

Protein Modification

Flow-Based Enzymatic Ligation by Sortase A**

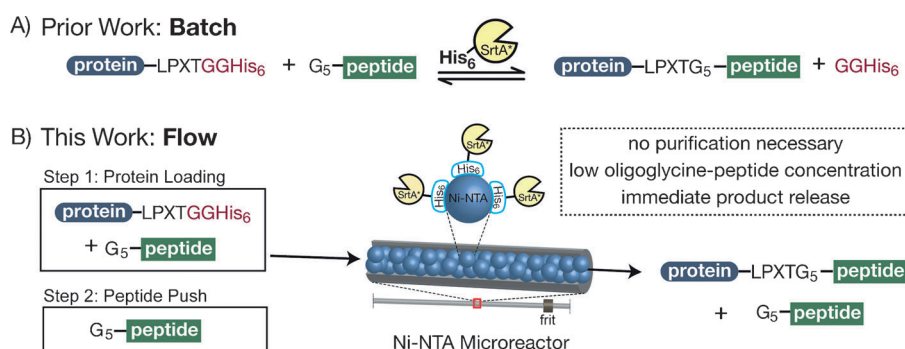
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Abstract: Sortase-mediated ligation (sortagging) is a versatile, powerful strategy for protein modification. Because the sortase reaction reaches equilibrium, a large excess of polyglycine nucleophile is often employed to drive the reaction forward and suppress sortase-mediated side reactions. A flow-based sortagging platform employing immobilized sortase A within a microreactor was developed that permits efficient sortagging at low nucleophile concentrations. The platform was tested with several reaction partners and used to generate a protein bioconjugate inaccessible by solution-phase batch sortagging.

Adantages conferred by flow-based methodology have rapidly accelerated the development of flow technology in biological and chemical research over the past decade. Ease of process intensification, limited exposure to dangerous reagents, and higher reaction yields are among the multitude of reasons flow chemistry has become more common.^[1] One class of flow-based platforms relies upon immobilized enzymes to convert substrates to products in a continuous manner. However, many of the flow-based enzymatic transformations reported to date focus on the conversion of small molecule substrates.^[2] In this work, we expand the scope of flow-based enzymatic reactions to include sortase A-mediated ligation (sortagging). The reported flow-based sortagging platform has granted access to a synthetic

bioconjugate inaccessible by standard solution-phase sortagging techniques.

C-terminal sortagging is a versatile strategy in protein engineering, allowing the site-specific conjugation of a peptide or protein substrate bearing a C-terminal LPXTG recognition motif to another substrate containing an N-terminal polyglycine nucleophile (Scheme 1a).^[3] Sortase A,^[4] a calcium-dependent cysteine transpeptidase derived from *Staphylococcus aureus*, site-specifically cleaves the threonine-glycine bond of LPXTG to generate a sortase-thioester intermediate



Scheme 1. a) Traditional batch mode sortagging reaction between a substrate containing a C-terminal LPXTGG-His₆ motif and a nucleophile containing five N-terminal glycine residues. The reaction reaches equilibrium so large excesses of glycine nucleophile are often used. b) Flow-based immobilized enzyme sortagging reaction. A “load” solution of polyglycine nucleophile and protein-LPXTGG-His₆ is first flowed through the reactor. Unreacted protein-LPXTGG-His₆ binds the microreactor column while transpeptidation products are released. A peptide “push” solution is used to drive the sortagging reaction toward product formation.

resolved by an N-terminal polyglycine nucleophile. Sortagging methodology plays a vital role in the synthesis of complex bioconjugates with important structural and biological properties. Lipids,^[5] fluorophores,^[6] affinity tags,^[6] reactive handles,^[7] sugars and GPI anchors,^[8] polymer initiators,^[9] peptide nucleic acids,^[10] and thioesters^[11] have all been successfully conjugated. Methods such as surface modification^[12] and protein immobilization,^[13] protein^[14] and peptide^[15] cyclization, N–N and C–C protein fusion synthesis,^[16] chemoenzymatic protein synthesis,^[17] CRECS strategies (combined recombinant, enzymatic, and chemical synthesis),^[18] combined protein purification and modification,^[19] site-specific antibody labeling,^[20] functionalization of PEGylated capsules,^[21] intracellular^[22] and cell-surface labeling,^[23] protein-dendrimer formation,^[24] sortase-mediated hydrazinolysis,^[25] and production of receptor-directed chimeras,^[26] among others,^[27] have all utilized sortase A-mediated ligation.

