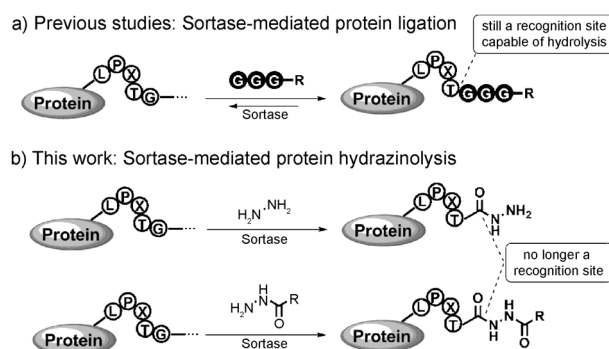


Irreversible Site-Specific Hydrazinolysis of Proteins by Use of Sortase**

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Abstract: Sortase-mediated hydrazinolysis of proteins with hydrazine or its derivatives was developed for the production of recombinant protein hydrazides. This process provides an alternative approach for protein semisynthesis through the use of recombinant protein hydrazides as thioester surrogates. It also provides an alternative method for C-terminal modification of proteins with functional units as well as for the preparation of C-to-C fusion proteins.

Protein modification with a either reporter group, an immobilization tag, or a synthetic polymer is an enabling tool for studies of protein biophysics and pharmaceuticals.^[1] Although many bioconjugation reactions have been developed for this purpose,^[2] chemoenzymatic protein modification often shows higher efficiency.^[3] One emerging chemoenzymatic process is sortase-mediated protein ligation, which can be used to modify proteins bearing a short recognition sequence (usually LPXTG; Scheme 1 a).^[4] The active-site Cys of sortase cleaves between LPXT and G to produce a thioester intermediate, which reacts with a nucleophile containing one to five Gly residues to afford the ligation product. Up to now the sortase-mediated ligation has been successfully applied to many fields such as protein lipidation^[5] and PEGylation,^[6] protein immobilization,^[7] histone semisynthesis,^[8] and others.^[9] Efforts have also been made to extend its scope (e.g. transpeptidation with oligoglycine thioesters)^[10] and to overcome its constraints (reaction reversibility and hydrolysis).^[11]



Scheme 1. Sortase-mediated protein modification reactions.

Herein we report a sortase-mediated protein hydrazinolysis reaction with hydrazine and derivatives (Scheme 1 b).^[12,13] This reaction proceeds with site specificity and high yields under mild reaction conditions. Its product (a protein hydrazide) is no longer a substrate of the transpeptidase and therefore, sortase-mediated hydrazinolysis is irreversible and exhibits less hydrolysis. Consistent with our previous studies,^[14] protein hydrazides produced by the sortase-mediated reaction are useful reagents for the semisynthesis of functionally active proteins. By using hydrazine derivatives as nucleophiles, we show that the sortase-mediated process can also be used for protein C-terminal modification as well as preparation of chimeric proteins. Thus the new hydrazinolysis process extends the concept and usefulness of sortase-mediated protein ligation.

We started with sortase A (SrtA)-mediated hydrazinolysis of the model peptide QALPETGEE (100 μ M). Under initial reaction conditions (NH_2NH_2 0.2 M, SrtA 10 μ M) the hydrazinolysis proceeded smoothly to afford QALPET-NHNH₂ in an hour (Figure 1 a). The yield, as determined by HPLC, was greater than 95 % whereas the hydrolysis product was not detected (Table 1, entry 1). When 0.05 M NH_2NH_2 was used, the reaction needed 3 hours to reach a greater than 95 % yield (entry 2). When 0.5 M NH_2NH_2 was used the yield was greater than 95 % after 1 hour (entry 3). Decrease in the loading of SrtA to 1 μ M reduced the reaction efficiency to less than 5 % product as observed after 5 hours (entry 4). Ca^{2+} was beneficial but not essential to the reaction because without addition of Ca^{2+} the hydrazinolysis reaction took 5 hours to reach greater than 95 % yield (entry 5). Besides the canonical sequence (LPXTG), LPKAG and LPAAG could also be used as the hydrazinolysis sites (entries 6–7). Collectively, our results showed that SrtA-mediated hydrazinolysis is an enzyme-dependent transformation with high efficiency.

