

Nonenzymatic Polyubiquitination of Expressed Proteins

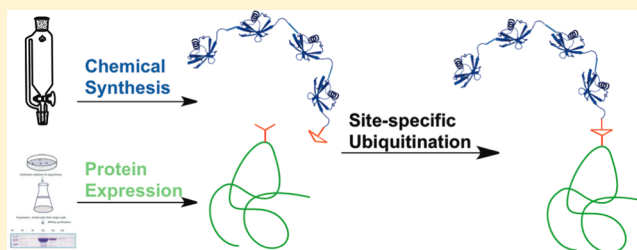
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Supporting Information

ABSTRACT: Ubiquitination is one of the most ubiquitous posttranslational modifications in eukaryotes and is involved in various cellular events such as proteasomal degradation and DNA repair. The overwhelming majority of studies aiming to understand ubiquitination and deubiquitination have employed unanchored ubiquitin chains and mono-ubiquitinated proteins. To shed light on these processes at the molecular level, it is crucial to have facile access to ubiquitin chains linked to protein substrates. Such conjugates are highly difficult to prepare homogeneously and in workable quantities using the enzymatic machinery. To address this formidable challenge we developed new chemical approaches to covalently attach ubiquitin chains to a protein substrate through its Cys residue. A key aspect of this approach is the installation of acyl hydrazide functionality at the C-terminus of the proximal Ub, which allows, after ubiquitin chain assembly, the introduction of various reactive electrophiles for protein conjugation. Employing α -globin as a model substrate, we demonstrate the facile conjugation to K48-linked ubiquitin chains, bearing up to four ubiquitins, through disulfide and thioether linkages. These bioconjugates were examined for their behavior with the USP2 enzyme, which was found to cleave the ubiquitin chain in a similar manner to unanchored ones. Furthermore, proteasomal degradation study showed that di-ubiquitinated α -globin is rapidly degraded in contrast to the mono-ubiquitinated counterpart, highlighting the importance of the chain lengths on proteasomal degradation. The present work opens unprecedented opportunities in studying the ubiquitin signal by enabling access to site-specifically polyubiquitinated proteins with an increased size and complexity.



1. INTRODUCTION

Chemical and semisynthesis of proteins offer unique opportunities to deliver the desired bioconjugates in high homogeneity and workable quantities to support biochemical and structural studies.^{1,2} These approaches become even critical in systems where biological methods fail to afford the target in great quality and quantities, as is the case with posttranslationally modified proteins.³ One such example is ubiquitination: the attachment of ubiquitin (Ub) or a polyubiquitin (polyUb) chain to a protein target employing a specific set of enzymes known as E1–E3.⁴ Ubiquitination is involved in a wide range of cellular processes in eukaryotes ranging from proteasomal degradation, trafficking, transcription, and DNA damage response. The aberrations in the ubiquitination process have been implicated in the pathogenesis of several diseases such as neurological disorders and cancer, thus understanding the Ub signal at the molecular level is highly important.

Polyubiquitination produces molecular signals that depend on which of the seven lysines in Ub (K63, K48, K33, K29, K27, K11, K6) is used for interlinking the consecutive Ub molecules in the Ub chain.^{5–7} For example, while the K48-linked Ub chain facilitates protein degradation by the 26S proteasome, the K63-linked Ub chain plays a crucial role in signal transduction,

receptor endocytosis, and DNA-repair processes.⁶ The evolving complexity of the Ub signal and its involvement in a wide range of biological functions continue to engage several research groups aiming to decipher the molecular bases of this signal and its importance in health and disease. While recent developments in chemical approaches offer a number of solutions to prepare unanchored Ub chains with various configurations and mono-ubiquitinated proteins,^{8–10} the facile synthesis of di-Ub and the longer chains conjugated to a protein is still in its infancy. Such a development is highly crucial to unravel the exact role of Ub chains in physiological processes, e.g., degradation. This is also important to examine the molecular bases of deubiquitinases (DUBs) recognition of Ub conjugates and the role of the substrate in this process.

The majority of the reports aimed at studying ubiquitinated proteins have been limited to conjugation of different proteins to mono-Ub employing several strategies that generate native or non-native linkage. For example, native isopeptide bond was used to link mono-Ub to histone H2B^{11–13} and α -synuclein (α -Syn),¹⁴ while unnatural linkages such as disulfide and triazole

Received: December 11, 2013

Published: January 17, 2014

were employed to link mono-Ub (or other Ub-like modifiers) to α -Syn,¹⁵ proliferating cell nuclear antigen (PCNA),^{16,17} histone H2B,^{13,18} and SUMO-specific E2 enzyme (Ubc9).¹⁹ Recently, we reported the first synthesis of di- and tetra-Ub linked α -Syn by employing isopeptide chemical ligation (ICL).²⁰ In this synthetic adventure, δ -thiolsine²¹ was used as the key tool for constructing the specific Ub chains and to site specifically link these chains to α -Syn, which was obtained via semisynthesis.

The preparation of di- and tetra-Ub linked α -Syn represents synthetic advances that are of great importance in the Ub system and will continue to be applicable and useful to various protein targets. However, the execution of these strategies demands the incorporation of δ -thiolsine at the desired ubiquitination position in the protein target.²² This requirement, in several cases, can be highly challenging to accomplish synthetically, semisynthetically, or even via the use of expression methods of proteins with unnatural amino acids, e.g., δ -thiolsine.²³ We reasoned that by taking advantage of a cysteine residue in proteins, one could modify it with a desired Ub chain possessing a designed electrophile at the proximal end (Scheme 1).