In the sortagging reaction the expelled leaving group can also serve as a nucleophile, attacking the sortase-thioester intermediate and driving the reaction in the reverse direction.

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[**] We are indebted to Alexander Vinogradov, Chi Zhang, Jingjing Ling, Surin Mong, Tatiana Berger, and Richard Chang for technical assistance and helpful discussions throughout the course of this work. We thank R. John Collier for providing some of the laboratory equipment used to perform this study. We are grateful to the MIT UROP program for providing funding to R.L.P. and H.K. and the NSF GRFP for A.E.R. We thank the MIT startup fund, the MIT S.O.S. Reed Fund, the Damon Runyon Cancer Research Foundation Award, the Sontag Foundation Distinguished Scientist Award, and the MIT Deshpande Innovation Grant for B.L.P.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201403582>.

Thus, the sortase reaction reaches an equilibrium dependent on the initial concentration of the polyglycine nucleophile. As a result, sortagging protocols often call for high concentrations of polyglycine nucleophile (0.5–2 mM) to drive the sortagging equilibrium toward product formation and achieve reasonable reaction yields.^[28] If the concentration of the desired nucleophile is too low, both water and lysine side chains become competitive nucleophiles, intercepting the acyl-enzyme intermediate and yielding hydrolyzed C-terminal LPXT proteins, intramolecular cycles, or cross-linked protein dimers (Supporting Information, Figure S1). The proportion of each of these sortagging side products is both protein specific and pH sensitive. Furthermore, side products cannot be suppressed by maintaining a large stoichiometric ratio of nucleophile to substrate in very dilute reactions. It is often challenging or impossible to separate the desired construct from reaction byproducts with standard techniques (if desired products and byproducts are of similar molecular weight). While sortagging is undoubtedly a powerful bioconjugation tool, its practical utility is often limited by achievable concentrations of the reaction partners. To overcome some of these challenges, researchers have used depsipeptides,^[29] diketopiperazine precursor peptides,^[30] detergents,^[5] and elevated reaction temperatures^[31] to drive product formation. A thorough study of sortagging side reactions demonstrated that 25 μ M oligoglycine and 10 μ M antibody-Fab-fragment-LPETGG can be successfully sortagged with wild-type sortase A, but 16 hour reaction times and elevated temperatures were required and small amounts of hydrolysis byproducts were observed.^[20a] To the best of our knowledge, successful sortagging with lower nucleophile concentration has not previously been reported.

In this work we describe a general strategy for efficient sortagging with dilute nucleophiles by using a flow-based immobilized enzyme microreactor. Because the sortagging reaction reaches equilibrium, we hypothesized that a flow-based sortagging platform would allow us to sortag at much lower nucleophile concentrations by: 1) immediately releasing the transpeptidation product from the microreactor to minimize the reverse reaction, 2) supplying a fixed concentration of nucleophile, and 3) limiting contact time with sortase A to decrease side reactions such as hydrolysis and dimerization. We rely upon an evolved version of sortase A (SrtA*) that has been shown to have faster reaction kinetics than the wild-type variant.^[32] Here we demonstrate that sortagging performed under flow conditions proceeds with high efficiency, yielding the desired transpeptidation product in less than thirty minutes with 10 μ M G_5 -nucleophile concentrations. This flow-based method allowed us to generate a protein bioconjugate that was inaccessible by batch model sortagging because of a poor side reaction profile.

