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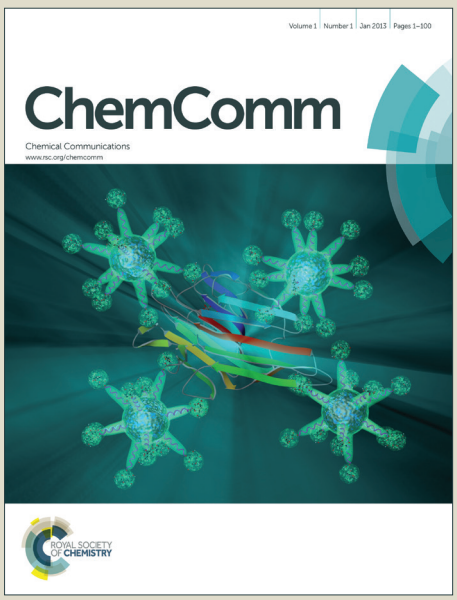

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Enhancing the Efficiency of Sortase-Mediated Ligations through Nickel-Peptide Complex Formation

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A modified sortase A recognition motif containing a masked Ni^{2+}-binding peptide was employed to boost the efficiency of sortase-catalyzed ligation reactions. Deactivation of the Ni^{2+}-binding peptide using a Ni^{2+} 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,[1] sortase A from *Staphylococcus aureus* ($SrtA_{staph}$), as well as evolved variants of this enzyme and homologs from other bacterial species,[2] have been used to generate a wide variety of protein derivatives.[3] Recent examples include the synthesis of antibody conjugates,[4] cyclic polypeptides,[5] modified viral particles,[6] protein-polymer conjugates,[7] and unique protein-protein fusions.[8] Sortases have further been exploited for generating isopeptide bonds,[9] immobilizing proteins on surfaces and particles,[10] and as a means for remodeling the surface of live cells.[11]

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 Ca^{2+}, $SrtA_{staph}$ cleaves the substrate between the threonine and glycine residues to generate a thioester linked acyl-enzyme intermediate.[12] 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

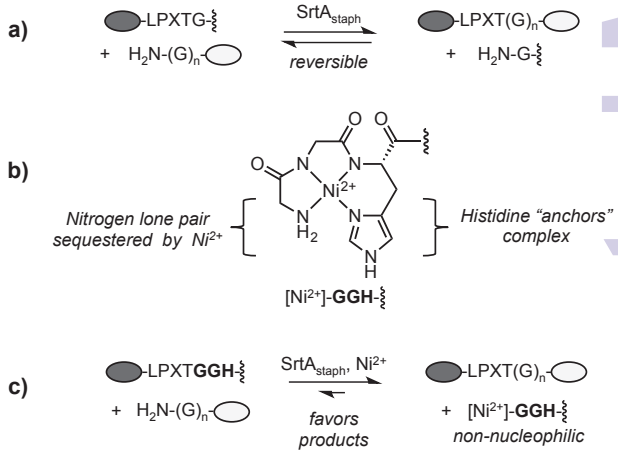

Fig. 1 a) Standard, reversible sortagging reaction catalyzed by $SrtA_{staph}$. b) Square planar complex formed between Ni^{2+} and N-terminal GGH motif. c) Sortagging reaction driven by deactivation of GGH fragment by Ni^{2+}.

one of the ligation partners is typically employed to drive the reaction to completion.[13] 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,[14] the formation of a nonreactive β-hairpin at the sortase ligation site,[15] the use of depsipeptides,[2b, 16] hydrazinolysis,[17] and affinity immobilization strategies combined with either sortase-substrate fusions[18] or a flow-based sortagging platform.[19] While all of these strategies are effective at blocking or reducing reaction reversibility, many still possess certain limitations.[2b, 14-17] 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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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 encodable modification of the standard $SrtA_{staph}$ 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 $SrtA_{staph}$ commonly employ substrates containing an LPXTGG motif. The inclusion of an additional glycine is known to significantly enhance *in vitro* reaction rates.[14b] 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 $SrtA_{staph}$ 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.[20] 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+}. Over the course of this reaction, 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 *in vitro*.[14b] Therefore, we anticipated no issues with the ability of $SrtA_{staph}$ to process the modified LPXTGGH substrate.

