Granting specificity for breast cancer cells using a hepatitis B core particle with a HER2-targeted affibody molecule

Received August 30, 2012; accepted October 30, 2012; published online December 11, 2012

Yuya Nishimura[1], Wakiko Mimura[1], Izzat Fahimuddin Mohamed Suffian[1], Tomokazu Amino[1], Jun Ishii[2], Chiaki Ogino[1] and Akihiko Kondo[1,*]

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

*Akihiko Kondo, Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan. Tel:/Fax: +81-78-803-6196, email: akondo@kobe-u.ac.jp

Capsid-like particles consisting of a hepatitis B core (HBc) protein have been studied for their potential as carriers for drug delivery systems (DDS). The hollow HBc particle, which is formed by the self-assembly of core proteins comprising 183 aa residues, has the ability to bind to various cells non-specifically via the action of an arginine-rich domain. In this study, we developed an engineered HBc particle that specifically recognizes and targets human epidermal growth factor receptor-related 2 (HER2)-expressing breast cancer cells. To despoil the non-specific binding property of an HBc particle, we genetically deleted the C-terminal 150–183 aa part of the core protein that encodes the arginine-rich domain (ΔHBc). Then, we genetically inserted a Z_{HER2} affibody molecule into the 78–81 aa position of the core protein to confer the ability of target-cell-specific recognition. The constructed Z_{HER2}-displaying HBc (Z_{HER2}-ΔHBc) particle specifically recognized HER2-expressing SKBR3 and MCF-7 breast cancer cells. In addition, the Z_{HER2}-ΔHBc particle exhibited different binding amounts in accordance with the HER2 expression levels of cancer cells. These results show that the display of other types of affibody molecules on HBc particles would be an expandable strategy for targeting several kinds of cancer cells that would help enable a pinpoint DDS.

Keywords: affibody/breast cancer cells/cell-specificity/hepatitis B core/HER2.

Abbreviations: DDS, drug delivery system; FBS, foetal bovine serum; HBc, hepatitis B core; HBV, hepatitis B virus; HER2, human epidermal growth factor receptor-related 2; ΔHBc, HBc deletion mutant lacking arginine-rich domain; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Anticancer drugs act against abnormal proteins in cancer cells and present a large treatment effect. However, they are often limited by their systemic toxicities and side effects (*1*). Therefore, targeting ability is an important factor for the development of drug delivery systems (DDS). To attain pinpoint delivery to target cells, studies have extensively focused on fusing targeting molecules with the drug itself (*2*) or on modifying the surface of the DDS carrier (*3*, *4*). As the targeting molecules, binding molecules such as antibodies (*5*), peptides (*6*) and aptamers (*7*) are often used.

As a binding protein, the affibody is an attractive molecule. An affibody is a small molecule that is based on the Z-domain derived from *Staphylococcus aureus* protein A (*8*). As a type of affibody, Z_{HER2} has the ability to bind to human epidermal growth factor receptor-related 2 (HER2) that is a type 2 epidermal growth factor receptor and is expressed on the surface of breast cancer cells and ovarian cancer cells (*9*, *10*). Since natural ligands against HER2 have yet to be found in nature (*11*), Z_{HER2} has been used as an alternative molecular probe to diagnose (*12*) or target HER2-expressing cells (*13*). In addition, various other types of affibodies such as Z_{WT}, Z_{440} and Z_{955} can be used as the binding molecules to the Fc regions of immunoglobulin G, insulin-like growth factor-1 receptor and epidermal growth factor receptor, respectively (*8*, *14*, *15*).

Hepatitis B virus (HBV) core (HBc) protein has been studied for developing viral genome-free particles as DDS carriers. The HBc is a 183-aa protein and assembles spontaneously into icosahedral capsid-like particles comprising 180–240 subunits (*16*). The important feature of HBc is to transiently dissociate and re-associate in the presence or absence of denaturants, thereby enabling it to enclose molecules such as drugs (*17*). In addition, the HBc can be produced in large quantities, because it can be expressed in *Escherichia coli* (*18*). The original HBc has been used as a permeable particle because it has the ability to bind to every cell (*16*), which is caused by an arginine-rich domain (150–183 aa) that recognizes the cell surface heparan sulphate proteoglycan with an electrostatic interaction (*19*). Additionally, foreign molecules (e.g. green fluorescence protein) have been successfully displayed on the surface of a HBc particle without drastically altering its structure via insertion into the 78–81 aa position of the original HBc core protein (*20*).

