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Corresponding Author:	Peng Zheng Nanjing University NANJING, Jiangsu CHINA
Corresponding Author's Institution:	Nanjing University
Corresponding Author E-Mail:	PENGZ@NJU.EDU.CN
Order of Authors:	Yibing Deng
	Bin Zheng
	Yutong Liu
	Shengchao Shi
	Jingyuan Nie
	Tao Wu
	Peng Zheng
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TITLE:

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

AUTHORS AND AFFILIATIONS:

Yibing Deng[1], Bin Zheng[1], Yutong Liu[1], Shengchao Shi[1], Jingyuan Nie[1], Tao Wu[1] and Peng Zheng[1]

[1]State Key Laboratory of Coordination Chemistry, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing, China

KEYWORDS:

single-molecule force spectroscopy, bio-conjugation, protein immobilization, atomic force spectroscopy, OaAEP1, protein engineering

SUMMARY:

Here, we present a protocol to conjugate protein monomer by enzymes forming protein polymer with a controlled sequence and immobilize it on the surface for single-molecule force spectroscopy studies.

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.

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[4-7]. 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[11-20]. Single-molecule AFM has been widely used in the study of protein (un)folding[21-25], the strength measurement of receptor-ligand interaction[26-35], inorganic chemical bond[20,36-43] and metal-ligand bond in metalloprotein[44-50]. Here, single-molecule AFM is used to verify the synthesized polyprotein sequence based on the corresponding protein unfolding signal.

PROTOCOL:

1. Protein production

1.1. Gene clone

1.1.1. 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).

1.1.2. 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.

1.1.3. Confirm all genes by direct DNA sequencing.

1.2. Proteins expression and production

1.2.1. Transform *E. coli* BL21(DE3) with the pQE80L-POI or pET28a-POI plasmid for expression.

1.2.2. 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.

1.2.3. 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.

1.2.4. 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.

1.2.5. 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.

1.2.6. 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.

1.3. **Purification of protein of interest**

1.3.1. 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.

1.3.2. Clarify the cell lysate at 19,000 x *g* for 40 min at 4 °C.

1.3.3. 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.

1.3.4. Pass the protein supernatant through the column by gravity flow for three times.

1.3.5. Pour wash buffer on the column with 50 CVs to move away contaminant proteins.

1.3.6. 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.

1.3.7. Analyze the sample by SDS-PAGE.

2. Functionalization of coverslip and cantilever surface

2.1. **Functionalized coverslip surface preparation**

2.1.1. Dissolve 20 g of potassium dichromate in 40 mL of ultrapure water. Slowly add 360 mL of concentrated sulfuric acid to the potassium dichromate 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.

2.1.2. 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.

2.1.3. Wash the coverslip with toluene and absolute ethyl alcohol and dry the coverslip with a stream of nitrogen.

2.1.4. Incubate the coverslip at 80 °C for 15 min and then cool down to room temperature.

2.1.5. 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.

2.1.6. Wash the coverslip with DMSO first and then with absolute ethyl alcohol to remove residual sulfo-SMCC.

2.1.7. Dry the coverslip under a stream of nitrogen.

2.1.8. Pipet 60 µL of 200 µM GL-ELP$_{50nm}$-C protein solution onto a functionalized coverslip and incubate for about 3 h.

2.1.9. 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.

2.2. **Functionalized cantilever surface preparation**

2.2.1. Clean the cantilevers at 80 °C for 10 min by chromic acid treatment.

2.2.2. 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.

2.2.3. Link the C-ELP$_{50nm}$-NGL to the surface with the maleimide group of sulfo-SMCC for 1.5 h.

2.2.4. Wash away the unreacted C-ELP$_{50nm}$-NGL on the coverslip by ultrapure water.

2.2.5. 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

3.1. Link the ligation unit Coh-tev-L-POI-NGL to the GL-ELP$_{50nm}$ immobilized on the coverslip surface by OaAEP1 for 30 min.

3.2. Use 15-20 mL of AFM buffer (100 mM Tris, 100 mM NaCl, pH 7.4) to wash away any unreacted proteins.

3.3. 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.

3.4. Use 15-20 mL of AFM buffer to wash away residual proteins.

3.5. Link the ligation unit Coh-tev-L-POI-NGL to the GL-Ub-NGL-Glass by OaAEP1 for 30 min.

3.6. 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

4.1. AFM measurements

4.1.1. Add 1 mL of AFM buffer to the chamber with 10 mM $CaCl_2$ and 5 mM Ascorbic Acid.

4.1.2. 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.

4.1.3. Attach the cantilever tip to the sample surface to form the Cohesin/Dockerin pair.

4.1.4. Retract the cantilever at a constant velocity of 400 nm·s^{-1} from the surface. In the meantime, record the force-extension curve at a sample rate of 4000 Hz.

