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Enhancing the Efficiency of Sortase-Mediated Ligations through Nickel-Peptide Complex Formation

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A modified sortase A recognition motif containing a masked Ni²⁺-binding peptide was employed to boost the efficiency of sortase-catalyzed ligation reactions. Deactivation of the Ni²⁺-binding peptide using a Ni²⁺ additive improved reaction performance at low to equimolar ratios of the glycine amine nucleophile and sortase substrate. The success of this approach was demonstrated with both peptide and protein substrates.

Sortase-mediated methods for protein modification, often termed sortagging, continue to increase in importance for protein engineering. Since the introduction of this technology in 2004, sortase A from Staphylococcus aureus (SrtAaph), as well as evolved variants of this enzyme and homologs from other bacterial species, have been used to generate a wide variety of protein derivatives. Recent examples include the synthesis of antibody conjugates, cyclic polypeptides, modified viral particles, protein-polymer conjugates, and unique protein-protein fusions. Sortases have further been exploited for generating isopeptide bonds, immobilizing proteins on surfaces and particles, and as a means for remodeling the surface of live cells.

While the range of applications enabled by sortagging continues to expand, the efficiency of these processes is limited by the reversibility of the ligation reaction. In a typical reaction, a substrate containing a LPXTG sequence is paired with an amine nucleophile possessing at least one N-terminal glycine (Fig 1a). When incubated in an aqueous buffer that contains Ca²⁺, SrtAaph cleaves the substrate between the threonine and glycine residues to generate a thioester linked acyl-enzyme intermediate. The intermediate is then attacked by the glycine amine nucleophile, which generates a new amide linkage and releases the desired ligation product. Due to the reformation of the LPXTG motif in the ligation product, as well as the release of a nucleophilic N-terminal glycine fragment from the original LPXTG substrate, the reaction is completely reversible. To overcome this reversibility, an excess of one of the ligation partners is typically employed to drive the reaction to completion. This necessity for excess reagents is problematic, particularly when the material used in excess is expensive, difficult to prepare, or challenging to purify away from the final ligation product. Strategies for circumventing the reversibility of sortagging reactions have been reported. These include dialysis, the formation of a nonreactive β-hairpin at the sortase ligation site, the use of depsipeptides, and hydrazinolysis, and affinity immobilization strategies combined with either sortase-substrate fusions or a flow-based sortagging platform. While all of these strategies are effective at blocking or reducing reaction reversibility, many still possess certain limitations. For example, dialysis schemes are only effective when a large molecular weight difference exists between the incoming glycine amine nucleophile and the fragment cleaved from the initial sortase substrate. Designed β-hairpins result in a substantial increase in the size of the sortase ligation site. Depsipeptides are only suitable for appending modifications to the protein N-terminus, and hydrazinolysis requires a large excess of hydrazine nucleophile. Overall, given the growing importance of sortagging as a strategy for protein engineering, there continues to

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**Fig. 1**

**a)** Standard, reversible sortagging reaction catalyzed by SrtAaph. Square planar complex formed between Ni²⁺ and N-terminal GGH motif. Sorting reaction driven by deactivation of GGH fragment by Ni²⁺.
be a need to further refine the efficiency of this process. Here, we describe a straightforward method for driving the equilibrium of sortagging reactions through selective deactivation of a reaction by-product using Ni\(^{2+}\) ions. This strategy relies on a genetically
codable modification of the standard SrtAstaph substrate motif and utilizes standard glycine amine nucleophiles. In this study we
demonstrate a significant increase in sortagging efficiency using model peptides with minimal contamination from reaction side
products. We also show that the approach is readily adapted to the modification of larger protein targets.

