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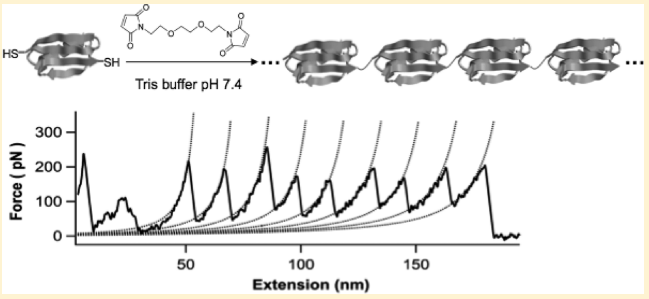
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Facile Method of Constructing Polyproteins for Single-Molecule Force Spectroscopy Studies

Peng Zheng, Yi Cao,† and Hongbin Li*

Department of Chemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z1, Canada

ABSTRACT: Constructing polyproteins consisting of identical tandem repeats of proteins provides an unambiguous method of investigating the mechanical properties of proteins at the single-molecule level using force spectroscopy techniques. Here we report a maleimide−thiol coupling-based facile method of constructing polyproteins for single-molecule force spectroscopy studies on the mechanical properties of proteins. This method allows for the construction of polyproteins in an efficient fashion under room temperature. The resultant thioether bonds are resistant to reduction and make it possible to carry out single-molecule force spectroscopy studies under various redox conditions. This novel method complements existing polyprotein engineering methods and can be easily applied to a wide variety of proteins.



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INTRODUCTION

Protein mechanics plays an important role in a wide variety of biological processes across different length scales.[1−3] Many proteins are subject to the mechanical stretching force under their biological conditions and play various structural and mechanical roles. Elastomeric proteins are one representative class of such mechanical proteins, and many of them are tandem modular proteins made of multiple individually folded domains.[4,5] They function as molecular springs under their biological settings to provide tissues with elasticity, extensibility, and strength[3,6] and also play important regulatory roles in a wide variety of biological processes.[2] The development of single-molecule force spectroscopy techniques, in particular, the atomic force microscopy (AFM)-based force spectroscopy techniques, over the last two decades has made it possible to measure the mechanical properties of elastomeric proteins at the single-molecule level and has provided tremendous insight into the mechanical design of elastomeric proteins.[4,7−11] Moreover, single-molecule AFM has evolved into a general method to characterize the mechanical unfolding/folding dynamics of proteins along a well-defined reaction coordinate at the single-molecule level.[7,10,12]

In single-molecule AFM studies, it is critical to unambiguously distinguish single-molecule stretching events of the protein of interest from nonspecific interactions between the AFM tip and sample as well as from non-single-molecule events.[13,14] Constructing polyproteins made of multiple identical tandem repeats of proteins of interest provides unambiguous fingerprints for identifying single-molecule events and has thus become a gold standard in single-molecule AFM studies of protein mechanics. Because polyproteins are made of identical tandem repeats of proteins, the resultant force−extension curves will exhibit characteristic sawtooth-like force−extension curves with identical contour length increments between consecutive sawtooth peaks. Such repetitive patterns allow one to identify single-molecule stretching events readily and unambiguously from a myriad of nonspecific interactions and complex multimolecule stretching events.[13,14]

The construction of polyproteins is typically accomplished using a recombinant DNA technology-based DNA concatamerization method.[15] This method provides precise control of the molecular structure and composition of the resultant polyprotein and has been the most widely used method.[15−17] However, it requires stepwise repetitive cloning and is laborious and expensive. To facilitate the faster construction of polyproteins, alternative methods have been pursued. A cysteine engineering-based method was developed for this purpose. This method was first successfully demonstrated in a solid-state synthesis of polyproteins based on the specific crystallographic arrangement of proteins in their crystals[18] and later was generalized to proteins in solution.[19,20] In this method, a pair of cysteines were introduced into the protein of interest at chosen locations. The subsequent oxidation of cysteines results in the polymerization of the protein via the formation of intermolecular disulfide bonds. This method provides an efficient alternative to the recombinant DNA technology-based polyprotein engineering approach and also allows the stretching of polyproteins in any defined pulling direction.[19] This method has found many applications in a variety of proteins.[21,22] However, the oxidation of cysteines is a slow process, and the disulfide linkage in the resultant polyproteins limits the use of this method of studying redox-dependent mechanical