Despite the modification of the native isopeptide bond between the Ub chain and the substrate, we believe that such a strategy would be appropriate for the following reasons: (1) Monoubiquitinated proteins with isopeptide mimic were prepared, and exciting results were extracted from those conjugates;^{15,16,18,24} (2) for various structural and biochemical analyses the isopeptide bond linking a specific chain with the protein substrate might play only a small role, therefore a logical replacement of this bond could have only a minimal effect on the properties of the bioconjugate for these studies; and (3) in our new strategy the isopeptide bonds linking Ubs within the Ub chain will remain unmodified, hence the dynamic and the structural features of these Ub chains are conserved. With these rationale and motivations, herein we report novel approaches to link a desired Ub chain with varying length to a specific protein possessing a Cys residue. We also show how such a strategy could be applied to α -globin ubiquitination with K48-linked Ub chain composed of up to four Ubs for biochemical studies.

2. EXPERIMENTAL METHODS

2.1. General. Solid-phase peptide synthesis (SPPS) was carried out manually in syringes, equipped with Teflon filters, and purchased from Torviq or by using an automated peptide synthesizer (CS336X, CSBIO). If not differently described, all reactions were carried out at room temperature (rt).

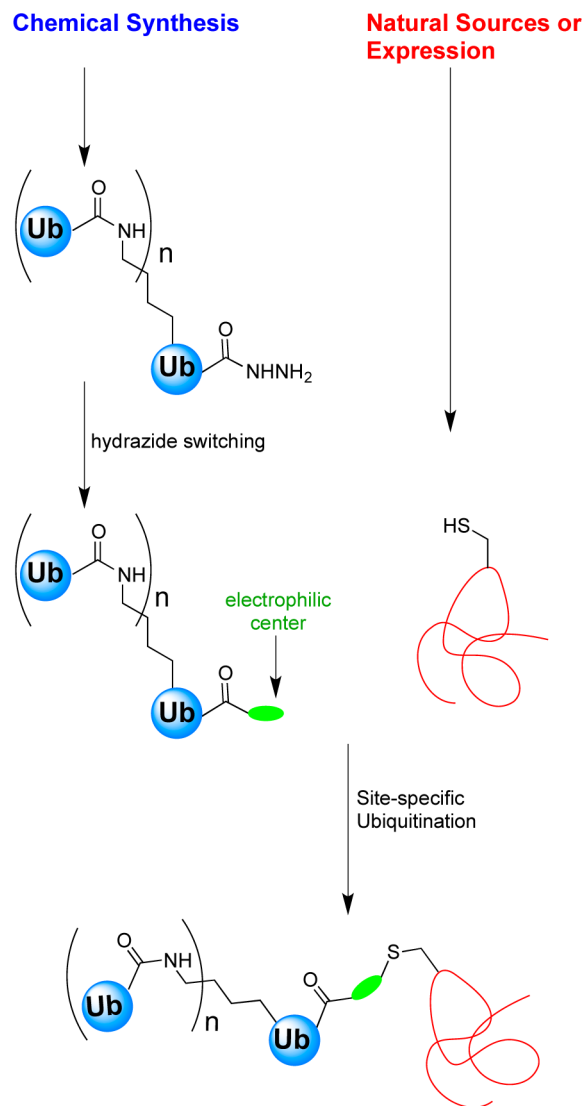
Analytical HPLC was performed on a Thermo instrument (Spectra System P4000) using an analytical column (Jupiter 5 μ m, C4 300 \AA , 150 \times 4.6 mm) and a flow rate of 1.2 mL/min. Commercial reagents were used without further purification. Resins, protected amino acids, and HBTU, HOBt, HCTU, HATU were purchased from Novabiochem, Aapptec, Luxembourg, and Chem-Impex. DMF was purchased in biotech grade.

HPLC Purification was performed on a Waters instrument using a semipreparative column (Jupiter 10 μ m, C4 300 \AA , 250 \times 10 mm) and a flow rate of 4 mL/min or a preparative column (Jupiter 5 μ m, C18/C4 300 \AA , 250 \times 22.4 mm) and a flow rate of 20 mL/min. Buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in acetonitrile.

Mass spectrometry analysis was carried out using LCQ Fleet Ion Trap (Thermo Scientific).

2.1.1. Synthesis of Ub-Aminothiols 10. Ub-NHNH₂ **3** (5 mg, 0.584 μ mol) was dissolved in 150 μ L of 6 M guanidine hydrochloride (Gn-HCl) buffer (2 mM, pH 3) and subjected to oxidation using 10 equiv

Scheme 1. General Strategy for Site-Specific Polyubiquitination of a Protein Possessing a Single Cys Residue

