Video Article

OaAEP1-Mediated Enzymatic Synthesis and Immobilization of Polymerized Protein for Single-Molecule Force Spectroscopy

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Abstract

Chemical and bio-conjugation techniques have been developed rapidly in recent years and allow the building of protein polymers. However, a controlled protein polymerization process is always a challenge. Here, we have developed an enzymatic methodology for constructing polymerized protein step by step in a rationally-controlled sequence. In this method, the C-terminus of a protein monomer is NGL for protein conjugation using OaAEP1 (*Oldenlandia affinis asparaginyl endopeptidases*) 1) while the N-terminus was a cleavable TEV (tobacco etch virus) cleavage site plus an L (ENLYFQ/GL) for temporary N-terminal protecting. Consequently, OaAEP1 was able to add only one protein monomer at a time, and then the TEV protease cleaved the N-terminus between Q and G to expose the NH_2-Gly-Leu. Then the unit is ready for next OaAEP1 ligation. The engineered polyprotein is examined by unfolding individual protein domain using atomic force microscopy-based single-molecule force spectroscopy (AFM-SMFS). Therefore, this study provides a useful strategy for polyprotein engineering and immobilization.

Video Link

The video component of this article can be found at https://www.jove.com/video/60774/

Introduction

Compared with synthetic polymers, natural multi-domain proteins have a uniform structure with a well-controlled number and type of subdomains[1]. This feature usually leads to improved biological function and stability[2,3]. Many approaches, such as cysteine-based disulfide bond coupling and recombinant DNA technology, have been developed for building such a polymerized protein with multiple domains[4,5,6,7]. However, the former method always results in a random and uncontrolled sequence, and the latter one leads to other problems, including the difficulty for the overexpression of toxic and large-size proteins and the purification of complex protein with cofactor and other delicate enzymes.

To meet this challenge, we develop an enzymatic method that conjugates protein monomer together for polymer/polyprotein in a stepwise fashion using a protein ligase OaAEP1 combined with a protease TEV[8,9]. OaAEP1 is a strict and efficient endopeptidase. Two proteins can be linked covalently as Asn-Gly-Leu sequence (NGL) through two termini by OaAEP1 in less than 30 min if the N-terminus is Gly-Leu residues(GL) and the other of which the C-terminus is NGL residues[10]. However, the use of OaAEP1 only to link protein monomer leads to a protein polymer with an uncontrolled sequence like the cysteine-based coupling method. Therefore, we design the N-terminus of the protein unit with a removable TEV protease site plus a leucine residue as ENLYFQ/G-L-POI. Before the TEV cleavage, the N-terminal would not participate in OaAEP1 ligation. And then the GL residues at N-terminus, which are compatible with further OaAEP1 ligation, is exposed after the TEV cleavage. Thus, we have achieved a sequential enzymatic biosynthesis method of polyprotein with a relatively well-controlled sequence.

Here, our stepwise enzymatic synthesis method can be used in polyprotein sample preparation, including sequence-controlled and uncontrolled, and protein immobilization for single-molecule studies as well, especially for the complex system such as metalloprotein.

Moreover, AFM-based SMFS experiments allow us to confirm the protein polymer construction and stability at the single-molecule level. Single-molecule force spectroscopy, including AFM, optical tweezer and magnetic tweezer, is a general tool in nanotechnology to manipulate biomolecule mechanically and measure their stability[11,12,13,14,15,16,17,18,19,20]. Single-molecule AFM has been widely used in the study of protein (un)folding[21,22,23,24,25], the strength measurement of receptor-ligand interaction[26,27,28,29,30,31,32,33,34,35], inorganic chemical bond[20,36,37,38,39,40,41,42,43] and metal-ligand bond in metalloprotein[44,45,46,47,48,49,50]. Here, single-molecule AFM is used to verify the synthesized polyprotein sequence based on the corresponding protein unfolding signal.

Protocol

1. Protein production

1. **Gene clone**
   1. Purchase genes coding for the protein of interest (POI): Ubiquitin, Rubredoxin (RD)[51], the cellulose-binding module (CBM), dockerin-X domain (XDoc) and cohesion from *Ruminococcus flavefacience*, tobacco etch virus (TEV) protease, elastin-like polypeptides (ELPs).
   2. Perform polymerase chain reaction and use three-restriction digestion enzyme system *BamH*I-*Bgl*II-*Kpn*I for recombining the gene from different protein fragments.
   3. Confirm all genes by direct DNA sequencing.

