Bioorganic & Medicinal Chemistry Letters xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Affibody-displaying bio-nanocapsules effective in EGFR, typical biomarker, expressed in various cancer cells

Yuya Nishimura [a], Ryosuke Ezawa [b], Jun Ishii [a], Chiaki Ogino [b], Akihiko Kondo [a,b,*]

[a] Graduate School of Science, Technology and Innovation, Kobe University, Japan
[b] Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, Japan

ARTICLE INFO

Article history:
Received 7 July 2016
Revised 11 October 2016
Accepted 11 November 2016
Available online xxxx

Keywords:
Epidermal growth factor receptor
Bio-nanocapsule
Affibody
Drug delivery
Anticancer

ABSTRACT

The expression of epidermal growth factor receptor (EGFR) across a wide range of tumor cells has attracted attention for use as a tumor marker in drug delivery systems. Therefore, binding molecules with the ability to target EGFR have been developed. Among them, we focused on affibodies that are binding proteins derived from staphylococcal protein A. By displaying affibody (Z_{EGFR}) binding to EGFR on the surface of a bio-nanocapsule (BNC) derived from a hepatitis B virus (HBV), we developed an altered BNC (Z_{EGFR}-BNC) with a high specificity to EGFR-expressing cells. We considered two different types of Z_{EGFR} (Z955 and Z1907), and found that the Z1907 dimer-displaying BNC ($[Z1907]_2$-BNC) could effectively bind to EGFR-expressing cells and deliver drugs to the cytosol. Since this study showed that $[Z1907]_2$-BNC could target EGFR-expressing cells, we would use this particle as a drug delivery carrier for various cancer cells expressing EGFR.

Epidermal growth factor receptor (EGFR) is a transmembrane protein belonging to the ErbB receptor kinase family.[1] EGFR regulates important signals in intracellular processes such as proliferation, apoptosis, differentiation and migration via activation of the intracellular tyrosine kinase domain due to dimerization triggered by binding with ligands.[2–4] Over-expressing EGFR is detected in a wide range of tumors such as cell lung cancer, esophageal cancer, gastric cancer, pancreas cancer, and kidney cancer, which makes it an attractive target of molecular imaging and treatment.[5,6]

Methods that can be used to target EGFR-expressing tumors have been studied, and the best-known targeting agents are monoclonal antibodies against EGFR. These agents are often used in receptor inhibitors that cause internalization of ligand-binding sites and decrease the available EGFR on the cell surface.[7] Recent reports have focused on binding proteins called affibodies that are based on the Z domain derived from staphylococcal protein A.[8] Affibodies have a binding ability that is similar to that of antibodies, and they are small (monomer; 7 kDa and dimer; 15 kDa) with an easy expression that is due to a lack of cysteine. Affibodies consist of 58 amino acid residues with three α helix structures. Various affibodies with different specificities were developed by randomizing 13 amino acids during the first and second of three α helixes.[9] Among them, Z955 and Z1907 are known as affibodies (Z_{EGFR}) with specificity to EGFR. The binding ability is K_D = 185 nM and K_D = 5.4 nM, respectively, and the dimer formulate is reported to result in an increased binding ability ($[Z955]_2$; K_D = 50 nM).[10,11]

A bio-nanocapsule (BNC) that is composed of the L protein of the hepatitis B virus (HBV) surface antigen (HBsAg) and a lipid bilayer shows high specificity for human hepatocytes.[12] Therefore, BNCs have been studied as a possible drug delivery system (DDS) that can incorporate drugs and genes.[13] Several varieties of specificity-altered BNCs have been generated by deleting the hepatocyte-specific recognition site (located in the preS region) in the L protein and inserting binding molecules with the ability to target other cells. For example, a BNC with a ZZ domain[14] (dimer of Z domain derived from Staphylococcal protein A) inserted will bind to the Fc region of immunoglobulin G (IgG). Also, the insertion of biotin[15] permits the recognition of various types of cells through streptavidin and biotinylated antibodies that bind to receptors. Furthermore, we have developed a BNC displaying affibody, Z_{HER2} (Z342), which has the ability to specifically bind to HER2.[16,17] However, while full-length antibody-displaying BNCs[14] and heavy-chain antibody-displaying BNCs[15] mediated by the ZZ domain and the biotin–streptavidin interaction, respectively have already been reported as carriers targeting EGFR, a Z_{EGFR}-displaying BNC has not been developed yet. The advantage of this particle is that we can get the binding molecules-displaying particles without addition and fusion of them later on. To recognize target cells,

