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Specific Protein Delivery to Target Cells by Antibody-displaying Bionanocapsules

Naoya Kurata[1], Takuya Shishido[2], Masaru Muraoka[2], Tsutomu Tanaka[3], Chiaki Ogino[4], Hideki Fukuda[3] and Akihiko Kondo[4,*]

[1]Department of Chemical Science and Engineering; [2]Department of Molecular Science and Material Engineering, Graduate School of Science and Technology; [3]Organization of Advanced Science and Technology; and [4]Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

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Bionanocapsules (BNCs) are nanoparticles with a high biocompatibility composed of the L protein of the hepatitis B virus surface antigen. BNC can deliver bioactive molecules to hepatocytes efficiently and specifically. However, delivery is limited to hepatocytes and incorporation of proteins into BNC is quite troublesome. Here, in order to alter the specificity of BNC and to achieve efficient protein delivery, we developed engineered BNC displaying the ZZ domain of protein A and incorporating enhanced green fluorescent protein (EGFP) inside the particles using an insect cell expression system. The ZZ domain displayed on the surface of BNC binds to anti-epidermal growth factor receptor (EGFR) antibodies, allowing specific delivery of EGFP to HeLa cells. The engineered BNCs are a promising and powerful tool for efficient and cell-specific protein delivery.

Key words: antibody-mediated targeting, bionanocapsule, insect cell expression system, protein delivery, ZZ domain.

Abbreviations: BNC, bionanocapsule; EGFP, enhanced green fluorescent protein; EGFR, epidermal growth factor receptor.

INTRODUCTION

Specific delivery of bioactive molecules, such as genes and proteins, to the desired cells and tissues efficiently is of significant importance in fields varying from fundamental research to medical applications. In protein delivery, use of intact therapeutic proteins has been limited by their inability to cross the plasma membrane. Protein transduction domains are very useful for efficient protein delivery into cells (*1–3*); however, cell-specific protein delivery is difficult with protein transduction domain-mediated protein delivery (*4*). Alternatively, liposomes have been established to encapsulate and deliver genes and proteins (*5–7*). Although liposomes are safe and can package therapeutic genes and proteins efficiently, they are not stable enough for *in vivo* use (*8*). In addition, there are few carriers to transfer macromolecules, especially proteins, that can target the appropriate cell. Therefore, development of a carrier that can deliver proteins specifically and efficiently to target cells is important.

Bionanocapsules (BNCs) are hollow particles in which L protein derived from the hepatitis B virus is embedded in yeast endoplasmic reticulum (ER) membrane-derived phospholipid vesicles (*9*). BNCs are an attractive carrier for human hepatocyte-specific drug delivery because several bioactive molecules such as genes and drugs are efficiently introduced inside and BNC has specific affinity and natural infectivity to human hepatocytes (*10*). The advantages of BNC-mediated bioactive molecule delivery are safety, high stability and high transformation efficiency, which allow to use BNC as a carrier *in vitro* and *in vivo* (*10*). To expand the utility of BNC for other types of cells, approaches to engineer BNC specificity have recently been developed. The PreS region, which has specific affinity for human hepatocytes (*11*, *12*), was genetically eliminated from the L-protein region. Then the ZZ domain of protein A, which has affinity for several antibodies (*13*, *14*), was inserted. BNC displaying the ZZ domain (ZZ-BNC) were prepared using yeast as the host and antibody-displaying BNC were demonstrated (*15*).

Another problem is that the incorporation of various molecules into BNC is difficult and time-consuming. Electroporation can be employed for incorporation of DNA and small compounds into BNC, but electroporated BNCs are significantly damaged and the incorporation efficiency is very low. To overcome these problems, a liposome-assisted incorporation method was recently developed (*16*); however, incorporation of protein is relatively troublesome compared to that of DNA and small compounds. Here we describe a one-step approach for efficient production of BNC incorporating the functional protein inside the particles. We employed enhanced green fluorescent protein (EGFP) as a model delivered protein. EGFP was genetically fused to the C-terminus of ZZ-BNC (ZZ-BNC-EGFP). We optimized expression of ZZ-BNC-EGFP using insect cells as a host. EGFP was easily incorporated into BNC with expression of ZZ-BNC-EGFP. Using an anti-epidermal growth factor receptor (EGFR)

*To whom correspondence should be addressed. Tel: +81 78 803 6196, Fax: +81 78 803 6196, E-mail: akondo@kobe-u.ac.jp

antibody as a model, we also demonstrated that antibody-displaying ZZ-BNC-EGFP could specifically deliver EGFP to HeLa cells without loss of viability. The engineered BNCs in this study are promising carriers for efficient and specific protein delivery to target cells.

