A trial for the construction of a novel drug-delivery system against HER2-positive cancer cells using anti-HER2 recombinant antibody

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CHAPTER 1   General Introduction

1.   Background of the construction of drug-delivery systems (DDSs) in our laboratory

Drug-delivery systems (DDSs) are considered to be effective treatment approaches for various diseases, particularly in the treatment of cancer so as to relieve the side effects caused by anti-cancer drugs on normal (non-target) cells, owing to their potential in targeting, controlling drug release and minimizing dose. In order to establish an effective DDS for cancer therapeutics, the development of novel DDSs against cancers has been attempted in our laboratory using functional proteins produced from bacteria. For instance, the bacterial pore-forming protein toxins called cholesterol-dependent cytolysins (CDCs) were investigated in order to use them for the construction of novel DDSs. This investigation led to the development of several model DDSs such as chimera CDCs carrying a lung tumor-binding peptide (LTBP) [Tabata *et al*., 2012] or IgG-binding domain of protein A (Z-domain) fused with CDCs [Tabata *et al*., 2013] as targeting modules, which are mentioned as below. In addition, a bacterial transpeptidase, sortase A (SrtA), derived from Gram-positive bacteria was also characterized in order to apply the bacterial functional protein in the direct modification of DDS drug carriers [Tabata *et al*., 2014a].

2.   Concepts of novel DDSs using bacterial functional proteins

2.1 Utilization of sortase A transpeptidation in the modification of DDS carriers

SrtA is considered to be a promising protein engineering tool in the development of DDSs, because SrtA recognizes the LPXTG motif in proteins, cleaves it between T and G, and

joins the C-terminal of the generated LPXT motif to the N-terminal of oligoglycine in mild conditions [Mao *et al.*, 2004; Tomita *et al.*, 2013; Beerli *et al.*, 2015]. In those studies, ligations between two proteins/molecules with individual functions were successfully performed using SrtA reaction, *e.g.*, the molecules such as green fluorescent protein (GFP) and various antibodies with LPXTG motif and the molecules with oligoglycine such as peptide/protein, toxin and fluorophore. The investigations proved that SrtA transpeptidation provides brilliant flexibility in protein modification without interference of protein activity. Therefore, we have attempted to apply the SrtA-mediated reaction in preparation of anti-cancer-DDSs by ligation of cancer-targeting molecules such as antibody, which can effectively recognize cancer cells, to oligoglycine-containing drug-carriers/effecters such as liposomes and toxins. Against such a background, we previously investigated SrtA with respect to the construction of a tool applicable for DDSs by modifying liposomes with a cancer-cell targeting module of LTBP [Tabata *et al.*, 2015].

2.2 Utilization of cholesterol-dependent cytolysins to the targeting domain of DDSs

Cholesterol-dependent cytolysins (CDCs) are pore-forming protein toxins produced from pathogenic Gram-positive bacteria [Hotze *et al.*, 2012]. Typical CDCs have four domains (domains 1-4) and some typical CDCs have five domains, domains 1-4 with an N-terminal additional domain [Tabata *et al.*, 2014b]. Domain 4 plays an essential role in the binding of CDCs

to their target cell membrane to recognize their receptor(s), cholesterol, human CD59, or both of them [Tabata *et al*., 2014b]. The interaction between CDCs and lipid membrane was also investigated and the information about interactions of CDCs with lipid membrane and the morphological observation of the pore formed on the lipid membrane was reported [Sonnen *et al*., 2014]. Liposomes are artificial lipid vesicles and widely used as drug-carriers in DDSs. Moreover, liposomes have a useful characteristic for the construction of the DDS in our study, that is, the receptor of CDCs (cholesterol or human CD59) can be incorporated or anchored in the liposome [Boyd *et al*., 2016]. Therefore, we have also attempted to apply CDCs to the construction of liposomal DDSs [Tabata *et al*., 2013], because of the simplicity in decorating of cholesterol-containing liposome with targeting modules just by co-incubation for a short time. Particularly, domain 4 of CDCs with different modes of receptor recognition will also be useful to prepare fusion/linked proteins with cancer cell-targeting peptides/proteins for the decoration of DDS carrier liposomes with targeting modules against cancer cells.

Scheme 1. Construction of the DDSs using bacterial functional proteins.

A: The model of the DDS prepared by SrtA transpeptidation. The targeting module with LPXTG motif is transferred to the surface of the liposome containing oligoglycine-derivatives. B: The model of DDS prepared by the cholesterol-binding property of CDC-domain 4. Cancer cell-targeting module fused/linked with domain 4 of CDC is directly bound to the surface of the liposome containing cholesterol.

CHAPTER 2   Introduction

In DDS development, antibodies and their derivatives are useful as DDS targeting modules (DDS-TM) against cancer cells. The antibodies applied in therapeutics, such as drug-conjugates and DDS, against various tumors have been investigated for many years [Weiner *et al*., 2015; Panowski *et al*., 2014]. Recently, recombinant fragment antibodies such as single-chain variable fragments (scFvs) and antigen-binding fragments (Fabs), also known as smaller derivatives of IgG possessing only one antigen-binding region [Weisser *et al*., 2009; Crivianu-Gaita *et al*., 2016], have attracted more attention than complete IgGs.

Liposomes are artificial spherical bilayer vesicles composed of phospholipid molecules, and they are biocompatible to be used as drug-carriers for DDSs and applied in drug therapeutics against cancers. Various anti-cancer drugs can be incorporated into the liposomes with optimal modifications, and they can be delivered to target cancer cells [Fang *et al*., 2018; Assanhou *et al*., 2015; Lakkadwala *et al*., 2018]. It is also easy to make surface-decoration on liposomes, which gives them improved characteristics, such as PEGylation that increases circulation of anti-cancer compounds *in vivo* [Bunker *et al*., 2016; Suk *et al*., 2016]. Particularly, ligand-targeted liposomes are supposed to enhance specificity of drug delivering against their targets such as cancer. These kinds of liposome are effectively modified with ligands such as antibody against cancer cells and their derivatives, or aptamers, and so on [Kirpotin *et al*., 2006; Srinivasarao *et al*., 2017].

In the present study, we focused on Trastuzumab (Herceptin) as the ligand of our model DDS, since this antibody is a recombinant humanized monoclonal antibody against human epidermal growth factor receptor 2 (HER2) that is over-expressed in breast and cervical cancers, which has been used for the clinical treatment of breast cancer [Elster *et al*., 2015; Oh *et al*., 2015; Goldenberg *et al*., 1999]. To develop our novel DDS, both the Trastuzumab-derived recombinant scFvs and Fab were prepared as the donor substrates for transpeptidation of SrtA, in which SrtA-recognition motif (LPXTG) is contained at their C-terminal. The recombinant scFvs and Fab were expressed in an *Escherichia coli* expression system and a Chinese hamster ovary (CHO) cells expression system, respectively. Both recombinant fragment antibodies were purified then evaluated for their yield, purity, and targeting ability towards HER2-positive cells. Moreover, we also prepared the SrtA recognition motif-decorated liposomes, which exposed penta-glycine-moiety of lipopeptides as the acceptor motif for SrtA transpeptidation on its surface, and evaluated their characteristics, such as their size, integrity, and lipopeptide content. In addition, Fab was used to label lipopeptide-containing liposomes, and the function of the product liposome against HER2-positive cancers was analyzed.