4.2. Data analysis

4.2.1. Use JPK data processing select force-extension traces.

4.2.2. 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.

4.2.3. 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 AND TABLE LEGENDS:

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.

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.

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.

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.

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.

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.

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.

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[4,6,52-61].

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.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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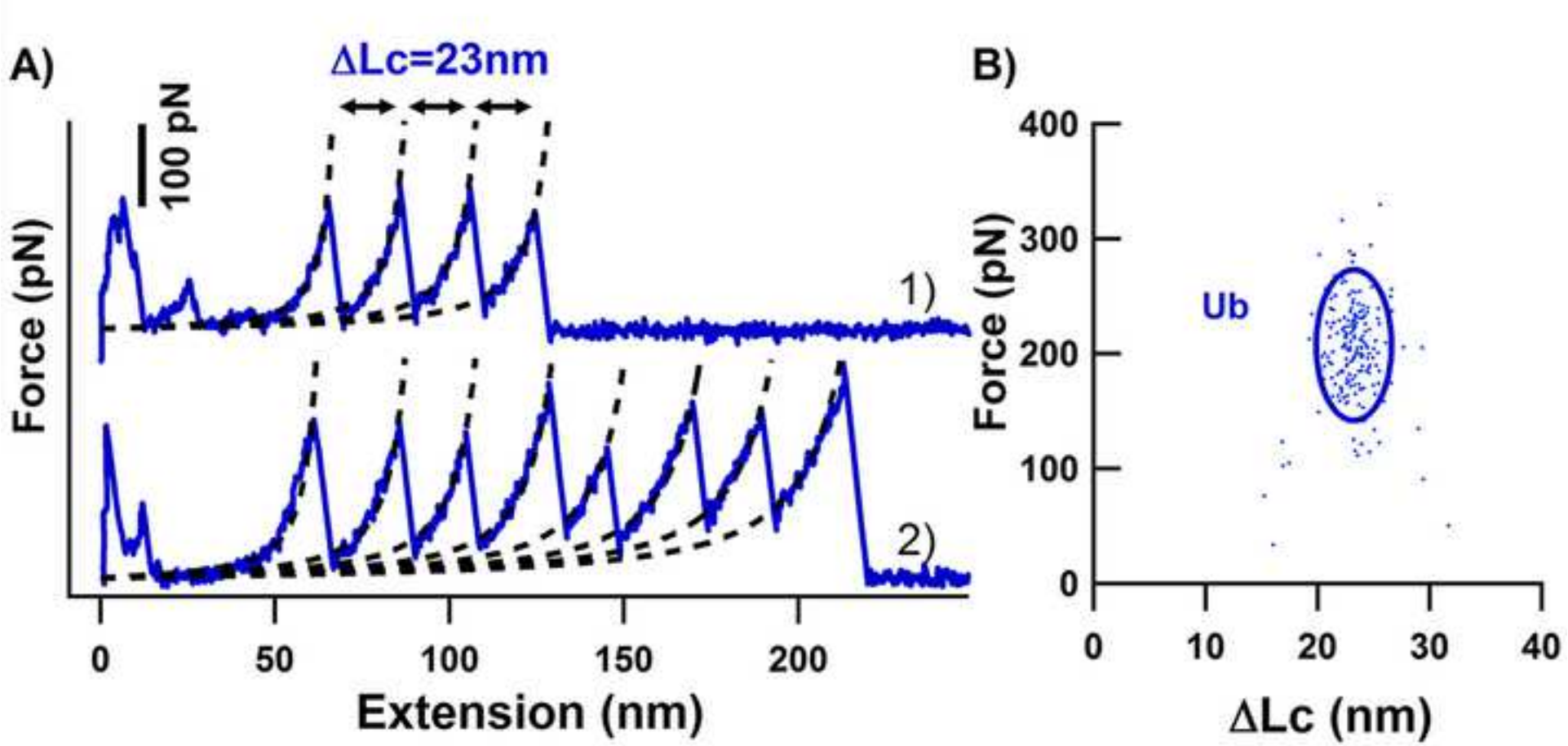

