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Synthesis of a Bis-thio-acetone (BTA) Analogue of the Lysine Isopeptide Bond and its Application to Investigate the Effects of Ubiquitination and SUMOylation on α -Synuclein Aggregation and Toxicity

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Graphical Abstract

The reversible modification of protein by the small protein ubiquitin and other ubiquitin-like modifiers plays important roles in virtually every key biological process in eukaryotic cells. The establishment of a range of chemical methods for the preparation of ubiquitinated proteins has enabled the site-specific interrogation of the consequences of these modifications. However, many of these techniques require significant levels of synthetic expertise, somewhat limiting their widespread application by the biological community. To overcome this issue, the creation of structural analogues of the ubiquitin–protein linkage that can be readily prepared with commercially available reagents and buffers is an important goal. Here we present the development of conditions for the facile synthesis of bis-thio-acetone (BTA) linkages of ubiquitinated proteins in high yields. Additionally, we apply this technique to the preparation of the aggregation prone protein α -synuclein bearing either ubiquitin or the small ubiquitin-like modifier (SUMO). With these proteins, we demonstrate that the BTA linkage recapitulates the previously published effects of either of these proteins on α -synuclein, suggesting that it is a good structural mimic. Notably, the BTA linkage is chemically and enzymatically stable, enabling us to study the consequences of site-specific ubiquitination and SUMOylation on the toxicity of α -synuclein in cell culture, which revealed modification and site-specific differences.

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Ubiquitin is the founding member of a family of small proteins that share a common three-dimensional structure, which also includes the small ubiquitin-like modifier (SUMO) and several others.^{1,2} Many of these proteins function as posttranslational modifications (PTMs) through their addition to free amines located at the N-terminus, or more commonly through isopeptide linkages to lysine side chains, of substrate proteins. These complex PTMs participate in almost every biological process within the cell. Ubiquitin alone is involved in proteasomal degradation of proteins, the mediation of protein–protein interactions necessary for signal transduction and the DNA damage response, and receptor endocytosis from the cell membrane. Given the key roles that ubiquitin and ubiquitin-like proteins play in cellular biology, understanding the site-specific consequences of individual modifications on particular substrate proteins is an important goal. Unfortunately, traditional enzymatic methods for the modification of proteins have not risen to the challenge. Ubiquitination and SUMOylation are both multistep processes carried out by three classes of enzymes.^{3,4} The protein modifiers are first activated at their C-termini by E1-activating enzymes as protein thioesters before being transferred to one of several E2 ubiquitin-conjugating enzymes to yield a second protein thioester.⁵ In the final step, site-specific substrate selection is carried out by a collection of E3 ligase enzymes (over 600 for ubiquitin).^{6,7} Exploiting this multistep enzymatic cascade has proven challenging for the production of site-specifically modified proteins for subsequent biochemical studies. Therefore, an ever expanding toolbox of chemical methods have been developed for the site-specific installation of ubiquitin and ubiquitin-like modifiers.^{4,8–10}

Several of these chemical methods result in the formation of native isopeptide bonds by taking advantage of the native chemical ligation reaction in combination with an ubiquitin-thioester and cleavable auxiliaries^{11,12} or unnatural amino acids,^{13–15} both of which are site-specifically incorporated using solid phase peptide synthesis. Alternatively, unnatural amino acid mutagenesis can be exploited for the incorporation of selectively protected lysine residues that enable the chemical transformations necessary to generate ubiquitinated proteins^{16–19} or the site-specific incorporation of lysine analogues that can undergo native chemical ligation reactions.²⁰ These methods are ideal for understanding the consequences of the native ubiquitin linkage, particularly for *in vitro* experiments where either deubiquitination is not a concern or alternatively the substrate selectivity of a deubiquitinase is under investigation. However, they may not be appropriate in experiments (e.g., cell lysates, cellular uptake, or micro-injection) where the stable effects of protein ubiquitination are of interest. Other strategies have been developed for the generation of isopeptide-bond analogues. These include the attachment of ubiquitin through both triazole-^{21,22} and oxime-based linkages.²³ Complementary strategies have relied on the unique reactivity of cysteine residues as lysine replacements in the substrate protein to perform disulfide-forming reactions,^{24,25} thiol–ene couplings,^{26,27} and ligations with electrophiles.²⁸ While all of these methods have been applied for the investigation of site-specific ubiquitination, they have certain drawbacks. Solid-phase peptide synthesis and unnatural amino acid strategies require a certain level of chemical expertise. Cysteine-targeted reactions circumvent the need for chemistry to prepare the substrate protein, but this disulfide strategy is limited to experiments in nonreducing conditions, and the preparation of activated ubiquitin proteins required for thiol–ene and electrophilic methods use aminolysis reactions with moderate

yields. Additionally, these analogues may not accurately recapitulate the consequences of the native isopeptide linkage. Therefore, significant thought should be given to which type of strategy is most suitable for a given experiment. For example, if chemical and enzymatic stability is a requirement, then an isopeptide analogue would be most appropriate.

The first method used to generate ubiquitin analogues was reported by Wilkinson and co-workers and required no prior chemical manipulation of either the ubiquitin or substrate proteins.²⁹ Specifically, they first used site-specific mutagenesis and recombinant expression to prepare both ubiquitin with a cysteine in place of the last glycine residue of ubiquitin (G76C) and a substrate protein with a cysteine at the site of modification. These two thiol-bearing proteins were then reacted with 1,3-dichloroacetone to obtain an isopeptide analogue that was stable to chemical and enzymatic cleavage (Figure 1A).²⁹ This approach requires fewer chemical transformations and is therefore less technically challenging; however, it does result in an analogue that is more divergent from the native isopeptide linkage. We report here the novel replacement of this C-terminal cysteine with a 2-amino-ethanethiol linker for the robust synthesis of stable bis-thio-acetone (BTA) analogues of ubiquitin and SUMO modifications that more accurately mimic the structure of the native isopeptide bond (Figure 1B). Notably, these analogues could not be prepared in good yields using the conditions reported by Wilkinson and instead required development of the reaction, which yielded robust conditions that can be readily applied by the biochemical community for the preparation of stable, site-specifically modified proteins. After optimization of these conditions, we explored the scope of our procedure by preparing the protein α -synuclein bearing site-specific ubiquitination at four and SUMOylation at two physiologically relevant sites. α -Synuclein forms the toxic protein aggregates that are closely associated with the progression of Parkinson's disease and is the substrate for a variety of posttranslational modifications that have the potential to directly affect this process. We previously described the consequences of both site-specific ubiquitination and SUMOylation on α -synuclein using the previously mentioned disulfide-linkage strategy.^{30–32} We found that these modifications have interesting site-specific effects on both protein aggregation and degradation, but due to the labile nature of the disulfide bond, we were unable to test their effects on the well-documented extracellular toxicity of α -synuclein when added to living cells in culture. Here, we demonstrate that the bis-thio-acetone analogues of ubiquitin-nated and SUMOylated α -synuclein are nearly identical to the corresponding disulfide analogues in terms of their effects on protein aggregation and that they have interesting site-specific differences in their ability to inhibit the toxicity of α -synuclein.

