

# Investigation on the Reaction Conditions of *Staphylococcus aureus* Sortase A for Creating Surface-modified Liposomes as a Drug-delivery System Tool

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**Abstract.** *Background/Aim:* This study aimed to determine the preferred conditions for the transpeptidase reaction of sortase A from *Staphylococcus aureus*, for the purpose of creating functional liposomes useful for a drug-delivery system (DDS). *Materials and Methods:* His-tagged recombinant sortase A with 59 amino acids deleted from the N-terminus (His- $\Delta N_{59}$ SrtA) was prepared using an *Escherichia coli* expression system. The pH dependency and sorting signal sequence dependency of the transpeptidase reaction of His- $\Delta N_{59}$ SrtA were analyzed by monitoring the transfer of model donor-substrates (i.e. His-tagged mutant green fluorescent proteins with a C-terminal LPxTG sorting signal) to model acceptor-beads with a GGGGGC peptide. In addition, using preferred conditions, the sortase A reaction was used to modify liposome surface. *Results and Discussion:* The transpeptidase reaction of His- $\Delta N_{59}$ SrtA was enhanced under weakly acidic conditions. Transfer efficiency, based on sorting signal recognition by His- $\Delta N_{59}$ SrtA, was similar to or higher than that obtained using several substrates with amino acids other than Glu in the sorting signal position "x". Furthermore, liposomes containing GGGGGC peptide-linked dipalmitoylphosphatidylethanolamine were successfully modified using the preferred conditions for His- $\Delta N_{59}$ SrtA determined in this study. *Conclusion:* Preferred conditions for the transpeptidase reaction of His- $\Delta N_{59}$ SrtA, especially in a weakly acidic environment to enhance reaction, was

established and successfully used to create functional liposomes applicable to DDS.

Sortase A is a bacterial transpeptidase produced by Gram-positive bacteria. It functions to anchor bacterial cell-surface components such as pili and surface proteins to the cell wall (1-6). The typical sortase A produced by *Staphylococcus aureus* recognizes the LPxTG sorting signal (where "x" indicates any amino acid) of target proteins and cleaves a peptide bond between threonine and glycine in this motif. Subsequently, the cleaved protein is transferred to the amino group of the pentaglycine of the peptidoglycan chains.

The *S. aureus* sortase A has been characterized, and many applications using the transpeptidase reaction of sortase A have been investigated for the development of novel methods to modify various materials, including *in vivo* labeling of cultured cells (7-20). The transpeptidase reaction of sortase A has also been used to generate novel products/methods for drug-delivery systems (DDSs) (21-24). One example of the application of the transpeptidase reaction of sortase A to DDS is to modify liposomes with an enhanced green fluorescent protein (EGFP)-based donor substrate (25). However, although the transfer of an EGFP-based donor substrate to liposomes was successful, the reaction was time-consuming and it was thought that further optimization of the transpeptidase reaction conditions is needed to create functional materials effectively.

In the present study, we determined the preferred conditions for the transpeptidase reaction of recombinant sortase A with 59 amino acids deleted from the N-terminus and replaced with a His-tag (designated as His- $\Delta N_{59}$ SrtA). Subsequently, a system was constructed to evaluate the preferred sequence of the sorting signal and to determine the efficiency of the transpeptidase reaction of various sortase As, including His- $\Delta N_{59}$ SrtA, using mutant GFP-based donor substrates with a C-terminal LPxTG sorting signal and pentaglycine-linked beads. A trial to directly modify the

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*Key Words:* Sortase A, *Staphylococcus aureus*, drug-delivery system, liposome.

Table I. Primers used in this study.

