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Affibody-displaying bionanocapsules for specific drug delivery to HER2-expressing cancer cells

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ABSTRACT

A novel HER2-targeted carrier was developed using bionanocapsules (BNCs). Bionanocapsules (BNCs) are 100-nm hollow nanoparticles composed of the L-protein of hepatitis B virus surface antigen. An affibody of HER2 was genetically displayed on the BNC surface (Z_{HER2}-BNC). For the investigation of binding affinity, Z_{HER2}-BNC was incubated with the cancer cell lines SK-BR-3 (HER2 positive), and MDA-MB-231 (HER2 negative). For analysis of HER2 targeting specificity, Z_{HER2}-BNC or Z_{WT}-BNC (without affibody) was incubated with both SK-BR-3 and MDA-MB-231 cells by time lapse and concentration. For the delivery of encapsulated molecules (calcein), fluorescence of Z_{HER2}-BNC mixed with liposomes was also compared with that of Z_{WT}-BNC and nude liposomes by incubation with SK-BR-3 cells. As a result, Z_{HER2}-BNC-liposome complex demonstrated the delivery to HER2-expressing cells (SK-BR-3) with a high degree of specificity. This indicates that genetically engineered BNCs are promising carrier for cancer treatment.

Most of studies about drug delivery system (DDS) are focused on three points; enhancement of targeting efficiency,[1] increase of drug encapsulation yield,[2] and effective release of drug into the target cell.[3] Among these topics, the research on improving target-efficiency is the most active field such as materials for carrier and homing molecule immobilization technique for active targeting. Although viral system is known as effective to target and deliver, there are limitations about safety problem such as high immunogenicity and possibility of introducing foreign viral gene. Therefore, the development of novel carrier should be required.

A bionanocapsule (BNC) is composed of hepatitis B virus (HBV) surface antigen (HBsAg) L-protein combined with a lipid bilayer derived from yeast endoplasmic reticulum (ER) membrane.[4] Bionanocapsules are attractive as carriers for targeted drug delivery due to their high stability in the blood, high internalized efficiency compared with original HBV, high capacity, and high specificity for human hepatocytes.[5–7] However, a BNC has original specificity only for hepatocytes. To surmount this limited targeting, the pre-S region (a human hepatocyte recognizing molecule) is genetically substituted with other bio-recognizable molecules, such as epidermal growth factor (EGF) or cell penetrating peptide (CPP).[5,8] However, the range of complex ligands that can be displayed on the surface of a BNC is limited. There is considerable interest in development of novel targeting molecules that are both simple in structure and have high specificity that can be engineered onto BNCs.

We focused on using affibodies as targeting molecules for BNC-mediated drug delivery. Affibodies are a new class of affinity ligands derived from one of the IgG-binding domains of staphylococcal protein A. Affibody molecules have simple and robust structures with lower molecular weight (6 kDa) than antibodies (about 150 kDa), and have both high binding affinity and high specificity. Affibody molecules that can bind a range of different proteins, such as insulin, TNF-α, EGFR, and HER2, have been identified.[9] HER2 (ErbB2) is a member of the human epidermal growth factor receptor (EGFR) family, and is highly expressed in several types of cancer, especially tumors of the breast (30%), and ovary (15–30%).[10,11] Over-expression of HER2 is associated with resistance to chemotherapy and poor prognosis,[12,13] thus making HER2 an attractive target for molecular therapy.[14–16] Among HER2-binding affibody variants, $Z_{HER2:342}$ shows very high affinity (K_D ~22 pM), and it has been applied to various carriers, including liposomes,[17] nanoparticles,[18] biopolymers,[19] and the adenovirus vector.[20] In this study, we constructed $Z_{HER2:342}$ displaying BNCs (Z_{HER2}-BNC) by genetically substituted the pre-S region with Z_{HER2} and the affibody-displaying BNCs-assisted drug delivery was demonstrated.

