Development of a Sortase A-mediated Peptide-labeled Liposome Applicable to Drug-delivery Systems

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Abstract. *Background/Aim: In order to develop an efficient drug-delivery system (DDS), a lipopeptide-loaded liposome that functions as a platform for the transpeptidase reaction mediated by sortase A (SrtA) was constructed and its stability, as well as cell-specific targeting were evaluated in the present study. Materials and Methods: Several lipopeptides possessing an acceptor peptide sequence (oligoglycine ≥ three residues) or donor peptide sequence (LPETG) for the SrtA-mediated reaction were chemically synthesized and then inserted into the liposome membrane composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol (DPPC-Chol-lipo) to obtain the lipopeptide-loaded liposomes. The transpeptidase reaction mediated by recombinant SrtA (His-ΔN_{59}SrtA) was employed to modify the peptide moiety on the liposomal surface using a fluorescently-labeled substrate peptide corresponding to the species of each loaded lipopeptide. Furthermore, lung tumor-binding peptide (LTBP)-labeled liposomes, prepared by this transpeptidase reaction, were investigated for selective targeting to lung cancer cells in vitro. Results and Discussion: The His-ΔN_{59}SrtA-mediated transpeptidation of fluorescently-labeled peptide on the lipopeptide-loaded DPPC-Chol-lipo was confirmed. The selective targeting of LTBP-labeled liposomes to the lung cancer cell line A549 was also observed in vitro. These results suggest that the labeling of acceptor or donor lipopeptide-loaded liposomes with the transpeptidase SrtA could be a useful method for developing a platform applicable to a cancer-targeting DDS.*

Bacteria produce various proteins in order to promote survival and growth in their habitat. Among these proteins, some, such as enzymes, have characteristics that could not be substituted for by any chemical or artificial processing methods. These functional bacterial proteins are expected to be applicable to the production or development of valuable and useful materials.

We have been investigating the function of bacterial proteins and their applicability to developing valuable and useful materials or techniques. One of our aims has been the development of novel tools applicable to drug-delivery systems (DDSs). We have developed a lung cancer cell-targeted DDS prototype: a drug-carrier liposome with the N-terminal lung tumor-binding peptide (LTBP) (1) fused, on its surface, with a pore-forming bacterial protein toxin, a cholesterol-dependent cytolysin (CDC) (2). In order to enhance the cell-targeting versatility of this tool for DDS, a conformation-restricted CDC fused with the Z-domain, which is a modified domain of protein A from *Staphylococcus aureus* (3) with the ability to bind to the Fc domain of IgG at the N-terminus, was also developed (4). Moreover, an investigation of the transpeptidase reaction using a recombinant version of the bacterial transpeptidase sortase A (SrtA) derived from *S. aureus* (His-ΔN_{59}SrtA) was performed in order to modify a liposome surface directly by a convenient labeling method (5). Transpeptidation by SrtA is one of the remarkably successful labeling reactions and many results suggesting the applicability of this reaction have been reported (6-13).

As one of the efforts to develop a useful DDS by direct modification of the surface of a drug-carrier liposome, a convenient technique to directly modify the liposome surface

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with a cancer-directed ligand using transpeptidation by His-ΔN_{59}SrtA was investigated in this study. Briefly, a series of lipopeptides with acceptor peptide (oligoglycine ≥ three residues) or donor peptide (LPETG) functions in the SrtA transpeptidase reaction were chemically synthesized and inserted into the lipid membrane of cholesterol-containing 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) liposomes (DPPC-Chol-lipo). Then, the stability of the prepared lipopeptide-inserted DPPC-Chol-lipo and the transpeptidase reaction mediated by His-ΔN_{59}SrtA on the lipopeptide-inserted DPPC-Chol-lipo were evaluated. Furthermore, liposomes labeled by His-ΔN_{59}SrtA with a lung cancer-binding peptide were developed and their targeted delivery to lung cancer cells was evaluated *in vitro*.