Figure 2

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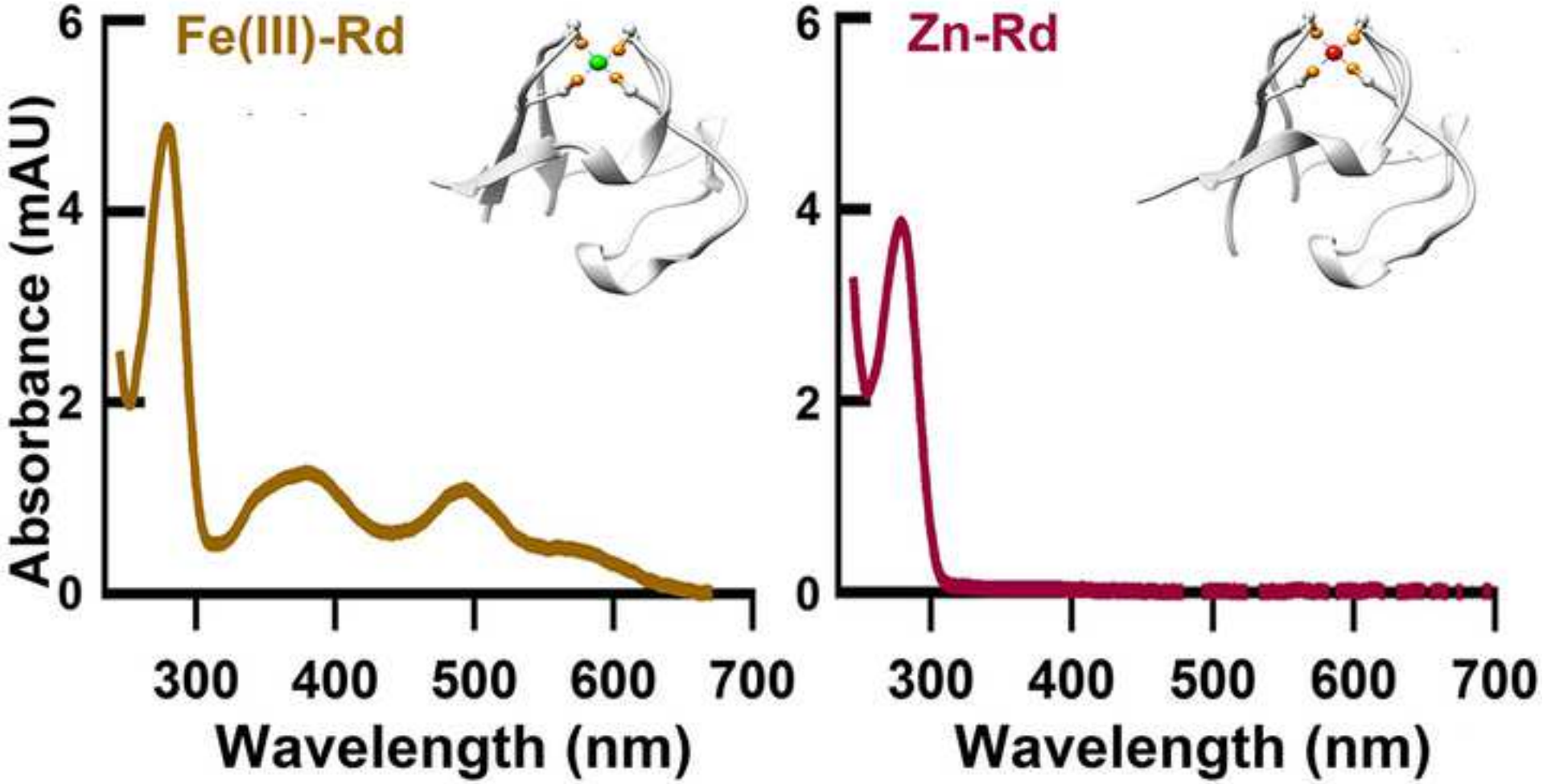