CHAPTER 3   Materials and Methods

1. **Preparation and characterization of anti-HER2 recombinant antibodies as targeting modules for DDSs**

1.1 Cell lines and culture conditions.

A human colorectal adenocarcinoma cell line, HCT-15 (TKG0504; Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan), and a human cervix epithelioid carcinoma cell line, HeLa (RCB0007; RIKEN BioResource Center, Tsukuba, Japan), were cultured in RPMI-1640 and EMEM, respectively, supplemented with 10 % (v/v) FBS and antibiotics (benzylpenicillin potassium and streptomycin sulfate salt) at 37 ºC in 5 % CO_2. In addition, two CHO cell lines, an EB-B2 cell line derived from CHO-K1 (Onitsuka *et al*., manuscript in preparation) and a CHO-HcD6 cell line [Onitsuka *et al*., 2015], were used for the transient expression of Trastuzumab Fab fragment and Trastuzumab IgG1, respectively.

1.2 Trastuzumab-derived scFvs expression system in *E. coli*.

The template plasmid, pFscFV(LH)-Fc (Onitsuka *et al*., manuscript in preparation), was constructed using a bacterial codon-optimized scFv(LH)-Fc fragment, which was synthesized according to the amino acid sequence of Trastuzumab (Drug bank ID: DB00072) and pFLAG-CTS™ Expression Vector (Merck KGaA, Darmstadt, Germany). In order to construct the scFv expression system in *E. coli*, the sequences coding V_H and V_L of Trastuzumab were first amplified

via PCR using PrimeSTAR® HS DNA Polymerase (TaKaRa Bio, Shiga, Japan) and pFscFV(LH)-

Fc as the template along with the primer sets described in Table 1 (No.1 and No.2 for V_H, and

No.3 and No.4 for V_L). Subsequently, a linker fragment encoding $(G_4S)_3$ was amplified using

PrimeSTAR® HS DNA Polymerase, an annealed oligonucleotide (No.5 and No.6 in Table 1) as a

template, and the No.7 and No.8 primer set (Table 1). The purified V_H, V_L, and $(G_4S)_3$ fragments

were joined via fusion PCR to produce inserts encoding V_H-$(G_4S)_3$-V_L (scFv_HL) and V_L-$(G_4S)_3$-

V_H (scFv_LH). Each fragment was inserted into the BamHI/SalI-digested cloning site of a pQE-

9 (Qiagen, Hilden, Germany) variant with deletion of N-terminal His-tag (6 residues of His)-

encoding sequences and addition of SrtA-recognition motif (LPXTG) and His-tag encoding

sequences between the SalI and HindIII restriction sites. Transformation of the SHuffle® T7

Express Competent *E. coli* (C3029H; New England Biolabs, MA, USA) by each expression

plasmid, pscFv_HL or pscFv_LH (Fig. 1A), as well as transformant selection were performed

according to the standard methods. After confirming the sequence of the expression vector from

the selected clone, the recombinant protein was expressed overnight in the presence of 0.1 mM

IPTG at 16 ºC according to the C3029H manual. Both recombinants were purified using nickel-

affinity chromatography as previously reported [Tabata *et al*., 2012].

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1.3 Trastuzumab-derived Fab expression system in CHO cells

Table. 1. Oligonucleotides used in this study.

No.	Name	Sequence (5'→3')
1	Trastuzumab V_H Fw	GAAGTTCAGCTGGTTGAATCAGGTGGCGGTTTGG
2	Trastuzumab V_H Bw	ACTACTGACGGTCACTAAGGTGCCTTGACCCCA
3	Trastuzumab V_L Fw	GACATTCAGATGACCCAGTCCCCGAGTAGTCTGTCT
4	Trastuzumab V_L Bw	TTTGATTTCGACTTTCGTGCCTTGGCCAAAGGTTGG
5	$(GGGGS)_3$-Linker2_sense	GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGG
6	$(GGGGS)_3$-Linker2_antisense	TGATCCGCCACCGCCAGAGCCACCTCCGCCTGAAC
7	Trastuzumab V_H-Linker fuseFw	CTTAGTGACCGTCAGTAGTGGTGGAGGCGGTTCAGGC
8	Linker- Trastuzumab V_L fuseBw	ACTGGGTCATCTGAATGTCTGATCCGCCACCGCCAGAG
9	Trastuzumab V_L-Linker fuseFw	GGCACGAAAGTCGAAATCAAAGGTGGAGGCGGTTCAGGCGGA
10	Linker- Trastuzumab V_H fuseBw	TGATTCAACCAGCTGAACTTCTGATCCGCCACCGCCAGAG
11	Trastuzumab V_L (BamHI) Fw	CCGGATCCATGGACATTCAGATGACCCAGTCCCCGAG
12	Trastuzumab V_H (SalI) Bw	CCCGTCGACTTTGATTTCGACTTTCGTGCCTTGGC
13	Trastuzumab sigHC (KpnI) Fw	CTTGGTACCGCCGCCACCATGGGCTCCCAGGTGCAC
14	Trastuzumab sigHC-Hinge9 (BamHI) Bw	CCGGATCCGGTCTTGTCGCAGCTCTTCGGGGGCTCCAC
15	BamHI-LPETGG-NotI Sense	CCGGATCCCTGCCCGAAACCGGCGGCTGAGCGGCCGCAC
16	BamHI-LPETGG-NotI Antisense	GTGCGGCCGCTCAGCCGCCGGTTTCGGGCAGGGATCCGG
17	huHER2 RT-qPCR Fw1*	TCCTGTGTGGACCTGGAT
18	huHER2 RT-qPCR Bw1*	TGCCGTCGCTTGATGAG
19	huGAPDH-Fw	GTCTTCACCACCATGGAGAAGGCT
20	huGAPDH-Bw	CATGCCAGTGAGAGCTTCCCGTTCA

*Perreard et al., 2006.

