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Ubiquitin Conjugates

Chemistry and Biology of the Ubiquitin Signal

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Dedicated to Professor Aaron Ciechanover

Ubiquitination is one of the most utilized posttranslational modifications in eukaryotes and is involved in a wide range of cellular processes, but is mostly known as a signal for proteasomal degradation. Recently, it has become clear that the ubiquitin signal is far more complex and is dictated by the ubiquitin component and the substrate. The remarkable diversity of the ubiquitin signaling process has triggered an incredible amount of effort to investigate the role of ubiquitination on biological processes. However, despite more than three decades of studies, several important questions remain unanswered. A major hurdle is the inability to obtain homogeneous ubiquitin bioconjugates in sufficient amounts from cells or by application of the enzymatic machinery. Recent breakthroughs in chemical and semisynthetic strategies, however, offer solutions to these challenges. In this Review, we survey the fundamental biological aspects of the ubiquitin signal and present the emerging non-enzymatic approaches for overcoming these obstacles.

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1. Introduction

Posttranslational modifications (PTMs) play an essential role in regulating protein structure and function for health and disease.[1] Ubiquitination, the attachment of a ubiquitin (Ub) monomer or a polyubiquitin (poly-Ub) chain to a protein target is one of the most utilized PTMs in eukaryotes, and is involved in a wide range of cellular processes. One such example is the role of ubiquitination in the degradation of protein by the 26S proteasome,[2–6] which is the best-characterized function and most recognized signal. The importance of this research is evident by the 2004 Noble Prize in Chemistry being awarded to Aaron Ciechanover, Avram Hershko, and Irwine Rose "for the discovery of ubiquitin-mediated protein degradation".[7–9] The key player, Ub,[10] is a highly conserved protein of 76 amino acids with a secondary structure composed of three and a half turns of an α helix, a short piece of 3_{10} helix, and a β sheet that contains five strands and seven reverse turns (Figure 1).[11] Interestingly, Ub was given its name as it was thought to be present ubiquitously in both prokaryotes and eukaryotes. However, this turned out not to be the case, as further studies revealed that it exists only in eukaryotes.[7] Ub is known to be highly stable at neutral pH, with a denaturation temperature of around 100 °C or higher.[12,13]

Ubiquitination involves three enzymes known as E1, E2, and E3. These enzymes coordinate their work to link the C-terminal Gly residue of Ub to the Lys side chain of the protein target through an isopeptide bond,[14,15] which is an amide linkage outside the main protein backbone. This process is initiated by E1, which activates the Ub C terminus in an ATP-dependent manner to form a thioester intermediate.[16] Subsequently, the conjugating E2 enzyme carries the Ub, also in the thioester form, for the following isopeptide formation step.[17] The E3 ligase catalyzes the transfer of one Ub molecule at a time or a Ub chain to a protein target.[18] This mechanism could involve the RING-domain E3 family,

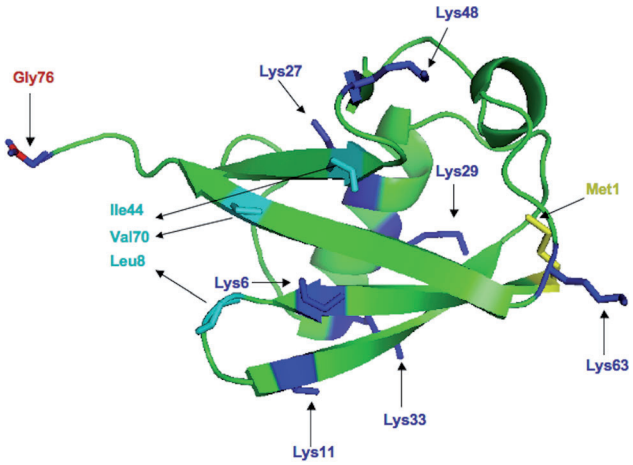

Figure 1. Ub structure (PDB code 1UBQ)[11] in ribbon representation with a ball and stick representation of all the Lys residues (K63, K48, K33, K29, K27, K11, and K6), the C and the N termini (G76 and M1), and the hydrophobic patch (L8, I44, V70), which mediate most of the Ub–protein interactions.

which brings the Ub thioester/E2 ensemble into proximity to the nucleophilic ε-amino group of a Lys residue to form the isopeptide bond between the target protein and Ub (Scheme 1). This family lacks a classical catalytic active site that often characterizes enzymes.[19] Alternatively, another group of the E3 family known as HECT E3 (homologous to the E6-AP C terminus) catalyzes the formation of the isopeptide bond by utilizing a catalytic Cys residue, which forms a transient thioester intermediate between Ub and the

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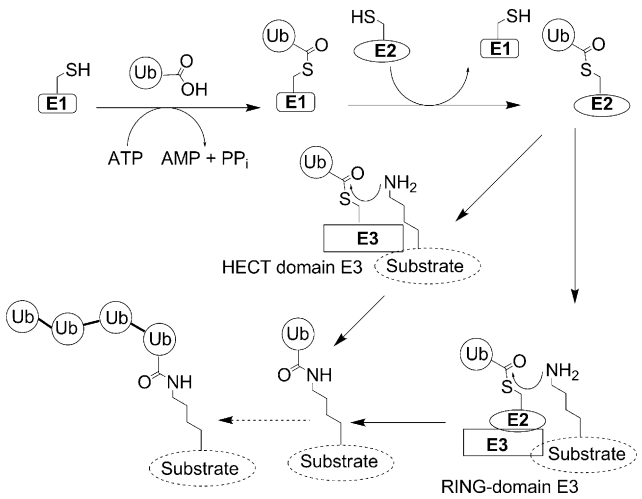

Scheme 1. Ubiquitination of a protein substrate assisted by the E1, E2, and E3 enzymes.

E3.[20] In general, although E3 is the main determinant of the substrate specificity, E2 has a major role in determining the linkage type, in particular when acting with RING-dependent E3.[17] The enzymatic machinery is composed of two E1 enzymes, 30–40 E2 enzymes, and several hundred E3 ligases.[19,21,22]

The conjugation of Ub to a protein target may involve one Ub molecule (monoubiquitination) or a chain of Ubs of various lengths and linkage types (polyubiquitination). Hence, these protein modifications produce a variety of molecular signals, in which the outcome depends on the nature of the ubiquitination, for example, monoubiquitination versus polyubiquitination. The latter is a highly complex and diverse modification since all the seven lysine residues in Ub (K63, K48, K33, K29, K27, K11, K6) can serve to link the consecutive Ub molecules to from a particular chain (Figure 2).[23] Recent proteomic studies revealed that all these chains exist in vivo and several of them have been found to affect various cellular pathways.[24–27] For example, proteomic analysis on unperturbed yeast cells revealed that the K48- and K11-linked chains are the most abundant chains (29% and 28%, respectively), while the K29- and K33-linked chains are the least abundant chains (3% each). The K63, K27, and K6 chains are present at 17%, 9%, and 11%, respectively.[24]

The K48-linked Ub chain is known as a typical chain, while chains conjugated through any one of the other six Lys residues are known as atypical chains.[28] The Ub chains can be homotypic, mixed-linkage (with several different Lys residues), or heterologous (in which Ub and Ub-like proteins, such as the small Ub-like modifier (SUMO), are utilized in the assembly of the chain). In addition to chains linked through the isopeptide bond, the N terminus of Ub can be used to form a head-to-tail linkage; such a chain is known as a linear chain (Figure 2).[28] Multiple monoubiquitination could also occur on the same protein, as was suggested, for example, in the case of p53, where such a modification targets this protein for degradation.[29] A different type of ubiquitination involves a so-called lysine-less site wherein Cys, Thr, Ser, and the N terminus of a protein serve as the anchoring sites for ubiquitination.[30–37]

The above-described diversity of ubiquitination, with different types, lengths, connectivities, and anchoring sites (e.g. Lys, N terminus, Ser), provides some clues as to the origin of the astonishing diversity in the Ub signaling process (Table 1). For example, while the K48-linked chain facilitates recognition by the 26S proteasome and the subsequent degradation of ubiquitinated protein,[4–6] the K63-linked chain plays an important role in signal transduction, endocytosis, and DNA repair processes.[23,38–40] Interestingly, new data also support a central role of the other chains in different biological processes. For example, it has recently been reported that the K33-linked chain plays a key role in T-cell activation and autoimmune responses,[41] while the K11-linked chain signals for cell signaling and endoplasmic reticulum-associated degradation (ERAD).[42–44] Notably, monoubiquitination is emerging as a significant form of ubiquitination, as evident by proteomic analysis.[25] It is also involved in a variety of biological processes, ranging from membrane transport to transcriptional regulation as well as protein degradation.[45–49]

2. Interpretation and Termination of the Ub Signal

In general, the molecular events leading to a specific signal subsequent to assembly of the enzyme start with recognition of the Ub binding protein through its Ub binding domain (UBD). The termination of a signal is achieved by

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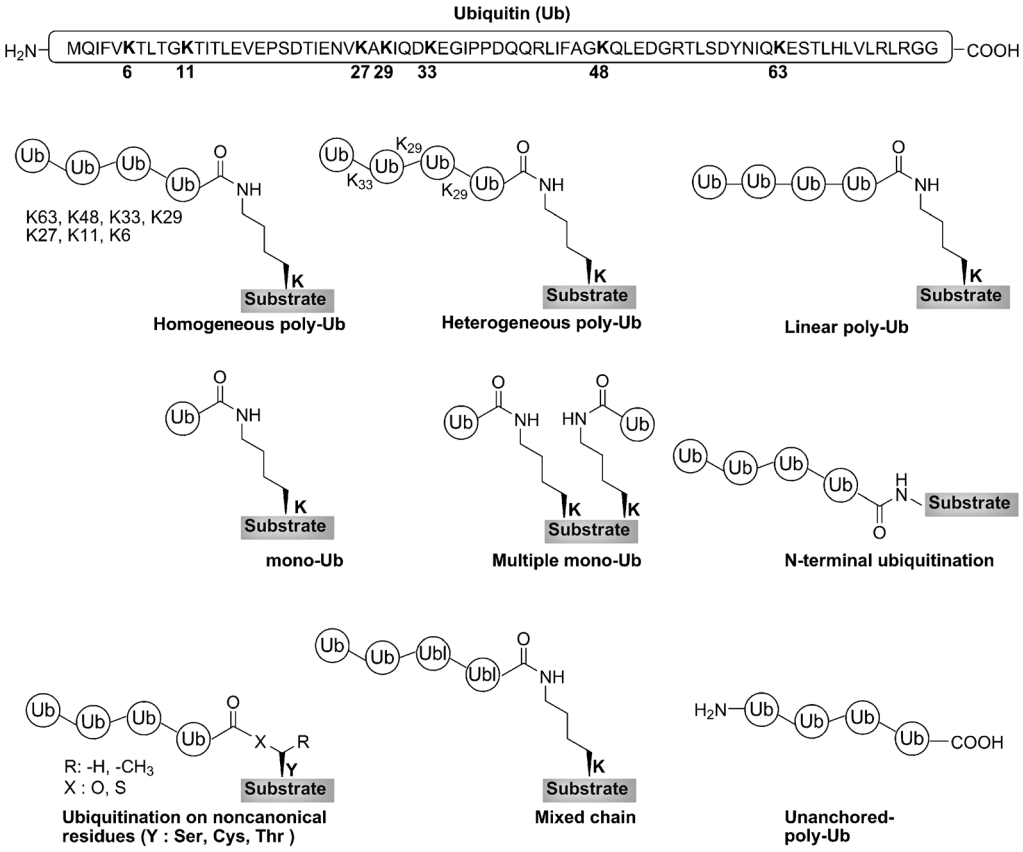


Figure 2. The diversity of Ub, showing the different compositions of Ub chains and their anchoring possibilities to a protein target.

detaching a part or the entire chain with deubiquitinases (DUBs). In the following sections, we will examine parts of this process in more detail.