Sortagging reactions using wild-type SrtAstaph commonly employ substrates containing an LPXTGG motif. The inclusion of an additional glycine is known to significantly enhance \textit{in vitro} reaction rates.\(^{149}\) We noted that by extending this motif with one additional histidine residue (LPXTGGH) that we would obtain a substrate that releases a GGH fragment upon SrtAstaph cleavage (Fig. 1c). This is significant because GGH represents a well-studied example of an amino terminal Cu\(^{2+}\) and Ni\(^{2+}\) binding (ATCUN) motif.\(^{25}\) Specifically, GGH has been shown to serve as a high affinity site for metal binding when present on the N-terminus of proteins or peptides.\(^{20}\)

We were intrigued by two features of the square planar complex that is formed when metals, such as Ni\(^{2+}\), complex the GGH unit (Fig. 1b). First, the complex is “anchored” by the imidazole side chain of the histidine residue. The presence of histidine in the third position leads to superior metal binding affinity,\(^{20b, 20c}\) suggesting that metals such as Ni\(^{2+}\) and Cu\(^{2+}\) would preferentially bind GGH over peptides in which the histidine was replaced by an amino acid with a non-coordinating side chain. Second, the metal center coordinates the nitrogen lone pair on the N-terminal glycine.\(^{20b, 20c}\)

For this reason, we hypothesized that this would block the nucleophilicity of this amine. With these features in mind, we envisioned a scheme where a sortagging reaction would be performed between a LPXTGGH substrate and a standard glycine nucleophile in the presence of Ni\(^{2+}\).

The released GGH fragment would be selectively deactivated by Ni\(^{2+}\), and in turn drive formation of the desired ligation product (Fig. 1c). Importantly, residues C-terminal to the LPXTGG motif have been shown to have modest impact on reaction rates \textit{in vitro}.\(^{149, 16}\) Therefore, we anticipated no issues with the ability of SrtAstaph to process the modified LPXTGGH substrate.

To test our hypothesis, we generated a model substrate / nucleophile pair consisting of peptide 1 (Ac-k(DNP)LPETGGGH) and
dansylated (Dns) monoglycine nucleophile 2 (Fig. 2a). A commercially available dinitrophenyl (DNP) lysine building block
was incorporated into 1 to provide a convenient chromophore for monitoring reaction progress by RP-HPLC. When 1 and 2 were combined in a 1:1 molar ratio in the presence of 10 mol\% SrtAstaph (standard Δ59 variant\(^{13}\)), the reaction reached equilibrium after
4 h at 37 °C, having generated only 58% of the desired ligation product (3) (Fig. 2b,c). The remaining balance of material consisted of 37% starting material 1, 0.7% hydrolysis product 4, and 4.0% covalent adduct (5) between SrtAstaph and the Ac-k(DNP)LPETGGG fragment of substrate 1. The identity of all species was confirmed by mass spectrometry (Fig. S6, ESI). In the case of 5, the observed mass was consistent with the acyl-enzyme intermediate.

The variant of SrtAstaph used in these studies also possesses a N-terminally modified SrtAstaph species. This material was not characterized further, and in either case only represented 4.0% of the total product mixture. We then examined the same model reaction in the presence of Ni\(^{2+}\). While 1 molar equivalents of NiSO\(_4\) resulted in a significant increase in reaction conversion, optimal results were obtained with 2 equivalents or higher (Fig. S7, ESI). Specifically, when 2 equivalents of NiSO\(_4\) were included, ligation product 3 was found to represent 85% of the total product mixture, with the remaining material consisting of 1 (7.5%), (2.9%), and 5 (4.1%) (Fig. 2b). When monitored over time, a reproducible boost in sortagging efficiency was observed when the