HBc deletion mutant lacking arginine-rich domain (ΔHBc) consisting of the first 149 aa residues of a core protein was developed as a deletion mutant lacking a non-specific binding ability (*21*). The ΔHBc particle is far more suitable for a DDS capsule than the original HBc because of the particle's capacity to incorporate drugs (*22*) and the avoidance of host-derived RNA/DNA-binding functions (*21*). However, despite the successful development of the ΔHBc particle, there have been no reports of a binding-molecule-fused ΔHBc.

In the present study, we developed a concept for constructing a DDS carrier that is based on the ΔHBc particle and that can specifically recognize target cancer cells. By genetically inserting a Z_{HER2} affibody between the 78 and 81 aa of a ΔHBc core protein, the engineered particle (Z_{HER2}-ΔHBc) specifically bound to HER2-expressing breast cancer cells.

Materials and Methods

Construction of plasmids for the expression of core particles

The plasmids for expression of HBc, ΔHBc and Z_{HER2}-ΔHBc were constructed as described later. Fragments 1 and 2 encoding HBc were amplified by polymerase chain reaction with the following primers: Fragment 1 (5′-GGG GCT AGC AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG ATG GAC ATT GAC CCG TAT AA-3′ and 5′-ATT CTC TAG ACT CGA GAT TAC TTC CCA CCC AGG TGG-3′) and Fragment 2 (5′-TAA TCT CGA GTC TAG AGA ATT AGT AGT CAG CTA TGT-3′ and 5′-CCC GTC GAC TTA GTG GTG GTG GTG GTG ACA TTG AGA TTC CCG AGA TT-3′). Then, the whole-length fragment encoding of HBc was amplified from Fragments 1 and 2 with the following primers: (5′-GGG GCT AGC AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG ATG GAC ATT GAC CCG TAT AA-3′ and 5′-CCC GTC GAC TTA GTG GTG GTG GTG GTG ACA TTG AGA TTC CCG AGA TT-3′). The amplified fragment was digested with NheI/SalI and ligated into the XbaI/SalI sites of pET-22b (+) (Novagen). The resultant plasmid was designated as pET-22b-HBc. Fragment encoding of ΔHBc was amplified from pET-22b-HBc with the following primers: (5′-TAA TCT CGA GTC TAG AGA ATT AGT AGT CAG CTA TGT-3′ and 5′-GGG GTC GAC AAG CTT TTA GTG GTG GTG GTG GTG GTG AAC AAC AGT AGT TTC CGG AA-3′). The amplified fragment was digested with XbaI/SalI and ligated into the same sites of pET-22b-HBc. The resultant plasmid was designated as pET-22b-ΔHBc. A fragment encoding Z_{HER2} was amplified from pGLDsLd50-Z_{HER2} (*23*) with the following primers: (5′-GGG CTC GAG GAC GGT GGT GGT GGT TCT GCG CAA CAC GAT GAA GCC GT-3′ and 5′-GGG TCT AGA ACC ACC ACC ACC TTT CGG CGC CTG AGC ATC AT-3′). The amplified fragment was digested with XhoI/XbaI and ligated into the same sites of pET-22b-ΔHBc. The resultant plasmid was designated as pET-22b-Z_{HER2}-ΔHBc.

Expression of core particles in E. coli

The plasmids for expression of HBc, ΔHBc and Z_{HER2}-ΔHBc were transformed into *E. coli* BL21. The culture of *E. coli* BL21 carrying each plasmid was diluted with 1 l of fresh LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) in the presence of 100 μg/ml ampicillin and grown to $OD_{600} = 0.7$ at 37°C and a shaking speed of 150 rpm. The culture was induced by adding isopropyl-β-thiogalactopyranoside to a final concentration of 0.1 mM at 25°C overnight. Cells were then collected at 3,000 rpm and 4°C for 15 min.

Purification of core particles

To purify core particles fused with a His6-tag, we followed the procedure described by Wizemann and von Brunn (*18*) with minor modifications. Briefly, the cell pellet was re-suspended in 50 ml of lysis buffer (pH 8.0) (50 mM Tris–HCl, 100 mM NaCl, 5 mM EDTA, 0.2% Triton X-100, 10 mM β-mercaptethanol). The cells were lysed on ice by three cycles of sonication for 1 min each with 2-min intervals to avoid heating of the material. The supernatant was removed by centrifugation at 15,000 rpm and 4°C for 30 min. The core particles in the pellet were washed in 50 ml of lysis buffer and collected by centrifugation at 12,000 rpm and 4°C for 15 min twice. The pellet containing *E. coli* proteins was dissolved in 50 ml of dissociation buffer (pH 9.5) (4 M urea, 200 mM NaCl, 50 mM sodium carbonate, 10 mM β-mercaptethanol) by overnight incubation in an ice-cold water bath. Then, the soluble fraction was separated by centrifugation at 15,000 rpm and 4°C for 20 min.