2.1. UBDs

These domains, which mediate most of the effects of protein ubiquitination, consist of structural units that bind noncovalently to Ub, thereby translating molecular events into particular cellular functions. At least 20 members of this family (e.g. motif interacting with ubiquitin (MIU), coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE)) with a wide range of structural folds, in particular of the α helix, have already been identified. These domains are relatively small (20–150 amino acids), usually exhibit a moderate to low binding affinity to mono-Ub ($K_d \approx 10$–$500\ \mu M$),[71–73] and are known to recognize various surfaces of Ub. The vast majority of studies on UBDs have focused on the interactions between a Ub monomer and a specific domain. Several X-ray and NMR structures have been reported, and molecular models of the recognition of the Ub monomer by many of these domains have been developed.[71–73] However, the recognition behavior of UBDs with most of the Ub chains is still unknown.[28,74] Moreover, a comprehensive picture of the mechanisms by which these domains are able to discriminate between the different Ub chains is still lacking. The recent development of the synthesis of all types of homogeneous Ub chains should allow the fundamental understanding of the molecular basis of the selective recognition of each chain by the different domains and expand our knowledge of the Ub signal process in terms of health and disease. For example, it was recently reported that a small molecule (Ubistatin) could bind to a Ub chain and interfere

Table 1: Cellular signaling processes based on different Ub species.[50]

Ubiquitination type	Cellular signaling	Selected references
Mono	DNA repair, endocytosis, histone regulation, virus budding, proteasomal and lysosomal degradation.	[45–49, 51–55]
K63	cell signaling/kinase activation and cytokine signaling, DNA damage response, endocytosis.	[39, 40, 56–58]
K48	proteasomal degradation.	[2–6]
K33	immune regulation.	[41]
K29	lysosomal degradation, Wnt signaling.	[59–61]
K27	the physiological function is unclear.	
K11	cell cycle/proteasomal degradation, ERAD, cell signaling such as TNF and Wnt signaling.	[61–63]
K6	the physiological function is still unclear; however, it has been suggested to inhibit proteasomal degradation and involvement in DNA damage repair.	[64, 65]
linear	regulation of NF-κB signaling in inflammation, protein degradation.	[66, 67]
multi-mono	endocytosis, proteasomal, and lysosomal degradation.	[29, 68]
mixed chains	MHC I endocytosis (K11/K63), kinase activation (K33/K29).	[69, 70]
lysine-less	proteasomal degradation.	[30–37]

with the interaction of UBDs.[75] Despite the poor pharmacokinetic properties of Ubistatin, the study provided a proof of concept that small molecules could be used to target such interactions and selectively manipulate distinct cellular processes with pharmaceutical relevance.

2.2. DUBs

Similar to several other PTMs, ubiquitination is a reversible process, in which a family of enzymes known as DUBs removes Ub from its target. Approximately 100 known DUBs identified by in silico efforts and activity-based profiling are encoded in the human genome.[76–78] DUBs are involved in a variety of regulatory processes and play key roles in various diseases, such as neurological disorders, infectious diseases, and cancer, thus they represent novel therapeutic targets.[79,80] DUBs can remove Ub or poly-Ub from proteins, process Ub precursors, and disassemble unanchored poly-Ub chains.[78,81–83] Mechanistically, DUBs are divided into two groups: The first group comprises cysteine proteases (majority), which are subdivided into Ub-specific proteases (USPs), Ub C-terminal hydrolases (UCH), ovarian tumor related proteases (OTUs), and the Josephin/Machado-Joseph diseases protease (MJD). The second group of DUBs is the metalloproteases containing the JAB1/MPN/Mov34 metalloenzyme (JAMM) domain.[81–83]

Several factors are known to determine the substrate recognition and specificities of DUBs as well as their mode of action (Figure 3). Although, some known DUBs do not discriminate between chain linkages, several others are chain-specific (Figure 3A). The cleavage of the chain could occur from the end (*exo*) or within the chain (*endo*), whereby the accommodation of the Ub molecules must precede the cleavage step (Figure 3B). In some cases, the DUBs are substrate-specific, such that a certain sequence of the target is recognized, thereby leading to chain removal in a single step (Figure 3C,D). Current scientific opinion holds that each Ub chain is disassembled by a specific DUB, but for several Ub chains the specific DUB is not known. Hence, a complete picture of the specificities of DUBs is still lacking. Such studies were hindered, until recently, mainly by the lack of availability of most of the well-defined chains and tools to address the above-described challenges. As will be discussed in the following sections, some of these tools are now available, and we anticipate that they will contribute significantly to the basic understanding of the role of DUBs in health and disease and ultimately be used in drug discovery.

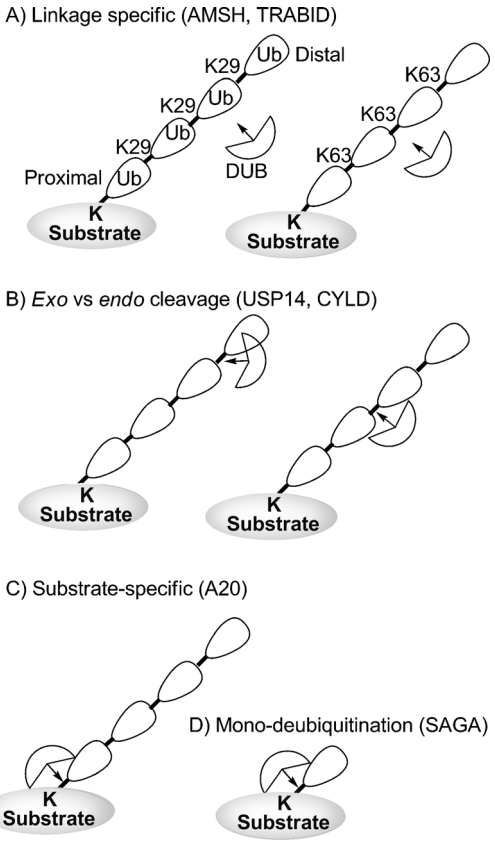


Figure 3. Examples of the different levels of DUB specificities: A) AMSH[84] and TRABID[85] are specific DUBs for K63- and K29-linked chains, respectively. B) USP14[86] cleaves K48-linked poly-Ub chains from the distal end, while CYLD[87] cleaves internal isopeptide linkages in K63-linked poly-Ub chains. C) A20[88] is TRAF6 specific which amputates the entire Ub chain. D) SAGA cleaves mono-Ub from the H2B protein.[89–91]

2.3. The Effect of Linkage Type on the Structure and Recognition by DUBs and UBDs

The diversity of Ub is believed to result in the formation of Ub bioconjugates with a large conformational space, thereby leading to a variety of different protein–protein interactions and affecting a wide range of biological signals. Indeed, X-ray structural analyses and NMR spectroscopic studies on K48- and K63-linked chains revealed major conformational differences between these chains. At physiological pH values,[92,93] the K48-linked chain adopts predominantly a closed conformation, in which the hydrophobic patch residues (L8, I44, V70) are sequestered at the interface between two adjacent Ub monomers. However, the K63-linked chain mainly adopts an extended conformation that lacks the contacts of the functionally important residues between the two monomers.[94–96] Table 2 provides several examples of the solved structures of Ub chains in their

Table 2: Examples of solved structures of Ub chains.

Chain type	Pdb code (NMR structure)	Pdb code (X-ray structure)
K63-linked di-Ub		2JF5,[97] 3H7P,[98]
K63-linked tri-Ub		3H7S[98]
K63-linked tetra-Ub		3HM3[95]
K48-linked di-Ub	2PE9,[99] 2PEA,[99] 2BGF,[100]	3AUL,[101] 3M3J,[102] 1AAR[103]
K48-linked tetra-Ub	2O6V[92]	2O6V,[92] 1TBE,[104] 1F9J[105]
K11-linked di-Ub		3NOB,[42] 2XEW[106]
K6-linked di-Ub		2XK5[85]
linear (di-Ub)		2W9N,[97] 3AXC[107]

unanchored forms, as determined by NMR spectroscopic and/or X-ray analyses. Notably, it has been shown that K48-linked chain significantly alters its conformation upon a change in the pH value.[93]

The above-described structural differences of Ub chains could have a major impact on the selective recognition of these chains by different protein partners. An example is the ubiquitin associate domain (UBA2) from the human homologue of yeast Rad23 (hHR23A) bound to K48-linked di-Ub, and K63-linked di-Ub bound to the ubiquitin-interacting motif (UIM) from the receptor associate protein 80 (RAP80). The latter protein is involved in the recruitment of BRCA1 (breast cancer type 1 susceptibility protein) to DNA damage sites,[64] while Rad23 is a Ub receptor that is associated with substrate targeting to the 26S proteasome.[108] The UBA2 domain in hHR23A was found to bind with 3.6-fold higher affinity to the K48-linked tetra-Ub compared to the K63-linked chains.[109] The structure of the complex of K48-linked di-Ub and UBA2 of hHR23A revealed that this domain binds in the center of two Ub molecules, and it appears to also interact with the K48 linkage. Together, the domain has more binding surfaces in the K48 chain compared to Ub or K63-linked di-Ub (Figure 4A), which explains its preference for

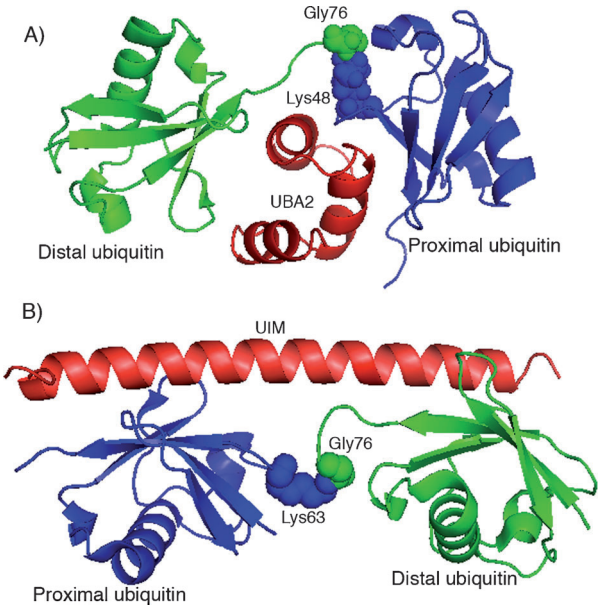


Figure 4. Examples of structures of Ub chains bound to different UBDs. A) UBA2 domain of hHR23A (red) bound to K48-linked di-Ub (PDB code 1ZO6[110]). B) K63-linked di-Ub bound to UIM of RAP80 (red; PDB code 3A1Q[112]).

this linker.[96,110] On the other hand, the structure of the K63-linked di-Ub bound to the UIM of RAP80 explains the preference of K63- over the K48-linked chain (Figure 4B).[111] Here, the stretched helix of the UIM domain takes advantage of the extended conformation of the chain and interacts with both Ubs to increase its binding affinity beyond that of the mono- or K48-linked di-Ub.

In addition to the tertiary structure and dynamics that affect the recognition and specificity of DUBs and UBDs, the residues flanking the isopeptide linkage also play an important role in these events. For example, while K63-linked and linear poly-Ub chains are known to adopt similar open conformations with no contact between the monomers, the various DUBs and UBDs exhibit different specificities.[97] The diverse specificities have been attributed to the chemically distinct isopeptide linkage in K63 (as opposed to the peptide linkage in the linear chain). Very recently it was reported that the K11-linked di-Ub chain adopts a compact conformation distinct from that of the K48-linked di-Ub chain and is hydrolyzed preferentially by the DUB Cezanne.[106] Although the origin of this specificity is still unknown, the sequences around the isopeptide linkage could be an important factor. These studies emphasize the effect of different linkages on the globular conformation of the chains and their importance on the intrinsic specificity of the Ub system with DUBs and UBDs. However, a comprehensive understanding of the structural and functional properties as well as the specificities of the different DUBs, UBDs, and their substrates is still missing.

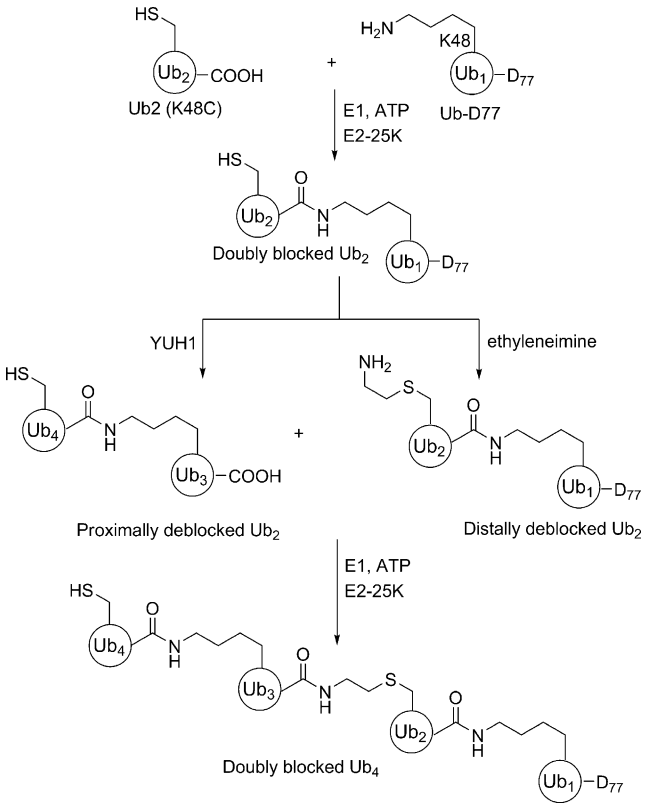
3. Challenges and Limitations in Studying Ub Systems

Research in the field of Ubs, including structural and functional analyses as well as the development of reagents (e.g., antibodies that recognize specific chains),[113,114] has focused mainly on K48- and K63-linked chains (di- and tetra-Ub chains). This is due to the unavailability of the E2/E3 enzymes to reconstitute the remaining chains in vitro (expect for K11) in their free or conjugated form to a specific substrate.[115] Hence, the identification, isolation, and characterization of E2/E3 enzymes for a specific target have been the limiting steps in these studies. Moreover, these enzymes do not often operate, in vitro, in a highly efficient and specific manner to furnish the desired Ub conjugate with great homogeneity and in sufficiently large quantities for the desired biochemical studies. Even though more studies are now being conducted on the other chains, for example, K6[85] and K11,[43,44] the inability for many years to prepare these chains in quantities for structural and biochemical studies in vitro has obstructed the elucidation of the Ub signaling process.[23,116] As a result, several fundamental questions in the field remain unanswered, and a comprehensive picture of the Ub signaling process has been unattainable.