To test our hypothesis, we generated a model substrate / nucleophile pair consisting of peptide **1** (Ac-K(DNP)LPETGGHG) 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% $SrtA_{staph}$ (standard Δ59 variant[13a]), the reaction reached equilibrium after 3 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% of covalent adduct (**5**) between $SrtA_{staph}$ and the Ac-K(DNP)LPET 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 $SrtA_{staph}$ used in these studies also possesses a N-terminal glycine, and so it is also possible that the observed adduct represents a N-terminally modified $SrtA_{staph}$ 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 equivalent 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%), **4** (2.9%), and **5** (4.1%) (Fig. 2b). When monitored over time, a reproducible boost in sortagging efficiency was observed when the

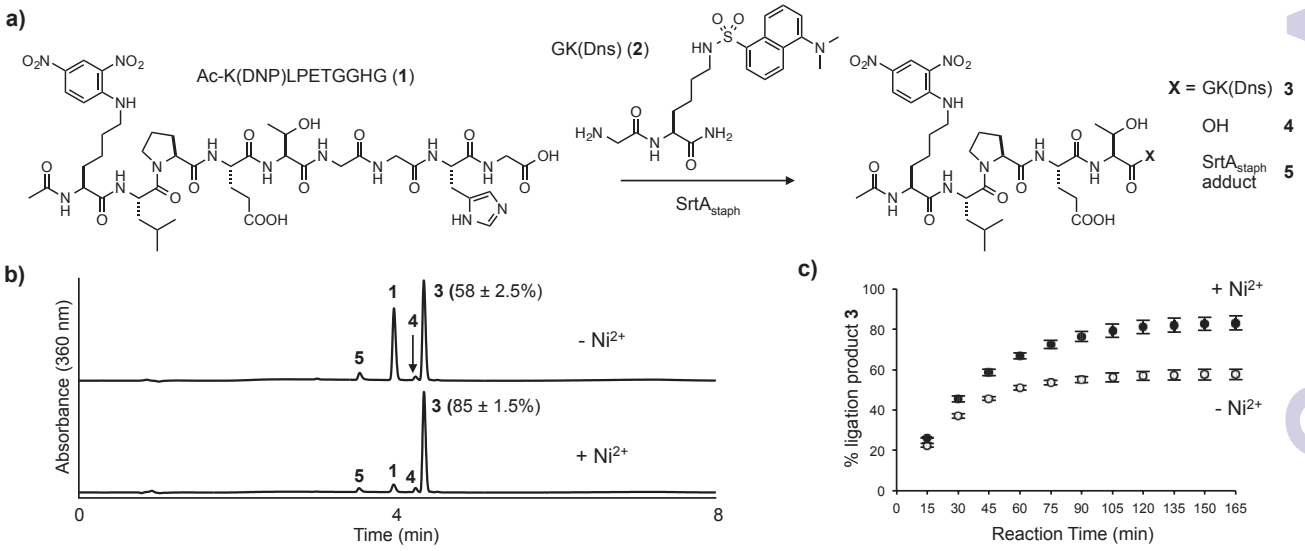

Fig 2. a) Model reaction for studying the effect of Ni^{2+} on a substrate containing a masked Ni^{2+}-binding motif (**1**). b) RP-HPLC chromatograms demonstrating improved ligation efficiency in the presence of Ni^{2+}. Conditions: 100 μM **1**, 100 μM **2**, 10 μM $SrtA_{staph}$, 50 mM Tris pH 7.5, 150 mM NaCl, 10 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.