Contaminating proteins were separated from the core particle proteins using Ni^{2+}-chelate affinity chromatography. A column with 10 ml of Ni^{2+}-chelate agarose (Nacalai Tesque, Kyoto, Japan) was pre-equilibrated with a 5-fold volume of dissociation buffer. The prepared sample was loaded into a column and washed with 30 ml of dissociation buffer. Then, bound proteins were eluted with 30 ml of elution buffer (pH 9.5) (4 M urea, 200 mM NaCl, 50 mM sodium carbonate, 10 mM β-mercaptethanol, 1 M imidazole). The eluate was fractionated in 1-ml aliquots. The aliquots of each fraction were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie brilliant blue to analyse their purity. The proteins of purified fractions were polymerized to core particles by the removal of the urea in a polymerization buffer (pH 7.0) (500 mM NaCl, 50 mM Tris–HCl, 0.5 mM EDTA).

SDS–PAGE and western blotting

The expression of each core monomer was confirmed by western blotting. The purified core particles were analysed by SDS–PAGE and electrotransferred onto a polyvinilidene fluoride membrane. For the detection of the His6-tag, rabbit anti-6-His antibodies (Bethyl Laboratories, Montgomery, TX, USA) were used as a primary antibody for immunoblotting, followed by anti-rabbit antibodies conjugated with alkaline phosphatase (Promega, Madison, WI, USA) used as a second antibody. For the detection of 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 (Promega) used as the second antibody. The membrane was stained with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Promega).

Atomic force microscopy analysis of purified core particles

A gold chip (100-nm thickness of Au wafer; KST world, Fukui, Japan) was covered with 200 μl of solution containing core particles at room temperature for 1 h. The gold chip was then washed with 10 ml of polymerization buffer. After washing, the core particles adsorbed onto the surface of the gold chip were measured using an SPA400-Nanonavi atomic force microscopy unit (SII Nanotechnology Inc., Chiba, Japan) with a cantilever (BL-RC150VB-C1 from Olympus, Tokyo, Japan) at 0.8 kHz scan speed according to the manufacturer's procedure.

Dynamic light scattering analysis of purified core particles

The size of the purified core particles was determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK), following the manufacturer's procedure.

Cell culture

SKBR3 cells (human breast carcinoma) were maintained in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS) at 37°C in 5% CO_2. MCF-7 cells (human breast carcinoma) and HeLa cells (human cervical carcinoma) were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS at 37°C in 5% CO_2.

Flow cytometric evaluation

Purified core particles were reacted with Alexa Fluor 488 Succinimidyl Esters (Invitrogen Life Technologies, Carlsbad, CA, USA) (2.6 mol equiv) in phosphate-buffered saline for 1 h at room temperature. The mixture then was dialysed against polymerization buffer overnight to remove free Alexa Fluor 488. Approximately 2×10^5 of SKBR3, MCF-7 and HeLa cells were seeded in individual 12-well plates. After washing with serum-free medium, indicated volumes of Alexa Fluore 488-labelled core particles were added to the medium, each of which was adjusted to a volume of 1 ml, followed by culturing of the cells for 1 h. After washing with serum-free medium twice, the cells were incubated with FBS-containing medium

for 2 h. Cells were suspended into 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 analysed using the BD FACSDiva software v5.0 (BD Biosciences).

Confocal laser scanning microscopy observation

Approximately 5×10^4 of SKBR3, MCF-7 and HeLa cells were seeded in individual 35-mm glass-bottom dishes. After washing with serum-free medium, Alexa Fluore 488-labelled core particles (10 μg/ml) were added and then cells were cultured for 1 h. After washing with serum-free medium twice, the cells were incubated with FBS-containing medium for 2 h. The cells were observed using a LSM 5 PASCAL laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 63-fold oil immersion objective lens with excitation by the 488-nm line of an argon laser and emission collection by a 505-nm long-pass filter.

Results and Discussion

Expression of core proteins and formation of particles

As shown in Fig. 1, we first constructed the plasmid to express the His6-tag-fused HBc, which consisted of 183 aa residues of full-length core proteins. To eliminate the non-specific binding property of a core protein, we constructed the plasmid to express a ΔHBc consisting of 149 aa residues by deleting the corresponding sequence to the C-terminal arginine-rich domain (Fig. 1). Finally, we constructed the expression plasmid for Z_{HER2}-ΔHBc, in which a Z_{HER2} affibody molecule was inserted between the 78 and 81 aa positions of the ΔHBc (Fig. 1). As described in the 'Materials and Methods' section, we introduced these plasmids into *E. coli* and produced each kind of HBc particle.