* Corresponding author at: Graduate School of Science, Technology and Innovation, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe 657-8501, Japan.
E-mail addresses: nyuya@landscape.kobe-u.ac.jp (Y. Nishimura), 134t410t@stu.kobe-u.ac.jp (R. Ezawa), junjun@port.kobe-u.ac.jp (J. Ishii), ochiaki@port.kobe-u.ac.jp (C. Ogino), akondo@kobe-u.ac.jp (A. Kondo).

http://dx.doi.org/10.1016/j.bmcl.2016.11.038
0960-894X/

Biotin-displaying BNC needs biotinylated binding molecules and streptavidin or streptavidin fused with binding molecules and ZZ domain-displaying BNC needs antibodies with Fc region. Although these particles have the advantages that they could change target cells by altering the kind of binding molecules, it would be thought that they have the demerits such as expansion of particle diameter, variation in amount of binding molecules on the particle surface and desorption of binding molecules in the blood. Therefore, it would be thought that this approach can be the best possible option in the case of EGFR-targeting BNCs. In this study, we developed two types of Z_{EGFR}-displaying BNC that display Z955 or Z1907 with differing affinity for EGFR on the particle surface and demonstrated the binding ability to EGFR-expressing cells.

We prepared [dimer of Z_{EGFR}]-displaying BNC, $[Z955]_2$-BNC and $[Z1907]_2$-BNC, from *S. cerevisiae* AH22R$^-$ harboring the plasmids pGLDsLd50-$[Z955]_2$[18] and pGLDsLd50-$[Z1907]_2$,[19] respectively, as described previously.[12]

First, in order to check the expressions of each L protein inserted into the dimers of Z955 or Z1907 in *S. cerevisiae*, we performed western blot analysis[20] (Fig. 1B). Since the desired bands appeared at about 45 kDa, the expressions of L protein inserted into the dimers of Z955 or Z1907 were confirmed. The band (<37 kDa) for $[Z955]_2$-BNC was thought to be the S protein fusing the monomer of Z955.

Second, to examine each $[Z_{EGFR}]_2$-BNC-formed particle structure, we measured the particle diameters via dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) (Fig. 1C). The diameters of $[Z955]_2$-BNC and $[Z1907]_2$-BNC were 127 and 86 nm, respectively. We determined that $[Z_{EGFR}]_2$-BNC formed the conventional particle size appropriate for a drug delivery system (<200 nm).

To demonstrate cell-specificity to EGFR-expressing cells[21] for $[Z_{EGFR}]_2$-BNC, Alexa Fluor 488-labeled[22] $[Z955]_2$-BNC and $[Z1907]_2$-BNC were added to EGFR-positive HeLa cells,[23] A431 cells, and EGFR-negative MCF-7 cells.[24] After incubation for 3 h, we measured the fluorescent intensity of these cells via flow cytometry[25] (Fig. 2). The addition of each particle promoted clear fluorescence for HeLa and A431 cells in a dose-dependent manner, while it never exhibited fluorescence for MCF-7 cells. This result indicated that $[Z955]_2$-BNC and $[Z1907]_2$-BNC specifically targeted EGFR-expressing cells, and $[Z1907]_2$-BNC showed an ability to recognize EGFR-expressing cells that was higher than that of $[Z955]_2$-BNC.

Then, to demonstrate the internalization of Alexa Fluor 488-labeled $[Z1907]_2$-BNC showing high cell-specificity, we observed the time course of internalization to EGFR-positive HeLa and EGFR-negative MCF-7 cells using LSCM[26] (Fig. 3). Binding to the cell membranes of HeLa was observed after incubation for 30 min, and the internalization into HeLa cells was observed after incubation for 120 min. Additionally, it was confirmed that $[Z1907]_2$-BNC had the ability of cell specificity, because neither binding nor internalization were observed for MCF-7 after incubation for 180 min.