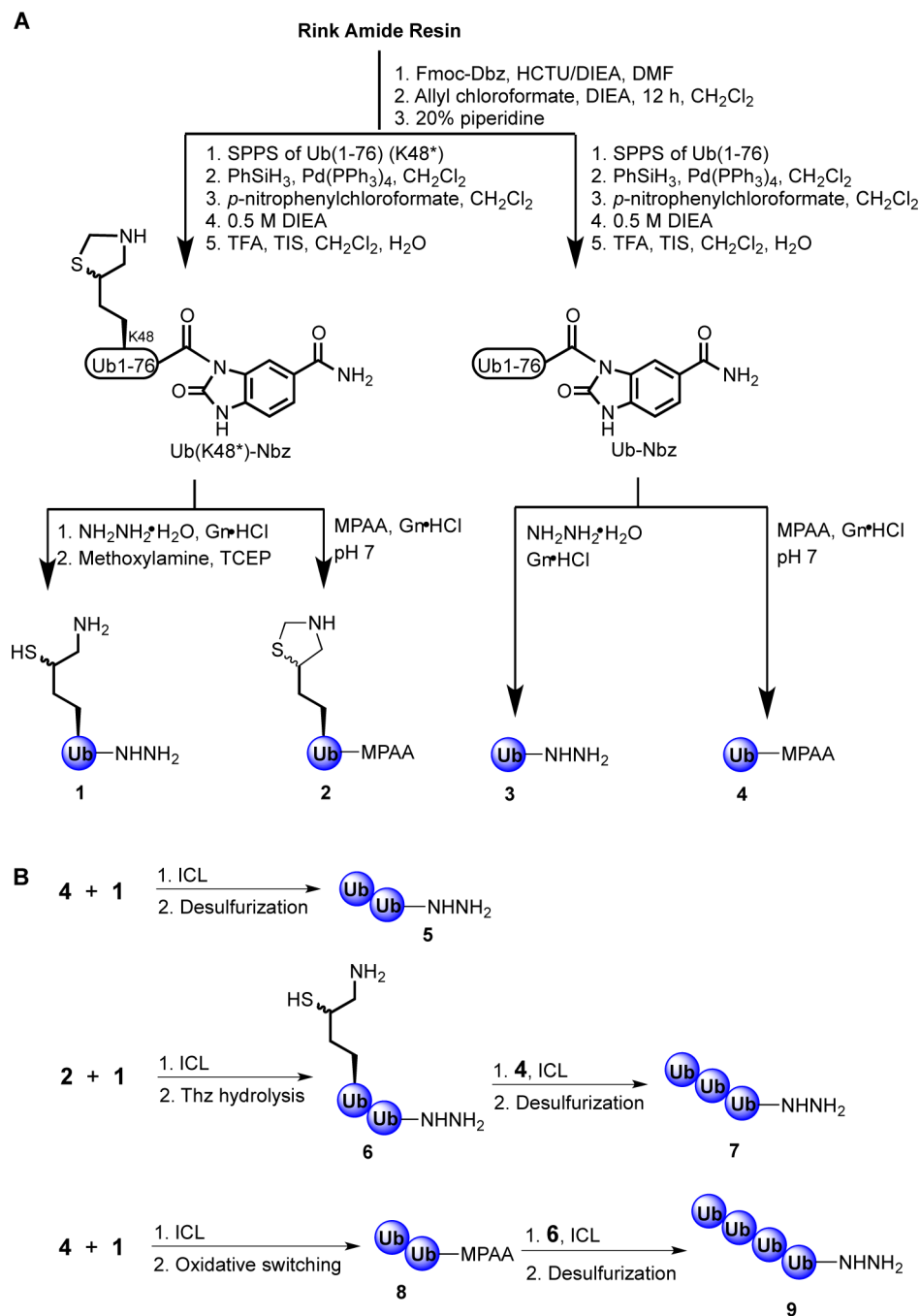


of aqueous NaNO₂ (200 mM) at -15 $^{\circ}$ C for 20 min. Subsequently, 2-aminoethanethiol (50 equiv, 400 mM) was added, and the pH was adjusted to 7. This step was allowed to proceed for 20 min at 25 $^{\circ}$ C with occasional shaking. The reaction was followed using an analytical HPLC and a gradient of 5–55% B over 60 min. For preparative HPLC, the same gradient was used to afford the Ub-aminothiols **10** in \sim 60% yield (\sim 3 mg).

2.1.2. Conjugation of Ub to α -Globin via Disulfide Linkage. Ub-aminothiols **10** (2 mg, 0.23 μ mol) and α -globin-DTNP disulfide adduct **15** (1.1 equiv, 3.9 mg) were dissolved in 6 M Gn-HCl buffer (pH 7, 0.5 mM). Reaction was allowed for 1 h at 25 $^{\circ}$ C with occasional shaking and was analyzed using C4 analytical HPLC and a linear gradient of 30–70% B over 30 min. After the completion of the reaction, the product **16** was isolated via preparative HPLC using a similar gradient in 42% yield (\sim 2.33 mg).

2.1.3. Synthesis of Ub(1–75)-bromoacetamide 25. Ub(1–75)-NHNH₂ **24** (8.3 mg, 0.97 μ mol) was dissolved in 488 μ L of 6 M Gn-HCl buffer (2 mM) and subjected to oxidation using 10 equiv of aqueous NaNO₂ (400 mM) at -15 $^{\circ}$ C (pH \sim 3.0) for 15 min. Subsequently, the bromo compound **20** (14 mg, 50 equiv) was added, and the pH was adjusted to \sim 7, and the reaction was kept at rt for 30 min. The reaction was followed using an analytical HPLC and a gradient of 5–55% B over 60 min. For preparative HPLC, the same

Scheme 2. Synthesis of Ub Building Blocks (A) and Ub Chains Equipped With C-Terminal Hydrazide Functionality (B)



gradient was used to afford the Ub(1-75)-bromoacetamide **25** in ~53% yield (4.5 mg).

2.1.4. Preparation of Ub(1-75)- α -Globin via Thioether Linkage. Ub(1-75)-bromoacetamide **25** (3 mg, 0.34 μ mol) and α -globin **14** (5.7 mg, 0.38 μ mol) were dissolved in Gn-HCl buffer (173 μ L, 2 mM, pH 8) containing NaI (5.19 mg, 100 equiv). After 1 h, the reaction was checked via analytical HPLC using a gradient of 5–70% B over 30 min and then purified using a similar gradient to afford the product **26** in 67% yield (5.5 mg).

