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Enhancing native chemical ligation for challenging chemical protein syntheses

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Abstract

Native chemical ligation has enabled the chemical synthesis of proteins for a wide variety of applications (e.g., mirror-image proteins). However, inefficiencies of this chemoselective ligation in the context of large or otherwise challenging protein targets can limit the practical scope of chemical protein synthesis. In this review, we focus on recent developments aimed at enhancing and expanding native chemical ligation for challenging protein syntheses. Chemical auxiliaries, use of selenium chemistry, and templating all enable ligations at otherwise suboptimal junctions. The continuing development of these tools is making the chemical synthesis of large proteins increasingly accessible.

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Native chemical ligation, Auxiliary-mediated ligation, Templated ligation, Traceless templated ligation, Chemical protein synthesis, Peptide ligation, Solid-phase peptide synthesis, Mirror-image proteins, Selenocysteine, Diselenide-selenoester ligation.

Abbreviations

NCL, Native Chemical Ligation; CPS, Chemical Protein Synthesis; KAHA, α-Ketoacid-Hydroxylamine; STL, Ser/Thr Ligation; MPE, 2-mercapto-2-phenethyl; HPLC, High-Performance Liquid Chromatography; TCEP, Tris(2-carboxyethyl)phosphine; rDSL, Reductive Diselenide-Selenoester Ligation; DSL, Diselenide-Selenoester Ligation; Sec, Selenocysteine; Fmoc, 9-Fluorenylmethoxycarbonyl; Boc, *tert*-Butoxycarbonyl; Mob, *p*-Methoxybenzyl; Oligos, Oligonucleotides; PNA, Peptide Nucleic Acid; UV, Ultraviolet; CAN, Click-Assisted NCL; SPAAC, Strain-Promoted Alkyne–Azide Cycloaddition; DBCO, Dibenzocyclooctyne.

Introduction

Proteins produced using chemical protein synthesis (CPS) enable atomic-level customization that is not readily accessible with recombinant methods. For example, synthetic proteins can be built with diverse nonnatural functionalities including mirror-image (D-) amino acids and complex posttranslational modifications. Synthetic proteins have been used to answer interesting biological questions, create new mirror-image proteins, and develop D-peptide therapeutics [1–4]. Solid-phase peptide synthesis can produce high-quality peptide segments of up to ~50 amino acids. Larger synthetic proteins require chemoselective ligation methods.

Native chemical ligation (NCL) [5] is the most commonly used ligation method because of its simplicity in creating a natural amide bond between two unprotected peptide segments (for a comprehensive review see Ref. [3]). NCL requires the combination of a C-terminal peptide thioester with a peptide containing an N-terminal Cys residue. After the initial transthioesterification reaction, an irreversible S-to-N acyl shift occurs through a 5-membered ring intermediate, linking the two peptides. To overcome the relatively low abundance of Cys in proteins, Ala residues can be temporarily replaced with Cys for NCL, followed by desulfurization to regenerate Ala [6,7]. More recently, unnaturally thiolated amino acids have been synthetically prepared and used to expand possible ligation junctions, as reviewed [8]. However, these have several drawbacks that have limited broad usage (e.g., slow ligation kinetics and complex reagent syntheses).

Although CPS has been used to produce many interesting proteins, its scope is still limited, especially for larger protein targets (>300 residues). This challenge is mainly because of the slow kinetics of NCL, which requires high (mM) peptide concentrations [3]. The poor and unpredictable solubility of some peptide segments (e.g., colloid formation observed in the synthesis of DNA ligase [9]) presents a serious challenge. This issue precludes obtaining the required concentrations, resulting in poor product yields and complex purifications because of competing side-reactions (e.g., thioester hydrolysis, side-chain oxidation, and aspartimide formation). As a result, only a few large proteins have been successfully made through CPS (DapA [10], Dpo4

[11,12], tetraubiquitins with various linkages [13–15], tetraubiquitinated α-globin [14,16], and branched pentaubiquitin and hexaubiquitin [15]). Two general strategies have emerged to address these challenges. Poorly soluble peptides can be functionalized with solubilizing groups that enable optimal NCL concentrations to be reached (see recent reviews [14,17–19]). Alternatively, as covered in this review, otherwise suboptimal NCL junctions can be rescued using chemical auxiliaries, selenium-based chemistry, or templating. Here, we focus primarily on NCL, as it is the most commonly used ligation technique.