MATERIALS AND METHODS

Plasmid Construction—The expression plasmid for ZZ-BNC-EGFP was constructed as described below. The plasmid pGLDLIIP39-RcT contains the HBV envelope L gene (*9*). The gene encoding the deletion mutant L (Δ33-159) was prepared by a QuikChange Site Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) using pGLDLIIP39-RcT as template and the following primers: 5′-CAATCCAGATTGGGGCGGCCGCCCTGCACCGAAC-3′ and 5′-GTTCGGTGCAGGGCGGCCGCCCCAATCTGGATTG-3′. A fragment encoding the dimer of the Z domain derived from *Staphylococcus aureus* protein A was amplified using the plasmid pWI3αZZ as template (*17*). The primers utilized were 5′-GGGGGCGGCCGCGCGCAACACGATGAAGCCGTAGAC-3′ and 5′-GGTTGAGATAAAAGAGCTTTTGGCGCGGCCGCCTTT-3′. The amplified fragment was digested with NotI and ligated into the NotI site of pGLDLd33 and the insertion orientation was confirmed by DNA sequencing. The resulting plasmid was designated pGLDLd33-ZZ.

The L-ZZ gene tethered to honeybee melittin secretion signal peptide sequence was amplified from the plasmid pGLDLd33-ZZ by PCR using the following primers: 5′-GGGGGATCCACCATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTTATGGTCGTATACATTTCTTACATCTATGCCATGGGGACGAATCTTTCTGTTCCC-3′ and 5′-CACCACCACCAGAACCACCACCACCAATGTATACCCAAAGACAAA-3′. A fragment encoding the EGFP gene was amplified using the plasmid pX-GFP (*18*) as template with the following primers: 5′-TGGTGGTTCTGGTGGTGGTGGTTCTATGGTGAGCAAGGGCGAGGA-3′ and 5′-GGGCCGCGGGTCGACTTACTTGTACAGCTCGTCCATGC-3′. Both amplified fragments were mixed and PCR was carried out using the following primers: 5′-GGGGGATCCACCATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTTATGGTCGTATACATTTCTTACATCTATGCCATGGGGACGAATCTTTCTGTTCCC-3′ and 5′-GGGCCGCGGGTCGACTTACTTGTACAGCTCGTCCATGC-3′. The amplified fragment encoding the ZZ-EGFP gene fusion was digested with BamHI/SacII and ligated into pXIHAbla (*18*). The resulting plasmid was designated pXIHAbla-Ld33-ZZ-EGFP.

Expression and Purification of the ZZ-BNC-EGFP—*Trichoplusia ni* BTI-TN-5B1-4 insect cell line (High Five; Invitrogen, Carlsbad, CA, USA) was maintained in a serum-free medium (Express Five SFM; Invitrogen) supplemented with 0.26 g/l L-glutamine and 10 mg/l gentamicin (Invitrogen) at 27°C. HeLa (human cervical carcinoma) and HepG2 (human hepatocellular carcinoma) cells were obtained from Riken BioResouse Center (Tsukuba, Japan). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nakalai Tesque, Kyoto, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in 5% CO_2.

ZZ-BNC-EGFP was expressed using High Five cells as described previously (*20*). Briefly, High Five cells were seeded on a 35-mm dish at a density of 2×10^5 cells/ml for 24 h before transfection, then the cells were transfected with 2 μg of pXIHAbla-Ld33-ZZ-EGFP using FuGENE HD transfection reagent (Roche Ltd, Basel, Switzerland) following the manufacturer's procedure. At 72 h after transfection, expression of ZZ-BNC-EGFP was confirmed by western blotting. The supernatant was fractionated by 12.5% SDS–PAGE and electrotransferred onto a poly vinilidene difluoride (PVDF) membrane. The blots were incubated with an anti-protein A antibody (Rockland, PA, USA), anti-S protein antibody from an IMx enzyme immunoassay (EIA) kit (Abbott Laboratories, Abbott Park, IL, USA) and anti-EGFP antibody (MBL, Nagayo, Japan), then stained using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Promega, Madison, WI, USA). The amount of expressed ZZ-BNC-EGFP in the culture supernatant was analysed using the IMx kit, following the manufacturer's procedure.

