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Sortase-Mediated Ligation: A Gift from Gram-Positive Bacteria to Protein Engineering

Shinya Tsukiji[a] and Teruyuki Nagamune*[a, b, c]

1. Introduction

The field of protein engineering has always played a central role in biological science, biomedical research, and biotechnology. In particular, in the current postgenomic era, much research requires chemically modified proteins or protein bioconjugates that are impossible to prepare by standard ribosomal synthesis. Thanks to the contributions of various chemists, an array of techniques to precisely (site specifically) modify proteins with diverse natural and unnatural functionalities has been developed in the last two decades. These methods range from classical bioconjugation reactions[1] to more sophisticated approaches such as the biomimetic transamination reaction,[2,3] affinity-based protein surface labeling,[4,5] peptide-/protein-tag fusions,[6,7] and nonsense suppression mutagenesis.[8,9] Protein ligation is another powerful protein engineering technique, which allows fully unprotected synthetic and recombinant polypeptides to be regioselectively joined together to build up a target protein molecule.[10–13] The major strength of this approach is the combination of the ability of chemical (peptide) synthesis to access any desired modification and the flexibility of recombinant DNA technology to produce any size of protein, thus permitting the semisynthesis of even large proteins. To date, several chemical and enzymatic peptide ligation methods have been reported. Early examples of the former include thioether- and hydrazone-/oxime-forming reactions (classical chemical ligation reactions have been reviewed in refs. [12]–[14]). Other water-compatible organic transformations such as the traceless Staudinger ligation,[15] Diels–Alder reaction,[16] and Huisgen cycloaddition[17] have recently been adopted as new peptide ligation strategies. On the other hand, enzymatic methods are few, and the representative one employs subtiligase,[18,19] a double-mutant form of subtilisin that is capable of catalyzing the ligation of peptide segments. Unfortunately, most of these approaches are cumbersome and make an unnatural linkage at the ligation sites. More significantly, these methods rely on the use of unnatural reactive groups that cannot be introduced into recombinant proteins by standard genetic means, severely restricting their application to proteins.

Currently, "native chemical ligation" (NCL),[20] developed in the Kent laboratory in 1994, has proven to be the most general and robust method for peptide/protein ligation (reviewed in ref. [21]). NCL is a chemoselective coupling reaction that links a peptide fragment containing an N-terminal Cys (α-Cys) residue and another peptide fragment bearing a C-terminal α-thioester group by a native peptide bond. This reaction proceeds efficiently under physiological conditions and is compatible with all natural amino acid side-chain functionalities. Therefore, through the recombinant preparation of proteins having an α-Cys residue, NCL can be used to generate proteins containing modifications at their N termini. Moreover, the utility of NCL for protein semisynthesis has been elegantly expanded by the emergence of the technique that provides access to α-thioester derivatives of proteins.[22,23] By genetically fusing proteins of interest to the N terminus of an engineered protein splicing domain (intein), recombinant protein α-thioesters can be obtained by thiolysis of the corresponding protein–intein fusions, thus allowing the C-terminal modification of the proteins by subsequent NCL. The semisynthetic version of NCL, in which one or both of the polypeptide building blocks are made by recombinant DNA expression, was dubbed by Muir "expressed protein ligation" (EPL, reviewed in refs. [12] and [24]). EPL has been applied to introduce a variety of modifications such as fluorophores, unnatural amino acids, isotopic labels, and post-translational modifications into a large number of proteins. Additionally, the EPL system has been successfully used not only in test tubes but also in cultured cells.[25,26] Although undoubtedly powerful, NCL and EPL also have some drawbacks. First, the preparation of synthetic peptide α-thioesters is still technically difficult, especially for nonspecialists. Second, since the ligation process is a chemical reaction, the use of higher concentrations (in most cases, more than several mM) of both or either of the reactants is required, which is not always easy to achieve. Third, in the preparation of protein α-thioesters, the need for high concentrations (usually more than several tens of mM) of thiol derivatives to induce thiolysis of the protein–intein fusions could restrict or complicate the application of EPL to many disulfide bond-containing proteins. Fourth, the expression of intein-based fusion constructs often results in

[a] *Dr. S. Tsukiji, Prof. T. Nagamune*
Department of Chemistry and Biotechnology
School of Engineering, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)

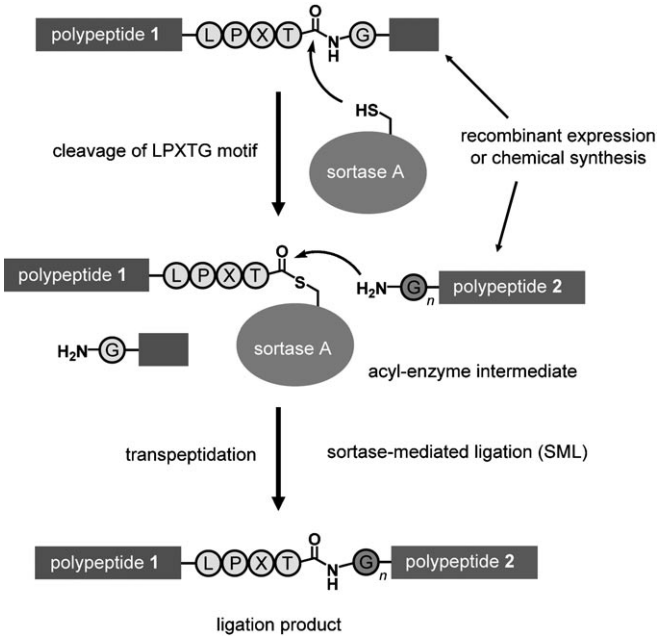
[b] *Prof. T. Nagamune*
Department of Bioengineering
School of Engineering, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)
Fax: (+81) 3-5841-8657
E-mail: nagamune@bioeng.t.u-tokyo.ac.jp

[c] *Prof. T. Nagamune*
Center for NanoBio Integration, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)

the formation of inclusion bodies due to the large protein sizes and poor solubility, which necessitates additional refolding steps.

"Protein *trans*-splicing" (PTS) has recently emerged as a new tool for protein ligation (reviewed in refs. [24] and [27]). This is based on split inteins that mediate the linking of N- and C-terminal exteins by a native peptide bond in *trans* with concomitant removal of the intein complex. Because inteins are promiscuous with respect to the extein sequences, any polypeptide, either synthetic or recombinant in origin, can be placed in the extein regions and linked together through PTS. Among several *trans*-splicing inteins reported so far, the naturally split *Synechocystis* sp. (*Ssp*) DnaE intein,[28,29] each fragment of which can spontaneously associate with high affinity, has been the most characterized and exploited for various protein modification purposes.[24] Artificially split *Ssp* DnaB mini-intein[30,31] and its small-molecule-integrated version[32] have also been created and shown to be applicable to protein semisynthesis in test tubes as well as on the surface of living cells. The PTS-based ligation is an autocatalytic process, and thus overcomes several of the shortcomings of EPL described above. However, as with EPL, expressing split intein fusions in a soluble form is often challenging. Another severe drawback is that synthetic motifs to be introduced must always be fused with an intein fragment (in the shortest cases, the N-terminal half of the split DnaB mini-intein is 11 amino acids, and the C-terminal half of the split DnaE intein is 36 amino acids), inevitably making their preparation more demanding.