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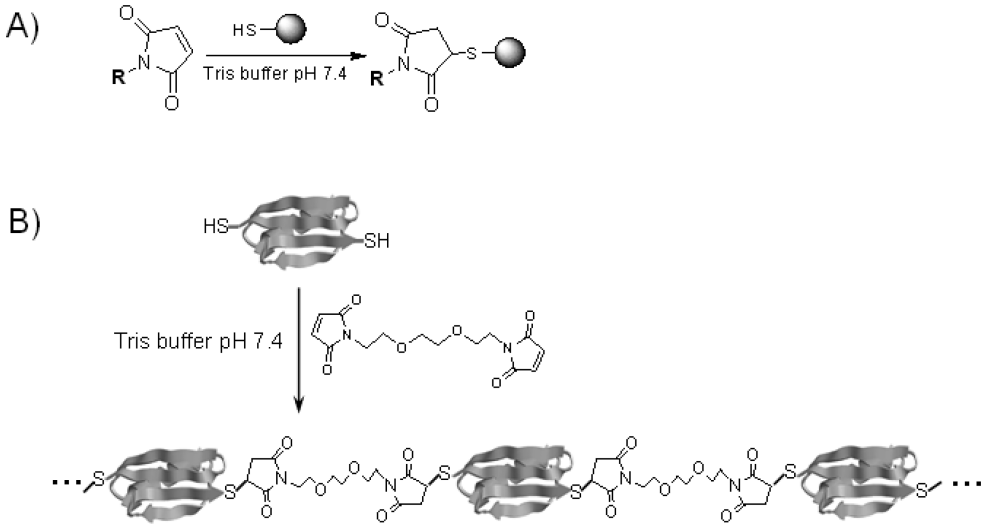


Figure 1. Construction of polyprotein using maleimide−thiol coupling chemistry. (A) The maleimide group can specifically react with the sulfhydryl group, forming a covalent thioether bond that cannot be cleaved under reducing conditions. (B) Construction of the polyprotein $(GB1)_n$ using the maleimide−thiol coupling chemistry. $BM(PEO)_3$ can link two GB1s together by reacting sequentially with the sulfhydryl groups on the protein via maleimide groups on both ends to form stable thioether bonds and further extension leading to the formation of polyprotein $(GB1)_n$.

properties of proteins.[20] Here, we report a facile method based on maleimide−thiol coupling chemistry to construct polyproteins to complement these existing polyprotein engineering methods.

MATERIALS AND METHODS

Protein Engineering. The model proteins we used in this study are GB1, the B1 IgG binding domain of protein G from *Streptococcus*, and rubredoxin from *C. pasteurianum* (cpRD). The gene coding cys-GB1-cys was amplified by using the polymerase chain reaction (PCR) and subcloned into the expression vector pQE80L (Qiagen). The gene encoding cys-cpRD-GB1-cys was constructed using standard molecular biology techniques and cloned into expression vector pQE80L between *BamH*I and *Kpn*I sites. The sequences of both genes were verified by direct DNA sequencing. Both constructs were overexpressed in *Escherichia coli* strain DH5α and purified using Co^{2+}-affinity chromatography with a TALON His-Tag purification resin (Clontech). The purified protein was kept in PBS (phosphate-buffered saline) buffer at 4 °C with a concentration of ∼2 mg/mL.

