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A New Method for Site-Specific Modification of Liposomes with Proteins Using Sortase A-Mediated Transpeptidation

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Abstract

A new method was developed for site-specific modifications of liposomes by proteins via sortase A (SrtA)-mediated transpeptidation reactions. In this regard, the enhanced green fluorescent protein (eGFP) was biologically engineered to carry at its polypeptide C-terminus the LPATG motif recognized by SrtA and used as the protein donor for linking to liposomes that were decorated with phospholipids carrying a diglycine motif as the other SrtA substrate and the eGFP acceptor. Under the influence of SrtA, eGFP was efficiently attached to liposomes, as proved by analyzing the enzymatic reaction products and the resultant fluorescent liposomes. It was observed that increasing the concentration and the distance of the diglycine motif on and from the liposome surface could significantly improve the efficiency of liposome modification by proteins. It is anticipated that this strategy can be widely useful for the modification of liposomes by other proteins.

Introduction

Liposomes have been widely used as simple models of cells or cell membranes in biological studies, as vehicles for targeted drug delivery, and so on, due to their structural versatility in terms of size, composition, and surface property.^{1, 2} In this context, numerous methods have been developed for liposome modification and functionalization.³ For example, bioactive molecules and other molecular tags were attached to the liposome surface by amine-carboxylic acid condensation reactions,⁴ disulfide⁵ or hydrazine⁶ formation reactions, thiol addition to maleimide,^{7–10} and amine-amine crosslinking.¹¹ However, an important issue about these coupling methods is that they are not regiospecific, especially when macromolecules such as proteins are involved, thus resulting in heterogeneous products and non-specific liposome modification. Moreover, biological and chemical compatibility is another potential concern. For instance, the presence of one protein on the liposome surface may affect the attachment of another protein by these coupling methods. To address these issues, several biologically compatible and chemically selective coupling methods, such as those based on “click” reaction,¹² the Staudinger ligation,^{13, 14} and native chemical ligation (NCL),^{15–17} have been developed. Although these coupling methods have been proved useful, their application to large proteins has been hindered by the difficult access to proper substrates and reactive intermediates. Consequently, more flexible and site-specific coupling methods are desirable for protein attachment to liposomes. For this purpose, we developed here a novel strategy based on sortase-catalyzed transpeptidation.

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Supporting Information: Data of the dynamic light scattering analysis of liposomes and NMR and MS spectra of all synthetic intermediates and target molecules. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

Sortases are a family of transpeptidases found in gram-positive bacteria¹⁸ and responsible for anchoring surface proteins to the bacterial cell wall via the so-called “sorting reaction”.¹⁹ Sortase A (SrtA) of *Staphylococcus aureus* origin is a typical member of the sortase family, which recognizes a unique pentapeptide LPXTG, where X is variable, at the upstream of the C-terminal domain of target proteins, cleaves the amide bond between T and G to form a reactive thioester linkage with T, and finally transfers the carboxylic group of T to substrate peptidoglycans carrying an *N*-terminal glycine to give the transpeptidation products (Scheme 1A).²⁰ It has been demonstrated that SrtA is rather substrate promiscuous and has been used to mediate the coupling of proteins to a variety of amino nucleophiles.^{21–24} Accordingly, we envisioned that SrtA may be utilized to site-specifically attach proteins to liposomes, which are equipped with glycine residues, in the presence of other proteins or biomolecules, as outlined in Scheme 1B. As such, proteins to be attached to liposomes need to carry the LPXTG motif near their *C*-termini, which can be very easily achieved via gene engineering and recombinant protein techniques. In this work, we have demonstrated the feasibility of the new strategy for liposome functionalization by preparing modified liposomes bearing glycine residues, expressing proteins carrying the *C*-terminal LPXTG motif, and attaching the recombinant proteins to the modified liposomes by SrtA.