2.1.5. Switching of Ub(1-75)-NHNH₂ **24 to Ub(1-75)-maleimide **29**.** To a cooled solution (–15 °C) of Ub(1-75)-NHNH₂ **24** (2.5 mg, 0.29 μ mol) in Gn-HCl buffer (145 μ L, 2 mM, pH 3.0), a solution of NaNO₂ (10 equiv, 400 mmol) was added and kept at the same temperature for 15 min. Then, a solution of *N*-(aminoethyl)maleimide **21** (2.6 mg, 50 equiv) in 15 μ L Gn-HCl buffer was added, and the pH of the reaction was adjusted to 7 and kept at rt for 30 min. Progress of

the reaction was followed by HPLC and mass spectrometry analyses. The desired Ub(1-75)-maleimide **29** was isolated using semipreparative HPLC and a gradient of 5–70% B over 30 min, in 64% yield (1.6 mg).

2.1.6. Reaction of Ub(1-75)maleimide **29 with α -Globin **14**.** Ub(1-75) maleimide **29** (1.5 mg, 0.175 μ mol) and α -globin **14** (2.9 mg, 0.19 μ mol) were dissolved in 90 μ L of Gn-HCl buffer (2 mmol, pH 7.2) and the reaction was checked after 5 min at which a complete consumption of the starting material was observed. The desired product **30** was isolated via semipreparative HPLC using a gradient of 5–70% B over 30 min in 67% yield (2.8 mg).

2.1.7. Enzymatic hydrolysis using USP2. Stock solution of the USP2 (Catalytic Domain), human recombinant (BostonBiochem), (2 μ M) was prepared by diluting 50 μ g of the enzyme in 50 mM Tris buffer (pH 7.54) containing 0.1 mM EDTA and 5 mM DTT. The enzymatic assay was initiated by incubating each substrate (5 μ M) with

USP2 (50 nM) at 37 °C. The enzymatic reaction was quenched using 10% v/v HCl (2 N). Enzyme activity was detected by SDS-PAGE.

2.1.8. Degradation Monitoring the Stability of α -Globin and Ubiquitinated α -Globin in a Cell Free Reconstituted System. Purified α -globin (~1 μ g), mono-, and di-ubiquitinated α -globin **30** and **32** were incubated at 37 °C for the indicated times in a reaction mixture containing in a final volume of 12.5 μ L: 0.5 μ g purified 26S proteasome (bovine 26S proteasome UBPBio cat. no. A1200), supplemented with ATP and ATP-regenerating system. When indicated, the proteasome inhibitor MG132 was added in a concentration of 100 μ M. Reactions were quenched by the addition of three-fold concentrated sample buffer. Boiled samples were resolved via SDS-PAGE (15%), and proteins were visualized after blotting to a nitrocellulose membrane using antihemoglobin α -antibody (rabbit polyclonal antihemoglobin α cat. no. SC21005, Santa Cruz) and chemiluminescence. Band intensities were quantified using the Total Laboratories TL100 1D gel analysis software.

3. RESULTS AND DISCUSSION

Taking advantage of the unique nucleophilicity of the thiol group, we sought to develop a facile and chemoselective route for bioconjugation of Ub chains to a protein through its Cys residue. This would require the installation of a suitable electrophile at the C-terminus of the proximal Ub. The challenging aspect is to properly functionalize the C-terminus of the proximal Ub with a handle that is stable during the synthesis of a Ub chain and upon chain assembly can be switched to an electrophilic moiety to allow site-specific conjugation. In this regard, we were pleased to come across the utility of the acyl hydrazide as a latent thioester group in native chemical ligation (NCL).²⁵ We reasoned that such a moiety should allow smooth tailoring of Ub chains and facilitate incorporation of a desired electrophile via oxidative switching.

3.1. Synthesis of Mono-Ub Building Blocks. For the preparation of a Ub chain with a C-terminal electrophile we initially investigated the synthesis of Ub or Ub chain possessing acyl hydrazide. Liu and co-workers reported the direct synthesis of peptide hydrazides via SPPS starting from hydrazine functionalized Wang resin.²⁵ Having this in mind and with our optimized SPPS protocol for full length Ub,²⁶ we began to assemble Ub equipped with C-terminal hydrazide (Ub-NHNH₂). However, our efforts to adopt this protocol for synthesizing Ub-NHNH₂ were not fruitful since we often observed the addition of two Gly residues to the resin bound hydrazine moiety leading to a chain branching. Changing the resin to the 2-Cl-Trt and using allyloxycarbonyl (Alloc) protection of the hydrazide moiety led to a successful synthesis of Ub-NHNH₂, without any branching (Supporting Information), however the yield of the crude material was significantly lower (14%) than the synthesis on Rink amide (75%), forcing us to search for an alternative route.

