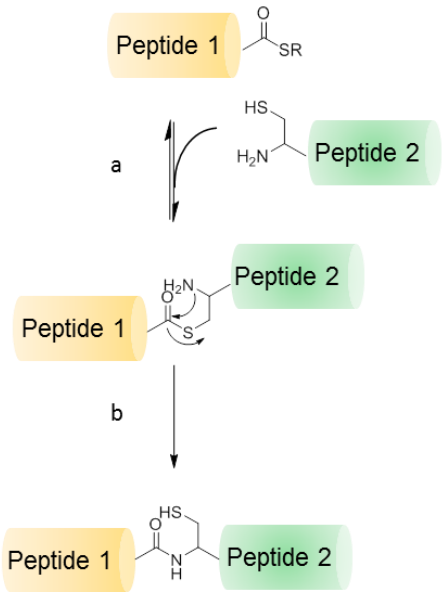
Native Chemical Ligation and Beyond: Recent Developments in Chemical Protein Synthesis

Can Araman

Gorlaeus Laboratories, Einsteinweg 55, 2333 CC Leiden, Netherlands, e-mail: m.c.araman@lic.leidenuniv.nl

The total chemical synthesis of proteins has been one of the most challenging topics of organic chemistry in the 20th century. Among others, one method has revolutionized the field of chemical protein synthesis: In 1994, Kent and coworkers developed this technique called native chemical ligation (NCL), [1] thorough which two unprotected peptide segments can be conjugated in a chemoselective fashion. The reaction was based on Wieland`s observation made in 1953. [2] The researchers reported that under aqueous conditions highly reactive peptide arylthioesters can react with cysteine and its derivatives under the formation of a native peptide bond.

In NCL, typically one unprotected peptide carries a C-terminal thioester, which can be reacted with another unprotected peptide harboring an N-terminal cysteine leading to the formation of a native peptide bond. In the first and reversible step (Scheme 1a), a thioester intermediate is formed, which then undergoes a spontaneous S-to-N acyl shift, yielding the peptide bond (Scheme 1b). The discovery of NCL has expanded the scope of chemical protein synthesis immensely over the past two decades, enabling the synthesis of proteins with more than 300 amino acids (aa). Moreover, proteins containing posttranslational as well as artificial modifications have been synthesized. [2] In this commentary, I will give brief information on some limitations of NCL and recent developments to overcome those, namely deprotection of orthogonal side-chain protecting groups (Maity *et al. Angew. Chem. Int. Ed. Engl.*, 2016, **55**, 8108-8112.) and HPLC-free purification of peptides and proteins (Loibl *et al. Chem. Sci.*, 2016, **7**, 6753-6759).



Scheme 1: *Native Chemical Ligation (NCL)*

Despite its major impact in chemical protein synthesis, NCL has its limitations such as the demand for a cysteine residue at the ligation junction site. Cysteine is the second least abundant amino acid and is, therefore, not predominantly found in common protein sequences. To overcome this issue, distinct laboratories developed metal-assisted [3] and metal-free [4] reductive desulfurization methods to reduce cysteine residues to native alanine after NCL, which is the second most abundant amino acid. However, these methods require the use of orthogonal protecting groups on other cysteines distinct from the one on the N-terminus. Acetamidomethyl (Acm) is a commonly used, orthogonal cysteine protecting group for reductive desulfurization. Nevertheless, Acm cleavage occurs under harsh conditions, is relatively time consuming, and cannot be performed without isolating the peptide prior to deprotection. Recently, Maity *et al.* obtained a solution to these problems through the development of a fast and mild one-pot Acm deprotection strategy using $PdCl_2$ (Figure 1). [5]

Briefly, the authors tested the Acm cleavage efficiency of two Pd(II) complexes using a designed peptide CLYRAGC(Acm)LYRAG in Guanidine-HCl (Gdn-HCl), at pH=7.0-7.2. Two interesting observations were made; 1) $PdCl_2$ gave a superior cleavage efficiency compared to $[Pd(allyl)Cl]_2$ and 2) $PdCl_2$ was more soluble in Gdn-HCl than in H_2O. Based on these results, a series of one-pot sequential NCL reactions with short peptide sequences and subsequent Acm deprotection have been carried out to optimize the cleavage conditions. Employing optimal conditions, the authors could to synthesize a ubiquitin-like protein 5 (UBL-5).