No.	Name	Sequence (5'-3')*
1	Staph ΔN SrtA-Fw	CAAGCTAAACCTCAAATTCCGAAAG
2	Staph ΔN SrtA-Bw( <i>Pst</i> I)	CCC <u>TGCAGT</u> TATTGACTTCTGTAGCTAC
3	mutant GFP-Fw( <i>Bam</i> HI)	GGGGATCCATGAGTAAAGGAG
4	mutant GFP-Bw( <i>Sal</i> I)	CCGGT <u>CGACT</u> TTGTATAGTTC
5	LPETGG-Fw	TCGACTTACCGGAAACCGGTGGTA
6	LPETGG-Bw	AGCTTACCACCGGTTTCCGGTAAG
7	LPSTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTGGACGGTAAGTC
8	LPTTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTGGTCGGTAAGTC
9	LPGTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTGCCGGTAAGTC
10	LPATGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTGCCGGTAAGTC
11	LPDTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTATCCGGTAAGTC
12	LPKTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTTTTCCGGTAAGTC
13	LPRTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTGCCGGTAAGTC
14	LPHTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTATGCCGGTAAGTC
15	LPVTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTCCCGGTAAGTC
16	LPLTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTCCAGCGGTAAGTC
17	LPITGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTAATCCGGTAAGTC
18	LPFTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTAACCGGTAAGTC
19	LPYTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTATACGGTAAGTC
20	LPWTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTCCACGGTAAGTC
21	LPNTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTGTTCCGGTAAGTC
22	LPQTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTCTCCGGTAAGTC
23	LPMTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTATCCGGTAAGTC
24	LPCTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTGCACGGTAAGTC
25	LPPTGG-Bw	ATTA <u>AAGCTT</u> ACCACCGGTCCGGCGGTAAGTC

\*Restriction sites are underlined.

liposome surface using the His-ΔN<sub>59</sub>SrtA transpeptidase reaction under the preferred conditions determined in this study demonstrated that the method can serve as a functional tool for creating DDSs.

## Materials and Methods

*Expression and purification of His-ΔN<sub>59</sub>SrtA and His-mGFP-x.* Sortase A derived from *S. aureus* was prepared as the N-terminal His-tagged recombinant protein with 59 amino acids deleted from the N-terminal (26) and was designated as His-ΔN<sub>59</sub>SrtA. The expression vector for His-ΔN<sub>59</sub>SrtA was constructed based on the N-terminal His-tagged expression vector pQE-1 (Qiagen, Hilden, North Rhine-Westphalia, Germany) as follows: the insert encoding ΔN<sub>59</sub>SrtA with a *Pst*I site at the downstream end was prepared with polymerase chain reaction (PCR) performed using PrimeSTAR™ HS DNA polymerase (TaKaRa, Otsu, Shiga, Japan) and the primer sets Staph ΔN SrtA-Fw and Staph ΔN SrtA-Bw(*Pst*I) (Table I). A colony suspension of the *S. aureus* strain IFO 12732 was used as the template (27). The amplified fragment was digested with *Pst*I and the purified fragment was phosphorylated using T4 polynucleotide kinase (TOYOBO, Osaka, Osaka, Japan) and then ligated into pQE-1 and double-digested with *Pvu*II and *Pst*I using the Mighty Mix DNA ligation kit (TaKaRa, Otsu, Shiga, Japan). *Escherichia coli* JM109 was transformed with the constructed vector and transformants were selected with ampicillin. Sequencing of the expression vector purified from the selected clone was outsourced to

BEX Co. Ltd. (Itabashi, Tokyo, Japan). Expression of His-ΔN<sub>59</sub>SrtA induced by isopropyl-β-D-thiogalactopyranoside was confirmed by coomassie brilliant blue R-250 (CBB) staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the transformed cells. Subsequently, both expression and purification of His-ΔN<sub>59</sub>SrtA were conducted as described previously (28).

Mutant green fluorescent protein (mGFP)-based donor substrates for sortase A with a C-terminal LPxTGG sorting signal ("x" indicates any amino acid) were constructed based on the N-terminal His-tagged expression vector pQE-9 (Qiagen, Hilden, North Rhine-Westphalia, Germany). The expression vector of a model donor substrate with a LPETGG-type sorting signal (designated as pHis-mGFP-E) was constructed as follows: the insert encoding mGFP with a *Bam*HI site at the upstream end and a *Sal*I site at the downstream end was prepared by PCR. The PCR amplification was conducted using PrimeSTAR™ HS DNA polymerase (TaKaRa, Otsu, Shiga, Japan) with the primer sets mutant GFP-Fw(*Bam*HI) and mutant GFP-Bw(*Sal*I) (Table I). pGreen (29) was used as the template and the amplified fragment was digested by *Bam*HI and *Sal*I. The insert encoding the LPETGG-type sorting signal was prepared by a cassette ligation method (28) using two phosphorylated oligonucleotides (LPETGG-Fw and LPETGG-Bw). The resultant fragment had *Sal*I at the 5'-end and *Hind*III at the 3'-end. Two fragments prepared as above were inserted into the *Bam*HI- and *Hind*III-digested pQE-9 using the Mighty Mix DNA ligation kit (TaKaRa, Otsu, Shiga, Japan). *E. coli* JM109 was then transformed with the vector. After selection of transformants with ampicillin, sequencing of the recombinant vector purified from the selected clone was outsourced to BEX Co. Ltd. (Itabashi, Tokyo, Japan).