In order to perform flow-based sortagging we first assembled the platform outlined in Scheme 1b. A microreactor was constructed by packing a small length of tubing with Ni-NTA agarose resin pre-loaded with 10 μ g His₆-SrtA* (Figure S4–S6). With this system in hand, we performed a model sortase-catalyzed transpeptidation of protein substrate **1** (eGFP) and model peptide **2** under flow conditions (Figure 1a). Biotinylated peptide **2** (Figure 1b) was used so

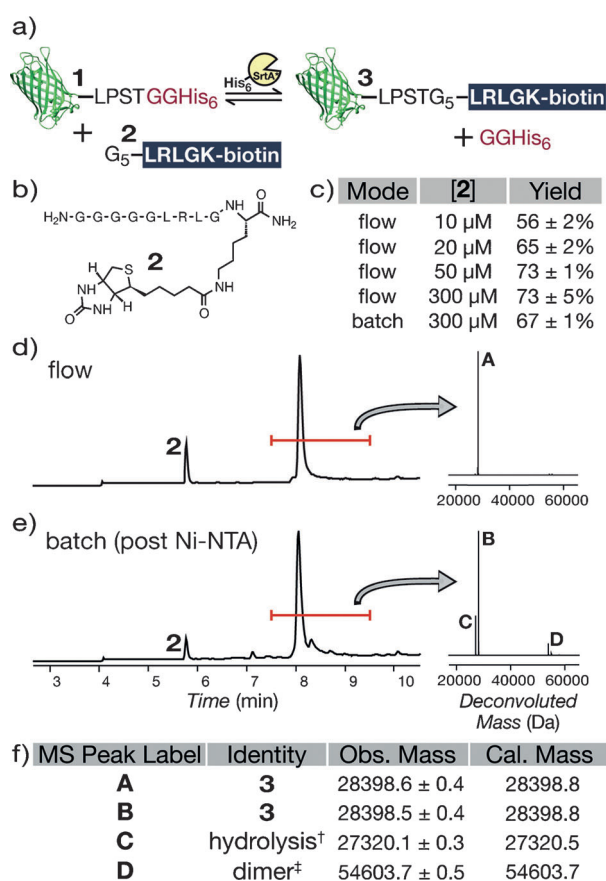


Figure 1. a) Sortagging reaction between protein substrate **1** and pentaglycine nucleophile **2**. b) Structure of **2**. c) Isolated reaction yields of model flow reactions at several nucleophile concentrations and a “best case” batch reaction (see Supporting Information). Yield values represent isolated yields quantified by UV absorbance at 280 nm. Percentages in table represent the average of three independent reactions and are shown as mean $\pm$ standard deviation (s.d.). d) LCMS trace (TIC; total ion current) and deconvoluted mass spectrum (t_{decon} = 7.5–9.5 min, red bar) of reactor effluent for model flow reaction between **1** and **2**. Reaction conditions: Step 1 (load): 7 μ M **1** and 10 μ M **2** in 800 μ L of sortase flow buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl₂, 15 mM imidazole, pH 7.5) was passed through the SrtA* microreactor at 65 μ L min^{−1} by syringe pump. Step 2 (push): 1 mL of 10 μ M **2** in sortase flow buffer was passed through the microreactor at 65 μ L min^{−1} by syringe pump. e) LCMS trace and deconvoluted mass spectrum (t_{decon} = 7.5–9.5 min) of model batch reaction after Ni-NTA pull-down. Reaction conditions: 5 μ M **1**, 10 μ M **2**, 3.5 μ M SrtA*, sortase buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl₂, pH 7.5), t_{rxn} = 30 min. At t = 20 min, 3.33 μ L of buffer exchanged Ni-NTA resin per μ g protein **1** was added and reaction was allowed to proceed for another 10 min. f) Mass spectrometry (MS) data for insets in panels (d) and (e). Protein masses calculated with ExPASy or ApE. †: Sortase-mediated hydrolysis of **1** or **3** (Figure S1). ‡: Sortase-mediated dimerization of **1** or **3** (Figure S1). Observed mass (obs. mass) and calculated mass (cal. mass) are both expressed in Daltons (Da).