The culture supernatant (30 ml) of transfected insect cells was collected and mixed with polyethylene glycol 6,000 solution (33%, w/v). After 6 h incubation, the mixture was centrifuged at 10,000 g for 30 min at 4°C and the precipitate was dissolved in 2.8 ml of phosphate-buffered saline (PBS). The solution was layered onto a discontinuous cesium chloride (CsCl) gradient [11 ml, concentration: 10–40% (w/v) in buffer A (0.1 M sodium phosphate, 15 mM EDTA)] and centrifuged at 24,000 r.p.m. for 16 h at 15°C in a himac CP70MXX centrifuge (Hitachi, Tokyo, Japan). The amount of ZZ-BNC-EGFP in each fraction was analysed using the IMx kit and ZZ-BNC-EGFP was dialysed against PBS. After dialysis, the ZZ-BNC-EGFP solution was layered onto a discontinuous sucrose gradient [11 ml, concentration: 10–50% (w/v) in buffer A] and centrifuged at 24,000 r.p.m. for 10 h at 4°C. Fractions containing ZZ-BNC-EGFP were dialysed against PBS and stored at 4°C. The concentration of ZZ-BNC-EGFP was determined by IMx enzyme immunoassay kit according to manufacture's procedure. As the standard protein, wild type of BNC, which was obtained from the recombinant yeast (*9*), was used.

Dynamic Light Scattering Analysis of Purified ZZ-BNC-EGFP—The size of the purified ZZ-BNC-EGFP was determined by dynamic light scattering using a Zetasizer Nano particle size analyser (Malvern Instruments Ltd, Worcestershire, UK), following the manufacturer's procedure.

Target Cell-Specific Delivery of ZZ-BNC-EGFP via Anti-EGFR Antibody—ZZ-BNC-EGFP was mixed with an equal amount of anti-EGFR polyclonal antibody (Lab Vision, CA, USA) for 30 min, and prepared as anti-EGFR-BNC-EGFP. As a negative control, we employed an anti-biotin polyclonal antibody (Bethyl Laboratories, TX, USA) and prepared anti-biotin-BNC-EGFP in a similar way. HeLa or HepG2 cells (10^4 cells) were seeded on a 35-mm glass base dish and cultured for 20 h. After washing with serum-free DMEM, anti-EGFR-BNC-EGFP was added to the HeLa cells (final concentration: 30 and 70 nM) and they were cultured for 1 h. The cells were washed with serum-free DMEM twice and subsequently incubated with FBS-containing DMEM for appropriate time intervals. The cells were observed with a 5 Pa laser-scanning confocal microscope (LSCM) (Carl Zeiss, Oberkochen, Germany).

Fluorescence 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 oil immersion objective.

Internalization of ZZ-BNC-EGFP and Alexa Fluor 647 EGF Complex via EGFR—Anti-EGFR-BNC-EGFP or ZZ-BNC-EGFP was added to HeLa cells at final concentration of 30 nM with incubation for 1 h. After washing with serum-free DMEM twice, 1.5 nM Alexa Fluor 647 EGF complex (Invitrogen) was added with incubation for 1 h. The cells were washed twice and subsequently cultured for appropriate times (0 or 4 h) in serum-containing DMEM. After washing with serum-free DMEM twice, the cells were observed with a LSCM. Fluorescence images were acquired using the 488 nm line of an Ar laser for excitation and a 505–530 nm band pass filter for emission and with the 633 nm line of a He–Ne laser for excitation and a 650 nm band pass filter for emission. The specimens were viewed using a 63-fold oil immersion objective.