2. **Proteins expression and production**
   1. Transform *E. coli* BL21(DE3) with the pQE80L-POI or pET28a-POI plasmid for expression.
   2. Pick one single colony into 15 mL of LB medium with respective antibiotics (e.g., 100 µg/mL ampicillin sodium salt or 50 µg/mL, kanamycin). Keep shaking the cultures at 200 rpm at 37 °C for 16-20 h.
   3. Dilute the overnight cultures into 800 mL of LB medium (1:50 dilution). For rubredoxin, centrifuge the culture at 1,800 x *g*, then resuspend in 15 mL of M9 medium (supplemented with 0.4% glucose, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$), and then dilute it into 800 mL of M9 medium.
   4. Incubate the culture at 37 °C while shaking at 200 rpm, until the culture reaches an optical density at 600 nm (OD_{600}) of 0.6. Save 100 µL sample of the culture as the pre-induction control for testing protein expression.
   5. Induce protein expression by adding IPTG to a final concentration of 1 mM and shake the culture at 37 °C for 4 h at 200 rpm. Reserve a 100 µL sample of the culture as the post-induction control for testing protein expression.
   6. Centrifuge the culture at 13,000 x *g* for 25 min at 4 °C and store at -80 °C before purification.
      NOTE: The protocol can be paused here.

3. **Purification of protein of interest**
   1. Resuspend the cells in 25 mL of lysis buffer (50 mM Tris, 150 mM NaCl, pH 7.4 containing DNase, RNase, PMSF) and use a sonicator (15% amplitude) to lyse it for 30 min on ice.
   2. Clarify the cell lysate at 19,000 x *g* for 40 min at 4 °C.
   3. Pack 1 mL (bed volume) of Co-NTA or Ni-NTA affinity column and wash the column with 10 column volumes (CV) of ultrapure water and then 10 CVs of wash buffer (50 mM Tris, 150 mM NaCl, 2 mM imidazole, pH 7.4) by gravity flow.
   4. Pass the protein supernatant through the column by gravity flow for three times.
   5. Pour wash buffer on the column with 50 CVs to move away contaminant proteins.
   6. Elute the bound protein with 3 CVs of ice-cold elution buffer (20 mM Tris, 400 mM NaCl, 250 mM imidazole, pH 7.4). When it comes to rubredoxin proteins, further anion exchange purification using anion exchange column at pH 8.5 at 4 °C is necessary.
   7. Analyze the sample by SDS-PAGE.

2. Functionalization of coverslip and cantilever surface

1. **Functionalized coverslip surface preparation**
   1. Dissolve 20 g of potassium chromate in 40 mL of ultrapure water. Slowly add 360 mL of concentrated sulfuric acid to the potassium chromate solution with glass rod stir gently and to prepare the chromic acid.
      CAUTION: The chemical used here and the final chromic acid is strongly corrosive and acidic. Work with proper protective equipment. The solution releases heat when add concentrated sulfuric acid, which means slow adding and proper pause for cooling down.
   2. Clean and activate a glass coverslip at 80 °C for 30 min by chromic acid treatment. Completely immerse the coverslips in 1% (v/v) APTES toluene solution for 1 h at room temperature while protecting them from light.
   3. Wash the coverslip with toluene and absolute ethyl alcohol and dry the coverslip with a stream of nitrogen.
   4. Incubate the coverslip at 80 °C for 15 min and then cool down to room temperature.
   5. Add 200 µL of sulfo-SMCC (1 mg/mL) in dimethyl sulfoxide (DMSO) solution between two immobilized coverslips and incubate for 1 h protected from light.
   6. Wash the coverslip with DMSO first and then with absolute ethyl alcohol to remove residual sulfo-SMCC.
   7. Dry the coverslip under a stream of nitrogen.
   8. Pipet 60 µL of 200 µM GL-ELP_{50nm}-C protein solution onto a functionalized coverslip and incubate for about 3 h.
   9. Wash the coverslip with ultrapure water to remove the unreacted GL-ELP_{50nm}-C.
      NOTE: Functionalized coverslips are capable for about two weeks under storage at 4 °C.