Owing to the advances in NCL, EPL, and PTS, it has become possible to precisely introduce a variety of nongenetically encoded functionalities into proteins. However, there is still an obvious need for other general procedures that permit the regioselective assembly of synthetic and/or recombinant polypeptides. Recently, a novel, versatile, enzymatic protein ligation tool, sortase, has emerged from Gram-positive bacteria, which has great potential to make a profound contribution in the field of protein engineering by aiding in the preparation of engineered/labeled proteins. This minireview describes the outline of this new technique, "sortase-mediated ligation" (SML, Scheme 1) and the current state of its applications.



Scheme 1. Principles of sortase-mediated ligation (SML). Sortase A first recognizes an LPXTG sequence within polypeptide 1 and cleaves the amide bond between the Thr and the Gly with an active-site Cys184, generating a covalent acyl-enzyme intermediate. The thioester intermediate is then attacked by an amino group of the oligoGly-containing polypeptide 2, which allows the ligation of the two polypeptides by a native peptide bond.

2. General Descriptions of Sortase

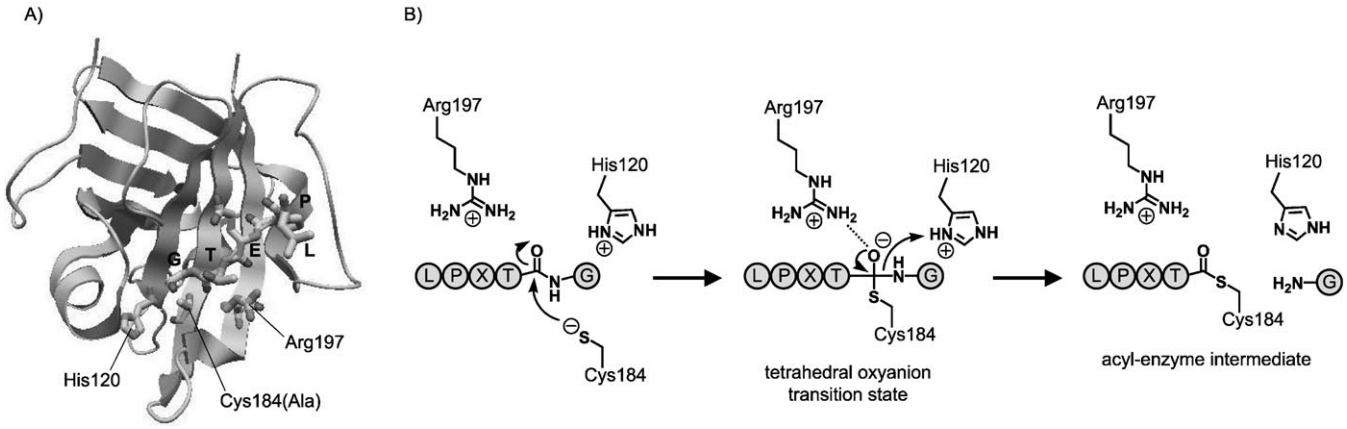
Sortases are transpeptidase enzymes found in most Gram-positive bacteria.[33–35] They are positioned at the plasma membrane and are responsible for covalently anchoring a variety of surface proteins to the cell wall envelope, a process that is important in the physiology and pathogenesis of the bacteria. The enzyme was named after its role in "sorting" the surface proteins between the cell wall and other compartments.[36] Among several isoforms and homologues discovered so far, the *Staphylococcus aureus* sortase A (SrtA) is the focus of this article. Since it was first identified in 1999 by Schneewind and co-workers,[37] much effort has been devoted to elucidate the molecular basis of the SrtA-catalyzed transpeptidation reaction. In particular, the following three important findings provide the foundation of the SML technology (for more general information on the cell wall sorting processes in *S. aureus* and the physiological roles, biochemistry, structure, and inhibitors of SrtA, see an excellent recent review in ref. [35]).

2.1 Recombinant soluble sortase

Wild-type SrtA is a polypeptide of 206 amino acids with an N-terminal membrane-spanning region, that is, it is a type II membrane protein. However, gratifyingly, a truncated catalytic core of SrtA that lacks the N-terminal membrane-anchoring motif can be expressed at high levels and purified as a soluble functional form from *E. coli*.[38,39] Truncated soluble versions of SrtA (in most cases, SrtA devoid of the N-terminal 59 amino acid residues) have been used throughout the in vitro studies and applications described below.

2.2 Transpeptidation mechanism

SrtA recognizes substrates that contain an LPXTG sequence and catalyzes the cleavage of the amide bond between the threonine and the glycine by means of an active-site cysteine (Cys184) residue (see also Scheme 1). This process generates a covalent acyl-enzyme intermediate. The carboxyl group of Thr of the thioester intermediate then undergoes nucleophilic attack by an amino group of oligoglycine substrates (in *S. aureus*, a pentaglycine (Gly_5) cross-bridge on a branched lipid II precursor), producing ligated products. In the absence of oligoglycine nucleophiles, the acyl-enzyme intermediate is hydrolyzed by a water molecule.



Scheme 2. A) Crystal structure of SrtA (Cys184Ala) complexed with the LPETG peptide. The structure was generated from atomic coordinates deposited in the Protein Data Bank, PDB ID: 1T2W.[40] B) Proposed reverse protonation mechanism for formation of the acyl-SrtA intermediate.[42,43]

The crystal structure of an active-site mutant (Cys184Ala) of SrtA complexed with its substrate LPETG peptide has been solved[40] and has revealed that the leucine and proline residues of the LPXTG motif are held in position by hydrophobic contacts, whereas the glutamic acid at the X position points out into the solvent (Scheme 2 A). The scissile Thr–Gly bond is positioned between the active-site Cys184(Ala) and Arg197 residues. In addition to Cys184 and Arg197, His120 is also conserved among sortases from Gram-positive bacteria and is essential for SrtA catalysis.[41] In the latest "reverse protonation model" made by McCafferty and co-workers (Scheme 2 B),[42,43] the LPXTG substrate binds to SrtA, in which Cys184 and His120 are reverse protonated (as a Cys184 thiolate and His120 imidazolium pair). The Cys184 thiolate attacks the carbonyl of the Thr–Gly bond, resulting in the formation of a tetrahedral oxyanion transition state, which is stabilized by a hydrogen bond with Arg197.[43] The protonation of the leaving group (Gly) by the His120 imidazolium facilitates the collapse of the transition state and the formation of the acyl-enzyme intermediate (and unprotonated His120). Although the details are not yet clear, His120 might also deprotonate the incoming N-terminal amine of oligoglycine substrates. Interestingly, SrtA has a binding site for a calcium ion, by which the catalytic activity is stimulated eightfold through a mechanism that could facilitate substrate binding.[39]

2.3 Substrate specificity

SrtA is highly specific for both the LPXTG motif and N-terminal Gly repeats (α-Gly$_n$) with a free amino group.[44,45] All natural amino acid residues except Cys and Trp (not tested so far) have been confirmed as acceptable at the X position.[44] Although peptides carrying only a single N-terminal glycine have been shown to participate in the sortase-catalyzed transpeptidation, maximum reaction efficiency is generally obtained with substrates in which two or more glycines are incorporated.[45–47] On the other hand, and more significantly, SrtA is promiscuous with respect to the flanking sequences. As we can see in the next section, various molecules can be used as substrates for SrtA by fusing them to the LPXTG and α-Gly$_n$ tags.[46–59] As another interesting feature, SrtA is also capable of using several primary amine derivatives such as hydroxylamine[38] and alkylamines[49,58] as α-Gly$_n$ surrogates, though these non-peptidic substrates appear to be less efficient than oligoglycine derivatives.