Materials and Methods

Synthesis of acceptor and donor lipopeptides for modification of liposome surface by recombinant SrtA. All lipopeptides, the acceptor-type lipopeptides (AcLPs) 1-3 and the donor-type lipopeptides (DoLPs) 1 and 2, were synthesized by microwave-assisted solid-phase synthesis according to the fluorenylmethyloxycarbonyl (Fmoc) strategy. H-Rink amide TentaGel® resin was swollen with *N,N*-dimethylformamide (DMF) and Fmoc-amino acids (620 mmol) were serially coupled onto the resin by *N,N*-diisopropylcarbodiimide/1-hydroxybenzotriazole in DMF at 70˚C using a microwave (<70 W) for 15 min. Fmoc de-protection was performed using 20% piperidine/DMF. After completion of the synthesis, the resulting resin was dried *in vacuo*. The resin was treated with trifluoroacetic acid (TFA)/triisopropylsilane/water (92:4:4) for 2 h at room temperature. The resin was removed by filtration and TFA was then evaporated to obtain an oily residue, which was washed with diethyl ether and then dissolved in aqueous acetonitrile. The crude lipopeptide solution was purified by preparative high performance liquid chromatography and dried to yield an amorphous powder. The pure lipopeptides were analyzed by electronspray ionization-mass spectrometry (ESI-MS). AcLP1: H-GGGGGSGG-miniPEG-K(Pal)-NH_2, ESI-MS (TOF) *m/z*: 1015.6144 ($C_{45}H_{82}N_{12}O_{14}$ consistent with calculated $[M+H]^+$ 1015.2046). AcLP2: H-GGGGGSGG-$(miniPEG)_2$-K(Pal)-NH_2, ESI-MS (TOF) *m/z*: 1160.6854 ($C_{51}H_{93}N_{13}O_{17}$ consistent with calculated $[M+H]^+$ 1160.3610). AcLP3: H-GGGGGSGG-K(Pal)-NH_2, ESI-MS (TOF) *m/z*: 870.5325 ($C_{39}H_{71}N_{11}O_{11}$ consistent with calculated $[M+H]^+$ 870.0481). DoLP1: H-K(Pal)-miniPEG-GGSGGLPETGGG-NH_2, ESI-MS (TOF) *m/z*: 1398.8235 ($C_{63}H_{111}N_{15}O_{20}$ consistent with calculated [M+H]+ 1398.6439). DoLP2: H-K(Pal)-$(miniPEG)_2$-GGSGGLPETGGG-NH_2, ESI-MS (TOF) *m/z*: 1543.8983 $C_{69}H_{122}N_{16}O_{23}$ consistent with calculated [M+H]+ 1543.8004).

Insertion of synthesized lipopeptides into liposome membrane. DPPC-Chol-lipo was prepared as described previously (2) and the DPPC concentration of the prepared DPPC-Chol-lipo suspension was measured using a Phospholipids C-test Wako Kit (Wako, Osaka, Japan). A 0.1 molar ratio of each synthesized lipopeptide was mixed with the DPPC-Chol-lipo suspension (containing the prepared DPPC-Chol-lipo corresponding to 5 mM of DPPC) and incubated at 37˚C for 1 h. After ultra-centrifugation (40,000 × *g*, 30 min, 4˚C), the pellet was washed once with imidazole-buffered saline (IBS; 10 mM imidazole, 150 mM NaCl, pH 6.5). The supernatant was discarded after a second ultra-centrifugation step (40,000 × g, 30 min, 4˚C) and the resulting pellet was dissolved in 10% (w/v) sodium dodecyl sulfate (SDS) solution. The lipopeptide contents, both in the supernatant after the first centrifugation and in the final pellet fraction prepared as an SDS solution, were determined using a Protein Assay Bicinchoninate Kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol.

DPPC-Chol-lipo in which 10 mM fluorescein sodium salt (uranine) and 10 mM 5-fluorouracil were encapsulated was prepared as reported previously (4) and the insertion of lipopeptide into this liposome was conducted as described above. The prepared liposome was washed once with IBS by ultra-centrifugation (40,000 × *g*, 30 min, 4˚C) and the fluorescence intensity of the IBS suspension was measured using a fluorescence microplate reader (Infinite M200; TECAN, Männedorf, Switzerland) at 480 nm excitation and 520 nm emission. After measurement of the fluorescence intensity, the sample was retrieved and stored at 4˚C in the dark. The procedure from ultra-centrifugation to fluorescence intensity measurement was repeated each day in order to examine liposomal stability.