Auxiliary-mediated peptide ligations

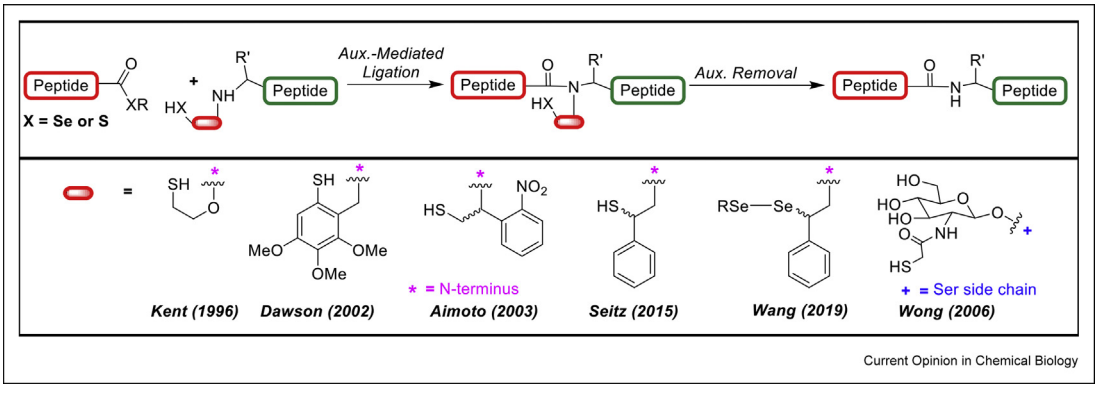
In recent years, novel amide-forming reactions have been developed to complement and expand peptide ligation beyond traditional NCL junctions [20,21]. To be widely applicable, these new reactions must be chemoselective to terminal functional groups and amenable to general CPS conditions (e.g., acidic and neutral aqueous buffers containing high denaturant). To maintain NCL's excellent chemoselectivity, a popular approach is to expand S-to-N acyl shift options, though larger transition-state ring size and increased steric bulk hamper reactivity. Chemical auxiliaries or nonnative thiol-containing modifications for peptide ligation were first documented by the Kent group at Xaa-Gly and Gly-Xaa junctions. They used an N-terminal oxyalkyl moiety that transitions through a 6-membered ring and can be removed with Zn [22] (Figure 1). Numerous auxiliaries have since been developed, most of which are attached to peptides via the N-terminal amine [23]. Some auxiliaries of note include Dawson's 4,5,6-trimethoxy-2-mercaptobenzyl scaffold, which can be removed with trifluoroacetic acid [24]. The Aimoto and Muir labs expanded on this mercaptobenzyl design by adding nitro groups to the benzyl ring, which renders the auxiliary photolabile [25,26]. Wong's sugar-based auxiliary goes through a 14- or 15-membered transition state ring and allows ligation with *O*- or *N*-linked Ser, Thr, and Asn residues [27,28,29].

