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Chemical Methods for Protein Ubiquitination

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Abstract In eukaryotic cells, many proteins undergo extensive post-translational modifications (PTMs) such as methylation, acetylation, phosphorylation, glycosylation, and ubiquitination. Among these, ubiquitination is a particularly interesting PTM from both structural and functional viewpoints. In ubiquitination, the C-terminal carboxyl group of the small ubiquitin protein is attached to the ε-amine of a lysine residue of a substrate protein through an isopeptide bond. Ubiquitination has been shown to be involved in the regulation of many cellular processes including protein degradation and gene expression. And dysfunction of these processes is implicated in many human diseases. Despite many years of intensive research, a large number of protein ubquitination events remain poorly characterized. The challenge lies with the tremendous difficulties in isolating homogeneously modified proteins from biological samples for structural and functional studies. Enzymatic ubiquitination in vitro often has limited practical value due to the large number of substrate-specific E3 ligases and the difficulties in identifying or isolating these enzymes. Chemical approaches to the preparation of ubiquitinated proteins provide a powerful solution, and the development of such approaches has been the subject of intense research by many research laboratories. This review summarizes the methodological developments of protein chemical ubiquitination in recent years.

Keywords Post-translational modification · Ubiquitin · Ubiquitination · Site-specific modification · Peptide ligation

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Contents

1 Introduction

As a fundamental mechanism to modulate protein function in the cell, post-translational modification (PTM) regulates almost all biological processes [1, 2]. The side chain ε-amine of lysine provides a platform for several important PTMs such as methylation, acetylation, and ubiquitination [1, 2]. Among these, ubiquitination is a particularly interesting PTM from both structural and functional viewpoints [3–9]. In contrast to most other PTMs such as phosphorylation, methylation, and acetylation, in which the modifying groups are rather simple, ubiquitination involves the transfer of an entire, albeit small, protein onto a protein substrate [3–9]. Ubiquitin is a 76-amino acid protein ubiquitously distributed in all tissues of eukaryotic organisms. In the post-translational ubiquitination process, the C-terminal carboxyl group of ubiquitin becomes attached to the ε-amine of a lysine residue of the substrate protein through an isopeptide bond [3–9]. More interestingly, as any of the seven lysine residues or the amino terminus of ubiquitin can still accept another ubiquitin molecule, the modified proteins often carries polyubiquitin chains of the same or mixed linkages [9]. This gives a great number of possibilities to ubiquitinating cellular proteins. These different ubiquitin modifications have different structural effects which are interpreted by the various effector proteins in the cell and thereby impart many different outcomes to the targeted proteins [9]. For example, K48 polyubiquitination signals for their degradation via the proteasome system, whereas other forms of polyubiquitination or monoubiquitination can change their function, alter their cellular location or trafficking, or inhibit/enhance their interactions with other proteins in a signaling pathway [10, 11]. Biologically, ubiquitination is achieved through the consecutive action of three enzymes – ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) [3–9]. As with other PTMs, ubiquitination is reversible. The biochemical processes of ubiquitination and deubiquitination are tightly controlled, and their dysfunction is implicated in many human diseases. However, despite many years of intense research, a large number of protein ubquitination events remain poorly characterized. A great challenge in the structural and functional analysis of this PTM lies with the tremendous difficulties in isolating

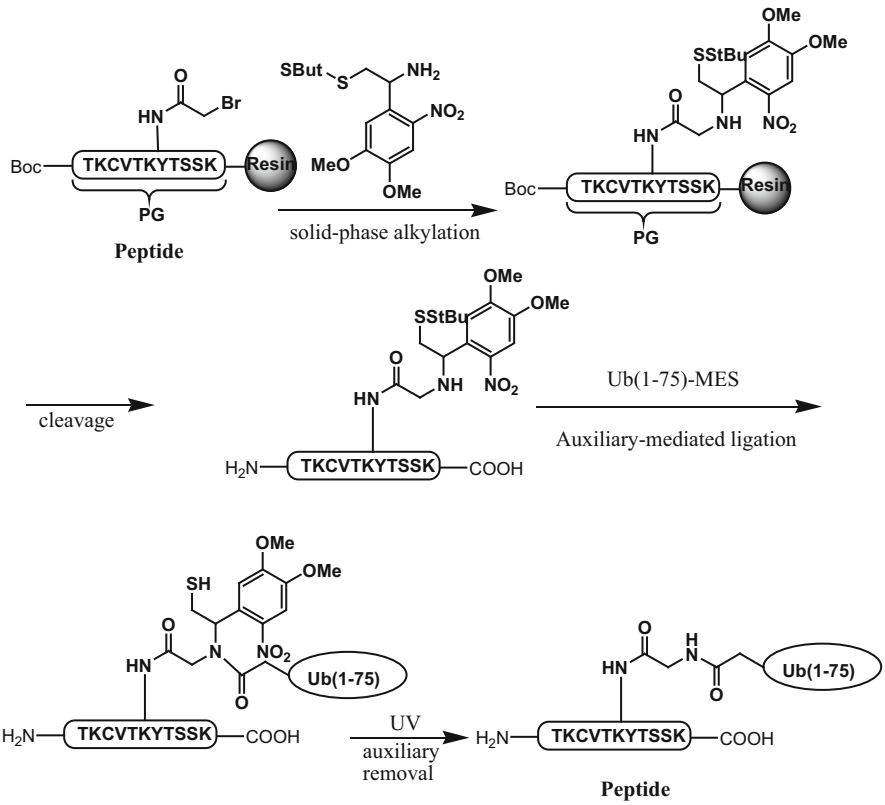
homogeneously modified proteins from biological samples. Because of the difficulties in identifying or isolating the substrate specific E3 ligases, enzymatic ubiquitination in vitro often has limited practical value. Chemical approaches to the preparation of ubiquitinated proteins provide a powerful solution, and the development of such approaches has been the subject of intense research by many research laboratories. This review summarizes the methodological developments of protein chemical ubiquitination in recent years.