Next, we thought to utilize the *N*-acylbenzimidazolinone (Nbz) approach developed by Dawson and Blanco-Canosa²⁷ to prepare Ub-Nbz as a reactive intermediate from which Ub-NHNH₂ can be achieved by hydrazinolysis. For this, we started SPPS on a Rink amide resin functionalized with Alloc protected diaminobenzoic acid (Dbz)²⁸ to avoid peptide branching on the Dbz functionality. Using this approach, two separate Ub syntheses were accomplished by using either protected δ -thiolsine (K48*) or Lys at position 48 (K48) in Ub (Scheme 2A). After peptide cleavage and lyophilization, the crude Ub-Nbz was treated with hydrazine hydrate in 6 M guanidine hydrochloride (Gn·HCl) buffer at rt for 20 min to afford the

pure Ub-NHNH₂ **3** in 10% yield (for all steps described above).¹²

On the other hand, Ub-Nbz was also treated with a solution of 4-mercaptophenylacetic acid (MPAA) in Gn·HCl buffer (pH 7) to obtain Ub-MPAA **4** (Scheme 2A), which was later used as the distal Ub component during Ub chain assembly. The Ub(K48*)-Nbz was also treated with hydrazine hydrate as described above to obtain Ub(K48*)-NHNH₂. Notably, during the hydrazinolysis of the Nbz, removal of the δ -thiolsine protecting group also occurred to a certain extent (~35%) and was driven to completion by the addition of methoxylamine, affording the unprotected Ub(K48*)-NHNH₂ **1** (Scheme 2A). In the context of convergent synthesis of tetra-Ub,^{26,29} this monomer can be used as Ub1 and Ub3. In addition, a portion of Ub(K48*)-Nbz was also switched to Ub(K48*)-MPAA **2**. Notably, a single synthesis of Ub(K48*)-Nbz afforded three different monomers simply by converting the Nbz group into hydrazide or thioester while keeping the mercaptolysine either protected or in the free form as per the requirement.

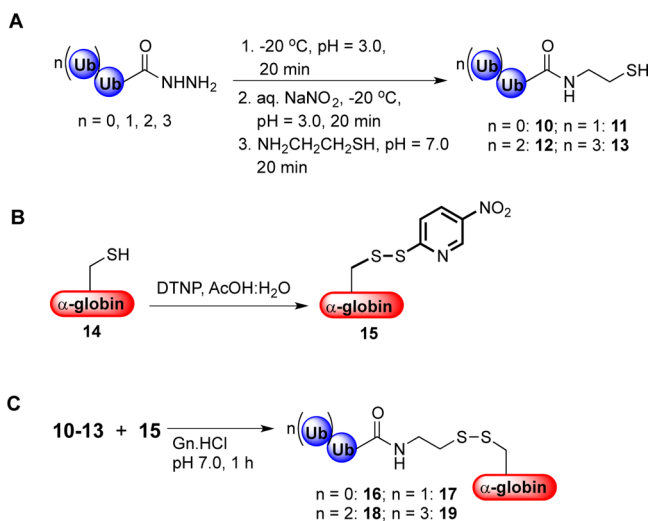
3.2. Ub Chain Assembly. Having the Ub building blocks in hand, we then turned our attention to the synthesis of Ub chains to enable the introduction of C-terminal electrophile taking advantage of the stability and tunability of the hydrazide moiety. To obtain di-Ub-NHNH₂ **5**, Ub-MPAA **4** was ligated to Ub(K48*)-NHNH₂ **1** under standard ICL conditions followed by desulfurization using the free radical approach³⁰ (Scheme 2B). Compared to the Ub-alkyl thioesters such as 3-mercaptopropionic acid, methyl 3-mercaptopropionate, or sodium mercaptoethanesulfonate, the preformed Ub-aryl thioester, i.e., Ub-MPAA **4** underwent ligation in just 30 min at rt³¹ (Scheme 2B). Interestingly, under these reaction conditions, no hydrolysis of the thioester was observed. For the preparation of tri-Ub-NHNH₂ **7**, initially Ub-MPAA **2** was ligated with Ub(K48*)-NHNH₂ **1**. After the completion of the reaction, methoxylamine was added to unmask the δ -thiolsine, and the resulting di-Ub-NHNH₂ **6**, after isolation, was reacted with Ub-MPAA **4**. Desulfurization of this ligation product afforded tri-Ub-NHNH₂ **7** (Scheme 2B). Finally, for the preparation of tetra-Ub-NHNH₂ **9**, a convergent approach was followed wherein, initially di-Ub-NHNH₂ **5** was treated with a solution of NaNO₂ at pH 3 (-15 °C) followed by the addition of a solution of MPAA to the *in situ* generated peptide acyl azide (pH 6, 30 min) to generate the di-Ub-MPAA **8**.³² In the next step, di-Ub-MPAA **8** was ligated with di-Ub-NHNH₂ **6** and desulfurized to afford tetra-Ub-NHNH₂ **9** (Scheme 2B). Under the ligation and desulfurization conditions, the acyl hydrazide moiety remained intact, and no undesired side reactions were observed.

3.3. Ub Chain Conjugation to α -Globin. **3.3.1. Conjugation via Disulfide Bond.** To demonstrate our proposal of chemically tagging the Ub chain to a protein target, we initially tested whether we could conjugate α -globin to the Ub chains through a disulfide linkage. We chose α -globin as a model substrate for the following reasons: (1) Deficiency of β -subunit and concomitant accumulation of α -subunit in hemoglobin (which is composed of two of each subunits) causes β -thalassemia in humans.³³ One way that nature counters this disorder is by enhancing the intercellular proteasomal degradation of the α -subunit, i.e., α -globin, in an ATP-dependent manner involving the Ub system;³⁴ (2) α -globin, composed of 141 residues, makes it an excellent target to further examine our hypothesis that mono-ubiquitination of small proteins could be enough for proteasomal degradation;³⁵

and (3) the presence of a single Cys at position 104 in the sequence makes α -globin a practical substrate to test the proposed chemical strategies. Interestingly, this protein can be isolated in very large quantities by simple HPLC separation of commercially available and inexpensive lyophilized powder of hemoglobin.

