv-BNC or Bio-BNC was added directly to A431 cells, respectively. Both StAv-BNC and Bio-BNC were not detected at either the cell surface or within the cell after 3 h of incubation. These results show that la1-displayed BNC had both of specific targeting ability and internalization ability, suggesting as a superior carrier to expand the target cells of drug delivery by conjugation of numerous targeting ligands that are otherwise difficult to attach to the BNC surface, including other Nanobodies (*17*), antibodies, synthetic functional peptides (*18–20*) and single-stranded nucleic acids (aptamers) (*21–23*).

To evaluate the internalization property of la1-BNC, we investigated la1-StAv internalization property. Alexa488-labelled streptavidin was conjugated with la1-Bio (la1-StAv) and incubated with A431 cell for 1–3 h (Fig. 7A). Although almost all of la1-StAv was localized at the cell surface even after 3 h of incubation, la1-BNC was mostly internalized after 3 h of incubation. These results imply that BNC itself enhances internalization into the cytoplasm, which corresponding to previous report (*24*). Therefore, we successfully demonstrated re-targeting BNC using biotin–streptavidin interaction without loss of internalization ability of BNC.

In conclusion, we developed a novel type of BNC, which displayed BAP on its surface. Using a biotin ligase, BirA, BAP-BNC was efficiently biotinylated and subsequently conjugated with biotinylated ligands *via* streptavidin. We also demonstrated that Bio-BNC could be retargeted by displaying a biotinylated anti-EGFR Nanobody, and efficiently internalized by target cells. By virtue of their ability to display diverse ligands, Bio-BNC has the potential to be versatile carriers for drug delivery to target cells.

ACKNOWLEDGEMENTS

We thank Prof. Shun'ichi Kuroda (Osaka University), Prof. Masaharu Seno (Okayama University) and Prof. Masakazu Ueda (Keio University) for their helpful discussion.

FUNDING

This work was supported in part by the Ministry of Health, Labor and Welfare, Japan, and Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe), MEXT, Japan.

CONFLICT OF INTEREST

None declared.

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