The plasmid construction and BNC preparation were carried out as follows. The pGLDLIIP39-RcT plasmid contains the HBV

Abbreviations: BNCs, bionanocapsules; HBV, hepatitis B virus; HBsAg, hepatitis B virus surface antigen (HBsAg).

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envelope L gene.[4] A fragment encoding GLD promoter and cloning sites was amplified using pGLDLIIP39-RcT as a template. The primers utilized were 5′-GGGAGATCTGCGAGCTTACCAGTTCTCAC-3′ and 5′-GGGGCGGCCGCGGATCCCCGCGGTGTTTTTATACTCGACCTCG-3′. The amplified fragment was digested with *Bgl*II/*Not*I and ligated into *Bam*HI/*Not*I sites in pGLDLd50.[8] The resulting plasmid was named pGLDsLd (1–159). The fragment encoding the chicken-lysozyme signal peptide and a part of L gene (from 1 to 49 a.a.) was subsequently amplified from pGLDLIIP39-RcT using the following primers: 5′-GGGCCGCGGATGAGATCTTTGTTGATCTT-3′ and 5′-GGGGCGGCCGCCGGATCCACCCGCTCCTACCTGATTTGCCT-3′. The amplified fragment was digested with *Sac*II/*Not*I and ligated into pGLDsLd (1–159). The resulting plasmid was named pGLDsLd50. The gene encoding synthetic EZ, which consists of the first six amino acids of domain E and Z derived from *Staphylococcus aureus* protein A, was amplified from pXIHAbla-Ld33-ZZ-EGFP[21] using the following primers: 5′-GGGGGATCCGCGCAACACGACGAAGC CGTAGACAACAAATTCAACAA-3′ and 5′-GGGGCGGCCGCCTTTCGGC GCCTGAGCATCAT-3′. The amplified fragment was digested with *Bam*HI/*Not*I and ligated into pGLDsLd50. The resultant plasmid was named pGLDsLd50-Z_{WT}. The gene encoding amino acid residues 1–25 of $EZ_{HER2:342}$, which includes the E domain and the two α-helix of the $Z_{HER2:342}$ domain, was prepared by annealing the following synthetic oligonucleotides: 5′-GTAGACAACAAATT CAACAAAGAAATGAGAAACGCGTACTGGGAGATCGCTTTGTTACCTAA CTTAAACAACCAA-3′ and 5′-TTGGTTGTTTAAGTTAGGTAACAAAGC GATCTCCCAGTACGCGTTTCTCATTTCTTTGTTGAATTTGTTGTCTAC-3′. A fragment encoding residues 19–58 of $EZ_{HER2:342}$, which includes the third α-helix of the $Z_{HER2:342}$ domain, was amplified from pGLDsLd50-Z_{WT} using the following primers: 5′-TTACCTAACTT AAACAACCAACAAAAGAGAGCCTTCATCAGAAGTTTATACGATGACCC AAGCCAAAGCGC-3′ and 5′-GGGGCGGCCGCCTTTCGGCGCCTGAG CATCAT-3′. Both amplified fragments were mixed and PCR was carried out using the following primers: 5′-GGGGGATCCGCGCAA CACGACGAAGCCGTAGACAACAAATTCAACAA-3′ and 5′-GGGGCGGC CGCCTTTCGGCGCCTGAGCATCAT-3′. The amplified fragment encoding $EZ_{HER2:342}$ in its entirety was digested with *Bam*HI/*Not*I and ligated into pGLDsLd50. The resulting plasmid was designated