First, to check the expression and purification of each core protein, we performed western blot analysis with anti-His6 antibody. Since three His6-specific bands appeared at each desired position (HBc, 21 kDa; ΔHBc, 17 kDa and Z_{HER2}-ΔHBc, 24 kDa), successful expression and purification of the core proteins were confirmed (Fig. 2, left). When using anti-protein A antibody that can specifically bind to the Z-derived affibodies, we detected the single band only for Z_{HER2}-ΔHBc (24 kDa), as expected (Fig. 2, right). Furthermore, secondary structural formation of each core protein was confirmed by circular dichroism spectra analysis (data not shown).

Second, to examine whether each core protein-formed particle could be attributed to the capsid-like structure, we analysed the purified core proteins by dynamic light scattering (Fig. 3) and atomic force microscopy (Fig. 4). Two measurement analyses suggested similar results, and it was strongly supported that every core protein showed same particle-like structure that was ~50 nm in diameter. According to previously reported for wild-type HBc particles analysis (*20*, *21*), it was assumed that the deletion of the arginine-rich domain and the insertion of the Z_{HER2} affibody did not affect the self-assembly of the engineered core proteins.

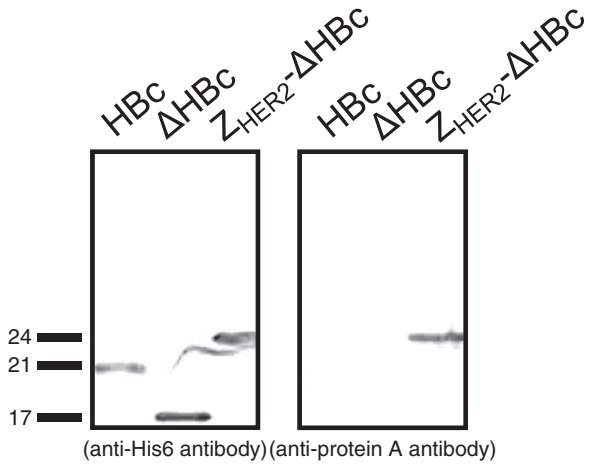

Fig. 2 Western blotting analyses of purified core particles. Purified samples (HBc, ΔHBc and Z_{HER2}-ΔHBc) were subjected to SDS–PAGE followed by immunoblotting using anti-His6 antibody (for His6-tag, left image) and anti-protein A antibody (for Z_{HER2} affibody, right image).

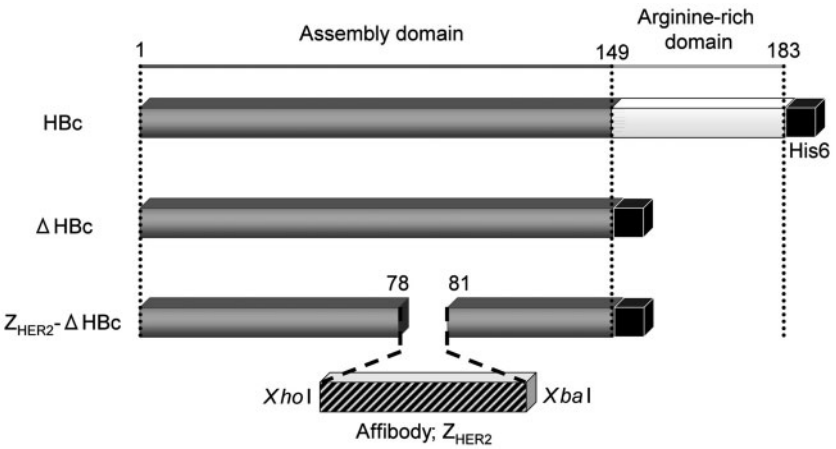

Fig. 1 Schematic representations of constructed core proteins (HBc, ΔHBc and Z_{HER2}-ΔHBc). Wild-type HBc core protein consisted of an assembly domain (grey prismatic body) and an arginine-rich domain (white prismatic body). For ΔHBc core protein, the arginine-rich domain (150–183 aa) was deleted. For the Z_{HER2}-ΔHBc core protein, Z_{HER2} affibody (diagonal prismatic body) was inserted at the XhoI and XbaI sites between 78 and 81 aa. For all constructs, His6-tag (black prismatic body) was fused to the C-termini.