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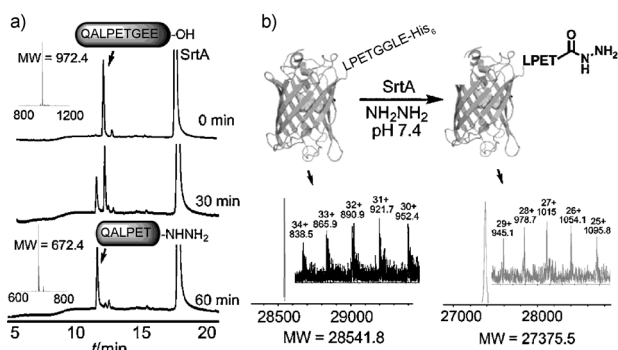


Figure 1. a) HPLC traces for SrtA-mediated hydrazinolysis of QALPETGEE for $t=0$, 30, and 60 min. Reaction conditions: 100 μM QALPETGEE, 0.2 M NH_2NH_2 , 10 μM SrtA, 10 mM CaCl_2 , 50 mM Tris, 150 mM NaCl, pH 7.4. b) SrtA-mediated hydrazinolysis of eGFP bearing a C-terminal LPETGGLE sequence. ESI-MS mass for eGFP-LPETGGLE is 28541.8 Da (calc. 28541.1). ESI-MS mass for eGFP-LPET-NHNH₂ is 27375.5 Da (calc. 27375.9).

Table 1: Sortase-A-mediated hydrazinolysis reaction.^[a]

$\text{QALPETGEE} \xrightarrow[\text{pH 7.4, RT}]{\text{SrtA hydrazine}} \text{QALPET-NHNH}_2$				
Entry	Hydrazine conc. [M]	Enzyme conc. [μM]	t [h]	Yield [%]
1	0.2	10	1.0	> 95
2	0.05	10	3.0	> 95
3	0.5	10	1.0	> 95
4	0.2	1	5.0	< 5
5 ^[b]	0.2	10	5.0	> 95
6 ^[c]	0.2	10	6.0	93
7 ^[d]	0.2	10	6.0	> 95

[a] All reactions were carried out in aqueous buffer containing 50 mM Tris, 150 mM NaCl, 10 mM CaCl_2 , pH 7.4. Yield was determined by HPLC. [b] No CaCl_2 was added. [c] Peptide sequence: QALPKAGGE. [d] Peptide sequence: QALPAAGG.

To test SrtA-mediated hydrazinolysis of proteins, we expressed enhanced green fluorescent protein (eGFP) bearing a C-terminal LPETGGLE-His₆ sequence (Figure 1b). When this protein (20 μM) was treated with NH_2NH_2 (0.2 M) and SrtA (20 μM) at pH 7.4, we observed a complete conversion of the starting material after 3 hours. The product was purified by HPLC (yield of isolated product: 64%) and determined by ESI-MS mass to be the desired hydrazide (eGFP-LPET-NHNH₂, Figure 1b). Thus the SrtA-mediated hydrazinolysis reaction can be used to prepare recombinant protein hydrazides with high efficiency and operational simplicity.

An application of the above process is to site-specifically modify the C termini or linker regions of proteins in a modular manner by the use of sortase-mediated ligation and native chemical ligation. To test this idea we examined the model peptide LALPETGE, which was converted into LALPET-NHNH₂ smoothly after treatment with SrtA and NH_2NH_2 (Figure 2a). LALPET-NHNH₂ was activated with NaNO_2 at pH 3 and then ligated with Cys at pH 7 in the presence of excess 4-mercaptophenylacetic acid (MPAA). The final

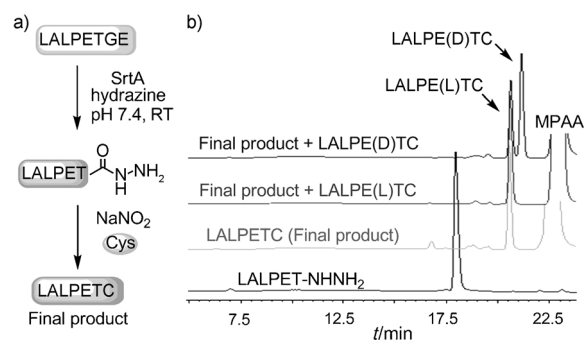


Figure 2. a) Sequential sortase-mediated ligation and native chemical ligation. b) HPLC traces after SrtA-mediated hydrazinolysis, crude reaction mixture of final ligation, and final product co-injected with either LALPE(L)TC or LALPE(D)TC.

product was obtained in a nearly 100% HPLC yield (Figure 2b). Through co-injection analysis with substrates containing either L- or D-Thr (Figure 2b), we confirmed that both SrtA-mediated hydrazinolysis and hydrazide-based ligation were epimerization free.