RESULTS AND DISCUSSION

Development of Conditions for the Synthesis of Bis-thiol-acetone Isopeptide Analogues.

The original conditions developed by the Wilkinson lab used 1,3-dichloroacetone in sodium borate buffer to generate diubiquitin analogues for biochemical analysis.²⁹ To determine if these reaction conditions were applicable to α -synuclein, we first recombinantly expressed and purified ubiquitin(G76C) and α -synuclein with a lysine to cysteine mutation at residue 23, α -synuclein-(K23C). We then proceeded in a stepwise fashion (Figure 2A).

Ubiquitin(G76C) (Figure S1 in the Supporting Information (SI)) was first reacted with 1,3-dichloroacetone in 71.4 mM sodium borate, resulting in the corresponding C-terminal chlorothiol-acetone. Notably, the unreacted starting material and the product co-eluted on reverse-phase HPLC (RP-HPLC). We overcame this issue by incubating the mixture with N-(aminoethyl)malimide, which reacted with the starting material and caused its retention time to shift. Using this two-step purification, the product was isolated in 18% yield (SI, Figure S2). This activated ubiquitin was then incubated with α -synuclein(K23C) in borate buffer with or without 3 M guanidine HCl (GuHCl) to prevent protein aggregation. Unfortunately, we did not observe the formation of any detectable product by RP-HPLC followed by ESI-MS (SI, Figures S3, S4). Despite this disappointing result, we decided to move on to the aminoethanethiol linker (Figure 2B). Accordingly, ubiquitin(1–75) was recombinantly expressed as a in-frame fusion to the AvaDNAE intein,³³ followed by introduction of the C-terminal linker by treatment with 2-aminoethanethiol. The resulting ubiquitin C-terminal thiol was incubated with 1,3-dichloroacetone in borate buffer. In contrast to ubiquitin(G76C), the reaction was easily monitored by HPLC (SI, Figure S5), and we allowed the reaction to proceed for 24 h. These conditions gave 30% product by HPLC (B, SI, Figure S5), suggesting that the activation yield of ubiquitin-(G76C) could be improved with longer reaction times. Next, to avoid the potential formation of ubiquitin disulfides, which would compete with product formation, we repeated the same reaction conditions with different reducing reagents (Figures 2B ad SI Figures S6–S9). Interestingly, under all of these conditions, we observed a different amounts of reductive dechlorination to give the C-terminal ketone product (C in SI, Figures S6–S9), which again resulted in only moderate yields. Despite this, we chose to move onto the second step: the coupling of ubiquitin to α -synuclein. Activated ubiquitin was incubated with α -synuclein(K23C) under the same conditions as described above (Figures 2B, SI, Figures S10, S11). No detectable product was formed without the addition of GuHCl; however, in its presence, we were pleased to observe conversion of the starting materials to the BTA-ubiquitinated α -synuclein, which was isolated in 61% yield.

Given the low overall yield (~30% over two steps) of BTA-ubiquitinated α -synuclein using dichloroacetone, we next decided to explore a more electrophilic linker, 1,3-dibromoacetone. Again, the reactions were performed in a stepwise fashion (Figure 3). Ubiquitin C-terminal thiol was incubated with 1,3-dibromoacetone in 71.4 mM sodium borate, resulting in essentially complete conversion to the corresponding activated ubiquitin (B in SI, Figure S12) in 95% isolated yield after only 1 h. To determine whether the reaction conditions affected the stability of ubiquitin, we determined the melting temperature of both the C-terminal thiol starting material and the product using circular dichroism (CD) spectroscopy as previously described.³⁴ Importantly, we found that the reaction had essentially no effect on the stability of ubiquitin in the product (SI, Figure S13). Next, α -synuclein-(K6C) was incubated with this product in 71.4 mM sodium borate with or without 3 M GuHCl. Without GuHCl, we observed the formation of the BTA product (SI, Figure S14). However, it was not readily separable from the symmetric α -synuclein(K6C) disulfide that also formed during the reaction. In the presence of GuHCl at two different pHs, the reaction produced only moderate yields of the desired product due to poor solubility or slow reaction kinetics (SI, Figures S15, S16). Interestingly, Brik and co-workers recently reported the replacement

of the bromine in a bromoacetamide-containing protein with a chlorine directly from the chloride ions from GuHCl in solution.²⁸ They subsequently overcame this unwanted side reaction by the addition of 100 equivalents of sodium iodide. We explored an alternative approach by replacing GuHCl with GuHBr. Accordingly, GuHBr was readily synthesized from guanidine carbonate. After replacement of the GuHCl with GuHBr, we were delighted to see a large product peak that was isolated in 62% yield (SI, Figure S17).

Synthesis and Characterization of BTA-Ubiquitinated α -Synuclein Analogues.

We next set out to synthesize ubiquitinated α -synuclein analogues at four different residues. Monoubiquitination is a major modifier of α -synuclein in the aggregates isolated from Parkinson's disease patients.^{35–38} It has been shown to be modified by ubiquitin at up to nine different positions using a variety of experimental techniques, including *in vitro* modification and cell culture experiments. We previously used disulfide analogues of ubiquitination to demonstrate that site-specific ubiquitination at the majority of these sites inhibits the formation of α -synuclein fibers.³⁰ However, as mentioned in the introduction, the disulfide bond will be reversed under reducing conditions, preventing the application of these proteins to cell culture. To test whether BTA-ubiquitinated α -synuclein is also resistant to aggregation, we used our optimized conditions to also modify α -synuclein at residues 23, 43, and 96 in isolated yields of 52%, 56%, and 59% over two steps, respectively (SI, Figures S18–S20). These proteins were characterized by RP-HPLC and ESI-MS (SI, Figure S21), as well as SDS-PAGE analysis (SI, Figure S22). We next analyzed the effect of BTA-ubiquitin on the structure of monomeric α -synuclein using CD and dynamic light scattering (DLS). The CD spectra of all four ubiquitinated proteins were highly similar and resembled the expected combination of the random-coil structure of α -synuclein and the ubiquitin fold (SI, Figure S23), indicating that the linkage does not have any consequences on either protein in solution. Characterization by DLS showed that all four proteins had Stokes radii of less than 10 nm (SI, Figure S24), consistent with monomeric protein in solution.