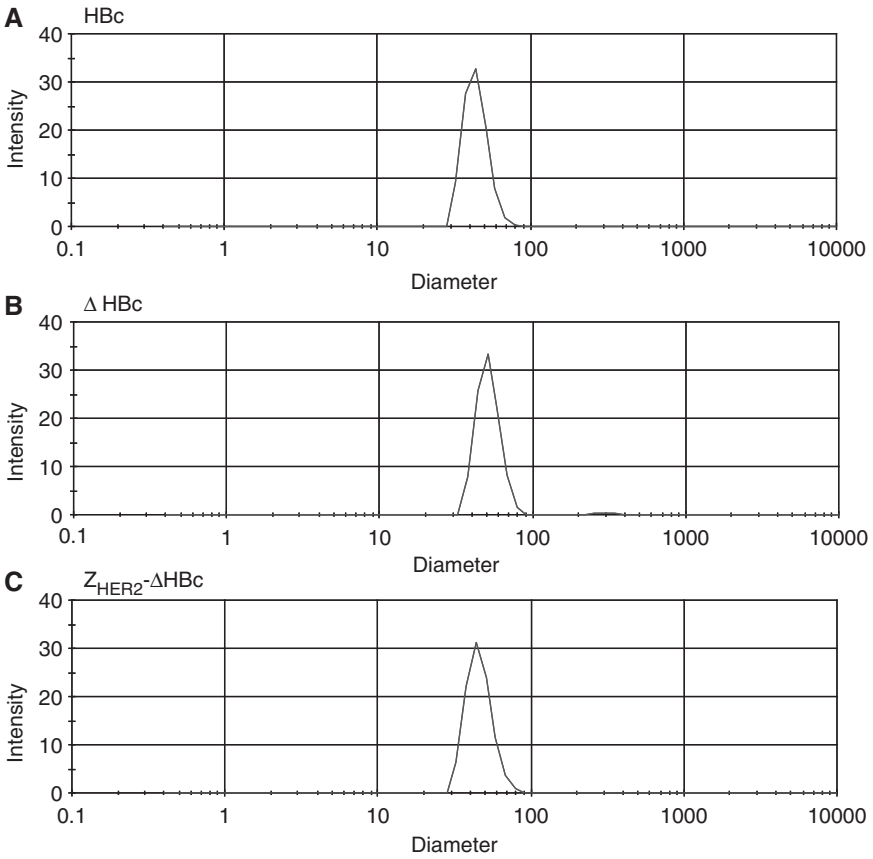

Fig. 3 Particle size distribution analysis by dynamic light scattering. The average sizes of (A) HBc, (B) ΔHBc and (C) Z_{HER2}-ΔHBc are 55.38 ± 10.84 nm, 47.41 ± 10.07 nm and 45.05 ± 1.50 nm, respectively.

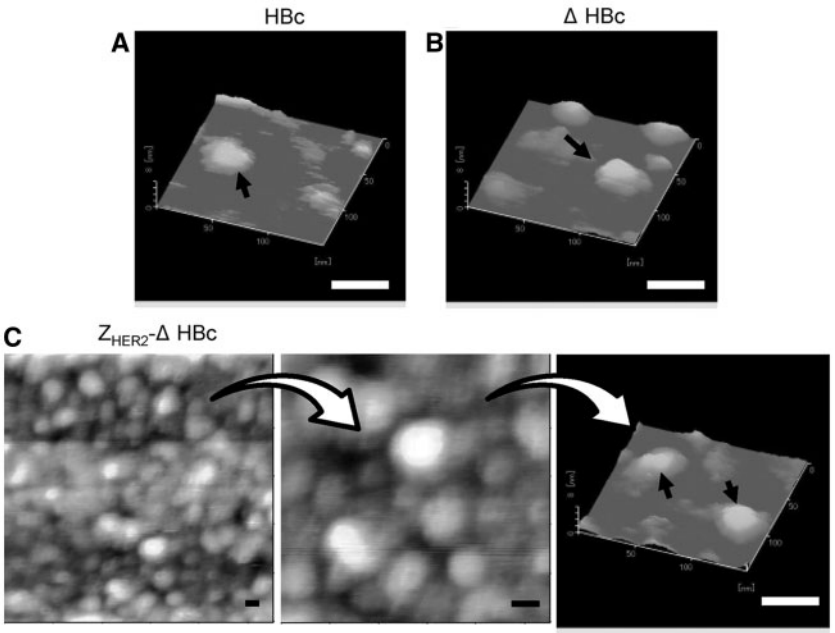

Fig. 4 AFM analyses of purified core particles. The macro and micro photographs show 2D and 3D images, respectively. HBc core particles were analysed on the surface of the gold chip. (A) HBc, (B) ΔHBc and (C) Z_{HER2}-ΔHBc. Scale bars, 50 μm.