Although auxiliaries have been used during many CPS projects, issues remain that limit their general use. For example, after ligations, the acidic removal conditions required for some auxiliaries can cause a reversed N-to-S acyl shift. Also, many auxiliaries allow ligations at Gly-containing junctions but proceed much more slowly at bulkier sites. In a recent attempt to design an auxiliary with broader utility, the Seitz group introduced the 2-mercapto-2-phenethyl (MPE) design. MPE can be removed after ligation in one pot using Tris(2-carboxyethyl)phosphine (TCEP) and morpholine at basic pH and was used to synthesize the antimicrobial peptides dermcidin DCD-1L (Gly-Gly) and opistoporin-2 (Ser-Glu) [30]. It was also used in the clever HPLC-free synthesis of various MUC1 domains (His-Gly) [31]. To investigate the effect of 5- and 6-membered transition states, as well as α- and β-substituents, a variety of auxiliaries were compared with MPE [32]. MPE appeared to be the most efficient design and expanded the possible ligation junctions past Gly (e.g., Leu-Asn). The simple installation, comparable kinetics to traditional NCL (using low mM peptide), and facile removal make MPE an attractive option when combating difficult peptide ligations. In future CPS projects, MPE and other auxiliaries can help access proteins that otherwise would be hampered by poor ligation junctions.

Substituting sulfur with selenium increases ligation reactivity

Chemical ligation efficiency can be enhanced by substituting the thiol with the more reactive selenium (Se). Compared with S, Se has increased nucleophilicity and lower reductive potential [33,34]. As a result, sterically hindered junctions, such as Val, Ile, or Pro C-terminal thioesters [35], can be ligated more quickly. Additionally, Se-peptides exist as diselenide dimers that remain unreactive until the addition of an external reductant, and the Se–C bond can be chemoselectively cleaved without desulfurization of unprotected Cys [34,36,37].

Figure 1



Summary of CPS auxiliaries discussed in this review.

Se ligations proceed through a mechanism similar to NCL, but Se is much faster, enabling use of lower peptide concentrations. The Payne group recently demonstrated this concept with reductive diselenide-selenoester ligation (rDSL) performed with model peptides at extraordinarily low concentrations (50 nM) [37]. Initially, they noted their additive-free diselenide-selenoester ligation (DSL) [34,38–41] performed better than NCL at mM peptide concentrations, but this advantage was lost at μM levels [37]. They postulated that an increased amount of the phenylselenoate species that liberates the active selenopeptide would increase the rate of DSL at lower concentrations, as was observed in the presence of TCEP and diphenyldiselenide. Similar success was observed with the lipidated therapeutic tesamorelin (at 125 μM) and two palmitylated variants of the membrane protein phospholemman (at 60 μM). After rDSL, deselenization can be performed with a one-pot several-minute photolytic Se–C cleavage (254 nm, 35 W) to produce the native Ala. Payne recently reported the synthesis of other selenated amino acids (e.g., Phe, Leu, Pro, Glu, and Asp), though these are not yet commercially available [38–41]. While Se-based ligations appear quite promising, several issues hamper their immediate utility. First, selenocysteine (Sec) derivatives are expensive (~$1000/g for either Boc- or Fmoc-Sec(Mob)-OH), and unnatural selenated amino acid derivatives require complex syntheses. Selenol intermediates are also more sensitive to oxidative side reactions and thus require more careful anaerobic conditions or the use of oxygen scavengers during handling [41,42]. Despite these issues, Se-based ligations offer significant improvements over traditional NCL and, with improved availability, will likely enjoy more use in CPS.

Using the advantages of Se reactivity and auxiliary-mediated ligation, the Wang group demonstrated successful ligations at numerous sterically hindered junctions [43]. By substituting the thiol in Seitz's MPE with Se (Figure 1), they first tested short peptides and reported a rapid Ile-Leu ligation followed by a mild TCEP/morpholine auxiliary removal step. They then used this tool to synthesize a more difficult protein target: the 127-residue human granulocyte-macrophage colony-stimulating factor [44]. In this synthesis, they combined their Se auxiliary-mediated ligation (Gln-Glu) with traditional NCL to yield the full-length protein-bearing multiple *O*- and *N*-glycosylated residues, as well as two disulfide bonds.

