

Construction of an Improved Drug Delivery System Tool with Enhanced Versatility in Cell-targeting

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Abstract. *Background/Aim:* The aim of this study was to develop an improved drug delivery system (DDS) tool with enhanced versatility in the cell-targeting step using as Z-domain, a modified IgG binding domain of protein A from *Staphylococcus aureus*, as an IgG adapter domain. *Materials and Methods:* The chimera protein expression system composed of the Z-domain and chimeric cholesterol-dependent cytolysin mutant named His-Z-CDC(ss)^{IS} was constructed in *Escherichia coli*. His-Z-CDC(ss)^{IS} was purified by Ni-affinity chromatography, and its abilities for controlled pore formation, membrane binding, IgG binding, and target cell-specific delivery of liposomes carrying medicine were investigated. *Results and Discussion:* His-Z-CDC(ss)^{IS} purified by Ni-affinity chromatography indicated pore-forming activity only under disulfide bond reducing conditions. His-Z-CDC(ss)^{IS} also demonstrated an ability to bind both IgG and cholesterol-embedded liposomes via its Z-domain and domain 4, respectively. Furthermore, anticarcinoembryonic antigen (CEA) IgG-bound His-Z-CDC(ss)^{IS} indicated effective delivery of liposomes carrying drugs to CEA-expressing cells. *Conclusion:* His-Z-CDC(ss)^{IS} was revealed to be an improved DDS tool with enhanced versatility in cell targeting.

Proteins produced by bacteria are promising materials with the potential for a variety of applications. One such protein is cholesterol-dependent cytolysin (CDC), which is a pore-forming toxin secreted by many pathogenic Gram-positive

bacteria. Typical CDCs are composed of four domains: domain 1 (D1) to domain 3 (D3) contribute to pore formation and domain 4 (D4) contributes to target-membrane binding (1). The molecular mechanism of pore formation for CDCs has been actively investigated and these details as well as the structural information contributing to pore formation are becoming clearer (2-14).

Studies regarding the application of CDCs to various fields including medical applications have been reported (15-18). Most of the applications of CDCs have been reported mainly for intermedilysin (ILY) (especially for their D4), such as an immunotherapy against cancer (19), an adjuvant for monoclonal antibody (mAb)-mediated complement-dependent cytolysis (20-22), and enhanced complement-mediated virolysis (23). We have been investigating the therapeutic application of CDCs as a DDS tool, with special attention to the various useful functions of CDCs. In general, a successful DDS is accomplished by the combination of the techniques for targeting/delivery, controlled release, and controlled adsorption of drugs. Among these techniques, the useful functions of CDCs are applicable for (i) controlled release, and (ii) controlled adsorption of drugs. That is, (i) controlled release is achieved by conferring the ability for controlled pore formation by the introduction of a disulfide bond between D2 and D3 of CDCs, and (ii) controlled adsorption is also achieved by the introduction of an additional domain or short peptide tag onto the N-terminus of CDCs without a harmful effect on their controlled pore formation ability. According to these concepts, we successfully constructed a candidate chimera protein as a novel drug delivery tool using the structure of CDCs (24). However, it was also suggested that further improvement of its functional versatility for targeted delivery was necessary.

In the present study, we constructed a chimera protein as an improved DDS tool using the Z-domain, a modified domain with the ability to bind to the fragment crystallizable (Fc) domain of immunoglobulin G (IgG) of protein A produced from *Staphylococcus aureus* (25) (Figure 1A). The newly-constructed chimera protein (designated as His-Z-CDC(ss)^{IS})