Figure 3

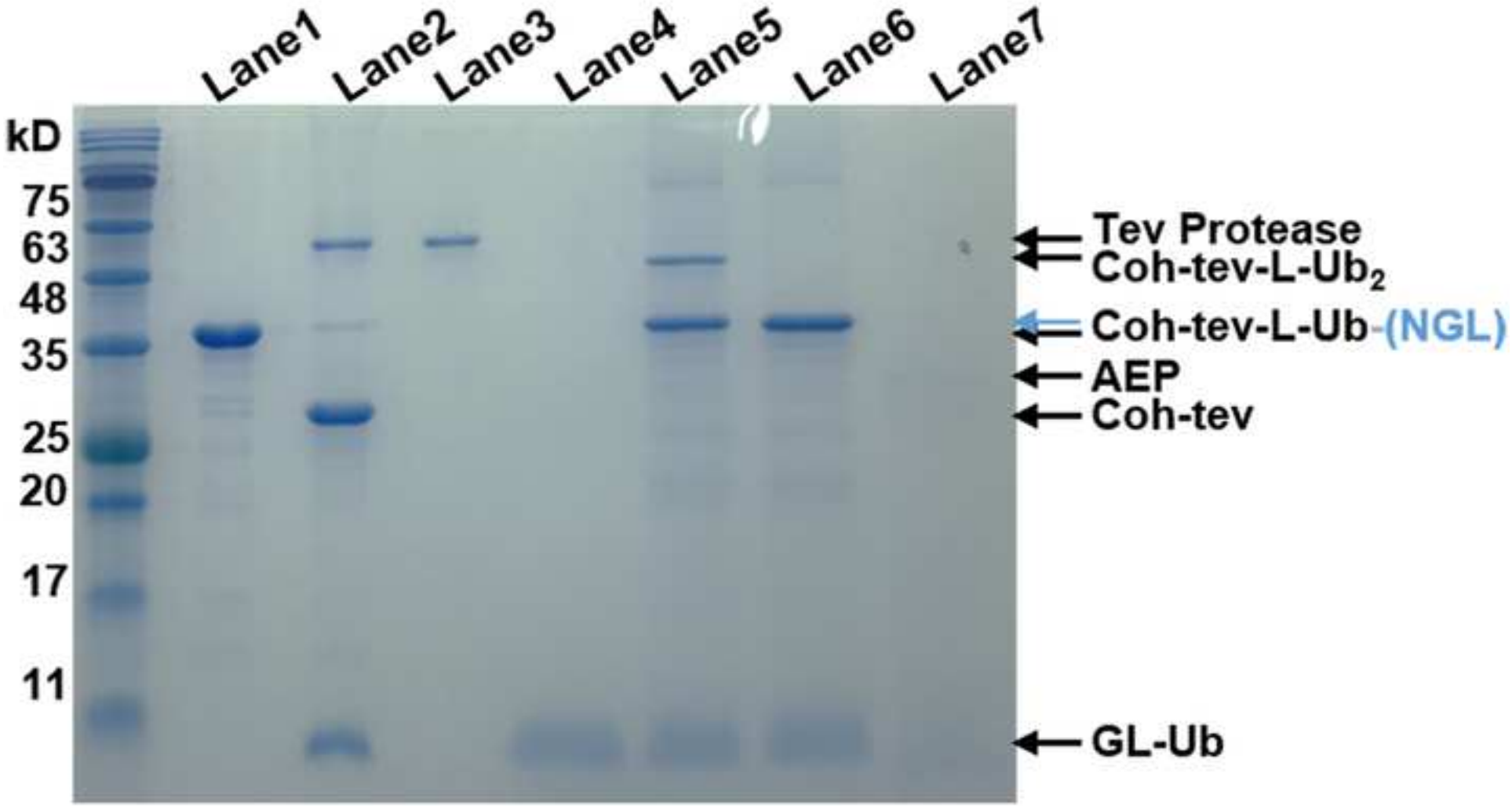