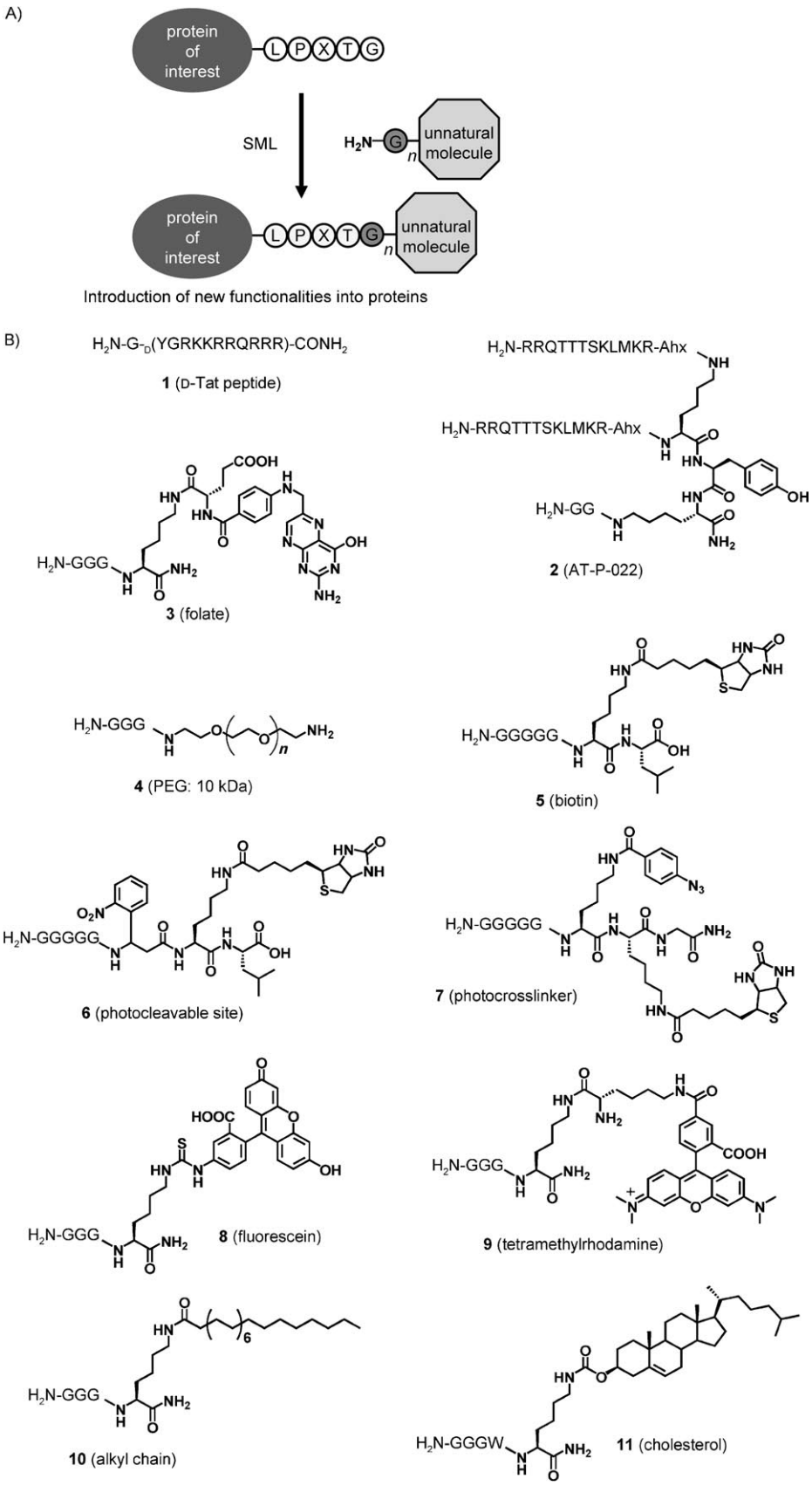
Distinct from previously reported peptide ligation methods (Section 1), both of the reaction groups for SrtA-mediated transpeptidation are very short, natural amino acid motifs that can be incorporated into polypeptides either by standard genetic means or chemical peptide synthesis. Benefiting from its simplicity and specificity, SrtA has begun to be applied for a wide variety of protein engineering and bioconjugation purposes. These applications are summarized in the following section.

3. Applications of SML

3.1. Introduction of new functionalities into proteins

One of the most straightforward and important applications of SML is as a method for introducing unnatural functionalities into proteins. With this new technique, various molecules such as small chemical probes, synthetic peptides, and polymers have been incorporated into LPXTG-tagged recombinant proteins (Scheme 3).

In 2004, Mao and co-workers demonstrated for the first time that SrtA can be used as a new protein ligation tool.[46] A recombinant green fluorescent protein (GFP) containing an LPETG-His$_6$ sequence at its C terminus (GFP-LPETG-His$_6$) was used as a model protein. By using SrtA, the LPETG-tagged GFP was site-specifically modified with not only native peptide sequences but also structurally diverse nonnative peptide fragments, including a D-peptide (**1**, D-isomer of Tat peptide) and a branched peptide (**2**, AT-P-022; Scheme 3 B). AT-P-022 possesses strong protein transduction activity, that is, it allows linked proteins to be delivered into eukaryotic cells. Indeed, flow cytometry analysis showed that the obtained GFP-LPET-[AT-P-022] conjugate can be taken up by NIH3T3 cells with high efficiency. In addition, a small molecule (folate) was introduced



Scheme 3. A) Schematic illustration of the strategy for introducing new functionalities into proteins with SML. B) Examples of unnatural compounds that have been incorporated into recombinant proteins with this approach. **1**–**3**, Mao et al., to GFP;[46] **4**, Boder et al., to GFP;[49] **5**–**11**, Ploegh et al., to MHC H-2K^b, CXCl14, CD154, neuraminidase, and GFP.[50,51]

into the LPETG-tagged GFP by derivatizing it with an α-Gly$_3$ tag via a lysine (**3**), generating a GFP-folate conjugate. The protein-peptide conjugation yields were approximately 50% after 6 h and increased to 90% within 24 h at 37 °C (70 μM GFP-LPETG-His$_6$, 350 μM α-Gly$_n$-peptide, 20 μM SrtA, pH 7.5). This demonstrates that SML is sufficiently efficient.

In another example, Boder and co-workers used SrtA as a molecular "stapler" for the specific attachment of α-Gly$_3$-terminated polyethylene glycol (**4**) to GFP-LPETG-His$_6$, so-called PEGylation.[49]

Ploegh and co-workers further extended the range of applications of SML as a protein labeling technique (they termed the method "sortagging").[50] A soluble form of the mouse class I major histocompatibility complex (MHC) H-2K^b molecule was tagged with a C-terminal LPETG motif and complexed with an octapeptide ligand. The LPETG-attached H-2K^b monomer was successfully labeled in the presence of SrtA with a variety of α-Gly$_n$-based probes (**5**–**9**). The reactions proceeded in almost quantitative yields in 1 hour with relatively high concentrations of probe (5 mM) and SrtA (150 μM). The introduction of an *o*-nitrobenzyl linker (**6**) allowed for photomediated cleavage of the polypeptide with concomitant release of the biotin label. When a phenylazide photocrosslinker (**7**) was incorporated, the engineered H-2K^b was capable of being crosslinked to β_2-microglobulin by light. The attachment of an organic dye, fluorescein (**8**) or tetramethylrhodamine (**9**), enabled the fluorescent visualization of the labeled H-2K^b. The method was generally applicable to other proteins including LPETG-tagged chemokine CKCl14 and human CD154 not only in a purified form but also

in a complex mixture such as cell lysates, as well as on the surface of living cells (see Section 3.8). Very recently, the same group also demonstrated the C-terminus-specific conjugation of lipids such as a long alkyl chain (**10**) and cholesterol (**11**) to LPETG-tagged GFP in high yields (60–90%).[51] It was shown that the generated semisynthetic lipoproteins associate strongly with the plasma membrane of mammalian cells.