4. Preparation of Ubiquitinated Peptides and Ub Chains by Enzymatic Methods

Since the discovery of Ub as a signal for protein degradation, great efforts have been invested to apply enzymatic approaches to assemble Ub chains of defined lengths and types. As described above, one of the main challenges in these approaches is the identification of the E2/E3 enzymes for a specific Ub conjugate. Another obstacle that could arise when adopting enzymatic methods is achieving a high level of control in the in vitro preparation of Ub

chains of defined type and length. Towards these goals, Pickart and co-workers successfully identified the conjugating factors E2-25K and Mms2/Ubc13 for the in vitro assembly of K48- and K63-linked chains, respectively.[115,117] However, some modifications were required to achieve a controlled synthesis over the chain length, such as blocking the C terminus of Ub1 and introducing K48C or K48R mutations in Ub2 (Scheme 2). After enzymatic coupling of these two Ub



Scheme 2. Enzymatic preparation of a K48-linked tetra-Ub chain.

monomers, the Cys residue of the Ub2–Ub1 was converted into a Lys mimic (*S*-aminoethylcysteine) by reaction with ethyleneimine to allow further chain elongation. In addition, the C terminus of the consecutive di-Ub (Ub4–Ub3) was cleaved from the extra C-terminal residue (D77) by using yeast Ub hydrolase 1 (YUH1) to give the deblocked di-Ub. Subsequently, these di-Ub building blocks were linked enzymatically to furnish the doubly blocked tetra-Ub chain (Ub_4).[118] Notably, this strategy produces Ub chains with an isopeptide mimic that could have different geometrical and electronic characteristics compared to the native linkage.

Recently, Fushman and co-workers prepared K48-linked chains with the native isopeptide linkage and of controlled lengths by employing genetically encoded Lys(Boc)-OH based on a pyrrolysyl-tRNA synthetase/tRNAPyl pair to generate the Ub building block UbK48Lys(Boc).[119] To prepare the K48-linked di-Ub chain, UbK48Lys(Boc) was treated, in the presence of E1 and E2-25K, with the proximal Ub having D77 at the C terminus to prevent polymerization. The Boc group was then removed to allow another ubiquitination step for further chain elongation.

Plough and co-workers used the same E2-25K to prepare a ubiquitinated peptide that consists of 13 residues linked through the isopeptide bond to HA-tagged Ub.[120] This substrate was used to shed light on the cleavage mechanism of UCH-L3, which is known to catalyze the removal of small adducts from the C terminus of Ub.[121–123]

Komander and co-workers have recently utilized the known E2 UbE2S[124] to assemble the unanchored K11-linked di-Ub chain in quantities sufficient to allow its characterization by NMR spectroscopy and X-ray crystallography. In addition, this allowed the research group to screen 14 DUBs, of which Cezanne was found to cleave the K11-linked chain preferably over K63, K48, and linear chains.[106] Dixit and co-workers have also prepared the K11-linked di-Ub chain enzymatically and studied its structure by X-ray crystallography.[42] Interestingly, this compact structure was different from that determined in the study by Komander and co-workers in terms of the residues that are involved in the Ub–Ub interface. This finding indicates that the same chain could adopt two distinct conformations in solution, which reflects the dynamic nature of the Ub chains.[93,95]

More recently, a new approach based on reconstituting the ubiquitination machinery in bacteria was reported for the preparation of ubiquitinated proteins such as monoubiquitinated Rpn10.[125] The search for new enzymatic machanisms is still required for the preparation of the remaining Ub chains (K6, K33, K27, K29).

5. Non-Enzymatic Methods for the Preparation of Ub Conjugates

The total chemical synthesis and semisynthesis of proteins offer unique opportunities to obtain highly homogeneous posttranslationally modified proteins as well as proteins with unusual connectivities as a result of the high level of control that they afford at an atomic level.[126–128] In terms of ubiquitination, the chemical methods in particular avoid the E1–E3 enzymatic machineries, the identification of which is considered to be the main challenge. Moreover, the advantages of the synthetic strategies stem from the ability to produce unique analogues of Ub bioconjugates, in addition to the naturally occurring ones (e.g. peptides and proteins ubiquitinated with a specific chain). In principle, virtually unlimited variations (e.g. specific labeling) could be performed in a highly controlled manner at essentially any desired position in a particular Ub bioconjugate. Nonetheless, the presence of the isopeptide bond and the relatively large size of the Ub chains presented a formidable task to synthetic chemists, and new methods had to be invented and utilized in combination with the available synthetic tools. The following sections will survey the application of chemical approaches to assemble the native isopeptide bond and its mimetics for the efficient synthesis of ubiquitinated peptides and proteins as well as the Ub chains.

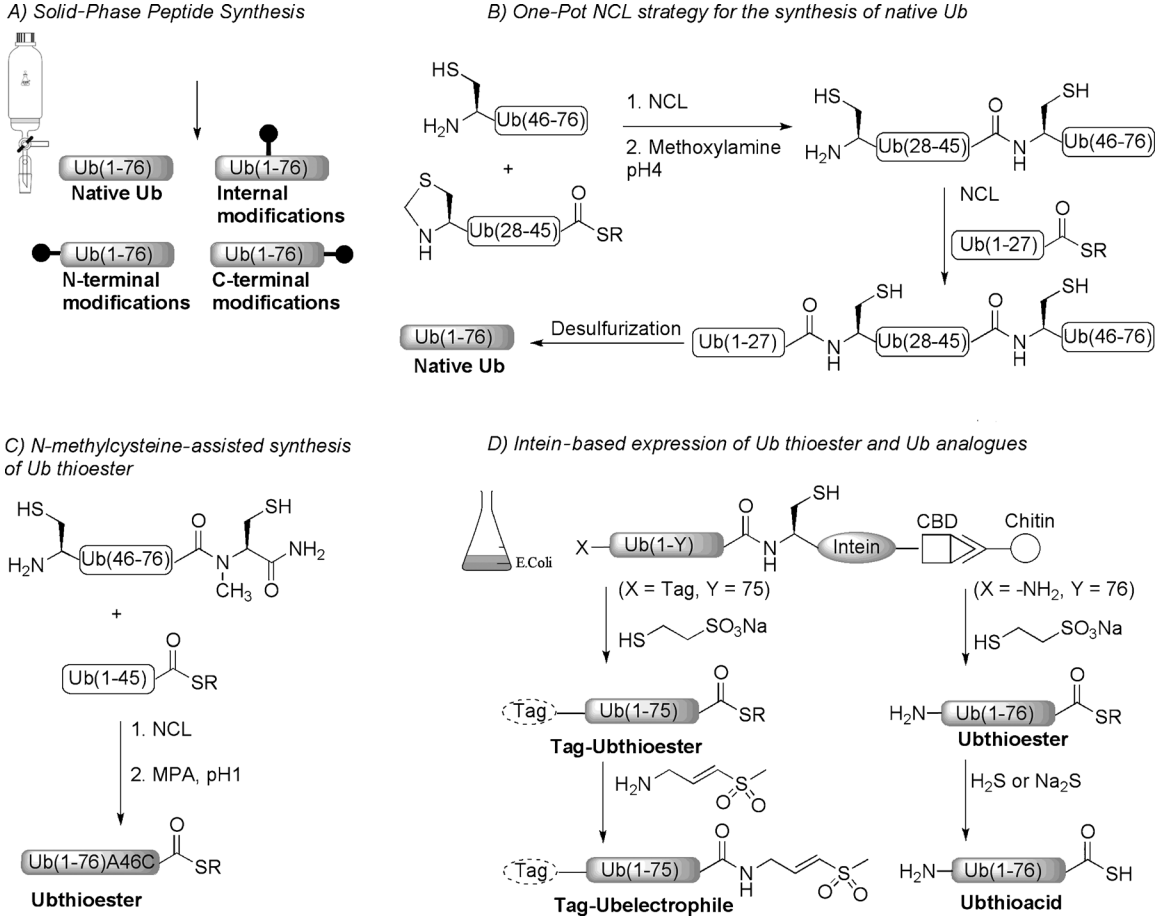
5.1. Chemical and Semisynthesis of the Ub Monomer and its Derivatives

The relatively small size of Ub (76 amino acids) allows its direct chemical synthesis by solid-phase peptide synthesis (SPPS). Ramage et al. were the first to successfully achieve such a synthesis by using Fmoc-SPPS (Scheme 3A).[129,130] By using this approach, they were able to prepare Ub with specifically fluorinated Leu residues and other unnatural amino acids at various positions. These analogues were further characterized by circular dichrosim (CD), NMR spectroscopy, and X-ray structure analysis, and their behaviors tested with the reticulocyte activating and conjugating enzymes.[131,132] These studies revealed that the different Ub analogues exhibited a similar structure and biological properties compared to the unmodified protein, despite an altered stability, which indicated that the whole structure and not the stability is the important element for recognition.

Ovaa and co-workers also accomplished the linear synthesis of mono-Ub by applying four different pseudoproline dipeptides and two dimethoxybenzyl dipeptides.[133] Various Ub fusions, such as N- and C-terminal modifications, for example, HA epitope and AMC, respectively, were generated using the PyBOP/DIEA coupling conditions. More recently, our research group further improved the direct synthesis of Ub by applying only two dipeptide analogues and the coupling reagents HCTU/DIEA for all the coupling steps. Milligram quantities of different Ub building blocks were produced, which allowed the preparation of ubiquitinated peptides with increasing lengths of different Ub chains (see Section 5.4 for more details).[134]

The preparation of Ub and its analogues was also accomplished by ligation strategies. Kent and co-workers reported a one-pot strategy to prepare Ub from three fragments (Scheme 3B) by taking advantage of Ala46 and Ala26, which were temporarily mutated to Cys to facilitate the native chemical ligation (NCL) steps.[135] Subsequently, these Cys mutations were converted into the original Ala residues by applying the desulfurization reaction developed by Yan and Dawson.[136] The Kent research group used Ub as a protein model and prepared the Ub diastereomer (D-Q35-Ub) for crystallographic studies to determine the effect of incorporating D-amino acid residues on protein folding and structure. The X-ray structure revealed a great similarity between the molecular structures of native Ub and its diastereomer. These results provided experimental support that the replacement of a native Gly residue, which has a left-handed conformation, with D-amino acids, as in the case of G35 in Ub, does not induce major perturbations of the local or global conformation of a protein molecule.

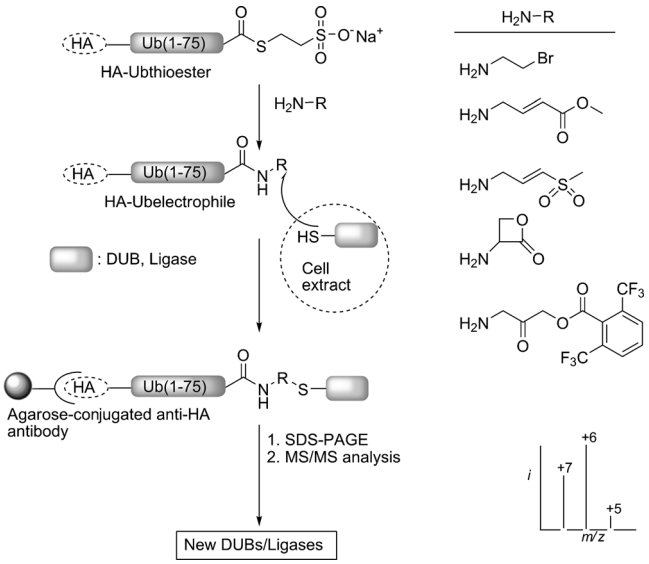
Our research group recently reported the chemical synthesis of the Ub thioester by NCL and by using *N*-



Scheme 3. Methods for the chemical and semisynthesis of Ub and its analogues.

methylcysteine as the N-S acyl transfer device (Scheme 3C).[137] In this synthesis, Ub was divided into two fragments, Ub(1–45) and Ub(46–76) with a C-terminal thioester and an N-terminal Cys residue, respectively, for the NCL step. The C-terminal fragment, Ub(46–76), was also equipped with *N*-methylcysteine to allow N-S acyl transfer, subsequent to the ligation step, to facilitate thioester formation with an external thiol, for example, 3-mercaptopropionic acid (MPA).[138] The ability to chemically prepare the Ub thioester allows the possibility to incorporate unnatural amino acids in this precursor. The importance of this strategy and its utilization in the synthesis of Ub chains will be described in Section 5.4.