The pOTC vector, prepared by inserting the PCR-amplified *oriP* region from pEBMulti-Neo (Wako Pure Chemical, Osaka, Japan) into the SspI site of pCAG-Hyg TARGET tag-C (Wako Pure Chemical), was employed to prepare the recombinant antibodies. First, to prepare a Trastuzumab IgG, two vectors denominated as pOTC-tHC and pOTC-tLC were constructed by

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inserting the synthesized Trastuzumab heavy-chain or light-chain coding sequences into pOTC between the KpnI and BamHI restriction sites, respectively (Onitsuka *et al.*, manuscript in preparation). Next, in order to prepare the Trastuzumab Fab carrying LPETGG sequence which turns the Fab into a substrate of SrtA transpeptidation reaction, pOTC-tLC (light-chain vector) was directly used, while heavy-chain vector was re-constructed as follows: A cassette for LPETGG, prepared by annealing the No.15 and No.16 oligonucleotides (Table 1), was inserted between the BamHI and NotI restriction sites of pOTC. Subsequently, the Trastuzumab V_HC_{H1} coding sequence with the nonapeptide hinge area in pOTC-tHC was amplified using PrimeSTAR$^®$ HS DNA Polymerase and the primer set No.13 and No.14 (Table 1) followed by KpnI and BamHI digestion. The purified fragment was then inserted between the KpnI and BamHI restriction sites of pOTC with LPETGG encoding sequence to construct pOTC-tHChg9. The transformation of *E. coli* DH5αZ1 with pOTC-tHChg9 and transformant selection on LB agar plate containing 100 μg/ml of hygromycin B were performed according to the standard methods. After confirming the pOTC-tLC and pOTC-tHChg9 sequences from the selected clones, the plasmids (Fig. 2A) were used to transform EB-B2 cells.

The constructed EB-B2 cells were cultured in duplicates in 500-ml Erlenmeyer flasks containing 100 ml of BalanCD Transfectory CHO medium (Irvine Scientific, Santa Ana, CA, USA) with 2 mM L-glutamine. When viable cell density reached 4×10^6 cells/ml, the culture medium was replaced with fresh BalanCD Transfectory CHO medium and the cells were

transfected with 320 μg of expression plasmid mixture (3:1, pOTC-tHChg9: pOTC-tLC) using

Polyethylenimine Max (Polysciences Inc, Warrington, PA, USA). The transfected cells were then

incubated at 32 °C and 80 % humidity with shaking at 120 rpm, and the culture was maintained

for 12 days. At day 2 of incubation, 10 ml of the nutrient medium transfectory supplement (Irvine

Scientific) was added. Recombinant Fab was purified from the culture supernatant via HiTrap™

Protein L affinity chromatography using an ÄKTAprime™ Plus system (GE Healthcare, Chicago,

Illinois, USA) with a one-step pH gradient from pH 7.0 to pH 2.5 ~ pH 2.7, and the eluate was

neutralized with equilibration buffer (1 M Tris-HCl, pH 8.0). After dialysis against PBS

containing 1 mM EDTA, the purity of the recombinant was confirmed using SDS-PAGE.

1.4 Semi-quantitative real-time PCR analysis

Total RNA from HCT-15 and HeLa cells was prepared using the NucleoSpin Plus

extraction kit (Macherey-Nagel, Düren, Germany) and cDNA was synthesized using the

PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa Bio). Real-time PCR for HER2 cDNA

was performed on the Thermal Cycler Dice Real Time System Lite (TaKaRa Bio) by a standard

2-step PCR protocol using the No.17 and No.18 primer set shown in Table. 1 [Perreard *et al*.,

2006]. The results were analyzed using the in-built TP700 software (TaKaRa Bio) against the

gene encoding human GAPDH as the standard.

1.5 Immunofluorescence imaging

HCT-15 (1.0×10^6 cells/well) and HeLa (0.4×10^6 cells/well) cells were seeded onto sterile cover glasses in 6 well-plates and incubated for 2 days until 80 % or higher confluency. The cells were then washed with FBS- and antibiotic-free culture medium, fixed with 4 % (w/v) paraformaldehyde at room temperature for 15 min, and washed with PBS. Next, the cells were treated with blocking solution [PBS containing 1 % (w/v) BSA] for 1 h at 4 ℃. Subsequently, the fixed cells were incubated for 1 h at 4 ℃ with 1 μM of recombinant antibody (scFv_HL, scFv_LH, or Fab) as the primary antibody, purified Trastuzumab IgG1 as the positive control, or blank PBS as the background control. After washing with PBS, the cells were probed with Alexa Fluor 488-labeled goat anti-human IgG (H+L) (Thermo Fisher Scientific, Waltham, MA, USA) or Alexa Fluor 488-conjugated anti-His-tag (Qiagen) secondary antibodies at 4 ℃ for 1 h. After washing with PBS again, each sample was mounted on a glass slide using Ultramount Aqueous Permanent Mounting Medium (Dako North America, Carpinteria, CA, USA). The cells were observed using the IX71 inverted microscope (Olympus, Tokyo, Japan) and the images were acquired using the cellSens software (Olympus).

2. Preparation and evaluation of liposomes as the drug-carrier in novel DDSs

2.1 One-step preparation of the lipopeptide-containing liposome

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids, Inc., Alabaster, AL, USA) and cholesterol (Wako pure chemical) were used for the preparation of the liposome by the thin-film hydration method as previously reported [Tabata *et al*., 2012]. Briefly, DPPC and cholesterol were dissolved in chloroform with molar ratio of DPPC: cholesterol of 1:1. For the preparation of the liposome with oligoglycine on its surface, lipopeptide 3 (LP3), a short synthesized peptide with mini-PEG and penta-glycine (G_5) at its C-terminal (H-G_5SGG-(miniPEG)$_2$-K(Pal)-NH$_2$), was dissolved in methanol and added to the lipid solution described above with the molar ratio DPPC: LP3 of 1:100. A thin lipid film was formed by the evaporation of the solvent for 2 h at room temperature using an evaporator. Then, 1 ml of pure water was added and sonicated at 60 °C, then treated with a freezing (by liquid nitrogen) and thawing procedure five times. The lipid suspension was extruded through a 0.05 μm or a 0.1 μm polycarbonate membrane (Avanti Polar Lipids, Inc., Alabaster, AL, USA) 21 times using mini-Extruder (Avanti Polar Lipids, Inc.). After purification using HiTrap™ Desalting column (GE Healthcare, Chicago, Illinois, USA), liposomes were concentrated by ultracentrifugation (24,200 ×g, 4 °C, 30 min, or 54,300 ×g, 4 °C, 15 min) using Optima TL Ultracentrifuge with TLA-100.3 Rotor (Beckman Coulter, Inc., Brea, CA, USA), then re-suspended in pure water. If it was necessary, liposomes were also prepared in different buffer systems as follows: PBS (pH 7.4) or

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2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (10 mM MES and 150 mM NaCl) containing

100 μM uranine was used to prepare the fluorescent-loaded liposome for model DDS, and MES

buffer (pH 6.0) was used to prepare the liposome for SrtA transpeptidation. All the prepared

liposomes were stored at 4 °C until use.

2.2 Determination of liposome size

The liposomes prepared by the pure water system were used for size determination.

Briefly, the liposomes were diluted in pure water and the hydrodynamic radius of the liposomes

was measured by the dynamic light scattering system (ALV/CGS-3/7002, ALV GmbH, Langen,

Germany).