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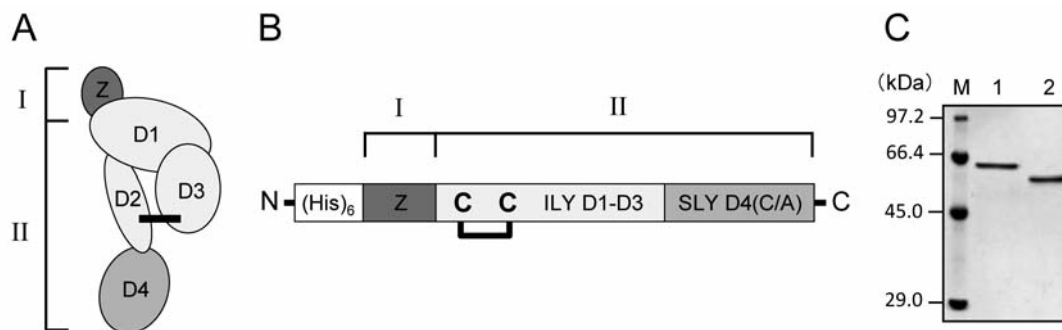


Figure 1. An improved drug delivery system (DDS) tool, His-Z-CDC(ss)^{IS}. A: Schematic representation of His-Z-CDC(ss)^{IS}. This chimera protein is composed of a Z-domain for binding of IgG (sector I) and chimera CDC with controlled pore-forming activity (sector II). B: Primary structural abstract of His-Z-CDC(ss)^{IS}. This recombinant protein has an N-terminal hexa His-tag to facilitate its purification, a Z-domain for IgG binding, D1 to D3 of ILY with two Cys substitutions to control the conformational change required for pore-formation, and D4 of SLY to bind the cholesterol-containing liposomes with the C456A point mutation for stabilization of the molecule. C: sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) image of purified His-Z-CDC(ss)^{IS} after coomassie brilliant blue (CBB) R250-staining (lane 1, 1 µg/lane) and His-LTBP-CDC(ss)^{IS} (lane 2, 1 µg/lane).

bound the antibody to carcinoembryonic antigen (CEA) by its Z-domain. Using the resultant complex, a model DDS was built by binding the D4 end onto the drug-carrying liposome in order to investigate its usefulness as a DDS against CEA-positive cells *in vitro*.

Materials and Methods

Expression and purification of chimera proteins. The expression vectors for the recombinant chimera protein His-Z-CDC(ss)^{IS} (Figure 1A and B) and the reference chimera protein His-LTBP-CDC(ss)^{IS} were constructed based on the N-terminal His-tagged expression vector pQE-1 (Qiagen, Hilden, Germany). Firstly, the expression vector for His-LTBP-CDC(ss)^{IS} was constructed as follows: the insert encoding lung tumor binding peptide [LTBP; (26)] was prepared as previously described (24). The other inserts encoding chimera CDCs were prepared by fusion polymerase chain reaction (PCR). Briefly, the preparation of D1 to D3 of ILY with two point mutations (G83C and S217C) was carried out as described previously (24). The amplification and introduction of a single-point mutation of C456A in the fragment encoding D4 of suilysin (SLY) was also carried out by PCR with the cloned *sly* gene from *S. suis* GTC430 as a template. These fragments were joined to have a *NotI* site at the upstream end and a *SphI* site at the downstream end by fusion PCR. The resultant fragment encodes a chimera CDC designated CDC(ss)^{IS}. The fragments encoding LTBP (a *Bam*HI site at the upstream end and a *NotI* site at the downstream end) and CDC(ss)^{IS} were ligated at the *NotI* site and then inserted into the multi-cloning site-modified pQE-1 (24) at the *Bam*HI and *SphI* sites. Next, the expression vector for His-Z-CDC(ss)^{IS} was constructed as follows: the insert encoding the Z-domain with a *Bam*HI site at the downstream end was amplified by PCR using the genomic DNA of *S. aureus* IFO 12732 as the template, and then the amplified fragment was digested by *Bam*HI and purified. The DNA fragment encoding CDC(ss)^{IS} with a *Bam*HI site at the upstream end and a *PstI* site at the downstream end was also amplified by PCR using the constructed gene encoding CDC(ss)^{IS} described above as the template, followed by digestion of the amplified fragment with *Bam*HI and *PstI*. Fragments were inserted into the *Pvu*II-*PstI* double-digested pQE-1

