JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Article

pubs.acs.org/JACS

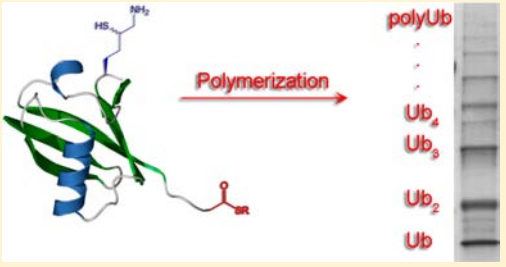
Polymerization Behavior of a Bifunctional Ubiquitin Monomer as a Function of the Nucleophile Site and Folding Conditions

Tal Moyal, Sudhir N. Bavikar, Subramanian Vedhanarayanan Karthikeyan, Hosahalli P. Hemantha, and Ashraf Brik*

Department of Chemistry, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel

Supporting Information

ABSTRACT: Biopolymers with repeating modules composed of either folded peptides or tertiary protein domains are considered some of the basic biomaterials that nature has evolved to optimize for energy efficient synthesis and unique functions. Such biomaterials continue to inspire scientists to mimic their exceptional properties and the ways that nature adopts to prepare them. Ubiquitin chains represent another example of nature's approach to use a protein-repeating module to prepare functionally important biopolymers. In the current work, we utilize a novel synthetic strategy to prepare bifunctional ubiquitin monomers having a C-terminal thioester and a nucleophilic 1,2-aminothiol at a desired position to examine their polymerization products under different conditions. Our study reveals that such analogues, when subjected to polymerization conditions under different folding states, afford distinct patterns of polymerization products where both the dynamic and the tertiary structures of the chains play important roles in such processes. Moreover, we also show that the presence of a specific ubiquitin-binding domain, which binds specifically to some of these chains, could interfere selectively with the polymerization outcome. Our study represents the first example of examining the polymerization of designed and synthetic repeating modules based on tertiary protein domains and affords early lessons in the design and synthesis of biomaterial. In regards to the ubiquitin system, our study may have implications on the ease of synthesis of ubiquitin chains with varying lengths and types for structural and functional analyses. Importantly, such an approach could also assist in understanding the enzymatic machinery and the factors controlling the assembly of these chains with a desired length.



1. INTRODUCTION

Biopolymers with repeating modules, composed of either folded peptides (e.g., elastin, collagen) or tertiary protein domains (e.g., titin), are considered some of the basic biomaterials that nature has evolved to optimize for energy efficient synthesis and unique functions. Such biomaterials continue to inspire scientists to mimic their exceptional properties and the ways that nature adopts to prepare them. These endeavors are being approached by combining lessons learned from cellular processes, organic chemistry, our understanding of the molecular forces that govern conformation changes (e.g., self-assembly, protein folding), and nature's machineries (e.g., enzymes) that are involved in such processes. In this regard, the employment of strategies to prepare biopolymers composed of repeating modules that are based on folded peptides has been the only successful approach so far.[1] This is because repeating modules based on folded peptides can be straightforwardly prepared by solid-phase peptide synthesis (SPPS), while the preparation of a bifunctional protein as the repeating unit is not often trivial and requires a set of advanced chemical tools that are often neither available nor simple to exercise for such a task.[2]

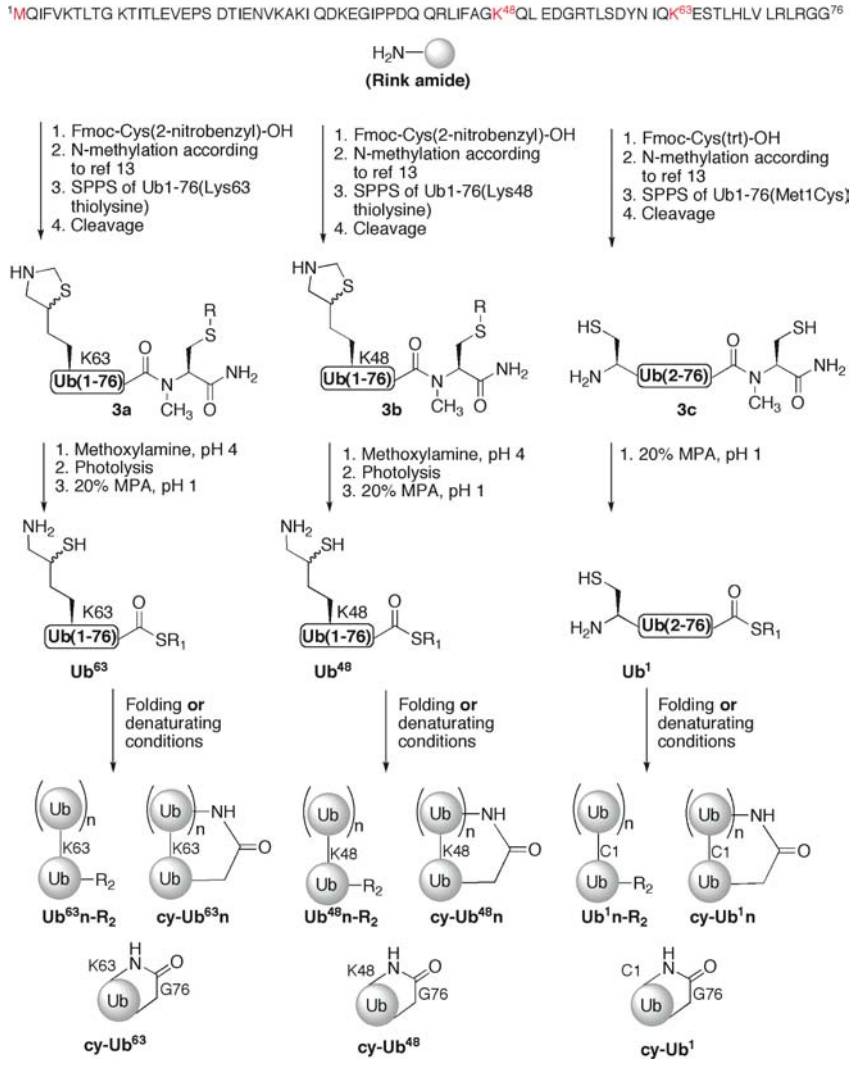
Ubiquitin (Ub) chains represent another example of nature's approach to use a protein-repeating module to prepare functionally important biopolymers. These chains serve as signals in various biological pathways, contrary to several other known biopolymers composed of protein-repeating module that serve in mechanochemical functions (e.g., muscle contraction).[3,4] Ub chains are known to form biopolymers of various lengths and linkage types in which eight different homogeneous linkages could be formed. Here, any of the seven Lys residues (K63, K48, K33, K29, K27, K11, K6) of one Ub could be used to link the C-terminus of the consecutive Ub via an isopeptide bond. In addition, the N-terminus of Met1 of Ub could also serve as a nucleophile to form a head to tail chain, known as the linear chain. Remarkably, the diversity of the Ub chains leads to their involvement in numerous signaling pathways.[4] At the molecular level, structural analyses revealed that different chains could adopt distinct structures and dynamics. For example, K48-linked di-Ub chain predominantly adopts a closed conformation in neutral pH where the hydrophobic patches (L8, I44, V70) interact with each other.[5] On the other hand, the K63-linked and linear chains adopt open conformations in which the functionally important residues of the two monomers lack any contacts.[6]