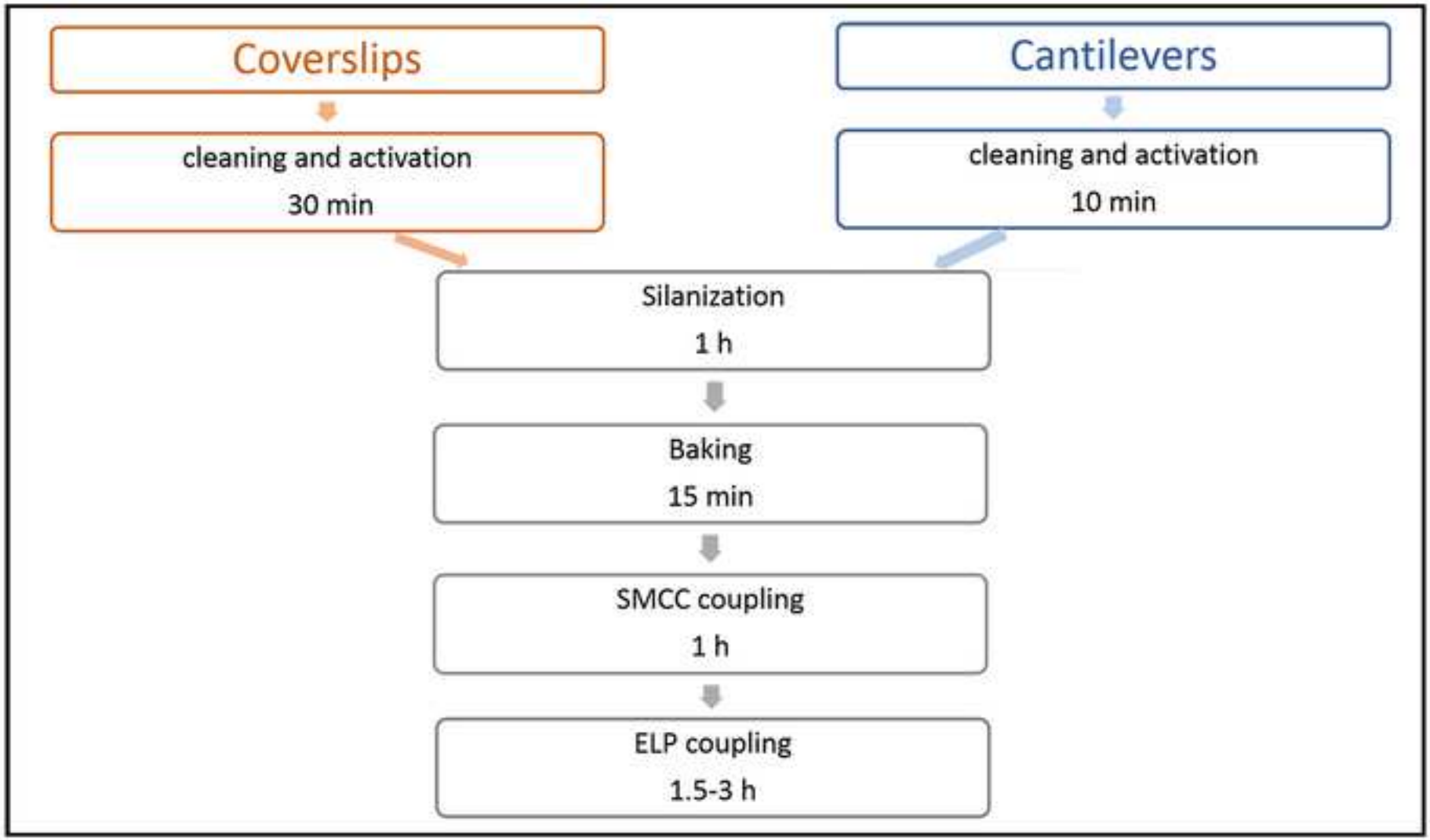