Expression vectors with other LPxTGG-type sorting signals were constructed as follows: the insert encoding mGFP with each LPxTGG sequence at the C-terminus was prepared by PCR performed using PrimeSTAR™ HS DNA polymerase (TaKaRa, Otsu, Shiga, Japan) and the primer sets mutant GFP-Fw (*Bam*HI) and LPxTGG-Bw (“x” indicates any amino acid) (Table I, no.7-25). Purified pHis-mGFP-E was used as the template and the amplified fragment was digested with *Bam*HI and *Hind*III. Each of these fragments was inserted into the *Bam*HI- and *Hind*III-digested pQE-9 using the Mighty Mix DNA ligation kit (TaKaRa, Otsu, Shiga, Japan). *E. coli* JM109 was then transformed with each vector. After selection of transformants with ampicillin, sequencing of the recombinant vectors purified from the selected clone was outsourced to BEX Co. Ltd. (Itabashi, Tokyo, Japan). Confirmation of expression and the purification of recombinant proteins were conducted as described above. To quantify the fluorescent intensity of the amount of purified His-mGFP-x, the fluorescence intensity of each His-mGFP-x was measured using a fluorescent microplate reader (Infinite M200; TECAN, Männedorf, Zürich, Switzerland).

*Time of flight-mass spectrometry (TOF-MS) analysis of His-ΔN<sub>59</sub>SrtA.* The exact molecular weight of His-ΔN<sub>59</sub>SrtA was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using an Autoflex (Bruker Daltonics Inc., Billerica, MA, USA) instrument. The purified fraction of His-ΔN<sub>59</sub>SrtA was desalted using ZipTip C18 (EMD Millipore, Billerica, MA, USA). A portion of the eluate was mixed with sinapic acid as a matrix and applied onto the sample plate. The measurement was conducted using a positive reflector mode.

*Preparation of acceptor beads with pentaglycine.* Polystyrene beads with amino groups on their surface (designated as amino beads; diameter of 3.00 μm, Polyscience, Warrington, PA, USA) were used for the transpeptidase reaction with His-ΔN<sub>59</sub>SrtA. An acceptor peptide GGGGGC (G<sub>5</sub>C, synthesized by GenScript, Piscataway, NJ, USA) was attached to the amino beads by a heterobifunctional linker *N*-(4-maleimidobutyryloxy)succinimide (GMBS; Dojindo, Kamimashiki, Kumamoto, Japan). Briefly, amino beads were reacted with 1 mM GMBS for 1 h at room temperature in 0.2 ml of 10 mM NaPi (pH 8.0) containing 1 mM EDTA. After discarding of the supernatant, the amino beads were incubated in 1 ml of 10 mM NaPi (pH 8.0) containing 10 mM glycine (to mask the remaining unreacted GMBS) for 0.5 h and washed six times with 1 ml of 10 mM NaPi (pH 7.0) containing 1 mM EDTA. The amino beads were suspended in 0.2 ml of 10 mM NaPi (pH 7.0) containing 1 mM EDTA and reacted with 50 μl of 10 μM G<sub>5</sub>C for 2 h at 37°C. After discarding the supernatant, the amino beads were resuspended in 0.2 ml of 10 mM NaPi (pH 7.0) containing 1 mM dithiothreitol (DTT) and further incubated for 0.5 h at room temperature. The resultant acceptor beads (G<sub>5</sub> beads) were washed six times with 1 ml of 10 mM imidazole-HCl containing 150 mM NaCl (imidazole-buffered saline, IBS; pH 6.5). Sodium azide, as a preservative, was then added to a final concentration of 0.1% (w/v) and the beads were stored 4°C until use. Prior to use, the G<sub>5</sub> beads were suspended in IBS (pH 6.5) containing 1% (w/v) bovine serum albumin (BSA) and washed three times with IBS (pH 6.5). The particle density of G<sub>5</sub> beads in suspension was estimated using a Guava PCA system (GE Healthcare, Fairfield, CT, USA).