RESULTS AND DISCUSSION

Expression and Purification of ZZ-BNC-EGFP—The aim of this study was to establish target cell-specific protein delivery of BNC. The PreS region, which binds specifically to human hepatocytes, was replaced by the ZZ domain, which has high affinity to the Fc region of various antibodies. EGFP was selected as the model delivered protein, because it is easily visible and can thus be used for tracing of BNC inside cells; EGFP was genetically fused to the C-terminus of ZZ-BNC (Fig. 1). These engineered BNCs are hardly expressed in yeast and mammalian cells (*19*). Therefore, we chose insect cells as the host cells to produce ZZ-BNC-EGFP because insect cells, which have high protein productivity, have been an attractive host for BNC expression (*20*). In addition, the advantages of insect cells are a serum-free expression system and a simple purification step due to secretory expression.

Figure 2 clearly shows that ZZ-BNC-EGFP was expressed and secreted in the medium 72 h after transfection, as confirmed by western blotting. The degradation of ZZ-BNC-EGFP was hardly observed compared to a yeast expression system (data not shown), indicating the superiority of the secretory expression system using insect cells. From Fig. 2, there are two major bands in each western blotting analysis, which might be caused by glycosylation of ZZ-BNC-EGFP as previously reported (*21*).

ZZ-BNC-EGFP particle formation was assayed by sucrose gradient ultracentrifugation in the purification step. After ultracentrifugation, the fluorescence of each fraction was evaluated. Figure 3A shows that the amount of ZZ-BNC-EGFP in each fraction corresponded to the fluorescence intensity, suggesting that ZZ-BNC-EGFP has potential to form particle. We also confirmed that other impurities were separated from purified samples by SDS–PAGE and silver stain (data not shown). The yield of purified ZZ-BNC-EGFP was 15.3 μg from 30 ml culture supernatant, which is 120 times higher than that of BNC-EGFP produced using COS-7 cells (data not shown). These results show that the insect cell expression system is appropriate for the production and purification of engineered BNC. In addition, size of the purified ZZ-BNC-EGFP was analysed using dynamic light scattering. Figure 3B shows that the diameter of ZZ-BNC-EGFP was about 130 nm, which is larger than that of the original BNC expressed in yeast cells (about 80 nm) (*22*). The large size may be caused by EGFP that has fused inside the particles or by properties of insect cell-derived phospholipids.

Target Cell-specific Delivery of ZZ-BNC-EGFP via Anti-EGFR Antibody—To form anti-EGFR-BNC-EGFP, which is a BNC displaying an antibody *via* the ZZ domain, ZZ-BNC-EGFP was incubated with an equal amount

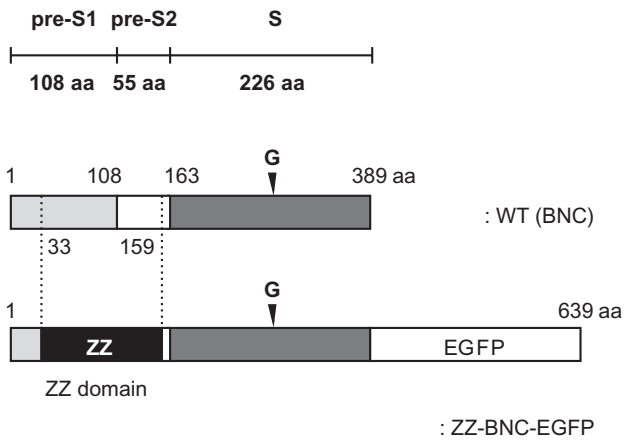

Fig. 1. **Schematic representation of ZZ-BNC-EGFP.** The hepatocyte-specific region corresponding to amino acids 33–159 of L-protein was replaced with the ZZ domain, which can bind to the Fc fragment of antibodies. EGFP was also fused to the C-terminus of L protein. N-linked glycosylation site at position 309 aa of the S protein was indicated with 'G'.