3.2. Covalent immobilization of proteins onto solid supports

The ability to attach proteins onto solid supports in their native state is essential to produce a variety of technological tools, such as supported catalysts, protein sensor chips, and protein microarrays. Many protein modification strategies have been adopted to achieve the site-specific, covalent immobilization of proteins.[60] SML might also become an attractive alternative approach (Scheme 4).

Boder and co-workers reported the first use of SML for protein immobilization.[49] Amine-terminated polystyrene beads were derivatized with triglycine. In the presence of SrtA, GFP-LPETG-His_6 was covalently attached to the α-Gly_3-conjugated beads. The unmodified, amine-terminated beads could also be used for protein immobilization, although it required a higher concentration of SrtA (40 μM compared to 4 μM for the α-Gly_3-conjugated beads). In addition, the researchers demonstrated that crude *E. coli* lysates can be directly used to immobilize the expressed protein onto the beads without the need for prepurification steps.

Neylon and co-workers further demonstrated that SML is generally applicable to the attachment of various proteins onto a range of solid supports including cross-linked polymer beads, affinity resins, and flat glass surfaces.[47] When Tus-LPETG-His_6, a sequence-specific DNA binding protein, was immobilized on α-Gly_4-conjugated methacrylate beads, it retained almost full binding affinity and specificity toward its cognate TerB DNA ligand. Very recently, Proft and co-workers also used SML to fabricate a protein sensor chip on which fibronectin-binding protein (Fba) was covalently attached.[52] The chip allowed the researchers to analyze the interaction between the Fba and its ligand, factor H, by surface plasmon resonance.

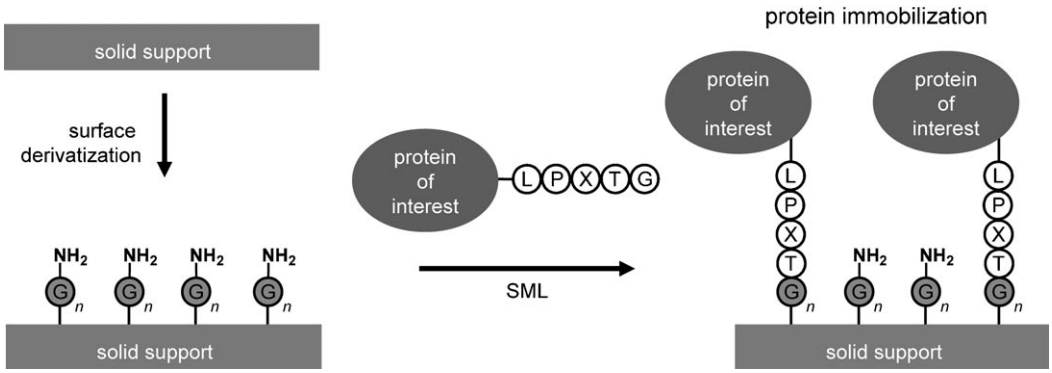
3.3. Protein–protein bioconjugation

As represented by antibody-enzyme complexes used in various immunological assays, linking two or more proteins with different properties is another important approach to generate new functional molecules.[1] Although protein–protein fusion can be readily achieved by genetic manipulation, the bacterial expression of the chimeric proteins is often problematic and low-yielding because of the formation of inclusion bodies. Also, there are many situations in which the genes of interest are not available (particularly for antibodies), or unnatural linkages such as a branched topology[54,61] are required. Previously, SML has been successfully applied to the preparation of several multiprotein conjugates that are difficult or impossible to make otherwise (Scheme 5).

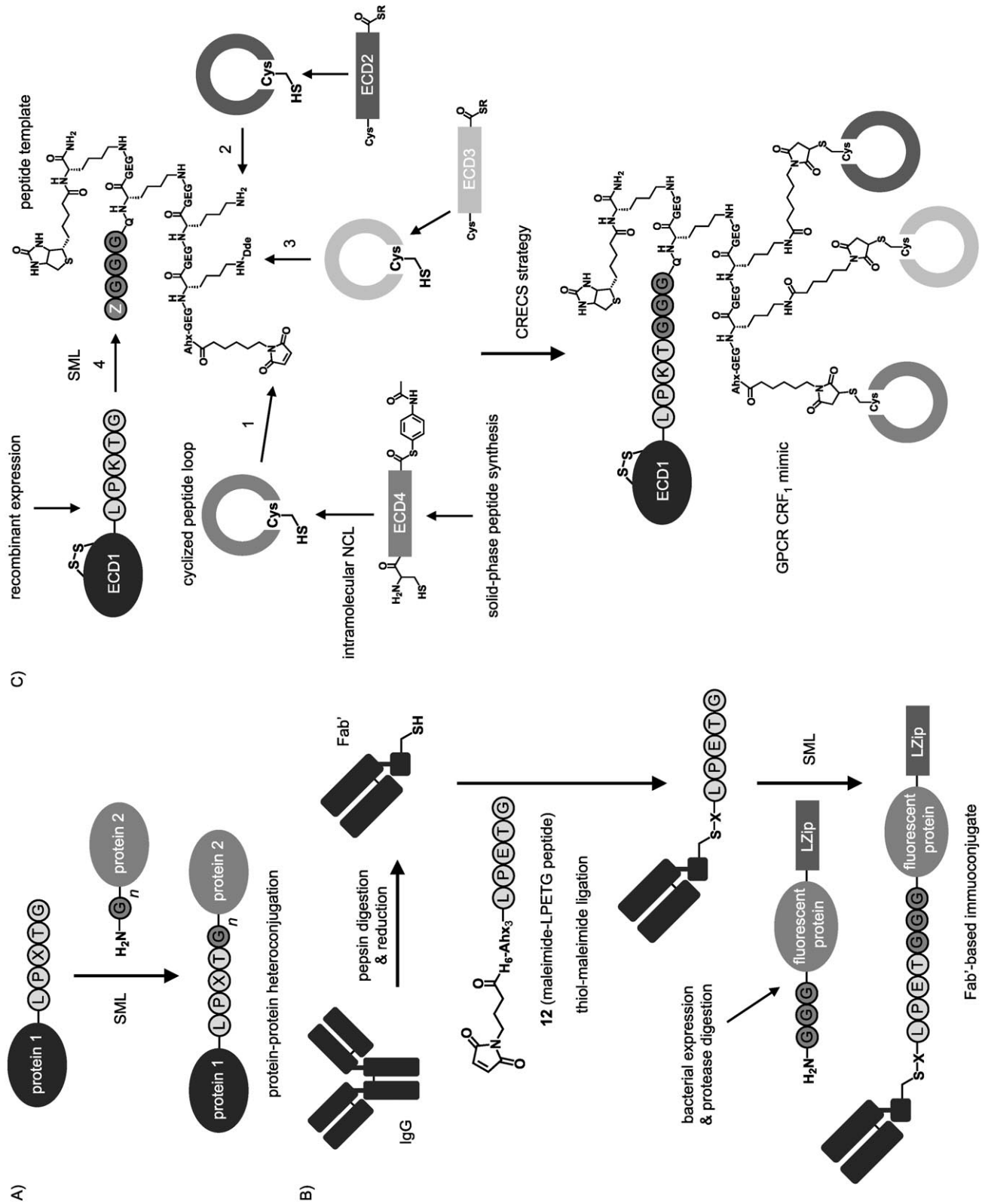
In early studies, Mao and co-workers demonstrated SML-based protein–protein dimerization by ligating GFP-LPETG-His_6 to another GFP variant with an N-terminal Gly (Scheme 5 A).[46] Boder and co-workers further extended the approach to protein oligomerization (Scheme 6).[49] The incubation of a dual-tagged GFP, that is, α-Gly_3-GFP-LPETG-His_6, in the presence of SrtA led to the formation of a mixture of oligomers (up to pentamer).