*pH dependency of the transpeptidase reaction by His-ΔN<sub>59</sub>SrtA.* To investigate the pH dependency of the transpeptidase reaction by His-

ΔN<sub>59</sub>SrtA, the reaction was carried out in three different buffer systems: IBS (pH 5.5-8.5), 2-morpholinoethanesulfonic acid (MES)-buffered saline (10 mM MES, 150 mM NaCl, pH 5.5-7.0), or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.0, 7.5, and 8.5). Twenty microliters of the reaction mixture containing 5 μM His-ΔN<sub>59</sub>SrtA, 1 mM DTT, and 5 mM CaCl<sub>2</sub> in each buffer system was pre-incubated at 37°C for 15 min, mixed with 50 μl of 3 μM His-mGFP-E, and incubated at 37°C for 1 h in the dark. After addition of 150 μl of the G<sub>5</sub> beads suspension (ca. 4.4 × 10<sup>6</sup> particles), the mixture was incubated at 37°C for a further 2 h in the dark. The beads were then washed once with each of the buffered saline described above and twice with Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 9.0) to reduce nonspecific binding of His-mGFP-E to the G<sub>5</sub> beads. The washed beads were resuspended in 150 μl of TBS (pH 7.5) and diluted ten-fold with TBS (10 mM Tris, 150 mM NaCl, pH 7.5) before analysis with FACSVerse (Becton Dickinson, Franklin Lakes, NJ, USA; excitation at 488 nm and emission at 527 nm). The results of the FACSVerse analysis were evaluated using the FACSuite software (Becton Dickinson, Franklin Lakes, NJ, USA).

*Construction of an evaluation system for determining the preferred sorting signal sequence of His-ΔN<sub>59</sub>SrtA.* The construction of an evaluation system for determining the preferred amino acid at the “x” site in the sorting signal sequence of a donor substrate for His-ΔN<sub>59</sub>SrtA was conducted in the imidazole buffer system. The transpeptidase reaction conditions were according to the method described above except for the concentration of His-ΔN<sub>59</sub>SrtA, which was 3 μM. The results of the transpeptidase reaction have been displayed as a histogram with the fluorescent intensity on the horizontal axis and the forward scatter on the vertical axis. The median value for G<sub>5</sub> beads linked with His-mGFP-x was compared to that for the background control of the reaction mixture without His-ΔN<sub>59</sub>SrtA in order to evaluate the efficiency of transpeptidation.

*Modification of liposome surface by His-ΔN<sub>59</sub>SrtA in the imidazole buffer system.* Preparation of the biotinylated LPETGG peptide (designated Bio-LPETGG) involved dissolving 1.2 mM LPETGG (GenScript, Piscataway, NJ, USA) in 5 mM NaPi (pH 7.4) and then reacting this with 2 mM of *N*-succinimidyl D-biotin (Dojindo, Kamimashiki, Kumamoto, Japan) at 30°C for 1 h. The reaction was terminated by the addition of Tris-HCl (pH 9.0) to a final concentration of 2 mM. After centrifugation (20,600 × g, 5 min) of the reaction mixture, the supernatant was applied to a Jupiter Proteo column (Shimadzu GLC, Taito, Tokyo, Japan) equilibrated with running buffer [0.1% w/v trifluoroacetic acid (TFA)], and eluted with a linear gradient of acetonitrile using the running buffer and the elution buffer (95% v/v acetonitrile and 0.1% w/v TFA) in an AKTA purifier (GE Healthcare, Fairfield, CT, USA). The purified fraction was dried in a Savant Speedvac (SUC-100H; Savant Instruments, Farmingdale, NY, USA). The purified Bio-LPETGG was dissolved in sterilized Milli-Q water.

Liposomes with G<sub>5</sub>C on their surface (G<sub>5</sub> liposomes) were prepared as follows: 3.2 mM of L-α-phosphatidylethanolamine, dipalmitoyl (DPPE; Wako, Osaka, Osaka, Japan) and 4.0 mM GMBS were mixed in the reaction solvent (chloroform:anhydrous methanol, 5:3) and reacted overnight at room temperature. The remaining unreacted GMBS was absorbed by Amino-TOYOPEARL beads (TOSOH, Minato, Tokyo, Japan). The mixture was then centrifuged

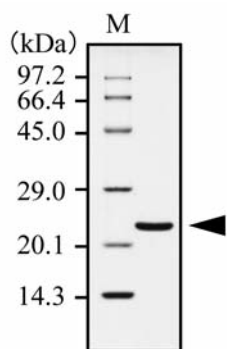


Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis image of coomassie brilliant blue R-250-stained purified recombinant sortase A with 59 amino acids deleted from the N-terminus and replaced with a His-tag (1  $\mu$ g/lane).