$NiSO_4$ 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 Ni^{2+}. Reactions utilizing Ac-K(DNP)LPETGGGG generated ~59% **3** in both the presence and absence of Ni^{2+}, 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 glycine nucleophile was explored (Table 1). As expected, increasing the concentration of **2** improved ligation efficiency for reactions both with and without Ni^{2+}. Reactions containing Ni^{2+} reached a maximum conversion of ~90% at 1.2 equivalents of **2** and above. Reactions lacking Ni^{2+} 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 Ni^{2+} failed to match the efficiency observed when **2** and **1** were combined in equimolar ratios in the presence of Ni^{2+}. 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 Ni^{2+} consistently outperformed those that lacked the Ni^{2+} 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 Co^{2+} nor Cu^{2+} were found to be suitable substitutes for Ni^{2+}. The use of Co^{2+} resulted in complex reaction mixtures, whereas Cu^{2+} was actually found to inhibit the sortagging reaction (Fig. S10, ESI). While the exact mechanism of Cu^{2+} inhibition is unclear, we speculate that it involves direct Cu^{2+} coordination of the cysteine active site, which has recently been proposed for the cysteine protease SpeB.[21]

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

Nucleophile	% ligation product (+ Ni^{2+})	% ligation product (- Ni^{2+})
1.0 eq GK(Dns)	85 ± 1.5%	58 ± 2.5%
1.2 eq GK(Dns)	89 ± 0.6%	61 ± 1.0%
1.5 eq GK(Dns)	91 ± 0.5%	68 ± 0.5%
2.0 eq GK(Dns)	91 ± 1.3%	75 ± 1.4%
1.0 eq GGK(Dns)	86 ± 2.6%	59 ± 0.8%
1.0 eq GGGK(Dns)	87 ± 2.8%	57 ± 2.4%

[a]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 $CaCl_2$, 0 / 200 µM $NiSO_4$, 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 LPETGGHG motif was constructed. When MBP-LPETGGHG 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 Ni^{2+}, 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 $NiSO_4$, 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 Ni^{2+}-containing reactions that were incubated substantially longer than 16 h (data not shown).

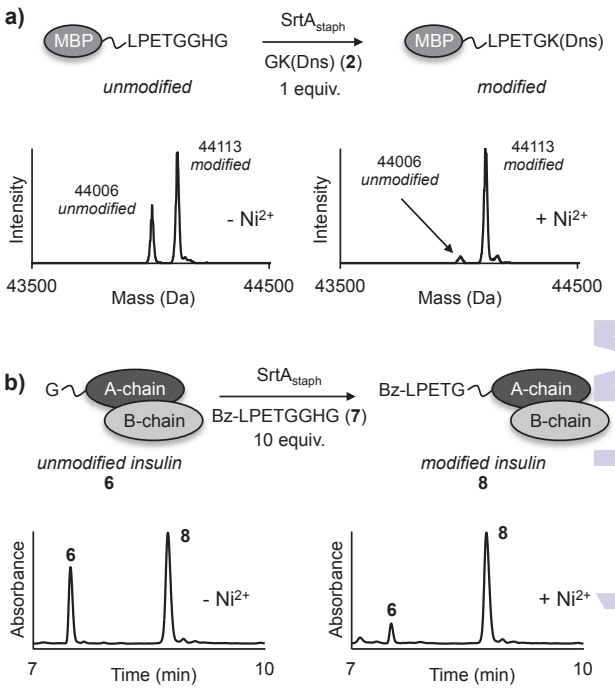

Fig 3. Ni-enhanced sortagging with protein targets. a) C-terminal modification of MBP using 100 µM MBP-LPETGGHG, 100 µM **2**, and 10 µM $SrtA_{staph}$ in the presence or absence of 200 µM $NiSO_4$ (16 h at 37 °C). Reconstructed ESI-MS spectra revealed a clear increase in the ratio of modified MBP (calculated MW = 44112 Da) versus unmodified MBP (calculated MW = 44003 Da). b) N-terminal modification of insulin using 50 µM **6**, 500 µM **7**, and 5 µM $SrtA_{staph}$ in the presence or absence of 400 µM $NiSO_4$ (8 h at 37 °C for $+Ni^{2+}$ reaction, 8.5 h at 37 °C for $-Ni^{2+}$ reaction). A significant boost in reaction conversion was observed by RP-HPLC (280 nm).