2 Preparation of Ub-Proteins with Native Isopeptide Bonds

2.1 Nα-Auxiliary-Mediated Ubiquitination

In 2007, Muir et al. reported the first method of chemical ubiquitination of a synthetic peptide through Nα-auxiliary-mediated ligation [12]. In this method, site-specific ubiquitination was achieved through the ligation at Gly75–Gly76 junction between a ubiquitin(1–75)-thioester and the side chain of a lysine residue pre-acylated by Gly76 bearing an Nα ligation auxiliary in the substrate peptide. After ligation, the photolabile auxiliary was removed by UV irradiation to generate the native isopeptide linkage. With this method, they initially synthesized a ubiquitinated peptide NH_2-**TKCVTKYTSSK**-COOH corresponding to the residues 115–125 of human histone H2B (Scheme 1). Later, they also applied this strategy to the synthesis of full-length Lys120-ubiquitinated H2B via multi-step ligations (Scheme 2) [13]. This method represents the first chemical method for the synthesis of native ubiquitinated peptides/proteins. The only limitation of the method is that the auxiliary-mediated ligation is sluggish as it occurs at a sterically hindered secondary amine.

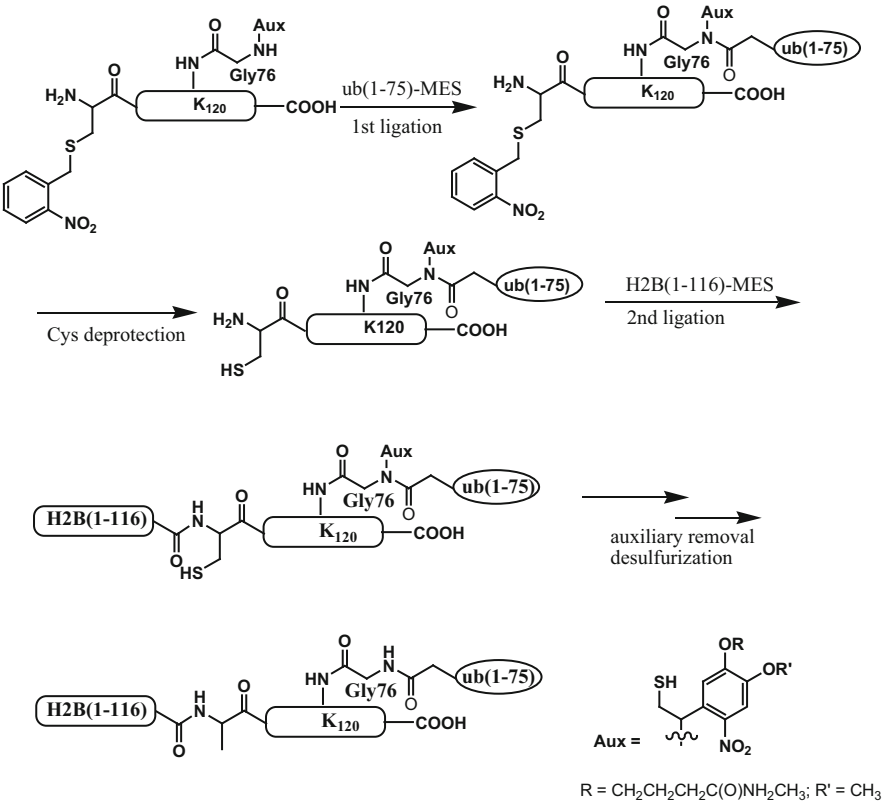
In Muir's auxiliary-mediated ubiquitination approach, the auxiliary has to be introduced onto peptides during solid-phase peptide synthesis (SPPS). When using this method for the synthesis of ubiquitinated proteins, one or more ligation steps may be required to construct the full-length substrate protein bearing the auxiliary. Recently, our group developed an auxiliary-mediated ubiquitination method in which the ligation auxiliary could be introduced onto recombinant proteins genetically (Scheme 3) [14]. In our method, the lysine involved in ubiquitination was first genetically incorporated as azidonorleucine (Anl) into the recombinant protein of interest as mediated by an evolved methionyl-tRNA synthetase. After protecting all the amines with *t*-Boc, Anl was reduced to lysine under mild conditions. A Gly derivative bearing the ligation auxiliary at its α-amine was installed on this lysine side chain through the active OSu ester. Site-specific ubiquitination was then achieved through auxiliary-mediated ligation between ub(1–75)-thioester and the Boc-deprotected acceptor protein. After ligation, the auxiliary group was removed by treating the ligation product with a cocktail of trifluoroacetic acid/triisopropylsilane/H_2O (95/2.5/2.5) (Scheme 3).



Scheme 1 General scheme of Nα-auxiliary-mediated ubiquitination

Besides the synthesis of ubiquitinated peptides and proteins, the Nα-auxiliary-mediated ubiquitination method has also been used for the synthesis of ubiquitin C-terminal derivatives. Recently, Liu et al. reported the semi-synthesis of ubiquitin C-terminal conjugate of 7-amino-4-methylcoumarin (ub-AMC) with such a method [15]. First, a ubiquitin hydrazide with residues 1–75 was generated through hydrazinolysis of a ubiquitin-intein fusion protein. The ub(1–75)-hydrazide was then ligated with Nα-(auxiliary)-Gly-AMC through Nα-auxiliary-mediated ligation. After ligation, the auxiliary was removed to generate the ub-AMC with a native peptide bond (Scheme 4).