(20,600  $\times$  g, 10 min, 4°C) and the resultant supernatant (GMBS-PE) was used for the preparation of liposomes. One micromole of GMBS-PE and 10  $\mu$ mol of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; Avanti Polar Lipids, Alabaster, AL, USA) were dissolved in the reaction solvent, and liposomes were prepared as described previously (30) except for the use of IBS to prepare the liposome suspension (IBS-L; 20 mM imidazole, 140 mM NaCl, pH 6.5). Subsequently, 1.2  $\mu$ mol of G<sub>5</sub>C was added to the liposome suspension and incubated at 30°C for 2 h. Unreacted GMBS residue was blocked by incubation for 1 h with DTT at a final concentration of 1 mM. After ultracentrifugation of the reaction mixture at 40,000  $\times$  g for 30 min at 4°C (Optima TL; Beckman Coulter Inc., Brea, CA, USA), the supernatant was discarded and the pellet was washed with IBS-L, subjected to ultracentrifugation as above, and resuspended in IBS-L. Then, the liposomes were preincubated with 5  $\mu$ M His- $\Delta$ N<sub>59</sub>SrtA, 1 mM DTT, and 5 mM CaCl<sub>2</sub> in IBS-L at 37°C for 15 min. Bio-LPETGG (5  $\mu$ M) was then reacted with G<sub>5</sub> liposomes for 3 h at 37°C. The pellet was collected by ultracentrifugation (40,000  $\times$  g, 30 min, 4°C), washed twice with IBS-L using the same centrifugation conditions, and resuspended in IBS-L.

The transpeptidase reaction on the liposome surface was confirmed using an enzyme-linked streptavidin assay (ELSA). Briefly, 2  $\mu$ g avidin was added in 50  $\mu$ l of IBS to each well of an enzyme immunoassay plate (IWAKI; AGC Technoglass, Haibara, Shizuoka, Japan) and the mixture was incubated for 2 h at 37°C. The avidin solution was then discarded and the wells were treated with blocking solution (IBS-L with 1% w/v BSA) for 1 h at room temperature. After washing with IBS, the Bio-LPETGG-linked G<sub>5</sub> liposome suspension was added and the wells incubated for 2 h at room temperature. Unbound liposomes were discarded and the wells were washed with IBS-L. Subsequently, 50  $\mu$ l of diluted horseradish peroxidase (HRP)-labeled streptavidin (Vector Laboratories, Burlingame, CA, USA) was added to each well and incubated for 1 h at room temperature. Bound liposomes were detected by incubation with 50  $\mu$ l of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate solution [2 mM ABTS, 0.002% v/v H<sub>2</sub>O<sub>2</sub>, and 50 mM sodium phosphate (pH 4.5)] after removing the unreacted HRP-labeled streptavidin by washing with IBS-L. The HRP reaction was evaluated by measuring absorbance at 415 nm in a microplate reader (Model 550; Bio-Rad, Hercules, CA, USA).