Experimental Procedures

General Methods

NMR spectra were recorded on a 400 MHz spectrometer with chemical shifts reported in ppm (δ) in reference to SiMe₄ if not specified otherwise. Coupling constants (*J*) are reported in hertz (Hz). High-resolution (HR) ESI MS was performed on a Micromass Autospec Ultima magnetic sector mass spectrometer or on Waters GCT time-of-flight mass spectrometers. MALDI-TOF MS was obtained on a Bruker Ultraflex mass spectrometer. Thin layer chromatography (TLC) was performed on silica gel GF254 plates detected by charring with phosphomolybdic acid in EtOH solution. Commercial anhydrous solvents and other reagents were used without further purification. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(poly(ethylene glycol))2000] (NH₂-PEG-DSPE), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), and cholesterol were purchased from Avanti Polar Lipids, Inc, USA.

***N*-[*tert*-Butyloxycarbonyl-L-glycinyl-L-glycinyl] 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (5)**—After 2-(*N*-*tert*-butyloxycarbonyl-L-glycinyl-L-glycinylamino ethyl) phosphonate **4** (200 mg, 0.426 mmol) and 1,2-distearoyl-*sn*-glycerol **4** (293 mg, 0.469 mmol) were co-evaporated with dry pyridine three time and dried at high vacuum for 2 h, the mixture was dissolved in dry pyridine (3 mL). A solution of pivaloyl chloride (54 μ L, 0.426 mmol) in pyridine (2 mL) was added to the solution at rt under N₂. The reaction was monitored by TLC until it was finished (5 h). The reaction mixture was cooled to 0 °C, and a solution of I₂ (108.2 mg, 0.426 mmol) in 1.1 mL of pyridine and water (10:1) was added. The reaction was completed in 3 h and quenched by addition of saturated aqueous Na₂S₂O₃ solution. The mixture was extracted with chloroform three times, and the organic phase was combined, dried, and concentrated. The residue was purified by a silica gel column to give **5** (254 mg, 62%). ¹H NMR (CDCl₃:CD₃OD 3:1, 400 MHz): δ 4.99 (s, 1H), 4.17 (m, 1H), 3.92 (m, 1H), 3.73 (s, 5H), 3.63 (s, 2H), 3.23 (s, 2H), 3.13 (m, 1H), 2.96 (q, CH₂ of Et₃N, 1.35 H), 2.08 (m, 4H), 1.38 (s, 4H), 1.24 (s, 9H), 1.16 (t, CH₃ of Et₃N, 3.5H), 1.10 (m, 60H), 0.66 (t, *J* = 6.4Hz, 6H); ¹³C NMR (CDCl₃:CD₃OD 3:1, 100 MHz): δ 173.7, 173.3, 79.9, 70.2, 62.4, 45.7, 34.0, 33.9, 31.7, 29.5–28.9(m), 28.0, 24.7, 24.6, 22.5, 13.8, 8.2; ³¹P NMR (CDCl₃:CD₃OD 3:1, 161 Hz): δ –3.42; HRMS (negative mode): calcd for C₅₀H₉₅N₃O₁₂P, 960.6653, found, 960.6653.

N-[L-Glyciny]L-glyciny] 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (NH₂-Gly-Gly-DSPE) (1)—Compound **5** (50 mg, 0.052 mmol) was dissolved in a mixture of TFA and DCM (1:1, 2 mL) containing 20 μ L of triethylsilane. The reaction mixture was stirred at rt for 2 h. Then, solvents were removed under reduced pressure, and the crude product was washed with diethyl ether to afford **1** (40.3 mg, 90%). ¹H NMR (CDCl₃:CD₃OD 3:1, 400 MHz): δ 5.02 (s, 1H), 3.96 (m, 1H), 3.76-3.70 (broad, 5H), 3.54 (broad s, 2H), 3.22 (broad s, 2H), 3.16 (s, 1H), 2.98 (q, CH₂ of Et₃N, 1.22 H), 2.11 (m, 4H), 1.40 (s, 4H), 1.13-1.01 (m, 60H), 0.68 (t, *J* = 6.4 Hz, 6H); ¹³C NMR (CDCl₃:CD₃OD 3:1, 100 MHz): δ 173.8, 173.4, 70.1, 63.5, 62.7, 62.3, 46.0, 41.2, 40.5, 34.0, 33.8, 31.7, 29.5-28.9(m), 24.7, 24.6, 22.4, 13.7, 8.2; ³¹P NMR (CDCl₃:CD₃OD 3:1, 161 Hz): δ 0.733; MALDI-TOF MS: calcd for C₄₅H₈₈N₃O₁₀P, 861.6; found, 862.4 (M+H⁺); HR MS (negative mode): calcd for C₄₅H₈₇N₃O₁₀P, 860.6129; found, 860.6130.