Previously, the research groups of Muir and Zhuang independently reported the ubiquitination of H2B histone and PCNA, respectively, via the disulfide linkage.^{16,18} In these studies, 2-aminoethanethiol was introduced at the C-terminus of the mono-Ub via aminolysis of the Ub-intein intermediate followed by conjugation to the target protein by activating the thiol group on either of the two proteins. We chose to incorporate 2-aminoethanethiol at the C-terminus of Ub or Ub chains by making use of the acyl hydrazide handle. Initially, the optimization of the switching reaction of acyl hydrazide with the 2-aminoethanethiol was carried out over mono-Ub-NHNH₂ **3**, (Scheme 3A). In a typical reaction, a solution of

Scheme 3. (A) Switching of Acyl Hydrazide with 2-Aminoethanethiol; (B) Activation of α -Globin With DTNP; and (C) Conjugation of Ub Chains to α -Globin via the Disulfide Linkage



3 in Gn·HCl buffer (pH 3.0) was treated with NaNO₂ solution to allow conversion of acyl hydrazide to highly active acyl azide. Addition of excess 2-aminoethanethiol and elevation of pH to ~7 yielded the desired Ub-aminothiol **10**. In a similar fashion, this protocol was implemented to obtain aminothiol functionalized di-, tri-, and tetra-Ub chains as well (**11–13**, Scheme 3A and Supporting Information). On the other hand, the Cys residue of α -globin **14** was activated by treating with 2,2-dithiobis(5-nitropyridine) (DTNP) in AcOH:H₂O, and the corresponding adduct **15** was isolated via semipreparative HPLC (Scheme 3B, Supporting Information).

In the next step, mono-Ub-aminothiol **10** and α -globin derivative **15** were mixed in 6 M Gn·HCl buffer (pH ~7) and kept at rt to allow conjugation via the disulfide bond. The reaction was complete within 1 h as evidenced by analytical HPLC/ESI-MS and afforded the disulfide linked Ub- α -globin conjugate **16** in 42% yield (Scheme 3C). In a similar manner, di- and tri-Ub-aminothiols **11** and **12** were reacted with **15** to obtain the corresponding disulfide conjugates **17** and **18** in 39 and 21% yields, respectively (Scheme 3C, Figure 1). However, in the case of tetra-Ub-aminothiol **13**, the reaction with α -

globin-DTNP was less efficient and afforded the desired product **19** in only 12% yield, while the majority of starting material remained unreacted. Extending the reaction time and increasing the temperature to 37 °C did not drive the reaction further (Supporting Information). This can be explained perhaps due to the bulkiness of the Ub chain as well as the accessibility of the Cys residue. It remains to be tested if a different protein substrate or variation of the Cys position in α -globin might lead to better reactivity.

3.3.2. Conjugation of α -Globin to Ub Chains via Thioether Bond. Although the disulfide chemistry worked relatively well to assemble polyubiquitinated α -globin conjugates, we realized that this linkage might not be suitable for certain enzymatic/biochemical studies, especially when reaction conditions could reduce the disulfide linkage. Hence, we sought other alternatives to the disulfide bond approach that would furnish Ub conjugates that are stable to reducing conditions. In this quest, thioether linkage appeared worth pursuing and to achieve this, we picked two classes of electrophiles to install at the C-terminus of proximal Ub to allow conjugation of α -globin: the α -bromo acetamide **20** and the maleimide **21** (Scheme 4A). Since these two molecules have an ethylamine (NH₂-CH₂-CH₂-) tail to facilitate their attachment to the C-terminus of Ub chain, they will also increase the number of atoms between Ub and the substrate. To counterbalance this, the proximal Ub was assembled without the Gly76 residue to afford after SPPS and hydrazinolysis, Ub(1-75)(K48*)-NHNH₂ **22**. This served as proximal Ub in the preparation of di-Ub-NHNH₂ **23** (Scheme 4B). In a similar way mono-Ub(1-75)-NHNH₂ **24** was also prepared.

Initially, Ub(1-75)-NHNH₂ **24** was subjected to oxidative switching with **20** to obtain the bromoacetamide Ub derivative **25** in 53% yield (Scheme 5). To achieve the Ub- α -globin conjugate, a mixture of **25** and **14** in ligation buffer was incubated at 37 °C for 30 min. In this reaction, we observed a rapid exchange of the bromide with the chloride ions present in the buffer, which hampered the progress of the reaction considerably. To overcome this, an excess of sodium iodide was added to the reaction mixture where the bromo-iodo exchange generated a more reactive electrophilic center through which the reaction proceeded to near completion and afforded **26** in 67% yield after purification (Supporting Information). In a similar fashion, the di-Ub **23** was attached to α -globin to afford **28** in 30% yield. However, extension of this synthetic strategy to tri-Ub chain was not successful due to a much slower reaction rate in spite of use of excess sodium iodide in the reaction.