that formation of the desired bioconjugate could be confirmed by Western blot (Figure S8) in addition to high-resolution LCMS analysis. His₆ affinity tags on SrtA* and **1** ensured that both proteins remained resin bound; only upon effective transpeptidation was the desired ligation product **3** released from the microreactor. To perform the flow-based

sortagging reaction, 800 μL of protein “load” solution (7 μM **1**, 10 μM **2**) in sortase flow buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl_2 , 15 mM imidazole, pH 7.5) was first flowed through the microreactor at 65 $\mu\text{L min}^{-1}$. A “push” solution was then passed through the microreactor at 65 $\mu\text{L min}^{-1}$ to drive the reaction toward completion. This flow-based reaction gave high purity ligation product in 56% isolated yield (Figure 1 d, S7). After flow-based sortagging with 10 μM **2** proved successful, we explored ligation efficiency at different nucleophile concentrations. Flow-based sortagging readily proceeded at nucleophile concentrations as low as 10 μM under flow conditions, and ligation yields increased with increasing nucleophile concentration (Figure 1 c, Table S4). A “best case” batch reaction between 50 μM **1** and 300 μM **2** (3.5 μM SrtA*, sortase buffer: 50 mM Tris, 150 mM NaCl, 10 mM CaCl_2 , pH 7.5) produced the desired bioconjugate **3** in 67% isolated yield, very similar to flow reactor performance with 20 μM **2** (65% isolated, Figure 1 c, Table S7).

Based on our results that 10 μM G_5 -nucleophile was sufficient for flow-based sortagging, we sought to determine if sortagging with 10 μM **2** could be performed in a batch mode reaction. Specifically, we performed model batch sortagging reactions between **1** and **2** (Figure 1 a) and analyzed the side reaction profile for each by high resolution LCMS (Table 1). These studies revealed significant amounts of hydrolysis byproducts in the batch reactions, even when a stoichiometric excess of peptide **2** to protein **1** was maintained (Figure 1 e, Table 1). Because hydrolysis is a sortase-mediated process, we

Table 1: Side reaction profile for model reactions between **1** and **2** evaluated by LCMS in both batch and flow mode.^[a]

Mode	[SrtA*] [μM]	[1] [μM]	[2] [μM]	Product ^[b] [%]	Hydrolysis and cyclization ^[c] [%]	Dimer ^[d] [%]
Batch	3.5	10	10	36 $\pm$ 1.1	25 $\pm$ 1.5	7.1 $\pm$ 2.4
Batch	3.5	5	10	56 $\pm$ 2.2	22 $\pm$ 3.1	2.0 $\pm$ 0.6
Batch	3.5	2.5	10	65 $\pm$ 1.0	19 $\pm$ 0.7	1.2 $\pm$ 0.2
Batch	0.35	2.5	10	31 $\pm$ 1.3	1.1 $\pm$ 0.4	n. d. ^[e]
Flow	[f]	[g]	10	[h]	1.5 $\pm$ 0.1	4.1 $\pm$ 1.2
Flow	[f]	[g]	20	[h]	0.8 $\pm$ 0.1	3.5 $\pm$ 0.5

[a] Batch reaction conditions: substrate and SrtA* concentrations specified in table, sortase buffer, pH 7.5, 30 min. Flow conditions: Step 1 (load): 7 μM **1** and 10 or 20 μM **2** in 800 μL of sortase flow buffer was passed through the SrtA* microreactor at 65 $\mu\text{L min}^{-1}$ by syringe pump. Step 2 (push): 1 mL of 10 μM **2** in sortase flow buffer was passed through the microreactor at 65 $\mu\text{L min}^{-1}$ by syringe pump. All percentages in table represent the average of three independent reactions and are shown as mean $\pm$ s.d. [b] Percent product expressed as ratio of product deconvoluted peak height ($\text{DPH}_{\text{product}}$) to sum of $\text{DPH}_{\text{starting material}}$, $\text{DPH}_{\text{product}}$, $\text{DPH}_{\text{hydrolysis \& cyclization}}$ and $\text{DPH}_{\text{dimer}}$. [c] Percent hydrolysis & cyclization expressed as ratio of $\text{DPH}_{\text{hydrolysis \& cyclization}}$ to $\text{DPH}_{\text{product}}$. [d] Percent dimer expressed as ratio of $\text{DPH}_{\text{dimer}}$ to $\text{DPH}_{\text{product}}$. [e] None detected. [f] The sortase flow reactor was pre-loaded with 10 μg SrtA* on 50 μL of buffer exchanged Ni-NTA resin containing 50% Ni-NTA beads by volume. [g] The concentration of **1** in the “load” fraction was 7 μM . [h] The method of calculating percent product via MS peak height ratios is only applicable to batch mode reactions because the flow reactor sequesters His₆-tagged starting material. Isolated flow yields are presented in Figure 1 c. Percent hydrolysis & cyclization and percent dimer are reported relative to amount of product formed to allow for cross comparison between batch and flow mode reactions.