![Fig 2. a) Model reaction for studying the effect of Ni\(^{2+}\) on a substrate containing a masked Ni\(^{2+}\)-binding motif (1). b) RP-HPLC chromatograms demonstrating improved ligation efficiency in the presence of Ni\(^{2+}\). Conditions: 100 μM 1, 100 μM 2, 10 μM SrtAstaph, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM CaCl\(_2\), 0 / 200 μM NiSO\(_4\), 0.2% glycerol (v/v), 5-6% DMSO (v/v), 3 h at 37 °C. Values in parentheses represent the average of three independent reactions and are reported as mean ± standard deviation. c) Time course demonstrating increased formation of ligation product 3 (as estimated by RP-HPLC peak areas) in the presence of Ni\(^{2+}\). All data points were measured in triplicate.](image-url)
NiSO₄ additive was included (Fig. 2c). Notably, this increase in reaction conversion was dependant on the presence of the histidine anchor residue. Control substrates lacking histidine (Ac-K(DNP)LPETGGGG and Ac-K(DNP)LPETGG) showed no change in reaction conversion upon inclusion of Ni²⁺. Reactions utilizing Ac-K(DNP)LPETGGGG generated ~59% 3 in both the presence and absence of Ni²⁺, while reactions employing Ac-K(DNP)LPETGG produced ~66% 3 (Fig. S8, ESI).

With our model system in hand, we proceeded to optimize certain reaction variables. First, the structure and loading of the glycan nucleophile was explored (Table 1). As expected, increasing the concentration of 2 improved ligation efficiency for reactions both with and without Ni²⁺. Reactions containing Ni²⁺ reached a maximum conversion of ~90% at 1.2 equivalents of 2 and above. Reactions lacking Ni²⁺ continued to generate more 3 as the loading of 2 was increased. Notably, even at 2.0 molar equivalents of 2 relative to 1, reactions lacking Ni²⁺ failed to match the efficiency observed when 2 and 1 were combined in equimolar ratios in the presence of Ni²⁺. We also evaluated diglycine and triglycine derivatives of 2. These extended nucleophiles were found to give identical results to those obtained with 2, and reactions including Ni²⁺ consistently outperformed those that lacked the Ni²⁺ additive (Table 1, Fig. S9, ESI). In all cases, hydrolysis (4) never exceeded 3%, while SrtA_staph adduct (5) formation peaked at 4.1% (Table S2, ESI). Alternate metal ions were also evaluated. Neither Co²⁺ nor Cu²⁺ were found to be suitable substitutes for Ni²⁺. The use of Co²⁺ resulted in complex reaction mixtures, whereas Cu²⁺ was actually found to inhibit the sortagging reaction (Fig. S10, ESI). While the exact mechanism of Cu²⁺ inhibition is unclear, we speculate that it involves direct Cu²⁺ coordination of the cysteine active site, which has recently been proposed for the cysteine protease SpeB.

Table 1. Effect of Nucleophile Loading and Structure[3a]  

<table>
<thead>
<tr>
<th>Nucleophile</th>
<th>% ligation product (+ Ni²⁺)</th>
<th>% ligation product (- Ni²⁺)</th>
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</thead>
<tbody>
<tr>
<td>1.0 eq GK(Dns)</td>
<td>85 ± 1.5%</td>
<td>58 ± 2.5%</td>
</tr>
<tr>
<td>1.2 eq GK(Dns)</td>
<td>89 ± 0.6%</td>
<td>61 ± 1.0%</td>
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<td>1.5 eq GK(Dns)</td>
<td>91 ± 0.5%</td>
<td>68 ± 0.5%</td>
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<td>2.0 eq GK(Dns)</td>
<td>91 ± 1.3%</td>
<td>75 ± 1.4%</td>
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<tr>
<td>1.0 eq GGK(Dns)</td>
<td>86 ± 2.6%</td>
<td>59 ± 0.8%</td>
</tr>
<tr>
<td>1.0 eq GGGK(Dns)</td>
<td>87 ± 2.8%</td>
<td>57 ± 2.4%</td>
</tr>
</tbody>
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[3a]Conditions: 100 μM 1, 1.0-2.0 equivalents of nucleophile, 10 μM SrtA_staph, 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 0 / 200 μM NiSO₄, 0.2% glycerol (v/v), 5-11% DMSO (v/v), 3 h at 37 °C. Percent ligation product was calculated from RP-HPLC peak areas. Values represent three independent reactions and are reported as mean ± standard deviation.