(Qiagen): the Z-domain with the blunt end at the upstream end and a *Bam*HI site at the downstream end, and CDC(ss)^{IS} with a *Bam*HI site at the upstream end and a *PstI* site at the downstream end. After confirmation of the DNA sequence of the constructed vectors, *E. coli* DH5α Z1 was transformed with each recombinant vector. Subsequently, the expression and purification of recombinant chimera proteins were conducted as described previously (24).

Evaluation of binding ability to cholesterol-containing membrane. The preparation of liposomes with or without cholesterol was conducted according to the method previously reported using egg yolk lecithin (24). One microgram of purified His-Z-CDC(ss)^{IS} and prepared liposomes were mixed in phosphate-buffered saline (PBS) and incubated at 37°C for 1 h, then centrifuged (13,200 ×g, 10 min, 4°C). Each of the resultant supernatants and precipitates were mixed with 4× sample buffer [20% (v/v) 2-mercaptoethanol, 8% (w/v) sodium dodecyl sulfate (SDS), 20% (w/v) sucrose, 0.02% (w/v) bromophenol blue, 250 mM Tris-HCl, pH 6.8] and boiled for 5 min to prepare the sample for SDS-polyacrylamide gel electrophoresis (PAGE). After SDS-PAGE and transblotting of the separated proteins to a polyvinylidene difluoride (PVDF) membrane, immunoblotting for the His-tag was conducted by standard method using His-probe (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as a first antibody and peroxidase-labeled affinity purified antibody against mouse IgA+IgG+IgM (H+L) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) as a secondary antibody.

Measurement of hemolytic activity. The hemolytic activity of His-Z-CDC(ss)^{IS} was measured according to the method described elsewhere (27).

Evaluation of IgG binding ability. Human and rabbit IgG were purified using HiTrap Protein G HP (GE Healthcare, Tokyo, Japan). Briefly, each blood plasma sample was applied to HiTrap Protein G HP, washed with PBS, and eluted by 0.1 M sodium citrate buffer (pH 2.5). The IgG fraction was neutralized by 1.0 M Tris-HCl buffer (pH 9.0) and dialyzed against PBS. The purity of the prepared IgG was checked by coomassie brilliant blue (CBB) R250 staining after SDS-PAGE.

The IgG binding ability of His-Z-CDC(ss)^{IS} was evaluated by enzyme immunoassay (EIA) as described below. The purified IgG was fixed onto a 96-well EIA plate at 1 µg/well by drying overnight at 37°C, then the wells were blocked with blocking solution [PBS containing 1% (w/v) bovine serum albumin] at room temperature for 1 h. After washing with PBS, 50 µl of the serial dilution of His-Z-CDC(ss)^{IS} (10 ng/ml-10 µg/ml) was dispensed into the wells and reacted at room temperature for 1 h. As a negative control for the reaction, His-LTBP-CDC(ss)^{IS} without IgG binding ability was used in the assay. After the reaction, the solutions containing chimera protein were discarded and the wells were washed three times with PBS, then incubated with 100-fold diluted culture supernatant of monoclonal antibody to SLY secreting hybridoma in blocking solution, and then with a 2,000-fold diluted solution of horseradish peroxidase-conjugated goat IgG fraction against mouse immunoglobulins (MP Biomedicals, Inc., Santa Ana, CA, USA) in blocking solution at room temperature for 1 h. The peroxidase reaction was carried out using the substrate solution [50 mM sodium phosphate buffer (pH 4.5) containing 2 mM 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonic) acid and 0.002% (v/v) H₂O₂] and the absorbance was measured at 415 nm in a microplate reader (Model 450; Bio-Rad, Hercules, CA, USA).