N-[L-Glyciny]L-glyciny]L-amino(poly(ethylene glycol)) 2000] 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (NH₂-Gly-Gly-PEG2000-DSPE) (2)—After NH₂-PEG2000-DSPE (**7**) (100 mg, 35.8 μ mol) was dissolved in DCM (5 mL) under an atmosphere of argon, BocNH-Gly-Gly-OSu (**6**, 24 mg, 71.67 μ mol) and triethylamine (10 μ L, 71 μ mol) were added to the solution. The reaction was stirring at rt overnight. The solution was concentrated under reduced pressure, and the crude product was purified by silica gel column chromatography to give **8** (106 mg, ~100%). Compound **8** (106 mg, 35.1 μ mol) was dissolved in a mixture of TFA and DCM (2 mL, 1:1) containing 20 μ L of triethylsilane, and the reaction mixture was stirred at rt for 2 h. Then, solvents were removed, and the crude product was washed with diethyl ether to afford **2** (86 mg) as a mixture with a molecular weight range of 2,665–3,369 (see Supporting Information).

Protein expression and purification

Fluorescent protein vector pcDNA3-EmGFP was purchased from Invitrogen Company. Primer pairs FF-NdeI (5'-GATATTCCATATGGTGAGCAAGGGCGA-3') and RF-XhoI (5'-CTCGAGGCCACCGGTCGCCGGCAGCTTGTACAGCTCGTCCAT-3) were used to PCR amplify EmGFP from the pcDNA3-EmGFP. RF-XhoI primer contains the SrtA-recognized sorting signal sequence LPATG. Both the PCR product and pET23a were excised with NdeI and XhoI. After purification with 1% agarose gel purification kit (Invitrogen), the products were ligated together with T4 DNA ligase overnight at 4 °C, and then transformed into DH5 α host strain and selected on LB-ampicillin agar plates. DNAs were sequenced to prove their identity. Positive clones were finally picked up for protein expression. EmGFP with SrtA-signal was expressed in BL21-DE3 *E. coli* (Invitrogen). *E. coli* cultures were allowed to reach an OD₆₀₀ of 0.7 before induction of expression by the addition of 1 mM of isopropyl thiogluconate (IPTG, Fisher). After further incubation at 37 °C for 3 h, the cultures were pelleted by centrifugation. B-per solution (Pierce) was added to the thawed pellet to solubilize the cells, and the manufacturer's protocol was followed closely to isolate soluble protein. Protein purification using the His₆ affinity tag and a Ni-NTA resin column followed the procedure outlined by Qiagen. Protein-enriched fractions were combined and dialyzed against Tris buffer (50 mM, pH = 8) containing 150 mM of NaCl, using a Slide-A-Lyzer dialysis cassette (Pierce) with a MWCO of 10 kDa for 48–60 h with buffer changed at least three times. The calculated MW of enhanced green fluorescent protein (eGFP) is 28.760 kD that was observed with MALDI-TOF MS.