The Nagamune laboratory has long been interested in creating (semi)artificial fusion proteins with useful functions through post-translational protein–protein crosslinking.[61–67] As an extension of our previous work on an enhanced fluorescence resonance energy transfer (FRET) immunoassay,[66,67] we used SML to prepare immunoconjugates in combination with chemical and genetic techniques (Scheme 5 B).[53] Antibody fragment F(ab′)$_2$, prepared by the pepsin digestion of IgG, was reduced and modified with a chemically synthesized LPETG peptide (**12**) containing an N-terminal maleimide group. By incubating the resulting Fab′-LPETG (10 μM) with bacterially expressed, α-Gly_3-tagged, fluorescent protein–leucine zipper fusion protein (40 μM) and SrtA (50 μM) at 30 °C for 12 h, a highly homogenous Fab′-based immunoconjugate was obtained in excellent yield (90%).

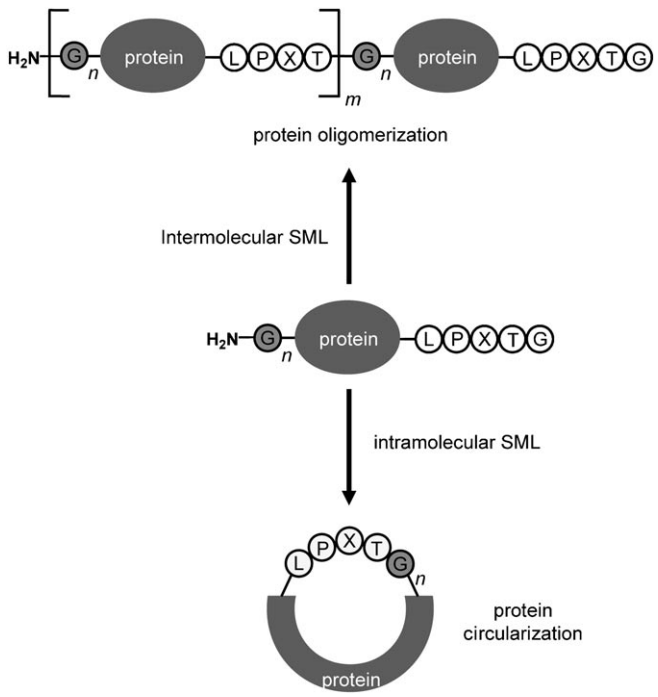
Recently, Beyermann and co-workers synthesized a protein mimic with nonlinear backbone topology by a strategy termed CRECS (combinations of recombinant, enzymatic, and chemical synthesis; Scheme 5 C).[54] Based on the concept of template-



Scheme 4. SML-based immobilization of proteins onto solid supports.[47,49,52]



Scheme 5. A) Protein–protein bioconjugation with SML method. B) The strategy taken by Nagamune et al. for preparing Fab′-based immunoconjugates.[53] Ahx = aminohexanoic acid. C) The CRECS strategy used to synthesize a protein mimic of GPCR CRF_1.[54]



Scheme 6. Protein oligomerization or circularization of dual-tagged proteins by an inter- or intramolecular transpeptidation reaction.[49,55]

assembled synthetic proteins (TASP),[68] four ectodomains—the receptor N terminus ECD1 and three loops (ECD2, 3 and 4) of a G protein-coupled receptor, CRF_1—were regioselectively attached to a peptide scaffold. ECD2–4 were totally synthesized and cyclized by intramolecular NCL. The cyclic ECDs were assembled into the template in a stepwise manner by thiol-maleimide ligation by use of orthogonal deprotection and maleimide attachment procedures. Soluble ECD1 containing three disulfide bridges was obtained as a fusion with a C-terminal LPKTGGRR sequence by bacterial expression and in vitro folding, and was subsequently introduced to the three-loop template by SML. The CRF_1 receptor mimic showed high-affinity binding toward natural peptide agonists, sauvagine and urcortin 1.

3.4. Protein circularization

There is growing interest in the protein engineering community in generating head-to-tail (backbone) circular proteins, in which the two terminal ends are joined together.[69] Several cyclic proteins prepared so far showed a higher stability or ligand-binding affinity than their linear counterparts.[12,69] As another notable application, Ozawa and co-workers recently designed a cyclic protein-based sensor for the detection of protease activities in living cells and animals.[70] It is now well established that protein cyclization can be achieved with an intramolecular version of NCL, EPL, or PTS techniques.[12,69] Therefore, not surprisingly, SML can also serve as a tool for this purpose (Scheme 6).

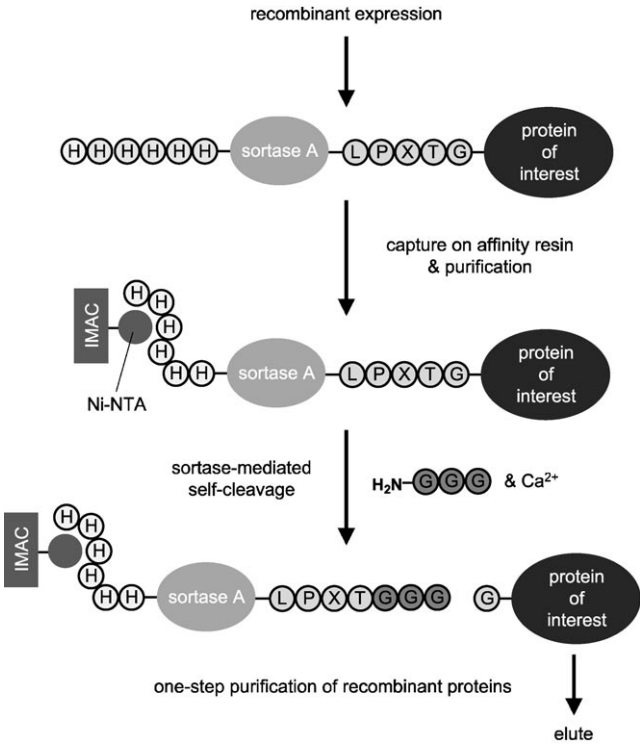
The first examples of protein cyclization by SML can be seen in the report by Mao as minor byproducts of the self-cleavable sortase fusion system (see Section 3.5).[56] Later, in their effort to engineer protein–protein bioconjugations, Boder and co-workers found that a bifunctional EGFP, that is, α-Gly_3-EGFP-LPETG-His_6, reacts in the presence of SrtA to give not only oligomers but also a cyclic GFP in moderate to low yields.[49] Unfortunately, direct evidence for the cyclization is lacking in these two reports.