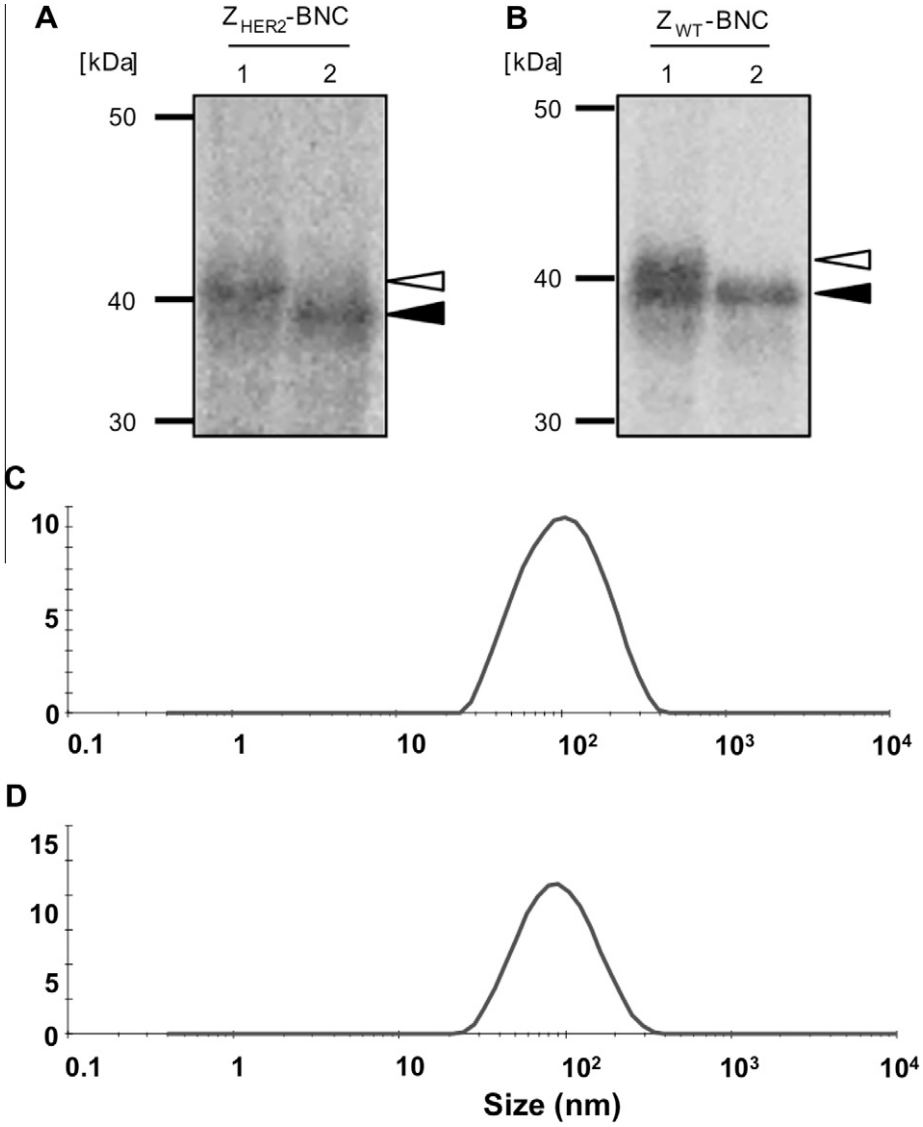

Figure 1. Western blot analysis and DLS of affibody-displaying BNCs. Purified affibody-displaying BNCs were analyzed with anti-HBsAg antibody. (A) Lane 1: Z_{HER2}-BNCs; Lane 2: PNGase F-treated Z_{HER2}-BNCs. (B) Lane 1: Z_{WT}-BNCs; Lane 2: PNGase F-treated Z_{WT}-BNCs. The two bands detected corresponded to unglycosylated (closed arrow) and glycosylated (opened arrow) forms of each affibody-displaying BNC. (C) Size distribution of Z_{HER2}-BNC. (D) Size distribution of Z_{WT}-BNC