2.3 Determination of the lipopeptide contents in the lipopeptide-containing liposome

The contents of LP3 in the liposomes were quantified by *o*-phthalaldehyde (OPA)

protein/peptide assay to measure the oligoglycine contents. To quantify LP3 in the prepared

liposomes, 50 μl of liposome suspension was pelleted by ultracentrifugation (24,200 ×g, 4 °C, 30

min, or 54,300 ×g, 4 °C, 15 min) and lysed with 1 % (w/v) SDS in sodium phosphate buffer (pH

7.0) for 15 min at room temperature. Then, each 10 μl of the lysate or LP3 solution (for standard

curve, final concentration of 0~500 ng/μl) was added to 96-well plates in triplicate. Next, 300 μl

of OPA reagent [0.08 % (w/v) OPA (in 95 % ethanol), 0.20 % β-Mercaptoethanol, 1.00 % (w/v)

SDS, 0.05 M sodium tetraborate] was added and the plate was incubated under shaking with

Thermo-shaker (PST-100HL, Biosan Ltd., Riga, Latvia) at 400 rpm for 4 min at room temperature.

Finally, the fluorescent intensity was measured at $\lambda_{Ex/Em}$=340nm/455nm using a plate reader

(Infinite M200, TECAN, Männedorf, Switzerland).

2.4 Integrity of the prepared liposome

Uranine (Honeywell International Inc., Morris Plains, NJ, USA)-loaded liposomes

prepared in PBS (pH 7.4) were used in this study. Fifty-μl of each liposome prepared as described

above was pelleted by ultracentrifugation (24,200 ×g, 4 °C, 30 min, or 54,300 ×g, 4 °C, 15 min).

The pellet was re-suspended in 150 μl of PBS and the suspension was dispensed into a 96 well-

plate, then the fluorescent intensity was measured at $\lambda_{Ex/Em}$=480nm/520nm (Infinite M200,

TECAN, Männedorf, Switzerland). This assay was performed from the day of liposome

preparation (Day 0) to Day 4, and the liposome suspensions were stored at 4 °C until the assay.

2.5 Transpeptidation of mutant GFP to the prepared liposome

To investigate whether the transpeptidation by the recombinant SrtA derived from

Streptococcus pyogenes occurs on the liposome containing LP3 (LP3_Lipo) prepared by the one-

step preparation method, a previously constructed model ligand based on the mutant GFP

possessing LPETGG sequence (SrtA recognition peptide) at its C-terminal (designated the model

ligand as His-mGFP-E) [Tabata *et al*., 2014a] was adopted. The liposome prepared in MES buffer

(pH 6.0) was used for the transpeptidation by SrtA, and the reaction was performed based on a method previously reported [Tabata *et al.*, 2014a]. LP3_Lipo was added to the reaction mixture containing 5 μM His-mGFP-E and 5 μM SrtA or 15 μM His-mGFP-E and 7.5μM SrtA in MES buffer (pH 6.0) with 1 mM dithiothreitol (DTT) and 5 mM $CaCl_2$. These mixtures were incubated at 40 °C for 2 h in darkness and the liposome was pelleted by ultracentrifugation (54,300 ×g, 4 °C, 15 min) then washed twice and re-suspended in MES buffer (pH 6.0). The reaction mixtures without SrtA were also prepared as the background controls. In addition to the LP3_Lipo prepared by the one-step method, another LP3_Lipo prepared by a two-step method, in which liposome without LP3 was prepared firstly then LP3 was inserted into the liposome [Tabata *et al.*, 2014a], was used as the positive control. To evaluate the transpeptidation by SrtA, 50 μl suspension of each SrtA-treated liposome was dispensed into a black 96-well plate in duplicate and the fluorescent intensity was measured at $\lambda_{Ex/Em}$=480nm/520nm using a plate reader (Infinite M200, TECAN, Männedorf, Switzerland).

3. Preparation and evaluation of liposomes modified with anti-HER2 recombinant antibodies on their surface

3.1 Modification of the prepared liposome with Trastuzumab-derived Fab

To prepare the novel model DDS against HER2-positive cancer cells, transpeptidation by SrtA was adopted to transfer the Trastuzumab-derived Fab (Fab) possessing the LPETGG

donor sequence onto the LP3_Lipo possessing acceptor sequence (penta-glycine). First, Fab

labeled with fluorescein-4-isothiocyanate (FITC-I, Dojindo Laboratories, Kumamoto, Japan) was

prepared and used to check the transfer of Fab onto LP3_Lipo. SrtA transpeptidation was

performed as follows: Briefly, 15 µM of FITC-labeled Fab and 15 µM LP3_Lipo were mixed with

7.5 µM SrtA in MES buffer (pH 6.0) containing 5 mM $CaCl_2$, then the mixture was incubated for

2 h at 40 °C in darkness. After incubation, the liposome was washed twice and re-suspended in

MES buffer (pH 6.0), the volume of which was the same as that of the initial reaction mixture.

Then 50 µl of the suspended mixture was dispensed into a black 96-well plate in duplicate and

the fluorescent intensity was measured at $\lambda_{Ex/Em}$=495nm/525nm using a plate reader (Infinite

M200, TECAN). The reaction mixture without SrtA was also prepared as a background control.

Next, to check the transfer of Fab onto the liposome further, immunoblotting to Fab was also

conducted. A hundred µl of the prepared liposome suspension treated by SrtA transpeptidation as

described above was pelleted by ultracentrifugation (54,300 ×g, 4 °C, 15 min) and re-suspended

in 15 µl of MES buffer (pH 6.0), then the sample for SDS-PAGE was prepared according to the

standard Laemmli's method [Laemmli *et al*., 1970]. The prepared sample was electrophoresed by

SDS-PAGE and the separated proteins were transferred onto a PVDF membrane [Kurien *et al*.,

2006]. Subsequently, the membrane was reacted with 1:10,000 dilution of peroxidase-conjugated

rabbit anti-human IgG(H+L) (Jackson ImmunoResearch Inc, West Grove, PA, USA) and the

substrate mixture for peroxidase (Immobilon™ Western Chemiluminescent HRP Substrate,

Merck Millipore, Burlington, Massachusetts, USA), then the band with chemiluminescence was detected using a CCD imager (LAS-4000mini, FUJIFILM, Tokyo, Japan).