2. **Functionalized cantilever surface preparation**
   1. Clean the cantilevers at 80 °C for 10 min by chromic acid treatment.
   2. Functionalize the cantilever by amino-silanization with 1% (v/v) APTES toluene solution and then bake the cantilever at 80 °C for 15 min before conjugating to sulfo-SMCC.
   3. Link the C-ELP_{50nm}-NGL to the surface with the maleimide group of sulfo-SMCC for 1.5 h.
   4. Wash away the unreacted C-ELP_{50nm}-NGL on the coverslip by ultrapure water.
   5. Immerse a functionalized cantilever in 200 µL of 50 µM GL-CBM-XDoc protein solution containing 200 nM OaAEP1 at 25 °C for 20-30 min. Then use AFM buffer (100 mM Tris, 100 mM NaCl, pH 7.4) to wash away unreacted protein.

NOTE: The surface chemistry of the cantilevers and the coverslip are similar.

3. Stepwise polyprotein preparation with controlled sequences

1. Link the ligation unit Coh-tev-L-POI-NGL to the GL-ELP_{50nm} immobilized on the coverslip surface by OaAEP1 for 30 min.
2. Use 15-20 mL of AFM buffer (100 mM Tris, 100 mM NaCl, pH 7.4) to wash away any unreacted proteins.
3. Add 100 µL of TEV protease (0.5 mM EDTA, 75 mM NaCl, 25 mM Tris-HCl 10% [v/v] glycerol, pH 8.0) to cleave the protein unit at the TEV recognize site for 1 h at 25 °C.
4. Use 15-20 mL of AFM buffer to wash away residual proteins.
5. Link the ligation unit Coh-tev-L-POI-NGL to the GL-Ub-NGL-Glass by OaAEP1 for 30 min.
6. Repeat steps 3.3 to 3.5 N-1 times to build protein construct GL-$(Ub)_n$-NGL on the glass surface. Omit the last TEV cleavage reaction to reserve cohesin on the protein-polymer as Coh-tev-L-$(Ub)_n$-NGL-Glass.

4. AFM Experiment measurement and data analysis

1. **AFM measurements**
   1. Add 1 mL of AFM buffer to the chamber with 10 mM $CaCl_2$ and 5 mM Ascorbic Acid.
   2. Choose the D tip of the functionalized AFM probe for the experiment. Use the equipartition theorem to calibrate the cantilever in AFM buffer with an accurate spring constant (*k*) value before each experiment.
   3. Attach the cantilever tip to the sample surface to form the Cohesin/Dockerin pair.
   4. Retract the cantilever at a constant velocity of 400 $nm \cdot s^{-1}$ from the surface. In the meantime, record the force-extension curve at a sample rate of 4000 Hz.

2. **Data analysis**
   1. Use JPK data processing select force-extension traces.
   2. Use software to analyze the traces. Fit the curves with the worm-like-chain (WLC) model of polymer elasticity and obtain unfolding force and contour length increment for individual protein unfolding peak.
   3. Fit the histograms of unfolding forces with the Gaussian model to obtain the most probable values of unfolding force ($<F_u>$) and contour length increment ($<\Delta L_c>$).

Representative Results

The NGL residues introduced between adjacent proteins by OaAEP1 ligation will not affect protein monomer stability in the polymer as the unfolding force ($<F_u>$), and contour length increment ($<\Delta L_c>$) is comparable with the previous study (**Figure 1**). The purification result of the rubredoxin protein is shown in **Figure 2**. To prove the protein after TEV cleavage is compatible with the following OaAEP1 ligation to construct protein polymer with a control sequence and the construction is high-efficiency, **Figure 3** provides an SDS-PAGE image as a reference. The steps of the functionalized cantilever and coverslip preparation are described in **Figure 4**. The stepwise enzymatic biosynthesis and immobilization of polyprotein on the coverslip are shown in **Figure 5**. Use this protocol, a protein polymer with the controlled sequence can be built and suitable for AFM-based SMFS experiments.