Next, we used a combination of thioflavin T (ThT) fluorescence and transmission electron microscopy (TEM) to investigate the effects of BTA-ubiquitination on α -synuclein aggregation. Specifically, unmodified and the four different ubiquitinated analogues of α -synuclein were incubated at a concentration of 50 μ M at 37 °C with constant agitation. To measure the kinetics of aggregation, aliquots of the aggregation reactions were removed after different lengths of time, and the extent of β -sheet rich fiber formation was measured using ThT fluorescence (Figure 4A). Despite the clear aggregation of unmodified α -synuclein, all four of the BTA-ubiquitinated proteins showed essentially no formation of ThT-positive aggregates over the course of the assay. Importantly, analysis of the proteins at the end of the aggregation reactions by RP-HPLC and mass spectrometry showed that the BTA linkage remained intact throughout the experiment (SI, Figure S25). At the termination of the aggregation reactions, samples were also analyzed by TEM to visualize any aggregates that formed (Figure 4B). A large number of mature fibers were observed in the unmodified α -synuclein reaction. In contrast, BTA-ubiquitination at residues 23, 43, and 96 completely blocked the formation of any fibrous structures and only resulted in disperse, amorphous structures. Notably, in the TEM images from α -synuclein modified at residue 6, we found some structures that resemble irregular, short fibers that are distinct from both

unmodified α -synuclein and the other sites of ubiquitination. We attribute the lack of detectable ThT fluorescence to a combination of the relatively small number of fiber structures that were formed and the somewhat irregular structure of the fibers, which may provide a less-than-ideal binding site for ThT. This is partially consistent with previous data showing that preformed α -synuclein fibers can be enzymatically ubiquitinated at lysine 6.³⁷ Additionally, the data from BTA-ubiquitination at residue 23 does not recapitulate what we previously observed using the disulfide-modification approach, where ubiquitination at residue 23 inhibited the kinetics of aggregation but not the formation of fibers.³⁰ In this previous publication, the protein was subjected to harsher aggregation conditions of 100 μ M protein concentration and stir-bar agitation, which have been shown to accelerate fiber formation.³⁹ We therefore chose to simultaneously compare the effects of both linkages at residue 23 on the aggregation of α -synuclein using ThT fluorescence. First, disulfide-ubiquitinated α -synuclein was prepared as previously described and both proteins were incubated at 50 μ M concentration at 37 °C with agitation in a thermomixer. Analysis using ThT fluorescence showed that both types of linkages result in complete inhibition of α -synuclein aggregation (Figure 4C). When the same proteins were subjected to the more aggressive aggregation conditions, the disulfide-linked material showed less inhibition (Figure 4C), consistent with our previous experiments. This highlights the fact that different analogues of the isopeptide bond can result in different experimental results, so caution should be taken in the selection of the specific chemistry that will be used. In the case of α -synuclein aggregation, the conditions used throughout this paper more closely mirror the concentration of the protein at synapses, suggesting that ubiquitination at residue 23 will inhibit aggregation and toxicity in Parkinson's disease.

These *in vitro* experiments demonstrate that ubiquitination has an inhibitory effect on the formation of α -synuclein fibers. However, they do not completely rule out the possibility that other toxic species are being formed. One way to test the toxicity of ubiquitinated or SUMOylated α -synuclein is the overexpression in cell culture of mutant proteins (i.e., lysine to arginine) that cannot be modified. Unfortunately, the over-expression of α -synuclein in primary neurons in culture and cell-line models is not always toxic.^{40–43} For example, the very high levels of protein expression driven by a tetracycline-inducible promoter was necessary for observable toxicity in a SH-SY5Y cell line.⁴⁴ One way to overcome this limitation that has been widely adopted is the exogenous addition of α -synuclein to cells in culture.^{43,45–50} Importantly, the extracellular toxicity of α -synuclein is supported by observations in human patients⁵¹ and has been translated to *in vivo* animal models.^{52–55} As stated above, our previous studies with disulfide-linked ubiquitin analogues could also not be extended to these type of toxicity measurements in cell culture due to the reducing environment. To determine if the BTA-ubiquitinated analogues were stable to culture conditions, we incubated α -synuclein that had been ubiquitinated at residue 43 with either a disulfide or BTA linkage in cell culture media and analyzed the stability of the protein using SDS-PAGE (SI, Figure S26). The disulfide-linkage was readily reduced over the course of 48 h, while the majority of the BTA-ubiquitinated α -synuclein remained intact, although we did observe the formation of some new bands at intermediate molecular weights that might be attributable to nonspecific degradation. Given this higher degree of stability, we next moved on to test the consequences of ubiquitination on the extracellular toxicity of α -

synuclein. Aggregation reactions were again initiated with either unmodified or BTA-ubiquitinated α -synuclein. After 168 h, any aggregates that formed were collected by centrifugation, followed by resuspension in cell culture media by sonication. To test for the possibility of any soluble toxic-species, the supernatant was also concentrated by lyophilization before addition of culture media and sonication. These preparations were then added to SH-SY5Y cells for 60 h. SH-SY5Y cells are a neuroblastoma cell-line derived from dopaminergic neurons that express endogenous α -synuclein and therefore can serve as a reasonable surrogate for primary neurons in these toxicity measurements.^{56,57} The toxicity of the proteins was measured by treatment with the small-molecule ethidium homodimer (Figure 4D). Ethidium homodimer is excluded from healthy intact cells but will intercalate into the DNA of damaged cells, which increases its fluorescence. The aggregates from unmodified α -synuclein had a large effect on ethidium homodimer signal, consistent with its toxicity previous extracellular culture experiments. In contrast, the BTA-ubiquitinated proteins induced very little toxicity in the cells. In general, the soluble fractions of the aggregation reactions also did not result in a much toxicity, with only modification at residue 43 showing any signal that was statistically significant. Together, these results indicate that ubiquitination of α -synuclein has largely an inhibitory effect on both aggregation and toxicity.

Synthesis and Characterization of BTA-SUMOylated α -Synuclein Analogues.

α -Synuclein is also SUMOylated in Parkinson's disease patients, and the modification has been localized to two residues, lysines 96 and 102, in cell culture.^{58,59} Like ubiquitin, we previously used the disulfide strategy to prepare α -synuclein with site-specific SUMOylation at either residue 96 or 102.³² Interestingly, we found that, despite being only 6-residues away from each other in the primary sequence of α -synuclein, SUMOylation at residue 102 had a much stronger inhibitory effect on protein aggregation and that modification at residue 96 resulted in the formation of large, irregular, and ThT positive aggregates. Again, however, we were unable to determine the consequences of SUMOylation on the toxicity of α -synuclein because of the fragility of the disulfide linkage. To perform this experiment, we first recombinantly expressed a SUMO3-AvaDNAE intein fusion in *Escherichia coli* and subsequently generated the SUMO C-terminal thiol by treatment with 2-aminoethanethiol (SI, Figure S1). Using the conditions optimized for ubiquitin above, this product was incubated with 1,3-dibromoacetone followed by coupling to α -synuclein(K96C) or α -synuclein(K102C) (SI, Figures S27–S29). These protein conjugates were then characterized by RP-HPLC and ESI-MS (SI, Figure S30), in addition to SDS-PAGE analysis (SI, Figure S31). Again, we first used CD spectroscopy and DLS to determine the consequences of the BTA linkage on the initial structure of SUMO and α -synuclein. The CD spectra of both SUMOylated proteins resembled the combination of each protein in isolation (SI, Figure S32), and DLS showed only protein with stokes radii of less than 10 nm (SI, Figure S33). Together, these data demonstrate that, like ubiquitination, BTA-SUMOylation has no significant effect on the structure of the monomeric proteins in solution.