To release drugs into the cytosol following cellular uptake, we confirmed whether it would be possible to grant the ability of endosomal escape to $[Z1907]_2$-BNC by forming a complex with LP containing 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), which is a helper lipid that enhances fusing with the endosomal membrane via the pH response. We prepared a complex carrier ($[Z1907]_2$-BNC/LP) encapsulating green fluorescence calcein.[27] Then, we added a particle to EGFR-expressing HeLa cells and observed the time course of endosomal escape using LSCM[28] (Fig. 4). This merge is the image overlapping the green fluorescence of calcein and the red fluorescence of endosome stained with LysoTracker, and the merge shows a yellow color if calcein is localized in the endosome. Since the merge image after incubation for 3 h showed yellow endosomes, this indicated that calcein was located in the endosomes. However, after incubation for 24 h, there were cells with green fluorescent cytosol due to the endosomal escape of calcein. This result indicated that $[Z1907]_2$-BNC/LP gained the

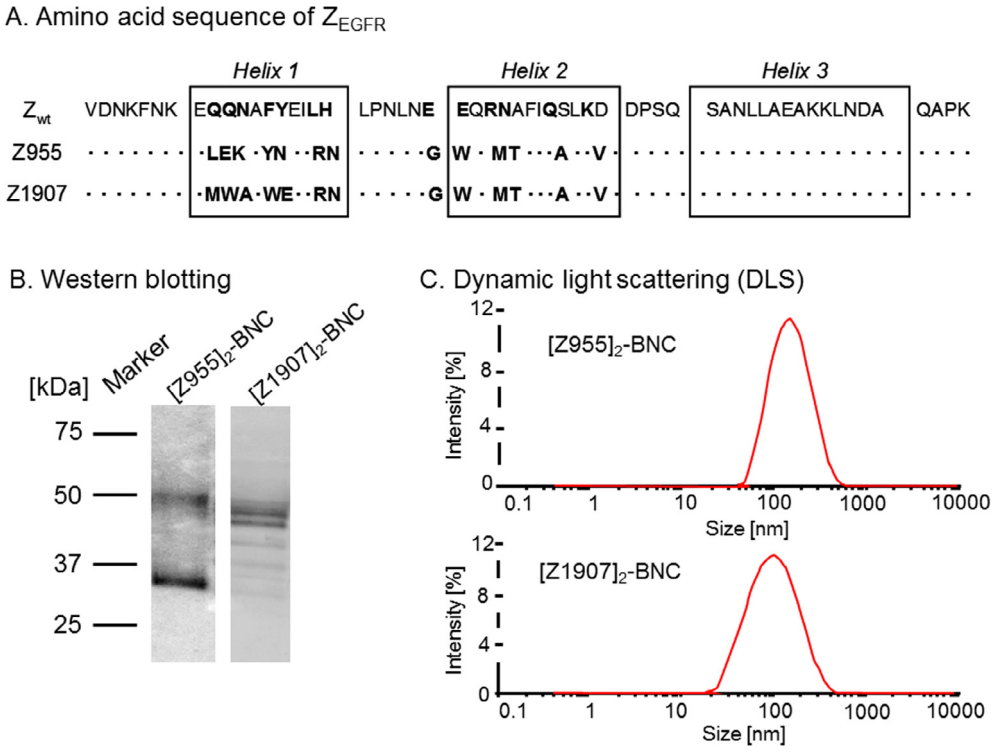

Fig. 1. Construction and characterization of Z_{EGFR}-BNCs. (A) amino acid sequence of Z_{WT}, Z955 and Z1907. (B) Western blotting analyses of Z_{EGFR}-BNCs. Purified $[Z955]_2$-BNC (left image) and $[Z1907]_2$-BNC (right image) were analyzed with anti-protein A antibody. (C) Size distribution using DLS analysis.