The Ub thioester was also prepared by intein-based expression (Scheme 3D).[139–141] The usefulness of this strategy in peptide and protein ubiquitination as well as in the preparation of di-Ub chains will be discussed in Sections 5.2–5.5. Plough and co-workers were the first to use expressed Ub thioester (Scheme 3D), whereby they prepared Ub analogues containing C-terminal electrophiles by direct aminolysis of recombinant Ub thioester, generated by thiolysis of the corresponding Ub-intein fusions (Scheme 3D). These analogues were used as activity-based probes for the identification of members of the Ub-proteasome system that utilize an active Cys residue (Scheme 4).[77,142,143] Specifically, 23 active



Scheme 4. Activity-based profiling used to identify new DUBs.

DUBs were identified, two of which tightly interact with the proteasome 19S regulatory complex, and one DUB that has no sequence homology with any of the known DUBs.[142] On the basis of this study and by fine-tuning the electrophilic component to generate second-generation probes, a new group of DUBs was further identified. Notably, these activity-based probes also allowed the identification of E3 ligases from the HECT family.[143]

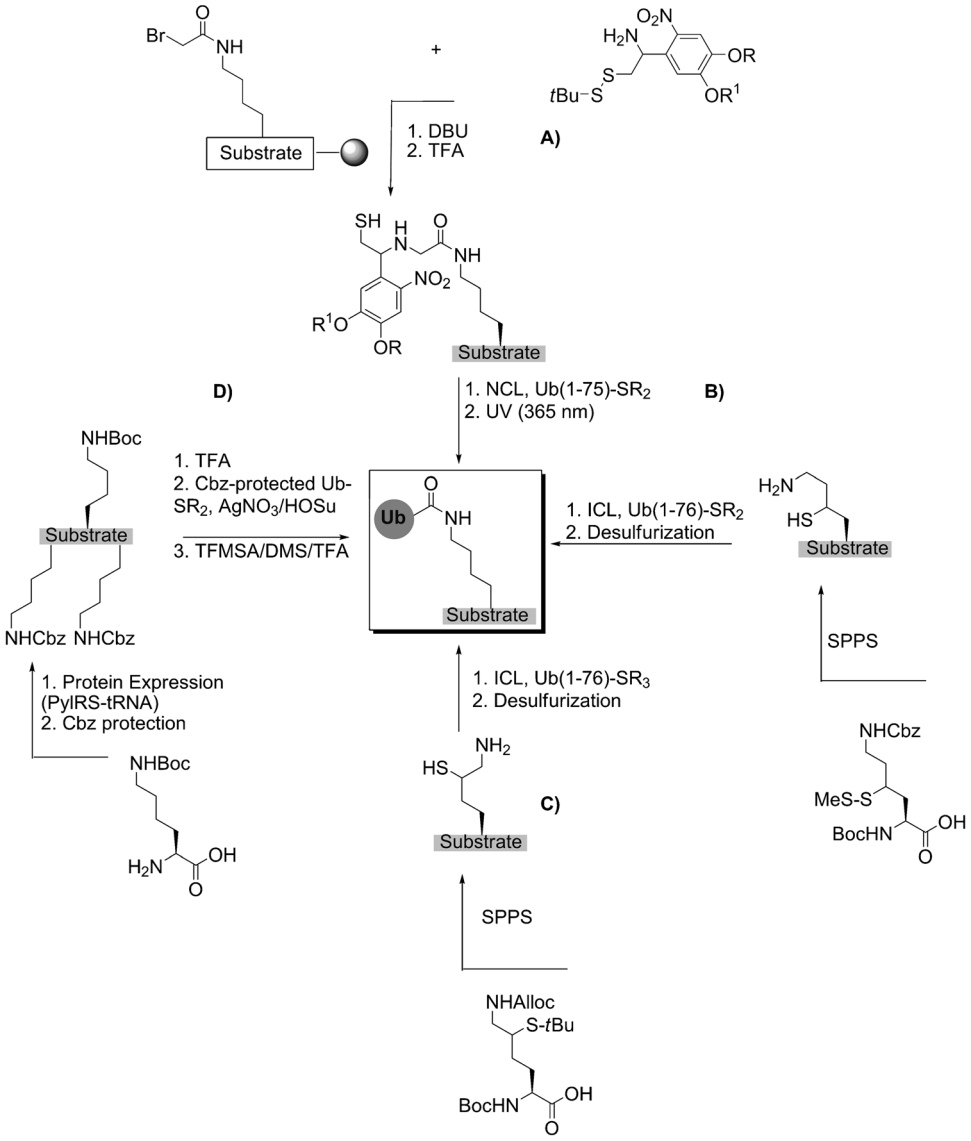
One drawback of this approach is related to the use of only one Ub unit in a probe, hence the effect of the chain structure and dynamics as well as the sequence surrounding the isopeptide bond are excluded. Such structural elements are very important in the recognition of protein partners, and could play a vital role in the selective identification of the specific enzymatic mechanisms. With the synthetic methods available today, this strategy could be extended to the longer chains to enable identification of new components in the Ub system that are more selective to a specific chain.

5.2. Chemical Methods for Constructing the Isopeptide Bond

During the last few years several novel methods have been developed to construct the native isopeptide bond in Ub-based bioconjugates.[144,145] Muir and co-workers reported a strategy based on auxiliary-mediated ligation and intein expression to assemble a ubiquitinated peptide derived from H2B (Scheme 5A).[146] In this seminal work, the H2B(115–125) peptide was prepared by Fmoc-SPPS, in which K120 was orthogonally protected with iv-Dde (1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl). After removal of iv-Dde, bromoacetic acid was coupled to the free amine of K120 and the photocleavable auxiliary was attached to enable ligation with the expressed Ub(1–75) thioester. However, the ligation step was considerably slow (5–7 days), presumably because of steric hindrance at the ligation junction. Subsequently, the auxiliary was removed by irradiation with He/Cd laser light of 325 nm to give the desired ubiquitinated peptide. The slow rate and moderate yield of the ligation step have limited the applicability of this method,[147] but has encouraged several research studies in the field.

In an effort to device a new method to generate ubiquitinated peptides efficiently, Liu and co-workers and our research group independently reported two strategies based on mercaptolysine derivatives to introduce the isopeptide bond. Liu and co-workers used orthogonally protected γ-mercaptolysine, which enabled sequential ligation to the α- and ε-amines through a six-membered-ring intermediate. This synthetic residue was incorporated in a short peptide to allow ligation of the isopeptide bond (ICL) with an expressed Ub thioester.[148] A final desulfurization under metal-free conditions (VA-044, TCEP (TCEP = tris(2-carboxyethyl)phosphine), *t*BuSH)[149] afforded the desired ubiquitinated peptides in good yields (Scheme 5B). Our approach positioned the thiol handle at the ε-carbon atom of the lysine residue (that is, in the form of a δ-mercaptolysine) to enable ligation to the ε-amine through a five-membered-ring intermediate (Scheme 5C).[150,151] We also extended the use of the δ-mercaptolysine for Boc- and Fmoc-SPPS as well as sequential ligation by developing five different analogues of the δ-mercaptolysine bearing various orthogonal protecting groups (Figure 5, **1**–**5**).[152] Recently, Chin and co-workers reported a genetically encoded δ-mercaptolysine, from which analogues **6**–**9** (Figure 5) were prepared to examine their efficient and site-specific incorporation into recombinant proteins, by evolution of a pyrrolysyl-tRNA synthetase/$tRNA_{CUA}$ pair. The usefulness of the method was demonstrated in the synthesis of K6-linked di-Ub and ubiquitinated SUMO.[153]

Ovaa and co-workers recently reported the use of δ-mercaptolysine in the synthesis of labeled Ub and Ubl-based (Ubl = ubiquitin-like) assays. In their study, the fluorescent



Scheme 5. Current methods for the formation of the isopeptide bond. R = $CH_2CH_2CH_2C(O)NHCH_3$, $R^1 = CH_3$, $R^2 = CH_2CH_2SO_3H$, $R^3 = CH_2CH_2CO_2H$. DBU = 1,5-diazabicyclo[5.4.0]undec-5-ene, TFA = trifluoroacetic acid, Cbz = carbobenzyloxy, HOSu = *N*-hydroxysuccinimide, Boc = *tert*-butyloxycarbonyl.

moiety TAMRA (5-carboxytetramethylrhodamine) was conjugated to the N terminus of the dipeptide δ-mercaptolysine-Gly, which was ligated with Ub or Ubls to generate fluorescence polarization assays. This assay was found to be useful for reporting on the activities of several DUBs, such as UCH-L3, USP7, and USP21, as well as for determining their kinetic parameters.[154]

The research groups of Komander and Chin developed a method to form the isopeptide bond by applying genetically encoded orthogonal protection and activated ligation (GOPAL).[85] In this strategy (Scheme 5D), genetically encoded Lys(Boc) was introduced at the desired position in a specific substrate while the remaining Lys residues were protected with *N*-(benzyloxycarbonyloxy)succinimide (Cbz-OSu). Subsequently, the Boc group was removed to allow direct coupling with the Ub thioester (obtained by intein expression) in which all the free amine groups were protected with Cbz groups. The isopeptide bond was formed by selective acylation of the thioester in the presence of HOSu (*N*-(hydroxy)succinimide) and Ag^I.[155] Finally, the protecting groups were removed to yield the Ub conjugates. One noticeable drawback of this method is the use of several protection and deprotection steps, which are needed to allow site-specific ligation between specific Lys residues and the C terminus of the consecutive Ub, thus resulting in a low yield of product. The use of this method in the synthesis of K6- and K29-linked di-Ub chains for structural and functional analyses will be discussed in Section 5.4.

To expedite the synthesis of ubiquitinated peptides we developed a method based on SPPS and NCL, without the need for mercaptolysine analogues (Scheme 6).[156] Our strategy relied on the use of the orthogonally protected Lys(iv-Dde), used also by the research groups of Muir[146] and Przybyski[157] for the formation of the isopeptide bond. Upon completion of the peptide synthesis, the ε-amine protecting group was selectively removed to allow the formation of the isopeptide bond and further peptide elongation of Ub(46–76) with an N-terminal Cys residue. The complementary fragment, Ub(1–45), was prepared with the thioester functionality at the C terminus by Fmoc-SPPS and N-acylurea-based reactions.[158] These two fragments were ligated using NCL, followed by a desulfurization step to give the desired ubiquitinated peptide in excellent yields. By using this approach, we studied the effect of the length of the peptide substrates on the cleavage efficiency of the isopeptide bond by UCH-L3. Previous biochemical and structural analyses revealed that this enzyme uses a disordered active-site crossover loop of 20 residues, which imposes substrate filtering and restricts access of larger substrates.[120,159–162] To further support these studies, we assembled four different ubiquitinated peptides of various lengths (8-, 15-, 21- and 31-mer) derived from the C terminus of H2B. Examination of these substrates with UCH-L3 revealed that peptides with up to about 20 residues are the preferred substrates for this enzyme, which