Examination of the ability of a Z_{HER2}-ΔHBc particle to recognize HER2-expressing breast cancer cells

Next, to evaluate the binding ability to HER2-expressing breast cancer cells, we labelled the particles with Alexa Fluor 488. Then, each kind of particle was added to two types of HER2-positive human breast cancer cells (SKBR3, expressing an abundant amount of HER2, and MCF-7, expressing a tiny amount of

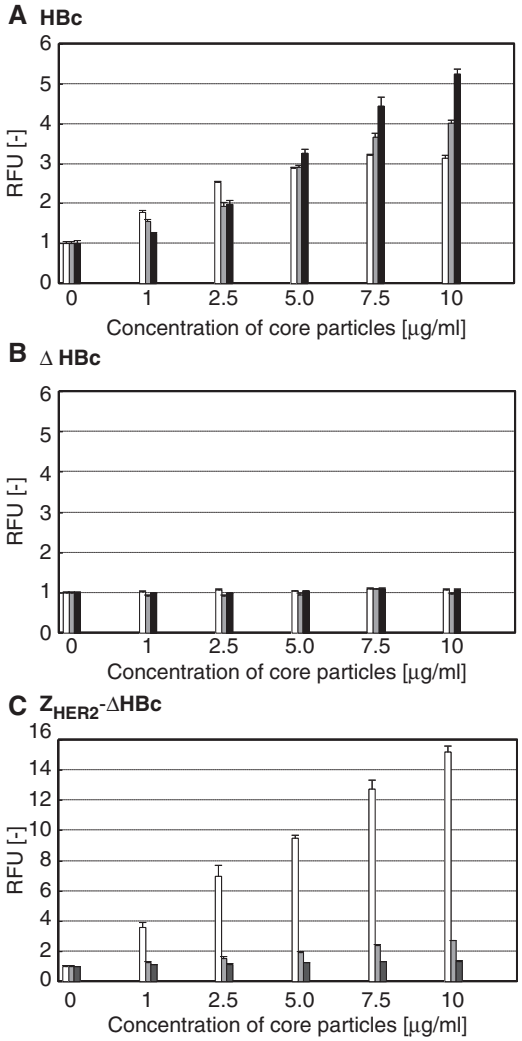

Fig. 5 Relative fluorescence units (RFUs) of HER2 positive and negative cells treated with several concentrations of Alexa Fluor 488-labelled HBc core particles. RFUs were determined by FACS measurement. (A) HBc, (B) ΔHBc and (C) Z_{HER2}-ΔHBc. White bars, SKBR3 (HER2, +++); grey bars, MCF-7 (HER2, +) and black bars, HeLa (HER2, −).

HER2) and a HER2-negative human cervical cancer cell (HeLa) at several concentrations. After 3 h of incubation, we measured the fluorescence intensity of these cells by FACS.

As shown in Fig. 5A, the addition of a wild-type HBc particle caused a dose-dependent increase in fluorescence for all three kinds of cancer cells (SKBR3, MCF-7 and HeLa), indicating that the original HBc particle binds to the cells in a non-specific manner. The addition of a ΔHBc particle did not exhibit fluorescence for all three cells, supporting the theory that the deletion of the arginine-rich domain can cancel the non-specific binding ability of the original HBc (Fig. 5B). In contrast, the addition of a Z_{HER2}-ΔHBc particle promoted an apparent fluorescence for SKBR3 cells in a dose-dependent manner (white bars), whereas it never exhibited fluorescence for the HeLa cells (black bars) (Fig. 5C). Additionally, the addition of Z_{HER2}-ΔHBc to MCF-7 showed a weaker fluorescence than in the case of SKBR3 (Fig. 5C, grey bars). These results indicate that Z_{HER2}-ΔHBc specifically recognized HER2-expressing breast cancer cells and that the binding amount of Z_{HER2}-ΔHBc differed in accordance with the HER2 expression levels in the cells.

Finally, to visually observe the binding ability of these particles to the cells, Alexa Fluor 488-labelled core particles were added into the cell cultures (SKBR3, MCF-7 and HeLa) to give a final concentration of 10 μg/ml. After 3 h of incubation, the cells were observed using a confocal laser scanning microscope (Fig. 6). These results were consistent with the results of the FACS analyses (Fig. 5), demonstrating that the Z_{HER2}-ΔHBc particle has the ability to specifically bind to HER2-expressing breast cancer cells.