The pure Fe(III) form of Cys-cpRD-GB1-Cys was obtained by further purification using anion-exchange chromatography. First, using a 9K MWCO pierce concentrator (Thermo Scientific), the protein chimera cpRD-GB1 solution was buffer exchanged into a 40 mM Tris (Tris-(hydroxymethyl)aminomethane) buffer at pH 8.5 and concentrated to ∼8 mg/mL. The Fe form of the cpRD-GB1 protein was eluted first at around 120 mM NaCl using a linear gradient elution (0−300 mM NaCl in 40 mM Tris buffer with 1 mM TCEP (Tris(2-carboxyethyl)-phosphine) at pH 8.5) with a Mono-Q 5/50GL anion-exchange column (GE Healthcare) in an AKTA FPLC system (GE Healthcare) at a flow rate of 2 mL/min (Figure 4A). The UV−vis spectrum of purified Fe(III)-cpRD-GB1 was recorded on a NanoDrop ND-1000 absorption spectrometer (Thermo Scientific), and the purity of Fe(III)-cpRD-GB1 was estimated to be >90% (based on its molar extinction coefficient of 8.2 mM^{-1} cm^{-1} at 490 nm).[23] The protein was kept in Tris buffer at pH 7.4.

Construction of Polyproteins through a Maleimide−Thiol Coupling Reaction. In a typical experiment, 1 mL of freshly purified Cys-GB1-Cys (or Cys-cpRD-GB1-Cys) at a concentration of 2 mg/mL was reacted immediately with 40 μL of a 10 mM concentrated stock solution of $BM(PEO)_3$ (1,8-bis-maleimido-$(PEO)_3$, Molecular Biosciences) in Tris buffer at pH 7.4. The solution was incubated at room temperature for ∼2 h, and then the resultant polyprotein solution was directly used in AFM experiments.

Characterization of Polyproteins Constructed Using Maleimide−Thiol Coupling Chemistry. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to examine the molecular weight of the resultant polyproteins. The polymerization efficiency of GB1 was also analyzed using a gel filtration chromatography technique with a Superdex 75 10/300GL column in an ATKA FPLC system (GE Healthcare). The buffer contains 50 mM sodium phosphate plus 100 mM sodium chloride at pH 6.5, and the flow rate was 0.5 mL/min.

Single-Molecule AFM Experiments. Single-molecule AFM experiments were carried out on a custom-built AFM as described.[24] Each Si_3N_4 cantilever (MLCT, Bruker Corp.) was calibrated in solution using the equipartition theorem before each experiment to obtain the spring constant (typically around 40 pN/nm). All experiments were done in Tris buffer at pH 7.4 at room temperature.

In a typical experiment, 2 μL of the polyprotein sample (at a concentration of ∼2 mg/mL) was added to a clean glass coverslip covered with ∼50 μL of Tris buffer. The protein was allowed to absorb for ∼5 min before starting the AFM measurements. During the experiment, the cantilever was brought into contact with the substrate at a contact force of ∼1 nN to pick up proteins. The pulling speed was 400 nm/s.

For experiments on the Fe(II)-cpRD-GB1 polyprotein, 10 μL of 200 mM dithioreitol (DTT) was added to the solution after the $(Fe(II)$-cpRD-$GB1)_n$ protein was absorbed on the glass coverslip. AFM experiments started after incubation for ∼20 min. The same amount of DTT solution was added every hour thereafter to ensure a reduced environment.