The covalent assembly of the Ub chains is achieved via an enzymatic process that is controlled by a set of enzymes known

Received: August 8, 2012
Published: September 10, 2012

 © 2012 American Chemical Society dx.doi.org/10.1021/ja3078736 | J. Am. Chem. Soc. 2012, 134, 16085−16092

Scheme 1. Synthetic Scheme of Ub^{63}, Ub^{48}, and Ub^{1} and Their Possible Polymerization Products under Folding and Denaturating Conditions[a]



[a]The native Ub sequence is also shown (top). R = 2-nitrobenzyl, R_1 = $-CH_2-CH_2-COOH$, R_2 = $-COOH$ or MPA.

as the E1, E2, and E3.[7] The E2 enzyme, which undergoes transthioesterification with the Ub-E1 to carry the Ub in the thioester form, is believed to be responsible for dictating the chain linkage types.[8] In such a process, the enzymatic assembly could lead to Ub chains of various lengths, where as many as eight Ub units were observed.[9] Moreover, it was reported that in the case of the enzymatic preparation of K48-linked chain via E2-25K, cyclization of polyUb (>tri-Ub) was observed.[10] Such a step was confirmed to occur via the reaction of Lys48 from the distal Ub with Gly76 of the proximal Ub, which is linked via a thioester bond with the E2. These observations along with the available knowledge of the current structures of the different chains hint that chemical polymerization of bifunctional Ub could lead to different products upon changing the position of the nucleophilic 1,2-aminothiol relative to the C-terminal thioester functionality. These functionalities are known to react with high chemoselectivity in aqueous media to form an amide bond.[11] Hence, we reasoned that the Ub system could serve as an excellent model to study some of the factors that could aid the design of biomaterials based on a protein-repeating module.[1] In addition, this may have implications on the ease of synthesis of Ub chains with varying lengths and types for structural and functional studies. Such an endeavor could also assist in understanding the functions of the E2 ligases and the factors controlling the assembly of these chains with a desired length. Here, we report on the initial efforts and findings of chemically polymerizing three different Ub monomers, each bearing C-terminus thioester functionality and thiolysine at position 48 or 63 or N-terminal Cys.

2. EXPERIMENTAL METHODS

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

Analytical HPLC was performed on a Thermo instrument (Spectra System P4000) using an analytical column (Jupiter 5 μm, C18/C4 300 Å 150 × 4.6 mm) at a flow rate of 1.2 mL/min.

Preparative HPLC was performed on a Waters instrument using a semipreparative column (Jupiter 10 μm, C4 300 Å, 250 × 10 mm and a flow rate of 5 mL/min or a preparative column (Jupiter 5 μm, C18/C4 300 Å, 250 × 22.4 mm) at a flow rate of 25 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).

dx.doi.org/10.1021/ja3078736 | J. Am. Chem. Soc. 2012, 134, 16085−16092

Gel filtration was performed on the AKTA purifier FPLC system using a Superdex 75 10/300 GL column and Tris buffer (150 mM NaCl, 50 mM Tris, pH 7.54).

SDS-PAGE analysis was carried out on 4−20% gradient NuSep gels, using standard sample buffer and TGS buffer. The polymerization products were quantified using ImageJ imaging software.

Commercial reagents were used without further purification. Resins, protected amino acids, and HBTU, HCTU, and HATU were purchased from Novabiochem, Aapptec, and Chem-Impex. DMF was purchased in biotech grade.

Synthesis of Ub^{48}, Ub^{63}, and Ub^{1} Monomers. The synthesis of each Ub monomer was carried out on Rink amide resin (0.44 mmol/g, 0.1 mmol scale). Unless specified otherwise, for each coupling cycle, amino acid and the coupling agent (HCTU or HATU) were used in 4-fold excess, while DIEA was used in 8-fold excess to the initial loading of the resin.

For the synthesis of $\mathbf{Ub^{48}}$ and $\mathbf{Ub^{63}}$ monomers (Scheme 1), the preswollen resin was treated with 20% piperidine in DMF (5−10−5 min) to remove the Fmoc-protecting group. Fmoc-Cys(*o*-nitrobenzyl)-OH activated with HCTU was coupled to the resin for 1 h. Subsequently, the resin was washed with DMF (3 × 5 mL), and the Fmoc-protecting group was removed using 20% piperidine (4−7−3 min). In the next step, a solution of collidine (264 μL, 20 equiv) in CH_2Cl_2 (1.5 mL) was added to the resin, followed by *o*-nitrobenzenesulfonyl chloride (*o*-NBS-Cl, 442 mg, 20 equiv) in 1.5 mL of CH_2Cl_2. The resin was shaken for 2 h at room temperature and washed using CH_2Cl_2 (3 × 5 mL) and DMF (3 × 5 mL). To carry out the *N*-methylation of the resin-bound Cys, DBU (74 μL, 5 equiv) in 1.5 mL of DMF was added followed by methyl-4-nitrobenzenesulfonate (108 mg, 5 equiv) in 1.5 mL of DMF. The resin was then shaken for 1 h at room temperature and was washed with DMF (3 × 5 mL). Finally, *o*-NBS group was removed by treating the resin with DBU (74 μL, 5 equiv) and β-mercaptoethanol (70 μL, 10 equiv) in DMF for 30 min at room temperature followed by a DMF wash (3 × 5 mL). The Gly76 was manually coupled using HATU/DIEA for 45 min (2×). The remaining amino acids were coupled on automated peptide synthesizer using 4 equiv of AA/8 equiv of DIEA/4 equiv of HCTU (45 min) corresponding to the initial loading of the resin. Fmoc-removal was achieved using 20% piperidine with 5−10−5 min cycles. Pseudoproline dipeptides Leu-Ser and Asp-Gly(Dmb) were manually coupled at positions Leu56-Ser57 and Asp52-Gly53 junctions using 2.5 equiv of Fmoc-Leu-Ser($\psi^{Me,Me}$Pro)-OH and Fmoc-Asp(O^{t}Bu)-(Dmb)Gly-OH, respectively. The thiazolidine (Thz)-protected mercaptolysine was also manually coupled for 2 h at the position K48 or K63 using 2.5 equiv of this residue and HATU, while DIEA was used in 5-fold excess to the resin initial loading. Analytical cleavage and HPLC analysis were performed after these couplings to ensure complete reaction. Met1 in ubiquitin was replaced with norleucine to avoid oxidation during synthesis and handling.