As a final example of promoting sortagging efficiency using Ni^{2+}, we explored the N-terminal modification of bovine insulin (**6**) (Fig. 3b). The A-chain of the bovine insulin heterodimer naturally possesses a N-terminal glycine. To facilitate monitoring of the reaction by RP-HPLC, **6** was paired with an alternate substrate (**7**, 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 sortase-mediated ligations.[16a] In our hands, we found bovine insulin to be relatively unreactive. For example, when **6** was combined with 1 equivalent of peptide **7**, reactions lacking Ni^{2+} gave ~20% conversion to the desired ligation product (**8**), while those containing Ni^{2+} improved only slightly to ~35% (data not shown). We attribute the low reactivity of bovine insulin to the self-assembly properties of this polypeptide.[22] 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 N-terminus. Despite this inherently low reactivity, we were ultimately

able to obtain high sortagging yields using an excess of peptide **7**, and once again the use of the Ni^{2+} 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 Ni^{2+}. When the reaction was repeated with Ni^{2+}, conversion increased to 89% (Fig. 3b). The use of Ni^{2+} 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 sortagging reactions based on the deactivation of a reaction by-product using Ni^{2+}. This strategy requires a simple modification of the standard $SrtA_{staph}$ substrate motif, and uses standard sortagging nucleophiles to generate standard sortagging products. A key advantage of this approach is the fact that the latent Ni^{2+}-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 sortagging applications, as well as the growing number of evolved variants and natural homologs of $SrtA_{staph}$.