explored lowering the SrtA* concentration 10-fold (to 0.35 μM) in an attempt to mitigate this side reaction. While side reactions were effectively suppressed, the reaction mixture contained ca. 68% unreacted starting material. Flow reactions employing 10 μM **2** exhibited much lower levels of hydrolysis and cyclization byproducts relative to batch reactions run with 10 μM **2** and 3.5 μM SrtA*. Increasing the concentration of **2** to 20 μM in flow mode both improved the overall isolated yield and diminished side reactions (Figure 1 c, Table 1).

To confirm that our observations were neither protein nor peptide specific, we explored the sortagging reaction between the N-terminal domain of anthrax toxin lethal factor (LF_N ; **4**) and another model peptide, G_5LRL (**5**; Figure 2 a). Using 10 μM **5**, high-purity ligation product **6** was obtained in 39% isolated yield (Figure 2 b). The comparable batch reaction between **4** and **5** was contaminated with significant amounts of hydrolysis and cyclization byproducts (Figure 2 c).

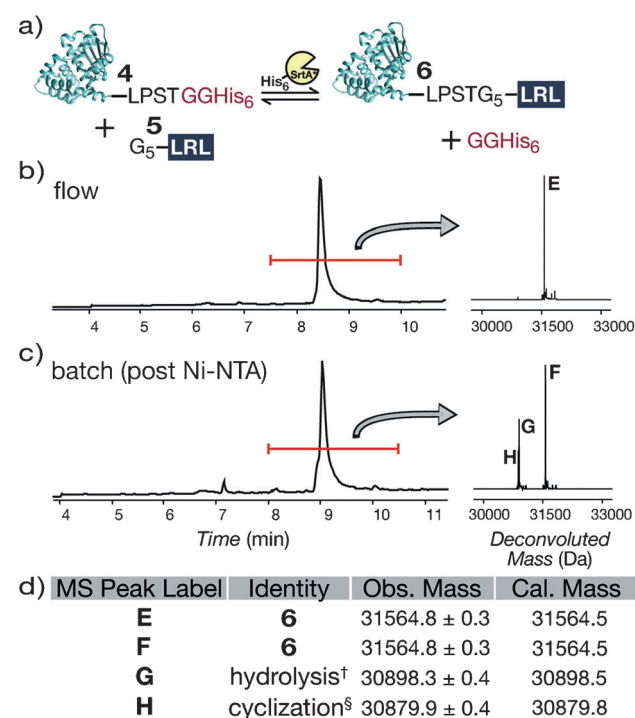


Figure 2. a) Sortagging reaction between protein substrate **4** and pentaglycine nucleophile **5**. b) LCMS trace (TIC) and deconvoluted mass spectrum of flow-based sortagging reaction between **4** and **5** ($t_{\text{decon}} = 7.5\text{--}10.0$ min). Reaction conditions: Step 1 (load): 7 μM **4** and 10 μM **5** in 800 μL of sortase flow buffer (pH 7.8) was passed through the SrtA* microreactor at 65 $\mu\text{L min}^{-1}$ by syringe pump. Step 2 (push): 1 mL of 10 μM **5** in sortase flow buffer (pH 7.8) was passed through the microreactor at 65 $\mu\text{L min}^{-1}$ by syringe pump. c) LCMS trace and deconvoluted mass spectrum of batch mode sortagging reaction between **4** and **5** ($t_{\text{decon}} = 8.0\text{--}10.5$ min). Reaction conditions: 5 μM **4**, 10 μM **5**, 3.5 μM SrtA*, sortase buffer (pH 7.8), $t_{\text{rxn}} = 30$ min. At $t = 20$ min, 3.33 μL of buffer exchanged Ni-NTA resin per μg protein **4** was added and reaction was allowed to proceed for another 10 min. d) Mass data for insets in panels (b) and (c). [†]: Sortase-mediated hydrolysis of **4** or **6** (Figure S1). [‡]: Sortase-mediated cyclization of **4** or **6** (Figure S1). Obs. mass and cal. mass are both expressed in Da. Retention times vary slightly between panels (b) and (c) because LCMS column had to be changed during the course of this work.