Unmodified α -synuclein and the two BTA-SUMOylated proteins (50 μ M concentrations) were then subjected to aggregation conditions. After different lengths of time, aliquots of the

reaction were removed and the presence of protein fibers was ascertained using ThT fluorescence (Figure 5A). Over the course of 120 h, unmodified α -synuclein produced a large amount of fibers and SUMOylation at residue 102 was essentially completely inhibitory. In contrast, modification at residue 96 produced an intermediate amount of ThT fluorescence. Next, at the termination of the assay, we used TEM to visualize the aggregate structures. As in our previous aggregation experiment, unmodified α -synuclein formed a large number of mature fibers (Figure 5B). SUMOylation at residue 96 resulted in the formation of irregular fibers that were much more rigid than the scattered, amorphous material visualized from α -synuclein modified at residue 102. Notably, these data are very consistent with our previously published experiments using disulfide-linked SUMOylation, where SUMOylation at residue 96 also resulted in the formation of ThT positive fibers, while modification at residue 102 was completely inhibitory.³² Analysis of the proteins at the end of the aggregation assay once again showed that the BTA linkage was stable during the aggregation reaction (SI, Figure S34). Next, we set out to determine if any of the structures formed from BTA-SUMOylated α -synuclein were toxic to cells in culture. First, we incubated either disulfide-linked or BTA-linked SUMOylated α -synuclein (residue 96) in cell culture media and observed reduction of the disulfide linked modification over the course of 48 h but no breakdown of the BTA linkage (SI, Figure S35). Aggregation reactions were then initiated with either modified or BTA-SUMOylated α -synuclein at residue 96 or 102, and the aggregates and soluble material were resuspended as described above for the ubiquitinated proteins. SH-SY5Y cells were then treated with these preparations for 60 h before analysis of cellular toxicity using ethidium homodimer fluorescence (SI, Figure 5C). The aggregate fraction of unmodified α -synuclein induced a large amount of toxicity. Notably, SUMOylation at residue 96, which forms irregular fibers, did not effectively inhibit the induction of cellular toxicity. In contrast, modification at residue 102 was significantly less toxic. Together, these data support our previous experiments and reveal site-specific differences in the consequences of SUMOylation on both aggregation and toxicity.

CONCLUSION

Chemical synthesis and semisynthesis has enabled the site-specific preparation and biochemical characterization of ubiquitinated proteins and transformed the type of experiments that can be performed. However, many of these techniques require expertise in peptide and/or small molecule synthesis, reducing their widespread adoption by the biological community. Therefore, the continued development of robust and simple ubiquitination methods is an important goal. We report here the incorporation of ubiquitin and the ubiquitin-like modifier SUMO into proteins using a bis-thio-acetone (BTA) linkage, which takes advantage of only recombinantly expressed proteins and commercially available reagents. Our approach builds on the pioneering work by Wilkinson and co-workers²⁹ but results in a more structurally accurate isopeptide linkage and proceeds in a much higher overall yield. While the yield of the first step of the Wilkinson method could undoubtedly be increased by longer incubation times, we did not observe any appreciable product formation in the coupling to α -synuclein. The original Wilkinson procedure produced ubiquitin dimers, and we speculate that the high solubility of ubiquitin over α -synuclein may explain their success by increasing the reaction kinetics. During the development of our optimized

synthetic conditions, we made several other interesting observations. The reactions involving 1,3-dichloroacetone and the less sterically hindered animoethanethiol-functionalized ubiquitin were sluggish, and our attempts to optimize the reaction conditions only led to the competitive formation of side-products. To address these issues, we took advantage of 1,3-dibromoacetone as a more electrophilic reagent. The activation of ubiquitin with this reagent proceeded in excellent yield to give the corresponding product; however, the coupling of this protein to α -synuclein in GuHCl was inefficient, which we attribute to both limited solubility of α -synuclein and potentially to in situ replacement of the bromide with chloride.²⁸ Switching the solubilizing agent to GuHBr solved both of these problems and enabled the synthesis of four different ubiquitinated and two different SUMOylated α -synuclein analogues.

We previously used a disulfide-based strategy to investigate the consequences of either ubiquitination or SUMOylation on the aggregation of α -synuclein and found that different modification sites all largely inhibit aggregation but to different extents depending on the site of modification.^{30–32} However, we were unable to test the toxicity of these semisynthetic proteins in cell culture due to the labile nature of the disulfide bond. Importantly, we show that BTA-ubiquitination of α -synuclein at residues 6, 43, and 96 behaves almost identically to the disulfide-linked material. All three modification sites inhibit aggregation to mature fibers as measured by a lack of ThT fluorescence, but modification at residue 6 is capable of forming some small irregular fiber structures that were visible by TEM. In contrast, modification at residue 23 generated somewhat different results under aggressive aggregation conditions, demonstrating that different linkage analogues are not completely interchangeable in all experiments and that the subsequent confirmation of any results using other biochemical or cellular methods is key. We then used the chemical stability of the BTA linkage to enable the treatment of SH-SY5Y cells with the aggregation reactions to investigate the effect of ubiquitination on α -synuclein toxicity. Notably, the three tested ubiquitination sites inhibited the toxicity of α -synuclein, while aggregates formed by the unmodified protein induced significant cell death. These results add additional key evidence that indicates a protective role for ubiquitination in Parkinson's disease. This is somewhat counterintuitive, as a large fraction of deposited α -synuclein in the brains of Parkinson's disease patients is ubiquitinated. However, we favor a model where ubiquitination of α -synuclein occurs as an early response in the disease where it slows the rate of protein aggregation, but this protective function is overcome during the progression of neurodegeneration resulting in ubiquitinated protein becoming "trapped" in the large neuronal aggregates. In the case of SUMOylation, the disulfide linked proteins also both inhibited the aggregation of α -synuclein. However, they were not equivalent in their inhibitory potency, with modification at residue 96 being significantly less effective than SUMOylation at residue 102. Here, we observed the exact same trend with the BTA analogues. Aggregation of α -synuclein SUMOylated at residue 96 resulted in the formation of some ThT-positive fibers that appear shorter than those formed by unmodified protein. In contrast, SUMOylation at residue 102 completely inhibited aggregation, with only amorphous aggregates visible by TEM. Consistent with these *in vitro* results, treatment of SH-SY5Y cells with the different aggregation reactions showed that SUMOylation at residue 96 is still quite toxic to cells despite being less so than unmodified protein, with

modification at residue 102 is largely nontoxic. These results suggest that the addition of these protein modifiers does not simply have a large steric effect that blocks aggregate formation, as ubiquitination at residue 96 is quite different from SUMOylation despite being proteins of similar size and shape. Furthermore, they encourage the efforts to identify which enzyme is responsible for the removal of SUMOylation at residue 102, as it could be a potential drug target in Parkinson's disease. Finally, this approach relies on the absence any cysteine residues in the proteins of interest. However, ubiquitin has no native cysteine residues and SUMO has only one such amino acid that can be mutated, as in this study, to serine without affecting the protein structure. Additionally, α -synuclein has no native cysteines, making it particularly amenable to this strategy.

METHODS

General Information.

Cysteamine was purchased from Tokyo Chemical Industry CO, Ltd. All of the other commonly used chemicals and solvents were purchased from commercial sources (Sigma-Aldrich, VWR, EMD, Novagen, Invitrogen, Fluka) and used without any further purification. Antibiotic (ampicillin Na salt, EMD) was prepared as a stock solution (100 mg mL^{-1} or 500 mg mL^{-1}) and stored at -20°C . Stock solution of ethidium homodimer (3 mM) was prepared and stored at -20°C . Growth media (Luria–Bertani Broth, Miller) was prepared, sterilized, and stored based on the manufacture instruction. Agilent Technologies 1200 Series HPLC with diode array detector was used for reverse phase high performance liquid chromatography (RP-HPLC). The buffer system is as following: buffer A (water with 0.1% TFA), buffer B (90% acetonitrile, 10% water, 0.1% TFA). Mass spectra were obtained on either an API 3000 LC/MS-MS System (Applied Biosystems/MDS SCIEX) or an API 150EX system (Applied Biosystems/MDS SCIEX).