The aforementioned Nα-auxiliary-mediated ubiquitination methods all employed substituted benzylamine-derived auxiliaries. Recently, Chatterjee et al. reported an auxiliary-mediated ubiquitination method employing 2-mercaptoethoxyl group as the auxiliary [16]. The 2-mercaptoethoxyl auxiliary was introduced to the α-amine of Gly76 which had been attached to the lysine side chain during SPPS. The ubiquitination was achieved through the reaction between

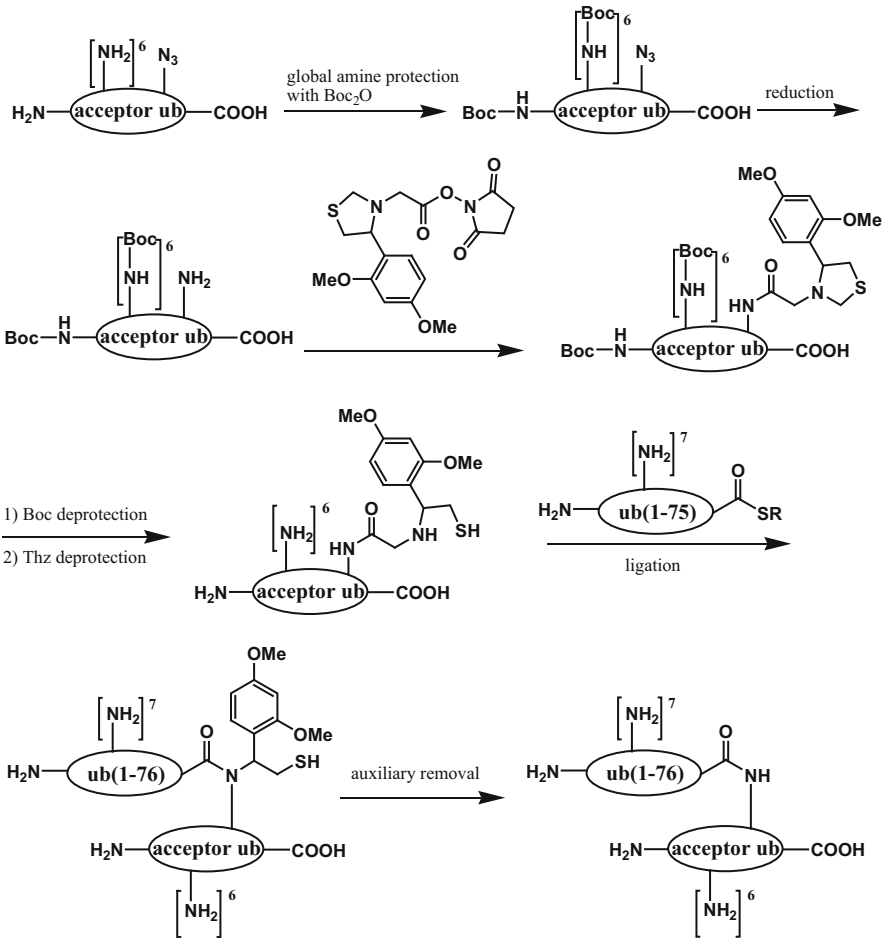


Scheme 2 General scheme for the synthesis of K120 ubiquitinated human H2B through N-α-auxiliary-mediated ubiquitination

ub(1–75)-thioester and the peptide with the auxiliary. After ligation, the auxiliary could be removed by activated Zn in acidic HPLC buffer containing 6 M guanidine hydrochloride (Scheme 5).

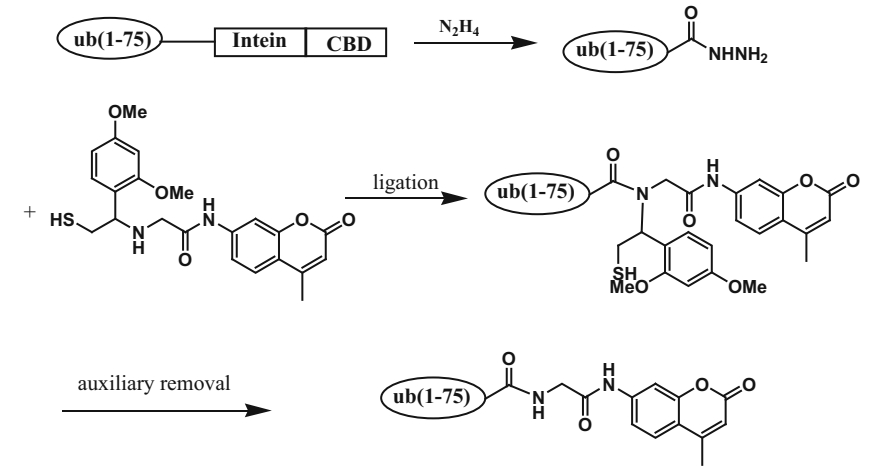
2.2 Thiolated-Lysine-Mediated Ubiquitination

Since the development of the Nα-auxiliary-mediated ubiquitination method, many researchers have been actively seeking the development of alternative and more efficient chemical ubiquitination methods. Our group [17, 18] and Brik's group [19–21] have independently reported the γ- and δ-thiolysine-mediated ubiquitination methods, respectively. In these two approaches, site-specific ubiquitination was achieved through chemical ligation involving full-length ubiquitin(1–76) thioester and the ε-amine of a lysine residue in the peptide