Functional assessment of a model DDS. The cholesterol-embedded 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) liposome containing 10 mM 5-fluorouracil and 10 mM fluorescein disodium salt, designated as 5FU-FL-liposome, was prepared according to the method reported elsewhere (24). In order to prepare a model DDS, the 5FU-FL-liposome was mixed with His-Z-CDC(ss)^{IS} (molar ratio for DPPC to His-Z-CDC(ss)^{IS} was 400:1) in PBS and reacted at 25°C for 30 min. After centrifugation (20,600 ×g, 10 min, 4°C), the supernatant was discarded and the 5FU-FL-liposome with His-Z-CDC(ss)^{IS} was resuspended in the PBS solution containing antibody to CEA rabbit IgG (Abbotec, San Diego, CA, USA), and then reacted at 25°C for 1 h. After centrifugation (20,600 ×g, 10 min, 4°C), the supernatant was discarded and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS).

A normal, human fibroblast cell line (NB1RGB; Riken bioresource center, Tsukuba, Ibaraki, Japan) was inoculated into a 12-well cell culture plate at 2×10⁴ cells/well and cultured for two days at 37°C in an atmosphere at 5% (v/v) CO₂. In order to prepare a mixed culture of NB1RGB and HepG2 cells, the culture supernatant of NB1RGB cells was discarded and HepG2 cells were inoculated into the NB1RGB-cultured wells at 5×10⁴ cells/well. Then the cells were further cultured for two days. The prepared model DDS or 5FU-FL-liposome only was added to the cells and which were then incubated for 2 h, then washed three times with DMEM. The cells were observed using a fluorescence microscope (IX71; Olympus, Tokyo, Japan) and the images were recorded by a digital camera (DP72; Olympus).

Results

Expression and purification of chimera proteins. Purification of the recombinant chimera proteins was conducted by standard nickel-affinity chromatography using HisTrap HP (GE Healthcare, Tokyo, Japan) and AKTApurifier Plus (GE Healthcare) with the preset program for this column. Each purified fraction containing His-Z-CDC(ss)^{IS} or His-LTBP-CDC(ss)^{IS} was sufficiently pure as shown by CBB staining of the SDS-PAGE gel (bands observed were 62.5 kDa for His-Z-

CDC(ss)^{IS} in lane 1, Figure 1C, and 58.2 kDa for His-LTBP-CDC(ss)^{IS} in lane 2, Figure 1C).

Controlled pore-forming activity of His-Z-CDC(ss)^{IS} under reducing conditions. In order to investigate the controlled pore formation of His-Z-CDC(ss)^{IS}, the hemolytic activity was measured in the presence and absence of 10 mM dithiothreitol (DTT). Similarly to the results described previously (24), His-Z-CDC(ss)^{IS} did not exhibit hemolytic activity even at a concentration of 1 µg/ml under non-reducing conditions. However, under reducing conditions with 10 mM DTT, obvious hemolysis was exhibited at a level of activity comparable to that of the parental molecules, ILY (27) and SLY (28) (Figure 2A).

The controlled pore-forming activity was also investigated in the presence of 3 mM of the reduced form of glutathione (GSH) for physiological/intracellular reducing materials as described previously (24). Under these conditions, His-Z-CDC(ss)^{IS} exhibited about 20% hemolysis compared to that in the presence of the same concentration of DTT (Figure 2B).

Ability of His-Z-CDC(ss)^{IS} to bind cholesterol-embedded liposomes. The binding ability of His-Z-CDC(ss)^{IS} for cholesterol-embedded liposomes was investigated as described previously (24). As shown in Figure 2C, His-Z-CDC(ss)^{IS} was detected mainly in the pellet fraction when in the presence of cholesterol-embedded liposomes, although the chimera protein was detected in the supernatant in the absence of cholesterol-embedded liposomes. These results indicate that His-Z-CDC(ss)^{IS} sufficiently maintains its ability to bind to membrane cholesterol.

