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Tailored-polyproteins using sequential staple and cut

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Abstract

Polyproteins, clusters of smaller proteins fused covalently in tandem, have been evolved as a promising tool for measuring the dynamic folding of bio-macromolecules in single-molecule force spectroscopy. However, the synthetic routes of preparing polyproteins have been a bottleneck, and urge for developing in-vitro methods to knit individual protein units covalently into polyprotein. Employing two enzymes of orthogonal functionalities periodically in sequence, we synthesized monodispersed polyproteins on a solid surface. We used Sortase A (SrtA), the enzyme known for sequence specific transpeptidation, to staple protein units covalently through peptide bonds. Exploiting the sequence-specific peptide cleaving ability of TEV protease, we controlled the progress of the reaction to one attachment at a time. Finally, with unique design of the unit proteins we control the orientation of proteins in polyprotein. This simple conjugation has the potentiality to staple proteins with different functionalities and from different expression systems, in any numbers, at any positions in the polyprotein and above all, via irreversible peptide bonds.

Introduction

Deciphering the mechanics of proteins has become an interesting area of research for their active involvements in biological and cellular processes ranging from mechanotransduction^{1,2}, replication³, transcription^{4,5}, translocation⁶, and degradation⁷. During these processes, biomolecules experience mechanical tension, and many-a-times undergo through mechanical unfolding⁸. Single-molecule force spectroscopy (SMFS) has aptly been evolved to directly map the dynamic response of these molecules under tension, and provided insights to design mechano-responsive polymers^{9,10}. However, one of the major hurdles in SMFS measurements is to unambiguously distinguish the specific stretching events of the molecule of interest over non-specific contributions from various impurities and non-single molecule events.

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Author Contributions

S.R. has supervised the project. S.G., G.S.S. and S.Y. did the cloning, expression, and purification. S.G. and G.S.S. performed all the experiments. S.R., S.G. and G.S.S. analyzed all the data. S.G. and G.S. made the figures. S.R. and S.G. wrote the manuscript. S.G., G.S.S., and S.R. edited the manuscript.

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Synthetic polyproteins, the tandem repeats of the protein of interest (POI) has successfully overcome the issue with non-specificity, and become a standard protocol in SMFS^{11,12}. The typical saw-tooth pattern in the force-distance curves has been the fingerprinting for the specific force-stretching events. Therefore, an extensive research is ongoing on the synthesis of polyproteins^{13,14,15}. The traditional and widely accepted method of polyprotein synthesis has been the recombinant DNA based technology. Two ways the recombinant DNA for the polyprotein is constructed, one is polymerase chain reaction (PCR) based directional DNA concatemerization (DC) method¹⁶ and lately, utilizing Gibson Assembly (GA) cloning¹⁷. Both these methods produce monodisperse polyproteins, however, deal with laborious, expensive and extensive cloning, expression, and often refolding steps. Alternative to recombinant DNA based method is the cysteine based conjugations¹⁸ either by direct oxidation of cysteines to disulphide bonds or by forming thiosuccinimidyl adducts through Michael addition reactions between cysteinyl thiol and maleimide^{19,20}. These methods are far simpler than the recombinant DNA technology, and do not handle large DNA or protein constructs. However, the major drawbacks of these methods are, (i) long reaction-time that leads to uncontrolled progress of the reaction thus generating polydispersity in chain-length, and (ii) the identical chemistry at both the terminals (C- and N-terminal) that reduces the orientation-specificity of POIs in polyprotein chains. More adversely, the reduction of disulphide bonds, and the breakdown of thiosuccinimidyl adducts via thiol-exchange reactions constrain the time of the SMFS experiments, use of buffer, and the use of POIs containing constitutional cysteines²¹. Here, we developed a method of synthesizing polyproteins on a surface by stapling one protein unit at a time via covalent peptide bonds, thus making the construct free from constraints including lifetime of the linking bonds, buffer, biochemical properties of the POIs.