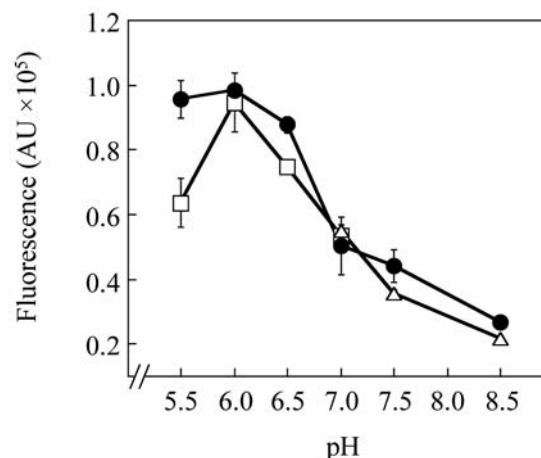


Figure 2. Transpeptidase reaction of recombinant sortase A with 59 amino acids deleted from the N-terminus and replaced with a His-tag (His- $\Delta$ N<sub>59</sub>SrtA) under weakly acidic conditions. The transpeptidase reaction of His- $\Delta$ N<sub>59</sub>SrtA was observed by measuring mutant green fluorescent protein (mGFP) fluorescence using a FACSVerse cell analyzer. The results show the transfer of His-tagged mGFP with a LPETGG-type sorting signal (His-mGFP-E) to G<sub>5</sub> beads under different pH. Closed circle, IBS (10 mM imidazole, 150 mM NaCl, pH 5.5-8.5); open square, MES-buffered saline (10 mM MES, 150 mM NaCl, pH 5.5-7.0); open triangle, HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.0, 7.5, and 8.5). Data are expressed as the mean  $\pm$  difference.

## Results

**Expression and purification of His- $\Delta$ N<sub>59</sub>SrtA.** His- $\Delta$ N<sub>59</sub>SrtA was purified by single-step Ni-affinity chromatography with a high yield; approximately 10 mg of His- $\Delta$ N<sub>59</sub>SrtA was obtained from 1 L of *E. coli* culture. The purity of the His- $\Delta$ N<sub>59</sub>SrtA was confirmed by CBB staining after SDS-PAGE (Figure 1). Reference markers from the CBB staining image allowed an estimated molecular weight of purified His- $\Delta$ N<sub>59</sub>SrtA of 22.0 kDa although the calculated molecular weight is 17.8 kDa. Therefore, MALDI TOF-MS was performed to determine the exact molecular weight of purified His- $\Delta$ N<sub>59</sub>SrtA. This analysis showed that the exact molecular weight of the purified His- $\Delta$ N<sub>59</sub>SrtA was 17.8 kDa.

**Expression and purification of His-mGFP-x.** All His-mGFP-x substrates were purified by single-step Ni-affinity chromatography. After SDS-PAGE, a single band was observed for all substrates by CBB staining (data not shown). Purified His-mGFP-x substrates indicated no significant difference in fluorescence intensity per amount of protein (F/protein) with each other.

**Evaluation of the effect of weakly acidic pH on the transpeptidase reaction of His- $\Delta$ N<sub>59</sub>SrtA.** The pH dependency of the transpeptidase reaction of His- $\Delta$ N<sub>59</sub>SrtA