Transpeptidase reaction on lipopeptide-inserted DPPC-Chol-lipo. A schematic illustration of the strategy for evaluating the transpeptidase reaction mediated by His-ΔN_{59}SrtA on both AcLP- and DoLP-inserted DPPC-Chol-lipo is described in Figure 1. The lipopeptide-inserted DPPC-Chol-lipo was prepared as described above and suspended in IBS. The labeling with the substrate peptides (CAHHHHHHA LPETGG and GGGGGC, synthesized by GenScript, Piscataway, NJ, USA) modified by Alexa 488-maleimide (Life Technologies, Carlsbad, CA, USA) was conducted as follows. After pre-incubation of 5 μM His-ΔN_{59}SrtA with 1 mM dithiothreitol (DTT) and 5 mM $CaCl_2$ at 37˚C for 15 min, 1 μM Alexa 488-labeled peptide was added and the reaction mixture was incubated at 37˚C for 1 h. Subsequently, the lipopeptide-inserted DPPC-Chol-lipo was added to this reaction mixture, which was further incubated at 37˚C for 2 h. After ultra-centrifugation (40,000 × *g*, 30 min, 4˚C), the pellet was washed twice with IBS by ultra-centrifugation (40,000 × *g*, 30 min, 4˚C). The washed pellet was resuspended in IBS and the fluorescence was measured using a fluorescence microplate reader (Infinite M200; TECAN) at 492 nm excitation and 524 nm emission. The results are shown as net fluorescence intensity; the background fluorescence intensity (the fluorescence intensity measured in the sample with lipopeptide and without both His-ΔN_{59}SrtA and Alexa 488-labeled peptide) was subtracted from the fluorescence intensity of samples.

In vitro investigation for targeted delivery of liposomes labeled with cancer cell-targeting peptide. The human lung cancer cell line A549 (RCB0098; Riken Bioresource Center, Tsukuba, Japan) and the human normal fibroblast cell line NB1RGB (RCB0222; Riken Bioresource Center) were cultured according to a previously reported method (2). NB1RGB cells were inoculated into a 12-well cell culture plate at 2.0×10^4 cells/well and cultured overnight. After discarding the culture supernatant, A549 cells were inoculated at 1.0×10^4 cells/well and the co-culture incubated overnight. DPPC-Chol-lipo encapsulating 10 mM uranine and 10 mM 5-fluorouracil was prepared as reported previously (4). In this investigation, AcLP2-inserted DPPC-Chol-lipo was adopted because it exhibited the highest acceptance efficiency in the His-ΔN_{59}SrtA-mediated transpeptidase reaction among all AcLP-inserted DPPC-Chol-lipo.