After demonstrating successful flow-based sortagging utilizing 10 μM G_5 -peptides, we hoped to utilize our system to access a construct inaccessible by solution phase sortagging chemistry because of susceptibility to the hydrolysis side reaction. Specifically, we sought to make construct **8** (Figure 3a), a fusion between LF_N and a modified MDM2 binding peptide^[33] (G_5 -2(D-pico), **7**; Figure 3b). In our experience, nucleophiles with low solubility are difficult to sortag because this limits achievable nucleophile concentrations. Solutions of **4** (7 μM in sortase buffer) containing **7** in greater than ca. 20 μM concentration readily formed a white, insoluble precipitate (Figure S10) that caused the batch mode reaction to fail under standard sortagging conditions. Working within the solubility limits of the system did nothing to remedy the

problem in batch mode, as the batch reaction between **4** and **7** yielded substantial amounts of both hydrolysis and cyclization byproducts in addition to the desired **8** (Figure 3d). Switching to a flow-based platform (using 20 μM **7**) allowed for the facile generation of **8** with low amounts of side-products (15 % isolated,^[34] Figure 3c).

It has been previously reported that sortase A catalyzes the transpeptidation reaction between C-terminal LPXTG proteins and N-terminal glycine containing proteins to generate protein–protein fusions.^[31] We demonstrated the versatility of our flow-based platform by generating protein–protein fusions. Model protein **4** was successfully sortagged under flow conditions with an α -helical immunoglobulin binding protein containing G_5 at the N-terminus (**9**, cal. M.W. 6925.6 Da) to generate the desired conjugate **10** (41 % by LCMS, Figure 4a,c). To further explore the scope of flow-based protein–protein sortagging, the β -sheet-containing 10th human fibronectin type III domain harboring G_5 at the N-terminus (**11**, cal. M.W. 11022.6 Da) was conjugated to protein **4** under flow conditions to generate desired conjugate **12** (35 % by LCMS, Figure 4b,d). Low concentration protein nucleophile (20 μM **9** or **11**) was used in both flow-based protein–protein conjugations with virtually no side reactions observed. Comparative batch reactions employing stoichiometric **4** and **9** or **11** (50 μM **4**; 50 μM **9** or **11**; 3.7 μM SrtA*) yielded the desired conjugates **10** and **12**, respectively, but were contaminated with sortase-mediated hydrolysis byproducts (Figure S11, Table S8).

Here we have demonstrated that a flow-based method enables high-efficiency sortagging at low nucleophile concentrations, even with an oligoglycine nucleophile that renders sortagging impossible in batch mode. By immediately releasing the desired transpeptidation product from the microreactor resin bed, contact time between the LPXTGG-containing protein and SrtA* is minimized, limiting side product formation. Moreover, our flow-based system allows us to hold nucleophile concentration at a low, fixed concentration and circumvent the decrease in nucleophile concentration that occurs in traditional batch chemistry. We have also demonstrated that SrtA* retains catalytic activity while resin bound via a simple His₆-affinity tag. While prior methods have covalently immobilized sortase for use in batch chemistry, such methods have relied upon chemical immobilization strategies that markedly decreased enzyme activity.^[35] Our flow-based sortagging platform is highly accessible, employing no specialized microfabrication techniques or multistep covalent enzyme immobilization strategies.