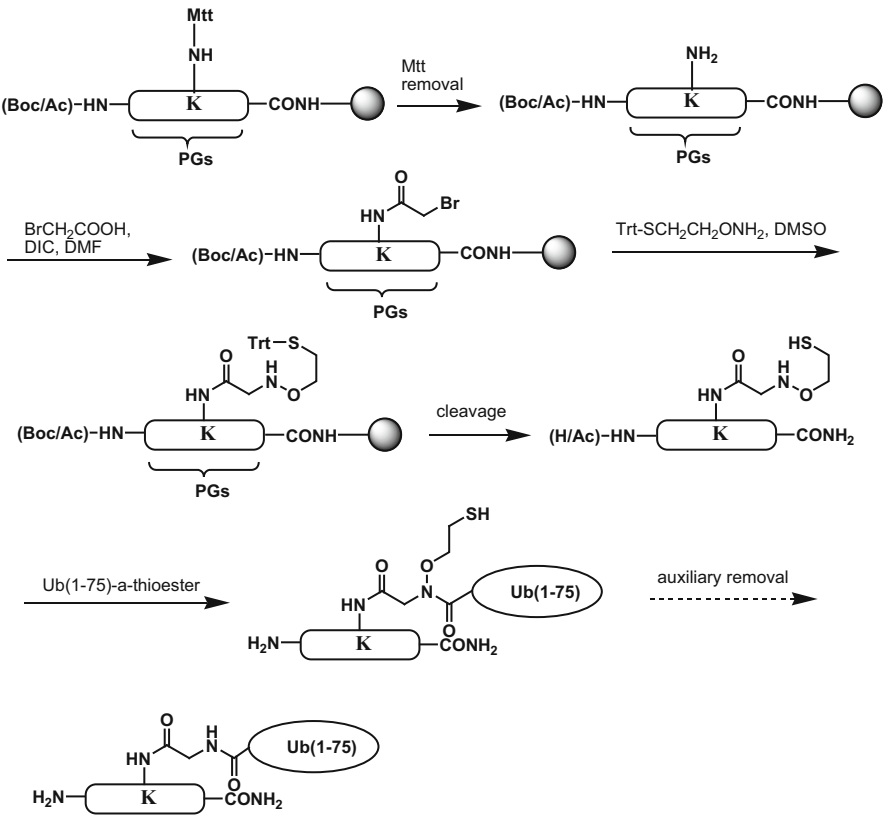


Scheme 3 General scheme for the synthesis of linkage-specific diubiquitins through genetic incorporation of azidonorleucine and Nα-auxiliary mediated ligation

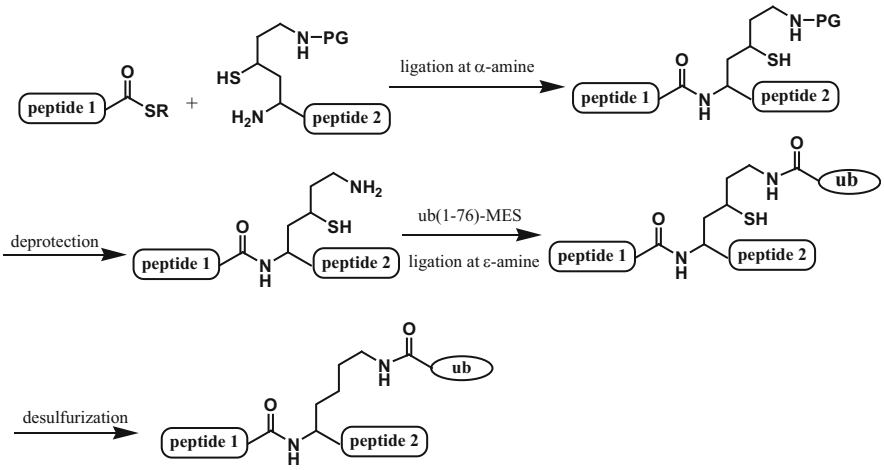
or protein substrate. In contrast to the Nα-auxiliary-mediated ubiquitination approach, this ligation process was mediated by a simple thiol group located at either the γ- (our approach) or δ- (Brik's approach) position of the lysine side chain. As the ligation occurred at the non-sterically hindered primary ε-amine, the reaction was very efficient (Schemes 6 and 7). With these methods, both ubiquitinated peptides and proteins were synthesized. Chin's group also reported a method for genetic incorporation of δ-thiolysine which therefore allows for ubiquitination of recombinant proteins [22]. The only limitation of the thiolated-lysine methods was that it took many steps to synthesize the unnatural thiolysine derivatives. Recently, Ovaa's group reported a simplified method for the



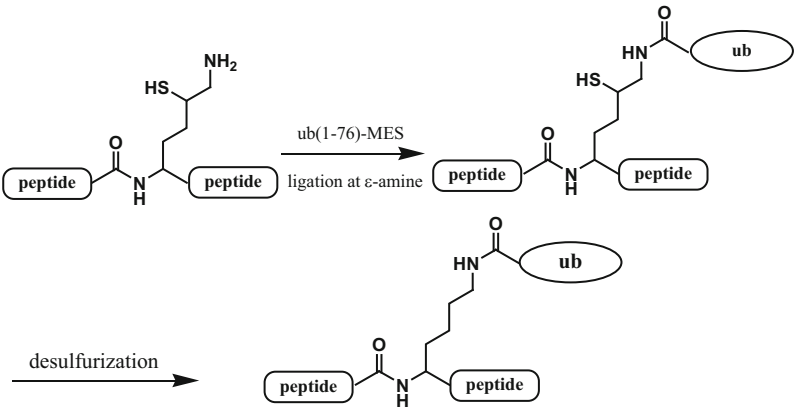
Scheme 4 Synthesis of ub-AMC through Nα-auxiliary-mediated ligation



Scheme 5 Nα-auxiliary-mediated ubiquitination employing 2-mercaptoethoxyl auxiliary



Scheme 6 General scheme of dual native chemical ligation at lysine for peptide ubiquitination



Scheme 7 General scheme of chemical ubiquitination through δ-thiolysine mediated ligation

synthesis of γ-thiolysines [23, 24]. This enabled the scale-up production of γ-thiolysine and can significantly enhance the application of our γ-thiolysine-mediated ubiquitination approach.