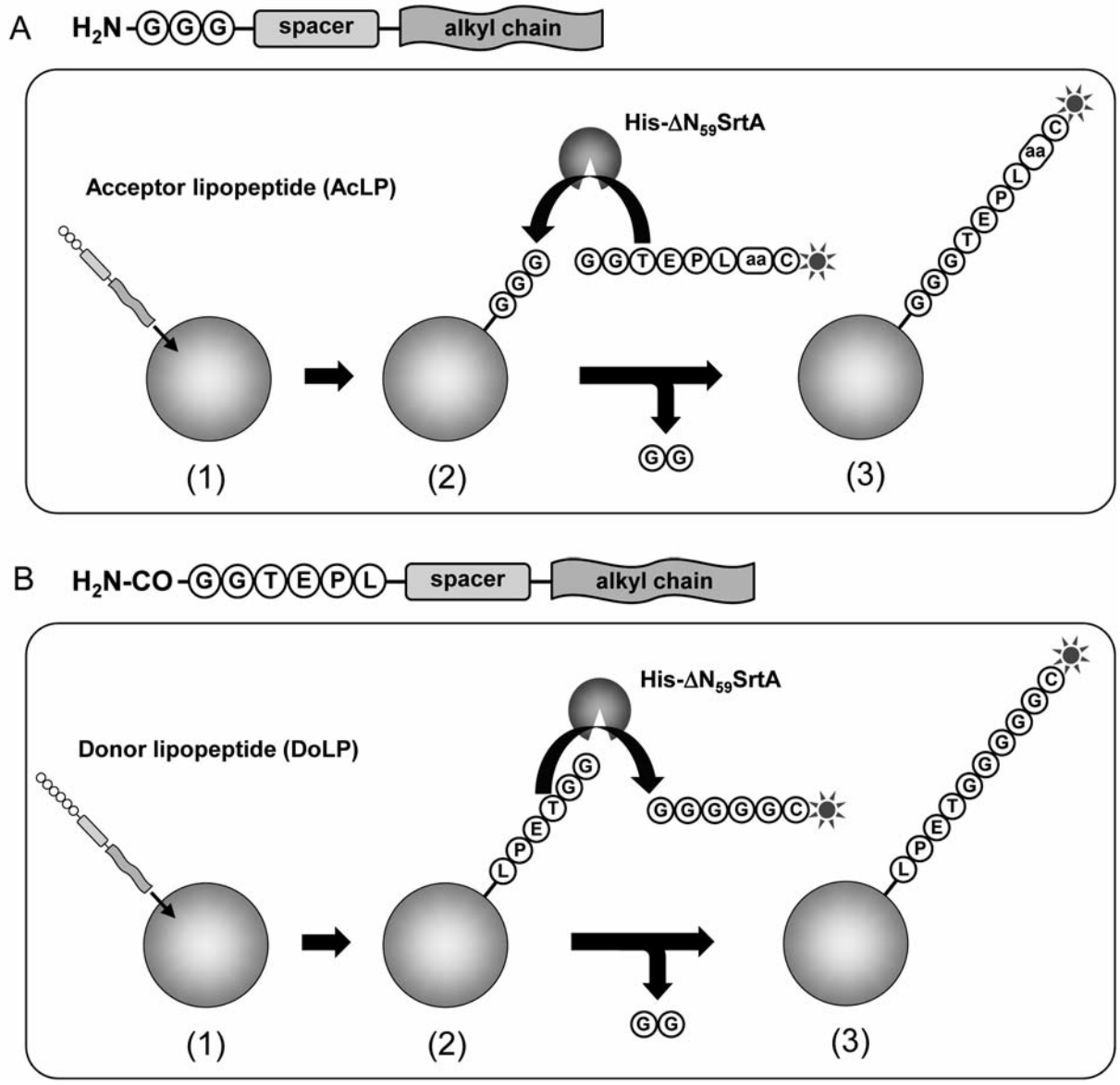

Figure 1. *Schematic illustration of lipopeptide structure and strategy for evaluating His-ΔN_{59}SrtA-mediated transpeptidation on lipopeptide-inserted DPPC-Chol-lipo. A: Acceptor lipopeptide (AcLP). The synthesized AcLP is composed of the acceptor oligoglycine and hexadecanoic acid with or without conjugated spacer (polyethylene glycol). Each AcLP was incubated with DPPC-Chol-lipo (step 1). Subsequently, the transpeptidase reaction mediated by His-ΔN_{59}SrtA using a fluorescently labeled peptide (including the SrtA recognition sequence LPETG where some amino acids are described as "aa") as donor substrate was conducted (step 2). Finally, the reaction product was evaluated by measuring the fluorescence intensity of resulting liposomes (step 3). In the present study, three AcLPs with different spacer structures were investigated. B: Donor lipopeptide (DoLP). The synthesized DoLP is composed of the donor sequence of SrtA (LPETG) and hexadecanoic acid with or without conjugated spacer (polyethylene glycol). Each DoLP was incubated with DPPC-Chol-lipo (step 1). Subsequently, His-ΔN_{59}SrtA-mediated transpeptidation of the fluorescently labeled acceptor oligoglycine peptide was conducted (step 2). The reaction product was evaluated as described in A (step 3). In the present study, two DoLPs with different spacer structures were investigated.*

The transpeptidase reaction conditions were similar to those described above except for two factors: final 1 μM recombinant GFP with LTBP-LPETGG peptide on its N-terminus was used instead of Alexa 488-labeled peptide and the reaction time was changed to 6 h. The prepared liposome designated as "model DDS" was washed twice with IBS by ultra-centrifugation (40,000 × g, 30 min, 4˚C) and the resulting pellet was resuspended in IBS. The IBS suspension containing the model DDS was mixed with cell culture medium and added to the NB1RGB/A549 co-culture, which was then incubated for 2 h. After incubation, the cells were washed three

times with fresh cell culture medium and the model DDS targeting was evaluated using a fluorescence microscope (IX71N-22FL/PH, Olympus, Shinjuku-ku, Tokyo, Japan) with an integrated DP72 digital camera (Olympus).