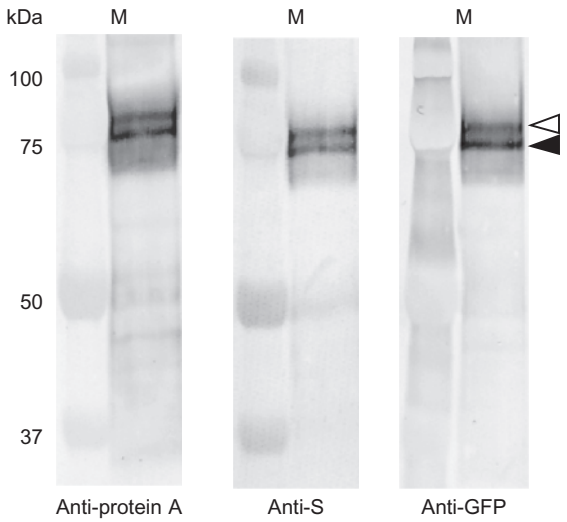

Fig. 2. **Western blot analysis of ZZ-BNC-EGFP.** The culture supernatant of transfected insect cells was analysed with anti-protein A antibody (left), anti-S protein antibody (middle) and anti-EGFP antibody (right). The detected two bands were corresponding to unglycosylated (closed arrow) and glycosylated (opened arrow) form of ZZ-BNC-EGFP.

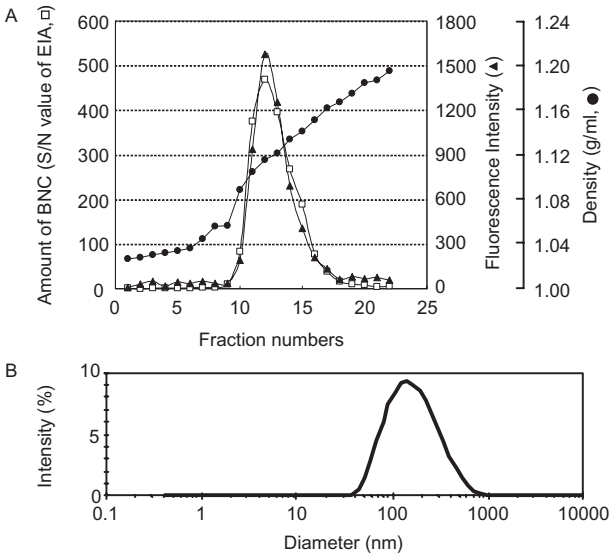

Fig. 3. **The evaluation of ZZ-BNC-EGFP after sucrose gradient centrifugation.** (A) After centrifugation, the density of each fraction was evaluated (closed circles), as amount of BNC (S/N value of EIA) was measured by IMx kit (open squares) and EGFP fluorescence (closed triangles). (B) ZZ-BNC-EGFP in the peak fraction was assayed by dynamic light scattering.

of anti-EGFR antibody for 30 min. Then anti-EGFR-BNC-EGFP was incubated with HeLa cells, which have been reported to display between 35,000 and 65,000 EGFRs per cell (*23*). After 1 h incubation with 30 nM anti-EGFR-BNC-EGFP, cells were washed and observed by LSCM. After 1 h incubation with anti-EGFR-BNC-EGFP, the fluorescence of EGFP was localized at the cell surface and internalization of anti-EGFR-BNC-EGFP was hardly observed. Internalization of anti-EGFR-BNC-EGFP was observed after 3 h and almost accomplished after 6 h (Fig. 4A). As a negative control, 30 nM ZZ-BNC-EGFP, which does not display the antibody, was incubated with the cells. ZZ-BNC-EGFP was not localized at the cell surface and was not internalized after 6 h incubation. We also employed an anti-biotin antibody as another negative control; 30 nM anti-biotin antibody displaying ZZ-BNC-EGFP, called anti-biotin-BNC-EGFP, was not internalized inside cells after 6 h incubation, suggesting that the anti-EGFR antibody displaying ZZ-BNC-EGFP was able to recognize HeLa cell. Additionally, when ZZ-BNC-EGFP (70 nM) was incubated with HepG2 cells for 6 h, no internalization was observed (data not shown). This result clearly shows that ZZ-BNC-EGFP lost specificity for human hepatocytes. Therefore, it indicated that the delivery of ZZ-BNC-EGFP depended on the antibody which was displayed on its surface. ZZ-BNC-EGFP, which displays suitable antibody against targeted cells, is expected to accomplish the specific delivery.