Figure 4



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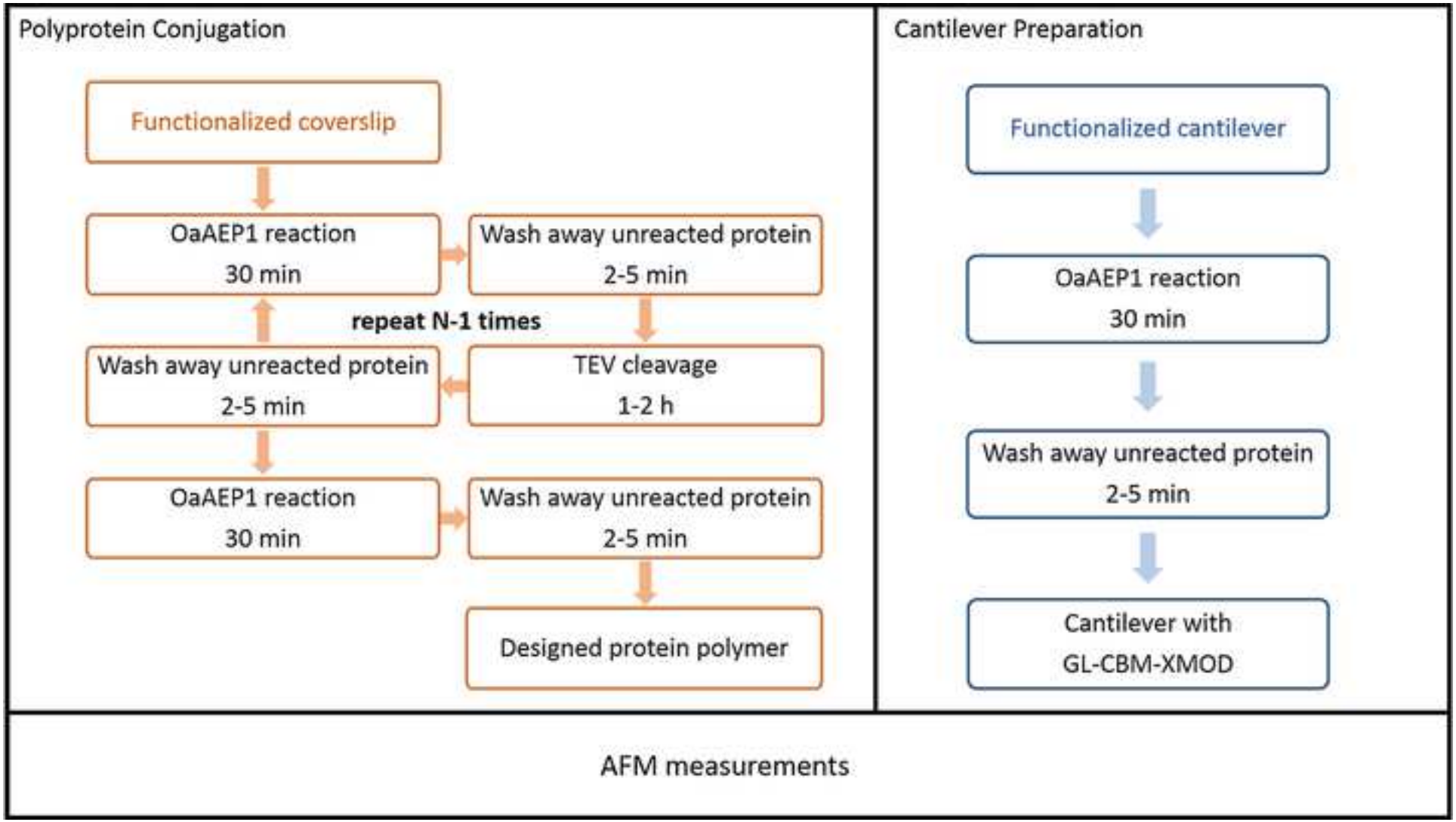
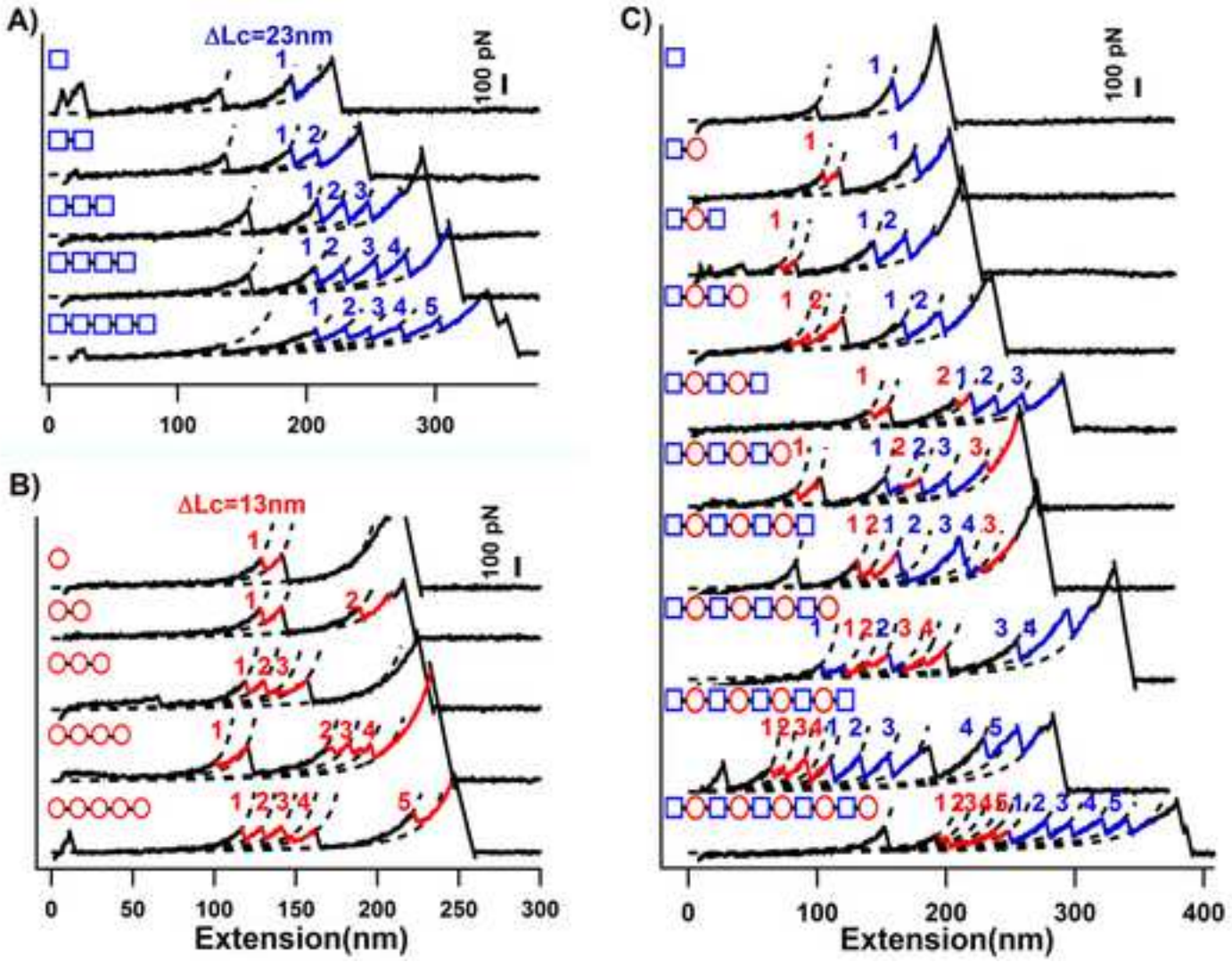