Results

Insertion efficiency of synthesized lipopeptides into DPPC-Chol-lipo. The insertion efficiency of synthesized AcLPs and DoLPs into DPPC-Chol-lipo is shown in Table I. All lipopeptides were successfully inserted into the lipid membrane with 53.0% (for DoLP2) to 65.6% (for AcLP1 and AcLP3) efficiency. Overall, higher insertion efficiency was observed with AcLPs than with DoLPs.

Stability of lipopeptide-inserted DPPC-Chol-lipo. In order to investigate the stability of lipopeptide-inserted DPPC-Chol-lipo, 10 mM uranine was encapsulated in the liposomes as an indicator of membrane integrity and liposomal stability was evaluated by measuring the fluorescence intensity of uranine retained in intact liposomes. The results for AcLP-inserted DPPC-Chol-lipo and DoLP-inserted DPPC-Chol-lipo are shown in Figure 2A and B, respectively. These results show that ≥80% uranine was retained both in AcLP-inserted DPPC-Chol-lipo and in DoLP-inserted DPPC-Chol-lipo, even after 2 days of storage at 4˚C. These results suggest that the prepared lipopeptide-inserted DPPC-Chol-lipo was sufficiently stable for our experimental purposes.

Transpeptidase reaction on lipopeptide-inserted DPPC-Chol-lipo. The results of His-ΔN_{59}SrtA-mediated transpeptidation on each lipopeptide-inserted DPPC-Chol-lipo are shown in Figure 3. The His-ΔN_{59}SrtA-mediated transpeptidation efficiency on AcLP3-inserted DPPC-Chol-lipo was low and the fluorescence intensity was nearly at background levels (Figure 3C). The other lipopeptide-inserted DPPC-Chol-lipo was significantly labeled with Alexa 488-fluorescent peptide in a His-ΔN_{59}SrtA-dependent manner, although some non-specific binding was observed with the AcLP1-inserted DPPC-Chol-lipo (Figure 3A). Moreover, overall higher fluorescence labeling was observed with all DoLP-inserted DPPC-Chol-lipo compared to all AcLP-inserted liposomes.

Targeted-delivery of liposomes labeled with cancer cell-targeting peptide in vitro. The targeted-delivery of LTBP-labeled AcLP2-inserted DPPC-Chol-lipo encapsulating 10 mM uranine and 10 mM 5-fluorouracil (model DDS) was investigated *in vitro*. After the application of the model DDS to the mixed culture of a normal fibroblast cell line (NB1RGB) and a lung cancer cell line (A549), the model DDS was selectively targeted to A549 and a negligible level of fluorescence was associated with NB1RGB, as shown in Figure 4.

Table I. *Insertion efficiency of synthesized lipopeptides into the liposome membrane*.

	Acceptor lipopeptide			Donor lipopeptide	
	AcLP1	AcLP2	AcLP3	DoLP1	DoLP2
Insertion efficiency (%) (S.D.)*	65.6 (4.0)	58.8 (1.1)	65.6 (6.6)	58.8 (0.6)	53.0 (7.1)

*Standard deviation (n=3).

Discussion

DDS is an excellent technique that is able to selectively deliver anticancer therapeutics to target cells/tissues. The DDS has been investigated since the 1980s for cancer treatment and it continues to attract attention because it has the potential to reduce adverse side-effects in normal cells/tissues and enhance the quality of life of cancer patients. In general, the vehicle that has been adopted most frequently as a drug carrier for DDS is the liposome (14). Liposomes are used not only as drug carriers but also as functionalized liposomes with various utilities in DDS: *e.g.*, PEGylated liposomes or stealth liposomes with reduced phagocytic clearance to prolong their circulation *in vivo* (15-17), targeting liposomes, such as immunoliposomes (18, 19), affisomes (20), proteoliposomes (21), liposomes with peptide tags (22-25), liposomes modified with lectins (26), and so on.