We also investigated the concentration dependence of anti-EGFR-BNC-EGFP delivery. In the case of 7 nM anti-EGFR-BNC-EGFP, localization and internalization were hardly observed (data not shown). After incubation with 70 nM anti-EGFR-BNC-EGFP, a lot of the particles were localized on the surface and were internalized after 6 h incubation (Fig. 4B). As expected, 70 nM ZZ-BNC-EGFP (without antibody) was not internalized after 6 h incubation (data not shown), showing that a significantly low concentration (70 nM) is enough to achieve specific and effective protein delivery to the target cells. In addition, the uptake of anti-EGFR-BNC-EGFP was faster compared with that of the original BNC uptake into HepG2 cells (*20*). Interestingly, longer incubation of anti-EGFR-BNC-EGFP with HeLa cells did not improve the internalization of anti-EGFR-BNC-EGFP (data not shown). Therefore, replacement of the medium after 1 h incubation with anti-EGFR-BNC-EGFP is appropriate to achieve efficient protein delivery while retaining cell viability.

Internalization of Anti-EGFR-BNC-EGFP and EGF via EGFR—In order to ensure that anti-EGFR-BNC-EGFP was introduced to normal living cells in which EGFR was functional, the internalization of fluorescently labelled EGF (*24*) was evaluated. The cells were incubated with anti-EGFR-BNC-EGFP for 1 h, then washed, and the cells were subsequently incubated for 1 h with EGF. Then the cells were washed again and observed by LCSM. The green fluorescence of anti-EGFR-BNC-EGFP was localized at the cell surface and the red fluorescence of the Alexa Fluor 647 EGF complex was observed in the cytosol (Fig. 5A). After incubation with EGF, cells were incubated for an additional 4 h in serum-containing fresh medium and observed by LCSM. Figure 5B shows that both EGF and anti-EGFR-BNC-EGFP were internalized inside the cells, suggesting efficient protein delivery without loss of cellular function. In the case of ZZ-BNC-EGFP, although the fluorescently labelled EGF was internalized after 6 h incubation, ZZ-BNC-EGFP was not internalized (Fig. 5C). These results indicate that ZZ-BNC-EGFP can achieve specific protein delivery while retaining cellular functions, such as ligand–receptor interaction and internalization.

Anti-EGFR antibody, which has high affinity against the extracellular domain of EGFR, competes with EGF for binding to EGF receptors and also induces internalization of plasma membrane receptors (*25*, *26*). Thus, liposomes conjugated with anti-EGFR antibody fragments are used to deliver genes and drugs into EGFR-overexpressing tumour cells (*27–31*). However, it has been reported that the EGF:EGFR complex is internalized through the endocytic pathway and broken down in lysosomes (*32*). As shown in Fig. 5B, the localization of anti-EGFR-BNC-EGFP and EGF seemed to differ slightly. This result implies that anti-BNC-EGFP escapes from endosome as well as wild type of BNC reported previously (*33*), although additional experiments are needed to elucidate the endosomal escape of ZZ-BNC-EGFP.

Conclusively, we produced novel particles named ZZ-BNC-EGFP, which display the protein A ZZ domain and include EGFP, using an insect cell expression system with high yield. Antibody-displaying ZZ-BNC-EGFP can target specific cells and deliver the proteins effectively. ZZ-BNC will be a powerful tool for therapeutic protein delivery to desired cells *in vivo* as well as *in vitro* (*15*).