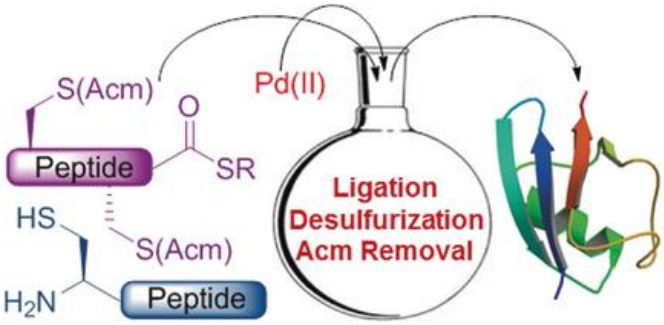
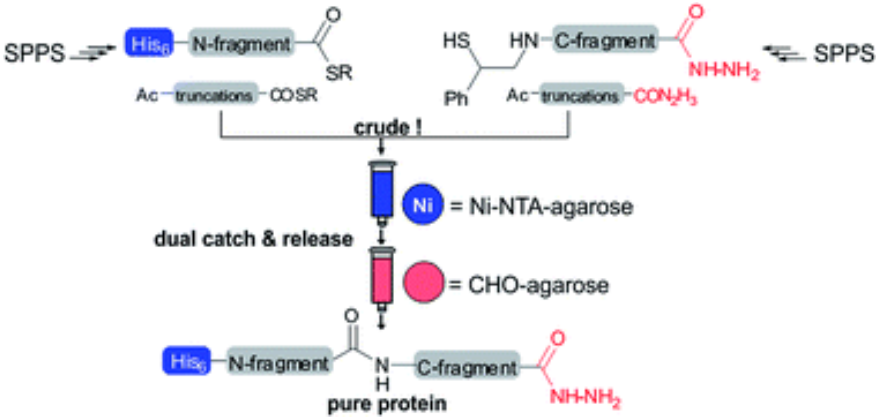


Figure 1: *Pd(II) mediated one-pot ligation, desulfurization and Acm removal strategy* [5]

Two segments, a UBL-5 thioester carrying the first 32 aa (1-32) and harboring native cysteines (Cys6 and Cys18, both Acm protected) as well as UBL-5(33-73) with Ala33→Cys33 mutation were synthesized. Subsequently, both segments were ligated, desulfurized and the Acm groups were removed in a one pot reaction. The deprotection of Acm occurs in less than 15 min. In 2016, Brik and coworkers published three articles on the utilization of Pd(II) complexes for cleavage of protecting groups such as thiazolidine, propargyloxycarbonyl and Acm. [5-7] The fast and mild Acm removal reaction is not only a versatile method for orthogonal protecting group chemistry but also opens up new possibilities for biorthogonal cysteine protection.

One of the other requirements of NCL is the necessity to purify starting materials and products via HPLC. However, HPLC purification is time-consuming and is associated with low yields. Hence, one-pot sequential ligations with HPLC-purified peptides and a final HPLC purification of the product have been shown to be a solution to this problem. However, no HPLC-free chemical protein synthesis was reported until recently. In their publication, Oliver Seitz`s lab reported the chemical synthesis of MUC1 repeat proteins without using any HPLC purification, neither for peptides nor the final product. [8] The method is based on the purification of the crude peptide α-thioester via immobilized metal affinity chromatography (IMAC) and the peptide hydrazide carrying a thiolated auxiliary via hydrazone ligation on aldehyde functionalized agarose beads (Scheme 2).



Scheme 2: *HPLC-free chemical protein synthesis* [8]

The first part of purification involves the attachment of an IMAC purification tag, His_6-tag respectively. In general, capped truncation products can easily be washed out using aqueous buffers. The peptide harboring the His_6-tag can remain bound to the resin and be used for further NCL. In a small-scale cleavage reaction, the researchers showed that the peptide thioester purified via IMAC could be observed in great purity.

Subsequently, the NCL reaction was performed. Here, a peptide hydrazide loaded with a thiolated auxiliary was synthesized, which was previously developed by this research group and shown to be successfully cleaved after ligation.[9] The crude product of NCL was purified via immobilization of the hydrazide peptides on an aldehyde functionalized resin. Unreacted starting materials were eliminated via washing steps. Seitz and coworkers wanted to push the limits of their method. To do that, they aimed the chemical synthesis of MUC1 repeat proteins with distinct tandem repeat lengths. The researchers could obtain 46 to 126 amino acid long MUC1 tandem repeat proteins in only three days, without using RP-HPLC purification. Moreover, the overall yields and the purity of these proteins were similar to those of HPLC-purified proteins, whereby the synthesis and purification time was significantly decreased. Even though non-native modifications such as His_6-tag and a C-terminal hydrazide are present, the method provides very short synthesis times, good to excellent purity and yields (8-33%). Taken together, Seitz and coworkers have introduced a valuable and elegant method which may enable the synthesis of protein libraries in relatively short times.

In conclusion, I discussed two recent publications regarding their asset on total chemical protein synthesis. Brik and coworkers have succeeded in development of a fast and orthogonal Acm deprotection method, whereas Seitz lab performed the first HPLC-free chemical synthesis of a protein. Both methods deliver fast reaction/deprotection times simplifying the synthesis of modified protein libraries.

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