Because the sortagging reaction is of such great value to the chemical biology community, an alternative, flow-based sortagging approach could prove useful to researchers working with difficult substrates prone to hydrolysis, dimerization, or other side reactions. While several examples were presented in this work, flow-based sortagging could be applied to many substrates containing the necessary LPXTGG-His₆ and G_5 motifs. Several experimental variables, such as pH, imidazole concentration, sortase loading on the microreactor, G_5 -nucleophile concentration, NaCl concentration (to vary ionic strength of the flow buffer), and syringe pump flow rate

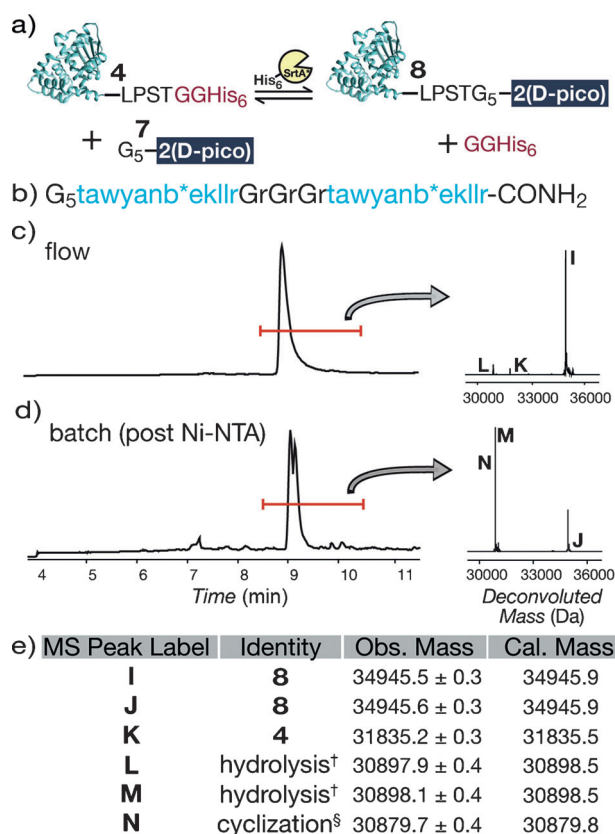


Figure 3. a) Sortagging reaction between protein substrate **4** and pentaglycine nucleophile **7**. b) Structure of **7**. Unnatural amino acid b* is 4-(trifluoromethyl)-D-phenylalanine and lower case indicates D-amino acid. c) LCMS trace (TIC) and deconvoluted spectrum of reactor effluent for model flow reaction between **4** and **7** ($t_{\text{decon}} = 8.0$ –10.0 min). Reaction conditions: Step 1 (load): 7 μM **4** and 20 μM **7** in 800 μL of sortase flow buffer (pH 7.8) was passed through the SrtA* microreactor at 65 $\mu\text{L min}^{-1}$ by syringe pump. Step 2 (push): 1 mL of 20 μM **7** in sortase flow buffer (pH 7.8) was passed through the microreactor at 65 $\mu\text{L min}^{-1}$ by syringe pump. d) LCMS trace and deconvoluted spectrum of model batch reaction between **4** and **7** ($t_{\text{decon}} = 8.0$ –10.0 min). Reaction conditions: 10 μM **4**, 20 μM **7**, 3.5 μM SrtA*, sortase buffer (pH 7.8), $t_{\text{rn}} = 30$ min. At $t = 20$ min, 3.33 μL of buffer exchanged Ni-NTA resin per μg protein **4** was added and reaction was allowed to proceed for another 10 min. e) Mass data for insets in panels (c) and (d). [†]: Sortase-mediated hydrolysis of **4** or **8** (Figure S1). [§]: Sortase-mediated cyclization of **4** or **8** (Figure S1). Obs. mass and cal. mass are both expressed in Da.