2.3 *Ubiquitination Through Ag^{+}-Mediated Activation of Ubiquitin C-Terminal Thioester*

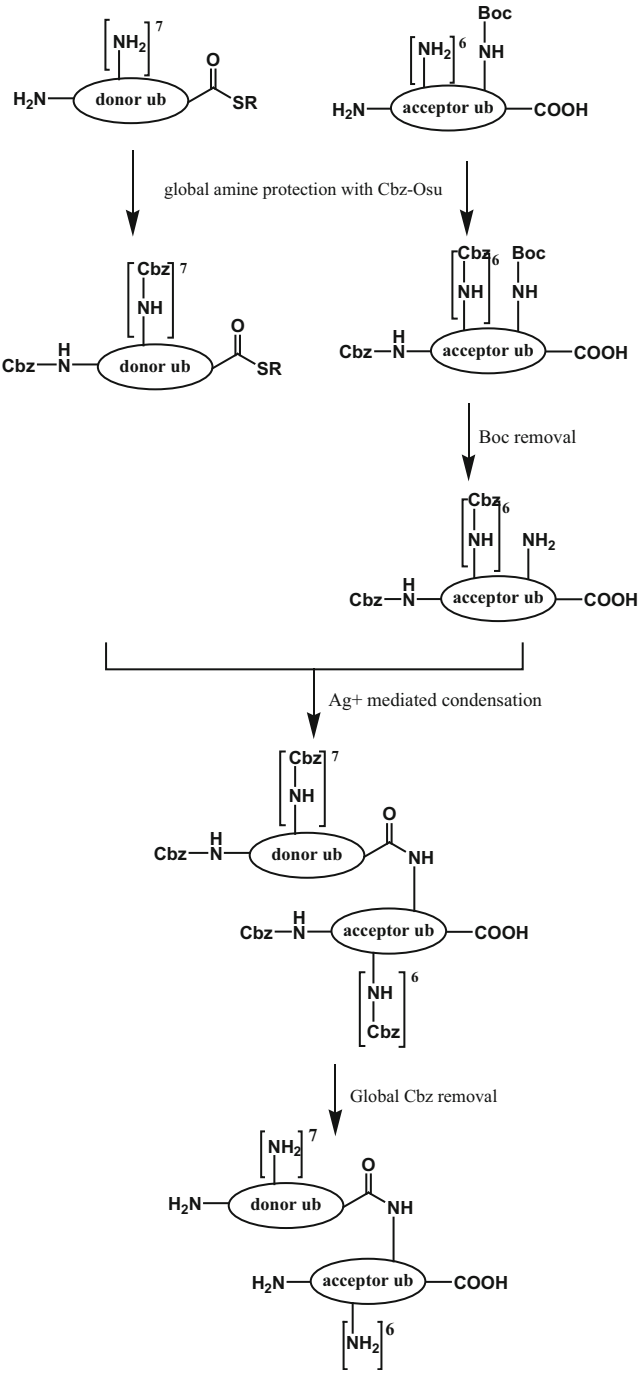
All these chemical ubiquitination methods mentioned above were through the chemoselective ligation between unprotected peptides or proteins. Recently, Chin

et al. reported the genetically encoded orthogonal protection and activated ligation (GOPAL) approach for the synthesis of K6- and K29-linked diubiquitins [25]. To synthesize diubiquitins using the GOPAL approach, the isopeptide bond was formed through Ag^{+}-mediated condensation between two partially protected (with Cbz) ubiquitin proteins (Scheme 8). One was a full-length ubiquitin thioester with all its amino groups protected. The other was the acceptor ubiquitin with all the amine protected except the ε-amine of the lysine where ubiquitination occurred. As the condensation occurred between two relatively large partially protected proteins, the reaction was less efficient than those chemical ligation approaches. For the acceptor ubiquitin, the lysine residue to be ubiquitinated was introduced genetically as Lys(Boc) which was deprotected after all other amines were protected with Cbz. More recently, Cropp and Fushman modified the approach and applied it to the synthesis of more complicated oligo-ubiquitins (Schemes 9 and 10) [26]. In their approach, Alloc, instead of Cbz, was used as the protecting group for the amines which were not involved in ubiquitination. At the end of the ubiquitin linkage construction, Alloc group could be globally removed by a ruthenium complex instead of strong acid treatment when Cbz was used as the protecting group. Another important modification was that the E1 ubiquitin activating enzyme was employed to thioesterify the C-terminal COOH of mono-, di-, or tri-ubiquitin to generate the reactive intermediates. Different from the protein splicing approach used by Chin, where a monoubiquitin thioester was generated through thiolysis of ubiquitin-intein fusion protein, their method could generate polyubiquitin thioesters, which was crucial for polyubiquitin construction.

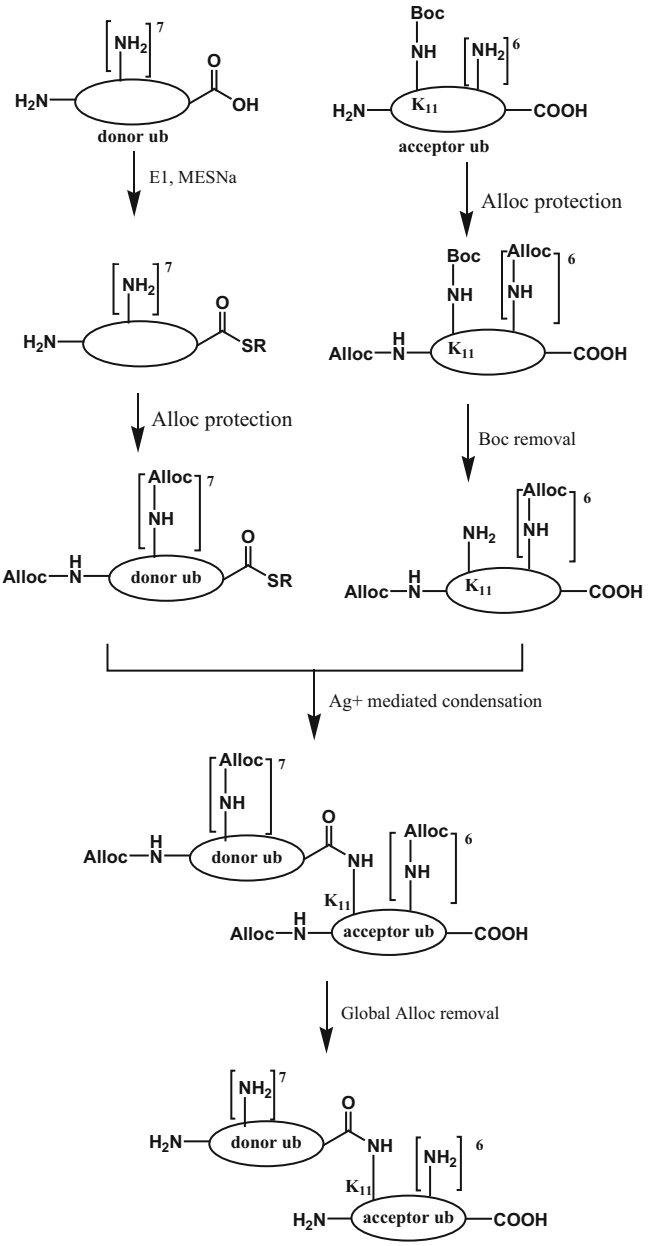
3 Preparation of Ub-Proteins with Non-Native Linkages