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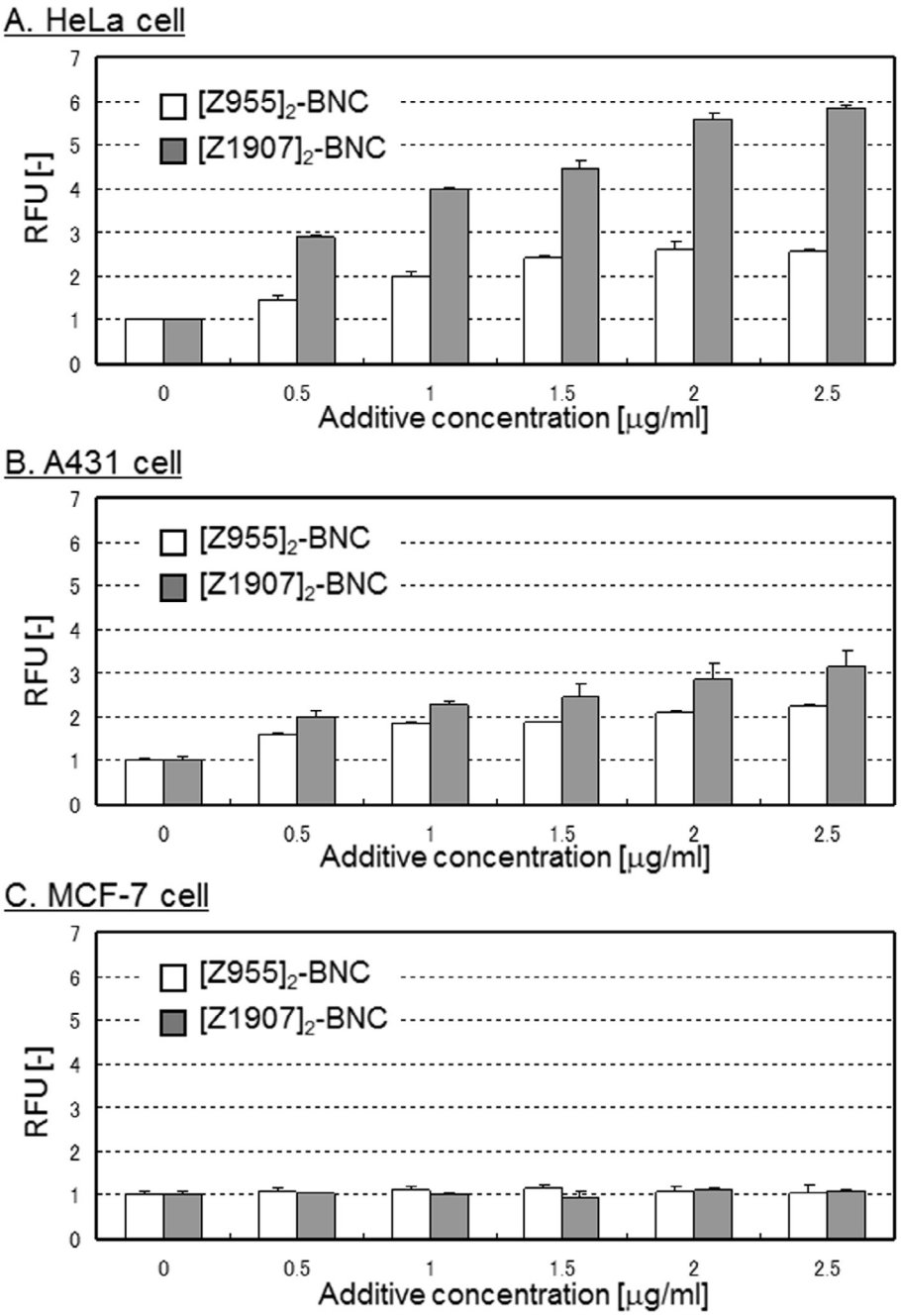

Fig. 2. Dose dependence of $[Z_{EGFR}]_2$-BNCs into (A) HeLa, (B) A431 and (C) MCF-7 cells. Cells were incubated with Alexa Fluor 488-labeled $[Z955]_2$-BNC (white bars) and $[Z1907]_2$-BNC (gray bars) (final concentration: 0.5–2.5 μg/ml) for 3 h.

ability of endosomal escape and would be available for drug delivery into the cytosol of EGFR-expressing cells.

Since it was indicated that $[Z1907]_2$-BNC/LP could release drugs into the cytosol, we demonstrated that $[Z1907]_2$-BNC/LP encapsulating doxorubicin (dox) showed cell-specific anticancer efficacy[29] (Fig. 5). The addition of dox only (white bars) showed non-cell-specific anticancer efficacy because dead cells were detected in both HeLa and MCF-7 cells. Conversely, there was no anticancer efficacy in either of the cells treated with LP encapsulating dox (gray bars). However, $[Z1907]_2$-BNC/LP encapsulating dox (black bars) showed anticancer efficacy in only HeLa cells. These results indicated that $[Z1907]_2$-BNC/LP could deliver drugs to EGFR-expressing cells and release drugs from the endosome to the cytosol with the retention of pharmaceutical activity.

