Protein Engineering Hot Paper

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Sortase-Mediated Multi-Fragment Assemblies by Ligation Site Switching

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Abstract: *Sortase-mediated ligation (SML) is a powerful tool of protein chemistry allowing the ligation of peptides containing LPxTG sorting motifs and N-terminal glycine nucleophiles. The installation of a sorting motif into the product prohibits the assembly of multiple fragments by SML. Here we report multi-fragment SML based on switchable sortase substrates. Substitution of the Leu residue by disulfide-containing Cys(StBu) results in active sorting motifs, which are inactivatable by reduction. In combination with a photo-protected N-Gly nucleophile, multi-fragment SML is enabled by repetitive cycles of SML and ligation site switching. The feasibility of this approach was demonstrated by a proof-of-concept four-fragment ligation, the assembly of peptide probes for bivalent chromatin binding proteins and oligomerization of peptide antigens. Biochemical and immuno-assays demonstrated functionality of these probes rendering them promising tools for immunology and chromatin biochemistry.*

Sortase-mediated ligation (SML) is a versatile tool for chemoselective ligations and modifications of peptides and proteins.[1] Ligation reactions are catalyzed by bacterial transpeptidase sortase A of *staphylococcus aureus* which recognizes the conserved sorting motif LPxTG (x = any amino acid) as substrate. The sorting motif is cleaved at the threonine residue followed by release of the downstream residues as leaving group. The formed enzyme-bound threonine thioester intermediate is ligated to a nucleophile constituted by an N-terminal glycine residue in a peptide or protein (Figure 1a). SML is widely applied in protein chemistry and can be combined with other protein modification strategies.[2] However, the ligation of multiple peptides remains a largely unresolved problem of SML. The ligation reaction reestablishes the LPxTG motif in the ligation product, which competes in a multi-fragment ligation with newly added LPxTG-substrates (Figure 1a) This results in

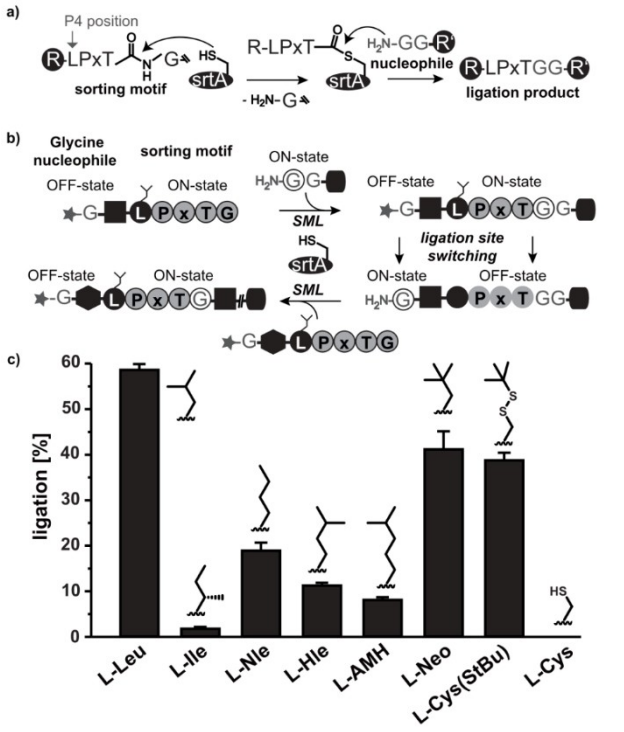

Figure 1. Sortase-catalyzed ligation of peptides and proteins. a) General scheme of sortase-mediated ligation. An LPxTG sorting motif in the substrate is cleaved at the threonine residue liberating the C-terminal Gly and downstream residues as leaving group. The sortase-bound thioester is ligated to a second substrate with N-terminal glycine nucleophile. b) SML with ligation-site-switching. The ligation scheme makes use of switchable sorting motifs in an active "ON-state" and N-terminal Gly nucleophile in an inactive "OFF-state". After ligation to a second substrate with active nucleophile, the sorting motif in the ligation product is switched from ON-state to OFF-state and the N-Gly nucleophile is subsequently activated. The switched ligation product can now be subjected to a further ligation reaction. c) Ligation assays with amino acid substitutions in the P4 position of the sorting motif of the donor substrate. The assays were performed for 1 hour with 500 μM of donor and 500 μM of acceptor substrate and 50 μM of sortase A.