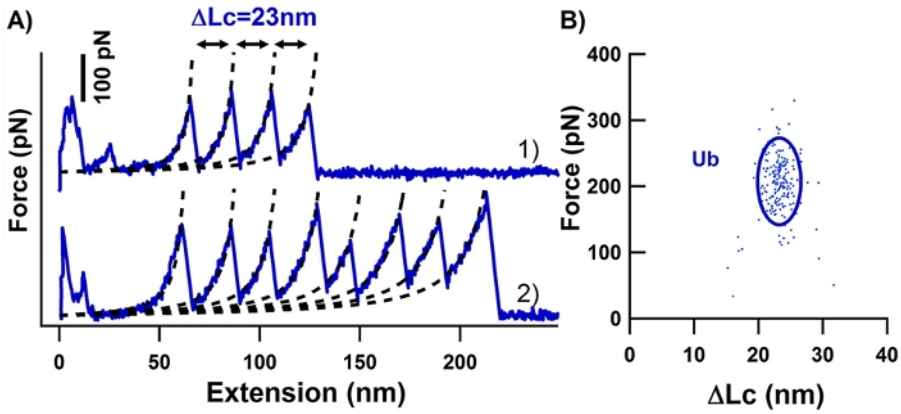


Figure 1: AFM-based SMFS measurements of polyprotein built by OaAEP1. (**A**) Typical sawtooth-like force-extension curves of Ub (curve 1 in blue) were shown with expected ΔLc of ~23 nm. (**B**) The scatter plot presents the relationship between Ub unfolding force (202 ± 44 pN, average ± s.d., n = 198) and ΔLc (23 ± 2 nm, average ± s.d.). This figure has been modified from Ref.8. Please click here to view a larger version of this figure.

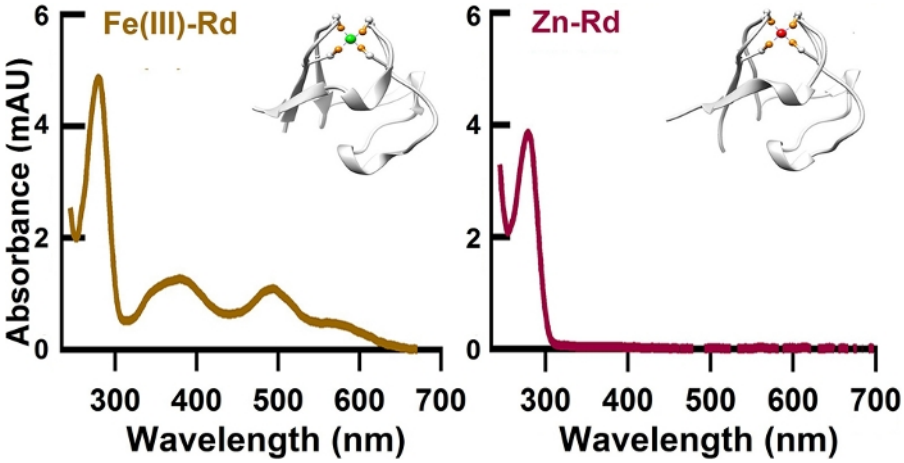


Figure 2: The UV-Vis absorbance spectra of GL-GB1-Fe(III)-Rd-NGL and GL-GB1-(Zn)-Rd-NGL. The Fe(III)-form Rd (Left spectrum, colored in brown, PDB code:1BRF) presented typical UV-Vis absorption peaks at 495 nm and 579 nm while the Zn-form did not (Right spectrum, colored in wine, PDB code: 1IRN). This figure has been modified from Ref.8. Please click here to view a larger version of this figure.