Synthesis of ubiquitinated peptides and proteins with the native isopeptide linkage is very challenging. During the development of chemical ubiquitination methods, researchers frequently need to balance the necessity for nativity of the ubiquitin linkage against the labor requirements of the synthetic task. Many relatively simple methods to generate non-native or mimetic ubiquitin conjugates have therefore been developed.

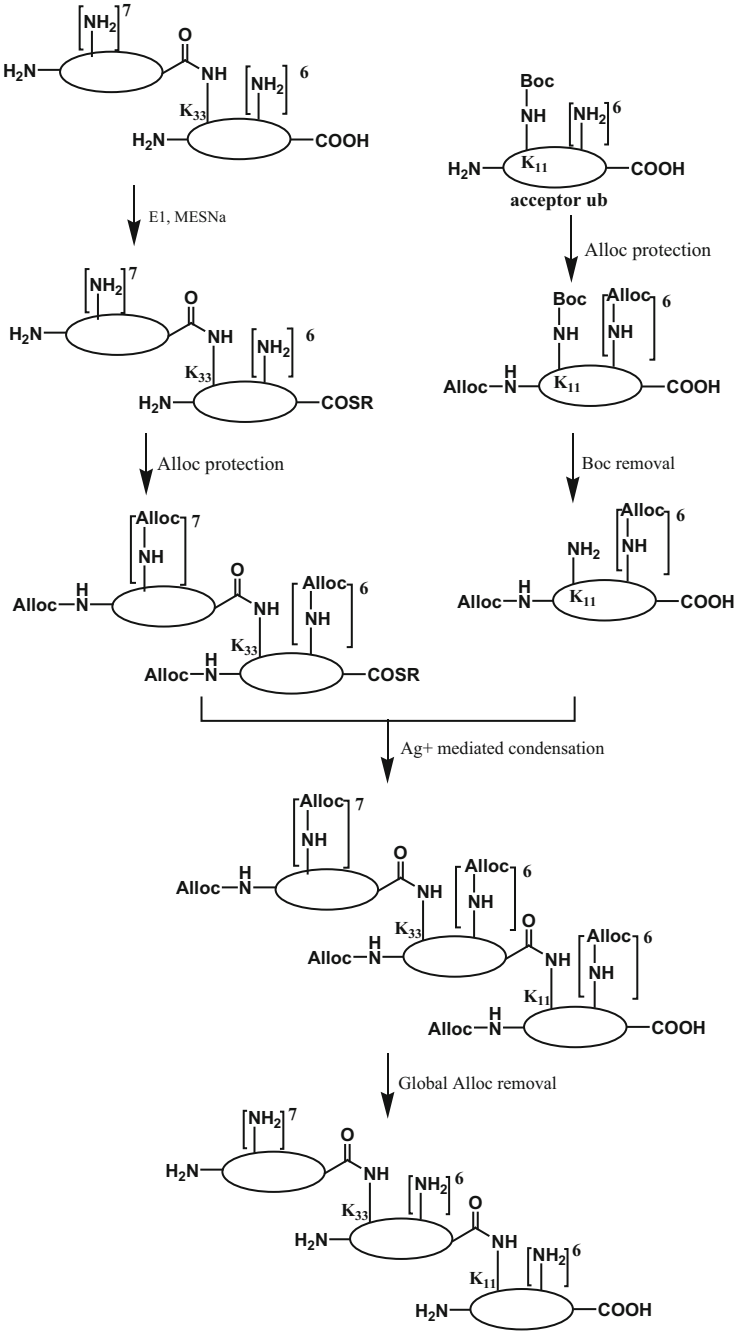
Ubiquitin conjugates with Gly76 to D-Cys or Ala mutation were synthesized by Chan's and Muir's groups, respectively (Scheme 11) [27–29]. Chan et al. reported the genetic incorporation of Nε-(D-cysteinyl)-L-lysine as an unnatural amino acid, which was mediated by the pyrrolysyl-tRNA synthetase and tRNA pair [27]. The Cys residue was employed to mediate native chemical ligation between ub(1–75)-thioester and the cysteinyl-lysine side chain on the acceptor ubiquitin. In Chan's method, the D-Cys was left unchanged after ligation. Muir's group attached the L-cysteine to the lysine side chain amine through solid phase peptide synthesis. After ligation, the thiol of cysteine residue could be converted to alanine through desulfurization [28, 29].



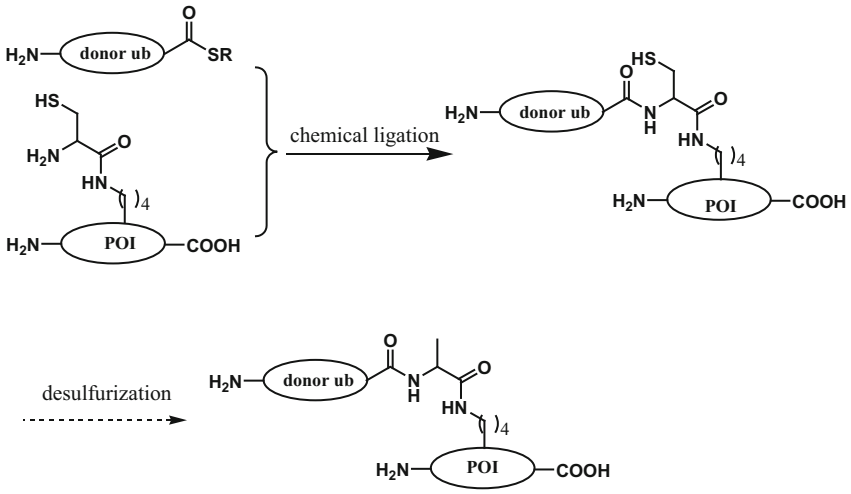
Scheme 8 General scheme of GOPAL approach for the synthesis of linkage-specific diubiquitins