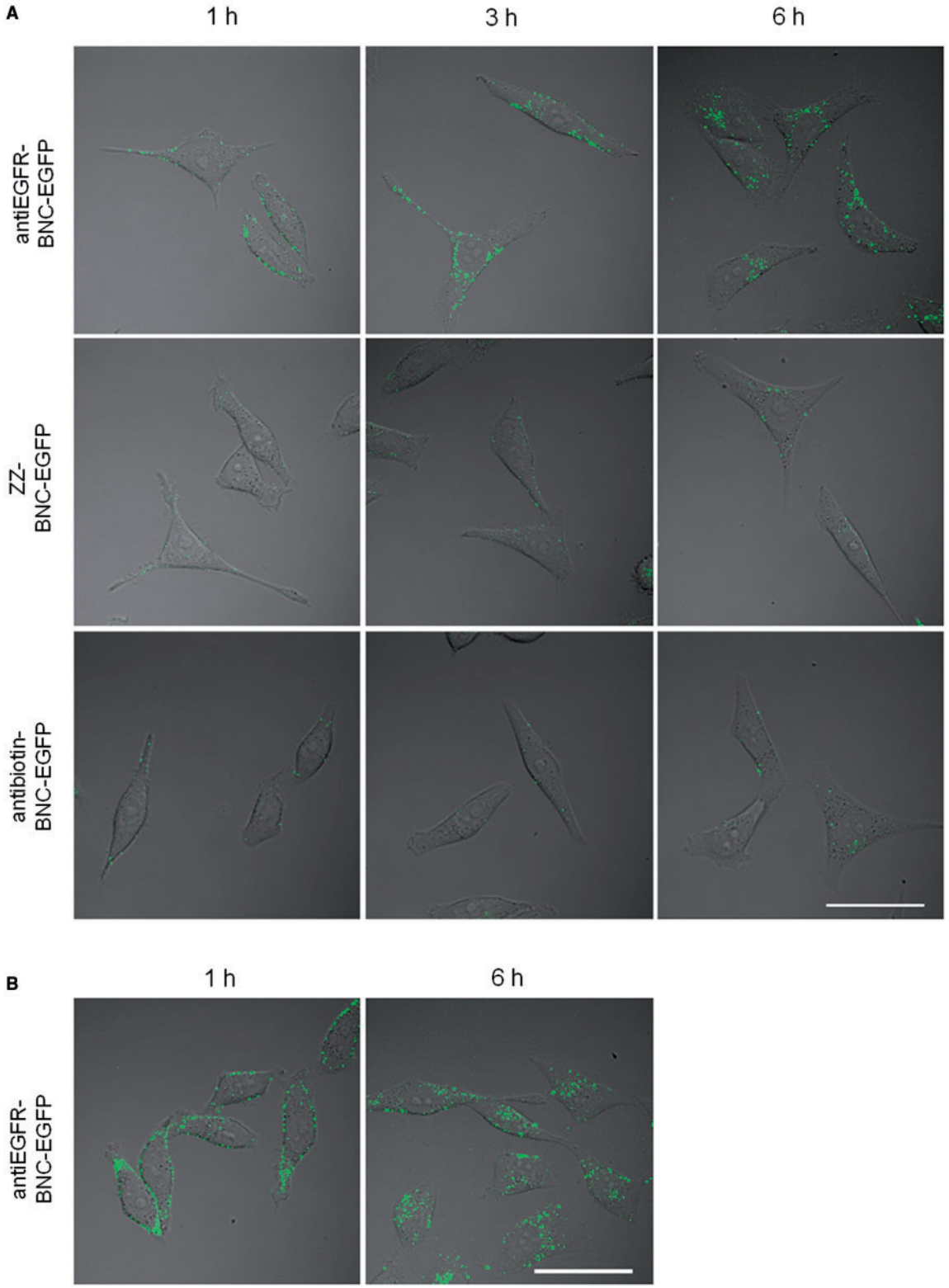

Fig. 4. **Target cell-specific delivery of ZZ-BNC-EGFP *via* an anti-EGFR antibody.** (A) The 30 nM ZZ-BNC-EGFP was incubated with anti-EGFR antibody (top panels), then the resulting anti-EGFR-BNC-EGFP was added to HeLa cells and the fluorescence observed. Negative controls were 30 nM ZZ-BNC-EGFP without antibody (middle panels) and ZZ-BNC-EGFP incubated with anti-biotin antibody (bottom panels) to form anti-biotin-BNC-EGFP. (B) The 70 nM anti-EGFR-BNC-EGFP was added to HeLa cells and the fluorescence was observed. Scale bar, 50 μm.