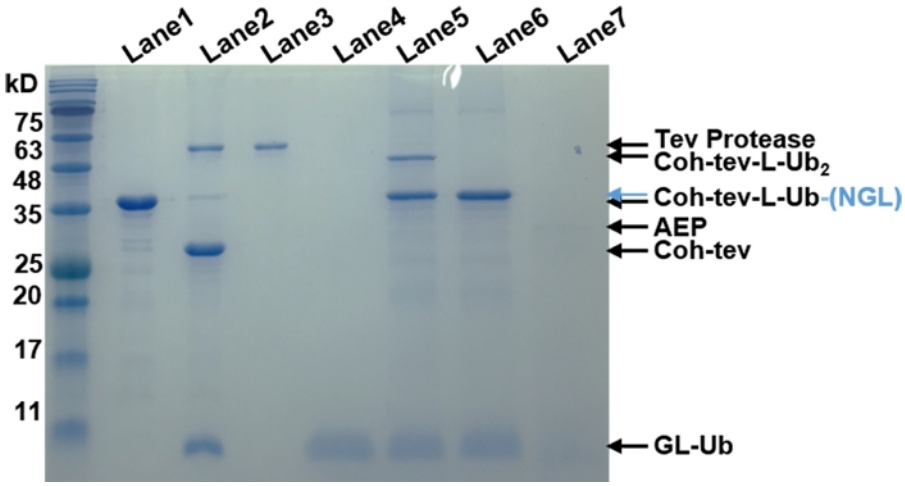


Figure 3: SDS-PAGE gel results of stepwise digestion and ligation using TEV protease and OaAEP1 to build the Ub dimer. Lanes 1–4 showed Coh-tev-L-Ub, the result protein mixture of TEV cleavage, pure *sf*GFP-TEV protease and purified product (GL-Ub). Lanes 5–7 showed the cleaved GL-Ub and Coh-tev-L-Ub-NGL ligation mixture with (Lane 5) or without (Lane 6) OaAEP1 and pure OaAEP1. This figure has been modified from Ref.8. Please click here to view a larger version of this figure.

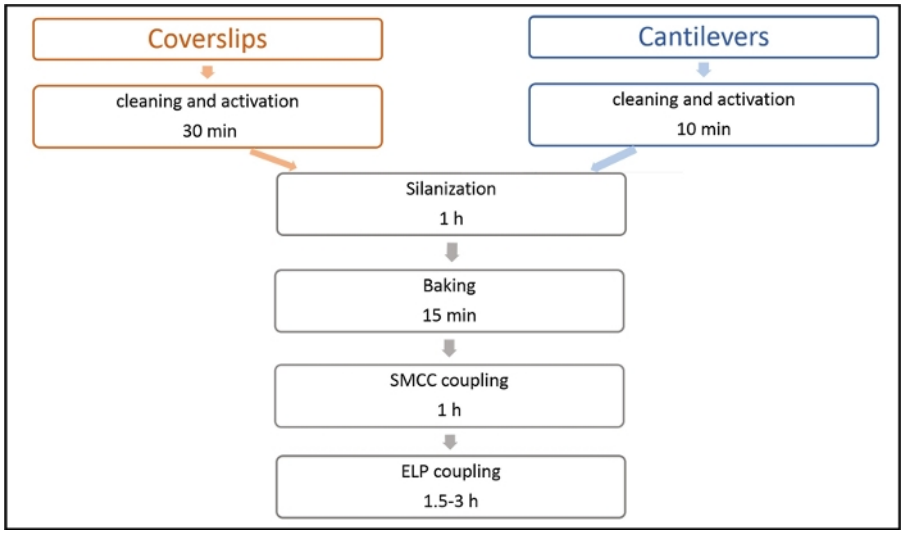


Figure 4: Process chart describing each step for functionalizing glass coverslips and cantilever. After cleaning and activation by chromic acid, coverslip and cantilever share similar functionalization process, except the last step in which GL-ELP$_{50nm}$-C couples with coverslip while C-ELP$_{50nm}$-NGL couples with cantilever. Please click here to view a larger version of this figure.