Liposome Preparation

A mixture of DSPC (15 mg, 20.4 μ mol), cholesterol (4 mg, 10.2 μ mol), and **1** (0.35 mg, 0.408 μ mol) or **2** (1.19 mg, 0.408 μ mol) (2% of total lipids) were dissolved in 3.0 mL of chloroform and methanol (1:1). Solvents were removed on an evaporator under reduced pressure to form a thin lipid film on the flask wall, which was kept in a high vacuum

overnight. The lipid film was swelled in 2.0 mL of Tris buffer (50 mM, pH = 7.4) to form a multilamellar vesicle suspension that was immersed in a 65 °C water bath for 1 h. The lipid suspension thus formed was extruded through a polycarbonate membrane (pore size 100 nm) to afford small unilamellar vesicles (SUVs), which was concentrated 4 times to give liposomes for ligation reactions. SUVs containing 5% of **1** (0.88 mg, 1.02 μmol) or **2** (2.98 mg, 1.02 μmol) were prepared by the same protocol.

Ligation of eGFP to functionalized-liposomes

Liposomes (42 μL) prepared above were mixed with 15 μL of 2 M Tris-HCl buffer (pH = 7.5) containing 10 μL of NaCl (1.5 M), 1 μL of 0.5 M CaCl₂, 2 μL of 0.1 M mercaptoethanol, and 10 μL of 1 mM eGFP. Then, 20 μL of SrtA (100 μM) was added. The solution was incubated at 37 °C overnight, followed by ultracentrifugation at 300,000 g and 15 °C for 1 h. The supernatant was removed, and the pellet containing liposomes was suspended in 100 μL of Tris-HCl buffer. This process was repeated twice. The pellet was then diluted to 100 μL with Tris-HCl buffer and finally analyzed with SDS-PAGE and a fluorometer. The fluorescence intensity of eGFP-modified liposomes was measured using an exciting wavelength of 360 nm.

RESULTS AND DISCUSSION

In this study, enhanced green fluorescent protein (eGFP) was employed as a model to probe the new liposome functionalization strategy outlined in Scheme 1B. eGFP as a protein donor was designed to contain a LPATG motif near its C-terminus. Furthermore, a histidine affinity tag (His₆), which is commonly used to facilitate the purification of recombinant proteins by a Ni²⁺ column, was linked to the LPATG motif. This design would assure the removal of the His₆ tag upon enzymatic coupling reactions (Scheme 2). eGFP-LPATG-His₆ was prepared by the conventional recombinant protein technique using *Escherichia coli* (*E. coli*), as described in detail in the experimental section.

We designed two lipid molecules as nucleophilic substrates of SrtA, which were incorporated in the liposome for SrtA-mediated site-specific transesterification reactions. One has a diglycine motif directly linked to the amino group of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) (**1**, Scheme 2), and the other has the diglycine motif attached to polyethylene glycol (PEG)-modified DSPE (**2**). Compounds **1** and **2** differ in that their diglycine motifs have different distances from the liposome surface. The comparison of their reactions would disclose how the bulky liposome might affect SrtA-mediated transesterification. We selected to incorporate a diglycine in these nucleophiles because previous studies showed that a single glycine is sufficient for the enzymatic reaction though a diglycine motif may be a slightly better substrate for SrtA.^{25, 26}

The synthesis of compounds **1** and **2** is outlined in Scheme 3. Phosphorylation of lipid **3** by freshly prepared **4** was achieved in a two-step one-pot manner to get phospholipid **5** in a moderate yield (62%), which was finally deprotected with 50% trifluoroacetic acid (TFA) in dichloromethane (DCM) to give the desired product **1** in an excellent yield (90%). Compound **2** was easily prepared from commercially available PEG2000-modified DSPE **7** in two separate steps, including coupling reaction with the activated ester of diglycine **6** in the presence of triethylamine and deprotection with 50% TFA in DCM. Both **1** and **2** were characterized by NMR and MS.