In conclusion, we developed a HBc particle displaying a Z_{HER2} affibody that specifically recognizes HER2-expressing breast cancer cells. It would be possible to incorporate drugs into Z_{HER2}-HBc particles by using the dissociation and association mechanism regulated by salt concentration (*24*) or urea denaturant (*18*), or by fusing peptidic drugs to C-terminal tail of core protein (*22*). By inserting other types of affibody

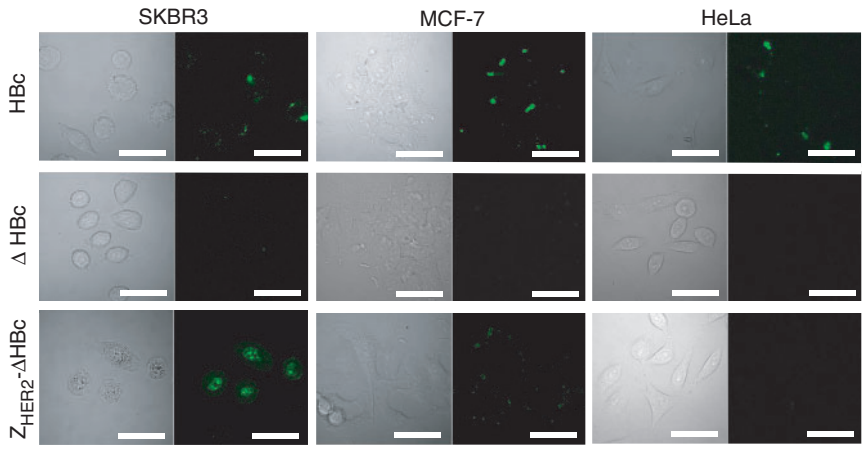

Fig. 6 Fluorescence images of SKBR3 (HER2, +++), MCF-7 (HER2, +) and HeLa (HER2, −) treated with Alexa Fluor 488-labelled HBc core particles (10 μg/ml). Cells were observed on a confocal laser scanning microscope. The fluorescence of Alexa Fluor 488 labeled HBc was shown in white. Scale bars, 50 μm.

molecules to the ΔHBc, these engineered HBc core particles could be used as pinpoint carriers to target various kinds of cancer cells.

Funding
This work was supported 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).

Conflict of interest
None declared.