Scheme 9 General scheme for the synthesis of K11-linked diubiquitin using the revised GOPAL approach



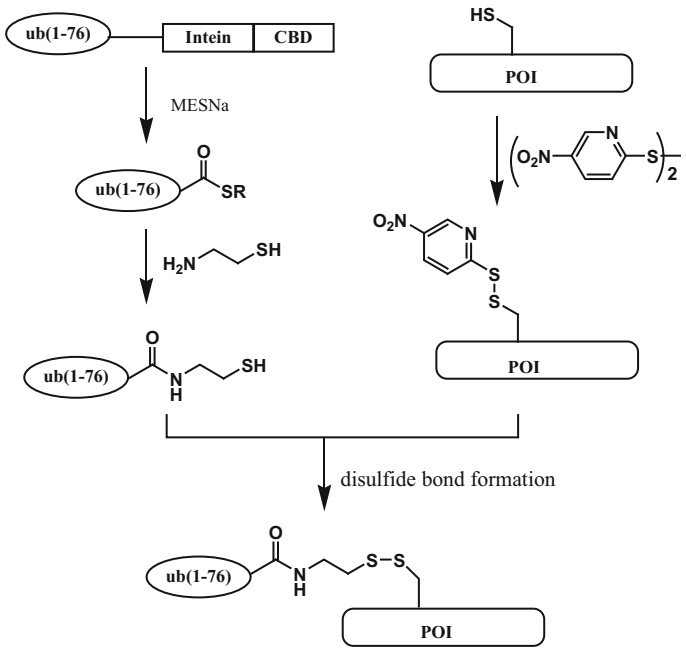
Scheme 10 General scheme for the synthesis of K33 and K11 mix-linked triubiquitin using the revised GOPAL approach



Scheme 11 General scheme for the synthesis of G76C or G76A ubiquitin conjugates. *POI* – protein of interest

Non-native ubiquitin conjugates with disulfide bond were also reported [30–33]. In this type of conjugates, a full-length ubiquitin with a C-terminal 2-mercaptoethyl amide moiety was first prepared by reacting cysteamine with ubiquitin thioester; the thiol at its C-terminal 2-mercaptoethyl amide was then linked to the cysteine residue of the substrate protein through a disulfide bond (Scheme 12). The method is very simple and suitable for the preparation of ubiquitinated proteins in large quantities as both reacting components can be easily obtained as recombinant proteins. The limitations of the method are that the linkage is much longer than the native lysine side chain and that the disulfide bond is also susceptible to reducing conditions.

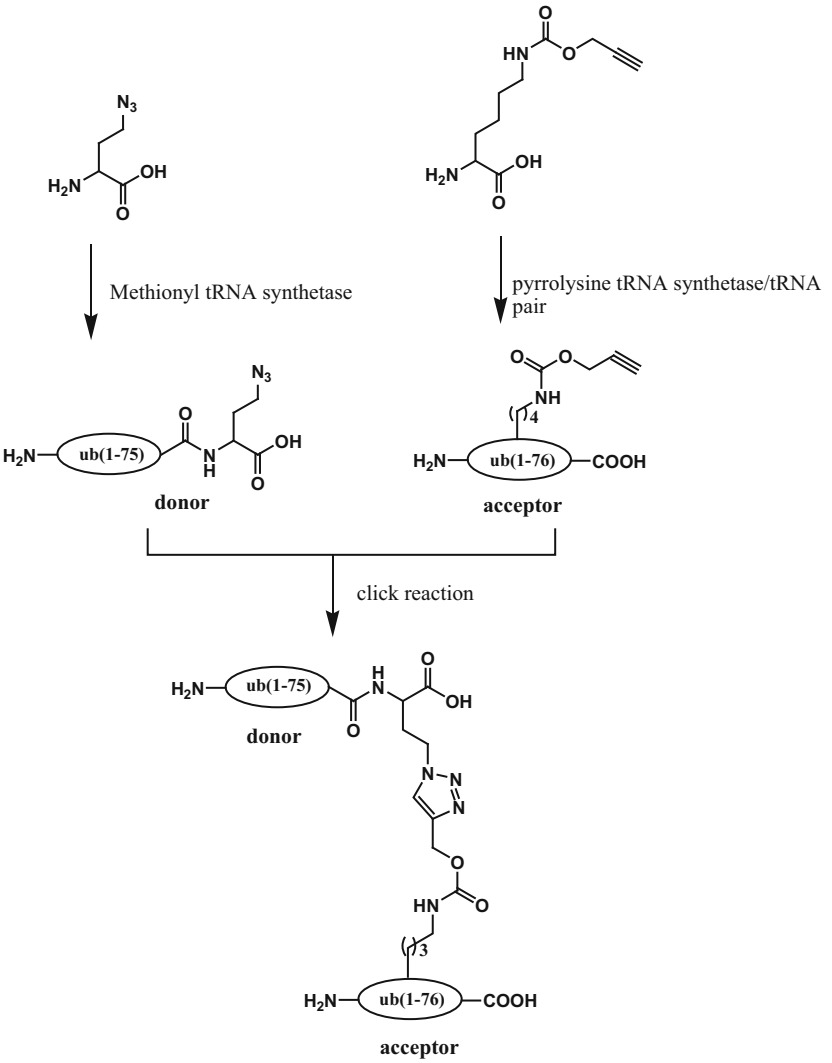
Triazole-linked ubiquitin conjugates were also demonstrated in the synthesis of proteins modified by ubiquitin or ubiquitin-like proteins (Schemes 13 and 14) [34–36]. The triazole linkage was formed through copper-catalyzed click reaction between an alkyne and an azide. Both the alkyne and azide are non-natural functional groups and have been introduced through genetic incorporation as part of the unnatural amino acids [34] or through post-expression chemical manipulation of recombinant proteins [35, 36]. In the genetic incorporation approach, an azide functional group was introduced when azidohomoalanine was incorporated into the donor ubiquitin at the C-terminal end. The incorporation was mediated by methionyl-tRNA synthetase in methionine auxotrophic bacterial strains grown in minimal media. The alkyne functional group was introduced when alkyne-bearing pyrrolysine analogs were incorporated into the receptor ubiquitin (Scheme 13). Mootz et al. reported another way to generate triazole-linked ubiquitinated/