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uncontrolled scrambling of fragments rather than the desired formation of a defined ligation product.

Reported strategies of sortase-catalyzed multi-fragment assemblies relay primarily on sortases recognizing alternative sorting motifs and nucleophiles.[3] Sortase A of *streptococcus pyogenes* has been used for this purpose because this enzyme recognizes peptides and proteins with N-terminal Ala residue as reaction partner, thus enabling stepwise SML of three fragments when combined with the *S. aureus* enzyme. Further investigations of the *S. pyogenes* enzyme have shown that this sortase also accepts variations of the sorting motif, but the canonical LPxTG motif and N-Gly nucleophile remain substrates, thereby complicating multi-fragment SML with native sortases.[4] Directed evolution of sortase substrate selectivity represents a promising alternative approach.[5] The generation of orthogonal *S. aureus* sortases accepting LAxTG and LPxSG sorting motifs represents an important step towards multi-fragment SML.[6] However, ultimately multi-fragment SML will require an orthogonal sortases for each ligation step, limiting the application range of multi-fragment SML to the number of available orthogonal sortases.

Here we report a different strategy for multi-fragment SML that relays on engineering of sortase substrates rather than evolved enzymes. The advantage of this SML strategy, which we referred to as ligation site switching, is that all ligation steps can be carried out with the same sortase enzyme. The general concept of ligation site switching SML bases on a sorting motif which can be switched from an ON-state, where it serves as sortase substrate, to an inactive OFF-state that no longer participates in SML reactions (Figure 1b). Upon ligation the sorting motif is switched to the OFF-state in the product. In addition, the substrates need to possess a latent N-terminal Gly nucleophile in OFF-state that remains inactive during the first ligation reaction. Upon activation in the ligation product a further SML reaction is enabled. In theory, this cycle of SML and ligation site switching can be repeated multiple times enabling multi-fragment assemblies without the need for orthogonal sortases (Figure 1b).

In order to establish a switchable sorting motif, we investigated sorting motif substrate selectivity of wild type *S. aureus* sortase with emphasis on residues which allow structural alterations by simple chemical reactions. We focused on the most N-terminal position of the sorting motif occupied by the leucine residue in native substrates (*P4* position). A set of potential substrates with various *P4* residues were synthesized (peptide pep1–pep8, Figure S1, Table S1) and subjected to transpeptidation assays (Figure 1c). As expected, the native L–Leu as *P4* residue resulted in the most potent sorting motif, whereas a substrate with L–Ile was only poorly processed. Substrates with *P4* residues L-norleucine (L-Nle), L-homoleucine (L-Hle) and L-2-amino-6-methylheptanoic acid (L-AMH) showed moderate transpeptidase activity. However, a L-α-Neopentylglycine (L-Neo) containing sorting motif was the most active substrate with a non-natural amino acid substitution at the P4 position. This finding prompted us to investigate a sorting motif with S-Thio-t-butyl-L-cysteine (L–Cys(StBu)) as P4 residue, which contained a tert-butyl group like L-Neo. The S-t-butylthio modified Cys residue is a well-established building block of solid-phase peptide synthesis (SPPS) and the disulfide bond in Cys(StBu) can be reduced by 2-mercaptoethanol liberating free cysteine.[7]