References

1. Wang, S., Placzek, W.J., Stebbins, J.L., Mitra, S., Noberini, R., Koolpe, M., Zhang, Z., Dahl, R., Pasquale, E.B., and Pellecchia, M. (2012) Novel targeted system to deliver chemotherapeutic drugs to EphA2-expressing cancer cells. *J. Med. Chem.* **55**, 2427–2436
2. Lorberboum-Galski, H. (2011) Human toxin-based recombinant immunotoxins/chimeric proteins as a drug delivery system for targeted treatment of human diseases. *Expert Opin. Drug Deliv.* **5**, 605–621
3. Liong, M., Lu, J., Kovochich, M., Xia, T., Ruehm, S.G., Nel, A.E., Tamanoi, F., and Zink, J.I. (2008) Multifunctional inorganic nanoparticles for imaging, targeting, and drug delivery. *ACS Nano.* **2**, 889–896
4. Nie, S., Xing, Y., Kim, G.J., and Simons, J.W. (2007) Nanotechnology applications in cancer. *Annu. Rev. Biomed. Eng.* **9**, 257–288
5. Ma, L.L., Tam, J.O., Willsey, B.W., Rigdon, D., Ramesh, R., Sokolov, K., and Johnston, K.P. (2011) Selective targeting of antibody conjugated multifunctional nanoclusters (nanoroses) to epidermal growth factor receptors in cancer cells. *Langmuir.* **27**, 7681–7690
6. Accardo, A., Salsano, G., Morisco, A., Aurilio, M., Parisi, A., Maione, F., Cicala, C., Tesauro, D., Aloj, L., Rosa, G.D., and Morelli, G. (2012) Peptide-modified liposomes for selective targeting of bombesin receptors overexpressed by cancer cells: a potential theranostic agent. *Int. J. Nanomed.* **7**, 2007–2017
7. Zhang, K., Sefah, K., Tang, L., Zhao, Z., Zhu, G., Ye, M., Sun, W., Goodison, S., and Tan, W. (2012) A novel aptamer developed for breast cancer cell internalization. *ChemMedChem.* **7**, 79–84
8. Nygren, P.-A. (2008) Alternative binding proteins: affibody binding proteins developed from a small three-helix bundle scaffold. *FEBS. J.* **275**, 2668–2676
9. Orlova, A., Magnusson, M., Eriksson, T.L.J., Nilsson, M., Larsson, B., Hoiden-Guthenberg, I., Widstrom, C., Carlsson, J., Tolmachev, V., Stahl, S., and Nilsson, F.Y. (2006) Tumor imaging using a picomolar affinity HER2 binding affibody molecule. *Cancer Res.* **66**, 4339–4348
10. Lee, S.B., Hassan, M., Fisher, R., Chertov, O., Chernomordik, V., Kramer-Marek, G., Gandjbakhche, A., and Capala, J. (2008) Affibody molecules for *in vivo* characterization of HER2-positive tumors by near-infrared imaging. *Clin. Cancer Res.* **14**, 3840–3849
11. Shojaei, S., Gardaneh, M., and Shamabadi, A.R. (2012) Target points in trastuzumab resistance. *Int. J. Breast Cancer* **2012**, 1–9
12. Gao, J., Chen, K., Miao, Z., Ren, G., Chen, X., Gambhir, S.S., and Cheng, Z. (2011) Affibody-based nanoprobes for HER2-expressing cell and tumor imaging. *Biomaterials.* **32**, 2141–2148
13. Puri, A., Kramer-Marek, G., Campbell-Massa, R., Yavlovich, A., Tele, S.C., Lee, S.B., Clogston, J.D., Patri, A.K., Blumenthal, R., and Capala, J. (2008) HER2-specific affibody-conjugated thermosensitive liposomes (Affisomes) for improved delivery of anticancer agents. *J. Liposome Res.* **18**, 293–307
14. Li, J., Lundberg, E., Vernet, E., Larsson, B., Hoiden-Guthenberg, I., and Graslund, T. (2010) Selection of affibody molecules to the ligand-binding site of the insulin-like growth factor-1 receptor. *Biotechnol. Appl. Biochem.* **55**, 99–109
15. Nordberga, E., Friedmanb, M., Gostringa, L., Adamsd, G.P., Brismare, H., Nilssona, F.Y., Stahlb, S., Glimeliusf, B., and Carlssona, J. (2007) Cellular studies of binding, internalization and retention of a radiolabeled EGFR-binding affibody molecule. *Nuc. Med. Bio.* **34**, 609–618
16. Cooper, A. and Shaul, Y. (2005) Recombinant viral capsids as an efficient vehicle of oligonucleotide delivery into cells. *BBRC.* **327**, 1094–1099
17. Leea, K.W. and Tana, W.S. (2008) Recombinant hepatitis B virus core particles: association, dissociation and encapsidation of green fluorescent protein. *J. Virol. Meth.* **151**, 172–180
18. Wizemann, H. and von Brunn, A. (1999) Purification of *E. coli*-expressed HIS-tagged hepatitis B core antigen by Ni^{2+}-chelate affinity chromatography. *J. Virol. Meth.* **77**, 189–197
19. Cooper, A. and Shaul1, Y. (2006) Clathrin-mediated endocytosis and lysosomal cleavage of hepatitis B virus capsid-like core particles. *JBC* **281**, 16563–16569
20. Kratz, P.A., Bottcher, B., and Nassal, M. (1999) Native display of complete foreign protein domains on the surface of hepatitis B virus capsids. *PNAS* **96**, 1915–1920
21. Birnbaum, F. and Nassal, M. (1990) Hepatitis B virus nucleocapsid assembly: primary structure requirements in the core protein. *J. Virol.* **64**, 3319–3330
22. Beteramsa, G., Bottcher, B., and Nassal, M. (2000) Packaging of up to 240 subunits of a 17 kDa nuclease into the interior of recombinant hepatitis B virus capsids. *FEBS Lett.* **481**, 169–176
23. Shishido, T., Mieda, H., Hwang, S.Y., Nishimura, Y., Tanaka, T., Ogino, C., Fukuda, H., and Kondo, A. (2010) Affibody-displaying bionanocapsules for specific drug delivery to HER2-expressing cancer cells. *Bioorg. Med. Chem. Lett.* **20**, 5726–5731
24. Kann, M. and Gerlich, W. (1994) Effect of core protein phosphorylation by protein kinase C on encapsidation of RNA within core particles of hepatitis B virus. *J. Virol.* **68**, 7993–8000