Our group independently studied the SML-based protein circularization approach.[55] By introducing α-Gly_5 and LPETG tags together to the N and C termini of proteins, respectively, various molecules including GFP, dihydrofolate reductase (DHFR), and the pleckstrin homology (PH) domain were cyclized by an intramolecular transpeptidation reaction. Interestingly, in contrast to the results of the Boder group, in all our cases, the circular forms were efficiently produced in over 90% yield with no detectable oligomerizations. This is probably due to more appropriate lengths of the tag linkers. The head-to-tail ligation was unambiguously characterized by peptide mass fingerprinting. It is noteworthy that the backbone-cyclized DHFR was more thermostable than was the linear form. In addition, the SML-based approach was successfully extended to generate circular proteins inside living bacteria cells.

3.5. A self-cleavable tag for one-step purification of recombinant proteins

The acquisition of "tag-free" recombinant proteins is essential in many fields of protein research but is often challenging. Therefore, fusing proteins with affinity tags, such as hexahistidine or glutathione *S*-transferase, is now the most widespread choice.[71] After purification, the tag is removed by a sequence-specific protease. A drawback of this approach is the requirement of additional chromatography steps to isolate the free target protein from other pieces and/or the protease. To overcome this limitation, the IMPACT system (New England Biolabs), which makes use of intein-mediated self-cleaving activity, has been developed.[72] This method enables the affinity purification, cleavage, and removal of the fusion partner in a one-step process. However, the use of intein is often limited due to the poor solubility of fusion constructs and the unpredictable occurrence of in vivo processing or inefficient post-purification cleavage.

The transpeptidase activity of SrtA is inducible by calcium and oligoglycine.[39] Using this property, Mao has developed a self-cleavable SrtA fusion tag for the one-step purification of recombinant proteins (Scheme 7).[56] Proteins of interest are fused to the C terminus of a His_6-SrtA-LPETG tag, expressed, and captured on Ni-nitrilotriacetic acid (NTA) resin for purification. The subsequent addition of calcium and triglycine activates the SrtA to induce intra- or intermolecular cleavage of the LPETG motif, allowing the tag-free target protein to be eluted with glycine as the only N-terminal modification. The sortase fusion approach was applied for the purification of the GFP, Cre, and p27 proteins. In all cases, the N-terminal SrtA module dramatically increased the expression level and solubility of the fusion constructs. Neither autocleavage nor transpeptidation with endogenous proteins in *E. coli* was observed. All



Scheme 7. A self-cleavable sortase fusion tag for the one-step purification of recombinant proteins, developed by Mao.[56] IMAC = immobilized metal-affinity chromatography.

of the above proteins were obtained with over 98% homogeneity in a single chromatography step. The sortase fusion approach provides a simple, efficient, and inexpensive tool that might be generally applicable to many proteins.

It should be added that, very recently, a self-processing module from *Neisseria meningitides*, FrpC protein, was introduced as the third self-cleavable fusion tag by Osicka and coworkers.[73]

3.6. Preparation of oligopeptide-nucleic acid hybrids

In recent years, there has been considerable interest in creating peptide/protein–nucleic acid hybrid molecules in the fields of biotechnology and gene therapy. For example, the sequence-specific hybridization properties of nucleic acids allow for the immobilization of proteins on DNA arrays, the regulation of protein activity, and the construction of biosensors toward DNA and RNA.[74,75] In a reverse way, cell-penetrating peptides (CPPs) are often used as carriers to deliver conjugated antigene or antisense oligonucleotides into live cells.[76] As NCL and EPL facilitated the preparation of homogenous covalent polypeptide-oligonucleotide conjugates,[77–79] SML should also be powerful in this area (Scheme 8).

Pritz and co-workers exploited SML for the synthesis of a peptide nucleic acid-(PNA)-CPP conjugate (Scheme 8B).[57] An 18-mer antisense PNA (**13**), which targets the aberrant splice site of a β-globin intron 2, was designed to possess a C-terminal LPKTGG motif. It should be noted that peptide–PNA chimeras can be readily prepared using standard, Fmoc-based, solid-phase peptide synthesis protocols. Model amphipathic peptide (MAP) was chosen as a CPP and N-terminally extended with Gly_3 residues (**14**). Ligation of the PNA and peptide by SrtA yielded the PNA–MAP conjugate. The splicing correction assay with HeLa cells revealed that the attachment of the MAP to the PNA led to an enhanced antisense activity in a dose-dependent manner, whereas the PNA alone remained ineffective.

The application of SML to recombinant protein–nucleic acid conjugations has yet not been demonstrated. However, we expect that the preparation of protein–PNA hybrids will be achieved as a direct extension of the above work. Additionally, the introduction of DNA strands into proteins should also be feasible by preparing LPXTG- or α-Gly_n-tagged oligo-DNAs with recently advanced methods for oligopeptide–DNA conjugate synthesis.[76–79]

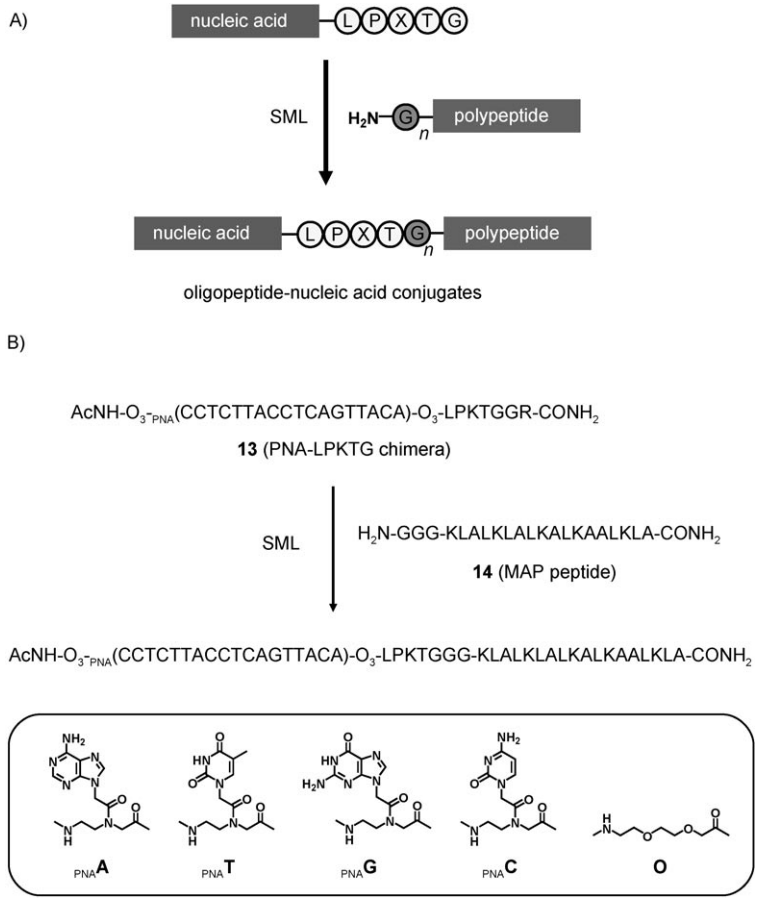
3.7. Chemoenzymatic synthesis of neoglycoconjugates