Transpeptidation assays showed that the Cys(StBu) containing sorting motif served as a potent sortase substrate in SML reactions and was processes with similar efficiency as the Neo-containing sorting motif (Figure 1c). In contrast, a CPxTG substrate with free cysteine was not ligated by sortase, indicating that reduction of Cys(StBu) can be used for switching this sorting motif from an On-state (C-(StBu)PxTG) to an OFF-state (CPxTG). Furthermore, cysteine residues can be converted to alanine by metal-free desulfurization protocols.[8] Previous reports have shown that a sorting motif with APxTG sequence is no substrate for wild type *S. aureus* sortase, enabling desulfurization as a further switching method.[5b] The switchable Glyine nucleophile was established by installing the photolabile o-Nitroveratryloxycarbonyl (Nvoc) protection group at the N-terminal glycine residue. The Nvoc group is cleaved by UV irradiation liberating an N-terminal Gly residue.[7] In summary, a sortase substrate for multi-fragment assemblies contains an active C(StBu)PxTG sorting motif in the C-terminal region and an inactive Nvoc-protected Glycine nucleophile at the N-terminus.

In a first step we demonstrated the feasibility of SML with ligation site switching in a proof-of-concept four-fragment assembly. This model system was optimized for simple investigation of ligation reactions by HPLC-MS: The C-terminal peptide (pep9) contained an active tri-glycine nucleophile and two Trp residues serving as hydrophobic anchor. The central fragment (pep10) consisted of a switchable C(StBu)PRTG sorting motif in ON-state and two Arg residues in the leaving group (Figure 2a and Figure S2). An Nvoc group blocked the N-terminal nucleophile keeping it in the OFF-state. The N-terminal fragment constituted the already synthesized pep1 contained a regular LPRTG sorting motif and an N-terminal dansyl modification. Upon ligation of pep9 and pep10 intermediate LP1 was detected, showing a retention time shift resulting from the release of the polar Arg moieties (Figure 2 and Figure S2). Desulfurization of LP1 followed by uncaging of the nucleophile resulted in switched ligation product LP2 that was subjected to a further SML reaction with the central fragment (pep10). Assembled intermediate LP3 was converted to LP4 by ligation site switching and subjected to a third SML reaction with N-terminal fragment pep1, furnishing the final ligation product LP5 (Figure 2 and Figure S3 and S4). To the best of our knowledge this ligation scheme represents the first reported four-fragment assembly by SML.

Encouraged by these findings we next applied ligation-site-switching SML for generating tools for chromatin biochemistry. Posttranslational modifications (PTMs) of histones constitute the epigenetic histone code by recruiting chromatin factors to the DNA template.[9] PTM containing peptides derived from the N-terminal tail regions of histones are powerful tools for investigating this PTM-dependent protein-protein interactions, but chromatin factors recogniz-