Cloning and Plasmids.

All constructions were prepared by following standard molecular cloning techniques. Generation of all plasmids were described in previous publications.^{30–32} A QuikChange mutagenesis kit (Stratagene) was used to generate glycine to cysteine point mutation on ubiquitin, Ubiquitin(G76C), and terminal glycine deletion on ubiquitin and SUMO3, ubiquitin(1–75) and SUMO3(1–92), respectively.

Ubiquitin C-Terminal Thiol Expression and Purification.

Ubiquitin(1–75)-aminoethanethiol was expressed and purified as described previously.^{30,31} Yield of 7.6 mg L^{-1} of culture was obtained. Pure protein product was characterized by ESI-MS ($M + H^+$). Expected mass is 8568.15 Da . Observed mass was $8567.7 \pm 1.3 \text{ Da}$.

SUMO3 C-Terminal Thiol Expression and Purification.

C47S mutated SUMO3(1–92)-aminoethanethiol was expressed and purified as described previously.³² Yield of 8.0 mg L^{-1} of culture was obtained. Pure product was characterized by ESI-MS ($M + H^+$). Expected mass is 10379.7 Da . Observed mass was $10380.8 \pm 0.18 \text{ Da}$.

UbiquitinG76C Expression and Purification.

UbiquitinG76C was expressed and purified as described^{30,31} with slight modifications. *E. coli* BL21(DE3) cells that had been transformed with the pTXB1-UbG76C-AvaDnaE-6XHis plasmid were grown in 1 L of LB medium containing ampicillin (100 $\mu\text{g}/\text{mL}$) at 37 °C. When the OD_{600 nm} reached 0.6, the culture was induced by addition of 0.5 mM IPTG and was then incubated for 16 h at 25 °C. The bacterial pellet was obtained by centrifugation (5000 rpm, 30 min, 4 °C). After resuspension of the pellet in lysis buffer (10 mL for 1 L of culture, 50 mM phosphate, 300 mM NaCl, 5 mM imidazole, pH 8.0, with complete protein inhibitor cocktail [mini-complete EDTA free, Roche]), cells were lysed by tip sonication (6 $\times$ 30 s on/off cycle, 4 °C). The cellular debris was pelleted by centrifugation (15000 rpm, 30 min, 4 °C). The resulting supernatant was then loaded on 1 mL HisTrap column (GE Healthcare). The protein was bound to the column by washing with 10 CV of buffer A (50 mM phosphate, 300 mM NaCl, 50 mM imidazole, pH 8.0). The protein was eluted with 4.5 CV of buffer B (buffer A with 250 mM imidazole). The elution fractions were analyzed by SDS-PAGE, and the pure fractions were pooled together, and dialyzed against buffer C (30 mM Tris, 150 mM NaCl, 1 mM EDTA pH 8.5). Intein cleavage by hydrolysis was accomplished by incubating in buffer C for 16 h at 25 °C. UbiquitinG76C was purified over a C4 semipreparative RP-HPLC using a 25–60% B linear gradient over 60 min and subsequently lyophilized to yield 6.0 mg L⁻¹ of culture. Pure UbG76C was characterized by ESI-MS ($M + H^+$). Expected mass is 8611.1 Da. Observed mass was 8612.2 $\pm$ 1.4 Da.

Activation of UbiquitinG76C with 1,3-Dichloroacetone.

All reagents were cooled to 4 °C before the reaction. Lyophilized UbiquitinG76C (2.0 mg) was dissolved in 10 mM HCl (200 μL), and sodium borate buffer (80 μL , 250 mM, pH 8.3) was added to the mixture. Then 10 equiv of 1,3-dichloroacetone (100 mM in DMF) were added to the reaction, and the resulting solution was rotated at 4 °C for 6 h. To efficiently separate unreacted starting material from product on RP-HPLC, 25 equiv of N-(2-aminoethyl)maleimide were then added and the resulting mixture was rotated for 2 h. The desired product was isolated by C4 semiprep RP-HPLC (25–60% B over 60 min). The isolated yield of the activated protein product was 18%. The activated ubiquitin product was characterized by ESI-MS ($M + H^+$). Expected mass is 8701.5 Da. Observed mass was 8701.9 $\pm$ 0.3 Da.

Coupling Reaction of Activated UbiquitinG76C and α -Synuclein(K23C) in Borate Buffer.

Lyophilized, activated ubiquitinG76C (0.5 mg, 2 equiv) and α -synuclein(K23C) (0.5 mg, 1 equiv) were resuspended in 10 mM HCl (100 μL), followed by addition of 40 μL of 250 mM sodium borate buffer (pH 8.3). The reaction mixture was rotated at 4 °C for 48 h. Before the HPLC injection, 20% v/v β -mercaptoethanol was added to the reaction mixture and heated for 5 min at 98 °C. The reaction was monitored by C4-analytical RP-HPLC (25–60% B linear gradient over 60 min).

Coupling Reaction of Activated UbiquitinG76C and α -Synuclein(K23C) in 3 M Guanidine-HCl Borate Buffer.

Lyophilized, activated ubiquitinG76C (2 equiv, 0.5 mg) and α -synuclein(K23C) (1 equiv, 0.5 mg) were resuspended in a reaction buffer (100 μ L, 3 M guanidine-HCl, 71.4 mM borate). The resultant mixture was rotated at 4 °C for 48 h. At the end of the reaction, 20% v/v β -mercaptoethanol was added to the mixture, and the resulting mixture was boiled at 98 °C for 5 min. The reaction was monitored by C4 analytical RP-HPLC (25–60% B linear gradient over 60 min).

Reaction of Ubiquitin C-Terminal Thiol and 1,3-Dichloro-acetone in Borate Buffer.

All reagents were cooled to 4 °C before the reaction. Lyophilized ubiquitin-aminoethanethiol (1.0 mg) was resuspended in 10 mM HCl (100 μ L), with a subsequent addition of sodium borate buffer (40 μ L, 250 mM, pH 8.3). Then 10 equiv of 1,3-dichloroacetone (100 mM in DMF) were added to the reaction mixture. The reaction was rotated at 4 °C for 24 h and then purified by C4 analytical RP-HPLC (25–60% B over 60 min) to yield 28% of the activated ubiquitin product that was characterized by ESI-MS ($M + H^+$). Expected mass is 8659.6 Da. Observed mass was 8658.5 ± 0.50 Da.

Reaction of 1,3-Dichloroacetone and Ubiquitin C-Terminal Thiol with 5 equiv of TCEP.