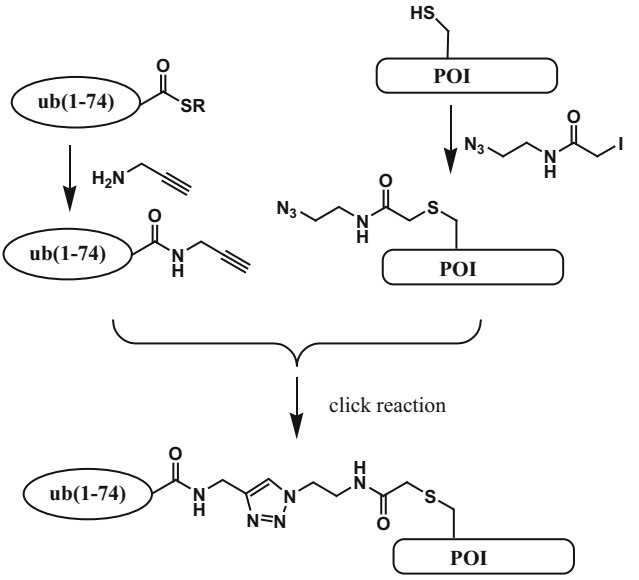
Scheme 12 General scheme for disulfide-directed site-specific ubiquitination. *POI* protein of interest

sumoylated proteins [36] (Scheme 14). In their approach, an alkyne group was introduced onto the C-terminus of ubiquitin(1–74) or ubiquitin-like proteins (such as SUMO) through reacting propargyl amine with the ubiquitin or SUMO thioester. An azide group was introduced to the side chain of a cysteine residue in the protein to be modified through alkylation with iodoacetamide ethyl azide. Click reaction was then performed to link ubiquitin (or SUMO) with the substrate protein. The triazole linkages formed were resistant to ubiquitin-deconjugating enzymes.

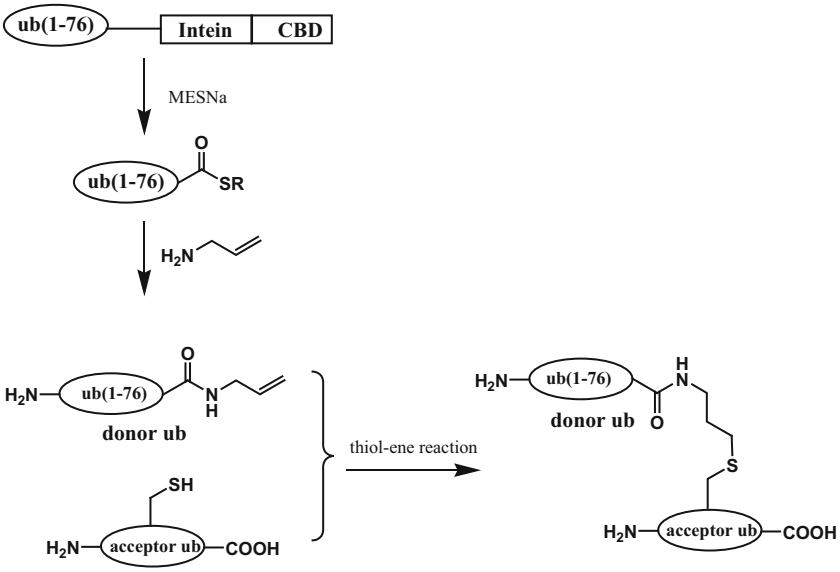
Ubiquitin conjugates with a thioether linkage have also been reported [37, 38]. The thioether linkage was formed through free-radical thiol-ene click reaction between an allyl group introduced at the C-terminus of donor ubiquitin and the thiol group of a cysteine residue of in the substrate protein (Scheme 15). The introduction of the ally group was achieved through reacting allylamine with the full-length ubiquitin thioester. The thioether linkage generated resembled very much the native isopeptide linkage of ubiquitination except that the former had a slightly longer "lysine" side chain. However, the thiol-ene reaction was inefficient in this setting and a yield of only about 30% was obtained. The probable reason was that the large size of the two reacting components imposed a molar concentration which was too low to sustain the radical chain reaction.



Scheme 13 Triazole-linked ubiquitin conjugate formed between genetically incorporated azide and alkyne



Scheme 14 Triazole-linked ubiquitin conjugate with post-translationally installed alkyne and azide. *POI* protein of interest



Scheme 15 General scheme for the synthesis of thioether-linked diubiquitins through thiol-ene click reaction

4 Conclusion

Chemical synthesis of site-specifically modified proteins has provided enabling techniques for protein structure-function studies. The development of the above methods has helped to make the difficult-to-obtain ubiquitinated proteins more available for biophysical and biochemical characterizations. Obviously, the ub-protein conjugates with non-native linkages are much easier synthetic targets and in many cases such conjugates are useful reagents for functional studies. Nevertheless, it is desirable to obtain ubiquitinated proteins with the native isopeptidic linkage to understand the complete and genuine structural/functional implications of this important post-translational modification. Without doubt, native chemical ligation [39] is still the most useful technique for the chemical installation of ubiquitin onto synthetic or recombinant proteins.

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