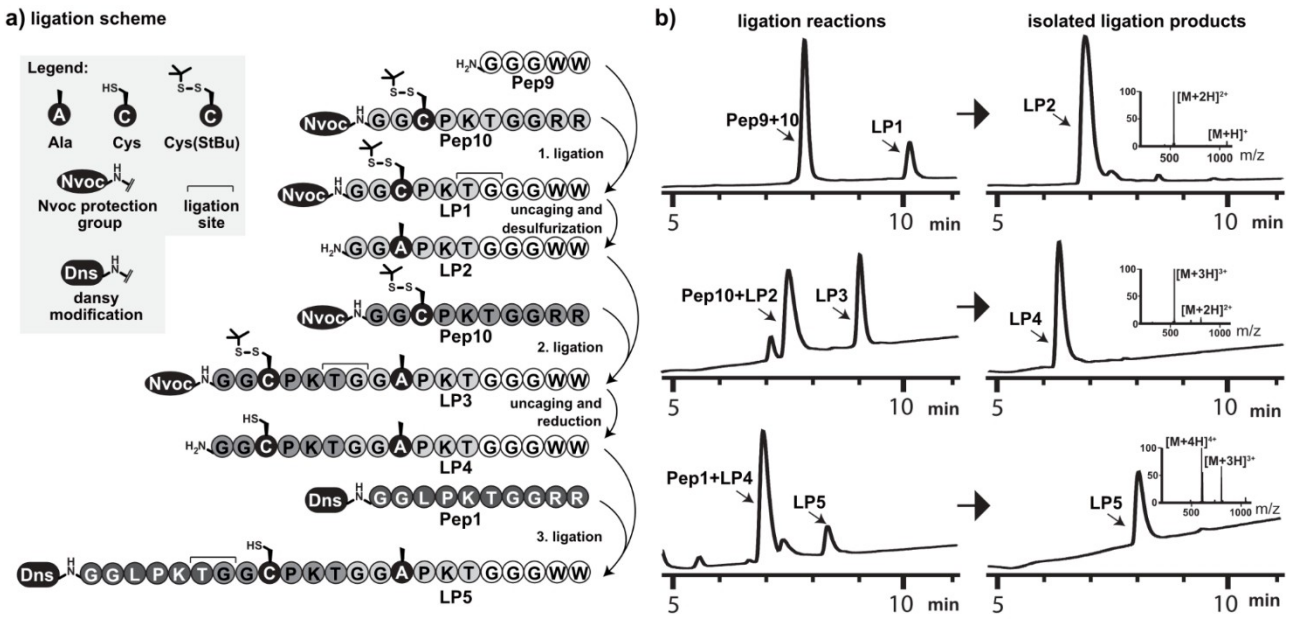

Figure 2. Proof of concept four-fragment ligation reaction. a) The ligation scheme starts with SML of C-terminal fragment pep9 (9.2 mg) and switchable central fragment pep10 (0.8 mg). Ligation site switching is enabled by a Leu to Cys(StBu) substitution at the P4 position of the sorting motif and protection of the N-Gly nucleophile by photolabile Nvoc. Ligation product LP1 obtained after 23 h ligation time is subsequently converted to switched LP2 (0.9 mg) by desulfurization of Cys(StBu) to Ala and uncaging of the nucleophile by UV irradiation. In the following LP2 is subjected to a further ligation reaction with pep10 (1.6 mg) yielding LP3 after 17 h followed by reduction of Cys(StBu) to Cys and activation of the N-Gly nucleophile resulting in LP4 (0.5 mg). A third ligation reaction with N-terminal fragment pep1 (0.5 mg) results in ligation product LP5 (0.2 mg) after 1 h reaction time. b) The ligation reactions were followed by HPLC. The Nvoc and StBu groups and the Arg residues in the leaving group governed the chromatographic properties of pep10, resulting in retention time shifts of primary ligation products L1 and LP3 and switched ligation products LP2 and LP4.

ing PTMs on different histones are difficult to address by this approach. The nucleosome-remodeling factor subunit BPTF represents one example for such a bivalent chromatin binder. The protein consists of a plant homeodomain (PHD) domain serving as binding module of trimethylated K4 of histone H3 and a bromodomain interacting with acetylated histone H4.[10] We used ligation site switching SML for generating peptide probes capable of coping with the bivalent binding mode of BPTF. To this end we grafted the H4-tail acetylated at K5, K8, K12, K16, and K20 (H4Ac5, pep11) and the H3 tail trimethylated at K4 (pep14) onto peptide template pep13 (Figure 3a, Figures S5, S6 and S7). The corresponding unmodified H4 (pep12) and H3 (pep15) tails were also synthesized and ligated to pep13 (Figure S5). Template peptide pep13 contained four glutamic acid residues in order to mimic the negatively charged DNA (Table S1). The template further contained a photo-caged N-terminal tri-Gly unit as nucleophile in OFF-state and an unprotected tri-Gly unit as active nucleophile (Figure 3a). In the first step of the ligation scheme the switchable H4 substrate was installed at the active tri-Gly nucleophile of the template, followed by reduction of Cys(StBu) with beta-mercaptoethanol and uncaging of the template's Nvoc group. The H3 substrate was subsequently ligated to the activated Glycine nucleophile furnishing the dual-histone-tail probes (Figure 3a). We synthesized all four combinations of the modified and unmodified tails and immobilized the probes on solid support (Figures S6 and S7). The probes were equipped with desthiobiotin for reversible immobilization as well as the Cys residue at the switched ligation site which we used for covalent attachment on iodocatyl-conjugated agarose (Figure S8). The pull-down experiments with BPTF showed no binding to the unmodified H3-H4 probe but strong interaction with methylated H3 K9me3-H4 (Figure 3b). Weaker interaction of BPTF with acetylated H3-H4Ac5 was observed and the binding properties of dual modified H3 K9me3-H4Ac5 were apparently governed by the strong interaction of the BPTF PHD domain with H3 K9me3. Collectively, the dual-histone-tail probes allowed binding of the PHD domain and the bromodomain of BPTF. Furthermore, the modular design enables simple probe assemblies from peptide building blocks which can be freely combined by ligation-site-switching SML.