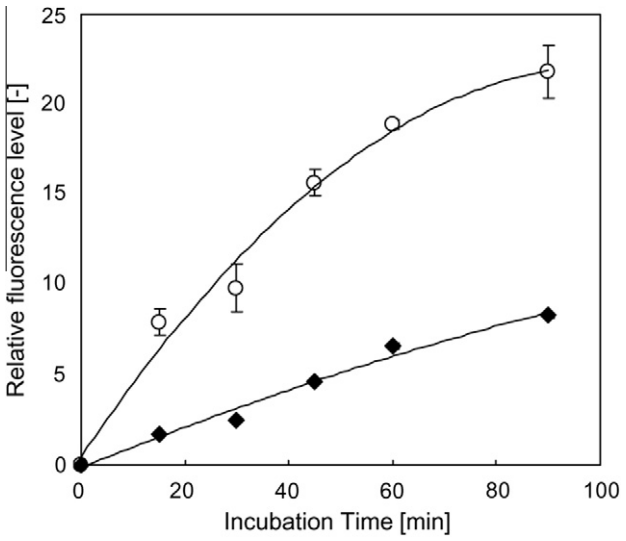

Figure 2. Time course of cellular uptake of Z_{HER2}-BNC into SK-BR-3 and MDA-MB-231 cells. Cells were incubated with Alexa488-labeled Z_{HER2}-BNC (final concentration: 1.0 μM) for 15, 30, 60, and 90 min. After washing three times with serum-free medium, cells was recovered and assayed by flow cytometry. Fluorescence intensity is given as the mean and standard deviation of three independent experiments. Open circle: SK-BR-3, Closed square: MDA-MB-231.

pGLDsLd50-Z_{HER2}. Z_{HER2}-BNCs and Z_{WT}-BNCs were obtained according to the previously described methods.[4]

Purified Z_{HER2}-BNCs were analyzed by western blot using anti-HbsAg (Fig. 1A and B). Two major bands were detected in each western blot analysis, which might have been caused by glycosylation of HbsAg.[4] The yield from 1 L of yeast culture was 2.80 mg of Z_{HER2}-BNCs and 3.50 mg of Z_{WT}-BNCs, a more efficient recovery that than reported for other genetically engineered BNCs.[5,8] The diameter of the affibody-displaying BNCs analyzed by dynamic light scattering was about 100 nm (Fig. 1C), which is almost the same as that of wild-type BNCs (Fig. 1D),[22] suggesting that affibody-displaying BNCs formed nano-size particles and size distributions of both BNCs were almost same. This size is suitable for use as a circulating carrier, since particles larger than 150 nm in diameter are known to be easily removed by macrophages.

To evaluate cellular uptake of Z_{HER2}-BNCs, the HER2 over-expressing SK-BR-3 human breast cancer cells (approximately 10^6 HER2 molecules per cell) were incubated with Alexa488-labeled $Z_{HER2:342}$-BNCs. Purified Z_{HER2}-BNCs were reacted with Alexa Fluor 488 succinimidyl esters (Invitrogen) as previously described.[8] The Alexa488 number labeled to Z_{HER2}-BNCs or Z_{WT}-BNCs were over104 molecules/BNC (0.95 molecule/protein). SK-BR-3 cells were obtained from Riken BioResource Center (Tsukuba, Japan) and Human breast carcinoma MDA-MB-231 cells were obtained from Dainippon Sumitomo Pharma (Osaka, Japan) and served as negative control cells. The labeled Z_{HER2}-BNCs (final concentration: 1.0 μM) were added to each well and cultured. After washing with serum-free medium, the green fluorescence was analyzed by flow cytometry (Fig. 2). The green fluorescence signal was exited with a 488-nm blue laser and collected through a 530/30-nm band-pass filter. The fluorescence of Z_{HER2}-BNCs was detected after 15 min incubation, which is much faster than the appearance of WT-BNC fluorescence against to human liver carcinoma cell.[5–7] The binding rate of SK-BR-3 is three times higher than that of MDA-MB-231 cells. The fluorescence level increased along with the incubation time, and 60 min incubation was sufficient for binding of Z_{HER2}-BNCs to HER2 on the surface of SK-BR-3 cells, a result similar to that obtained with Z_{HER2}-molecules.[24] In contrast, the fluorescence of MDA-MB-231 cells increased only slightly after 60 min incubation.