3.2 Evaluation of the novel model DDS against HER2-positive cancer cells

HCT-15 (1.0×10^4 cells/well) and HeLa (0.3×10^4 cells/well) were seeded respectively into 96-well plate and incubated for two days. Fab-labeled liposome prepared as described above was pelleted by ultracentrifugation (54,300 ×g, 4 °C, 15 min) and washed once with 1 ml of FBS/antibiotics-free culture medium, then re-suspended in the culture medium containing 10 %(v/v) FBS and antibiotics. When cell confluency reached 40~50 %, culture medium was removed and 50 μl of prepared Fab-labeled liposome suspension was added, then incubated at 37 ºC for 2 h in an incubator with humidified atmosphere of 5% CO_2. After the cells were washed once with culture medium without phenol-red, the observation of the cells was carried out using In Cell Analyzer 6000 system (GE Healthcare, Chicago, Illinois, USA). As negative controls, His-mGFP-E-labeled liposome and ligand-free liposome were also prepared and used for the assay.
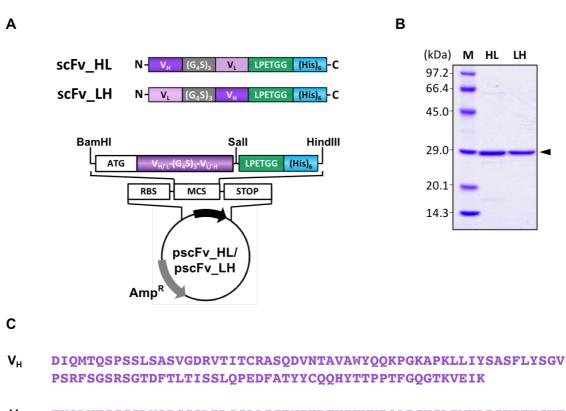
4. Statistics

Each experiment was repeated at least twice, and the typical results are shown in the figures. Error bars in the figures mean the standard deviation (n=3).

CHAPTER 4   Results

1. Preparation and characterization of anti-HER2 recombinant antibodies

1.1 Production and purification of Trastuzumab-derived scFvs with SrtA-recognition motifs

A

scFv_HL N—[V_H][$(G_4S)_3$][V_L][LPETGG][$(His)_6$]—C

scFv_LH N—[V_L][$(G_4S)_3$][V_H][LPETGG][$(His)_6$]—C

BamHI   SalI   HindIII
[ATG][$V_{H/L}$-$(G_4S)_3$-$V_{L/H}$][LPETGG][$(His)_6$]

[RBS][MCS][STOP]

pscFv_HL/
pscFv_LH

Amp^R

B

(kDa)   M   HL   LH
97.2
66.4
45.0
29.0 ◄
20.1
14.3

C

V_H DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGV
PSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIK

V_L EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT
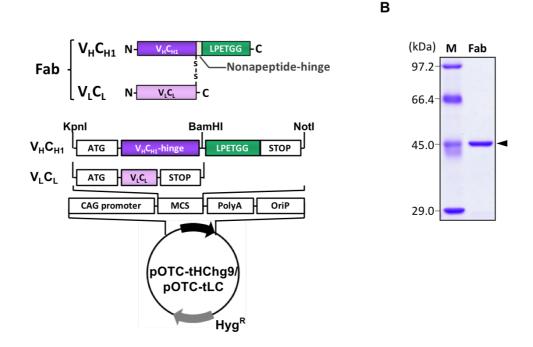RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLV
TVSS

Figure 1. Recombinant targeting modules (scFvs) prepared using a bacterial expression system.
A. Lower, map of expression vector for scFvs (pscFv_HL/ pscFv_LH). Upper, schematic diagram of recombinant scFvs possessing SrtA recognition sequence LPETG followed by His-tag for Ni-affinity purification. B: SDS-PAGE image (CBB staining) of the purified recombinant scFvs. Both scFv_HL and scFv_LH (indicated by arrowhead) were prepared to high purity and the calculated molecular weight of each antibody was observed to be 27.9 kDa. C. Amino acid sequences of Trastuzumab used for construction of recombinant scFvs.

To apply the Trastuzumab-derived scFvs as targeting modules for DDS (DDS-TM) against HER2-positive cancer cells, a bacterial expression system for scFvs was designed to contain LPETGG consisting of SrtA-recognition consensus motif (LPXTG) that is necessary for transpeptidation to the liposome with an oligoglycine motif, and His-tag for purification at the C-terminal side (Fig, 1A). In this study, two scFv expression systems were constructed *i.e.*, "V_H-linker-V_L" type (scFv_HL) and "V_L-linker-V_H" type (scFv_LH). These scFvs (calculated molecular weight: 27.9 kDa) were produced by *E. coli* and purified via nickel-affinity chromatography with sufficient purity (Fig. 1B). The scFv_HL and scFv_LH yields were calculated to be *ca.* 9.5 mg/l and 11.3 mg/l, respectively.

1.2 Production and purification of Trastuzumab-derived Fab with SrtA-recognition motif

A mammalian CHO cell expression system for Fab was also designed, which contained the LPETGG motif at the C-terminal of heavy-chain fragment (V_HC_{H1}) that followed the nonapeptide hinge (Fig. 2A). The Fab produced in the culture supernatant demonstrated high yield (*ca.* 80.0 mg/l) and superior purity according to CBB staining after SDS-PAGE under non-reducing conditions. Though the apparent molecular weight was smaller than the calculated value (53.8 kDa; Fig. 2B), gel filtration chromatography showed that Fab was highly purified and contained only a small amount of light-chain aggregates (data not shown).

A

V$_H$C$_{H1}$ N— V$_H$C$_{H1}$ — LPETGG —C
Fab
— Nonapeptide-hinge

V$_L$C$_L$ N— V$_L$C$_L$ —C

KpnI   BamHI   NotI
V$_H$C$_{H1}$ [ATG | V$_H$C$_{H1}$-hinge | LPETGG | STOP]
V$_L$C$_L$ [ATG | V$_L$C$_L$ | STOP]

[CAG promoter | MCS | PolyA | OriP]

pOTC-tHCHg9/
pOTC-tLC

Hyg^R

B

(kDa)   M   Fab
97.2
66.4
45.0 ◄
29.0

C

V$_H$C$_{H1}$ GSQVHLLSFLLLWISDTRAEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWV
RQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAV
YYCSRWGGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV
KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNV
NHKPSNTKVDKKVEPPKSCDKT

V$_L$C$_L$ MVLQTQVFISLLLWISGAYGDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWY
QQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHY
TTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQW
KVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP
VTKSFNRGEC

Figure 2. Recombinant targeting module (Fab) prepared using a mammalian cell expression system.
A. Lower, map of expression vector for Fab (pOTC-tHCHg9/pOTC-tLC). Only pOTC-tHCHg9 has a SrtA recognition sequence LPETG at the downstream of the sequence for hinge region. Upper, schematic diagram of recombinant Fab possessing SrtA recognition sequence LPETGG at the C-terminal of heavy chain fragment, which is connected with light chain by a disulfide bond. B: SDS-PAGE image (CBB staining) of the purified recombinant Fab. Fab (indicated by arrowhead) was prepared to high purity and the calculated molecular weight was observed to be about 45.0 kDa, which is lower than its calculated molecular weight (53.8 kDa). C. Amino acid sequences of Trastuzumab used for construction of recombinant Fab. Light blue: signal peptides. Dark grey: nonapeptide of hinge region. Underline: cysteines forming a disulfide-bond that links heavy chain region and light chain region of the recombinant Fab.