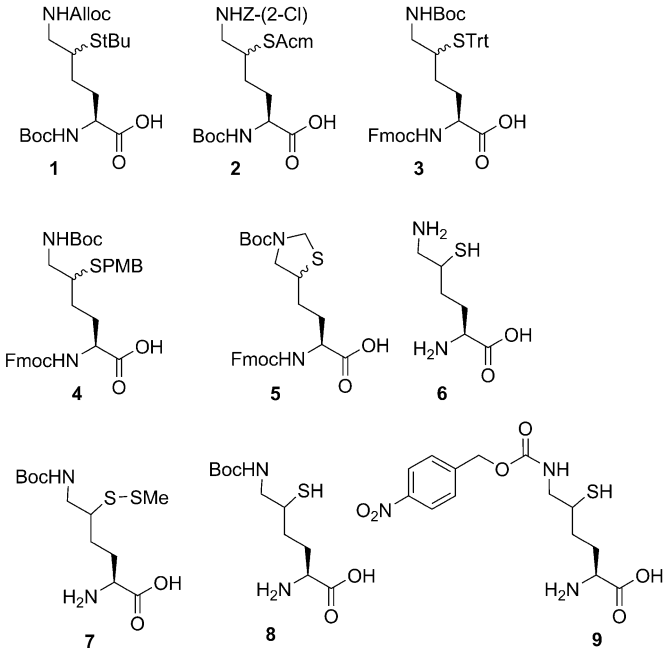
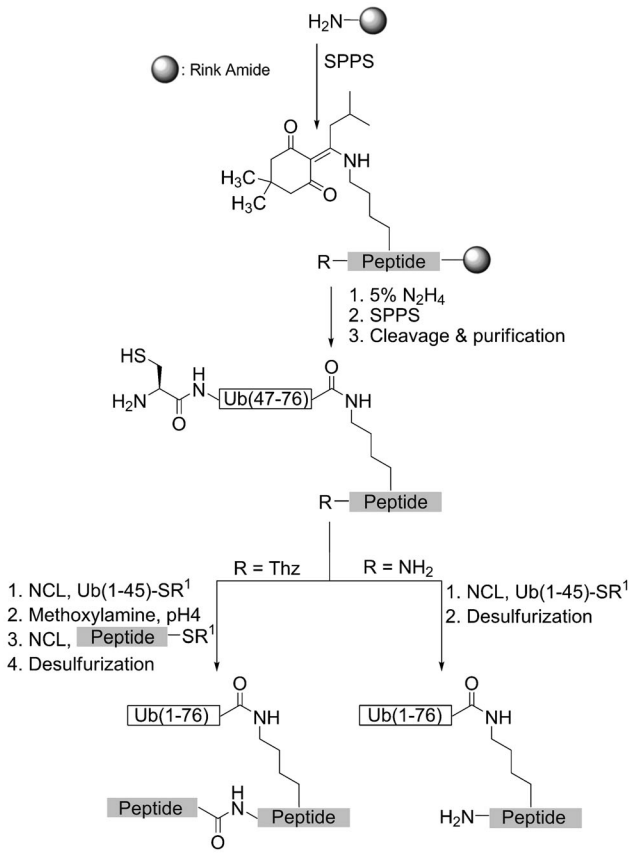


Figure 5. The δ-mercaptolysine analogues, which were prepared for Boc-SPPS, Fmoc-SPPS, and sequential ligation (**1**–**5**) as well as for incorporation into recombinant proteins (**6**–**9**).



Scheme 6. Application of SPPS and NCL/desulfurization for the synthesis of ubiquitinated peptides. $R^1 = CH_2CH_2CO_2CH_3$

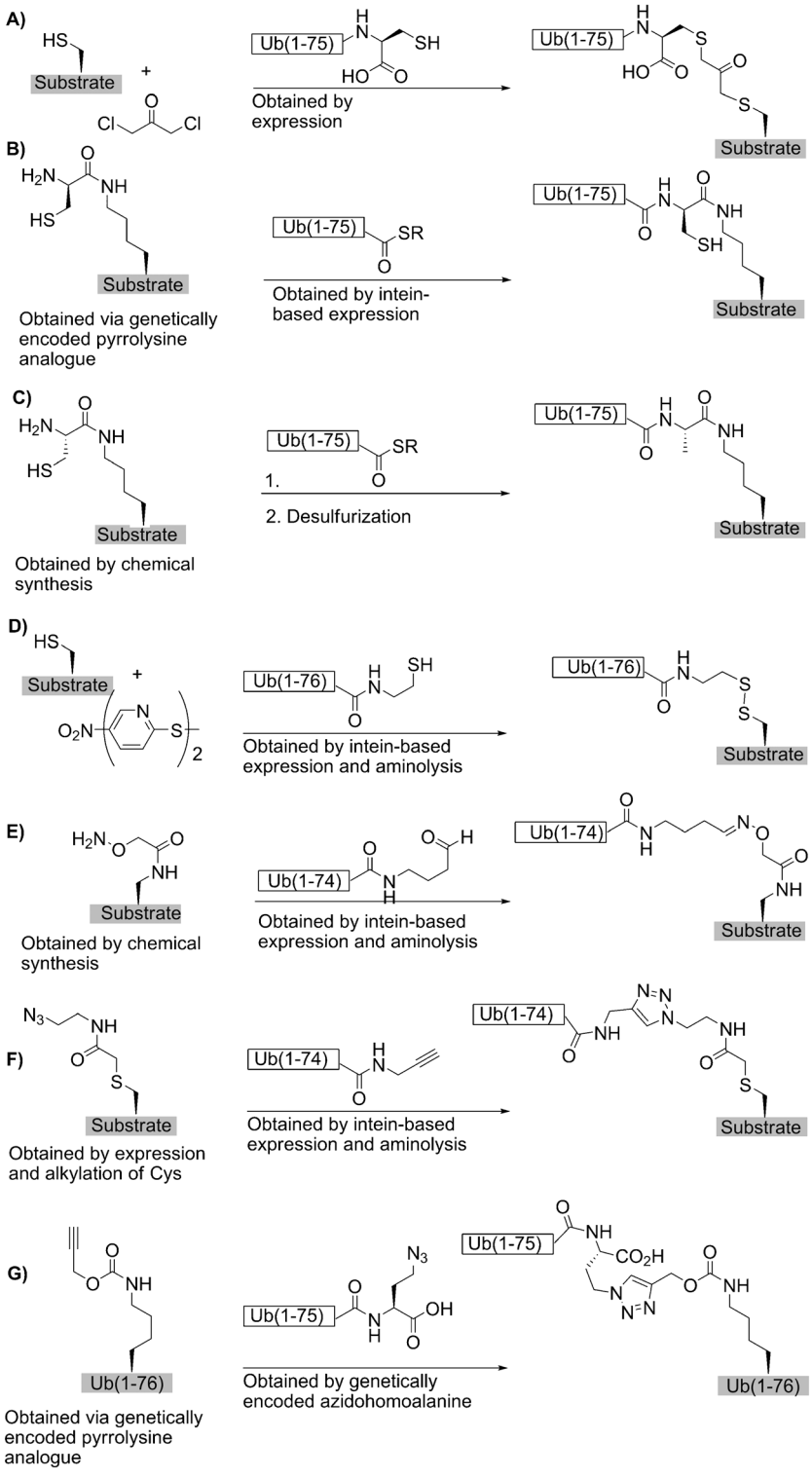
was consistent with previous structural and biochemical data.[120–123,159–162]

5.3. Isopeptide Bond Surrogates

Parallel to the studies that permitted the synthesis of Ub conjugates bearing the naturally occurring isopeptide bond, several research groups reported the development of chemical and semisynthetic methods to generate isopeptide mimics (Scheme 7). The main goals for such approaches are to ease the challenging synthesis of Ub bioconjugates and to produce stable analogues for a variety of studies such as activity-based probes for proteomics, DUB inhibitors, and for crystallography studies to shed light on mechanistic aspects of DUBs. However, these analogues cannot be used to investigate some biochemical aspects of DUBs, such as kinetic characterizations and specificity profile.

Wilkinson and co-workers were the first to design and prepare K63-, K48-, K29-, K11-, and head-to-tail linked di-Ub bearing a non-native isopeptide bond (Scheme 7 A).[163] In their strategy, a Cys residue was introduced at the desired positions in the proximal Ub, and Gly76 of the distal Ub was replaced with Cys. The two Ub monomers were reacted in the presence of 1,3-dichloroacetone to generate the isopeptide analogue. The biochemical studies performed with these nonhydrolyzable analogues revealed that they selectively inhibited the activity of several DUBs, such as UCH-L3 and IsoT (Isopeptidase T), in the cleavage of Ub-AMC. This study revealed that the linkage type of the di-Ubs has an important role in the recognition of the examined DUBs. It was also found that these di-Ub analogues have the ability to inhibit the enzymatic machinery of ubiquitination (i.e E1–E3). Moreover, the method was extended to the preparation of a K29-linked tetra-Ub chain attached to sepharose resin.[164] By incubating this bound chain with a yeast lysate and using affinity chromatography they identified two proteins that interacted very strongly with K29-linked poly-Ub. These are Ubp14, the yeast DUB that has similar enzymatic properties as the mammalian IsoT, and Ufd3, a cofactor related to the Ub-proteasome system.

In a more recent study, Chan and co-workers reported a pyrrolysine analogue for site-specific protein ubiquitination.[165] Specifically, the group genetically incorporated the pyrrolysine analogue (*S*,*S*)-D-Cys-ε-Lys, by using the UAG codon in the presence of pyrrolysyl-tRNA synthetase/$tRNA^{pyl}$, into the calmodulin protein (CaM). This protein is known to play an important role in the calcium signaling system in eukaryotes. The introduction of this chemical handle allowed site-specific ligation between this modified protein and the Ub(1–75) thioester to afford ubiquitinated CaM with a G76-D-Cys mutation (Scheme 7 B). Previous studies have shown that ubiquitination of CaM at K21 modulates its regulatory processes, for example, by decreasing CaM binding to kinases, thereby resulting in a decrease of its activity. The synthetic analogue of ubiquitinated CaM exhibited 85% decreased ability to modulate the kinase activity when compared to Wt-CaM, which was comparable to the enzymatically prepared ubiquitinated CaM. However,



Scheme 7. Strategies for the synthesis of isopeptide bond mimetics.

in another study examining the effect of the modified ubiquitinated analogue on the protein phosphatase 2B activity, a known protein phosphatase that is regulated by CaM, ubiquitination was found to have no effect on the ability of CaM to modulate the activity of phosphatase 2B.

In analogy to the use of D-Cys at the ligation junction, Muir and co-workers attached L-Cys chemically to a Lys residue of a peptide to allow NCL with the Ub(1–75) thioester. After the NCL step, the Cys residue was desulfurized to Ala to afford the ubiquitinated bioconjugate with the G76A mutation in Ub (Scheme 7C). The application of this method in the synthesis of ubiquitinated analogue of H2B protein[166] will be discussed in Section 5.5.

The research groups of Muir and Zhuang took advantage of the native Cys residue in a protein and independently reported the use of a disulfide bond as a replacement for an isopeptide bond (Scheme 7D).[167,168] This genetic method renders, in principle, any protein amenable to ubiquitination at a specific site. However, the ease of reduction of the disulfide bond in a biological setting and the requirement for reducing reagents in a variety of biochemical experiments could limit the applicability of the method. Moreover, the method is limited to proteins containing a single Cys residue. This strategy was used to examine the role of ubiquitination in two different proteins, and these examples will be discussed in detail in Section 5.5.

In a continuation of their efforts to introduce novel surrogates of isopeptide bonds, Ovaa and co-workers developed a method to prepare ubiquitinated peptides in which the native isopeptide bond was replaced by an oxime linkage (Scheme 7E).[169] Their strategy involved the synthesis of two short peptides derived from K48- and K63-linked di-Ub chains that mimic the surrounding of the isopeptide bond of the proximal Ub. The Lys residue in each peptide was replaced by aminoxyacetyl-L-diaminopropionic acid to introduce the non-native isopeptide in a site-specific manner. The Ub(1–74) with an aminobutyraldehyde functionality was treated with the aminoxy peptide to form the oxime bond. These bioconjugates were studied by surface plasmon resonance to determine the effect of the peptide sequence near the isopeptide bond on the specificity of several known DUBs such as USP7, USP4, and USP21. The binding study revealed that the tested DUBs discriminated between the various stable Ub bioconjugates, and that the peptide sequence flanking the isopeptide plays an important role in the selectivity.