In the next part of this study, we explored the conjugation of Ub chains to α -globin employing the maleimide functionality. Commercially available *N*-(2-aminoethyl)-maleimide **21** (Scheme 4A) was chosen and was accommodated at the C-terminus of mono-Ub **24** and di-Ub **23** via oxidative switching of acyl hydrazide. Gratifyingly, the reaction of maleimide functionalized Ub **29** with α -globin was complete within 5 min (Scheme 6, Figure 2) to give the product **30** in 67% yield. Compared to the above two strategies we employed for constructing Ub- α -globin conjugates (i.e., disulfide and thioether formation), the maleimide approach was much more rapid and high yielding. However, while the oxidative switching of acyl hydrazide to maleimide was smooth for mono-Ub, in the case of di-Ub-NHNH₂ a considerable hydrolysis side product was observed and eluted along with the di-Ub-maleimide **31** (Supporting Information). This mixture was

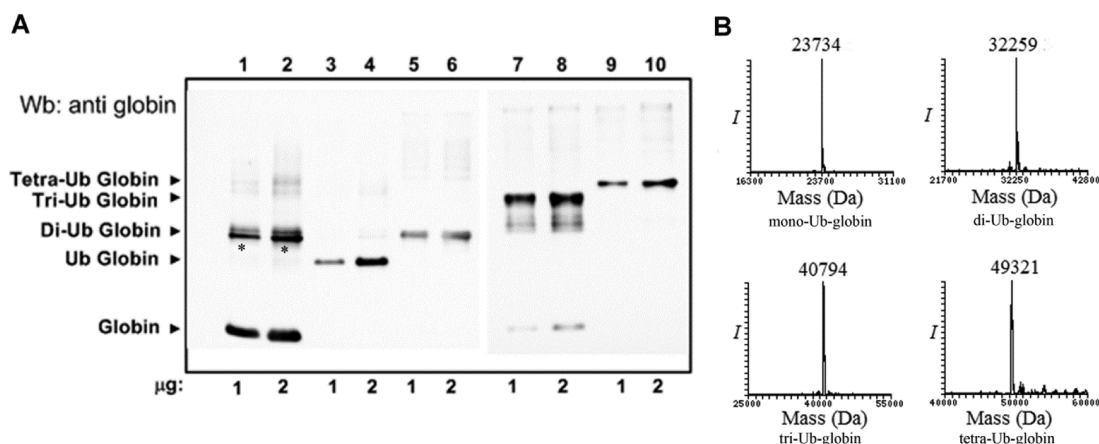
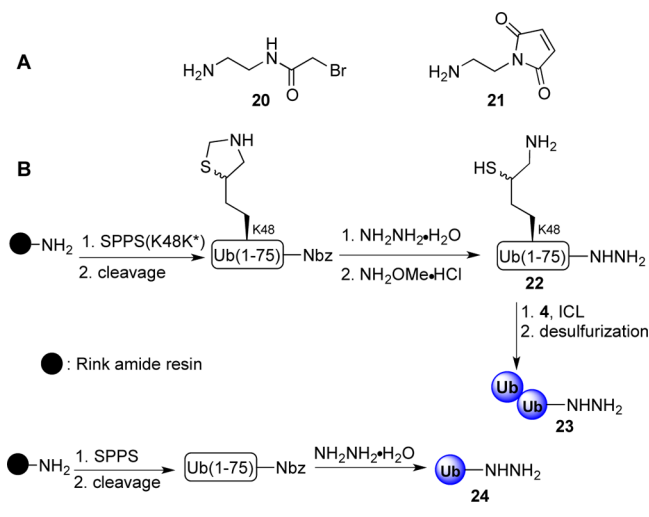


Figure 1. Characterization of ubiquitinated α -globin: (A) Western blot analysis of the four disulfide linked α -globin conjugates: 1 and 2 μ g of unconjugated (lanes 1 and 2), mono- (lanes 3 and 4), di- (lanes 5 and 6), tri- (lanes 7 and 8), and tetra- (lanes 9 and 10). Ubiquitinated α -globin analogues were resolved via SDS-PAGE. The gel was blotted onto a nitrocellulose membrane and was probed with an anti- α -globin antibody. * indicates globin dimerized via disulfide bond. (B) Mass spectrometry data of the purified materials showing the deconvoluted mass of each conjugate. Mono-Ub- α -globin having mass of 23 734 Da (calcd 23 731 Da); di-Ub- α -globin having mass of 32 259 Da (calcd 32 260 Da); tri-Ub- α -globin having mass of 40 794 Da (calcd 40 793 Da), and tetra-Ub- α -globin having mass of 49 321 Da (calcd 49 321 Da).

Scheme 4. (A) The Two Molecules Used to Introduce Electrophiles to the C-Terminus of Ub/di-Ub and (B) Synthesis of di-Ub-NHNH₂ 23 and Ub(1-75)-NHNH₂ 24



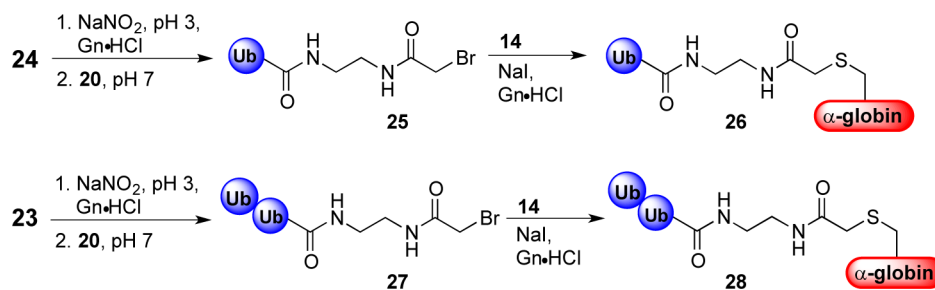
collected together and after lyophilization was reacted with α -globin. In this case also, the reaction was rapid, and the di-Ub- α -globin product 32 separated well from the hydrolyzed di-Ub carried from the previous step (Scheme 6 and Figure 3). Unfortunately, our effort to switch tri-Ub-NHNH₂ in a similar fashion largely suffered with hydrolysis, and a very low amount

of maleimide conjugate was formed. In principle, this can be overcome by first attaching the mono-Ub bearing protected δ -thiolysine to α -globin employing thioether formation described above followed by Ub chain elongation. Such a strategy is currently under investigation. Recently, Strieter and co-workers reported thiol-ene chemistry for site-specific coupling of Ub molecules to generate Ub chains. Modifying the proximal Ub with allyl group should also enable the execution of such a strategy to conjugate Ub chains to a protein via its Cys residue.³⁶