We have been investigating the properties of bacterial functional proteins and their application to the DDS platform. We have, thus far, developed a lung cancer cell-directed model DDS composed of DPPC-Chol-lipo with an LTBP-CDC chimeric protein on its outer leaflet (2) and another improved model DDS with enhanced versatility in targeting composed of DPPC-Chol-lipo with a Z-domain-CDC chimeric protein on its surface (4). In the present study, with the expansion of our strategy for the development of DDS, we intended to develop a novel system that allows modification of the liposome surface with a relevant targeting molecule (ligand) with greater facility. In order to accomplish this, we focused on the transpeptidase reaction mediated by SrtA, a representative transpeptidase secreted from Gram-positive bacteria (27, 28). We previously investigated the reaction conditions for transpeptidation by His-ΔN_{59}SrtA for the preparation of surface-modified liposomes (5).

In the present study, the lipopeptides with acceptor sequence (oligoglycine ≥ three residues) and donor sequence (LPETG), indispensable for the SrtA-mediated transpeptidase reaction, were chemically synthesized and inserted into DPPC-Chol-lipo to prepare the platform for

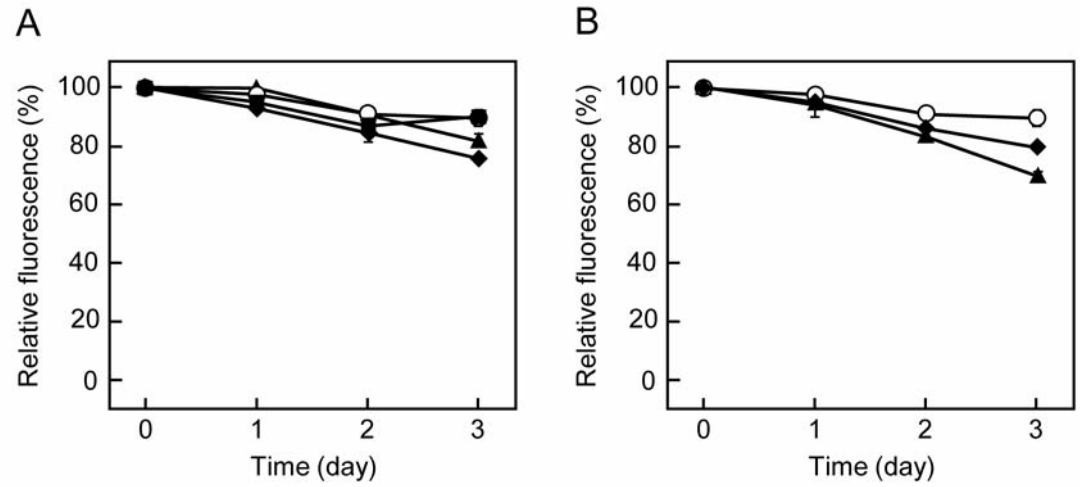

Figure 2. *Stability of lipopeptide-inserted DPPC-Chol-lipo. Each acceptor lipopeptide (AcLP1, AcLP2 and AcLP3) and donor lipopeptide (DoLP1 and DoLP2) was inserted into the lipid membrane of DPPC-Chol-lipo encapsulating a fluorescent dye (uranine) and 5-fluorouracil. Each lipopeptide-inserted DPPC-Chol-lipo was stored at 4˚C and its stability was evaluated by measuring the uranine-derived fluorescence retained in intact liposomes every 24 h for 3 days. A: Stability of AcLP-inserted DPPC-Chol-lipo. AcLP1 (solid triangle), AcLP2 (solid diamond) and AcLP3 (solid square). The DPPC-Chol-lipo without insertion of AcLP was also measured as a stability control (open circle). B: Stability of DoLP-inserted DPPC-Chol-lipo. DoLP1 (solid triangle), DoLP2 (solid diamond) and stability-control DPPC-Chol-lipo (open circle; same result shown in graph A). Error bars indicate±standard deviation of three independent measurements.*