1.3 Comparison of HER2 gene transcription level in HCT-15 and HeLa cells

Semi-quantitative real-time PCR was performed to compare HER2 gene transcription in HCT-15 and HeLa cells using a standard procedure described in the section "Materials and Methods". Consequently, the HER2 encoding gene was detected and was transcribed in both cell lines. However, the transcriptional level of the gene in HeLa was about 1/7 to 1/6 of that in HCT-15 (Fig. 3).
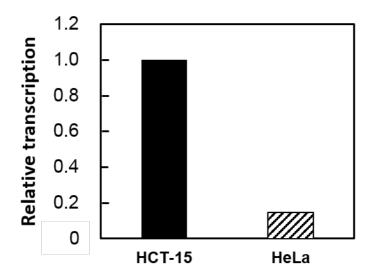

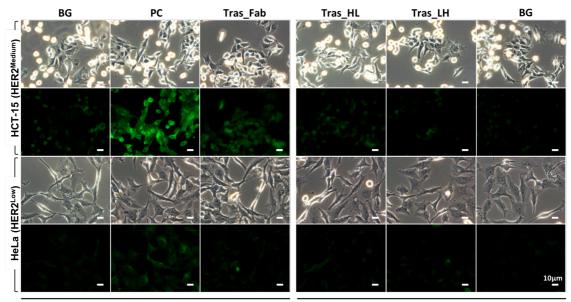*Figure 3. Transcription level of the HER2 gene in human cell lines.*
Transcription of the gene encoding HER2 was investigated in HCT-15 and HeLa cell lines. The gene encoding GAPDH was used as an internal standard for the assay. This experiment was conducted twice, and one of the results is shown.

1.4 Reactivity of Trastuzumab-derived recombinant antibodies to HER2-positive cells

The immunofluorescence imaging assay was performed to evaluate the reactivity of the three recombinant antibodies towards the tested cell lines. The results showed that the reactivity

of Fab towards HCT-15 expressing a high level of HER2 was superior to that of scFvs. On the

other hand, the reactivity of all of the recombinant antibodies towards HeLa (expressing lower

level of HER2) was either faintly detectable or almost undetectable (Fig. 4).

Figure 4. Reactivity of the anti-HER2 recombinant fragment antibodies against HER2-positive cells.
HCT-15 and HeLa cells were treated with anti-HER2 recombinant fragment antibodies as primary antibodies and Alexa Fluor 488-labeled secondary antibodies. The fluorescent image of Alexa Fluor 488 was observed using the IX71 fluorescence microscope (Olympus, Tokyo, Japan). BG: Samples prepared without anti-HER2 recombinant treatment. PC: Samples prepared by treating with Trastuzumab IgG as the primary antibody. Scale bars indicate 10 μm.

2. Preparation of the liposomes with SrtA-recognition motif for accepting transpeptidation

2.1 Hydrodynamic size of the prepared liposomes

The hydrodynamic size of LP3_Lipo and the liposome without LP3 (Cont._Lipo) was

determined by dynamic light scattering. All of the prepared liposomes showed a hydrodynamic

radius from several dozen to hundreds of nanometers. Moreover, the average hydrodynamic

diameters of LP3_Lipo prepared using polycarbonate membrane with 0.05 μm and 0.1 μm pore-

size were 134.87±2.87 nm and 140.12±5.12 nm, respectively, which are slightly larger than that

of Cont._Lipo, at 112.83±0.37 nm and 139.19±6.61 nm (Fig. 5). These results prove that the

liposomes with or without LP3 were successfully prepared according to the method adopted in
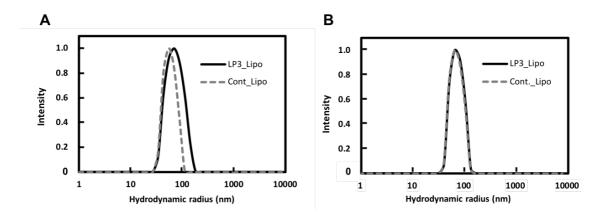
this study.

Figure 5. Hydrodynamic radii of the prepared liposomes.
Hydrodynamic radii of the prepared liposomes measured by dynamic light scattering. A. Hydrodynamic radius of Liposomes prepared using polycarbonate membrane with 0.05 μm pore-size. B. Hydrodynamic radius of Liposomes prepared using polycarbonate membrane with 0.1 μm pore-size. Solid line: LP3_Lipo. Dashed line: Cont._Lipo.

2.2 Measurement of the lipopeptide 3 contents in LP3_Lipo

The OPA protein/peptide assay was carried out to determine the contents of LP3 in the

prepared LP3_Lipo. According to the standard curve of LP3, the contents of LP3 in LP3_Lipo

were determined to be in the range of 1.31~5.66 x10^{-2} mM. This result indicates that LP3 was

successfully incorporated and the prepared LP3_Lipo can be applicable as an acceptor for SrtA transpeptidation. However, the actual ratio of LP3 in the outer liposome leaflet to LP3 in the inner liposome leaflet was unclear, though the even distribution of LP3 in spherical liposome was expected because of the one-step preparation method protocol.

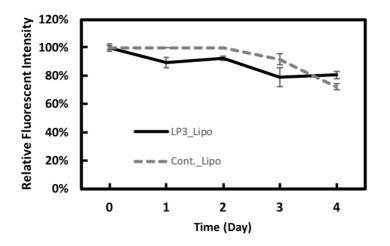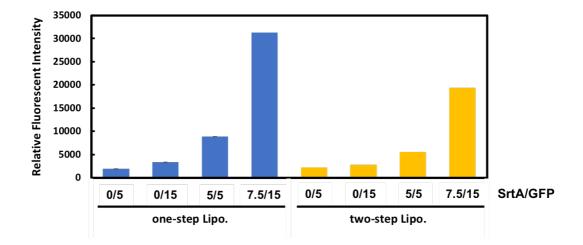## 2.3 Integrity of the LP3_Lipo

Figure 6. Integrity of the liposomes at 4 °C storage condition.

The fluorescent intensity measured at Day 0 was indicated as 100 % of intensity of the assay and the relative fluorescent intensity was measured at each day until the day 4. Solid line: LP3_Lipo. Dashed line: Cont._Lipo

The integrity of the LP3_Lipo was evaluated by the measurement of the leakage of fluorescent dye uranine from the liposomes as described in "Materials and Methods" (Fig. 6). Within the first two days, the relative fluorescent intensity was at a high level (approximately $\geqq$ 90 %) in both LP3_Lipo and Cont._Lipo. Then, the relative fluorescent intensity of both

liposomes started to decrease from day 3, however, the intensity of both liposomes was still higher than 70 % of initial intensity and this was maintained until day 4. As a result, about 80 % of fluorescent intensity was kept in LP3_Lipo even until day 4 under the storage condition of 4 °C. Therefore, LP3_Lipo showed comparative stability to Cont._Lipo during the assay period.