In this study, we developed a carrier targeting EGFR-expressing cells by displaying the dimer of Z955 or Z1907 with specificity to EGFR on the surface of BNC. As a result, the specific binding of both $[Z955]_2$-BNC and $[Z1907]_2$-BNC to EGFR-expressing cells was confirmed. Furthermore, Z1907 is reported to have an affinity to EGFR that is higher than that of Z955, and it showed a binding ability to EGFR-expressing cells that also was higher than that of Z955 in the case of the BNC surface display. Additionally, since we could grant the ability of endosomal escape to a $[Z1907]_2$-BNC by forming a complex carrier with LP, $[Z1907]_2$-BNC/LP would be a useful drug delivery carrier for various cancer cells. This study indicated that we could develop an affibody-displaying BNC with a high affinity for various targeted cells by displaying the dimer of an affibody on the BNC surface.

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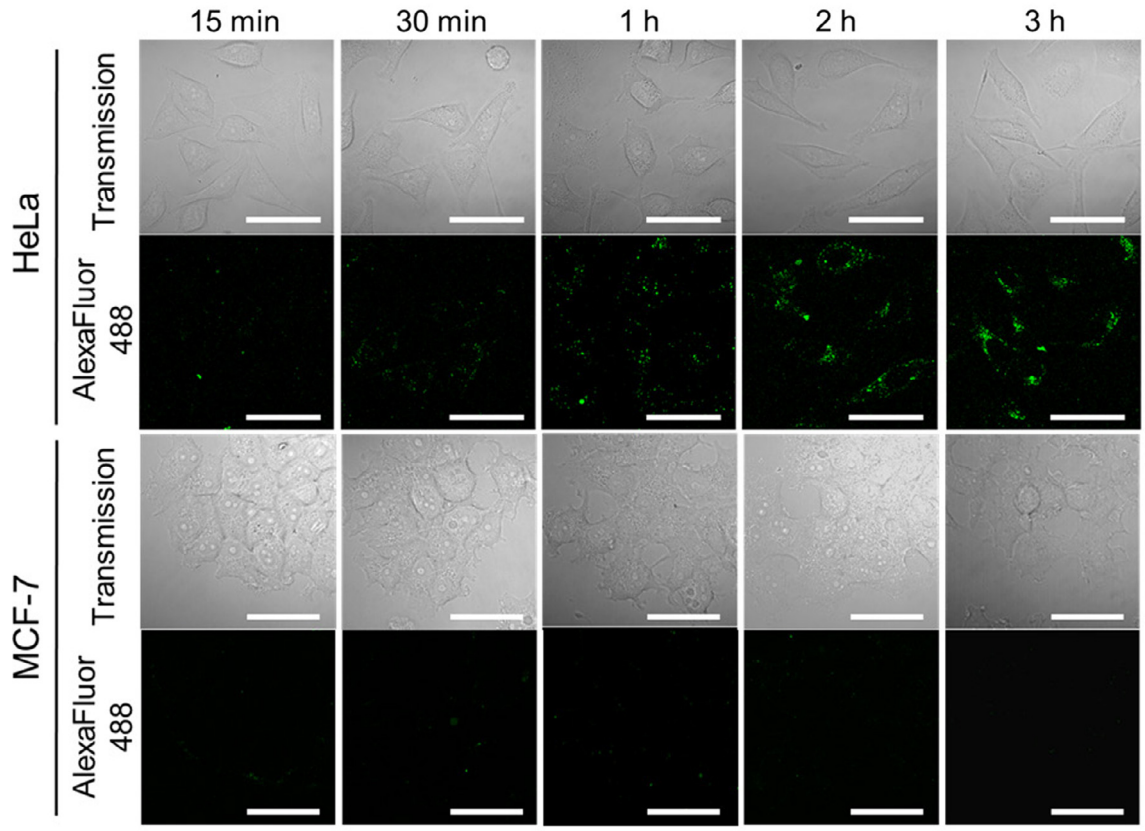

Fig. 3. Time course of Alexa Fluor 488-labeled $[Z1907]_2$-BNC into HeLa and MCF-7 cells. (final concentration: 9 μg/ml, scale bars: 50 μm).