To apply the method to a protein, we semisynthesized deoxy-D-ribose 5-phosphate aldolase (DERA).^[15] This protein (259 amino acids) has a structured C-terminal sequence (α -helix, 18 amino acids) which is connected to the protein by a flexible loop peptide suitable for the insertion of the SrtA recognition sequence (Figure 3a). Thus we expressed DERA-(1-239)-LPAAGG-His₆ and treated it (30 μM) with NH_2NH_2 (0.2 M) and SrtA (20 μM ; Figure 3b). Hydrazinolysis afforded DERA-(1-239)-LPAA-NHNH₂ in an almost quantitative HPLC yield (yield of isolated product: 58%). No hydrolysis was observed in this transformation (Figure 2b). DERA-(1-239)-LPAA-NHNH₂ was then ligated with CLKALGHGDGKSASSY by using the NaNO_2 /MPAA protocols (Figure 3c). This ligation afforded the final DERA after 2 hours with a yield of 46%. Semisynthetic DERA is different from wild-type (WT) DERA by four loop-region amino acids [DERA(241-244) was mutated from LASL to PAAC].

DERA was folded by successive dialysis.^[16] According to circular dichroism (CD; Figure 3d), semisynthetic DERA folded to a well-defined structure similar to that of WT DERA. To measure the enzyme activity of DERA in catalyzing the retro-aldol reaction of D-2-deoxyribose-5-phosphate (DR5P) to produce acetaldehyde and D-glycerinaldehyde-3-phosphate (G3P),^[15b] we employed the coupled assay using α -glycerophosphate dehydrogenase (α -GPD) and triose-phosphate isomerase (TPI).^[15b] In this assay G3P was reduced by GPD/TPI, thus causing consumption of NADH, which could be monitored at $\lambda = 340 \text{ nm}$ ($\epsilon = 6220 \text{ M}^{-1} \text{ cm}^{-1}$). The rates of DERA-catalyzed retro-aldol reaction were measured with a fixed amount of enzyme against different concentrations of DR5P. The Michaelis-Menten k_{cat} ($0.065 \pm 0.005 \text{ s}^{-1}$) and K_{M} ($3.68 \pm 0.3 \text{ mM}$) constants of semisynthetic DERA were obtained, and were comparable to k_{cat} ($0.056 \pm 0.002 \text{ s}^{-1}$) and K_{M} ($4.69 \pm 0.5 \text{ mM}$) of WT DERA. Thus semisynthetic DERA prepared by sequential sortase-mediated ligation and native chemical ligation was functionally active.

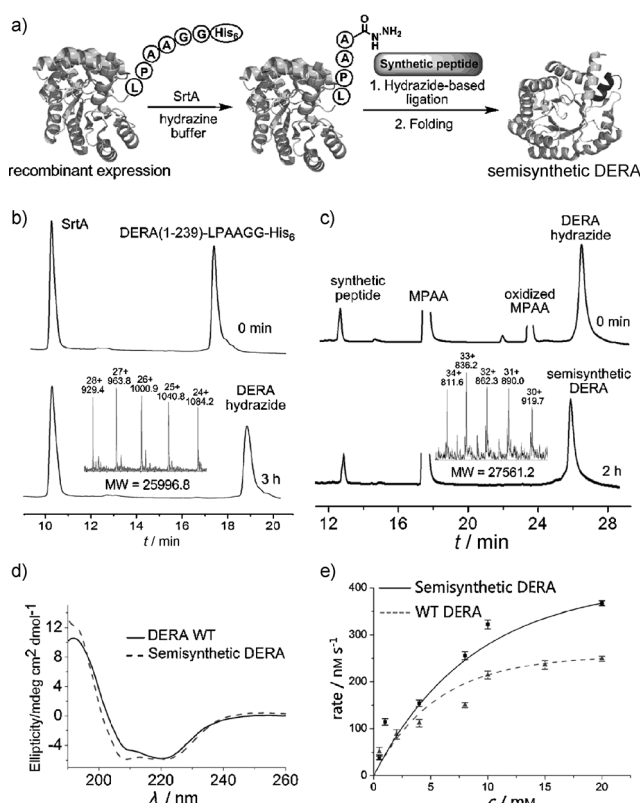


Figure 3. a) Sequential ligations for semisynthesis of DERA. b) HPLC traces for SrtA-mediated hydrazinolysis of DERA(1-239)-LPAAGG-His₆ at $t=0$ and 3 h. Observed ESI-MS mass for DERA(1-239)-LPAAGG-His₆ is 25996.8 Da (calc. 25998.1 Da). c) HPLC traces for hydrazide ligation. Observed ESI-MS mass for semisynthetic DERA is 27561.2 Da (calc. 27560.5 Da). d) CD spectrum of semisynthetic DERA as compared with that of recombinant WT DERA. e) Enzyme activities of semisynthetic DERA and WT DERA. The retro-aldol reaction was performed at 25 °C in 50 mM Tris buffer (pH 7.4) with 0.5–20 mM DR5P, 2U GPD/TPI, 0.3 mM NADH and 5 μ M DERA.