Saccharides and their derivatives such as glycoproteins play vital roles in cell function. Synthetic glycoconjugates are valuable not only as tools for probing biological processes but also as novel immunovaccines.[80] Therefore, the development of synthetic methods for introducing glycans into peptides and proteins has been one of the major challenges in glycochemistry. An obvious approach to obtain homogenous glycoconjugated polypeptides is to link presynthesized glycosylated building blocks to other peptide segments. Indeed, various glycolabeled peptides and proteins have been prepared by such a ligation-based route.[80]

Roy and co-workers took advantage of another feature of sortase, that is, its relaxed specificity for amine nucleophiles (Scheme 9).[58] The researchers considered that the aminomethylene (NH_2-CH_2-) moieties present in 6-aminohexoses might function as an N-terminal Gly surrogate in SML. As they expected, 6-deoxy-6-amino-glucose and -mannose, but not glucosamine, were successfully attached to a YALPETGK peptide in the presence of SrtA. The strategy was further extended to the conjugation of various aminoglycoside antibiotics, such as kanamycin, ribostamycin, and neomycin, to biologically relevant peptides including HIV-1 Tat and Rev peptides and Arg_9 sequences. The reaction yields were approximately 20–80% after 6 h of incubation (0.5 mM peptide, 2.5 mM sugar/antibiotics, 50 μM SrtA, pH 7.5, 37 °C). Importantly, the ligation was limited to the 6-amino site in the antibiotics despite the presence of other amino groups; this indicates rather strict specificity and selectivity for the sugar amino groups by SrtA. A neomycin-Rev peptide conjugate prepared by this method displayed approximately tenfold or higher affinity toward Rev-responsive element (RRE) RNA than did the unglycosylated Rev peptide. Moreover, the site-specific conjugation of tobramycin to Mrp protein nested with a C-terminal LPNTG tag was successfully achieved.

3.8. Cell-surface protein labeling/engineering

Introducing nongenetically encoded chemical probes such as fluorescent dyes and photoreactive agents into cellular pro-



Scheme 8. A) An approach to synthesize oligopeptide-nucleic acid hybrid molecules. The orientation of the polypeptides and nucleic acid strands can be reversed by exchanging the reaction tags. B) An example, by Pritz et al., of the sortase-mediated preparation of a PNA-cell penetrating peptide (MAP) conjugate.[57]

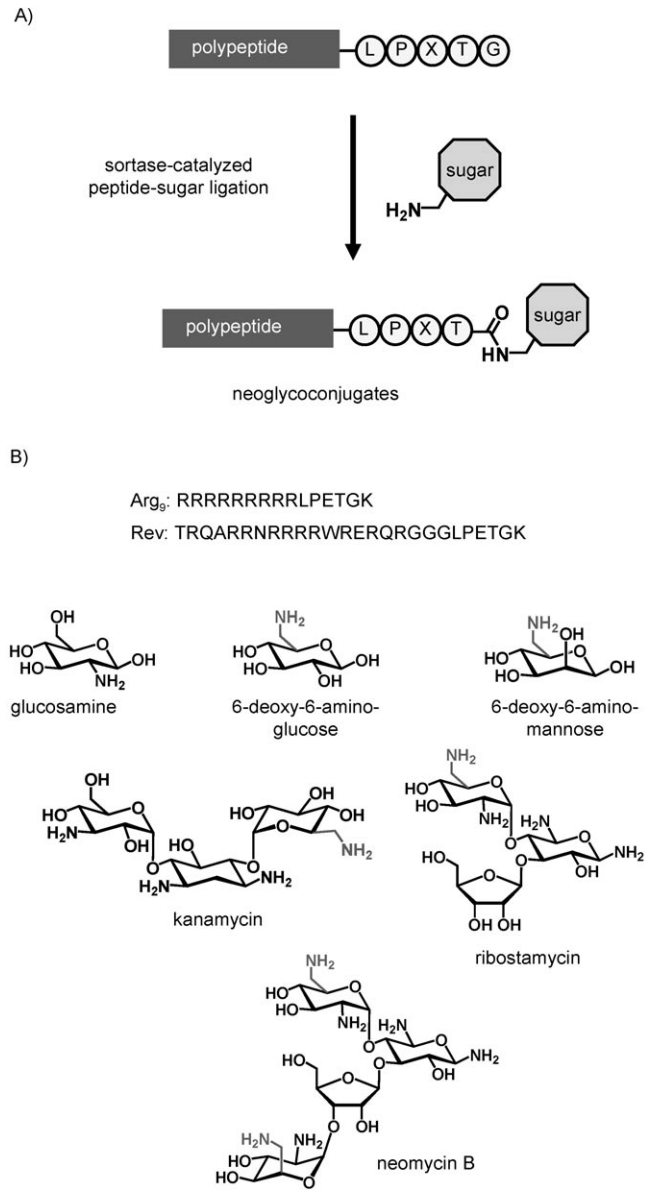
teins is now recognized as a powerful means to investigate fundamental biological issues that are not easily addressed by genetics-based methods.[81] Consequently, much effort has been directed to the development of new strategies for cellular protein labeling. Compared to recent significant advances in peptide-/protein-tag fusion[6,7] and nonsense suppression mutagenesis techniques, [8,9] the application of protein ligation to live cell systems has still been very limited.[25,26,32,82] SML should now be added to the toolbox for cellular protein ligation (Scheme 10).

Ploegh and co-workers reported the selective chemical labeling of cell-surface proteins using SML (sortagging).[50] Human CD154 (CD40L), a type II membrane protein, appended with a C-terminal LPETG tag, was expressed on the surface of HEK 293T cells and labeled with an α-Gly$_n$-derivatized biotin (**5**) or TAMRA probe (**9**) (Scheme 3B) in serum-supplemented medium containing SrtA. The biotin labeling of a surface-displayed influenza A/WSN/33 neuraminidase was also shown.

In a parallel effort, we applied SML to cell-surface protein engineering (Scheme 10B).[59] Osteoclast differentiation factor (ODF), a type II membrane protein also known as TRANCE or RANKL, was chosen as our target protein and equipped with an extracellular C-terminal LPETGG motif (ODF-LPETG). After it was expressed in HEK 293T cells, the selective attachment of α-Gly$_3$-tagged biotin (**15**) or AlexaFluor 488 (**16**) to ODF-LPETG was achieved on the cell surface with no cytotoxic effects. It is important to note that the incubation of the cells with just 10 μM of probe and 30 μM of SrtA at 37 °C for 4 h was enough to detect considerable levels of labeled products. Notably, the biotin-labeled ODF could be detected even after only 5 min of incubation. Thus, this technique could be used in the future for applications such as pulse-chase labeling and receptor trafficking experiments. This strategy was generally applicable to other cell lines including CHO and HeLa cells and could be performed in serum-containing or -free medium or in PBS. Interestingly, not only small molecule probes but also a large recombinant protein, α-Gly$_5$-GFP (**17**), was successfully ligated to ODF-LPETG, providing new opportunities for the semisynthesis of membrane proteins on living cells.