RESULTS AND DISCUSSION

General Principle. Maleimide−thiol coupling-based bioconjugation methods have been used extensively in biochemistry because of the rapid and specific reaction of maleimide with sulfhydryl groups under mild experimental conditions.[25,26] Here we employ this maleimide−thiol coupling chemistry to develop an efficient method of constructing polyproteins for single-molecule AFM studies. The basic experimental design is schematically

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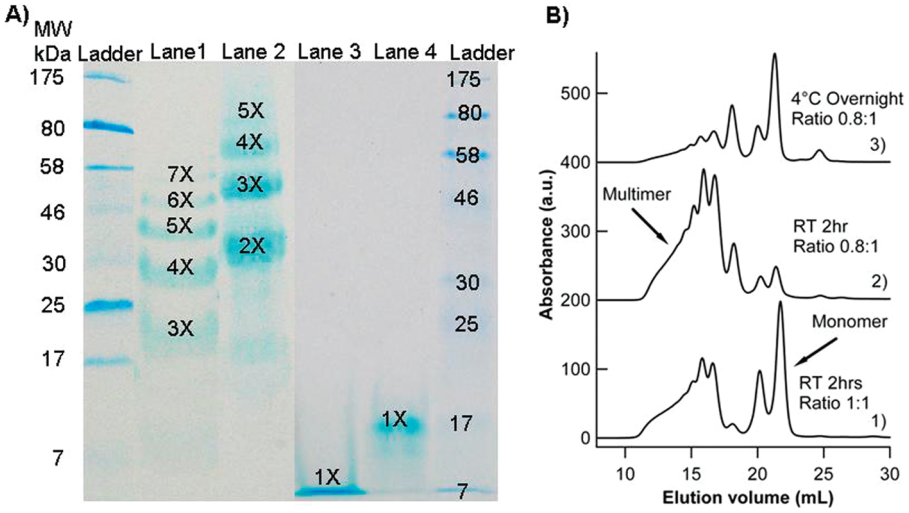


Figure 2. Characterization of polyproteins constructed using maleimide−thiol coupling chemistry. (A) SDS-PAGE analysis of the degree of polymerization of polyproteins $(GB1)_n$ (lane 1) and $(cpRD\text{-}GB1)_n$ (lane 2) constructed using maleimide−thiol coupling chemistry. Before being loaded into the gel, proteins were treated with β-mercaptoethanol. Monomeric proteins GB1 (lane 4) and cpRD-GB1 (lane 3) and protein makers are also shown. (B) Analysis of polymerization efficiency under different conditions by size-exclusion chromatography. Curve 1 shows a chromatograph of the products with the reaction being carried out at room temperature for 2 h at a molar ratio of 1:1 GB1/$BM(PEO)_3$; curve 2 corresponds to the chromatograph of the reaction carried out at room temperature for 2 h at a molar ratio of 0.8:1 GB1/$BM(PEO)_3$, and curve 3 is for the reaction carried out at 4 °C overnight.

depicted in Figure. 1. First, two cysteine residues will be introduced into the protein of interest via site-directed mutagenesis at well-defined, solvent-exposed positions to provide accessible sulfhydryl groups. Then, the protein will react with bifunctional maleimide compound $BM(PEO)_3$ in a molar ratio of 1:1, leading to the polymerization of the protein of interest via the formation of intermolecular thioether bonds. In bifunctional maleimide compound $BM(PEO)_3$, PEO serves as a flexible linker with increased solubility. $(PEO)_3$ has a contour length of ∼1.5 nm and can effectively minimize domain−domain interactions because of the formation of thioether bonds.[26,27]

Synthesis and Characterization of the Polyprotein $(GB1)_n$ Constructed via Maleimide−Thiol Coupling Chemistry. To validate the design of this maleimide−thiol coupling-based polyprotein engineering method, we first constructed a model polyprotein of GB1, which is an excellent model of an artificial elastomeric protein domain.[28,29] Polyprotein $(GB1)_8$ has been constructed using a recombinant DNA technology-based strategy, and its mechanical properties have been characterized in great detail in our previous single-molecule AFM studies.[28,29] Thus, constructing the GB1 polyprotein will provide a good test case for the proposed maleimide−thiol coupling approach.