The synthesis of $\mathbf{Ub^{1}}$ having all native Lys residues was carried out similarly to that of the previous two monomers, however, starting from resin loaded with Fmoc-Cys-(Trt)-OH, while Met1 was replaced with Boc-Cys(Trt)-OH (Scheme 1).

Cleavage from the Resin. The resin-bound peptide was washed with DMF, methanol, and DCM and dried. The cleavage cocktail (TFA, water, thioanisole, ethanedithiol, and phenol, 85:5:5:2.5:2.5) was added, and the reaction mixture was shaken for 2.5 h at room temperature. The combined filtrate was added dropwise to a 10-fold volume of cold ether and centrifuged. The precipitated crude peptide was dissolved in acetonitrile−water (1:1) and was further diluted to ∼30% with water and lyophilized. The HPLC analysis was carried out on a C4 analytical column using a gradient of 5−60% B over 30 min.

Thz Deprotection. The crude peptide was dissolved in 200 mM phosphate buffer (pH ∼7) containing 6 M guanidine·HCl (Gn·HCl) to a final concentration of ∼3 mM. This mixture was treated with methoxylamine (0.2 M) at pH 4 and tris-(2-carboxyethyl)-phosphine (TCEP) (30 equiv) for 12 h at 37 °C to unmask the δ-mercaptolysine. The reaction was monitored using an analytical column. Preparative HPLC using a linear gradient of 5−45% B over 40 min afforded the corresponding peptide in ∼11% yield (∼63 mg).

Photolysis and Thioesterification. The purified peptide (10 mg) from the previous step was dissolved in 566 μL (2 mM) of photolysis buffer (6 M Gn·HCl, 10 mM vitamin C, 10 mM 3-mercaptopropionic acid (MPA), 200 mM phosphate buffer pH ∼7). The reaction mixture was irradiated at 350 nm for 1 h and subsequently incubated with 20% MPA (v/v) at 40 °C for 20 h at pH 1. The reaction was monitored using a C4 analytical column and a gradient of 5−55% B over 30 min. Preparative HPLC using a linear gradient of 5−55% B over 30 min afforded the Ub monomer in 30% yield (3 mg).

Polymerization under Denaturation Conditions. The polymerization reaction was carried out with each monomer (400 μM) in 6 M Gn·HCl, 200 mM phosphate buffer (pH 7), containing 100 mM MPAA (4-mercaptophenylacetic acid) and 20 mM TCEP. The reaction was incubated at 37 °C for 1 h and was analyzed using C4 analytical HPLC and a linear gradient of 15−60% B over 30 min. Polymerization of $\mathbf{Ub^{1}}$ in denaturation conditions was also analyzed using SDS-PAGE.

Polymerization under Folding Conditions. The polymerization reaction was carried out in 100 mM phosphate buffer (pH 7) where each monomer was initially dissolved in 6 M Gn·HCl and then diluted with phosphate buffer followed by centrifuging and precipitation of the unfolded monomers. The protein concentration was determined using the BCA method and adjusted to 200 μM. To this solution were added 50 mM MPAA and 20 mM TCEP. The reaction was incubated at 37 °C for 2.5 h followed by dialysis against 100 mM phosphate buffer (pH 7) to discard MPAA. The reaction was analyzed using SDS-PAGE, FPLC, and LCMS as described in the general methods.

Polymerization in the Presence of UBA2 and under Folding Conditions. To a solution of 150 μM UBA2 and 100 μM $\mathbf{Ub^{48}}$ in Tris buffer (50 mM Tris, 150 mM NaCl, pH 7) were added 30 equiv of MPAA and 20 equiv of TCEP. The reaction was incubated at 37 °C and was analyzed at different intervals (1, 2, 4 h) using LCMS against the control (in the absence of UBA2). The reaction was also analyzed as described above using SDS-PAGE. The same procedure was repeated for the other monomers.

3. RESULTS AND DISCUSSION

3.1. Design and Synthesis of Ub Monomers. Recent advances in the chemical and semisynthesis of Ub bioconjugates have opened numerous opportunities for studying various aspects of the amazing Ub signal.[12] Particularly interesting to this study is our ability to chemically prepare Ub with C-terminal thioester moiety, enabling the introduction of additional functionalities within the same Ub unit.[13] Using this strategy, we were able to prepare Ub-thioester possessing protected δ-mercaptolysine[13b,c] as a replacement of any of the seven naturally occurring Lys residues to promote isopeptide chemical Ligation (ICL). These advances were a key to our previous successes in preparing the longer Ub chains.[14] Equipped with these tools, we designed three analogues of bifunctional Ub having a C-terminus thioester functionality and a thiolysine residue at positions 48 ($\mathbf{Ub^{48}}$), 63 ($\mathbf{Ub^{63}}$), and N-terminal Cys ($\mathbf{Ub^{1}}$) to examine the polymerization of these monomers under various conditions. The design of these analogues was based on the following: (1) The first was placing the 1,2-aminothiol nucleophile in an increasing distance relative to the C-terminal thioester of Ub (Figure 1). (2) The second was the known tertiary structures of the K48- and K63-linked di-Ub chains as well as the linear chains.[5,6] Because of the structural differences of the di-Ub chains, which are formed as the initial polymerization products in each case, the nucleophile in the distal Ub is expected to be at a different position relative to the thioester of the proximal Ub (Figure 1). Hence, we expect that these bifunctional di-Ub chains will afford different polymerization products (Scheme 1). (3) The third was the different interactions of these chains with various Ub binding

dx.doi.org/10.1021/ja3078736 | J. Am. Chem. Soc. 2012, 134, 16085−16092

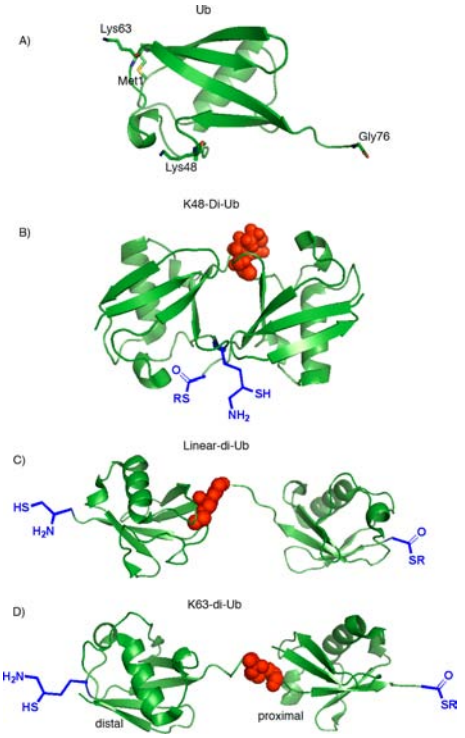


Figure 1. Structures of di-Ub linked via K48- or K63- and the linear chain (PDB codes: 2BGF,[5b] 2JF5,[6b] 2W9N,[6b] respectively) and Ub (PDB code: 1UBQ).[15] The structures also show the position of the nucleophile relative to the thioester.