Finally, we investigated the applicability of ligation-site-switching SML for the synthesis of immunological tools. Antigen-presenting cells (APCs) such as dendritic cells express major histocompatibility complex of class II (MHCII) presenting peptide antigens of exogenous or autophagic origin to T cells.[11] T cell receptors (TCRs) of CD4+ cells bind to cognate MHCII-antigen complexes, resulting in T cell activation and release of interleukins such as IL-2. Identifying and investigating the interaction of MHCII, peptide antigens, and TCRs is of major biomedical interest due to their roles in pathogen infections, autoimmune diseases and cancer immunotherapies.[12] Antigenic peptides bind to MHCII on the one hand utilizing conserved

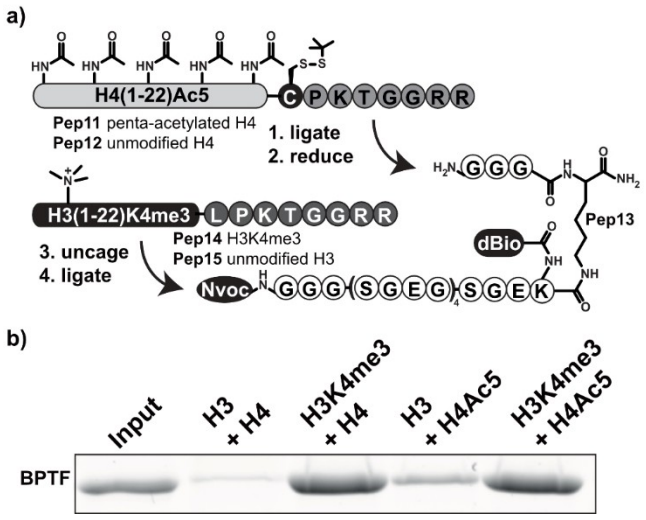

Figure 3. Peptide probes for bivalent chromatin binding proteins. a) Ligation strategy for probe assembly by SML with ligation-site-switching illustrated by the example of a dual modified probe. The histone H4 tail was synthesized in penta-acetylated form with acetylation marks at K5, K8, K12, K16, and K20 (pep11) and in unmodified form (pep12). The H4 peptides were further equipped with a switchable ligation site. The H4 peptides (16 mg and 19 mg) were ligated to peptide template pep13 (19.8 mg) for 43 h followed by reduction of the Cys(StBu) moiety and uncaging of the second acceptor nucleophile. Ligation of K4 trimethylated (pep14, 4.4 mg) and unmodified (pep15, 4.4 mg) H3 peptides furnished the peptide probes after 16 h to 96 h, yielding 2.5 mg to 3.3 mg of isolated material. Detailed ligation schemes are illustrated in Figures S6 and S7. All four combinations of modified and unmodified H3 and H4 peptide were generated. b) SDS-PAGE analysis of BPTF pull-downs with bivalent histone tail probes.