Ability of His-Z-CDC(ss)^{IS} to bind IgG. Binding of IgG mediated by the intramolecular Z-domain to His-Z-CDC(ss)^{IS} was confirmed by EIA. His-Z-CDC(ss)^{IS} indicated dose-dependent binding both to human and rabbit IgG (Figure 3A and B). In contrast with His-Z-CDC(ss)^{IS}, no obvious or specific binding of His-LTBP-CDC(ss)^{IS} to these IgGs was detected (Figure 3A and B).

Functional evaluation of His-Z-CDC(ss)^{IS}-based model DDS. A model DDS (5FU-FL-liposome coated with anti-CEA IgG-bound His-Z-CDC(ss)^{IS}) was applied to a mixed-culture of a CEA-positive human hepatoma cell line (HepG2) and a human normal fibroblast cell line (NB1RGB) in order to investigate the targeting specificity in cell delivery. As shown by microscopy, the model DDS was specifically delivered to the CEA-expressing HepG2 cells and not to NB1RGB cells (Figure 4A and B). In contrast, no 5FU-FL-liposomes were observed either on/in HepG2 or NB1RGB cells (Figure 4C and D). These results strongly suggest that anti-CEA IgG-bound His-Z-CDC(ss)^{IS} has the ability to deliver drug-carrying liposomes only to the target CEA-expressing cells.

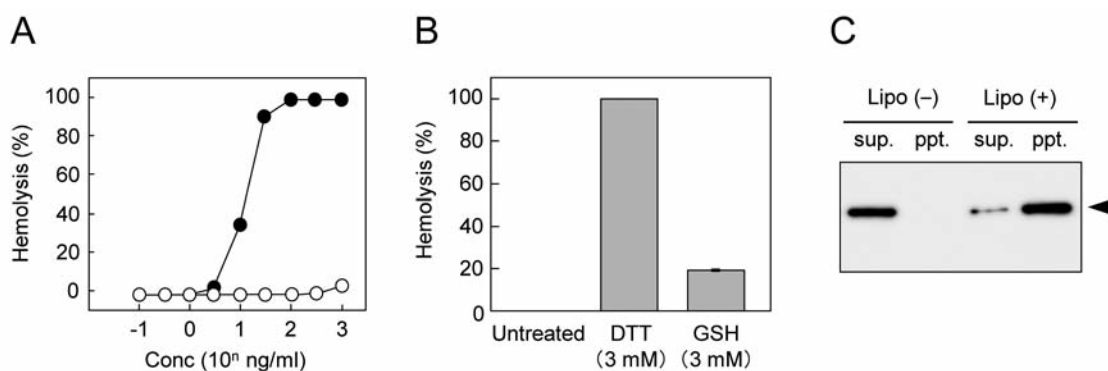


Figure 2. Controlled pore-forming ability of His-Z-CDC(ss)^{IS}. A: Hemolytic activity of His-Z-CDC(ss)^{IS} towards human erythrocytes under reducing (closed circle) and non-reducing (open circle) conditions. The data indicate the mean±difference (n=2). B: Hemolytic activity of His-Z-CDC(ss)^{IS} towards human erythrocytes in the presence of artificial or physiological/intracellular reductants [final concentration at 3 mM for dithiothreitol (DTT) and glutathione (reduced form) (GSH)]. The data indicate the mean±SD (n=3). C: Immunoblotting of His-Z-CDC(ss)^{IS} in the supernatant (sup.) and pellet (ppt.) fractions obtained from the His-Z-CDC(ss)^{IS} samples after incubation with and without cholesterol-embedded liposomes (Lipo) by centrifugation.