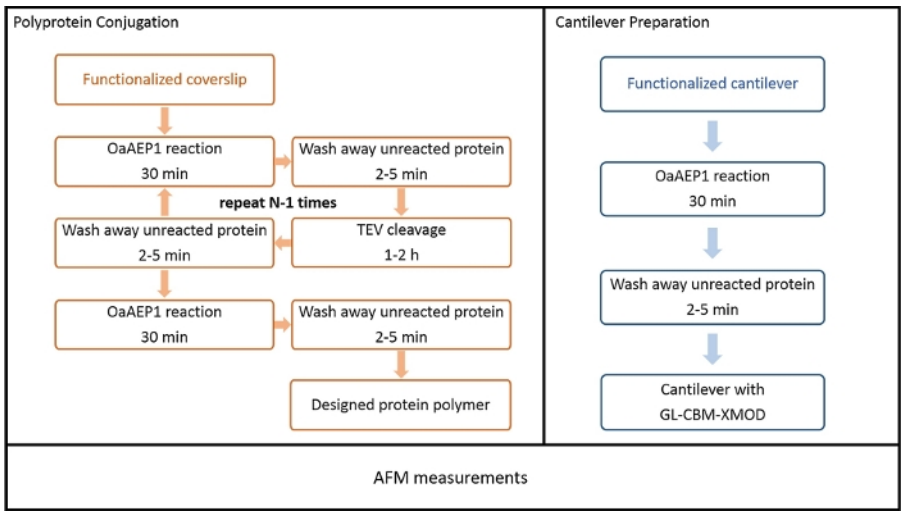


Figure 5: Process chart describing each step for polyprotein immobilization on the surface. Top left process flow diagram shows the stepwise building of polyprotein with controlled sequences on the coverslip. Top right diagram shows the preparation of the functionalized cantilever used in the AFM measurements. Please click here to view a larger version of this figure.

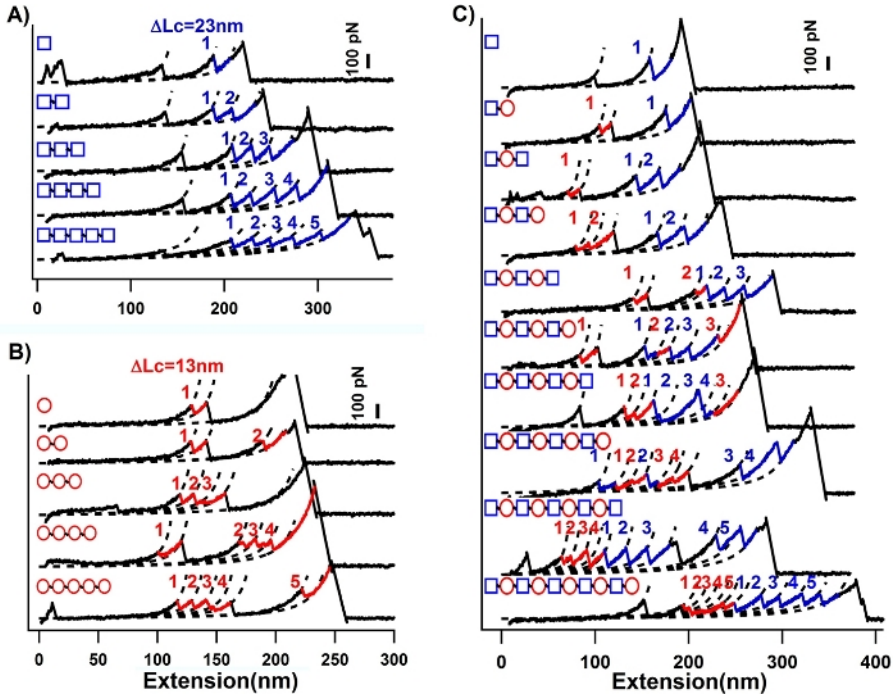
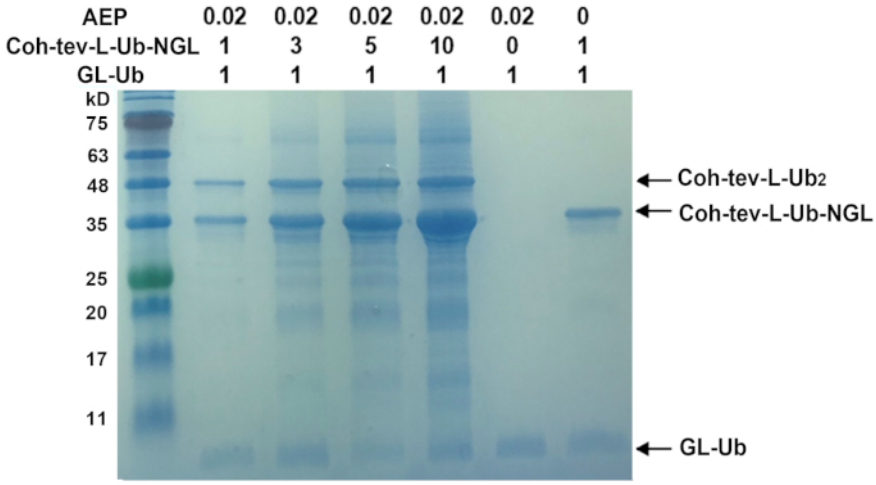