The dose dependence of Z_{HER2}-BNCs for cellular uptake was similarly examined. SK-BR-3 cells were incubated with 0.1, 0.5, 1.0, and 2.5 μM Alexa488-labeled Z_{HER2}-BNCs or Z_{WT}-BNCs for 60 min (Fig. 3A). In the case of SK-BR-3 cells, fluorescence intensity increased obviously with the concentration of Z_{HER2}-BNCs. Fluorescence intensity of Z_{WT}-BNCs used as negative controls, increased slightly due to non-specific uptake. In the case of MDA-MB-231 cells, there was no difference in fluorescence of Z_{HER2}- and WT-BNCs, and it did not increase under the various conditions tested (Fig. 3B). These results clearly demonstrate that Z_{HER2}-BNCs specifically bind only to HER2 over-expressing cells.

Then internalization of Z_{HER2}-BNCs was examined using time-course analysis. SK-BR-3 and MDA-MB-231 cells were incubated for 30 min, 1 h, and 3 h with Z_{HER2}-BNCs (1.0 μM), washed with PBS, then observed by laser-scanning confocal microscopy (Fig. 4). Localization of Z_{HER2}-BNCs on the surface of SK-BR-3 cells was observed after 30 min incubation, a similar result to that observed using Z_{HER2}-molecules.[14,24] After 1 h incubation, Z_{HER2}-BNC internalization was observed, and internalization increased after 3 h incubation, a result consistent with that observed for anti-EGFR antibody-displaying BNCs.[21] Significant fluorescence in MDA-MB-

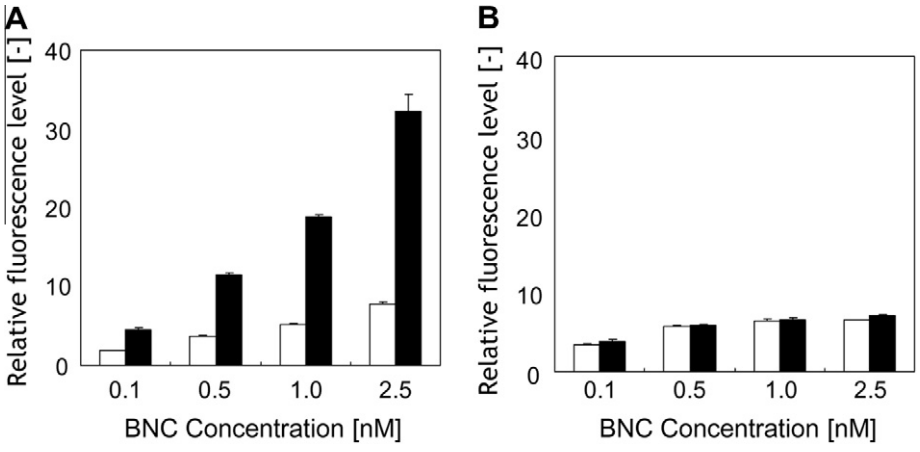

Figure 3. Dose dependence of Z_{HER2}-BNCs into (A) SK-BR-3 and (B) MDA-MB-231 cells. Cells were incubated with Alexa488-labeled Z_{HER2}-BNCs (final concentration: 0.1–2.5 μM) for 1 h. After washing three times with serum-free medium, cells were recovered and assayed by flow cytometry. The RLU (relative fluorescence level) is given as the mean and standard deviation of three independent experiments. Open bar: Z_{WT}-BNC; Closed bar: Z_{HER2}-BNC.