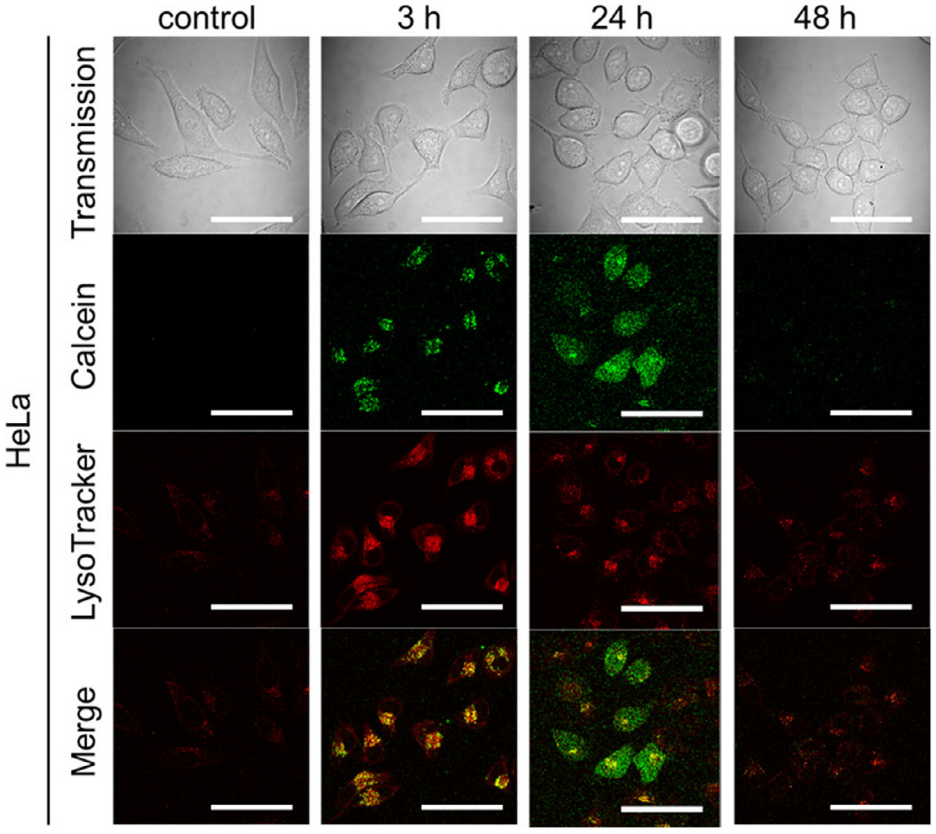

Fig. 4. Fluorescence images of HeLa cells treated with $[Z1907]_2$-BNC/LP complexes encapsulating calcein (100 μM). Scale bars: 50 μm.

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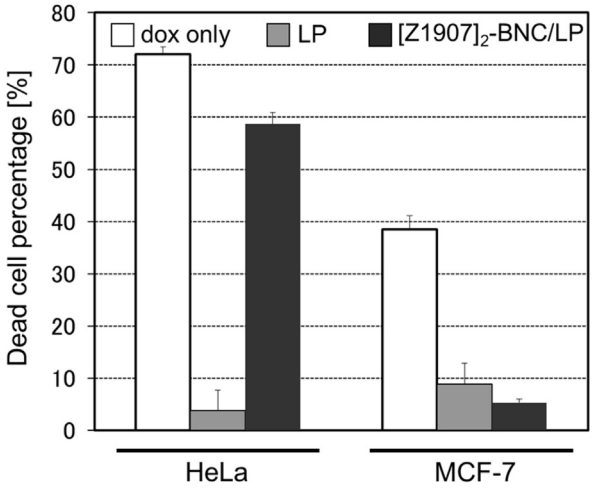

Fig. 5. Anticancer efficacy of $[Z1907]_2$-BNC/LP in HeLa and MCF-7 cells. White, gray and black bars showed dead cell percentage of HeLa and MCF-7 cells treated with doxorubicin (dox), LP encapsulating dox (LP) and $[Z1907]_2$-BNC/LP encapsulating dox ($[Z1907]_2$-BNC/LP), respectively.

Acknowledgements

The authors would like to thank Prof. Shun'ichi Kuroda for his advice on the BNC/LP conjugation method. This work was supported by JSPS KAKENHI Grant Numbers 15K21156 and in part by a Special Coordination Fund for Promoting Science and Technology, Creation of Innovative Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe) from the Ministry of Education, Culture, Sports and Technology (MEXT) to AK, and by Science Research Grants from the Ministry of Health, Labor and Welfare, Japan to AK.