Figure 6: Typical unfolding traces of the protein polymers with a rationally controlled sequence by AFM-based SMFS. (**A**) Typical sawtooth-like force-extension curves of Ub presented ΔL_c of ~23 nm as expected. (**B**) Typical force-extension curves of Rd presented ΔL_c of ~13 nm as expected. (**C**) Typical force-extension curves of $(Ub\text{-}Rd)_n$ protein mixture in which the blue peak means the unfolding events of Ub while the red means Rd. This figure has been modified from Ref.8. Please click here to view a larger version of this figure.



Supplementary Figure 1: SDS-PAGE gel results of the protein ligation efficiency under different ratio between two reactants. The ligation efficiency was 20% when the ratio is 1 to 1 and reached 75% at the ratio of 10 to 1. This figure has been modified from Ref.8. Please click here to view a larger version of this figure.

Discussion

We have described a protocol for enzymatic biosynthesis and immobilization of polyprotein and verified the polyprotein design by AFM-based SMFS. This methodology provides a novel approach to building the protein-polymer in a designed sequence, which complements previous methods for polyprotein engineering and immobilization[4,6,52,53,54,55,56,57,58,59,60,61].

Compared with the classic recombinant DNA methodology for polyprotein construction[7,62], our method bases on the ligation between small protein monomers. Thus, it allows the expression of large-sized or toxic protein molecules for polyprotein construction. Additionally, it allows the purification of the protein monomer before conjugation.

Compared with the widely-used bi-cysteine method forming intermolecular disulfide bond for protein polymerization[4], our enzymatic method using both OaAEP1 and TEV protease results in a polyprotein with a relatively controlled sequence and defined connection geometry. And it does not use cysteine, which is an essential functional residue for many proteins.

Our method is mostly similar to sortase-based protein conjugation[59]. The unique feature of our method is that the OaAEP1-based protein ligation is much more efficient, thus allows the construction of protein pentamer with a reasonable yield[10,53]. It also needs fewer residues for ligation and results in a shorter three-residues NGL linker. As a result, it shows no "linker effect" as the newly formed NGL linker does not affect the stability of individual protein monomer or induce any unnatural protein-protein interaction. Nevertheless, we believe that all methods have their own advantages and disadvantages. For example, the classic recombinant DNA method does not add any residue between protein monomer and not require the use of any enzyme for ligation. And the bi-cysteine method is simple and easy for protein polymerization. Thus, they can all be useful under different experimental requirements.

For our stepwise construction of polyprotein, it is crucial to remove the unreacted protein completely. Take enough volume of AFM buffer and enough time to clean the reacted surface carefully. Otherwise, the residual protein or protease will affect further synthesis reactions.

The efficiency of OaAEP1 ligation is a critical limit to our method as the TEV cleavage efficiency is almost complete (96%). It is critical to raise the ratio between the two reactants, GL-protein, and protein-NGL, to improve the ligation efficiency. Our study shows that when protein-NGL is tenfold to GL-protein, the efficiency increases from 20% (the ratio is 1 to 1) to 75% (**Supplementary Figure 1**). It is critical to consume the reactant, which was immobilized onto the surface as the free reactant can be moved away by washing with buffer. Additionally, whether the N- or C- terminus is exposed to the solution is also a crucial factor to ligation. It is an optional approach to expose the terminal by adding a linker containing the recognized site to the respective terminal.

In the end, our protocol is an enzymatic way to conjugate proteins in a designed sequence. It also provides an alternative approach to couple and immobilize protein samples in single-molecule studies.

Disclosures

The authors have nothing to disclose.

Acknowledgments

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