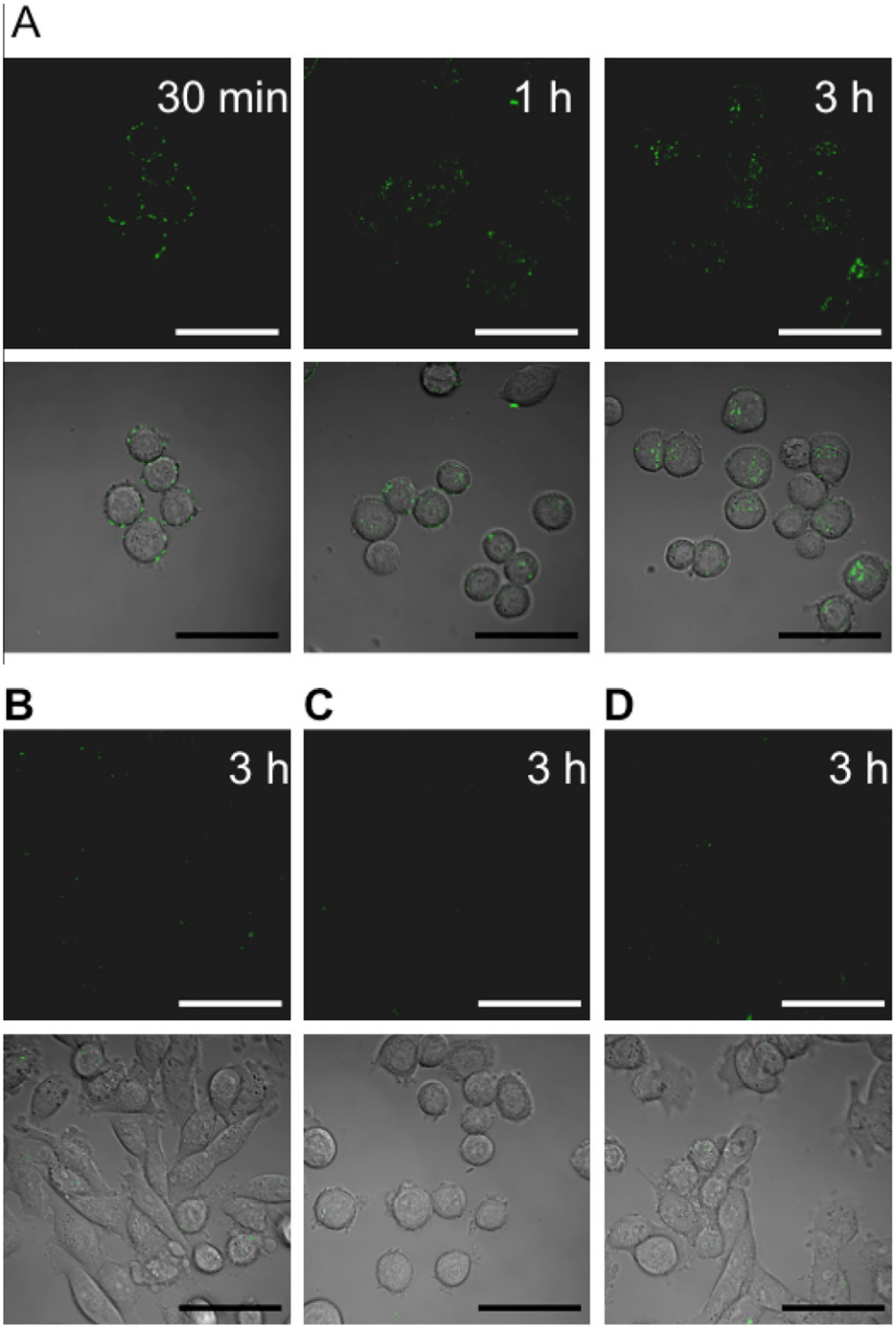

Figure 4. Fluorescence images of SK-BR-3 and MDA-MB-231 cells treated with affibody-displaying BNCs. Cells were incubated with 1.0 μM Alexa488-labeled Z_{HER2}-BNCs or Z_{WT}-BNCs. After incubation, cells were washed three times with serum-free medium and observed by laser-scanning confocal microscopy. (A) SK-BR-3 cells incubated with Z_{HER2}-BNCs for 30 min, 1 h, or 3 h. (B) MDA-MB-231 cells incubated with Z_{HER2}-BNCs for 3 h. (C) SK-BR-3 cells incubated with Z_{WT}-BNCs for 3 h. (D) MDA-MB-231 cells incubated with Z_{WT}-BNCs for 3 h. Scale bar = 50 μm.