hydrogen bonds between the MHCII side chains and the peptide backbone, and on the other hand by van der Waals and Coulomb interactions between peptide and MHCII side chains along the MHCII binding groove.[13] The N- and C-termini of MHCII-bound antigens usually do not contain specific features, but typically provide additional hydrogen bonds. Immunological tools for probing APC-antigen-TCR interactions have been developed. The most commonly used type of probes are the so-called tetramers, consisting of biotinylated antigen peptides bound to recombinant MHCII protein complexes (pMHCII) which are further conjugated to streptavidin-fluorophore molecules. Such oligomers exploit antigen multivalency effects.[14] Recombinant and synthetic linear antigen oligomers have been also shown to possess improved APC binding and T cell activation capacity.[15] This prompted us to establish access to synthetic antigen oligomers from monomeric antigen building blocks by ligation-site-switching SML. Assembly of oligomeric antigens from pre-made peptide building blocks should allow for flexible design of multivalent antigens including affinity tags, fluorescent probes, or other biophysical labels. We selected the well-established influenza hemagglutinin HA306-318 peptide that binds tightly to MHCII of the HLA-DRB1*01:01 (DR1) allotype as peptide antigen. A C-terminal fragment with nucleophile, a central fragment with switchable sorting motif and nucleophile, and an N-terminal fragment with active sorting motif were synthesized (Figure S9). The fragments were further equipped with PEG and aminohexanoic acid spacers bridging a distance of approximately 70 Å between the antigen peptides in the ligation products, as well fluorescein and desthiobiotin moieties (Figure S10). The fragments were assembled into antigen dimers and trimers (Figure 4a, Figures S10 and S11) and were subjected to functional assays. We observed that the binding efficiency of the corresponding antigens to APCs expressing DR1 increased gradually from antigen monomer to trimer over the concentrations assayed (Figure S12). We further tested the ability of these synthetic constructs to stimulate a hybridoma cell line (HA1.7) expressing a TCR restricted to the MHCII allotype DR1 in complex with the influenza peptide HA306-318 (Figure 4b and Figure S13). We assayed the IL-2 secretion by the HA1.7 cells as an indicator of T cell activation in the presence of APCs. We detected the production of IL-2 in an antigen-concentration dependent manner for the monomeric antigen. The dose-response curve with antigen dimer resulted in an EC50 value approximately one order of magnitude below that of the monomer and almost two orders of magnitude lower for the trimer (Figure 4b).