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18. *Construction of a plasmid for the expression of $[Z955]_2$-BNC*: A DNA fragment encoding the N-terminal moiety of Z955 was prepared by annealing an A1 and A2 primer pair (A1; 5′- GTA GAC AAC AAA TTC AAC AAA GAA TTG GAA AAG GCG TAC AAC GAG ATC AGA AAC TTA CCT AAC TTA AAC GGT TGG -3′ and A2; 5′- CCA ACC GTT TAA GTT AGG TAA GTT TCT GAT CTC GTT GTA CGC CTT TTC CAA TTC TTT GTT GAA TTT GTT GTC TAC -3′), and a DNA fragment encoding the C-terminal moiety of Z955 was amplified via polymerase chain reaction (PCR) using a B1 and B2 primer pair (B1; 5′- TTA CCT AAC TTA AAC GGT TGG CAA ATG ACC GCC TTC ATC GCT AGT TTA GTT GAT GAC CCC AGC CAA AGC GC -3′ and B2; 5′- GGG GCG GCC GCC TTT CGG CGC CTA AGC ATC AT -3′) from pGLDsLd50-Z_{WT}.[16] A DNA fragment encoding the entire Z955 molecule was amplified via an overlap PCR using an A1 and B2 primer pair and the amplified fragments. The amplified fragments were ligated into the EcoRV site of pBlueScript II KS(+) (Agilent Technologies, Inc. Santa Clara, CA, USA). The resultant plasmid was designated pBlue-Z955. Then, A DNA fragment encoding the N-terminal moiety of $[Z955]_2$ was amplified by PCR using a C1 and C2 primer pair (C1; 5′- GGG GGA TCC GCG CAA CAC GAT GAA GCC GTA GAC AAC AAA TTC AAC AA -3′ and C2; 5′- TGT TGT CTA CTT TCG GCG CCT GAG CAT CAT TTA GCT TTT T -3′) from pBlue-Z955, and a DNA fragment encoding the C-terminal moiety of $[Z955]_2$ was amplified via PCR using a D and B2 primer pair (D; 5′- GGC GCC GAA AGT AGA CAA CAA ATT CAA CAA AGA A -3′) from pBlue-Z955. A DNA fragment encoding an entire $[Z955]_2$ molecule was amplified via an overlap PCR using a C1 and B2 primer pair and the amplified fragments. The amplified fragments were digested with BamHI/NotI and ligated into the BamHI/NotI sites of pGLDsLd50-Z_{WT}. The resultant plasmid was designated pGLDsLd50-$[Z955]_2$.
19. *Construction of a plasmid for the expression of $[Z1907]_2$-BNC*: A DNA fragment encoding the entire Z1907 molecule was amplified via PCR using an E and B2 primer pair (E; 5′- GTA GAC AAC AAA TTC AAC AAA GAA ATG TGG GCT GCG TGG GAA GAG ATC AGA AAC TTA CCT AAC TTA AAC GGT TGG -3′) from pBlue-Z955. The amplified fragment was ligated into the EcoRV site of pBlueScript II KS(+). The resultant plasmid was designated pBlue-Z1907. Then, A DNA fragment encoding the N-terminal moiety of $[Z1907]_2$ was amplified via a PCR using a C1 and C2 primer pair from pBlue-Z1907, and a DNA fragment encoding the C-terminal moiety of $[Z1907]_2$ was amplified via a PCR using a D and B2 primer pair from pBlue-Z1907. A DNA fragment encoding the entire $[Z1907]_2$ molecule was amplified via an overlap PCR using a C1 and B2 primer pair and the amplified fragments. The amplified fragments were digested with BamHI/NotI and ligated into the BamHI/NotI sites of pGLDsLd50-Z_{WT}. The resultant plasmid was designated pGLDsLd50-$[Z1907]_2$.
20. *Western blotting*: The purified BNCs were analyzed via sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto a polyvinilidene fluoride (PVDF) membrane. For detection of the Z protein, Goat anti-protein A antibodies (Rockland Immunochemicals Inc, Gilbertsville, PA, USA) were used as the primary antibody for immunoblotting, followed by anti-goat antibodies conjugated with alkaline phosphatase (AP) (Promega, Madison, WI, USA), which were used as the secondary antibody. Blots were developed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Promega).
21. *Cell culture*: Gibco® Fetal bovine serum (FBS) and L-glutamine were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Dulbecco's modified Eagle medium (DMEM) was purchased from Nacalai Tesque (Kyoto, Japan). HeLa cells (human cervical carcinoma), A431 cells (human epidermoid carcinoma) and MCF-7 cells (human breast carcinoma) were maintained in DMEM medium supplemented with 10% FBS at 37 °C in 5% CO_2.