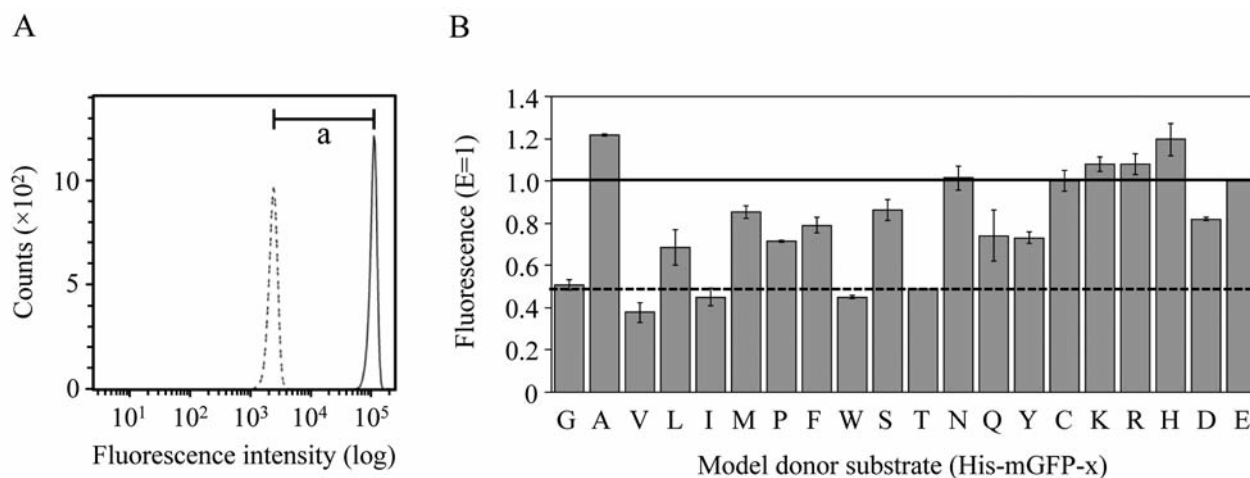


Figure 3. Evaluation of the preferred transpeptidase activity of recombinant sortase A with 59 amino acids deleted from the N-terminus and replaced with a His-tag (*His-ΔN<sub>59</sub>SrtA*). A: The histograms of the His-tagged mutant green fluorescent protein with a LPETGG-type sorting signal (*His-mGFP-E*) transferred onto *G<sub>5</sub>* beads and their background control were analyzed using a FACSVerse cell analyzer. A representative result for *His-mGFP-E* transpeptidation is shown. The difference between the median values of the histogram for the reaction mixture with and without *His-ΔN<sub>59</sub>SrtA* (shown as 'a') was calculated and used as the index of the transpeptidase reaction. B: The transpeptidase reaction of *His-ΔN<sub>59</sub>SrtA* with *His-mGFP-x* under preferred conditions. The result is shown as the ratio of the transferred *His-mGFP-x* to control *His-mGFP-E*.

was investigated using several buffer systems. Figure 2 shows that the transpeptidase reaction of *His-ΔN<sub>59</sub>SrtA* increased under weakly acidic conditions regardless of the buffer system. The greatest transpeptidase reaction was observed at pH 6.0 and was almost twofold higher than that observed under physiological conditions (pH 7.5). Furthermore, higher activity was observed in IBS than in the MES-buffered system. From these results, and considering the stability of the model donor substrates, the assay for the transpeptidase reaction of *His-ΔN<sub>59</sub>SrtA* was performed in 10 mM imidazole buffer (pH 6.5) containing 150 mM NaCl.

*Construction of an evaluation system for determining the optimal sorting signal sequence of His-ΔN<sub>59</sub>SrtA.* Using a peptide library, a previous study reported that sortase A derived from *S. aureus* exhibits substrate specificity (31). In order to determine the preferred condition for the transpeptidase reaction of *His-ΔN<sub>59</sub>SrtA* under weakly acidic conditions in IBS, further evaluation was conducted using *His-mGFP-x* substrates as donors and *G<sub>5</sub>* beads as acceptors. The activity of transpeptidase reaction was estimated by determining the difference between the median values of histograms for the reaction mixture with or without *His-ΔN<sub>59</sub>SrtA* (shown as "a" in Figure 3A). As shown in Figure 3B, the transpeptidase reaction of *His-ΔN<sub>59</sub>SrtA* varied depending on the amino acid in position "x" of the sorting signal sequence of *His-mGFP-x*. Similar or higher reactivity of *His-ΔN<sub>59</sub>SrtA* was observed with Ala-type (*His-mGFP-A*), Asn-type (*His-mGFP-N*), Lys-type (*His-mGFP-*

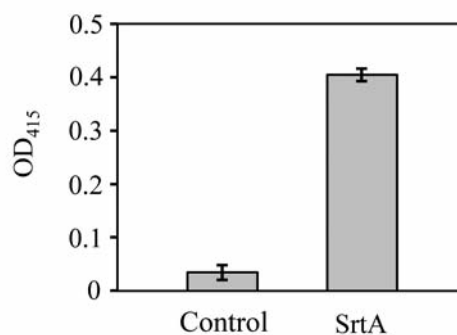


Figure 4. Surface modification of liposomes by recombinant sortase A with 59 amino acids deleted from the N-terminus and replaced with a His-tag (*His-ΔN<sub>59</sub>SrtA*) under the preferred condition. Biotinylated LPETGG peptide (*Bio-LPETGG*) was transferred onto the surface of *G<sub>5</sub>* liposomes by *His-ΔN<sub>59</sub>SrtA* at pH 6.5. The transferred *Bio-LPETGG* was detected by an enzyme-linked streptavidin assay using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as a chromogenic substrate after incubation with horseradish peroxidase-labeled streptavidin.

*K*), Arg-type (*His-mGFP-R*), and His-type (*His-mGFP-H*) model donor substrates, compared to the reactivity for the typical Glu-type (*His-mGFP-E*) substrate (Figure 3B). A Cys-type (*His-mGFP-C*) substrate had activity similar to that of the Glu-type substrate. However, a higher background was observed for the reaction with a Cys-type donor substrate. Interestingly, a Pro-type (*His-mGFP-P*) donor substrate that possesses a bend in the peptide backbone could also be used in the transpeptidase reaction.