All reagents were cooled to 4 °C before the reaction. Lyophilized ubiquitin-aminoethanethiol (4.3 mg) was resuspended in 10 mM HCl (400 μ L) followed by the addition of borate buffer (240 μ L, 250 mM sodium borate, 14.6 mM TCEP, pH 8.0). Finally, 10 equiv of 1,3-dichloroacetone (100 mM in DMF) were added to the mixture and the resulting reaction was rotated at 4 °C for 12 h and purified by C4 analytical RP-HPLC (25–60% B, 60 min). The major peak, ubiquitin thiopropanone, was characterized by ESI-MS ($M + H^+$). Expected mass is 8624.1 Da. Observed mass was 8622 ± 22 Da.

Reaction of 1,3-Dichloroacetone and Ubiquitin C-Terminal Thiol with 0.5 equiv of TCEP.

All reagents were cooled to 4 °C before the reaction. Lyophilized ubiquitin-aminoethanethiol (1.0 mg) was resuspended in 10 mM HCl (100 μ L) followed by the addition of sodium borate buffer (40 μ L, 250 mM sodium borate, 1.46 mM TCEP, pH 8.0). Finally, 10 equiv of 1,3-dichloroacetone (100 mM in DMF) were added to the mixture and the final mixture was rotated at 4 °C for 5 h and purified by C4 analytical RP-HPLC (25–60% B, 60 min) to yield 49% of the activated ubiquitin product that was characterized by ESI-MS ($M + H^+$). Expected mass is 8659.6 Da. Observed mass was 8658.5 ± 0.50 Da.

Reaction of 1,3-Dichloroacetone and Ubiquitin C-Terminal Thiol with 0.5 equiv TCEP in Basic pH.

All reagents were cooled to 4 °C before the reaction. Lyophilized ubiquitin-aminoethanethiol (2.0 mg) was dissolved in 10 mM HCl (200 μ L) followed by the addition of sodium borate buffer (80 μ L, 250 mM sodium borate, 28 mM NaOH, 1.46 mM TCEP, pH 8.96). Then 10 equiv of 1,3-dichloroacetone (100 mM in DMF) were added to the solution. The resulting solution was rotated at 4 °C for 5 h. The reaction was monitored by C4

analytical RP-HPLC (25–60% B linear gradient over 60 min). The 41% yield of the activated ubiquitin product was calculated from the RP-HPLC trace.

Reaction of 1,3-Dichloroacetone and Ubiquitin C-Terminal Thiol with Ascorbic Acid.

All reagents were cooled to 4 °C before the reaction. Lyophilized ubiquitin-aminoethanethiol (2.0 mg) was resuspended in 10 mM HCl (200 μ L) followed by the addition of sodium borate buffer (80 μ L, 250 mM sodium borate 5.84 mM ascorbic acid, pH 8.0). To the resulting solution, 10 equiv of 1,3-dichloroacetone (100 mM, DMF) were added. The reaction was incubated at 4 °C for 16 h. The reaction was monitored by C4 analytical RP-HPLC (25–60% B linear gradient over 60 min). The 51% yield of the activated ubiquitin product was calculated from the RP-HPLC trace.

Coupling Reaction of Activated Ubiquitin and α -Synuclein in Borate Buffer.

Activated ubiquitin (0.5 mg, 2 equiv) and α -synuclein(K23C) (0.5 mg, 1 equiv) were resuspended in a reaction buffer (80 mM sodium borate, pH 8.3) to give a final protein concentration of 10 mg mL⁻¹. The reaction mixture was rocked at RT for 24 h. The reaction was monitored by C4-analytical RP-HPLC (0–70% B linear gradient over 60 min).

Coupling Reaction of Activated Ubiquitin and α -Synuclein in 3 M Gu-HCl Borate Buffer.

Activated ubiquitin (0.5 mg, 2 equiv) and α -synuclein(K23C) (0.5 mg, 1 equiv) were resuspended in 3 M guanidine-HCl with 71.4 mM sodium borate buffer (pH 8.3) to give a final protein concentration of 10 mg mL⁻¹. The reaction mixture was rotated at 4 °C for 24 h. Before the injection to HPLC, the reaction was mixed with 20% v/v β -mercaptoethanol and boiled for 5 min at 98 °C. The reaction was purified by C4-analytical RP-HPLC (30–55% B linear gradient over 60 min). The 61% yield of BTA-ubiquitinated α -synuclein was calculated from the RP-HPLC and characterized by ESI-MS ($M + H^+$). Expected mass is 23,058.1 Da. The calculated mass was 23,059.2 $\pm$ 1.5 Da.

Synthesis of 1,3-Dibromoacetone.

1,3-Dibromoacetone is commercially available (Santa Cruz Biotechnology) but can also be prepared as follows. Bromine (10 mL, 194 mmol) was added dropwise to an acetone–methanol (6.08 mL of acetone in 72.9 mL of methanol, 82.8 mmol acetone) mixture with continuous stirring. The resulting mixture was stirred at RT for an additional 2 h, followed by cooling to –20 °C for 16 h. The resulting precipitate (~12 g) was collected and dissolved in 72 mL of water. Concentrated sulfuric acid (1 mL) was added then slowly, and the mixture was stirred at 48 h at 60 °C. The resulting mixture was then cooled to RT and extracted 3 $\times$ with CH₂Cl₂. The organic fractions were dried over Na₂SO₄, filtered, and concentrated under reduced pressure in a cool bath to give a 12% yield of 1,3-dibromoacetone as an orange oil.

Reaction of 1,3-Dibromoacetone with Ubiquitin or SUMO3 C-Terminal Thiols.

All reagents were cooled to 4 °C before the reaction. In a typical reaction, lyophilized ubiquitin or SUMO3 C-terminal thiol (2.0 mg) was resuspended in 10 mM HCl (200 μ L). Sodium borate buffer (80 μ L, 250 mM, pH 8.3) was added to the solution. Then 20 equiv of

1,3-dibromoacetone (DMF, 100 mM) were added to the mixture, and the solution was rotated at 4 °C for 1 h for ubiquitin or 2 h for SUMO3. The reaction mixture was purified over C4 semipreparative RP-HPLC (25–50%B over 60 min for ubiquitin, 30–50%B over 60 min for SUMO3). The purified ubiquitin product was characterized by C4 analytical RP-HPLC and ESI-MS ($M + H^+$). Expected mass is 8703.14 Da. Observed mass was 8702.0 ± 1.1 Da. Yield of Ubiquitin(BTA) was 95%. The purified SUMO3 product was characterized by C4 analytical RP-HPLC and ESI-MS ($M + H^+$). Expected mass is 10516.0 Da, and the observed mass was 10510.5 ± 1.2 Da. Yield of SUMO3(BTA) was 60.0%.

Protein Thermostability Measurements by Circular Dichroism.

The ubiquitin C-terminal thiol and the associated production after reaction with 1,3-dibromoactone were solubilized at the concentration of 7.5 μ M in 10 mM phosphate buffer (pH7.4). Samples in a 1 mm path length quartz cuvette were heated from 60 to 105 °C at the rate of 1 °C per min. The ellipticity at 220 nm was collected every 0.3 °C, with 2.0 mm slit width, and DIT of 8 s. The transition temperature (T_M) was calculated using Spectra Manager software. The calculated T_M for Ub-SH was 84.23 ± 0.14 °C. The calculated T_M for Ub-BTA was 82.44 ± 0.16 °C.