In addition to the use of hydrazides for protein semisynthesis, protein hydrazides have been shown to be useful for protein C-terminal modifications by bioconjugation with aldehydes or ketones.^[13b,c] Compared to the intein-based approach of generating protein hydrazides, an advantage of the sortase approach is the need for a much smaller tag (five amino acids versus an intein domain) at the POI (protein of interest).^[4c,17] To further expand the scope of the method, we tested SrtA-mediated hydrazinolysis reactions with hydrazine derivatives (Figure 4a). We expressed ubiquitin tagged with the SrtA recognition motif [i.e. Ub(1-76)-LPETG-His₆]. This protein (30 μ M) was treated with 5–10 mM NH₂NHCOR (R = functional unit) and SrtA (20 μ M) at pH 7.4 (Figure 4b). After 2–3 hours at room temperature, the starting materials were completely converted. The yields for the isolated Ub-alkyne and Ub-azide were both 45 %.

The above transformation enabled the attachment of a small molecule to the POI as an anchor for further modifications, for example, with polyethylene glycol (PEG) to improve the properties of the POI.^[18] Indeed, when Ub-azide was treated with DBCO-PEG-5000, we obtained the

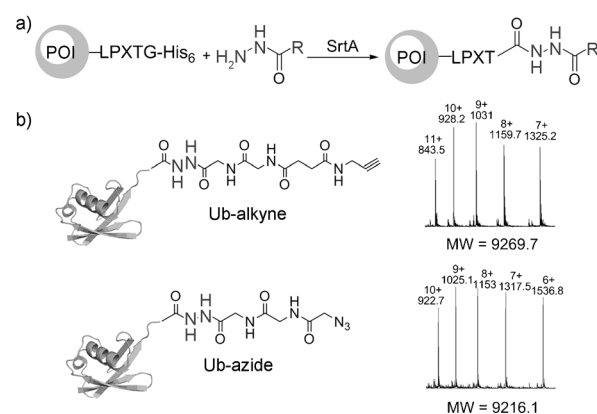


Figure 4. a) SrtA-mediated hydrazinolysis of proteins with hydrazine derivatives. b) Modified ubiquitins produced by the SrtA approach. Observed ESI-MS masses for Ub-alkyne and Ub-azide are 9269.7 Da (calc. 9270.3 Da) and 9216.1 Da (calc. 9216.3 Da), respectively.

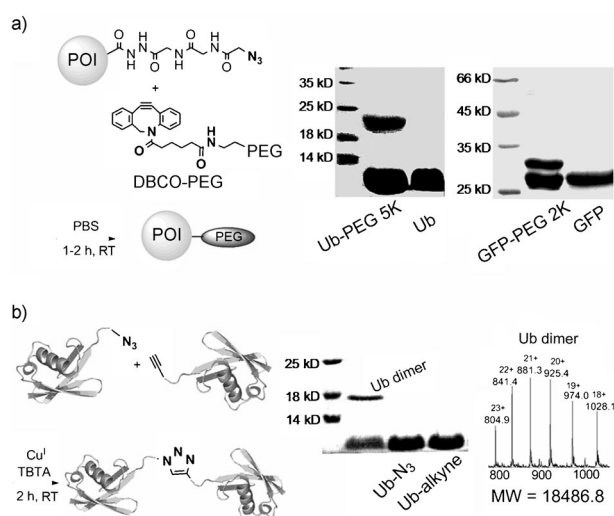


Figure 5. a) SDS-PAGE analysis of the PEGylation reactions of POI (POI = Ub or GFP) by strain-promoted azide-alkyne cycloaddition. b) SDS-PAGE analysis of the Ub dimer made between Ub-azide and Ub-alkyne by copper-induced azide-alkyne cycloaddition. Observed ESI-MS mass for Ub dimer is 18486.8 Da (calc. 18486.6 Da).

desired PEGylated protein (Figure 5a). Consistent with the previous studies,^[19] we found that this strain-promoted azide-alkyne cycloaddition process^[20] could not fully PEGylate the POI despite the addition of excess PEGylation reagents. Moreover, we could convert GFP-LPETGGG-His₆ into GFP-azide. GFP-azide was then PEGylated by treatment with DBCO-PEG2000.