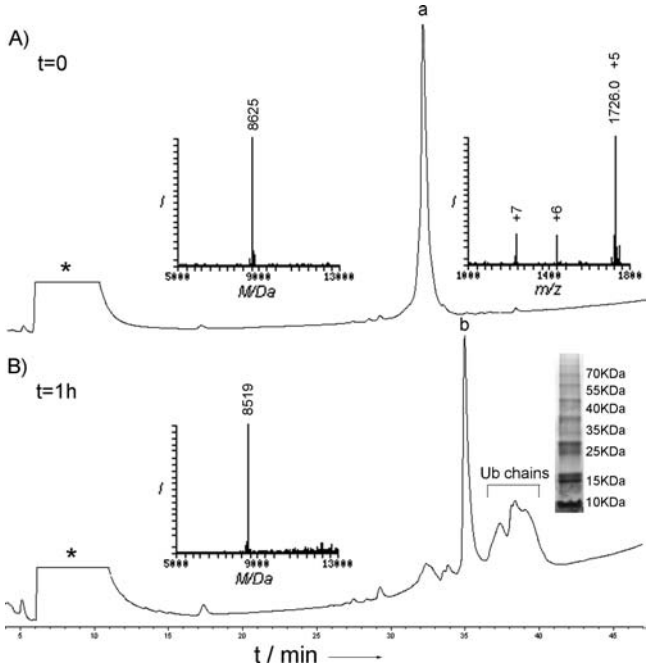


Figure 2. Analytical HPLC/ESI-MS of **Ub**[1] polymerization under denaturation conditions. (A) Analytical HPLC analysis of the polymerization reaction at t = 0. Peak a corresponds to **Ub**[1] with the observed mass 8625 Da (calcd 8625.8 Da). (B) Analytical HPLC analysis of the polymerization reaction after 1 h. Peak b corresponds to the cyclization product of the monomer with the observed mass 8519 Da (calcd 8519.8 Da). SDS-PAGE analysis was done after 1 h of polymerization reaction; peak "*" corresponds to thiol additives.

domains, wherein the presence of such a domain in the reaction mixture could interfere with the polymerization.

The synthesis of the three monomers was accomplished by slightly modifying our previously reported synthesis of Ub-thioester bearing protected δ-mercaptolysine[13a,14] (**3a**−**c**, Scheme 1). In the current synthesis, the crude peptide, corresponding to the entire Ub sequence, was treated with methoxylamine to unmask the 1,2-aminothiol functionality followed by photolysis and thioester formation to furnish the bifunctional Ub monomers **Ub**[48] and **Ub**[63]. The **Ub**[1] monomer was synthesized by coupling Boc-Cys(Trt)-OH to the N-terminus of Ub(2−76) followed by cleavage from the resin and thioester formation (Scheme 1). As a control experiment, Ub having only the C-terminus thioester functionality was also prepared. With these monomers in hand, we then studied the polymerization reactions under folding and denaturing conditions.

3.2. Polymerization Studies under Denaturation Conditions. To examine the polymerization of the three monomers under denaturing conditions, each analogue (400 μM) was incubated in 6 M Gn·HCl, 200 mM phosphate buffer (pH 7), in the presence of MPAA as a thioester activator[16] and TCEP at 37 °C for 1 h. Under these conditions, **Ub**[48] and **Ub**[63] afforded the intramolecular cyclization products, **cy-Ub**[48] and **cy-Ub**[63], without any measurable polymerization products (Supporting Information). These results are reflecting the relatively close position of the mercaptolysine to the C-terminus thioester even under denaturing conditions (Scheme 1).

In the case of **Ub**[1], we have also observed intramolecular cyclization products, **cy-Ub**[1], however, along with higher molecular weight products (Figure 2). Further analysis of the reaction mixture with SDS-PAGE indicated that, in this case, the longer Ub chains made of up to 10 Ub units were also formed (Figure 2). Under denaturing conditions, the two functional groups at C- and N-termini occupy the largest distance; hence, the intermolecular reaction to form the longer chains competes much more with the intramolecular reaction as compared to the cases of **Ub**[48] and **Ub**[63]. Yet, the formation of the **cy-Ub**[1] product in such a high percentage (42%) is interesting and indicates the close proximity of these regions even under unfolding conditions, which promotes the intramolecular reaction. Changing the temperature (25−37 °C) and the thiol additives to the less reactive thiols (benzyl mercaptan or thiophenol) did not induce detectable differences in these results. Finally, and as expected, incubation of the Ub-thioester lacking the 1,2-aminothiol functionality did not lead to any detectable polymerization or cyclization products.

3.3. Polymerization Studies under Folding Conditions. Next, we examined the behavior of the polymerization reaction of the three monomers (200 μM) under folding conditions (100 mM phosphate buffer, pH 7) and in the presence of 20 equiv of TCEP. After folding, MPAA was added to activate the alkylthiol and facilitate the polymerization. We first started with **Ub**[48], which upon ICL affords the K48-linked chains. Hence, **Ub**[48] was incubated for 2.5 h at 37 °C and analyzed by LC−MS (electrospray ionization), size exclusion chromatography (SEC), and SDS-PAGE. These analyses revealed that, contrary to the denaturation conditions, **Ub**[48] underwent polymerization under folding conditions to afford the longer chains. As indicated by the SDS-PAGE and the SEC analyses (Figure 3A), we were able to detect polymerization up to the tetra-Ub with the di-Ub chain being the major product