Templated ligations

An alternate strategy for increasing NCL efficiency is templating, in which two peptides are brought into close proximity through interactions with themselves or an external template (Figure 2). As a result, the effective concentration of the reactive groups is dramatically increased, enabling efficient peptide ligation at much lower concentrations, even for sterically hindered thioester ligation sites. Although several templated ligation strategies have been developed, they are not yet optimized for general CPS applications, as discussed below.

Protein-templated ligation

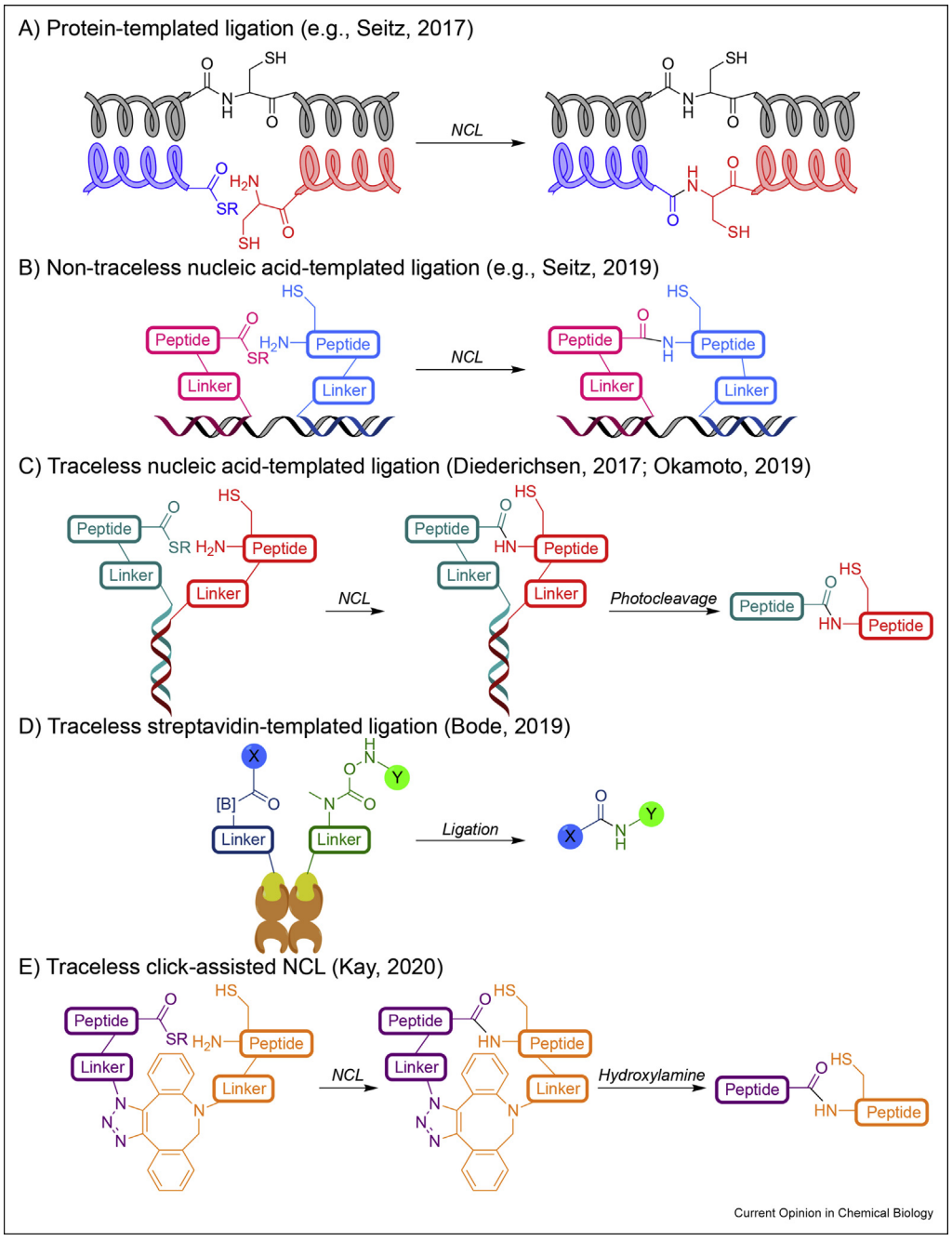
The concept of templated NCL was first demonstrated by the Ghadiri group [45,46] using a method known as protein-templated ligation. The ligated product autocatalyzed NCL, as interhelical hydrophobic interactions between the product and the two component peptides allowed the product to act as a template (Figure 2a). This methodology was extended to be triggered under acidic conditions [47], using β-sheets [48], or to enable the component peptides to template themselves [49–52]. Petszulat and Seitz recently developed fluorogenic NCL to determine ideal conditions for protein-templated ligations in which they rapidly analyze different reaction conditions at low peptide reagent concentrations (5 μM) [53]. The thioester-containing peptide has both a fluorophore (Lys functionalized with fluorescein at the C-terminus) and a quencher (integrated into the C-terminal thioester leaving group) such that NCL causes an increase in fluorescence. Using this technique, the Seitz group characterized this method's distance and linker flexibility requirements.