By reacting Cys-GB1-Cys, which was pretreated with β-mercaptoethanol at 4 °C overnight to prevent the formation of intermolecular disulfide bonds, with $BM(PEO)_3$ in a molar ratio of 1:1 in PBS buffer at room temperature for 2 h, we found that polyprotein $(GB1)_n$ readily formed. From the standard reducing SDS-PAGE picture (Figure. 2A), it is evident that the vast majority of monomeric GB1 has been converted to a multimer of GB1, and the formation of multimeric GB1 (up to 7) can be readily identified. At higher molecular weight, the protein band becomes smeared, indicating the formation of a mixture of higher-order multimers of GB1. From the intensity of the bands, it is possible to estimate the abundance of different multimers formed during this polymerization reaction.

The reaction of Cys-GB1-Cys and $BM(PEO)_3$ is similar to 2 + 2 condensation polymerization. The molecular weight of the resultant polymer depends on the stoichiometry of the two functional groups.[30] To obtain higher multimers of GB1, it is important to ensure that the two functional groups are present in a 1:1 molar ratio. However, because of the air oxidation of cysteine residues into intermolecular disulfide bonds, the molar ratio of GB1 versus $BM(PEO)_3$ may not be necessarily equal to that of sulfhydryl groups versus maleimide groups. Thus, fine-tuning the molar ratio of GB1 and $BM(PEO)_3$ is necessary. As shown in Figure 2B, because of the incomplete reduction of disulfide bonds, the apparent 1:1 molar ratio of GB1 versus $BM(PEO)_3$ led to a large fraction of monomeric GB1 remaining in the reaction mixture, as monitored by analytical size exclusion chromatography (Figure 2B, curve 1). By adjusting the apparent molar ratio of GB1/$BM(PEO)_3$ to 1:0.8, the fraction of remaining monomeric GB1 decreased dramatically after polymerization and the yield of multimeric GB1 increased significantly (Figure 2B, curve 2).

We also found that the reaction temperature is an important parameter affecting the formation of multimeric GB1. It can be seen that incubating the reaction mixture at 4 °C overnight led to the formation of a very small amount of oligomeric GB1, and a large amount of GB1 remained monomeric (Figure 2B, curve 3). However, reaction at room temperature for 2 h led to the efficient polymerization of GB1.

Thus, reacting Cys-GB1-Cys with $BM(PEO)_3$ at room temperature provides an efficient alternative method of constructing polyproteins for single-molecule AFM experiments.

Single-Molecule AFM Experiments Revealing That $(GB1)_n$ Shows the Same Mechanical Properties as $(GB1)_8$ Constructed on the Basis of the Recombinant DNA Approach. The formation of polyprotein $(GB1)_n$ is further confirmed by single-molecule AFM experiments. Stretching polyprotein $(GB1)_n$ results in characteristic sawtooth-like force−extension curves, where each individual sawtooth peak corresponds to the mechanical unfolding of individual GB1 domains in the polyprotein chain (Figure. 3A). The force−extension curve contained as many as

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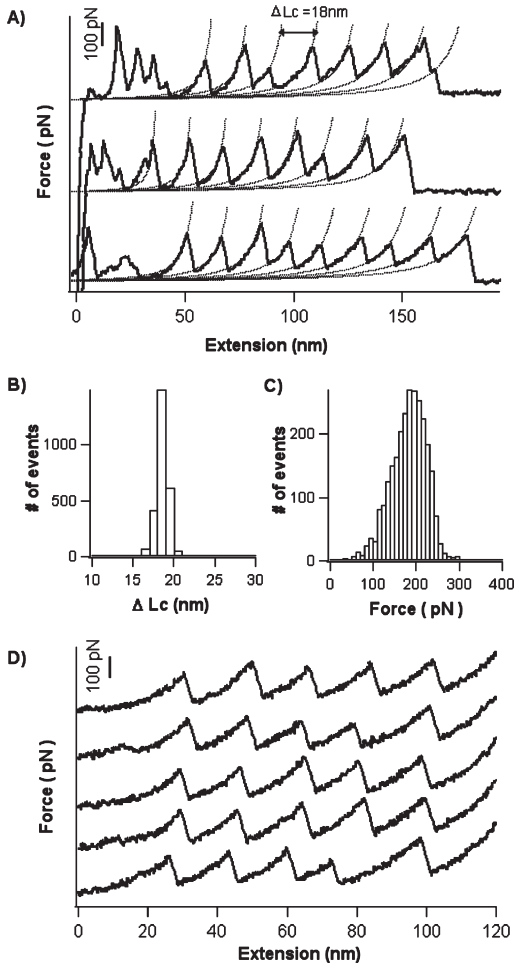