22. *Fluorescent labeling*: Purified BNCs were reacted with Alexa Fluor 488 Succinimidyl Esters (Invitrogen Life Technologies) (2.6 mol equiv) in phosphate-buffered saline (PBS) (Nacalai Tesque) for 1 h at room temperature. The mixture then was dialyzed against PBS overnight to remove the free Alexa Fluor 488.
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25. *Flow cytometry*: Approximately 1×10^5 HeLa, A431 and MCF-7 cells were seeded in 12-well plates. After washing with serum-free medium, Alexa Fluor 488-labeled BNCs with different concentrations were added to the medium, which was adjusted to 1 ml, and then the cells were cultured for 1 h. After washing with serum-free medium twice, cells were incubated with FBS-containing medium for 2 h. Cells were suspended in a sheath solution and subjected to a BD FACSCanto II flow cytometer equipped with a 488-nm blue laser (BD Biosciences, San Jose, CA, USA). The green fluorescence signal was collected through a 530/30-nm band-pass filter. The data were analyzed using the BD FACSDiva software v5.0 (BD Biosciences).
26. *Observation of time course*: Approximately 5×10^4 HeLa and MCF-7 cells were seeded in 35 mm glass-bottom dishes. After washing with serum-free medium, Alexa Fluor 488-labeled $[Z1907]_2$-BNC was added to the medium, which was adjusted to 2 ml, and then the cells were cultured for 1 h. After washing with serum-free medium twice, cells were incubated with FBS-containing medium for 3 h. Cells were observed using a LSM 5 Pa laser scanning confocal microscope (LSCM) (Carl Zeiss, Oberkochen, Germany) equipped with a 63-fold oil-immersion objective lens with excitation from the 488-nm line of an argon laser, and with emission collection provided by a 505-nm long pass filter.
27. *Preparation of BNC/LP complex*: Freeze-dried LPs (COATSOME EL-01-A; 2.2 mg and COATSOME EL-01-D; 1.5 mg) were dissolved in distilled water (1 ml) containing 100 mM of calcein. After incubation for 1 h at room temperature, gel-filtration chromatography was performed using a PD-10 (GE healthcare). Purified LP (200 μl) was added to freeze-dried $[Z1907]_2$-BNC (100 μg as protein) and incubated at room temperature for 1 h to form BNC/LP complexes. The resultant complex carrier was named $[Z1907]_2$-BNC/LP.
28. *Observation of endosomal escape*: Approximately 5×10^4 HeLa cells were seeded in 35 mm glass-bottom dishes. After washing with serum-free medium, $[Z1907]_2$-BNC/LP was added to the medium, which was adjusted to 2 ml and then the cells were cultured for 1 h. After washing with serum-free medium twice, cells were incubated with FBS-containing medium for 48 h. Cells were stained with Lysotracker® Red DND-99 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were observed using a LSCM with a 63-fold oil immersion objective lens with excitation from the 488-nm line of an argon laser, and emission collection provided by a 505–

530 nm band pass filter for calcein and from the 543-nm line of an He-Ne laser and emission collection provided by a 560-nm long pass filter for Lysotracker.

29. *Cell viability*: A complex carrier of $[Z1907]_2$-BNC and LP containing 500 μg/ml of doxorubicin (dox) (Wako Pure Chemical Industries, Osaka, Japan) was prepared. Approximately 5×10^3 HeLa and MCF-7 cells were seeded into a 96-well plate. Dox, LP and $[Z1907]_2$-BNC/LP containing dox were added to 100 μl of the medium (final concentration of dox: 10 μg/ml), and then the cells were cultured for 1 h. After washing with serum-free medium twice, cells were incubated with FBS-containing medium for 23 h. The dead-cell percentage was measured using a Cell Counting Kit-8 (DOJINDO LABORATORIES, Kumamoto, Japan) according to the manufacturer's instructions.

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