Having succeeded in improving sortagging performance using simple peptides, we next turned our attention to larger protein substrates. As an initial target, a derivative of maltose binding protein (MBP) containing the LPETGGGH motif was constructed. When MBP-LPETGGGH was combined with 2 in a 1:1 molar ratio, the formation of the expected ligation product was observed by LC-ESI-MS (Fig. 3a, Fig. S11, ESI). In the absence of Ni²⁺, the reaction reached a maximum ratio of modified to unmodified MBP of 2:1 based on peak areas from the reconstructed mass spectrum. When the reaction was repeated with 2 equivalents of NiSO₄, the ratio of modified to unmodified MBP increased substantially to 8:1. At the 16 h time point shown in Fig. 3, no evidence of MBP hydrolysis (by-product analogous to 4) or SrtA_staph-MBP adduct (by-product analogous to 5) was observed. With respect to MBP hydrolysis, trace amounts were observed in Ni²⁺-containing reactions that were incubated substantially longer than 16 h (data not shown).

As a final example of promoting sortagging efficiency using Ni²⁺, we explored the N-terminal modification of bovine insulin (Fig. 3b). The A-chain of the bovine insulin heterodimer naturally possesses a N-terminal glyrination. To facilitate monitoring of the reaction by RP-HPLC, 6 was paired with an alternate substrate (Bz-LPETGGHG) that lacked significant absorbance at 280 nm. It should be noted that an engineered version of insulin (Lispro) also possessing a N-terminal glycine was recently shown to serve as an excellent nucleophile in sortage-mediated ligations. As in our hands, we found bovine insulin to be relatively unreactive. For example, when 6 was combined with 1 equivalent of peptide 7, reaction lacking Ni²⁺ gave ~20% conversion to the desired ligation product (8), while those containing Ni²⁺ improved only slightly to ~35% (data not shown). We attribute the low reactivity of bovine insulin to the self-assembly properties of this polypeptide. Depending on solution conditions, bovine insulin is known to readily form dimers, hexamers, and higher order aggregates in solution, and we hypothesize that this reduces the accessibility of the A chain terminus. Despite this inherently low reactivity, we were ultimately...
able to obtain high sorting yields using an excess of peptide 7, and once again the use of the Ni²⁺ additive provided a significant advantage. Specifically, when a ten-fold molar excess of 7 was employed, the reaction plateaued at 66% conversion in the absence of Ni²⁺. When the reaction was repeated with Ni²⁺, conversion increased to 89% (Fig. 3b). The use of Ni²⁺ even allowed the loading of 7 to be reduced from ten to five equivalents while still providing improved ligation efficiency (79% conversion, Fig. S12, ESI). To confirm the identity of 8, this material was isolated by RP-HPLC and its molecular weight was verified by LC-ESI-MS (Fig. S13, ESI). We were further able to probe the site-selectivity of the ligation reaction by reducing 8 with excess DTT and characterizing the products by LC-ESI-MS. As expected, a single ligation product involving the A-chain was observed, while the B-chain was found to be completely unmodified (Fig. S13, ESI). Overall, these data are fully consistent with the selective modification of the N-terminal glycine residue of the insulin A-chain.

In conclusion, we have developed a straightforward method for enhancing the efficiency of sorting reactions based on the deactivation of a reaction by-product using Ni²⁺. This strategy requires a simple modification of the standard SrtA＜sub＞staph＜/sub＞ substrate motif, and uses standard sorting nucleophiles to generate standard sorting products. A key advantage of this approach is the fact that the latent Ni²⁺-binding unit is based entirely on native amino acids, and therefore it can be incorporated into protein targets at the genetic level. While the full scope of this methodology remains to be established with a wider range of large protein targets, we anticipate that it will be compatible with numerous sorting applications, as well as the growing number of evolved variants and natural homologs of SrtA＜sub＞staph＜/sub＞.

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Notes and References