231 cells was not observed even after 3 h incubation (Fig. 4B). In the case of Z_{WT}-BNCs, only slight fluorescence was observed from both SK-BR-3 and MDA-MB-231 cells (Fig. 4C and D). This result suggests that Z_{HER2}-BNCs have high specificity for HER2 and are capable of being efficiently internalized.

Encouraged by theses findings, we performed HER2-specific delivery using Z_{HER2}-BNCs. As a model compound, calcein was incorporated into Z_{HER2}-BNCs by the BNC-liposome complex method.[7] Briefly, freeze-dried liposomes (Coatsome EL-01-A, NOF, Tokyo, Japan) were dissolved in distilled water, and then the fluorescent material calcein (Dojindo, Kumamoto, Japan) was added (final concentration: 10 mM) and incubated for about 1 h at room temperature. Almost all liposomes might contain calcein inside because that excess amount of calcein was added during liposome

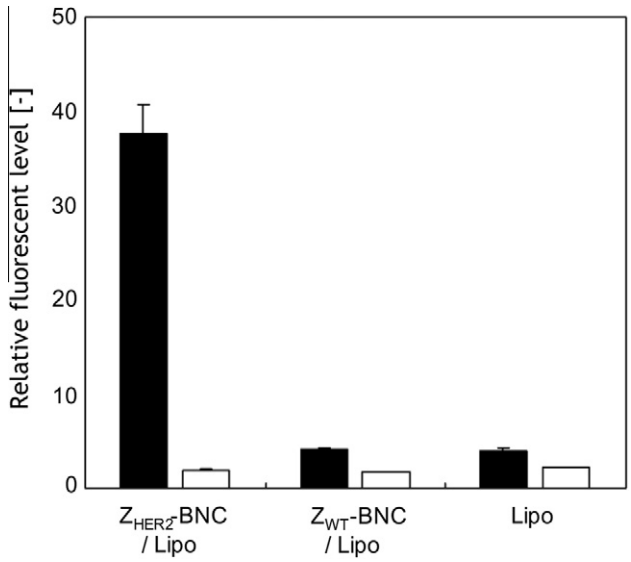

Figure 5. Transduction efficiency of calcein delivery using affibody-displaying BNCs. Cells were incubated with calcein-loaded Z_{HER2}-BNC-liposome complexes (Z_{HER2}-BNC/Lipo), Z_{WT}-BNC-liposome complexes (Z_{WT}-BNC/Lipo), or calcein-containing liposomes (Lipo) for 3 h. After three washes with serum-free medium, cells were recovered and assayed by flow cytometry. The RFL is given as the mean and standard deviation of three independent experiments. Closed bar: SK-BR-3; Open bar: MDA-MB-231.

formation. After gel filtration to remove non-incorporated calcein, freeze-dried Z_{HER2}-BNCs were added and incubated for 60 min to allow formation of the BNC-liposome complex. Large amount of calcein-containing liposome allowed that calcein was incorporated in almost all BNC-liposome complex. The complex was added to SK-BR-3 or MBA-MB-231 cells (final concentration of proteins: 100 μg/ml) and incubated for 3 h. Then cells were washed and analyzed by flow cytometry (Fig. 5). High intensity calcein fluorescence was detected only from SK-BR-3 cells treated with Z_{HER2}-BNC/Lipo. Only minute fluorescence was observed from SK-BR-3 and MDA-MB-231 cells treated with Z_{WT}-BNC-liposome complexes (Z_{WT}-BNC/Lipo) or calcein-containing liposomes (Lipo) as negative controls.