Protein-templated ligations are currently being used for a variety of applications, including specific labeling of proteins in live cells [50,51,54,55] and protein detection within cell lysates [56] (see recent reviews [57,58]). However, the use of protein-templated ligations for CPS has been limited, as the denaturant needed for peptide segment solubilization typically disrupts the protein structure required for templating. In addition, most peptide segments will not naturally interact with each other or an external protein template. Separate templating peptide tags could be incorporated but would need to be removed to produce the native protein.

Nucleic acid-templated ligation

Nucleic acids have also been used as a ligation template, as the specific base pairing between oligonucleotides (oligos) brings conjugated molecules into close proximity (Figure 2b). The first such example was presented by Joyce's group using a DNA template to direct the amide-forming ligation between a DNA oligo and a peptide–oligo conjugate [59]. While this templating strategy demonstrates that a low concentration (~3 μM) DNA oligo can ligate with a peptide–oligo conjugate, the DNA cannot be removed from the peptide after ligation. The Seitz group has extensively used peptide nucleic acids (PNAs) to template NCLs for a wide variety of applications [60–63], including detection of specific DNA/RNA sequences and formation of

Figure 2



Templated ligation strategies discussed in this review. (**a**) Protein-templated ligation (e.g., Seitz, 2017). (**b**) Non-traceless nucleic acid-templated ligation (e.g., Seitz, 2019). (**c**) Traceless nucleic acid-templated ligation (Diederichsen, 2017; Okamoto, 2019). (**d**) Traceless streptavidin-templated ligation (Bode, 2019). (**e**) Traceless click-assisted NCL (Kay, 2020).

bioactive molecules (e.g., proapoptotic peptides). Sayers, Payne, and Winssinger have also demonstrated the utility of PNA-templating for microRNA detection via a DSL ligation [64]. Excitingly, PNA-templating enables rapid, efficient ligations at nM-pM template concentrations, but this strategy has only been used with short peptide probes in nondenaturing conditions, and the PNA linkers cannot be removed after ligation.

In an effort to create traceless templated ligations (Figure 2c), Diederichsen's group used UV-cleavable ortho-nitrobenzyl linkers and PNAs to perform templated NCL with short model peptides [65]. The

Okamoto lab used a similar UV-cleavable linker with DNA handles to perform a one-pot carbodiimide-mediated ligation of three peptides [66]. Both groups observed successful ligations at low peptide concentrations (1−100 μM). However, the CPS scope of these strategies is limited because of complex linker syntheses and potential oxidative damage from UV irradiation and copper-catalyzed click reactions [67,68]. Additionally, because the carbodiimide ligation is not chemoselective or regioselective, it is not compatible with unprotected carboxylic acid or amine side chains.