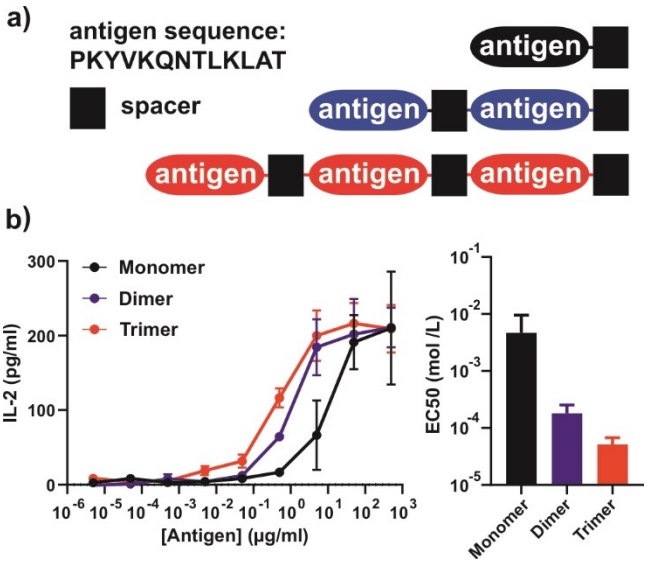

Figure 4. Synthesis and characterization of antigen oligomers. a) The influenza hemagglutinin derived antigen sequence was synthesized as monomer, assembled into a dimer by SML or trimer by SML with ligation site switching. After 24 h reaction time 4.2 mg dimer was isolated from the reaction of 8 mg and 10 mg of starting materials. The trimer was assembled from 11 mg and 16 mg of starting materials over 42 h yielding 4.9 mg of isolated intermediate, followed by a second 5 h ligation reaction of 2.4 mg intermediate and 2 mg monomer resulting in 1.5 mg of isolated antigen trimer. b) The antigen oligomers were tested in T cell activation assays. Antigen oligomers were incubated at various concentrations in presence of the DR1-restricted and HA-specific T cell hybridoma cell line HA1.7. T cell activation was monitored in three independent experiments by detection of interleukin IL-2 by ELISA. Mean values from three experiments are shown for each construct in the left panel with calculated EC50 values shown in the bar diagram in the right panel.

Importantly, the APC expressing DR1 did not stimulate a second RD4 hybridoma cell expressing a TCR restricted to an unrelated MHCII (DR4) in presence of the antigen oligomers (Figure S14a and S14b). We also observed stimulation of T cell hybridomas by the dimeric and especially the trimeric construct in absence of APCs under high concentrations of antigen oligomers, bearing the potential of unspecific T cell activation under these conditions (Figure S14c). No activation was detected with monomeric control antigen peptides (Figure S14b). These results indicate that under physiological conditions the antigen oligomers can be applied as potent tools for triggering T cell activation as long as antigen concentrations are chosen carefully.

In summary we have established a versatile strategy for multi-peptide ligations by ligation-site-switching SML. In comparison to other ligation strategies like native chemical ligation (NCL) which allows multi-fragment assemblies by utilizing latent or reactivity-modulated thioesters, the chemoenzymatic approach of SML does not require activated starting materials like thioesters. Protein trans-spicing (PTS) that bases on split inteins represents a further attractive chemoenzymatic ligation strategy allowing the assembly of more than two fragments. However, when compared to SML the required intein tags are very long and orthogonal inteins are required at each ligation site.[16] The method established here allows the assembly of multiple peptides with only wild-type sortase of *S. aureus* and bases on readily available building blocks for SPPS. We demonstrated the applicability of this method by the first four-fragment ligation by SML. In addition, we developed artificial nucleosome mimics for probing bivalent chromatin factors and antigen oligomers for probing APCs and T cell activation by ligation-site-switching SML. These findings indicate that ligation-site-switching SML is a broadly applicable and versatile tool for protein chemistry. A potential limitation of this approach might be its application in proteins where incorporation of Cys(StBu) is hardly achievable by standard techniques of molecular biology. However, there are several chemical techniques for installing disulfides into recombinant proteins that could pave the way for multi-fragment SML with recombinant protein fragments in the future.[17]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Chemical Biology · Peptide ligation · Protein bioconjugation · Protein semisynthesis · Sortase-mediated ligation

[1] a) M. W. Popp, J. M. Antos, G. M. Grotenbreg, E. Spooner, H. L. Ploegh, *Nat. Chem. Biol.* **2007**, *3*, 707–708; b) M. W. L. Popp, H. L. Ploegh, *Angew. Chem. Int. Ed.* **2011**, *50*, 5024–5032; *Angew. Chem.* **2011**, *123*, 5128–5137; c) M. Ritzefeld, *Chem. Eur. J.* **2014**, *20*, 8516–8529; d) L. Schmohl, D. Schwarzer, *Curr. Opin. Chem. Biol.* **2014**, *22 C*, 122–128; e) C. Freund, D. Schwarzer, *ChemBioChem* **2021**, *22*, 1347–1356.

[2] a) L. Schmohl, D. Schwarzer, *J. Pept. Sci.* **2014**, *20*, 145–151; b) T. J. Harmand, D. Bousbaine, A. Chan, X. Zhang, D. R. Liu, J. P. Tam, H. L. Ploegh, *Bioconjugate Chem.* **2018**, *29*, 3245–3249; c) R. E. Thompson, A. J. Stevens, T. W. Muir, *Nat. Chem.* **2019**, *11*, 737–743.