Weikart and Mootz used Cu^{I}-catalyzed click reactions to prepare SUMOylated and ubiquitinated substrates with the

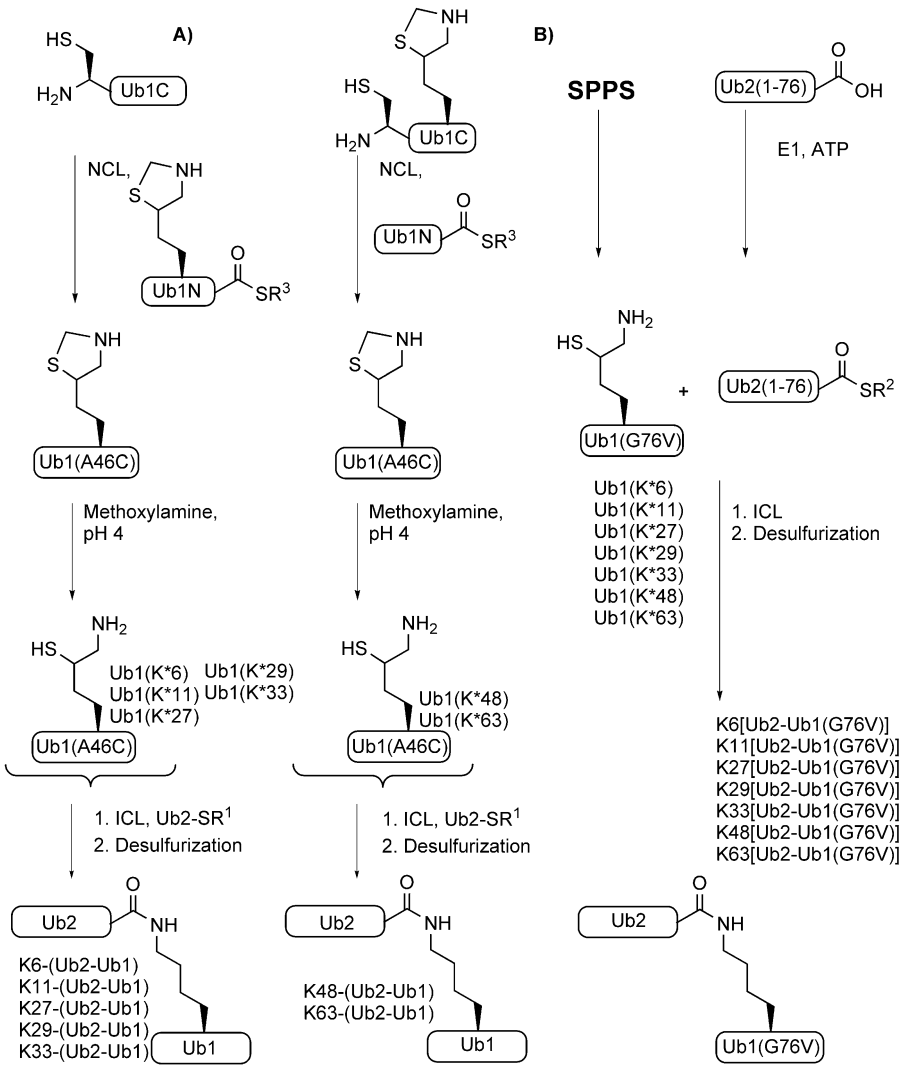
triazole linkage (Scheme 7F).[170] Their strategy involved intein-based expression of SUMO1, SUMO2, yeast Smt3, and Ub in which the two Gly residues at the C terminus were omitted, and a subsequent substitution of the thioester functionality by propargylamine. The side chain of the Cys residue in the substrate was alkylated with iodoacetamide ethyl azide to introduce the azide functionally and allow for the formation of the 1,4-triazole between the two precursors. This approach was extended to the synthesis of the SUMOylated SUMO-specific E2 enzyme (Ubc9) by using *p*-azidophenylalanine so as to elucidate the role of SUMO2 on the enzymatic activity of Ubc9.[171] The study revealed that SUMOylated Ubc9 with SUMO2 displayed an altered substrate preference toward Sp100 and RanGAP proteins, as uncovered earlier for SUMO1.[172] These results indicate that SUMOylation of Ubc9 could be a general mechanism to regulate the discrimination of this enzyme for its target.

Rubini and co-workers used a similar click chemistry approach to prepare all Lys-linked di-Ub chains.[173] In this strategy, the Lys residue of the proximal Ub was replaced with a propargyl-protected Lys residue, while the C-terminal Gly residue of the distal Ub was replaced with azidohomoalanine (Scheme 7G). These chains with the triazole linker were found to be recognized by the ubiquitination machinery. More recently, Mootz and co-workers also applied click chemistry to prepare all Lys-linked di-Ub analogues. This strategy allowed the preparation of these triazole-modified chains in hundreds of micrograms to a few milligrams, which enabled the investigation of their binding affinity with the UBA domain of the Mud1 protein.[174] Their study showed that the K48 analogue exhibited the strongest binding, the K27- and K11-triazole analogues had a weak binding, while the remaining chains showed no binding to this domain.

Since the specificities of the Ub chains are dictated to a large extent by their unique conformations, it is crucial that these modifications do not affect such proprieties. However, it is not yet clear what the influence of these isopeptide bond replacements on the dynamic and conformation of the specific chain. In principle, these modifications could affect structural properties, hence resulting in inaccurate analyses in different studies. As the Ub building blocks are obtained by expression methods in these synthetic strategies, this should enable their labeling (e.g. ^{15}N) for NMR studies to verify the effect of the isopeptide mimics on these aspects.

5.4. Synthesis of Ub Chains with Native Isopeptide Bonds

One of the major breakthroughs in the chemical biology of Ub stemmed from the development of different strategies to prepare highly homogeneous Ub chains without the use of the specific enzymatic machinery for each chain. In this regard, our research group was the first to prepare all Lys-linked di-Ub chains by a fully synthetic approach.[175] Our approach relied on the synthesis of the proximal Ub from two fragments, Ub1C (Ub46–76) and Ub1N (Ub1–45), in which the δ-mercaptolysine was incorporated at the desired position in each chain (Scheme 8A). The proximal Ub was then linked to the distal Ub thioester through the δ-mercaptolysine-mediated ICL step. Subsequently, a desulfurization step was applied to convert the Cys46 residues in each di-Ub chain into



Scheme 8. The synthesis of all Lys-linked di-Ub chains: A) Fully synthetic approach, B) enzymatic method combined with a chemical approach. K* = δ-mercaptolysine, $R^1 = CH_2CH_2CO_2H$, $R^2 = CH_2CH_2SO_3H$, $R^3 = CH_2CH_2CO_2Me$.

their native Ala46 along with the removal of the thiol handle at the isopeptide bond to furnish the native di-Ub structure. These chains were obtained in milligram quantities and high purity. The correct folding of all the synthetic di-Ub chains was confirmed by CD spectroscopy, and their activities with the two DUBs, UCH-L3 and IsoT, were studied. The latter enzyme is responsible for the disassembly of unanchored poly-Ub chains in vivo.[176,177] Our results showed that while the UCH-L3 exhibited no activity against any of the chains, IsoT was able to cleave most of these chains, thus supporting the wide substrate range of this enzyme.[175]

We used a semisynthetic approach to study the segmental isotopic labeling of Ub chains to unravel monomer-specific molecular behavior.[178] The weak interactions between monomers in Ub chains makes their structural characterization by X-ray crystallography a very challenging task; however, several studies have already been successfully carried out.[42,85,92,95,97,106] The difficulty arises because Ub chains either do not crystallize or the resulting crystal structures do not often represent the physiologically relevant conformations in solution.[23,92,93,95] Together, this makes NMR spectroscopy a highly important structural tool for studying the conformation and dynamics of these chains. Unfortunately, the homopolymeric nature of poly-Ub makes it almost impossible to resolve the NMR signals of each individual Ub unit in the chain, unless the Ubs are isotopically differentiated.

To address these challenges, we prepared K48- and K33-linked di-Ub chains with uniformly ^{15}N-enriched distal Ub. We then applied modern NMR methods based on isotope editing/filtering to selectively study the distal and proximal Ubs in the same chain.[175] This study allowed us to characterize, for the first time, the structure, local intramonomer dynamics, conformational properties, and ligand-binding properties of K33-linked di-Ub. The NMR data revealed relatively weak interdomain contacts in K33-linked di-Ub mediated by the hydrophobic patch on the distal Ub. Moreover, our data showed that UBA2 of hHR23A binds the K33-linked chain 10-fold weaker than the K48-linked di-Ub chain, which suggests that this chain may not serve as a signal for proteasomal degradation. One limitation of our approach is the use of proximal Ub that is synthesized chemically, as it is very difficult to label it in a similar manner to the expressed distal Ub. As a result, extracting important structural information on this Ub could be very challenging. The recent approach of genetically encoding δ-mercaptolysine should make this possible, with either the distal or the proximal Ub labeled as desired.[153]

Shortly after our report on the synthesis of all Lys-linked di-Ub, Ovaa and co-workers reported a semisynthetic methodology for the preparation of these targets (Scheme 8B).[133] Their proximal Ub was synthesized by direct synthesis on a solid support (see Section 5.1) and by using δ-mercaptolysine[150] to mediate the ICL step. A very exciting aspect of this synthesis is the use of the E1 enzyme to prepare the distal Ub in the thioester form. Gly76 in the proximal Ub was mutated to Val to prevent the activation of the C terminus as the thioester by the E1 enzyme.

The concept of using the E1 enzyme to prepare the Ub thioester was nicely extended by Fushman and co-workers for the preparation of the di- and tri-Ub thioester. The research group also modified the GOPAL strategy[85] by using the Alloc protecting group, which is removed under mild conditions, instead of Cbz. In this way they prepared the K11-linked di-, tri-, and tetra-Ub, K33-linked di-Ub, and a mixed linkage of K11 and K33 in the tri-Ub chain. By performing segmental labeling, the authors were able to characterize the middle Ub in the homogenously and mixed linkage K11 of the tri-Ub by NMR spectroscopy.[179]

The research groups of Liu and Liu also reported a semisynthetic method for the preparation of the K48-linked di-Ub chain. Their synthetic strategy for the proximal Ub was to prepare it from three fragments by sequential ligation, in which K48 was replaced with the γ-mercaptolysine to enable ligation of the backbone and isopeptide.[180]

The research groups of Chin and Komander used the so-called GOPAL strategy[85] described in Section 5.2 to prepare K6- and K29-linked di-Ub chains. This allowed the crystal structure of the K6-linked di-Ub chain to be solved for the first time, and revealed a new compact, asymmetric conformation wherein the known hydrophobic patch of the proximal Ub interacts with a new hydrophobic patch consisting of L71, I36, and L8 of the distal Ub. Moreover, they screened 11 DUBs from different families to study their proteolytic activities and specificities with these Ub chains. Interestingly, the TRABID enzyme, which was thought to be specific for K63-linked chains, exhibited a 40-fold higher cleavage efficiency toward K29-linked di-Ub.

The current possibility of having all of the di-Ub chains allows them all to be used in parallel to elucidate the relationship between the different chain structures and their binding behaviors and specificities with the different UBDs and DUBs. For example, Sixma and co-workers recently screened all the di-Ub chains against 12 USP isopeptidases and found that they could hydrolyze all the linkages without a high specificity towards a particular chain.[181] Similarly, Komander and co-workers also reported a comprehensive screen with all the di-Ub chains against the human ovarian tumor (OTU) domain of TRABID and found that this enzyme specifically hydrolyzes both the K29- and K33-linked chain. Interestingly, this study revealed a new UBD consisting of an ankyrin repeating unit, which was found to be crucial for the TRABID catalytic activity and specificity.[182]

The above-described studies on the di-Ub chains have provided useful information on several aspects of Ub biology and will continue to contribute to the Ub field in various ways. Nonetheless, the requirement for chains with different connectivities (Figure 2) and increasing numbers of Ub monomers beyond di-Ub is crucial. For example, several in vitro studies revealed that most UBDs bind to the Ub monomer, however, increasing numbers of Ub units in a specific chain can be used both as a mechanism to increase the binding and to enforce the cellular specificity of their interactions with other proteins such as with DUBs.[71–73,81,82] Moreover, the structural information that the di-Ub chains adopt may not fully represent the native structure of the longer chain, for example, tetra-Ub.[92] Another example of the importance of

the chain lengths and its effect on the fate of ubiquitinated protein can be found in the case of the K48-linked chain. It has been generally accepted that the minimal signal necessary for proteasomal degradation is a chain composed of four Ub molecules.[183] However, some relatively small proteins are degraded efficiently in their monoubiquitinated form.[5,47–49,184] For the other chains, the preferred length to promote a specific signal is still unclear. Hence, the preparation of the chains with various lengths, in their free or anchored forms, is crucial to address some of the open questions in the field.

Towards these goals, we have recently accomplished the first total chemical synthesis of an anchored K48-linked tetra-Ub chain. This chain is composed of 304 residues, and represents the longest protein made chemically to date.[185] Linear and convergent strategies were used to assemble this chain, where a key step in this synthesis was the ability to prepare di-Ub with a C-terminal thioester functionality (Scheme 9). However, such a synthetic strategy has several obstacles. For example, the requirement of several ligation steps to construct the different Ub building blocks and the final assembly of the tetra-Ub significantly lowered the yield of the synthesis. Moreover, the presence of seven thiols at the final stage made it difficult to desulfurize them completely. This forced us to desulfurize the Cys46 residue in each building block before their final assembly to yield the tetra-Ub having the correct fold and desired activity toward IsoT.

These obstacles were recently solved by preparing the different building blocks directly on a solid support, which reduced the number of ligation steps and the number of thiol groups for the final desulfurization step.[134] This enabled us to efficiently prepare a set of ubiquitinated peptides with various chain lengths (mono-, di-, tri-, and tetra-) and types (K63, K48) by employing the convergent strategy (Figure 6). For the first time, the activity of two DUBs, UCH-L3 and IsoT, were assessed with these sets of ubiquitinated peptides. Our study revealed that although the UCH-L3 can cleave the tripeptide from the proximal Ub of both types of chains (K48 and K63), a decrease in the efficiency of hydrolysis was observed as the length of chain increased. The decrease in the cleavage efficiency in the case of the K63-linked chains was more pronounced compared to that of the K48-linked chain.