dx.doi.org/10.1021/ja3078736 | J. Am. Chem. Soc. 2012, 134, 16085−16092

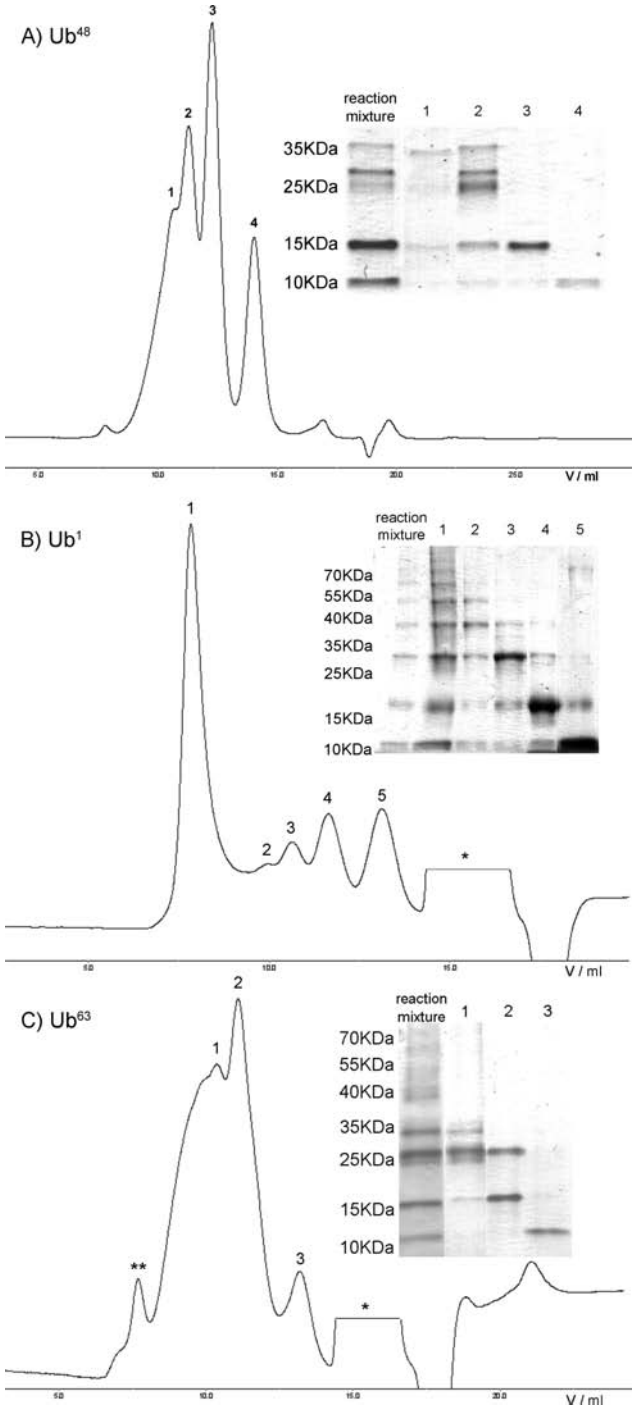


Figure 3. Analysis by SEC and SDS-PAGE of the polymerization of **Ub^{48}**, **Ub^{1}**, and **Ub^{63}** under folding conditions. "*" corresponds to TCEP, and "**" corresponds to unidentified products.

(48%), while the tri- and tetra-Ub were obtained in 19% and 6%, respectively. SEC separation of these peaks and further analyses by LC−MS revealed that under these conditions the starting material **Ub^{48}** (27% of the total mixture) underwent self-cyclization. The mono-Ub structure (Figure 1A) shows that, in principle, the C-terminal flexible region bearing the thioester could reach the mercaptolysine at position 48 and react with it. It is possible also that partially folded structures, formed during the folding process of synthetic Ub polypeptide, could bring these reactive units even to a closer proximity than the folded state and contribute to the cyclization process. However, the overall rigidity imposed by the folded structure favors the intermolecular over the intramolecular reaction of the monomer unit.

Interestingly, the di-Ub chain was observed only in the cyclized form, which occurs via intramolecular attack of the mercaptolysine of the distal Ub on the proximal Ub thioester. These results could be clearly explained by examining the K48-linked di-Ub structure where both functionalities are found in a very close proximity; hence, the high effective molarities of these groups favor the intramolecular reaction (Figure 1B).[17] These results are also consistent with the previous work of Fushman and co-workers,[18] wherein the cyclized di-Ub was prepared under folding conditions by cross-linking two Ub units bearing Cys residues at position 48 in the distal Ub and 76 in the proximal Ub with 1,6-hexane-bis-vinylsulfone. Such a synthesis enabled the group to study the effect of cyclization on conformational dynamics and binding properties of K48-linked di-Ub. Our approach, on the other hand, should enable after a desulfurization step straightforward access of cyclic di-Ub with natural linkages.

Interestingly also, analysis of the tri-Ub from the polymerization of **Ub^{48}** revealed that part of this chain was obtained in the cyclized form and migrated in the SDS-PAGE slightly lower as compared to the band corresponding to the uncyclized tri-Ub (Figure 3A). Such a behavior in the SDS-PAGE was also previously reported in the enzymatic studies where partial cyclization of the Ub chains was observed.[10] We have also observed such phenomena in the case of polymerization of **Ub^{1}** under denaturating conditions (Figure 2). In the latter case, despite di-Ub and tri-Ub are present under unfolding conditions, the proximity of their N- and C-termini is presumably still high enough to promote cyclization and competes with the intermolecular reaction.

Finally, the formation of chains of only up to tetra-Ub in the polymerization of **Ub^{48}** under folding conditions is interesting. This could be due to the cyclization of mono- and di-Ub, which reduces the concentration of the reactive species for further polymerization. Moreover, the closed conformation that this chain adopts could create steric hindrance that affects the intermolecular reaction. Notably, enzymatic assembly of K48-linked chains leads to the formation of polymers of higher order (>tetra-Ub),[10] which indicates that the involved enzymes are affecting the dynamics of these chains to allow further elongation.

Next, we examined the polymerization of **Ub^{1}** under folding conditions, which was designed to polymerize via native chemical ligation (NCL)[11,19] to give the linear chains. Under these conditions, this monomer polymerized to give the longer chains (up to 10 units) as was analyzed by SDS-PAGE (Figure 3B). Notably, the different chains that we were able to analyze by mass spectrometry were found to have only the hydrolyzed thioester of the proximal Ub without any detectable intramolecular cyclization products. Here, the rigidity of the open structures of these chains places the Cys far away from the thioester, leaving the latter moiety prone only to hydrolysis. Interestingly, when the polymerization reaction was analyzed using SEC we detected, in addition to the longer chains, a major peak that elutes at the void volume. Such a behavior was not affected by adding excess of TCEP to reduce any possibly formed intermolecular disulfide bonds. This peak was collected and further analyzed using SDS-PAGE (denaturating conditions), which gave a picture similar to that of the crude

dx.doi.org/10.1021/ja3078736 | J. Am. Chem. Soc. 2012, 134, 16085−16092

reaction. These results indicate the formation of soluble self-assembled oligomers, possibly through intermolecular Ub−Ub interactions that could occur through their exposed hydrophobic cores.