Templating with streptavidin binders

Recently, the Bode group developed an interesting ligation method using streptavidin to template biotinylated molecules (Figure 2d) [69]. Using acylboronates and *O*-acylhydroxylamines functionalized with desthiobiotin linkers, the two reagents are forced into proximity upon streptavidin binding. The acylboronate−hydroxylamine ligation then rapidly occurs to form an amide bond. Importantly, this method is traceless, as amide-bond formation causes cleavage of the desthiobiotin linkers and enabled efficient ligation, even at low reagent concentrations (500 nM). While streptavidin templating looks promising, its robust CPS applicability has not yet been established (e.g., using full-length peptide segments in the presence of denaturants).

Templating with click chemistry

Our group recently developed Click-Assisted NCL (CAN, Figure 2e), a unique templated ligation strategy [70]. In CAN, peptides are functionalized with traceless Lys linkers [71] containing copper-free strain-promoted alkyne−azide cycloaddition (SPAAC) handles (dibenzocyclooctyne [DBCO] or primary azide). The strain-promoted click reaction effectively templates two reagent peptides together, enabling rapid NCL. The click linkers are then removed through aqueous hydroxylamine treatment, generating the native ligated protein. Excitingly, efficient NCL was observed using 50 μM clicked model peptides. Using CAN, four reactions (SPAAC, NCL, desulfurization, and linker cleavage) were completed in one pot to synthesize the *E. coli* ribosomal subunit L32. Furthermore, CAN is broadly applicable for CPS applications, as the click linker synthesis is simple and robust, the strained-click reaction can be performed in typical peptide-solubilizing denaturants, the clicked linkers are stable to standard NCL conditions, and linker removal is chemoselective and traceless. Increased linker length and distance from ligation site modestly decreased templated ligation efficiency, indicating that linker positioning should be flexible. A current limitation of CAN is that DBCO peptide synthesis can result in minor Met oxidation, though this can be overcome by substitution with isosteric norleucine. While CAN has been demonstrated for a single ligation, the ultimate goal is to enable multiple concurrent ligations to avoid repeated yield-sapping HPLC purifications. Such a system will require a second pair of bioorthogonal, NCL-compatible reactive handles.

Conclusions

Improving NCL by expanding accessible ligation junctions and enhancing efficiency through the methods described here will widen the scope of CPS to enable the routine synthesis of large proteins. Thiolated and selenated amino acids are a promising avenue toward this goal, especially when considering the incredible rates exhibited by Payne's rDSL reaction [37]. However, the current lack of commercial availability of thiolated and selenated amino acid reagents hinders widespread use. Chemical auxiliaries offer another option to expand ligations to sterically unhindered ligation junctions, but further improvements are needed before widespread adoption (e.g., faster kinetics and commercial availability of reagents). Seitz's MPE [30] is a major advance in the auxiliary field because of its marked reactivity and simple removal conditions. Incorporating Se into chemical auxiliaries appears poised to increase auxiliary reaction kinetics even further [41]. Templated ligations can dramatically increase NCL efficiency, but their CPS application is often limited because of reaction incompatibilities and the inability to remove templates from the ligated product. Early developments of traceless templated ligations are promising but will require additional research for general CPS use.

Ultimately, these chemical auxiliaries, Se-based reagents, and templating approaches allow new ligation strategies using nontraditional junctions. Combining these distinct ligation chemistries with each other and other amide-forming ligations (e.g., Bode's α-ketoacid-hydroxylamine (KAHA) [72] or Li's Ser/Thr ligation (STL) [73]) opens new possibilities for one-pot multistep ligations. As the ligation chemistry toolkit expands, updated versions of computational tools, like our Automated Ligator (Aligator) [74], will be increasingly valuable for identifying optimal synthesis strategies from the enormous number of possibilities. While there is always room for improvement, the strategies described here provide more ligation junctions with improved kinetics, giving researchers additional options to tackle ambitious CPS projects.

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Declaration of competing interest

Nothing declared.

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