Figure 3. Mechanical unfolding and refolding of polyprotein $(GB1)_n$ constructed using maleimide−thiol coupling chemistry has an identical mechanical signature to that of $(GB1)_8$ constructed using the recombinant DNA technique. (A) Typical force−extension curves of the unfolding of $(GB1)_n$ show a characteristic contour length increment ΔL_c of ∼18 nm, which is identical to the result of recombinant-produced polyprotein $(GB1)_8$. Dotted lines are fits to the WLC model at a pulling speed of 400 nm/s. (B) Histogram of the contour length increment of $(GB1)_n$ showing 18.0 ± 0.7 nm (average ± standard deviation). (C) Histogram of the unfolding force showing an average force of 182 ± 42 pN (n = 2628). (D) Five consecutive force−extension curves of polyprotein $(GB1)_n$ from a repeated stretching and relaxation experiment that lasts 100 cycles.

17 unfolding events, suggesting that the polyprotein contained 17 or more GB1 domains (where the degree of polymerization is 17 or higher). Fitting force−extension curves using the wormlike chain (WLC) model of polymer elasticity[31] revealed that the contour length increment ΔL_c of GB1 is ∼18 nm, which is identical to that of polyprotein $(GB1)_8$ constructed using the recombinant approach.[28] The average unfolding force of GB1 is ∼180 pN, which is again close to that of $(GB1)_8$ (Figure. 3B). These results suggest that the thioether linkage between GB1 domains does not affect the structural integrity or mechanical properties of GB1. In addition, polymerized $(GB1)_n$ is also amendable for cyclic stretching−relaxation experiments in which the folding kinetics of GB1 domains can be readily measured. Figure 3D shows four consecutive stretching−relaxation curves from a $(GB1)_n$ polyprotein molecule that was repeatedly stretched

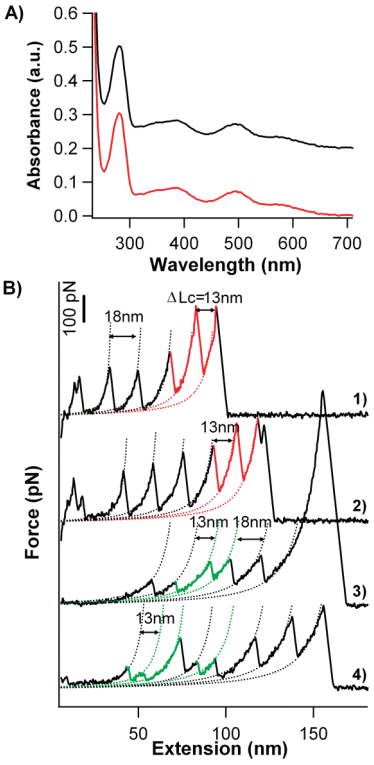


Figure 4. (A) UV−vis spectra of cpRD-GB1 before (top panel) and after (bottom panel) reacting with $BM(PEO)_3$. For clarity, the two spectra are offset from each other. The two proteins show identical spectral features, indicating that maleimide groups do not react with Fe-bound cysteine residues in rubredoxin. (B) Mechanical unfolding of $(cpRD\text{-}GB1)_n$ under normal and reduced environments shows that the polyprotein is stable under reduced conditions. Typical force−extension curves of Fe(III)-cpRD-GB1 (curves 1 and 2) and Fe(II)-cpRD-GB1 (curves 3 and 4) are shown. They both show characteristic contour length increments of $\Delta L_c \approx 18$ nm, which is from the unfolding of fingerprint GB1, and $\Delta L_c \approx 13$ nm, which is from the rupture of the $Fe-S_4$ center in cpRD, and a much lower unfolding force is observed in Fe(II)-cpRD-GB1 (in green). These results also reveal the robustness of the thioether linkage in the polyprotein under reducing conditions.