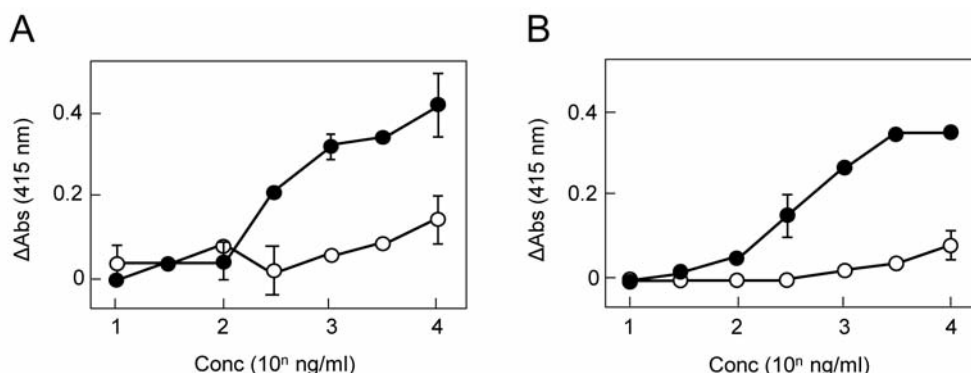


Figure 3. IgG-binding ability of His-Z-CDC(ss)^{IS}. Binding of His-Z-CDC(ss)^{IS} to human IgG (A) and rabbit IgG (B). Closed circle, His-Z-CDC(ss)^{IS}; open circle, a reference molecule His-LTBP-CDC(ss)^{IS}. The binding of chimera proteins to IgG was assayed by the method based on enzyme immunoassay (EIA). These data indicate the mean±SD (n=3).

Discussion

The strategy for antibody-based cancer therapies by applying specific antibodies to target cancer cells has been investigated in addition to the major strategies against cancer, such as surgery, anticancer chemotherapy, and radiation therapy. For example, monoclonal antibodies (mAbs) for cluster of differentiation 20 (CD20), human epidermal growth factor receptor type 2 (HER2), vascular endothelial growth factor (VEGF), and epidermal growth factor receptor (EGFR) have been approved as pharmaceutical agents in the United States and European countries (29). Furthermore, investigations on DDS using these mAbs have also been conducted. One of the representative subjects for antibody application is immunoliposome, a mAb-fixed liposome system (30). Because immunoliposomes can effectively deliver the drugs

to target cells while protecting against degradation and preventing *in vivo* side-effects of the drugs, this property is attractive for developing new, safer, and more effective procedures for anticancer therapies. Previously, we reported a novel DDS tool with LTBP as its targeting domain (24). However, this DDS tool was limited to use for a narrow range of target cells, such as lung tumor cells. In order to improve this defect in the previous DDS tool, an improved DDS tool was constructed with enhanced versatility in cell targeting ability by use of the Z-domain, a modified IgG-binding domain of protein A from *S. aureus*, as the IgG-adaptor domain (Figure 1), which was further characterized in this study. The Z-domain is an invaluable resource for mAb-mediated DDS because of its ability to bind the Fc domain of IgG. For example, one trial of DDS using the Z-domain has been reported for treatment of EGFR-overexpressing glioma