Figure 6



Name of Material

iron (III) chloride hexahydrate
Zinc chloride
calcium chloride hydrate
L-Ascorbic Acid
(3-Aminopropyl) triethoxysilane

sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
Glycerol
5,5'-dithiobis(2-nitrobenzoic acid)
Genes

Name of Equipment

Nanowizard 4 AFM
MLCT cantilever
Mono Q 5/50 GL
AKTA FPLC system
Glass coverslip
Nanodrop 2000
Avanti JXN-30 Centrifuge
Gel Image System

Company	Comments/Description
Energy chemical	99%
Alfa Aesar	100.00%
Alfa Aesar	99.9965% crystalline aggregate
Sigma Life Science	Bio Xtra, ⩾99.0%, crystalline
Sigma-Aldrich	⩾99%
Thermo Scientific	90%
Macklin	99%
Alfa Aesar	
Genscript	
JPK Germany	
Bruker Corp	
GE Healthcare	
GE Healthcare	
Sail Brand	
Thermo Scientific	
Beckman Coulter	
Tanon	

Dear Editor Dr. Bing Wu,

Many thanks for handling our manuscript (No. JoVE60774), previously entitled "Enzymatic synthesis and immobilization of polymerized protein verified at the single-molecule level". Please note we modify the title as "Enzymatic synthesis and immobilization of polymerized protein for single-molecule force spectroscopy".

A point-by-point response to the editorial and reviewers' comments is provided as follows: The requests are in italic.

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

Response: We have now double checked these issues.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response: We have added the citation in the figure legend. The original paper is published in Nat. Comm., which is an open access article allowing for unrestricted use. The link is:
https://s100.copyright.com/AppDispatchServlet?imprint=Nature&oa=CC%20BY&title=Enzymatic%20biosynthesis%20and%20immobilization%20of%20polyprotein%20verified%20at%20the%20single-molecule%20level&author=Yibing%20Deng%20et%20al&contentID=10.1038%2Fs41467-019-10696-x&publication=Nature%20Communications&publicationDate=2019-06-24&publisherName=SpringerNature&orderBeanReset=true

3. Please use 12 pt font and single-spaced text throughout the manuscript.
4. Please use h, min, s for time units.
5. Please add a Summary before Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"
6. Please add a one-line space between each of your protocol steps.
7. Step 1.1: Please write this step in the imperative tense.
8. 4.1.3: Please write this step in the imperative tense.
9. 4.2.3: Please write this step in the imperative tense.

Response: We have fixed all these issues now.

10. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Response: We have now added these discussions on page 7-8, Line 223-240.

11. Please avoid long steps (more than 4 lines).
12. Please highlight complete sentences (not parts of sentences) for filming.
13. Please do not abbreviate journal titles for references.
14. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please check the iThenticateReport attached to this email.

Response: We thanks for these comments and have fixed all the issues now.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This JoVE protocol reports the polyprotein assembly method based on protein ligation with OaAEP1 ligase and deprotection with TEV protease. The method was recently presented in a Nat. Comm. Paper [Ref 8 of current manuscript]. The method is interesting and innovative in the field of chemical biology, protein ligation and AFM SMFS. The introduction was well written and properly referenced as far as I can tell. I believe this is appropriate for JoVE with the following small corrections

Response: We appreciate the recognition of work by this reviewer. In this paper, we focus on reporting our new strategy using two enzymes for polyprotein preparation, which can be a sutiable sample for AFM-based SMFS studies. Thus, we cite most references from this perspective. We thank his/her support and understanding.

Minor Concerns:
Line 87: change to "Protein of interest" instead of "interest protein"
Response: We are sorry for this mistake and have corrected it now.

Line 90: Round the RCF value to a whole number. Ie., 19632xg implies a level of precision is required in this step when in fact it is not.
Response: We thank this comment, very educative.

Line 107: should be 'stream' not 'steam'
Response: We are sorry for this mistake and have corrected it now.

Line 120: it sounds like they are proposing baking the cantilevers at 80°C after

conjugation to SMCC. But I think the protocol they want to describe is baking the cantilevers at 80°C before conjugation to SMCC. Perhaps the authors can double check this detail.
Response: The reviewer is correct. We have modified our protocol to clarify this issue on Page 4, Line 119-121.

Line 159: English grammar needs correcting. It is not clear what they are trying to say about the 'linker effect'.
Response: We apology for the grammar issue, which impedes the understanding of our work. The “linker effect" means that the newly formed linker between the two protein monomers, for example the three-residues NGL linker in our work, affects the stability/structure of the protein monomer. This “linker effect” may lead to increased or decreased protein unfolding force or induce unnatural protein-protein interaction. Please see Page 8, Line 234-236.

Lines 162-163: English grammar needs correcting.
Response: We are sorry for this mistake and have corrected it now.