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Notes and References

1. H. Mao, S. A. Hart, A. Schink and B. A. Pollok, *J. Am. Chem. Soc.*, 2004, **126**, 2670-2671.
2. (a) B. M. Dorr, H. O. Ham, C. An, E. L. Chaikof and D. R. Liu, *Proc. Natl. Acad. Sci. U.S.A.*, 2014, **111**, 13343-13348; (b) J. M. Antos, G. L. Chew, C. P. Guimaraes, N. C. Yoder, G. M. Grotenbreg, M. W. Popp and H. L. Ploegh, *J. Am. Chem. Soc.*, 2009, **131**, 10800-10801; (c) T. Matsumoto, R. Takase, T. Tanaka, H. Fukuda and A. Kondo, *Biotechnol. J.*, 2012, **7**, 642-648; (d) K. Piotukh, B. Geltinger, N. Heinrich, F. Gerth, M. Beyermann, C. Freund and D. Schwarzer, *J. Am. Chem. Soc.*, 2011, **133**, 17536-17539.
3. (a) V. Haridas, S. Sadanandan and N. U. Dheepthi, *Chembiochem*, 2014, **15**, 1857-1867; (b) M. W.-L. Popp and H. L. Ploegh, *Angew. Chem. Int. Ed.*, 2011, **50**, 5024-5032; (c) M. Ritzefeld, *Chem. Eur. J.*, 2014, **20**, 8516-8529.
4. (a) B. M. Paterson, K. Alt, C. M. Jeffery, R. I. Price, S. Jagdale, S. Rigby, C. C. Williams, K. Peter, C. E. Hagemeyer and P. S. Donnelly, *Angew. Chem. Int. Ed.*, 2014, **53**, 6115-6119; (b) P. Kornberger and A. Skerra, *MAbs*, 2014, **6**, 354-366; (c) K. Wagner, M. J. Kwakkenbos, Y. B. Claassen, K. Maijoor, M. Bohne, K. F. van der Sluijs, M. D. Witte, D. J. van Zoelen, L. A. Cornelissen, T. Beaumont, A. Q. Bakker, H. L. Ploegh and H. Spits, *Proc. Natl. Acad. Sci. U.S.A.*, 2014, **111**, 16820-16825.
5. (a) M. Arias, L. J. McDonald, E. F. Haney, K. Nazmi, J. G. Bolscher and H. J. Vogel, *Biometals*, 2014, **27**, 935-948; (b) X. Jia, S. Kwon, C. I. Wang, H. Huang, L. Y. Chan, C. C. Tan, K. J. Rosengren, J. P. Mulvenna, C. H. Schroeder and D. J. Craik, *J. Biol. Chem.*, 2014, **289**, 6627-6638; (c) C. Stanger, T. Maurer, H. Kaluarachchi, M. Coons, Y. Franke and R. N. Hannoush, *FEBS Lett.*, 2014, **588**, 4487-4496.
6. (a) G. T. Hess, J. J. Cragnolini, M. W. Popp, M. A. Allen, S. K. Dougan, C. Spooner, H. L. Ploegh, A. M. Belcher and C. P. Guimaraes, *Bioconjugate Chem.*, 2012, **23**, 1478-1487; (b) G. T. Hess, C. P. Guimaraes, E. Spooner, H. L. Ploegh and A. M. Belcher, *ACS Synth. Biol.*, 2013, **2**, 490-496.
7. (a) Y. Qi, M. Amiram, W. Gao, D. G. McCafferty and A. Chilkoti, *Macromol. Rapid. Commun.*, 2013, **34**, 1256-1260; (b) J. Hu, W. Zhao, W. Gao, M. Sun, Y. Wei, H. Deng and W. Gao, *Biomaterials*, 2015, **47**, 13-19.
8. (a) A. T. Krueger, C. Kroll, E. Sanchez, L. G. Griffith and B. Imperiali, *Angew. Chem. Int. Ed.*, 2014, **53**, 2662-2666; (b) M. D. Witte, J. J. Cragnolini, S. K. Dougan, N. C. Yoder, M. W. Popp and H. L. Ploegh, *Proc. Natl. Acad. Sci. U.S.A.*, 2012, **109**, 11993-11998; (c) A. J. McCluskey and R. J. Collier, *Mol. Cancer. Ther.*, 2013, **12**, 2273-2281.
9. J. J. Bellucci, J. Bhattacharyya and A. Chilkoti, *Angew. Chem. Int. Ed.*, 2015, **54**, 441-445.