4. Summary and Outlook

As well-exemplified by fluorescent proteins,[83] protein splicing (inteins),[24] and the FKBP12-rapamycin-FRB complex,[84] the discovery of a novel type of protein or molecular process sometimes provides a significant breakthrough in making previously difficult or impossible tasks quite easy to perform and opens entirely new directions of application. Sortase might be such a case. The requirement for SML is two natural motifs, a C-terminal LPXTG sequence and an N-terminal glycine oligomer, both of which can be readily incorporated into small molecules by standard chemical (peptide) synthesis and into proteins by genetic means. SML proceeds under physiological conditions (pH 6.0–8.0) with reasonable concentrations of reactants (ca. 10–100 μM of protein and 10 μM–10 mM of probe) and SrtA (<200 μM). Typically, by adding an excess of the probe of interest, moderate to excellent yields are obtained in several hours. According to the report by Pritz and co-workers,[57] the presence of 20% DMSO or polyethylene glycol (PEG) does not affect sortase activity, which will be useful when the solubility of substrates in aqueous buffers is poor. In some cases, molecules bearing an aminomethylene (NH_2-CH_2-) group are accepted as a substrate surrogate, although more investigations are needed to reveal the structural specificity of SrtA toward this type of substrate. As we saw in Section 3, it is now possible to attach many kinds of natural/unnatural functionalities, ranging from small compounds to large proteins and solid supports, to polypeptides of interest by using SML. Transpeptidation can also be used as a tool for circularizing or specifically cleaving recombinant proteins. Most importantly, SML has proved to be applicable to engineer, that is, label or semisynthesize, proteins not only in test tubes but also in crude cell lysates and even inside bacterial cells and on the surface of living mammalian cells.



Scheme 9. A) Sortase-catalyzed peptide-sugar ligation to obtain neoglycoconjugates. B) Representative examples of oligopeptides, aminosugars and aminoglycoside antibiotics used in the study by Roy and co-workers.[58]

To date, the use of SML for protein labeling has been mostly limited to C-terminal modification with LPXTG-tagged proteins and α-Gly_n-derivatized probes. However, there appears to be no technical reason to prohibit N-terminal modification. The expression of proteins having an N-terminal glycine residue(s) might not always be an easy task, but should be accomplished by making use of (endogenous) methionyl amino-peptidase (MAP),[85] self-cleaving intein fusion,[86] or sequence-specific proteases such as factor Xa[87] and tobacco etch virus (TEV) protease.[88] Alternatively, as inspired by Mao's work (Section 3.5), the sortase-catalyzed, LPXTG motif cleavage reaction in the presence of oligoglycine (or hydroxylamine[38]) could also be used to generate α-Gly_n-appended proteins for subsequent SML reactions.

Although all the studies with SrtA reported to date have been proof-of-concept type experiments, SML has already established itself as a powerful platform for the chemical modification of peptides and proteins. One of the most important challenges remaining in this area is to extend its utility to intracellular proteins inside living mammalian cells. Unfortunately, our preliminary attempts to express SrtA or its fusion variants in cultured mammalian cells have so far been unsuccessful;[55] this suggests that additional engineering of the sortase appears to be required for the SML technique to be performed in mammalian cell systems. In addition, the inevitable incorporation of an extra LPXT$(G)_n$ motif could restrict the ligation sites to those near the N or C terminus or possibly within the loop regions of the proteins. Nonetheless, we can certainly anticipate that SML will facilitate advances in a number of research fields including chemical biology, proteomics, biomedicine, and biotechnology though the generation of various types of protein-/peptide-based molecular tools and therapeutic drugs. Of course, we can freely combine the approach with other protein engineering techniques. Finally, we add that sortases other than SrtA with distinct recognition motifs might be discovered in the future from Gram-positive bacteria[33,35] or through sortase engineering,[89,90] which should further enhance the potential of the SML technology.

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Keywords: bioconjugation · bioorganic chemistry · ligases · protein engineering · protein ligation

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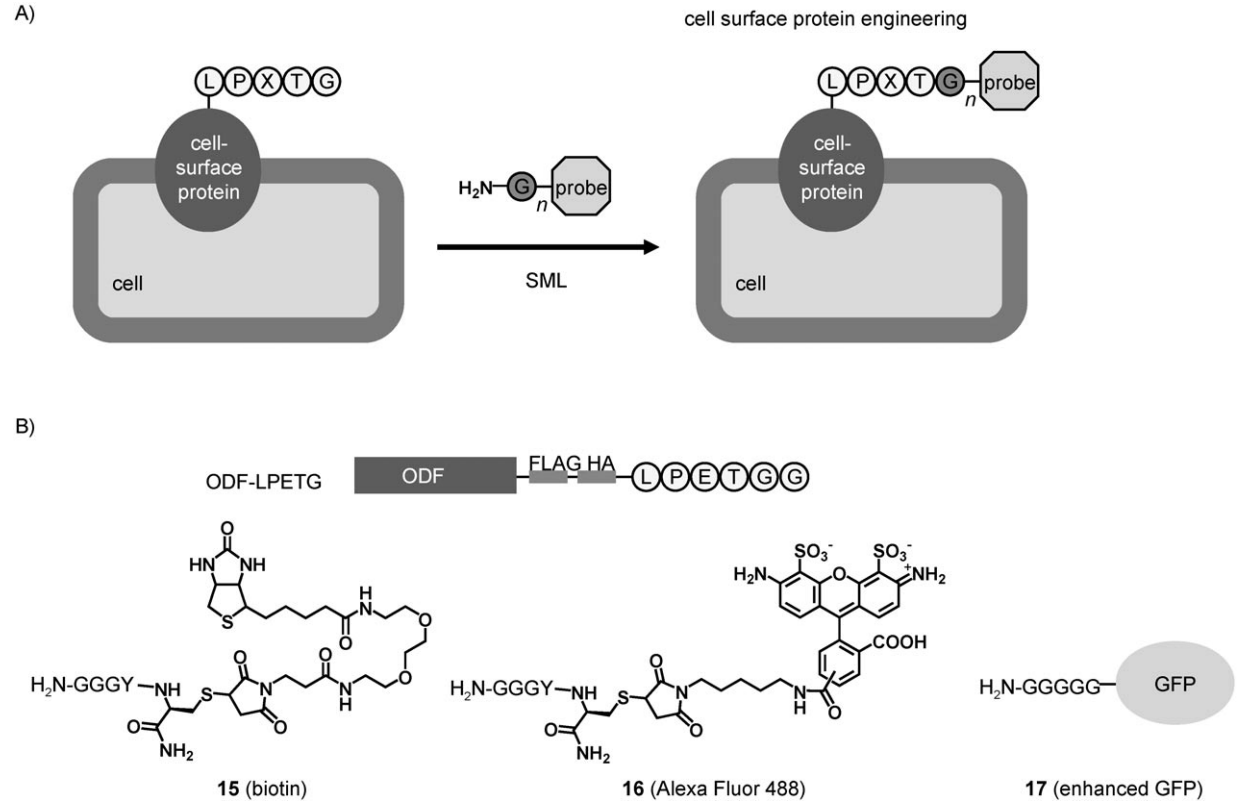

Scheme 10. A) Cell-surface protein engineering/labeling by SML on living cells. B) ODF construct and α-Gly$_n$-tagged probes used in the study by the Nagamune/Tsukiji group.[59] Ploegh et al. also demonstrated the labeling of cell-surface-expressed CD40L and neuraminidase with probes **5** and **9** shown in Scheme 3 B.

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