*Modification of the liposome surface by His- $\Delta N_{59}$ SrtA.* To ascertain whether the preferred condition for the transpeptidase reaction of His- $\Delta N_{59}$ SrtA determined in the present study (*i.e.* weakly acidic in the IBS system) would also be suitable for the surface modification of liposomes, the transfer of Bio-LPETGG to G<sub>5</sub> liposomes was evaluated by ELISA. As shown in Figure 4, under these conditions, there was an observable His- $\Delta N_{59}$ SrtA-dependent transpeptidase reaction to Bio-LPETGG. This result revealed that the transpeptidase reaction of His- $\Delta N_{59}$ SrtA for the modification of the liposome surface could proceed even under weakly acidic conditions in IBS.

## Discussion

In order to use the transpeptidase reaction of His- $\Delta N_{59}$ SrtA to create a functional DDS tool, the preferred condition for the transpeptidase reaction of His- $\Delta N_{59}$ SrtA was determined. As shown in Figure 2, the transpeptidase reaction of His- $\Delta N_{59}$ SrtA was enhanced under weakly acidic conditions. The *in vitro* transpeptidase reaction of *S. aureus* sortase A is usually conducted at neutral pH. One previous report using a Tris-buffer system showed that the optimal range for the sortase A transpeptidase reaction was pH 6.8 to pH 9.0, with maximal activity being observed at pH 8.2 (32). In the present study, a weakly acidic condition was shown to be preferable for the transpeptidase reaction of His- $\Delta N_{59}$ SrtA in all of the tested buffer systems (imidazole, MES, and HEPES). This result suggests an original property of His- $\Delta N_{59}$ SrtA in the transpeptidase reaction (Figure 2).

It is notable that higher transpeptidase activity was seen below pH 6.5 in the imidazole-buffer system. A detailed mechanism for the transpeptidase reaction of *S. aureus* sortase A has been proposed to involve the amino acids Cys184, His120, and Arg197 as the active site residues of sortase A (33, 34). A recent study suggested that the transpeptidase reaction of sortase A involves a reverse protonation mechanism and that additional residue Thr183 is also essential for the catalytic reaction of sortase A transpeptidase (35). The exact reason why the transpeptidase reaction of His- $\Delta N_{59}$ SrtA was enhanced under weakly acidic conditions, particularly in IBS, is under consideration. However, the enhanced protonation of imidazole in IBS under weakly acidic conditions might facilitate the transpeptidase reaction of His- $\Delta N_{59}$ SrtA.

Evaluation of the sorting signal sequence-dependent transfer of model donor substrates by His- $\Delta N_{59}$ SrtA showed comparable or higher transpeptidase activity for the substrates with A (Ala), N (Asn), K (Lys), R (Arg), and H (His) in the “x” position than for the substrate with E (Glu) in this position (Figure 3B). These results suggest that the choice of the amino acid within the sorting signal sequence LPxTG, which is added to the C-terminal side of donor substrates used for the

modification reaction, is important for determining the efficiency of the transpeptidase reaction when using this reaction to create a functional tool for DDSs.

The optimum pH range for the transpeptidase reaction of His- $\Delta N_{59}$ SrtA, determined in this study, was further investigated to determine whether it was applicable for the modification of liposomal surfaces. Significant His- $\Delta N_{59}$ SrtA-dependent modification of the liposome surface was observed under a weakly acidic condition, that is, at pH 6.5 (Figure 4). This result indicates that weakly acidic conditions in IBS are useful for the modification of inanimate materials such as liposomes. However, another report has shown that modification of the liposome surface by the transpeptidase reaction of sortase A can occur at neutral pH (25). Hence, it may be necessary to compare the reaction efficiency at neutral and weakly acidic pH conditions using various combinations of donor and acceptor substrates before final conclusions are made about the preferred condition for His- $\Delta N_{59}$ SrtA reactions.

In the present study, we determined the preferred conditions for the transpeptidase reaction of His- $\Delta N_{59}$ SrtA and succeeded in the construction of surface-modified liposomes as a model DDS tool under this condition. Moreover, we established an evaluation system for the preferential sorting signal sequence by using a panel of His-mGFP-x donor substrates and acceptor beads. This system may be useful for assessing whether other sortase A from other organisms are more effective and stable than that from *S. aureus* and to improve the present modification system depending on future needs. Using the reaction conditions determined in the current study, investigations for the application of the sortase A transpeptidase reaction in the creation of a novel functional tool for DDSs are in progress.

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