10. (a) Z. Qu, V. Krishnamurthy, C. A. Haller, B. M. Dorr, U. M. Marzec, S. Hurst, M. T. Hinds, S. R. Hanson, D. R. Liu and E. L. Chaikof, *Adv. Healthc. Mater.*, 2014, **3**, 30-35; (b) A. Tabata, N. Anyoji, Y. Ohkubo, T. Tomoyasu and H. Nagamune, *Anticancer Res.*, 2014, **34**, 4521-4527; (c) T. Hechler, H. Pham, F. Hammes, L. Thony-Meyer and M. Richter, *Bioconjugate Chem.*, 2014, **25**, 1492-1500; (d) R. K. Le, M. Raeeszadeh-Sarmazdeh, E. T. Boder and P. D. Frymier, *Langmuir*, 2015, **31**, 1180-1188; (e) W. C. Haller, E. Dai, X. Wang, C. E. Hagemeyer, D. R. Liu, K. Peter and E. L. Chaikof, *Angew. Chem. Int. Ed.*, 2015, **54**, 1461-1465.
11. (a) K. Park, J. Jung, J. Son, S. H. Kim and B. H. Chung, *Chem. Commun.*, 2013, **49**, 9585; (b) J. Shi, L. Kundrat, N. Pishesha, A. Bilate, C. Theile, T. Maruyama, S. K. Dougan, H. L. Ploegh and H. F. Lodish, *Proc. Natl. Acad. Sci. U.S.A.*, 2014, **111**, 10131-10136; (c) U. Tomita, S. Yamaguchi, T. Maeda, K. Chujo, K. Minamihata and T. Nagamune, *Biotechnol. Bioeng.*, 2013, **110**, 2785-2789; (d) L. K. Swee, S. Lourido, G. W. Bell, J. R. Ingram and H. L. Ploegh, *ACS Chem. Biol.*, 2015, **10**, 460-465.
12. H. Ton-That, G. Liu, S. K. Mazmanian, K. F. Faull and O. Schneewind, *Proc. Natl. Acad. Sci. U.S.A.*, 1999, **96**, 12424-12429.
13. (a) C. P. Guimaraes, M. D. Witte, C. S. Theile, G. Bozkurt, L. Kundrat, A. E. M. Blom and H. L. Ploegh, *Nat. Protoc.*, 2013, **8**, 1787-1799; (b) C. Theile, M. D. Witte, A. E. M. Blom, L. Kundrat, H. L. Ploegh and C. P. Guimaraes, *Nat. Protoc.*, 2013, **8**, 1800-1807.
14. (a) Y. Kobashigawa, H. Kumeta, K. Ogura and F. Inagaki, *J. Biomol. NMR*, 2009, **43**, 145-150; (b) S. Pritz, Y. Wolf, O. Kraetke, J. Klose, M. Bienert and M. Beyermann, *J. Org. Chem.*, 2007, **72**, 3909-3912; (c) M. A. Reddy, A. Combs, D. J. Kojetin, J. Cavanagh, C. Caperelli, M. Rance, J. Sapitro and P. Tsang, *J. Biomol. NMR*, 2011, **49**, 3-7.
15. Y. Yamamura, H. Hirakawa, S. Yamaguchi and T. Nagamune, *Chem. Commun.*, 2011, **47**, 4742.
16. (a) F. Liu, E. Y. Luo, D. B. Flora and A. R. Mezo, *J. Org. Chem.*, 2014, **79**, 487-492; (b) D. J. Williamson, M. A. Fascione, M. E. Webb and W. B. Turnbull, *Angew. Chem. Int. Ed.*, 2012, **51**, 9377-9380.
17. Y.-M. Li, Y.-T. Li, M. Pan, X.-Q. Kong, Y.-C. Huang, Z.-Y. Hong and L. Liu, *Angew. Chem. Int. Ed.*, 2014, **53**, 2198-2202.
18. R. Warden-Rothman, I. Caturegli, V. Popik and A. Tsourkas, *Anal. Chem.*, 2013, **85**, 11090-11097.
19. R. L. Policarpo, H. Kang, X. Liao, A. E. Rabideau, M. D. Simon and B. L. Pentelute, *Angew. Chem. Int. Ed.*, 2014, **53**, 9203-9208.
20. (a) F. Amini, C. Denison, H. J. Lin, L. Kuo and T. Kodadek, *Chem. Biol.*, 2003, **10**, 1115-1127; (b) I. Sóvágó, C. Kállay and K. Várnagy, *Coord. Chem. Rev.*, 2012, **256**, 2225-2233; (c) I. Sóvágó and K. Ősz, *Dalton Trans.*, 2006, **32**, 3841-3854.
21. K. Chella Krishnan, S. Mukundan, J. A. Landero Figueroa, J. A. Caruso and M. Kotb, *Infect. Immun.*, 2014, **82**, 2992-3001.
22. (a) A. K. Attri, C. Fernandez and A. P. Minton, *Biophys. Chem.*, 2010, **148**, 23-27; (b) A. K. Attri, C. Fernandez and A. P. Minton, *Biophys. Chem.*, 2010, **148**, 28-33; (c) M. R. DeFelippis, R. E. Chance and B. H. Frank, *Crit. Rev. Ther. Drug Carrier Syst.*, 2001, **18**, 201-264.