Coupling Reaction of Activated (Brominated) Ubiquitin and α -Synuclein in Borate Buffer.

Purified activated ubiquitin (4.2 mg) and α -synuclein(K6C) (2.7 mg) were resuspended in 10 mM HCl (700 μ L) to five a protein concentration of 10 mg mL⁻¹. Sodium borate buffer (250 mM at pH 8.3, 280 μ L) was added to the solution. The reaction was rocked for 2 h at RT and purified on C4 RP-HPLC (0–70% B over 60 min). The purified BTA-linked ubiquitinated α -synuclein(K6C) was characterized on SDS-PAGE.

Coupling Reaction of Activated (Brominated) and α -Synuclein in 3 M Gu-HCl Borate Buffer pH 8.3.

Purified activated ubiquitin (0.5 mg, 2 equiv) and α -synuclein(K6C) (0.5 mg, 1 equiv) were resuspended in 3 M guanidine-HCl with 71.4 mM sodium borate buffer (pH 8.3) to give a final protein concentration of 10 mg mL⁻¹. The reaction mixture was rotated at 4 °C for 48 h. β -mercaptoethanol (20% v/v) was added to the reaction mixture, and the resulting solution was heated at 98 °C for 5 min. The reaction was monitored by C4-analytical RP-HPLC (30–55% B linear gradient over 60 min). The 36% yield of BTA-ubiquitinated α -synuclein was calculated from the RP-HPLC and was analyzed on SDS-PAGE.

Coupling Reaction of Activated (Brominated) and α -Synuclein in 3 M Gu-HCl Borate Buffer pH 7.5.

Purified activated ubiquitin (0.5 mg, 2 equiv) and α -synuclein(K23C) (0.5 mg, 1 equiv) were resuspended in 3 M guanidine-HCl with 71.4 mM sodium borate buffer (pH 7.5) to give a final protein concentration of 10 mg mL⁻¹. The reaction mixture was rotated at 4 °C for 48 h. β -Mercaptoethanol (20% v/v) was added to the reaction mixture, and the resulting solution was heated at 98 °C for 5 min. The reaction was monitored by C4-analytical RP-HPLC (30–55% B linear gradient for 60 min). The 56% yield of BTA-ubiquitinated α -synuclein was calculated from the RP-HPLC and was analyzed on SDS-PAGE.

Synthesis of Guanidine HBr.

Guanidine HBr is commercially available (Santa Cruz Biotechnology) but can be prepared as follows. In a round-bottom flask, guanidine carbonate (100 g, 0.56 mol) was dissolved in 57 mL of water with continuous stirring and then cooled to 4 °C. In a separate beaker, concentrated hydrobromic acid (124 mL, 1.10 mol) was diluted with 177 mL of water and the diluted HBr was added slowly to the ice-cold guanidine carbonate solution. The resulting solution was removed from the ice and stirred overnight at RT. The solvent was removed by a rotary evaporator, resulting in rapid crystallization of the product, and crystals were dried on a filter paper (mp 178–180 °C).

Coupling Reaction of Activated (Brominated) Ubiquitin or Activated (Brominated) SUMO3 and α -Synuclein in 3 M Gu-HBr Borate Buffer.

Purified activated ubiquitin or SUMO3 and α -synuclein(K#C) (at a 2:1 molar ratio) were resuspended in a reaction buffer (3 M guanidine HBr, 71.4 mM sodium borate, pH 7.5) to give a final protein concentration of 10 mg mL⁻¹. The reaction mixture was rotated at 4 °C for 18 h. β -Mercaptoethanol (20% v/v) was added to the reaction mixture, and the resulting solution was heated at 98 °C for 5 min. The reactions were then purified by semipreparative C4 RP-HPLC (30–55% B over 60 min for Ubiquitin-linked or 30–50% B over 60 min for SUMO3-linked). The proteins were characterized by C4 analytical RP-HPLC, SDS-PAGE, and ESI-MS (M + H). Expected mass of BTA-ubiquitinated α -synuclein is 23,058.1 Da, and BTA-SUMOylated α -synuclein is 24 872 Da. Ubiquitination residue 6: yield = 62%, t_R = 27.92 min, observed = 23064.7 $\pm$ 2.71 Da. Ubiquitination residue 23: yield = 55%, t_R = 28.59 min, observed = 23065.6 $\pm$ 1.61 Da. Ubiquitination residue 43: yield = 60%, t_R = 27.92 min, observed = 23059.1 $\pm$ 2.8 Da. Ubiquitination residue 96: yield = 63%, t_R = 28.06 min, observed = 23,063.7 $\pm$ 1.78 Da. SUMOylation residue 96: yield = 33%, t_R = 27.47 min, observed = 24873.5 $\pm$ 1.4 Da. SUMOylation residue 102: yield = 41%, t_R = 27.52 min, observed = 24870.7 $\pm$ 1.2 Da).

Circular Dichroism (CD) Measurements.

Jasco-J-815 CD spectrometer was used to collect CD spectra. Sample protein solutions were diluted to 7.5 μ M in a reaction buffer without sodium azide (10 mM phosphate buffer, pH 7.4). The far UV-DC spectra (190–250 nm) were collected at 25 °C in a 1 mm path length quartz cuvette. For every spectrum, an average of three scans was obtained with a 0.1 nm data pitch, 1.0 m bandwidth, 50 nm min⁻¹ scanning speed, and data integration time of 4 s. The backgrounds of buffers were subtracted for all spectra, and the data were converted into mean residue ellipticity.

Dynamic Light Scattering.

Dynamic light scattering data were obtained with a Dynapro Titan temperature controlled microscopier (Wyatt). Aliquots from aggregation reactions (50 μ M, at time = 0 h) were analyzed with ten 10 s acquisition, at 25 °C, with laser power adjusted to give an intensity of 2.0×10^6 counts s⁻¹. Radii were calculated based on a Rayleigh sphere approximation.

***In Vitro* Aggregation Reactions.**

Proteins were resuspended to a concentration of 50 μM in phosphate buffer (10 mM phosphate, pH 7.4, 0.05% NaN_3) with bath sonication for 15 min. The supernatant was cleared by centrifugation (14000g, 15 min, 4 °C). The supernatant was then carefully aliquoted into triplicates (typical reaction volume was 200 μL). Samples were incubated at 37 °C under continuous shaking (1000 rpm) in an Eppendorf Thermomixer for indicated times. ThT signals were read as described below.

Aggressive Aggregation Reactions.

Disulfide linked ubiquitination residue 23 was prepared as previously described.³⁰ Proteins were then resuspended to a concentration of 100 μM in phosphate buffer (10 mM phosphate, pH 7.4, 0.05% NaN_3) with bath sonication for 15 min. The supernatant was cleared by centrifugation (14000g, 15 min, 4 °C). The supernatant was carefully aliquoted into 0.5 mL screw cap vials (Axygen scientific). To each tube, a $2 \times 2 \text{ mm}^2$ Teflon coated stir bar was added. All reactions were arranged symmetrically and placed on a magnetic stir plate in a 37 °C. All reactions were incubated with constant stirring for indicated times. ThT signals were collected as described below.

Thioflavin T Fluorescence.