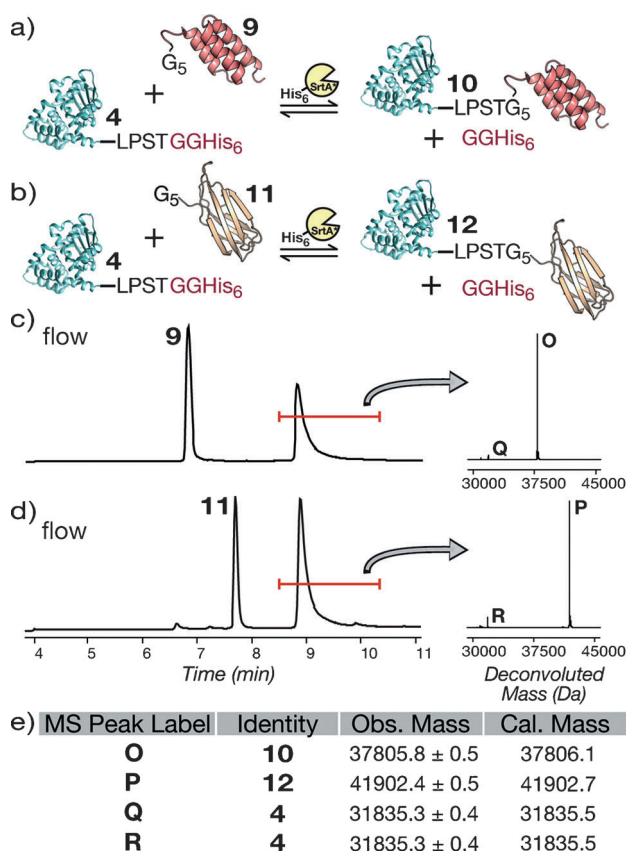


Figure 4. a) Model sortagging reaction between protein substrate **4** and protein nucleophile **9**. b) Model sortagging reaction between protein substrate **4** and protein nucleophile **11**. c) LCMS trace (TIC) and deconvoluted spectrum of reactor effluent for model flow reaction between **4** and **9** ($t_{\text{decon}} = 8.5\text{--}10.5$ min). Reaction conditions: Step 1 (load): $7\text{ }\mu\text{M}$ **4** and $20\text{ }\mu\text{M}$ **9** in $800\text{ }\mu\text{L}$ of sortase flow buffer (pH 7.8) was passed through the SrtA* microreactor at $65\text{ }\mu\text{L min}^{-1}$ by syringe pump. Step 2 (push): 1 mL of $20\text{ }\mu\text{M}$ **9** in sortase flow buffer (pH 7.8) was passed through the microreactor at $65\text{ }\mu\text{L min}^{-1}$ by syringe pump. d) LCMS trace and deconvoluted spectrum of reactor effluent for model flow reaction between **4** and **11** ($t_{\text{decon}} = 8.5\text{--}10.5$ min). Reaction conditions: Step 1 (load): $7\text{ }\mu\text{M}$ **4** and $20\text{ }\mu\text{M}$ **11** in $800\text{ }\mu\text{L}$ of sortase flow buffer (pH 7.8) was passed through the SrtA* microreactor at $65\text{ }\mu\text{L min}^{-1}$ by syringe pump. Step 2 (push): 1 mL of $20\text{ }\mu\text{M}$ **11** in sortase flow buffer (pH 7.8) was passed through the microreactor at $65\text{ }\mu\text{L min}^{-1}$ by syringe pump. e) Mass data for insets in panels (c) and (d). Obs. mass and cal. mass are both expressed in Da. A minimal amount of **4** passed through the microreactor unreacted (peaks **Q** and **R**). It was not removed by additional Ni-NTA treatment to accurately reflect microreactor performance.

can be optimized to decrease side reactions and increase reaction yield. We expect that an alternative flow-based ligation strategy for N-terminal protein labeling employing (molecule of interest)-LPXTGG-His₆ peptides and N-terminal-glycine-containing proteins could be readily developed as an extension of this work (Figure S13). We also anticipate that flow-based bioconjugation could be performed with other enzymes or that an alternative affinity handle strategy could be employed.

Received: March 21, 2014
Published online: July 2, 2014

Keywords: flow chemistry · immobilization · microreactor · protein modification · sortase A

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