3.4. Biochemical Characterization of Ub- α -Globin Conjugates.

Having the different Ub conjugates in hand, we then turned our attention to investigate the behavior of these chains with DUBs and in proteasomal degradation. The majority of the in vitro studies on DUBs activities were carried out on unanchored Ub chains. The attachment of a protein to the C-terminus of Ub chain generates Ub conjugates that are useful in providing insights into how the particular protein substrate affects the recognition and cleavage of the Ub chain with the specific DUB. As a model study, we tested with our di-Ub- α -globin conjugates with the USP2 enzyme, which is known to cleave various unanchored Ub chains and has been associated with cancer.³⁷ However, the disulfide bond linked conjugates were vulnerable to even a slightest amount of reducing agent (DTT) present in the enzymatic assay, contrary to the thioether-linked α -globin conjugates 26 and 28 (Figure 4). Notably, USP2 (catalytic domain) was able to cleave between the di-Ub in the di-Ub- α -globin 28 in a similar

Scheme 5. Conjugation of Mono- And Di-Ub to α -Globin via Thioether Linkage



affects the degradation of ubiquitinated α -globin. For this, we examined the degradation of Ub- α -globin conjugates **30** and **32** in a cell free reconstituted system. Interestingly, our results clearly show that mono-ubiquitination of α -globin at the C-terminal region is not sufficient to promote proteasomal degradation. On the other hand, as shown in Figure 5, when α -

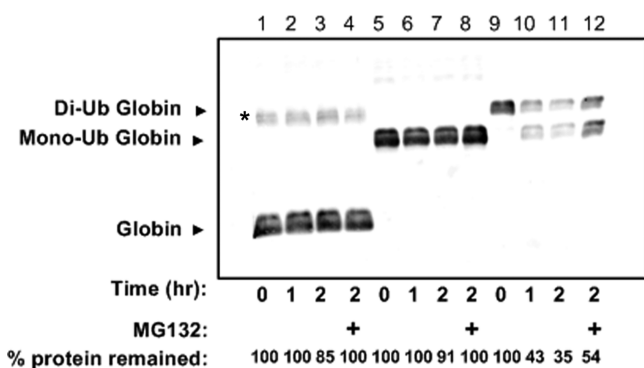


Figure 5. Degradation of unconjugated and of mono- and di-ubiquitinated α -globin **30** and **32** in a cell free reconstituted system. One μ g of unconjugated (lanes 1–4), mono- (lanes 5–8), and di- (lanes 9–12) ubiquitinated α -globin were incubated in the presence of purified 26S proteasome for the indicated times in the absence or presence of the proteasome inhibitor MG132 as specified. Following incubation, samples were resolved via SDS-PAGE. The gel was blotted onto a nitrocellulose membrane and was probed with antihemoglobin α antibody. The % of the remaining protein is shown. The bands that are labeled with * in lanes 1–4 correspond to dimerized globin via disulfide bond which has similar molecular weight of di-ubiquitinated globin.

globin is di-ubiquitinated, it is rapidly degraded. Previously we have shown that mono-ubiquitination of proteins made of up to \sim 150 residues could promote their proteasomal degradation, e.g., α -Syn.³⁵ It is possible that in the case of α -globin, additional factors such as the ubiquitination site or folding state deem the requirement of an additional Ub moiety (i.e., di-Ub) to promote its degradation,²⁴ though it is clear that a poly-Ub chain is not required in this case either.

4. SUMMARY

We have demonstrated the site-specific incorporation of Ub chains into protein via its native Cys residue. For this task, we introduced three different electrophiles to the C-terminus of the proximal Ub in the Ub chains, which allowed their facile attachment to α -globin via disulfide and thioether bonds. These conjugates were tested for their behavior with the catalytic domain of USP2, which was able to cleave the di-Ub- α -globin in similar efficiency to the unanchored di-Ub chain. Furthermore, we have also tested the proteasomal degradation of mono- and di-ubiquitinated α -globin and found that the latter was degraded efficiently, highlighting the importance of the chain lengths in this process. While the chemistry we introduced in this study is suitable to proteins with a single Cys residue, the rapid emergence of novel expression methods that allow the incorporation of noncanonical amino acids possessing diverse reactive groups (e.g., azide, ketone)^{43,44} applicable in bioorthogonal ligation should significantly expand the current approach beyond the Cys chemistry. With further optimization this should enable site-specific polyubiquitination of various proteins with an increased size and complexity and obtain large quantities of these precious bioconjugates for various

biochemical and structural studies. This should significantly boost the ongoing efforts aiming at deciphering the Ub signal at the molecular level.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods and HPLC and mass spectrometry analyses of the synthetic monomers and ligation products. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Israel Science Foundation (A.B.). H.P.H. thanks the Israel Council of Higher Education for a fellowship under the PBC program. Research in the laboratory of A.C. is supported by grants from the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (AMRF), the Israel Science Foundation (ISF), The TreatPolyQ Network funded by the EU, and the Deutsch-Israelische Projektkooperation (DIP). A.C. is an Israel Cancer Research Fund (ICRF) USA Professor.

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