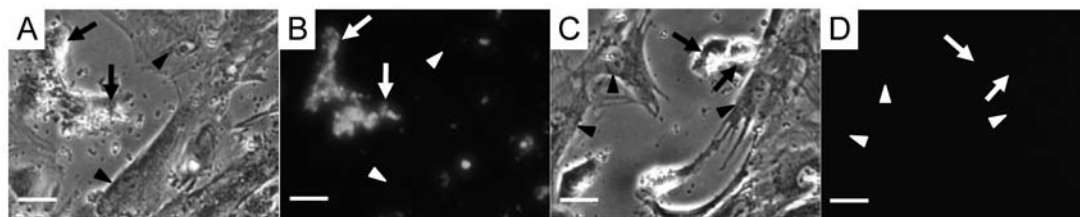


Figure 4. Targeting of a model drug delivery system (DDS) to carcinoembryonic antigen (CEA)-expressing HepG2 cells in vitro. CEA IgG antibody-bound His-Z-CDC(ss)^{IS} was attached to the cholesterol-embedded 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) liposome-enclosed fluorescein disodium salt and 5-fluorouracil, in order to construct a model DDS. This model DDS was incubated with a co-culture of human hepatoma HepG2 cells and human normal fibroblast cells NB1RGB, and then the delivered model DDS was observed under fluorescence microscopy. A: Bright-field image of a typical observed field of co-cultivated cells reacted with the model DDS. B: Fluorescent image of A. C: Bright-field image of a typical observed field of co-cultivated cells reacted with 5-fluorouracil–fluorescein disodium salt (5FU-FL)-liposomes. D: Fluorescent image of C. Arrows and arrow-heads indicate HepG2 and NB1RGB cells, respectively. Scale bars represent 40 μm .

cells: liposomes composed of nickel lipids were conjugated to antibody against EGFR by using the Z-domain as an adaptor and sodium borocaptate as the encapsulated effector drug (30). The His-Z-CDC(ss)^{IS} constructed in this study was also indicated to have sufficient ability to bind to IgG by introducing the Z-domain to the N-terminus of a chimera toxin protein (Figure 3). Moreover, because CDCs generally associate themselves on the membrane to form a large circular oligomer consisting of 40-50 molecules, the oligomerization of His-Z-CDC(ss)^{IS} seems to increase the avidity of this protein to IgG by circular clustering of the Z-domain on the surface of liposome membranes. Indeed, specific and effective delivery of model DDS to CEA-positive cells was observed by tight attachment of the antibody to CEA to the Z-domain in His-Z-CDC(ss)^{IS} (Figure 4A and B). Judging from the results described above, it is suggested that His-Z-CDC(ss)^{IS} is a promising molecule as a tool for antibody-based DDS.

The potential DDS should be evaluated not only by the delivery of drug carrier to the target cells but also by the efficiency of introduction of the drug into the target cells and not into the non-target normal cells. To achieve this controlled release of drugs only in the target cells, the chimera CDC used in the present model DDS (His-Z-CDC(ss)^{IS}) has an intramolecular disulfide bond to link D2 and D3 of CDC, thereby preventing conformational changes to membrane insertion and subsequently pore formation. This modification makes its pore formation controllable and enables pore formation to occur only under intracellular reducing conditions (Figure 2A and B). This modification prevents accidental pore formation on liposomes by His-Z-CDC(ss)^{IS}, inducing drug leakage in the extracellular, non-reducing conditions, and it is also helpful to reduce the non-specific cytotoxicity to normal cells/tissues. It is supposed that when the drug-carrying liposome with this DDS tool is delivered to the target cells, it will be endocytosed into the target cells and exposed to intracellular reducing conditions in the endolysosome. Under these conditions, the disulfide bond of His-Z-CDC(ss)^{IS} will

be reduced and open, and the drug-carrying liposome will be broken by the activated His-Z-CDC(ss)^{IS}. Due to this proposed mechanism, the drug enclosed in the liposome will be specifically released only in the targeted cancer cells. Recently, an investigation on immunoliposomes was reported, with application of listeriolysin O secreted from *Listeria monocytogenes*, which is specifically activated at lower pH conditions in endolysosomes (31). Thus, these CDCs will be promising materials to create more effective DDS tools if we can enhance their useful properties and control their harmful properties by genetic manipulation.

In the present study, the Z-domain with its ability to bind IgG was applied to a targeting domain for a new DDS tool, His-Z-CDC(ss)^{IS}. His-Z-CDC(ss)^{IS} was suggested to be highly versatile in selective targeting of cells/tissues by selecting the proper antibody for the cell-surface marker antigen expressed on the target cancer cells. To evaluate the usefulness of this new DDS tool, His-Z-CDC(ss)^{IS}, further investigations such as an *in vivo* assay using a mouse model, are necessary for practical DDS use.

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