[3] a) J. M. Antos, G. L. Chew, C. P. Guimaraes, N. C. Yoder, G. M. Grotenbreg, M. W. L. Popp, H. L. Ploegh, *J. Am. Chem. Soc.* **2009**, *131*, 10800–10801; b) G. T. Hess, C. P. Guimaraes, E. Spooner, H. L. Ploegh, A. M. Belcher, *ACS Synth. Biol.* **2013**, *2*, 490–496.

[4] a) L. Schmohl, J. Bierlmeier, F. Gerth, C. Freund, D. Schwarzer, *J. Pept. Sci.* **2017**, *23*, 631–635; b) K. D. Nikghalb, N. M. Horvath, J. L. Prelesnik, O. G. B. Banks, P. A. Filipov, R. D. Row, T. J. Roark, J. M. Antos, *ChemBioChem* **2018**, *19*, 185–195.

[5] a) I. Chen, B. M. Dorr, D. R. Liu, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 11399–11404; b) K. Piotukh, B. Geltinger, N. Heinrich, F. Gerth, M. Beyermann, C. Freund, D. Schwarzer, *J. Am. Chem. Soc.* **2011**, *133*, 17536–17539.

[6] B. M. Dorr, H. O. Ham, C. An, E. L. Chaikof, D. R. Liu, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 13343–13348.

[7] A. Isidro-Llobet, M. Alvarez, F. Albericio, *Chem. Rev.* **2009**, *109*, 2455–2504.

[8] Q. Wan, S. J. Danishefsky, *Angew. Chem. Int. Ed.* **2007**, *46*, 9248–9252; *Angew. Chem.* **2007**, *119*, 9408–9412.

[9] M. M. Müller, T. W. Muir, *Chem. Rev.* **2015**, *115*, 2296–2349.

[10] A. J. Ruthenburg, H. Li, T. A. Milne, S. Dewell, R. K. McGinty, M. Yuen, B. Ueberheide, Y. Dou, T. W. Muir, D. J. Patel, C. D. Allis, *Cell* **2011**, *145*, 692–706.

[11] E. R. Unanue, V. Turk, J. Neefjes, *Annu. Rev. Immunol.* **2016**, *34*, 265–297.

[12] M. Álvaro-Benito, E. Morrison, M. Wieczorek, J. Sticht, C. Freund, *Open Biol.* **2016**, *6*, 160165.

[13] a) M. Wieczorek, J. Sticht, S. Stolzenberg, S. Gunther, C. Wehmeyer, Z. El Habre, M. Álvaro-Benito, F. Noe, C. Freund, *Nat. Commun.* **2016**, *7*, 13224; b) M. Wieczorek, E. T. Abualrous, J. Sticht, M. Álvaro-Benito, S. Stolzenberg, F. Noe, C. Freund, *Front. Immunol.* **2017**, *8*, 292.

[14] M. M. Davis, J. D. Altman, E. W. Newell, *Nat. Rev. Immunol.* **2011**, *11*, 551–558.

[15] a) J. Mack, K. Falk, O. Rotzschke, T. Walk, J. L. Strominger, G. Jung, *J. Pept. Sci.* **2001**, *7*, 338–345; b) E. Piaggio, L. T. Mars, C. Cassan, J. Cabarrocas, M. Hofstatter, S. Desbois, E. Bergereau, O. Rotzschke, K. Falk, R. S. Liblau, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 9393–9398; c) K. Falk, O. Rotzschke, J. L. Strominger, *Eur. J. Immunol.* **2000**, *30*, 3012–3020.

[16] C. P. Hackenberger, D. Schwarzer, *Angew. Chem. Int. Ed.* **2008**, *47*, 10030–10074; *Angew. Chem.* **2008**, *120*, 10182–10228.

[17] C. Chatterjee, R. K. McGinty, B. Fierz, T. W. Muir, *Nat. Chem. Biol.* **2010**, *6*, 267–269.

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