Compounds **1** and **2** were used to prepare small unilamellar liposomes (SULs) by the extrusion method.²⁷ In brief, after DSPC and cholesterol (2:1 molar ratio) were mixed with **1** or **2** in a specific concentration (2% or 5% molar ratio of total lipids) in chloroform and methanol (1:1), the solvents were removed under reduced pressure to form a thin lipid film

on the flask wall. The lipid film was then swelled in Tris buffer in a 65 °C water bath to result in a suspension containing multilamellar vesicles that were extruded through a polycarbonate membrane (pore size: 100 nm) to form SULs. Dynamic light scattering analysis of the SULs revealed that they had an average diameter of about 110–130 nm with a rather narrow dispersion range (see Supporting Information).

The SULs obtained above were then employed to explore liposome modification by proteins via SrtA-mediated ligation reactions. In this context, SULs were mixed with eGFP-LPATG-His₆ in buffer followed by addition of SrtA, and the reaction mixture was incubated at 37 °C overnight. To verify the ligation reaction and assess the reaction efficiency, SULs were separated from SrtA and unreacted eGFP-LPATG-His₆ *via* ultracentrifugation, followed by washing with phosphate buffered saline (PBS), as described by Mulder and coworkers.¹⁵ The eGFP-modified liposomes, as well as the ligation product, were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorescence imaging analyses.

Shown in Figure 1 are the results of ligation reactions between eGFP-LPATG-His₆ and SULs containing 2% of **1**. SDS-PAGE analysis revealed that, after washed with PBS once, the SUL pellet was essentially free of soluble proteins (Figure 1A, lane 2). Nonetheless, PBS washing was repeated two to three times to ensure that SrtA and unreacted eGFP substrate were completely removed. The resultant SULs still showed a fluorescent protein band (Figure 1A, lane 5) with molecular weight consistent with that of the desired ligation product. However, the difference of the molecular weights of the substrate protein and the conjugate product is only 566 Da, thus they are difficult to distinguish by SDS-PAGE. Additional experimental proofs for the formation of the desired product are: (1) the band shown in lane 5 (Figure 1) was not observed in the control experiment, where SrtA was absent (data not shown), suggesting that the substrate protein did not adhere to SULs and was completely removed by washing and that the observed protein must have been covalently attached to SULs; and (2) the results of lipid **2** (as discussed in detail later) clearly showed that after washing SULs as described herein no substrate protein remained in the SUL pellet. Moreover, we observed that the isolated SULs showed strong fluorescence under a microscope (Figure 1B). All the results supported that eGFP was indeed attached to liposomes via SrtA-mediated ligation.

The ligation reactions between eGFP-LPATG-His₆ and liposomes containing 2% of **2** under the same conditions gave results (Figure 2) similar to that of **1**. Again, after washed with PBS once, the SUL pellet was basically free of soluble proteins (Figure 2A, lane 1). The washed SUL pellet showed a fluorescent protein band (Figure 2A, lane 3) that had the molecular weight in agreement with that of the desired ligation product (~30,217 Da) and significantly larger than that of SrtA (17,865 Da, lane 5) and eGFP-LPATG-His₆ (28,760 Da, lane 4). The molecular weight difference (~1,457 Da) between the ligation product of **2**, that is, eGFP-PEG2000-DSPE, and substrate protein eGFP-LPATG-His₆ was much larger than the difference (566 Da) between the ligation product of **1**, eGFP-DSPE, and the substrate protein, thus the product band was well separated from the substrate band in Figure 2A. Clearly, no substrate protein was detected in the SUL pellet (lane 3), proving that all soluble proteins were washed away and that the substrate protein itself did adhere to SULs. The isolated SULs were also observed to exhibit strong fluorescence under a microscope (Figure 2B). These results have unambiguously proved that eGFP was indeed covalently attached to **2** on liposomes in the presence of SrtA.