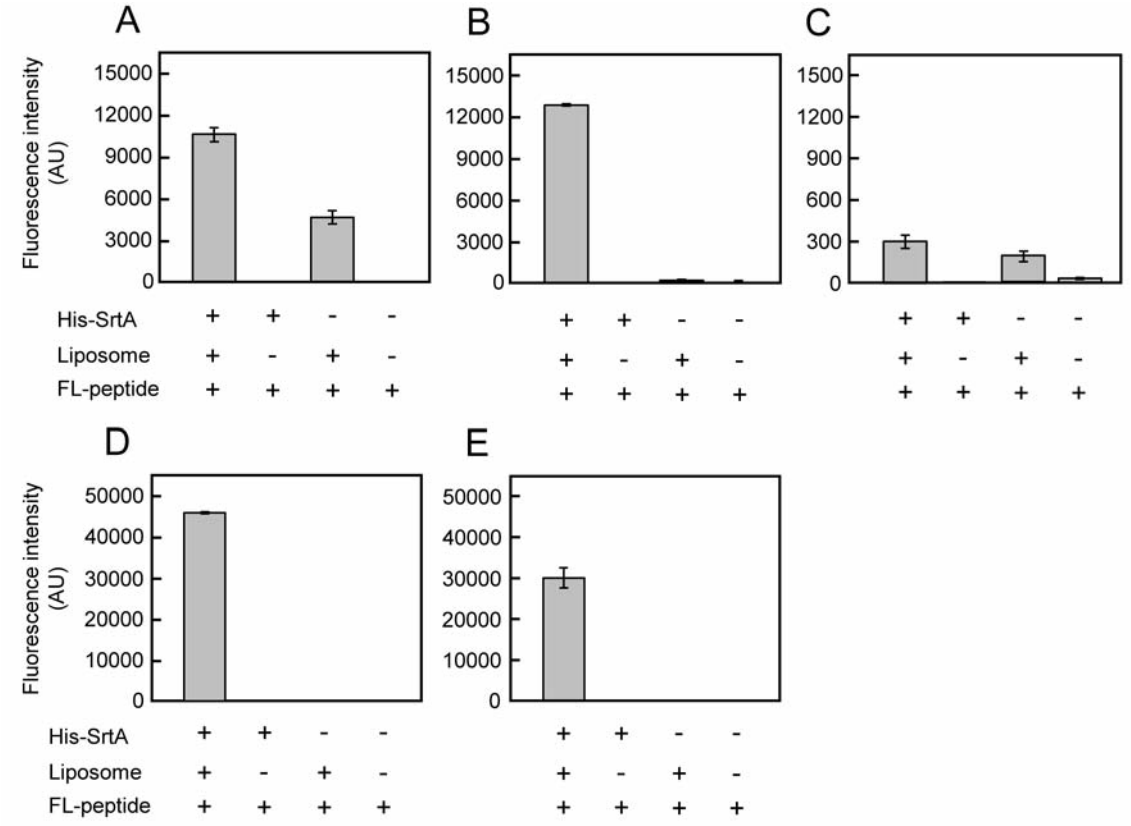

Figure 3. *Evaluation of the transpeptidase reaction mediated by His-ΔN_{59}SrtA on the lipopeptide-inserted DPPC-Chol-lipo surface. The His-ΔN_{59}SrtA-mediated transpeptidation of Alexa 488-labeled peptide onto the three acceptor lipopeptide (AcLP1, AcLP2 and AcLP3)-inserted DPPC-Chol-lipo and two donor lipopeptide (DoLP1 and DoLP2)-inserted DPPC-Chol-lipo was conducted as described in Materials and Methods. A: AcLP1-inserted DPPC-Chol-lipo, B: AcLP2-inserted DPPC-Chol-lipo, C: AcLP3-inserted DPPC-Chol-lipo, D: DoLP1-inserted DPPC-Chol-lipo, E: DoLP2-inserted DPPC-Chol-lipo. Error bars indicate±standard deviation of three independent measurements.*