2.4 Surface labeling of LP3_Lipo with His-mGFP-E by SrtA transpeptidation

Figure 7. Surface labeling of LP3_Lipo with His-mGFP-E by SrtA.
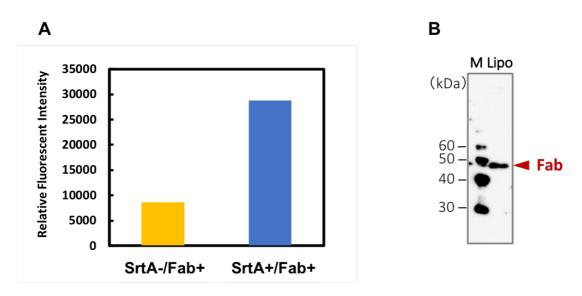
Surface labeling of both LP3_Lipos with His-mGFP-E was evaluated by measuring fluorescent intensity of GFP. "0/5", "0/15", "5/5", "7.5/15" represent final concentrations (μM) of SrtA/His-mGFP-E in the reaction mixture. Blue bar: LP3_Lipo prepared by the one-step preparation method. Yellow bar: LP3_Lipo prepared by the two-step preparation method.
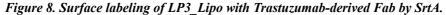
A fluorescent transfer substrate, His-mGFP-E, was transferred to two kinds of LP3_Lipo by SrtA transpeptidation reaction, and the resulting surface labeling of LP3_Lipo with His-mGFP-E was evaluated by measuring relative GFP fluorescent intensity in the LP3-Lipo fractions. It was confirmed that His-mGFP-E was successfully transferred onto the LP3_Lipo prepared by both

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the one-step preparation method (blue bar) and the two-step preparation method (yellow bar) in a

SrtA/His-mGFP-E dose-dependent manner (Fig. 7). Compared with the two-step preparation

method for LP3_Lipo, the one-step preparation method was more effective for the surface

labeling by His-mGFP-E. These results suggest that more LP3 was incorporated into liposomes

produced by the one-step preparation method than those produced by the two-step preparation

method, and that the former liposomes could be appropriate acceptors for SrtA transpeptidation.

3. Preparation and evaluation of the novel model DDS

3.1 Surface labeling of LP3_Lipo with Trastuzumab-derived Fab by SrtA transpeptidation

It was attempted to transfer the FITC-labeled Trastuzumab-derived Fab (FITC-Fab) to

the surface of LP3_Lipo by the transpeptidation of SrtA, and the surface labeling with FITC-Fab

was evaluated both by the measurement of the relative fluorescent intensity of FITC and by

immunoblotting against Fab. As shown in Figure 8A, the LP3_Lipo fraction treated with both

SrtA and FITC-Fab (blue bar) showed higher intensity of FITC-fluorescence compared with the

fraction treated without SrtA (yellow bar). This result indicates that the FITC-Fab was

successfully transferred onto the LP3_Lipo by SrtA transpeptidation. Moreover, the SrtA-

dependent transfer of Fab onto LP3_Lipo was also confirmed by the immunoblotting against Fab

(Fig. 8B).

Figure 8. Surface labeling of LP3_Lipo with Trastuzumab-derived Fab by SrtA.

A. Surface labeling of LP3_Lipo with Trastuzumab-derived Fab was evaluated by the fluorescent intensity according to the FITC-labeled Fab. Yellow bar: Reaction without SrtA (background). Blue bar: Reaction with SrtA. One representative result from triplicate assays is shown. B. Immunoblotting image of Trastuzumab-derived Fab. Trastuzumab-derived Fab (indicated by arrowhead) was detected using chemiluminescence after the specific reaction of peroxidase-conjugated rabbit anti-human IgG(H+L).

3.2 Delivery of liposome containing fluorescent dye uranine to HER2-positive cells by the novel model DDS

Fab-labeled LP3_Lipo containing a fluorescent dye, uranine, as a fluorescent marker and also as an anti-cancer drug model was reacted with HER2-positive cells in order to evaluate the targeting ability of the Fab-labeled LP3_Lipo. As shown in Figure 9, it was suggested that uranine was effectively delivered to the cells moderately expressing HER2 in the case of Fab-labeled LP3_Lipo. On the other hand, Cont. Lipo containing uranine but no surface Fab was hardly delivered to the target cells, though the His-mGFP-E-labeled LP3_Lipo showed some non-specific binding to HCT-15. These results showed that the labeling of LP3_lipo with

Trastuzumab-derived Fab by SrtA enhanced the specific delivery against HER2-medium positive

cancer cells, such as HCT-15.

Figure 9. Delivery of uranine to HER2-positive cells by novel model DDS.

HCT-15 and HeLa cells were treated with uranine-loaded Fab-labeled LP3_Lipo. The fluorescent image of uranine was observed using In Cell Analyzer 6000 system with the bright field image of the same region. Fab_Lipo: The cells treated with uranine-containing Fab-labeled LP3_lipo. mGFP_Lipo: The cells treated with His-mGFP-E-labeled LP3_lipo. Cont._Lipo: The cells treated with Cont._Lipo. Scale bars indicate 60 µm.

CHAPTER 5　Discussion

DDSs are thought to be highly promising in anti-cancer therapeutics owing to their selective drug delivery to target cancer cells. Using bacterial functional proteins, we have been investigating the construction of DDSs composed of drug-carrier liposomes with targeting modules on their surface. Previously, recombinant proteins prepared using bacterial expression systems have been used as DDS-TM [Tabata *et al*., 2012; Tabata *et al*., 2013], however, the selection of targeting modules is limited with these systems. In order to achieve better versatility of the DDS-TM, SrtA (a bacterial transpeptidase)-mediated transpeptidation was adopted to prepare a novel DDS, since a wide range of proteins or peptides with LPXTG (a SrtA-recognition motif) could be joined to the N-terminal of oligoglycine-introduced lipopeptides [Tabata *et al*., 2014a; Tabata *et al*., 2015]. Based on this background about the construction of novel DDSs using bacterial functional proteins, a novel DDS for the targeting of HER2 -positive cancer cells was planned.

In the present study, Trastuzumab-derived recombinant fragment antibodies (scFv and Fab) containing the SrtA-recognition motif (LPETG) at their C-terminal were designed and prepared (Fig. 1 and Fig. 2). The CHO cell expression system provided an 8-fold higher yield per liter of culture media and superior purity when compared with the *E. coli* expression system, although a much longer culture period is necessary for the CHO cell expression system. This is because the expression system of mammalian cells such as CHO undergoes appropriate post-

translational modifications, particularly with respect to mammal protein expression. Despite the time-consuming nature of the expression of recombinant proteins in mammalian cells, CHO cells are ideal for producing commercial therapeutic antibodies [Bandaranayake *et al*., 2014; Fischer *et al*., 2015]. On the other hand, bacterial expression systems such as those in *E. coli* are frequently adopted because they are effective in reducing costs and time with respect to target recombinant preparation. However, the target recombinants produced by *E. coli* expression systems often form inclusion bodies that are insoluble and difficult to purify as active proteins [Peternel *et al*., 2011]. Our experience also shows that purified scFvs are less stable, and Fab is more stable against freeze-thawing (data not shown). This may be because the constant domain of the antibody provides structural stability to Fab against temperature changes [Teerinen *et al*., 2006]. Moreover, in terms of HER2 recognition, Fab shows stronger recognition against HER2 expressed on the surface of HER2-positive cancer cells than both scFvs (Fig. 4). As a consequence, our results suggest that the CHO cell expression system exhibits superiority in the production of recombinant proteins, such as fragment antibodies, which can be applied to the targeting module of DDSs.