After having established the validity of the new strategy for liposome modification, we then assessed the efficiency of the ligation reaction based on the unique property of eGFP, namely that it emits fluorescence at 535 nm with an excitation wavelength of 360 nm. As

shown in Table 1, for liposomes containing 2% of the diglycine-lipid conjugates **1** and **2**, the fluorescent intensity of eGFP-modified liposomes of **2** was about 33% stronger than that of eGFP-modified liposomes of **1**, suggesting higher modification efficiency of **2** than that of **1** on the liposome surface under the given conditions.

We also compared the reactivity of liposomes containing different concentrations of **1** and **2**, and the results are outlined in Table 1. Clearly, eGFP-modified liposomes carrying 5% of **1** and **2** had significantly stronger fluorescent intensities than eGFP-modified liposomes carrying 2% of **1** and **2**, indicating that the former had more eGFP molecules attached to their surface. For example, the fluorescent intensity of eGFP-modified liposomes carrying 5% of **1** was *ca.* 50% higher than that of liposomes carrying 2% of **1**, whereas the fluorescent intensity of eGFP-modified liposomes carrying 5% of **2** was *ca.* 400% higher than that of liposomes carrying 2% of **2**.

These results suggest that both the concentration and the structure of the functionalized lipids used to decorate liposomes had a significant influence on their modification efficiencies. As expected, liposomes containing a higher concentration (5%) of substrate lipids **1** and **2** were more effectively modified with eGFP than liposomes containing a lower concentration (2%) of **1** and **2**. It was also observed that under the same conditions liposomes of **2** were much more effectively modified than liposomes of **1**. The latter observation may be the result of steric effect. The presence of a flexible PEG fragment in **2** may have extended the distance of the lipid diglycine motif from the liposomal surface so as to significantly reduce its steric hindrance, increase its accessibility, and improve the SrtA-mediated reactions, when compared to **1** that has the diglycine residue directly anchored onto the liposome surface.

In summary, we have demonstrated that SrtA can be utilized to efficiently modify liposomes via site-specific reactions between an eGFP substrate carrying the sorting signal LPXTG and liposomes carrying functionalized lipid substrates with a diglycine motif. To the best of our knowledge, this is the first report of using SrtA to site-specifically modify liposomes. This approach should be a useful alternative to other strategies for the preparation of functionalized liposomes and is expected to be widely applicable to the attachment of other proteins to liposomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. SDS PAGE and fluorescence imaging analyses of liposomes decorated with 2% of **1** upon reacting with eGFP-LPATG-His₆ and SrtA. (A) SDS PAGE results: Lane 1, molecular weight markers; Lane 2 and Lane 3: PBS solution obtained from the second and first washing of liposomes, respectively; Lane 4: supernatant of the reaction mixture; Lane 5: liposome pellet after 2 times of washing with PBS; Lane 6: standard eGFP substrate (eGFP-LPATG-His₆); Lane 7: SrtA. (B) Fluorescence imaging result of the eGFP-decorated SULs.

Figure 2.

SDS PAGE and fluorescence imaging analyses of liposomes decorated with 2% of **2** upon reacting with eGFP-LPATG-His₆ and SrtA. (A) SDS PAGE results: Lane 1, PBS solution obtained from the first washing of liposomes; Lane 2, supernatant of the reaction mixture; Lane 3, liposome pellet after 2 times of washing with PBS; Lane 4: standard eGFP substrate (eGFP-LPATG-His₆); Lane 5: SrtA; Lane 6: molecular weight markers. (B) Fluorescence imaging result of the eGFP-decorated SULs.

(A) SrtA-mediated anchorage of surface proteins onto the bacterial cell wall peptidoglycans **(B) Proposed site-specific attachment of proteins to liposomes**

Scheme 1.

Scheme 3.

Table 1 Fluorescence intensities of eGFP-modified liposomes containing 2% and 5% of **1** and **2**

UMLs carrying different concentrations of 1 or 2	Fluorescence intensities of eGFP-modified liposomes ^a
UML with 2% of 1	7007
UML with 2% of 2	9325
UML with 5% of 1	10464
UML with 5% of 2	46681

^a fluorescence intensity was the average of triplicate experiments