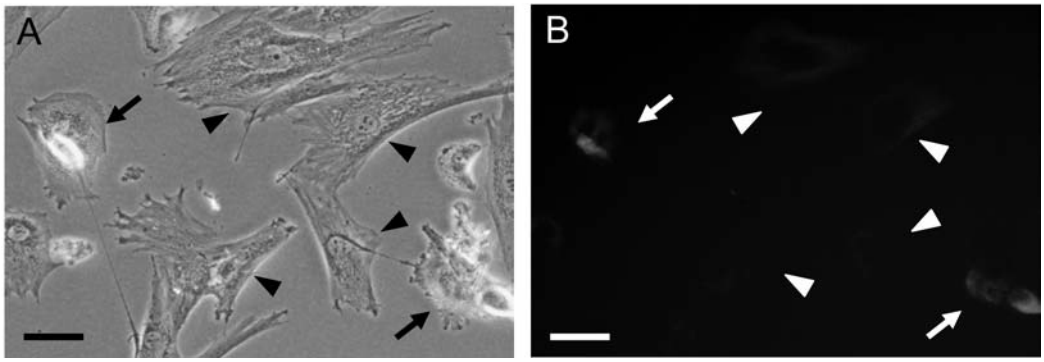

Figure 4. *Targeted delivery of a model DDS to the lung cancer cell line A549 in vitro. LTBP-labeled DPPC-Chol-lipo encapsulating uranine and 5-fluorouracil was incubated with a mixed culture of A549 and the human normal fibroblast cell line NB1RGB, followed by evaluation of the delivery of the model DDS by fluorescence microscopy. A: Representative bright-field image of A549 and NB1RGB co-culture incubated with model DDS. B: Fluorescence image of A. The arrows and arrowheads indicate A549 and NB1RGB cells, respectively. Scale bars are 50 μm.*

SrtA-dependent transpeptidase reactions (Figure 1). The insertion of the lipopeptides in DPPC-Chol-lipo produced stable SrtA-reactive liposomes (Figure 2), although the efficiency of lipopeptide insertion into DPPC-Chol-lipo was slightly higher for AcLPs than for DoLPs (Table I). Interestingly, the His-ΔN_{59}SrtA transpeptidation efficiency was superior in DoLP-inserted DPPC-Chol-lipo compared to AcLP-inserted DPPC-Chol-lipo (Figure 3). In addition, no significant non-specific interactions with substrate peptides were detected with DoLP-inserted liposomes (Figure 3). These results suggest that DoLP-type lipopeptides are more desirable than AcLP-type lipopeptides for the preparation of surface-modified liposomes using the His-ΔN_{59}SrtA-mediated transpeptidase reaction. Furthermore, the results shown in Figure 3C with the background level of transpeptidation indicate that the spacer structure/length between the alkyl chain and SrtA recognition sequence is important in the design of lipopeptides. In contrast, the efficiencies of the transpeptidase reaction with AcLPs were very similar despite the differences in spacer structure (Figure 3A and B); greater efficiency of the transpeptidase reaction was observed with a DoLP containing a shorter spacer than with one containing a longer spacer (Figure 3D and E). These results suggest that the efficiency of the transpeptidase reaction mediated by His-ΔN_{59}SrtA on the lipopeptide-inserted DPPC-Chol-lipo depends on the steric position between the enzyme active center and the peptide moiety of the lipopeptide on the liposome surface. Thus, it is expected that further investigation into the spacer structure of the lipopeptide will lead to optimization of the His-ΔN_{59}SrtA-mediated transpeptidation reaction on the liposome surface.

As shown in Figure 4, our *in vitro* investigation revealed that the model DDS with a lung cancer cell-targeting moiety was selectively delivered to target A549 cells. To evaluate the utility of this liposome-labeling system, we adopted the aptameric peptide LTBP with affinity for lung-cancer cells as an example. However, this DDS platform will be made more versatile by selection of the targeting molecule/ligand (such as peptides and proteins, including a single-chain Fv antibody) essentially rendering this system a made-to-order DDS. Although the model DDS liposomal platform used in this *in vitro* study was AcLP-inserted DPPC-Chol-lipo, more efficient labeling might be achieved using DoLP-inserted DPPC-Chol-lipo and the labeling molecule consisting of the N-terminal GGGGG sequence. This labeling, performed in the opposite direction, is also of interest to evaluate the recognition efficiency of target cells because the exposed molecular surface of labeling molecules will differ from those made in the original orientation. Further investigations into the optimization of the lipopeptide structure and the His-ΔN_{59}SrtA transpeptidation conditions on the lipopeptide-inserted liposome are ongoing.

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