Investigation of drug-carrier liposomes of DDSs applicable for surface modification and their actual labeling with a cancer-targeting module was also conducted. The prepared liposome, LP3_Lipo, produced by the one-step preparation system showed larger sizes than Cont._Lipo (Fig. 5), and this difference in size is thought to be due to the incorporation of LP3 into LP3_Lipo, according to the result of the OPA protein/peptide assay. These results suggest the successful

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introduction of the penta-glycine motif for SrtA transpeptidation into the liposome, even by the one-step preparation method using a short-synthesized peptide with mini-PEG and penta-glycine at its C-terminal (H-G$_5$SGG-(miniPEG)$_2$-K(Pal)-NH$_2$). In our previous study, a two-step preparation method was adopted to produce liposomes for SrtA transpeptidation, in which liposomes were prepared firstly then peptides with oligoglycine were inserted into the liposomes [Tabata *et al*., 2014a]. Compared with this previous system, the one-step preparation method adopted in this study is a more efficient method to produce the liposomes for SrtA transpeptidation. In addition, the size of LP3_Lipos was measured to be in the range of 100~200 nm in diameter. This result shows that the liposomes prepared by the one-step preparation method are probably able to deliver anti-cancer drugs to the target cancer tissues/cells with an enhanced permeability and retention (EPR) effect [Matsumura *et al*., 1986, Noble *et al*., 2014]. Furthermore, LP3_Lipo prepared by the one-step preparation method also showed sufficient integrity under 4 °C storage condition for at least 4 days (Fig. 6). This property will be suitable for the retaining of the anti-cancer drugs adequately in the LP3_Lipo under physiological pH conditions. The advantage of the LP3_Lipo prepared by the one-step method was demonstrated, since a larger amount of His-mGFP-E was labeled on the surface of LP3_Lipo prepared by the one-step preparation method than by the two-step preparation method (Fig. 7). Based on the results described above, we supposed that LP3_Lipo prepared by the one-step preparation method has enough potential to be the drug-carrier of the novel DDS we planned to construct. And as we expected, Trastuzumab-

derived Fab was successfully transferred onto the surface of LP3_Lipo by SrtA transpeptidation (Fig. 8). This Fab-labeled LP3_Lipo is a model DDS; Fab immobilized on LP3_Lipo enhanced delivery of liposomes containing uranine to HER2-positive cancer cells, especially HCT-15 expressing higher level of HER2 (Fig. 9). Although weak non-specific staining was observed in cells treated with His-mGFP-labeled liposomes, it is supposed that the non-negligible false signal from the liposome strongly double-labeled with internal uranine and external His-mGFP-E was caused by the non-specific interaction between the HCT-15 and His-mGFP-E. From this result, it is suggested that anti-cancer drugs loaded into liposomes are able to be delivered and may be released into HER2-positive cancer cells. Furthermore, it is considered that Trastuzumab-derived Fab produced from the CHO expression system has sufficient stability to function as a DDS-TM even after the treatment of SrtA transpeptidation because Fab on the liposomes maintains the property of specific recognition of HER2-positive cancer cells. And this Trastuzumab-derived Fab may also be adopted to modify other drug-carriers, such as nanoparticles and micelles, and so on [Alibakhshi *et al*., 2017]. The results described above suggest the Trastuzumab-derived Fab-labeled liposomes can function as a DDS against HER2-positive cancer cells, however, further investigations are necessary to apply this DDS in cancer therapeutics.

In summary, the findings of the present study are useful for the construction of novel DDSs using bacterial functional proteins (Gram-positive bacterial protein SrtA with transpeptidation activity) as follows: 1) a Trastuzumab-based Fab with a SrtA recognition

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sequence LPETG on its C-terminal for transpeptidation by SrtA was successfully and effectively prepared in a CHO cell expression system, 2) a penta-glycine containing liposome for SrtA transpeptidation was also successfully and effectively produced by a one-step preparation method, and 3) a penta-glycine containing liposome was successfully labeled with Trastuzumab-based Fab that can function to enhance targeting ability of HER2-positive cells by SrtA transpeptidation. Using this system, a novel DDS for the targeting of HER2-positive cancer cells, in which, anti-drugs or cytocidal effecter toxins are loaded instead of fluorescent dye, will be constructed in the near future, and this novel DDS will be developed to be a potent therapeutic method against HER2-positive cancers. Moreover, for DDS preparation, SrtA transpeptidation was confirmed as a potent/useful system with versatility as long as each targeting module and drug carrier is modified with LPXTG and an oligoglycine motif, respectively. Therefore, further application of SrtA for the preparation of other DDSs is also planned in our laboratory.

CHAPTER 6   Conclusion

Development of effective cancer therapeutics attracts the interest of many researches in medical and medicinal fields, especially investigating DDSs. Thus, we have been investigating the development of novel DDSs and the techniques for the construction of novel DDSs using various bacterial functional proteins such as CDCs and SrtA [Tataba *et al,* 2012; Tataba *et al,* 2013; Tataba *et al,* 2014a; Tataba *et al,* 2015]. In this study, to investigate the potential of SrtA transpeptidation in the construction of a novel DDS against HER2-positive cancers, anti-HER2 recombinant antibodies with the donor-sequence for SrtA reaction and liposomes with acceptor-sequence for SrtA reaction were prepared and characterized. Consequently, one of the prepared recombinant antibodies (Trastuzumab-based Fab) was proved to be a potential targeting module against HER2-positive cancer cells for the novel DDS. Liposomes with penta-glycine on the surface were also successfully prepared and confirmed to have appropriate properties to act as a drug-carrier in DDSs. In addition, by the SrtA transpeptidation as shown in Scheme 1A, the prepared liposomes were successfully labeled by the Trastuzumab-based Fab, and this constructed model DDS showed its potential to enhance the targeting to HER2-positive cancer cells. Moreover, in order to expand the potential of bacterial functional proteins such as SrtA and CDCs, the preparation of versatile targeting modules for DDSs with cancer-targeting ability and liposome/cell attachment ability, *e.g.*, small recombinant antibodies with other antigen specificities, such as scFv, nanobody, and Fab, genetically fused or enzymatically linked with

CDC domain 4, will also be conducted in the future. These trials should be helpful in developing

novel DDSs against various cancers (Scheme 1B).

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LIST OF PUBLICATION

I.   **<u>Qing Tang,</u>** Masayoshi Onitsuka, Atsushi Tabata, Toshifumi Tomoyasu and Hideaki Nagamune. (2018) Construction of anti-HER2 recombinants as targeting modules for a drug-delivery system against HER2-positive cells. Anticancer Res *38(7)*: 4319-4325. (Published)

I.

Construction of anti-HER2 recombinants as targeting modules for a drug-delivery system against HER2-positive cells

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