Samples from aggregation assay reaction were diluted in 96-well plate to a concentration of 1.25 μM with a reaction buffer (10 mM phosphate, pH 7.4, 0.05% NaN_3) containing 10 μM Thioflavin T. The plate was read by Synergy H4 hybrid reader (BioTek). The plate was shaken at 300 rpm for 3 min, followed by data collection ($\lambda_{\text{ex}} = 450 \text{ nm}$, 20 nm band path, $\lambda_{\text{em}} = 482 \text{ nm}$, 9.5 nm band path, reading from the bottom of a plate, gain = 100, read height was 5.00 mm). All of the readings were normalized to the ThT signal of buffer.

Transmission Electron Microscopy (TEM).

Formvar coated copper grids (150 mesh, Electron Microscopy Sciences) were incubated with the end of aggregation assay samples (10 μL) droplet for 5 min. The grids were negatively stained for 2 min with 1% uranyl acetate, with a subsequent wash 3 $\times$ with 1% uranyl acetate. Each time, the excess liquid was removed from the grids using a filter paper. The grids were dried for 48 h in a vacuum desiccator and then visualized using a JEOL JEM 2100 LaB6 transmission electron microscope operated at 200 kV, 60000 $\times$ magnification.

Cell Culture.

SH-SY5Y cells were maintained in 1:1 DMEM/F12 medium (Corning) supplemented with 10% fetal bovine serum and incubated at 37 °C, 5.0% CO_2 . Medium was changed every 2–3 days. Two days prior to an aggregate treatment, SH-SY5Y cells were plated in 96-well plate at a density of 12500 cells/well.

Cellular Toxicity Assay.

Lyophilized proteins were dissolved in phosphate buffer (10 mM NaPO_4H , 0.05% NaN_3) to a concentration of 50 μM and incubated at 37 °C with agitation (1000 rpm) in a Eppendorf Thermomixer for 7 days. On the day of a treatment, aggregates were pelleted by the

centrifugation (20000g, 1 h, 25 °C). The supernatant was collected and lyophilized as a soluble material fraction. Aggregates and soluble material fractions were resuspended in 1:1 DMEM/F12 media (supplemented with 10% FBS) to a concentration of 25 μ M protein, followed by bath sonication (20 min) and tip-sonication (7×1 s on/off cycle, 20% amp). Cells were treated with 100 μ L of synuclein containing medium and incubated for 60 h. After 60 h, 100 μ L of ethidium homodimer (3 μ M, in DPBS) was added to each well, and the plate was incubated at 37 °C for 40 min. Fluorescent signal was read by a plate reader (Synergy H4 hybrid reader, BioTek). The plate was shaken for 10 s at 300 rpm, followed by the data collection ($\lambda_{\text{ex}} = 530$ nm, bandwidth 20.0 nm, $\lambda_{\text{em}} = 620$ nm, bandwidth 20.0 nm, reading from top, gain 100, read height was 5.00 mm). Signals were normalized to background.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structure of native ubiquitination and the corresponding bis-thiol-acetone analogues. (A) Structure of the native ubiquitin isopeptide bond and the analogue utilized by Wilkinson and co-workers. (B) Structure of the bis-thiol-acetone (BTA) linkage developed here.

Figure 2. Synthesis of BTA-ubiquitinated α -synuclein using 1,3-dichloroacetone. #, Yield by HPLC; *, isolated yield.

Figure 3. Synthesis of BTA-ubiquitinated α -synuclein using 1,3-dibromoacetone. #, Yield by HPLC; *, isolated yield.

Figure 4.

Characterization of BTA-ubiquitinated α -synuclein. (A) Ubiquitination inhibits the aggregation of α -synuclein. Unmodified α -synuclein or the indicated BTA-ubiquitinated proteins were subjected to aggregation conditions (agitation at 37 °C) before analysis by ThT fluorescence ($\lambda_{\text{ex}} = 450 \text{ nm}$, $\lambda_{\text{em}} = 482 \text{ nm}$) at the indicated time points. y -Axis is fold-change in fluorescence compared to unmodified α -synuclein at $t = 0 \text{ h}$. Results are the mean $\pm$ SEM of three separate experiments. Statistical significance compared to unmodified α -synuclein (two-tailed, t test): ** $P = <0.01$, *** $P = <0.001$, **** $P = <0.0001$. (B) The same

reactions were analyzed by TEM after 120 h; scale bar, 500 nm. (C) The disulfide- and BTA-ubiquitination of α -synuclein are not equally inhibitory. Unmodified α -synuclein or the indicated ubiquitinated proteins were subjected to different aggregation conditions before analysis by ThT fluorescence ($\lambda_{\text{ex}} = 450 \text{ nm}$, $\lambda_{\text{ex}} = 482 \text{ nm}$) at the indicated time points. *y*-Axis is fold-change in fluorescence compared to unmodified α -synuclein at $t = 0 \text{ h}$. Results are the mean $\pm$ SEM of three separate experiments. (D) Ubiquitination inhibits α -synuclein extracellular toxicity. SH-SY5Y cells were treated for 60 h with vehicle or insoluble material or remaining soluble material ($25 \mu\text{M}$ based on monomer concentration) collected from aggregation reactions initiated with unmodified α -synuclein or the BTA-ubiquitinated proteins. Toxicity was measured with ethidium homodimer fluorescence ($\lambda_{\text{ex}} = 528 \text{ nm}$, $\lambda_{\text{ex}} = 617 \text{ nm}$). Results are the mean $\pm$ SEM of three separate experiments. Statistical significance compared to vehicle treated (two-tailed, *t* test): N.S. = $P \geq 0.05$, * $P < 0.05$, *** $P = < 0.0001$.

Figure 5. Characterization of BTA-SUMOylated α -synuclein. (A) SUMOylation has site-specific effects on the aggregation of α -synuclein. Unmodified α -synuclein or the indicated BTA-SUMOylated proteins were subjected to aggregation conditions (agitation at 37 °C) before analysis by ThT fluorescence ($\lambda_{\text{ex}} = 450 \text{ nm}$, $\lambda_{\text{em}} = 482 \text{ nm}$) at the indicated time points. y -Axis is fold-change in fluorescence compared to unmodified α -synuclein at $t = 0 \text{ h}$. Results are the mean $\pm$ SEM of three separate experiments. Statistical significance compared to unmodified α -synuclein (two-tailed, t test): ** $P = <0.01$, *** $P = <0.001$, **** $P = <0.0001$.

(B) The same reactions were analyzed by TEM after 120 h; scale bar, 500 nm. (C) SUMOylation has site-specific effects on α -synuclein extracellular toxicity. SH-SY5Y cells were treated for 60 h with vehicle or insoluble material or remaining soluble material (25 μ M based on monomer concentration) collected from aggregation reactions initiated with unmodified α -synuclein or the BTA-SUMOylated proteins. Toxicity was measured with ethidium homodimer fluorescence ($\lambda_{\text{ex}} = 528$ nm, $\lambda_{\text{ex}} = 617$ nm). Results are the mean $\pm$ SEM of three separate experiments. Statistical significance compared to vehicle treated (two-tailed, t test): N.S. = $P \geq 0.05$, * $P < 0.05$, *** $P = < 0.001$, **** $P = < 0.0001$.