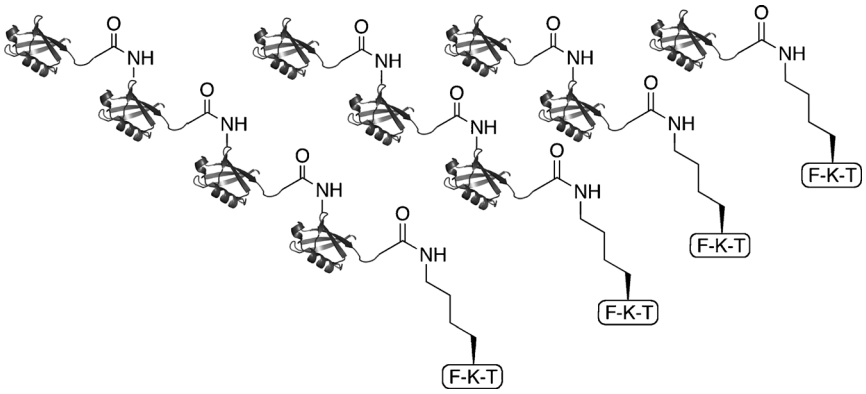
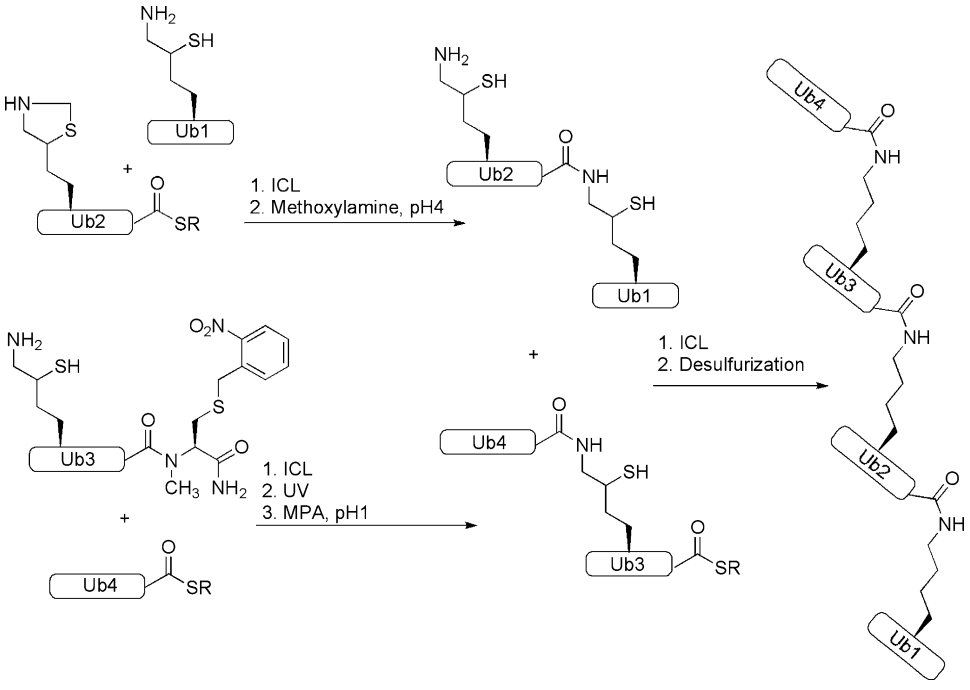


Figure 6. The tripeptide (FKT) linked to mono-, K48- or K63-di-, tri-, and tetra-Ub.



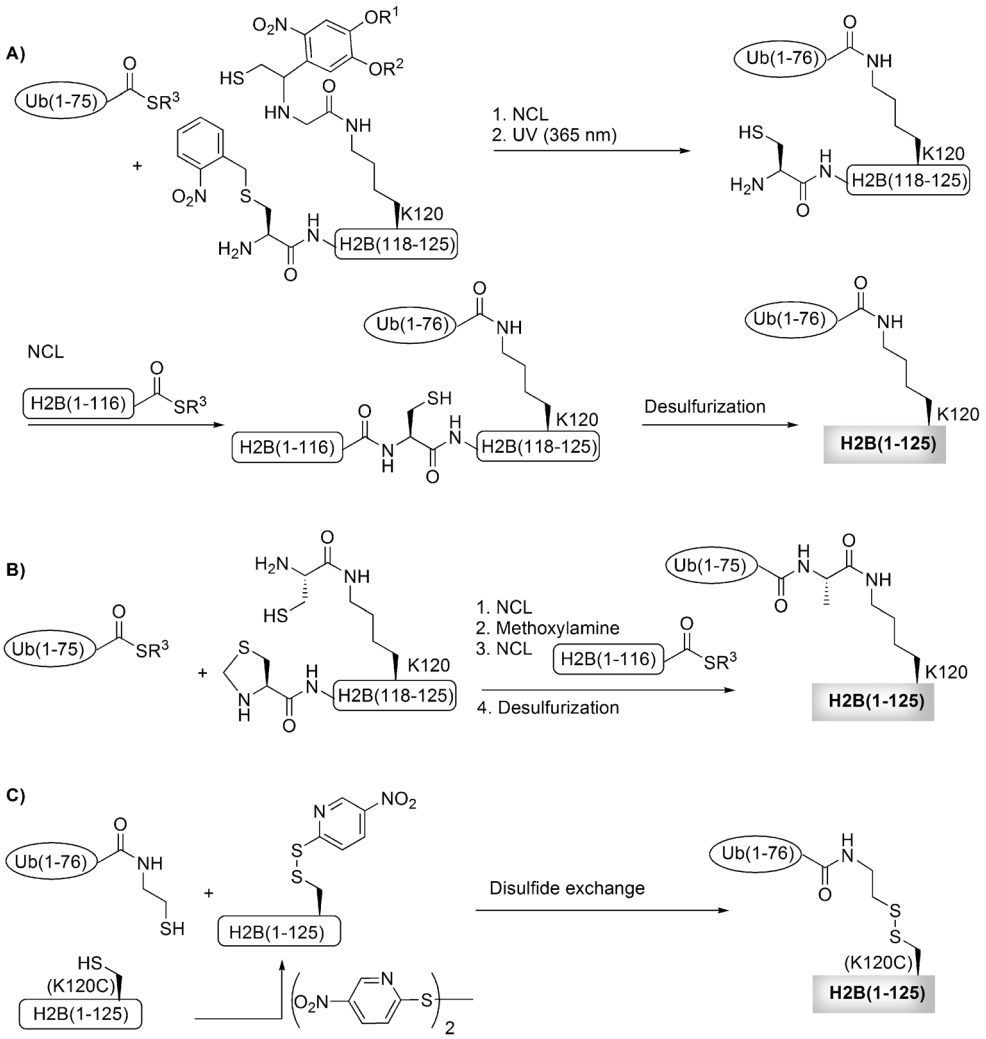
Scheme 9. The convergent approach to prepare tetra-Ub. The Ub building blocks 1–4 were prepared by chemical synthesis. R = $CH_2CH_2CO_2H$.

5.5. Synthesis and Semisynthesis of Monoubiquitinated Proteins

The above-described methods for constructing the native isopeptide bond and its mimics have also allowed the preparation of several monoubiquitinated proteins in a highly homogeneous manner and relativity large quantities for a variety of studies to determine the role of monoubiquitination on protein structure and function. Interestingly, recent proteomic studies found that monoubiquitination is a major form of the numerous Ub bioconjugates.[25]

The preparation of monoubiquitinated H2B represents the first example of using chemical methods combined

with semisynthesis to prepare monoubiquitinated protein. The H2B protein is one of the four histone proteins (H2A, H2B, H3, and H4), which constitute the nucleosome, and is wrapped by 147 base pairs of DNA in eukaryotic chromatin.[186] Histones undergo several PTMs such as phosphorylation, acetylation, and ubiquitination, mainly at their N- and C-terminal ends. These modifications play essential roles in regulating chromatin dynamics, gene expression, and DNA repair.[187,188] Histone H2B is monoubiquitinated at K120 in mammals and was recently reported to also undergo monoubiquitination at K34.[189] Despite extensive studies on the role of K120 ubiquitination, several questions remain open. For example, how is ubiquitinated H2B in chromatin recognized and processed by the known DUBs, is there any cross-talk between different PTMs, and how do these multiple modifications affect the regulation of gene expression? These studies have been hindered by the lack of suitable reagents based on homogeneously ubiquitinated H2B.



Scheme 10. The different strategies adopted to prepare Ub-H2B with native and non-native isopeptide bonds. $R^1 = (CH_2)_3C(O)NHCH_3$, $R^2 = CH_3$, $R^3 = CH_2CH_2SO_3H$.

One specific question is related to the cross-talk between the ubiquitination and methylation of K79 of histone H3. The latter modification is one of the main PTMs that affects the chromatin structure and regulates gene expression. Previous data have shown a correlation between ubiquitination and increased methylation of K79 catalyzed by K79-specific methyltransferase DotlL (disrupter of telomeric silencing-like).[190–193] However, the mechanism by how such cross-talk is achieved is unclear. It has been proposed that ubiquitination of H2B could induce K79 methylation directly, either by changing nucleosomal accessibility, or through the recruitment of enzymatic function.[194,195]

To shed light on this process, Muir and co-workers have reported a semisynthetic approach to prepare, for the first time, monoubiquitinated H2B (Scheme 10 A).[196,197] In their synthesis, the H2B was divided into two fragments: H2B(1–116) and H2B(117–125), in which the latter fragment was equipped with a photolytically active auxiliary (see Section 5.2). Moreover, this fragment was further decorated at the N terminus with an orthogonally protected Cys residue (originally Ala117). The assembly of ubiquitinated H2B started with an auxiliary-mediated ligation of the H2B(117–125) peptide with the Ub(1–75) thioester, in which exposure to light simultaneously removed the auxiliary and the 2-nitrobenzyl group to give ubiquitinated H2B(117–125) in a moderate yield. Subsequently, this fragment was ligated with expressed H2B(1–116) thioester to give the full-length ubiquitinated H2B(A117C). A final desulfurization step converted Cys117 into Ala117 and furnished the native ubiquitinated H2B with excellent purity.

The semisynthetic version of ubiquitinated H2B was successfully incorporated into the core histone octamer with recombinant Wt-H2A, H3, and H4. Other modified histone octamers were prepared containing the recombinant mutated H3(K79R) with and without the ubiquitinated H2B. Dinucleosomes with asymmetric insertion of ubiquitinated H2B and H3(K79R) were also formed. Biochemical analysis revealed that ubiquitinated H2B directly activates the methylation of H3K79 by Dot1L. Moreover, the asymmetrical dinucleosomes proved that efficient methylation of H3K79

requires the presence of ubiquitinated H2B in the same nucleosome, which provides evidence for a direct biochemical cross-talk between methylation and ubiquitination on separate histone proteins within a nucleosome.

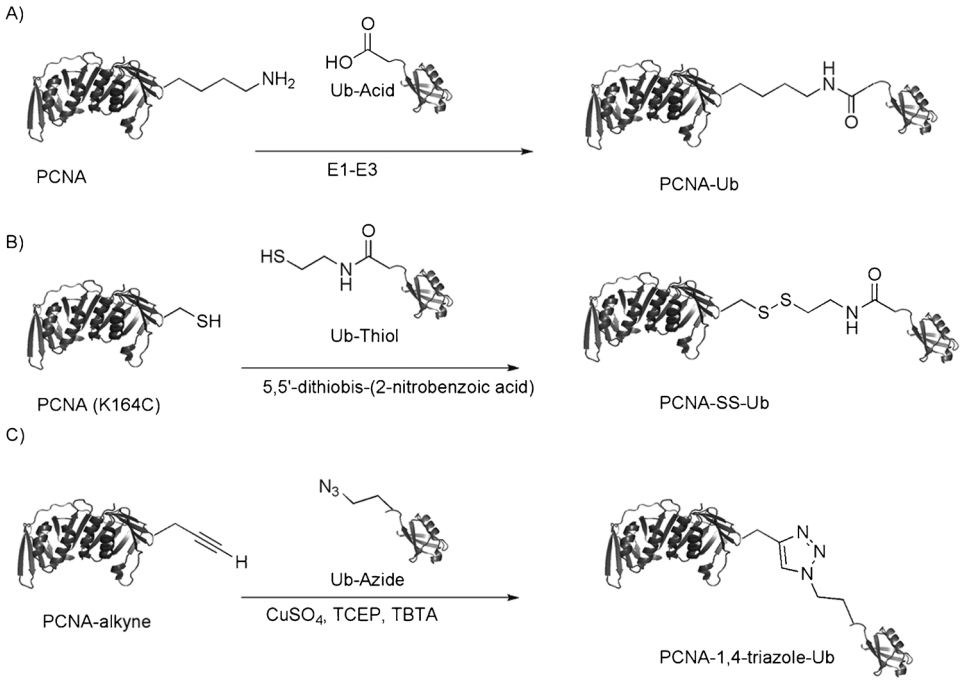
The moderate yield and extended time of the ligation step mediated by the photolabile auxiliary prompted Muir and co-workers to device alternative strategies for the more efficient synthesis of monoubiquitinated H2B (Scheme 10B,C). Two different strategies were developed that led to efficient syntheses, but compromised the native structure of the ubiquitinated H2B. In the first method, H2B(117–125) branched with Cys was used for the NCL with the Ub(1–75) thioester. The remaining steps, after NCL, were very similar to the previous strategy; however, the final desulfurization step afforded the ubiquitinated H2B construct with a G76A mutation (Scheme 10B).[166] Nevertheless, this analogue was found to stimulate Dot1L activity to comparable levels as that of the wild type. The authors were able to perform kinetic characterization of the activity of Dot1L by using the nucleosomes containing the ubiquitinated histone H2B, in contrast to previous studies that were performed on peptide and protein substrates. The study revealed a rate enhancement of the catalytic activity of Dot1L on nucleosomal substrates in addition to a 1:1 stimulation of methylation by ubiquitination. In an effort to assess the importance of the structural features of Ub on the activity of Dot1L, Ub was replaced with Smt3 (Ubl). This change led to a failure of Dot1L stimulation, which is presumably due to the specific recognition of the Ub surface. Interestingly, mutating two of the three residues of the hydrophobic patch in Ub (L8A/I44A) in addition to G76A did not affect the methylation step, which may indicate that the hydrophobic patch might not be involved in the recognition of Dot1L.