When examining the polymerization behavior of **Ub^{63}** under folding conditions, we also observed polyUb chains with varying lengths as evident by the SDS-PAGE and the SEC analyses (Figure 3C). However, differences in the polymerization pattern were also observed when compared to **Ub^{1}**. The first is related to the state of the polymerization products, that is, cyclized versus hydrolyzed chain (**Cy-Ub^{63}n** or **Ub^{63}n-COOH**, Scheme 1). While in the linear chain no cyclization products were observed in any of polyUb, in the case of **Ub^{63}** we also observed, as analyzed by mass spectrometry, cyclized di- and tri-Ub chains (**Cy-Ub^{63}2** or **Cy-Ub^{63}3**, Scheme 1), in addition to the uncyclized form **Ub^{63}2-COOH**, **Ub^{63}3-COOH**, Scheme 1). These results indicate that, although both chains adopt extended conformation, their dynamics are still distinct and apparently the K63-linked chains have more flexibility, which could explain the cyclization outcome observed here. Indeed, recent studies by Wolberger and co-workers to probe the conformation of K63-linked tetra-Ub chain in solution using small-angle X-ray scattering experiments and molecular dynamics simulations revealed that this chain adopts an ensemble of conformations that are more compact than the extended form in the crystal.[20]

A further difference we observed in the case of **Ub^{1}** is related to the self-assembly of the polymerization products. Such a behavior was not observed in the case of **Ub^{63}** despite the fact that both chains adopt open conformations in which the hydrophobic patches are solvent exposed. However, it has been suggested that the relative orientations of the consecutive molecules in these chains could vary wherein the hydrophobic patches in each monomer could point to the same side or to different directions.[6b,c] Such difference in the orientation could affect the self-assembly of these chains and could contribute to the observed outcome, where in the less symmetric structure the self-assembly could be less favored. Moreover, the formed cyclized chains in the case of the **Ub^{63}** could possibly interfere with the self-assembly and makes it a less favored process, contrary to the case of **Ub^{1}** where such cyclized products were not observed.

3.4. Polymerization Studies in the Presence of UBA2.

One of the consequences of the different structures adopted by the various Ub chains and the distinct sequences flanking the isopeptide bond is their ability to interact differently and selectively with the known Ub binding domains.[21] For example, it has been shown that the terminal Ub-associated domain (UBA2) of the hHR23a (the human homologue of protein Rad23), an extra-proteasomal shuttle that mediates the interactions of polyUb substrates with the 26S proteasome proteins, shows strong binding preference for K48-linked chains over the K63-linked and linear ones (Figure 4A).[6a,22] Hence, we reasoned that the presence of a specific domain in the polymerization reactions could interfere with the polymerization as a result of interactions with di-Ub and the longer chains.

To test whether we could interfere with the polymerization reaction in the presence of Ub binding domain, we chose the UBA2 as an example for such a study. The polymerization reactions were carried out with the three monomers (100 μM) and UBA2 (150 μM). These concentrations were chosen on the basis of the limited solubility of the UBA2. In addition,

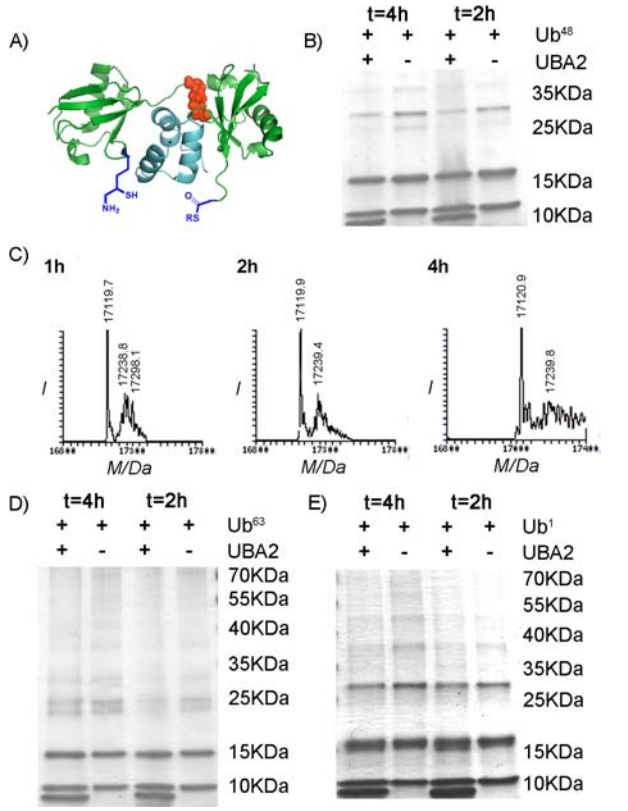


Figure 4. The polymerization of Ub monomers in the presence and absence of UBA2. (A) The structure of UBA2 bound to K48-linked di-Ub (green, the isopeptide bond is highlighted in red, PDB code 1ZO6). (B) SDS-PAGE analysis of the polymerization of **Ub^{48}** in the presence (+) and absence (−) of UBA2. (C) The mass spectrometry analysis of di-Ub product from the polymerization reaction of **Ub^{48}** in the presence of UBA2. The masses of 17 119.7, 17 119.9, and 17 120.9 Da correspond to cyclic di-Ub (calcd 17 122.6 Da), while the masses of 17 238.8, 17 239.4, and 17 239.8 Da, observed at different time points, correspond to di-Ub-MPA (calcd 17 238.6 Da). (D,E) SDS-PAGE analyses of the polymerization of **Ub^{63}** and **Ub^{1}** in the presence and absence of UBA2. The lowest band in the SDS-PAGE analyses corresponds to UBA2.