The image quality on the figures should be improved. They are not coming through very well in my PDF. Especially Figures 3, 4, and 5 have large black backgrounds that obscure the images. In fact, these images look corrupted and not correct in my PDF file.
Response: We apology for the figure issue and have updated new figures.

Reviewer #2:
Manuscript Summary:
This manuscript describes a method of synthesizing protein conjugates using a combination of enzymatic reactions

Major Concerns:
The manuscript has severe issues in hiding the literature and so the referencing.

Minor Concerns:
No minor comments

Response: In this work, we focus on reporting a new method using two enzymes for polyprotein preparation, which is especially suitable for AFM-based SMFS studies. Thus, we cite most references from this perspective. It is welcome if this reviewer could suggest some references from different perspective.

Reviewer #3:
Manuscript Summary:
The authors describe a useful protocol for enzymatically producing covalent

attachments between synthesized proteins. This protocol builds off their work that has been recently published in the last year so it is of current interest. Their protocol is easy to follow and should be able to reproduce in other labs.

Response: We appreciate the recognition of our work by this reviewer and his/her support.

Major Concerns:

- Authors briefly mention other methods for polyprotein conjugation. Can the authors specifically describe the advantages and disadvantages of each?

Response: Compared with the classic recombinant DNA methodology for polyprotein construction, 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, 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 important functional residue for many proteins.

Our method is mostly similar to sortase-based protein conjugation method. 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. It also needs fewer residues for ligation and results in a shorter three-residues NGL linker without the "linker effect".

We thank this reviewer's suggestion and have now added these discussions on Page 7-8, Line 223-240.

Minor Concerns:

- What is the composition of chromic acid in step 2.1.1?

Response: It contains potassium dichromate and sulfuric acid. We have added its description for preparation on page 4, Line 113-115.

- What is the composition of the "AFM buffer" in step 3.2?

Response: The AFM buffer contains 100 mM Tris and 100 mM NaCl under pH 7.4. We have now added its composition at step 3.2.

- Grammar is hard to understand, for example on line 159: "OaAEP ligation would not lead linker effect to protein monomer stability in the polymer" which I believe is meant to say that the ligated linker does not affect monomer stability.

Response: We apology for the grammar issue and appreciate this reviewer's advice. We have now corrected it accordingly, on page 6, Line 174-175, and Page 8, Line 234-236.

In summary, we thank all comments and suggestion from you and all three reviewers. We hope that the revised manuscript meets the quality requirements for publication in *Journal of Visualized Experiments*.

Sincerely yours,

Peng Zheng, Professor
School of Chemistry and Chemical Engineering, Nanjing University
No.163 Xianlin Ave., Nanjing, Jiangsu Province,
P.R. China 210023

Dear Editor Dr. Bing Wu,

Many thanks for handling our manuscript (No. JoVE60774) with comments. A point-by-point response to the editorial' comments is provided as follows: the requests are in italic.

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

Response: We tried our best to check the languages.

2.Please provide at least 6 keywords or phrases. There are only 5.

Response: We have added one more.

3.Figure 1: Please add a title for the figure in Figure Legend.

Response: We have added a tiltle for figure 1.

4. Figure 4: Please add a short description of the figure in Figure Legend.

Response: We have added a short description for figure 4.

5. Figure 5: Please add a short description of the figure in Figure Legend.

Response: We have added a short description for figure 5.

6. Figure S1: Please add a unit.

Response: We have added the unit (Dalton for molecular weight) for the ladder.

7. Step 1.1.1: Please write this step in the imperative tense.

Response: We changed it in the imperative tense as *"Purchase genes coding for the protein of interest (POI): Ubiquitin, Rubredoxin (RD), the cellulose-binding module (CBM), dockerin-X domain (XDoc) and cohesion from Ruminococcus flavefacience, tobacco etch virus (TEV) protease, elastin-like polypeptides (ELPs)."*

8. 1.2.3: Please ensure that all text is written in the imperative tense.

Response: We changed it in the imperative tense as *"Dilute the overnight cultures into 800 mL LB medium (1:50 dilution). For rubredoxin, centrifuge the culture (1800 x g) and then resuspend by 15 mL M9 medium (supplemented with 0.4% glucose, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$), and then dilute it into 800 mL M9 medium."*

9. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Examples of commercial language in your manuscript include Mili-Q, Mono Q, Genscript, etc.

Response: We changed these commercial products to generic terms.

10. Please avoid long steps (more than 4 lines).
Response: We have fixed this issue.

Lastly, we thank all help and comments from you. We hope that the revised manuscript meets the quality requirements for publication in *Journal of Visualized Experiments*.

Sincerely yours,

Peng Zheng, Professor
School of Chemistry and Chemical Engineering, Nanjing University
No.163 Xianlin Ave., Nanjing, Jiangsu Province,
P.R. China 210023

Figure S1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S1.psd