In order to insure that calcein was internalized into the targeted cells, these cells were observed by laser-scanning confocal microscopy (Fig. 6). After 3 h incubation, calcein fluorescence was observed at the cell surface and cytoplasm of SK-BR-3 cells, which is similar to results obtained using anti-EGFR antibody displaying BNCs.[21] In the case of Z_{WT}-BNC/Lipo or Lipo to MDA-MB-231 used as target cells, no fluorescence was observed. These results suggest that Z_{HER2}-BNC/Lipo could transfer calcein into targeted cells efficiently and specifically.

In conclusion, a Z_{HER2}-affibody displayed BNC was developed for use as a HER2-targeted carrier. Z_{HER2}-BNC began binding to the SK-BR-3 cell surface after 30 min and SK-BR-3 cells bind the BNCs at a rate three times higher than MDA-MB-231 cells. The uptake signal of Z_{HER2}-BNC/LIP was about seven times higher than that of nude liposomes. This result demonstrates the ease with which it is possible to modify the liposome surface with high target specificity. In addition, affibody-displaying BNCs have stability in PBS almost as same as that of wild-type BNCs, which is also superior as a DDS carrier (data not shown). Our results indicate that the genetically engineered BNC we describe is a promising drug delivery carrier for cell-specific delivery.

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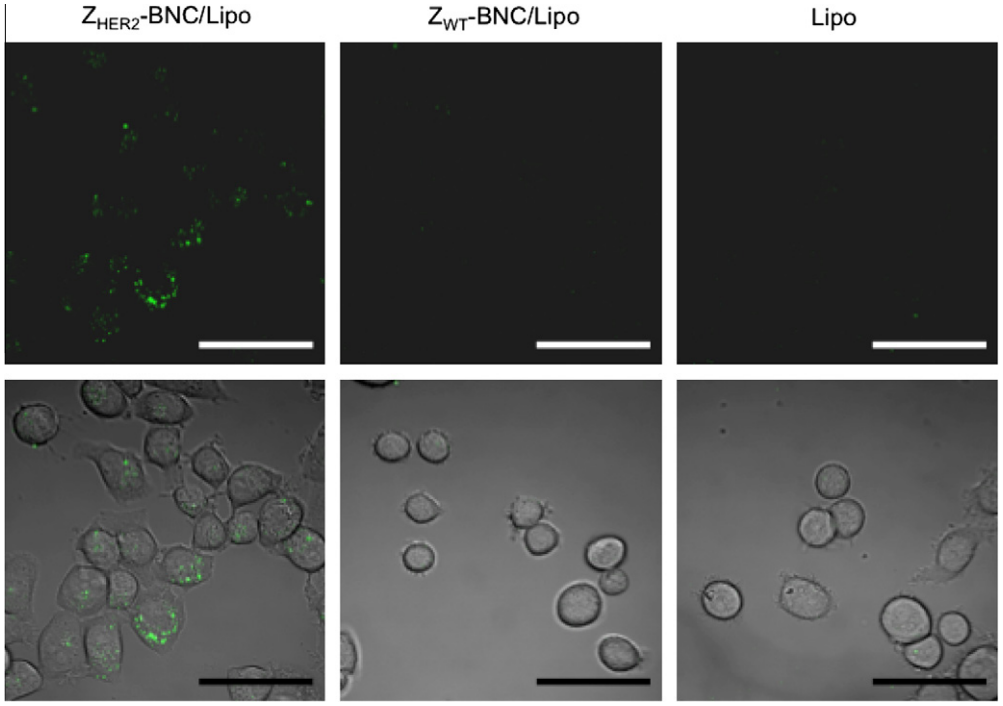

Figure 6. Fluorescence images of SK-BR-3 cells after treatment with calcein-loaded affibody-displaying BNCs. SK-BR-3 cells were incubated with calcein-loading Z_{HER2}-BNC/Lipo, Z_{WT}-BNC/Lipo, or Lipo for 3 h. After incubation, cells were washed three times with serum-free medium and then observed by laser-scanning confocal microscopy. Scale bar = 50 μm.

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