under these conditions and assuming the formation of a 1:1 complex (UBA2: di-Ub) for the case of K48-linked di-Ub, the concentrations of the formed di-Ub and the UBA2 are above the reported K_d (18 μM), which favor their binding.[21a] On the other hand, the reported K_d value for the K63-linked chains is 180 μM for the 2:1 complex of UBA2 and di-Ub, respectively.[22] Interestingly, the polymerization products of **Ub^{48}** were significantly different from the polymerization pattern for the same monomer under similar conditions yet in the absence of UBA2. These differences could be summarized as follows: (1) Analyzing the reaction mixture, after 2 h, by SDS-PAGE and mass spectrometry analyses revealed that the polymerization reaction led to the formation of significantly less tri-Ub and almost no tetra-Ub chains (Figure 4B). (2) While in the absence of the UBA2, we observed a cyclic tri-Ub product, in the presence of the domain, no such product was detected even after 4 h (Figure 4B). (3) Analyzing the formed di-Ub by mass spectrometry revealed that the cyclization step was slower, because after 2 h we observed that ∼25% of di-Ub did not undergo cyclization and the thioester of the proximal Ub was intact (Figure 4C). Such an intermediate (**Ub^{48}2-MPA**, Scheme 1) was never observed before without the UBA2, which

dx.doi.org/10.1021/ja3078736 | J. Am. Chem. Soc. 2012, 134, 16085−16092

indicates the interferences of UBA2 due to its binding in the center of two Ub molecules of the K48-linked chain (Figure 4A), hence inducing steric hindrance for the cyclization step. However, after 4 h, such an intermediate disappeared by further reaction (cyclization or polymerization) due to the dynamic nature of the interaction with the UBA2. (4) We also observed by LC−MS analysis that the mono-Ub retained its thioester functionality partially, even after 2 h, indicating that the presence of UBA2 interferes with the intramolecular cyclization of $\mathbf{Ub}^{48}$ and also slows the polymerization reaction. Notably, the polymerization of $\mathbf{Ub}^{63}$ and $\mathbf{Ub}^{1}$ in the presence and the absence of the UBA2 did not show detectable differences (Figure 4D and E), highlighting the specific interaction of the UBA2 with the K48-linked chains and the possibility of interfering with these polymerization reactions in a selective manner.

4. SUMMARY

We have demonstrated the use of bifunctional Ub monomers to examine the polymerization behavior as a function of the nucleophile site and folding conditions. In general, the current available X-ray and NMR structures of the Ub chains explain to a certain extent the polymerization behavior of each Ub monomer. Hence, K48-linked chains, which adopt a closed conformation, afforded mostly cyclized di-Ub structures due to the close proximity of the reactive moieties. On the other hand, the K63-linked and the linear chains polymerized to afford the longer chains. Surprisingly, in the K63-linked chains, we have also observed cyclized polyUb products but not in the linear chains, indicating that the dynamic of the K63-linked chains are different from the linear chains. Using this approach, one could explore the behavior of the remaining Ub chains to shed more light on the dynamic of these chains in solution based on the behavior of the polymerization reaction. Finally, in the presence of a specific ubiquitin-binding domain, one could also influence the lengths and states of these chains that are obtained in the polymerization reaction. While in the cases of $\mathbf{Ub}^{1}$ and $\mathbf{Ub}^{63}$, UBA2 had no influence on the polymerization products, with $\mathbf{Ub}^{48}$ we observed changes in the polymerization pattern. In principle, using other binding domains that have high binding affinities,[23] one could have a stronger influence on the polymerization products. It is also tempting to propose that in the assembly of Ub chains in cell, other factors could play a role in controlling the chain lengths because in vitro enzymatic assembly appears not to be fully controlled. In the general aspect of this work, which to the best of our knowledge represents the first example of examining the polymerization of repeating modules based on tertiary protein domains, it affords some early lessons on the design and synthesis of biomaterial for various goals.

■ ASSOCIATED CONTENT

Supporting Information

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

■ AUTHOR INFORMATION

Corresponding Author

abrik@bgu.ac.il

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Israel Science Foundation. We wish to thank Liat Spasser, Prof N. Gabriel Lemcoff, Dr. David Lukatsky, and Prof. Taleb Mokari for useful discussions as well as Dr. Alexandra Massarwa for proofreading this manuscript.

■ REFERENCES

(1) Kushner, A. M.; Guan, Z. *Angew. Chem., Int. Ed.* **2011**, *50*, 9026−9057.
(2) (a) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338−351. (b) Hackenberger, C. P. R.; Schwarzer, D. *Angew. Chem., Int. Ed.* **2008**, *47*, 10030−10074.
(3) Vogel, V. *Annu. Rev. Biophys. Biomol. Struct.* **2006**, *35*, 459−488.
(4) (a) Pickart, C. M.; Fushman, D. *Curr. Opin. Chem. Biol.* **2004**, *8*, 610−616. (b) Ikeda, F.; Dikic, I. *EMBO Rep.* **2008**, *9*, 536−542. (c) Komander, D. *Biochem. Soc. Trans.* **2009**, *37*, 937−953.
(5) (a) Ryabov, Y.; Fushman, D. *J. Am. Chem. Soc.* **2007**, *129*, 7894−7902. (b) van Dijk, A. D. J.; Fushman, D.; Bonvin, A. M. J. J. *Proteins: Struct., Funct., Bioinf.* **2005**, *60*, 367−381. (c) Eddins, M. J.; Varadan, R.; Fushman, D.; Pickart, C. M.; Wolberger, C. *J. Mol. Biol.* **2007**, *367*, 204−211.
(6) (a) Varadan, R.; Assfalg, M.; Haririnia, A.; Raasi, S.; Pickart, C. M.; Fushman, D. *J. Biol. Chem.* **2004**, *279*, 7055−7063. (b) Komander, D.; Reyes-Turcu, F.; Licchesi, J. D. F.; Odenwaelder, P.; Wilkinson, K. D.; Barford, D. *EMBO Rep.* **2009**, *10*, 466−473. (c) Rohaim, A.; Kawasaki, M.; Kato, R.; Dikic, I.; Wakatsuki, S. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2012**, *68*, 102−108.
(7) (a) Pickart, C. M. *Annu. Rev. Biochem.* **2001**, *70*, 503−533. (b) Weissman, M. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 169−178.
(8) Ye, Y.; Rape, M. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 755−764.
(9) Piotrowski, J.; Beal, R.; Hoffman, L.; Wilkinson, K. D.; Cohen, R. E.; Pickart, C. M. *J. Biol. Chem.* **1997**, *272*, 23712−23721.
(10) (a) Tingting, Y.; Robert, E. C. *J. Biol. Chem.* **2000**, *275*, 36862−36868. (b) Dong, K. C.; Helgason, E.; Yu, C.; Phu, L.; Arnott, D. P.; Bosanac, I.; Compaan, D. M.; Huang, O. W.; Fedorova, A. V.; Kirkpatrick, D. S.; Hymowitz, S. G.; Dueber, E. C. *Structure* **2011**, *19*, 1053−1063.
(11) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. *Science* **1994**, *266*, 776−779.
(12) For selected reviews that cover these approaches, see: (a) Fekner, T.; Li, X.; Chan, M. K. *ChemBioChem* **2011**, *12*, 21−33. (b) Spasser, L.; Brik, A. *Angew. Chem., Int. Ed.* **2012**, *51*, 6840−6862. (c) Strieter, E. R.; Korasick, D. A. *ACS Chem. Biol.* **2012**, *7*, 52−63. For selected examples on the synthesis of ubquitin conjugates with natural isopeptide linkage, see: (d) McGinty, R. K.; Kim, J.; Chatterjee, C.; Roeder, R. G.; Muir, T. W. *Nature* **2008**, *453*, 812−816. (e) Yang, R.; Pasunooti, K. K.; Li, F.; Liu, X.-W.; Liu, C.-F. *J. Am. Chem. Soc.* **2009**, *131*, 13592−13593. (f) El Oualid, F.; Merkx, R.; Ekkebus, R.; Hameed, D. S.; Smit, J. J.; de Jong, A.; Hilkmann, H.; Sixma, T. K.; Ovaa, H. *Angew. Chem., Int. Ed.* **2010**, *49*, 10149−10153. (g) Virdee, S.; Ye, Y.; Nguyen, D. P.; Komander, D.; Chin, J. W. *Nat. Chem. Biol.* **2010**, *6*, 750−757. (h) Hejjaoui, M.; Haj-Yahya, M.; Kumar, K. S. A.; Brik, A.; Lashuel, H. A. *Angew. Chem., Int. Ed.* **2011**, *50*, 405−409. (i) Castañeda, C.; Liu, J.; Chaturvedi, A.; Nowicka, U.; Cropp, T. A.; Fushman, D. *J. Am. Chem. Soc.* **2011**, *133*, 17855−17868. For selected examples on the synthesis of ubiquitin conjugates with isopeptide mimics, see: (j) Eger, S.; Scheffner, M.; Marx, A.; Rubini, M. *J. Am. Chem. Soc.* **2010**, *132*, 16337−16339. (k) Chatterjee, C.; McGinty, R. K.; Fierz, B.; Muir, T. W. *Nat. Chem. Biol.* **2010**, *6*, 267−269. (l) Chen, J.; Ai, Y.; Wang, J.; Haracska, L.; Zhuang, Z. *Nat. Chem. Biol.* **2010**, *6*, 270−272. (m) Shanmugham, A.; Fish, A.; Luna-Vargas, M. P. A.; Faesen, A. C.; El Oualid, F.; Sixma, T. K.; Ovaa, H. *J. Am. Chem. Soc.* **2010**, *132*, 8834−8835. (n) Sommer, S.; Weikart, N. D.; Brockmeyer, A.; Janning, P.; Mootz, H. D. *Angew. Chem., Int. Ed.* **2011**, *50*, 9888−9892. (o) Meier, F.; Abeywardana, T.; Dhall, A.; Marotta, N. P.; Varkey, J.; Langen, R.; Chatterjee, C.; Pratt, M. R. *J. Am. Chem. Soc.* **2012**, *134*, 5468−5471. (p) Valkevich, E. M.; Guenette, R. G.;