The facile access to Ub-azide and Ub-alkyne also allowed us to prepare a C-to-C fused Ub dimer (Figure 5b). When Ub-azide and Ub-alkyne were reacted with each other,^[21] the desired product was formed in about 40–50 % yield according to SDS-PAGE. Compared to the N-to-C and C-to-N fused recombinant proteins, nonnaturally linked C-to-C chimeric proteins were recently developed as interesting biological tools with potential therapeutic applications.^[22] The SrtA-

medicated hydrazinolysis reaction provided an alternative approach for the preparation of C-to-C fusion proteins.

Finally, to use the above method to make a practically useful protein, we prepared an anti-EGFR (epidermal growth factor receptor) nanobody labeled with fluorescein at the C terminus. This nanobody is a small (15 kD), stable, and well-soluble intact antigen-specific binding fragment isolated from camilidae.^[23] We expressed an anti-EGFR nanobody with the SrtA recognition motif [i.e. nanobody(1-142)-LPETG-His₆]. This protein was reacted with a hydrazide derivative of fluorescein through SrtA-mediated hydrazinolysis (Figure 6a), thus affording the labeled anti-EGFR

was not affected by the C-terminal labeling through the SrtA-mediated hydrazinolysis method. Thus our method was usable for making modified proteins with potential biomedical applications.

To conclude, we have reported a new sortase-mediated hydrazinolysis reaction of proteins. This process offers a practical enzymatic approach for the preparation of protein hydrazides with high yields and operational simplicity. Protein hydrazides not only are useful reagents for the hydrazone-type bioconjugation reactions, but also can be used as thioester surrogates for the semichemical synthesis of proteins through native chemical ligation. Furthermore, hydrazine derivatives can also be used in the sortase-mediated hydrazinolysis process. This process provided an alternative practical approach for the C-terminal modification of proteins with various functional units as well as for the preparation of nonnatural C-to-C fusion proteins.

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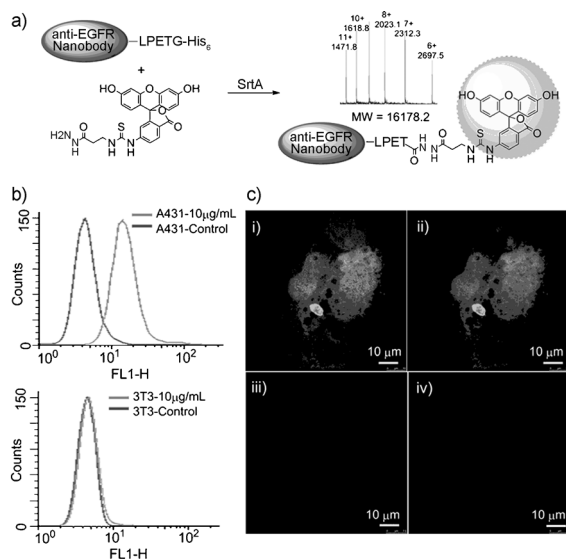


Figure 6. a) Fluorescent labeling of anti-EGFR nanobody by SrtA-mediated hydrazinolysis. Observed ESI-MS mass for fluorescein-labeled anti-EGFR nanobody is 16178.2 Da (calc. 16178.5 Da). b) FACS analysis of the cell line expressing EGFR (A431) and EGFR-negative cell line (3T3) stained with fluorescein-labeled anti-EGFR nanobody. c) Confocal laser scanning microscopy analysis of cell line expressing EGFR (A431, i and ii) and the EGFR-negative cell line (3T3, iii and iv) with fluorescein-labeled anti-EGFR nanobody. The cell nuclei were stained with DAPI (4,6-diamidino-2-phenylindole). Cells were visualized by Leica confocal microscope in the fluorescein channel (ii and iv). They were merged with the images from the DAPI channels to produce i and iii. For colored pictures, see the Supporting Information. Scale bar = 10 μ m.

nanobody in 35% yield upon isolation. It was used (at 10 μ g mL⁻¹) to stain both an EGFR-positive human epidermoid carcinoma cell line A431 and an EGFR-negative cell line 3T3 at 37 °C for 30 minutes. FACS (fluorescence-activated cell sorting) analysis indicated that in reference to the negative control, a significant shift of the fluorescence intensity was observed for the EGFR-positive cells (Figure 6b). This observation was consistent with the confocal microscopic imaging experiments on the binding of fluorescein-labeled anti-EGFR nanobody to both A431 and 3T3 cells (Figure 6c). Both the FACS and confocal microscopic imaging experiments confirmed the specific affinity of the fluorescein-labeled anti-EGFR nanobody to A431 cells. They also indicated that the specificity of the anti-EGFR nanobody

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