Despite this Ub-H2B analogue bearing the isopeptide bond, the replacement of G76A could affect the activities of DUBs that are known to be specific to Ub-H2B, for example, the SAGA deubiquitinating module.[89–91] This remained to be tested to expand the use of this Ub-H2B analogue for studies related to these DUBs.

The second strategy used by Muir and co-workers to expedite the synthesis of monoubquitinated H2B is based on a disulfide linkage as a replacement for the isopeptide bond (Scheme 10C).[167] To achieve this, Lys120 was mutated to Cys, while an intein-mediated transthioesterification with cysteamine generated a thiol handle at the C terminus of Ub. Subsequently, the H2B(K120C), after activation with 2,2′-dithiobis(5-nitropyridine) (DTNP), was treated with the Ub thiol to generate the disulfide analogue of ubiquitinated H2B. Notably, this analogue exhibited a stable methylation by the Dot1L. With this straightforward method to generate this bioconjugate, a small set of ubiquitinated H2Bs was prepared by relocating the ubiquitination position on H2B (108, 116, 125) and at position 22 of H2A, hence presenting Ub at various surface sites of the nucleosome. Interestingly, ubiquitination of H2B at positions 125 and 22 in H2A (which is nearby the site of Ub in native ubiquitinated H2B) stimulated most of the activity of Dot1L. However, ubiquitination at positions 108 and 116 significantly impaired its activity. Together, these results show that the Dot1L activity tolerates the ubiquitination sites and that ubiquitination at K125 could also serve for the stimulation of Dot1L activity.

By using the disulfide analogue of ubiquitinated H2B, Muir and co-workers also studied the effect of ubiquitination on the local and higher-order of chromatine fibers.[198] The dynamic structure between different structural states is a notable feature of chromatin and plays an important role in gene regulation. The primary structure of chromatin folds into a compacted secondary structure of 30 nm fibers and into large tertiary structures that eventually construct an entire chromosome. This fiber compaction is regulated by PTMs,[199] such as through the acetylation of K16 in H4.[200] Despite several proposals and models on the role of ubiquitination on nucleosome stabilization,[201,202] a detailed analysis of this modification on the chromatin compaction remains unsolved. Muir and co-workers used their chemically defined nucleosome arrays coupled with a fluorescence anisotropy based assay to shed light on this process. They found that ubiquitination interferes with chromatin fiber compaction in a way that led to an open and accessible fiber conformation. Two notable findings emerged from this study: 1) ubiquitinated H2B and acetylated H4 affect chromatin compaction through distinct mechanisms, and when both modifications are present they inhibit higher-order chromatin structure in a synergistic manner; 2) replacing Ub in Ub-H2B by Hub1, a Ubl which has a similar fold and shares 23% sequence homology with Ub, did not lead to a significant chromatin compaction. The latter finding shows that the fiber disruption is specific to Ub, presumably through its unique surface and is not due to steric hindrance.

Parallel to the studies of Muir and co-workers, where they applied the thiol exchange strategy to assemble Ub-H2B analogues, Zhuang and co-workers reported a very similar approach for the synthesis of the ubiquitinated proliferating cell nuclear antigen (PCNA).[168] This protein is known to be involved in DNA replication and repair, cell cycle control, and chromatin remodeling.[58] PCNA is also known to undergo monoubiquitination at position 164 (Scheme 11A), which plays a crucial role in eukaryotic transletion synthesis (TLS).[203] The process of DNA repair requires replicative polymerase to be replaced by a nonclassical polymerase, a process that is assisted by ubiquitinated PCNA. Specifically, ubiquitinated PCNA increases the affinity of TLS polymerases, such as Polη, which possesses a UBD for PCNA and recruits the TLS polymerases to the DNA damage site. In vitro reconstitution of ubiquitinated PCNA, by using the enzymatic machinery, afforded only several micrograms of this bioconjugate, which prevented a variety of biochemical, biophysical, and structural studies. Milligram quantities of a highly homogeneous ubiquitinated PCNA were generated by applying the disulfide method described above (Scheme 11B). This analogue showed normal activity, compared to the natively occurring one, in the polymerases exchange assay. Encouraged by these results, several analogues of PCNA were prepared that were ubiquitinated at different positions, including K164, K127, K107, and R44. Interestingly, changing the site of ubiquitination did not significantly affect the efficiency of the polymerase exchange, which could be attributed to Ub being able to occupy a large conformational



Scheme 11. The preparation of monoubiquitinated PCNA by using A) an enzymatic approach B) a thiol exchange strategy, and C) click chemistry.

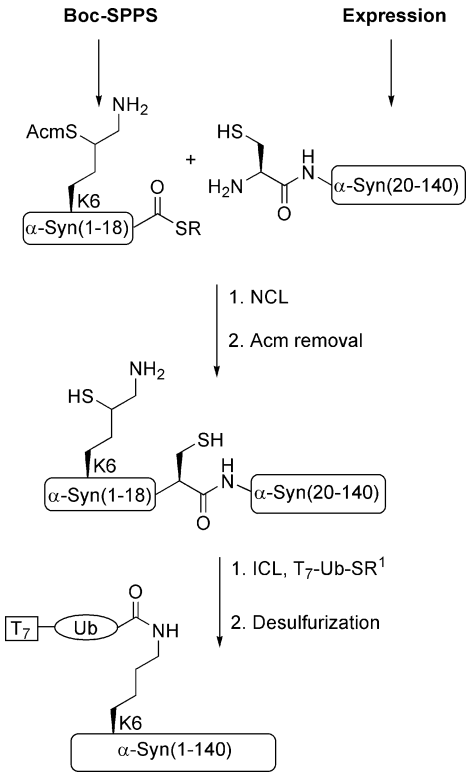
space as a result of the flexibility of the disulfide linker, hence allowing the formation of a functional ternary complex with the polymerases.

The Marx and Rubini research groups also utilized click chemistry to prepare a monoubiquitinated PCNA having the trizole linker as an isopeptide bond surrogate (Scheme 11 C).[204] This synthetic mimic of Ub-PCNA was able to stimulate DNA synthesis, albeit to a reduced extent compared to the unmodified PCNA.

In a joint effort with the Lashual research group, we accomplished the first semisynthesis of monoubiquitinated α-synuclein (α-syn) as a first step towards elucidating the role of ubiquitination in the pathogenesis of Parkinson's disease (PD).[205] Such a level of knowledge is crucial for understanding the biology of α-syn and may ultimately lead to the identification of novel therapeutic targets for the treatment of this disease.[206] The pathology of this disease is characterized by a loss of dopaminergic neurons and the presence of intracellular inclusions, known as Lewy bodies, composed mainly of α-syn. It has been reported that most α-syn species found in LBs are mono- or diubiquitinated, and to a lesser extent at multiple Lys residues.[207–209] Hence, understanding the role of ubiquitination in the regulation of α-syn aggregation and toxicity has been a major driving force for several research groups.

Site-specific ubiquitination of α-syn has not been possible by using enzymatic methods, thus it has been difficult to investigate the effect of ubiquitination at specific lysine residues.[210–212] To overcome these limitations, we recently reported the semisynthesis of monoubiquitinated α-syn at K6 (T7-Ub-α-syn(K6)). In our strategy, monoubiquitinated α-syn was prepared from a recombinantly expressed α-syn fragment, α-syn(19–140), bearing an N-terminal Cys residue and a synthetic peptide thioester α-syn(1–18) with an orthogonally protected δ-mercaptolysine at position 6 (Scheme 12).[152] Following the ligation step, the Acm protecting group was removed quantitatively to allow site-specific ubiquitination at K6. The final desulfurization step afforded the native monoubiquitinated α-syn T7-Ub-α-syn(K6) in high yield and homogeneity. Monoubiquitinated α-syn was then subjected to numerous biophysical studies, which all indicated correct folding. Interestingly, a thioflavin T (ThT) binding assay and transmission electron microscopy (TEM) revealed that ubiquitination at K6 resulted in a significant inhibition of the formation of α-syn fibrils. These findings support the hypothesis that the N-terminal ubiquitination of α-syn stabilizes the monomeric form of this protein, thus preventing its oligomerization and fibrillogenesis in vitro.

Previous studies have shown a close association between ubiquitination and the phosphorylation of α-syn at S129



Scheme 12. Semisynthesis of monoubiquitinated α-syn. $R = CH_2CH_2CONH_2$, $R^1 = CH_2CH_2SO_3H$.

within Lewy bodies.[207,208,213] However, the effect of ubiquitinated α-syn on phosphorylation has not been investigated. Interestingly, our results showed that ubiquitination at K6 did not affect the phosphorylation at S87 and S129 by several kinases known to phosphorylate α-syn at these residues.

This study demonstrated the utility of applying the semisynthesis of proteins to dissect the role of PTMs in modulating the α-syn function in health and disease. It remains to be tested whether the relocation of the Ub position from K6 to other residues, such as K23, has a similar effect on the activity of the kinases as well as for studying cellular aspects of these conjugates.

Pratt and co-workers used the disulfide approach to link Ub to a protein substrate to prepare nine ubiquitinated α-syn derivatives wherein K6, K10, K12, K21, K23, K32, K34, K46, and K96 in α-syn were mutated individually to Cys to enable the attachment of Ub bearing a thiol handle at its C terminus. This study demonstrated that different ubiquitination sites exhibit differential effects on the formation of α-syn fibrils.[214]

6. Summary and Outlook

The field of ubiquitin has entered a new era of research as a result of the development of novel synthetic methods. These strategies allow the preparation of precious bioconjugates to study previously unreachable aspects of Ub biology. Several ubiquitinated peptides and proteins have been prepared and are currently being used to answer fundamental questions. All the forms of Ub chains with defined lengths and types are now available and some are already commercialized. These bioconjugates were prepared with the native isopeptide bond and its surrogates to study various aspects of UBDs, DUBs, and E2/E3 ligases. Ultimately, this will greatly expand our knowledge on how the Ub machineries achieve their function and specificities at the molecular level.

Recent advances in the field of genetically encoded unnatural amino acids have allowed for the direct incorporation of δ-mercaptolysine in recombinant proteins, which in principle should allow the introduction of Ub and Ubl at any site of a protein regardless of its size. On the other hand, the beauty of chemical methods used for protein synthesis is that they allow full control over the atomic structure, which could be useful for a variety of studies such as for labeling the chains in a highly specific manner and for studying cross-talk between PTMs. With the current methods available, the synthesis of polyubiquitinated proteins should now be possible. Such an accomplishment should enable interesting findings related to the effect of the chain lengths and types on the Ub signal, for example, which protein substrates are degraded following mono- or polyubiquitination, and what characterizes them.

There are several similar elements in protein phosphorylation and ubiquitination, so one could draw on these modifications and their importance for drug discovery.[215] However, while the therapeutic potential of kinase inhibitors has been widely exploited, leading to the development of several drugs for use in cancer therapy, the pace of drug development in the Ub field is considerably slower. However, as a result of the high diversity of the Ub system, which exceeds phosphorylation, and the involvement of different components (e.g. E1–E3 enzymes, DUBs, 19S component of the proteasome), several approaches remain to be explored to unravel new opportunities for developing drugs that target the Ub system. In this regard, we believe that the chemical methods reviewed here could find great utility in the area of drug discovery, such as providing Ub-based probes and assays to facilitate the discovery process.[216] Collectively, we believe that these strategies will contribute significantly in ways that cannot be otherwise accomplished to unravel the mysteries of the Ub signal.

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