Sanchez, N. A.; Chen, Y.-c.; Ge, Y.; Strieter, E. R. *J. Am. Chem. Soc.* **2012**, *134*, 6916−6919.
(13) (a) Erlich, L. A.; Kumar, K. S. A.; Haj-Yahya, M.; Dawson, P. E.; Brik, A. *Org. Biol. Chem.* **2010**, *8*, 2392−2396. (b) Kumar, K. S. A.; Haj-Yahya, M.; Olschewski, D.; Lashuel, H. A.; Brik, A. *Angew. Chem., Int. Ed.* **2009**, *48*, 8090−8094. (c) Haj-Yahya, M.; Kumar, K. S. A.; Erlich, L. A.; Brik, A. *Biopolymers* **2010**, *94*, 504−510.
(14) (a) Kumar, K. S. A.; Spasser, L.; Erlich, L. A.; Bavikar, S. N.; Brik, A. *Angew. Chem., Int. Ed.* **2010**, *49*, 9126−9131. (b) Kumar, K. S. A.; Bavikar, S. N.; Spasser, L.; Moyal, T.; Ohayon, S.; Brik, A. *Angew. Chem., Int. Ed.* **2011**, *50*, 6137−6141. (c) Bavikar, S. N.; Spasser, L.; Haj-Yahya, M.; Karthikeyan, S. V.; Moyal, T.; Kumar, K. S. A.; Brik, A. *Angew. Chem., Int. Ed.* **2012**, *51*, 758−763.
(15) Vijay-Kumar, S.; Bugg, C. E.; Cook, W. J. *J. Mol. Biol.* **1987**, *194*, 531−544.
(16) Johnson, E. C. B.; Kent, S. B. H. *J. Am. Chem. Soc.* **2006**, *128*, 6640−6646.
(17) For examples on the occurrence of NCL via large membered ring, which is driven by proximity effect, see: (a) Kemp, D. S. *Biopolymers* **1981**, *20*, 1793−1804. (b) Beligere, G. S.; Dawson, P. E. *J. Am. Chem. Soc.* **1999**, *121*, 6332−6333. (c) Coltart, D. M. *Tetrahedron* **2000**, *56*, 3449−3491. (d) Birk, A.; Wong, C.-H. *Chem.-Eur. J.* **2007**, *13*, 5670−5675. (e) Lutsky, M.-Y.; Nepomniaschiy, N.; Brik, A. *Chem. Commun.* **2008**, *10*, 1229−1231.
(18) (a) Dickinson, B. C.; Varadan, R.; Fushman, D. *Protein Sci.* **2007**, *16*, 369−378. (b) Satoh, T.; Sakata, E.; Yamamoto, S.; Yamaguchi, Y.; Sumiyoshi, A.; Wakatsuki, S.; Kato, K. *Biochem. Biophys. Res. Commun.* **2010**, *400*, 329−333.
(19) For examples on the use of NCL in polymerization of peptides, see: (a) Paramonov, S. E.; Gauba, V.; Hartgerink, J. D. *Macromolecules* **2005**, *38*, 7555−7561. (b) Ryadnov, M. G.; Woolfson, D. N. *J. Am. Chem. Soc.* **2007**, *129*, 14074−14081.
(20) Datta, A. B.; Hura, G. L.; Wolberger, C. *J. Mol. Biol.* **2009**, *392*, 1117−1124.
(21) (a) Dikic, I.; Wakatsuki, S.; Walters, K. J. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 659−671. (b) Hurley, J. H.; Lee, S.; Prag, G. *Biochem. J.* **2006**, *399*, 361−372.
(22) Varadan, R.; Assfalg, M.; Haririnia, A.; Raasi, S.; Pickart, C. M.; Fushman, D. *Mol. Cell* **2005**, *18*, 687−698.
(23) Sims, J. J.; Scavone, F.; Cooper, E. M.; Kane, L. A.; Youle, R. J.; Boeke, J. D.; Cohen, R. E. *Nat. Methods* **2012**, *9*, 303−309.