and relaxed more than 100 times. These results clearly indicated that polyprotein $(GB1)_n$ constructed using maleimide−thiol coupling chemistry is the same as $(GB1)_8$, suggesting that this method is suitable for constructing polyproteins for single-molecule force spectroscopy studies.

Construction of the Pure Form Polymetalloprotein Rubredoxin. Many proteins require cofactors to carry out their biological functions. Metalloproteins are one representative class of such proteins.[32] Interactions between proteins and their cofactors can facilitate the folding of proteins and increase their thermodynamic stability. However, the existence of holo and apo forms of proteins leads to a mixture of proteins in different conformations. In addition, nonendogenous ligands can compete with endogenous ones, creating additional complexity for the composition of the protein.[33] For example, metalloprotein rubredoxin can accommodate different metal ions in its tetra-cysteine metal-binding center. When overexpressed in *E. coli*, two different forms, wt Fe-rubredoxin and Zn-substituted rubredoxin, coexpress naturally, leading to a mixture of the two different metal-containing rubredoxins.[23,34,35] These issues make it difficult, if not impossible, to use recombinant DNA methodology to construct

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a polyprotein in which all individual domains are in the same form for single-molecule AFM studies. Instead, the maleimide−thiol coupling method provides the possibility to overcome this technical hurdle. Figure 4 shows one example of using the maleimide−thiol coupling method to construct a polyprotein of metalloprotein rubredoxin from *Clostridium pasteurianum* (cpRD) for single-molecule AFM experiments.

Rubredoxin is the simplest iron−sulfur protein containing one Fe(III) bound by four cysteines[36,37] and has been used in our laboratories as a model system for investigating the mechanical unfolding/folding dynamics of metalloproteins. To construct pure Fe(III)-cpRD for single-molecule AFM experiments, we first constructed Cys-cpRD-GB1-Cys chimera as a building block for the construction of polyprotein $(cpRD\text{-}GB1)_n$. We used an anion-exchange chromatography technique to separate the Zn(II) and Fe(III) forms of cpRD and obtained Fe(III)-cpRD-GB1. Then, purified Cys-Fe(III)-cpRD-GB1-Cys was used to react with $BM(PEO)_3$ (molar ratio 1:1) at room temperature for 2 h to produce polyprotein $(cpRD\text{-}GB1)_n$, which contains Fe(III)-cpRD only. The formation of the polyprotein was verified by SDS-PAGE (Figure 2A). In addition, the maleimide−thiol coupling reaction did not alter the characteristic UV−vis spectrum of Fe(III)-cpRD (Figure 4A), which originates from the absorbance by the $Fe-S_4$ center, suggesting that the polymerization process does not affect the $Fe-S_4$ center, and $BM(PEO)_3$ reacted specifically with the cysteine residues at the N and C termini and the Fe(III)-coordinating cysteines did not react with maleimide groups.

Thus, we can now use single-molecule AFM to investigate the mechanical unfolding process of Fe(III)-cpRD in a well-controlled manner using polyprotein $(cpRD\text{-}GB1)_n$, where GB1 domains serve as single molecular fingerprints for identifying single-molecule stretching events as well as an internal force caliber.[38] Stretching polyprotein $(Fe(III)\text{-}cpRD\text{-}GB1)_n$ results in characteristic sawtooth-like force−extension curves, where the sawtooth peaks correspond to the mechanical unfolding of GB1 and Fe(III)-cpRD domains (Figure 4C, curves 1 and 2). Unfolding events of GB1 are characterized by $\Delta L_c \approx 18$ nm (in black), and unfolding events of $\Delta L_c \approx 13$ nm are due to the mechanical unfolding of Fe(III)-cpRD domains (in red), providing the characteristic signatures of the unfolding of cp-rubredoxin.