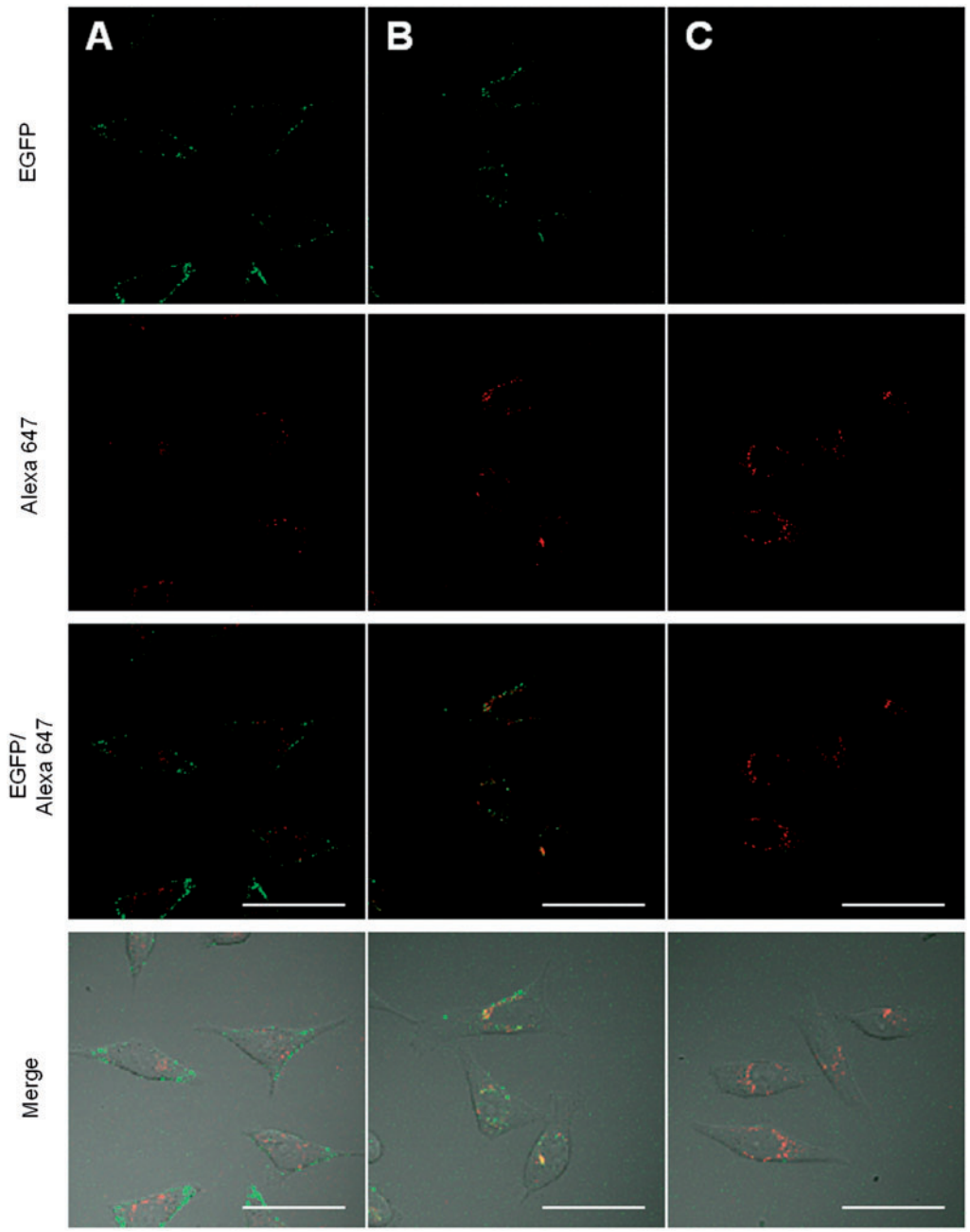

Fig. 5. **Internalization of ZZ-BNC-EGFP and Alexa Fluor 647 EGF *via* EGFR.** The 30 nM anti-EGFR-BNC-EGFP and Alexa Fluor 647 EGF were added to HeLa cells. Fluorescence was observed after 2 h (A) and 6 h (B) incubation. The green fluorescence results from EGFP and the red fluorescence results from the Alexa Fluor 647 EGF. In the negative control (C), 30 nM ZZ-BNC-EGFP and Alexa Fluor 647 EGF were added to HeLa cells and the fluorescence was observed after 6 h incubation. Scale bar, 50 μm.

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CONFLICT OF INTEREST

None declared.

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