DISCUSSION

In this letter, we have demonstrated a maleimide−thiol coupling-based facile method of constructing polyproteins for single-molecule AFM studies. This new method makes it possible to construct polyproteins in a much faster fashion compared with the traditional recombinant DNA technology-based method. This method allows for the construction of polyproteins made of pure forms of proteins to investigate the mechanical properties of each individual protein form in an unambiguous way. In addition, it is possible to use this method, which is similar to the disulfide bond-based polymerization method, to construct polyproteins to investigate the anisotropy of the mechanical stability of proteins. Compared with the traditional recombinant DNA technology-based method, the new method does not have precise control of the molecular weight of the polyprotein or the orientation of each individual domain in the polyprotein chain. In the polyprotein constructed using maleimide−thiol coupling method, the molecular weight of the polyprotein is polydisperse and the orientation of the individual protein domains are linked in either a head-to-tail or head-to-head (tail-to-tail) fashion, as compared to the monodisperse molecular weight and uniform head-to-tail orientation (N to C) in polyproteins constructed using the recombinant method. However, these drawbacks do not affect the measurement of the mechanical properties of the protein of interest.[20]

Although the method reported here is similar to the disulfide bond-based polymerization method in many ways, it does offer some unique features. First, the maleimide−thiol coupling chemistry is much more efficient. In 2 h at room temperature, high-molecular-weight multimeric proteins can readily form. In contrast, disulfide bond formation is much slower and the formation of polyproteins required a longer period of incubation.[20] Second, the PEO-linker sequences between the two maleimide groups provide a spacer between the two domains and may help to prevent the unfavorable domain−domain interactions in the resultant polyprotein. Third and probably the most important, the resultant thioether bond is no longer sensitive to the redox conditions (as evidenced by the fact that the polyprotein $(GB1)_n$ constructed using the maleimide−thiol coupling reaction remains intact in the reducing SDS-PAGE in Figure. 2A), making it possible to investigate the mechanical properties of the polyproteins under reducing as well as oxidizing conditions.[39,40] Figure 4B shows one such experiment in which the mechanical unfolding of Fe(II)-cpRD was investigated using single-molecule AFM. Under reducing conditions, Fe(III)-cpRD was reduced to Fe(II)-cpRD. Stretching $(cpRD\text{-}GB1)_n$ resulted in similar sawtooth-like force−extension curves with $\Delta L_c \approx 18$ and 13 nm, indicating the survival of polyprotein under reducing conditions (Figure 4B, curves 3 and 4). It is evident that the unfolding of Fe(II)-cpRD occurs at a much lower force as compared to that of Fe(III)-cpRD. Thus, the ferrous−thiolate bond is successfully measured and clearly shows that the polyprotein is robust for studies in a reducing environment.

CONCLUSIONS

We reported a novel, facile chemical coupling strategy to prepare polyproteins for single-molecule force spectroscopy studies. This maleimide−thiol coupling-based strategy offers a new alternative method that not only complements the existing strategies but also offers some unique advantages that will facilitate some special single-molecule AFM experiments that are otherwise difficult to study using current polyprotein construction strategies. We anticipate that the continuous development of a polyprotein engineering approach will greatly facilitate protein mechanics studies and help to develop the single-molecule AFM technique into a general biophysical tool for protein characterization.

AUTHOR INFORMATION

Corresponding Author

*E-mail: hongbin@chem.ubc.ca.

Present Addresses

[†]Department of Physics, Nanjing University, Nanjing, Jiangsu Province, PR China.

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