Results and Discussion

We performed successive ‘enzymatic stapling (ES)’ and ‘enzymatic cut’ (EC) to achieve polyproteins. Sortase A (SrtA) that attaches cell-adhesion molecules (CAM) on the cell wall of gram-positive *Staphylococcus aureus* via transpeptidation reaction, is used for ES. Naturally the transpeptidation reaction, commonly known as sortagging, occurs in multiple reaction-steps. First, SrtA recognizes a pentameric peptide sequence (-LPXTG) on the CAM, and forms a thioacyl-linked CAM-SrtA complex by the nucleophilic attack of sulfhydryl (S⁻) group of Cysteine¹⁸⁴ of SrtA to the peptide-carbonyl bond between threonine (T) and glycine (G) in -LPXTG. Next, a nucleophilic amine of polyglycine from the cell-wall precursor molecules cleaves the thioester-acyl complex and re-creates a peptide bond at T with G which eventually restores the sortase recognition sequence (-LPETGGG-). In order to mimic the sortagging protocol in-vitro, we modified the units of the polyprotein construct recombinantly. As shown in Figure 1A, from N-terminal to C-terminal of the protein-unit, we have 3G’s (nucleophile for cleaving the thioester-acyl complex), the protein construct, followed by the SrtA recognition sequence (LPETG) for ES, respectively. To control the progress of the ES reaction, we blocked the reactive nucleophile (3G’s at the N-terminal) with a cap, detachable by on-demand EC. TEV protease (TP) that specifically recognizes a heptameric peptide sequence (EXXXYXQG) for the enzymatic cleavage (EC) reaction, is chosen so that the protease cleavage at the QG site leaves another G succeeded

by 3G's at the N-terminal of the protein²². The 4 G's in sequence facilitates the rate of the nucleophilic attack at the final step of sortagging transpeptidation²³. For the affinity-based purifications, 6xHis-tag is attached at the front of N-terminal that would essentially be removed during EC (Figure 1A).

The scheme in Figure 1B, demonstrates the sequence of reactions towards the making of a polypeptide. Since this method involves successive ES and EC, it gives unique opportunity to make chimeric polypeptide constructs. As standard construct in SMFS, we aim to construct a chimeric-polypeptide by inserting one unit of the POI in-between elastomeric marker proteins. We selected a dimer of 27th-domain of Titin protein I27₂ as marker-unit of the polypeptide, and demonstrated the construction of an octamer using the sequential staple and cut (I27₈-ES). The selection of I27 was derived from its well understood force-stretching behavior which follows a worm-like chain (WLC) model with contour-length difference (L_C) of 28.4 ± 0.3 nm and persistence-length (l_p) of 0.39 ± 0.07 nm²⁴. Constructing octamer from a dimer allows one to systematically insert the POI at varying distal positions from the surface, and offers to study the effects of surface on protein stability and folding. The octamer from dimer units was achieved by four steps of Sortagging (ES), and three steps of protease reactions (EC)-(I27_n-ES; n=2 to 8). However, in most cases where the distance variations are not in the objectives, the reaction steps can be reduced to three times of ES and two times of EC by using higher polymeric form of I27 subunits (trimer or tetramer). This will constrain the position of the POI at the center or at the terminals of the polypeptide.

Attachment of the first layer of the protein unit, is done on polyglycine-coated coverslips (SI, Materials and Methods). This is the first ES step. In this step, we first prepared the thioester complex separately in solution by mixing SrtA and the protein unit I27₂, and subsequently incubated the polyglycine-coated coverslips with thioester complex in a Ca²⁺-free buffer (SI-Materials and Methods). This facilitated the installation of the 1st protein unit ((I27)₂) onto the coverslip substrate via transpeptidation between polyglycine and SrtA-(I27)₂ thioester complex. Sortagging restores the SrtA recognition sequence (LPETGGG-) in the linker which can serve as competitive substrate during subsequent ES. We therefore restricted the contact of active SrtA to surface by engaging the enzyme in the thioester complex before bringing the surface bound LPETG linker in contact, and secondly, by deactivating the enzyme-activity by the removal of Ca²⁺-ions during transpeptidation reaction²⁵. The extent of the thioesterification reaction in absence of any nucleophile is standardized using solution-based Ellman's assay²⁶ (SI Materials and Methods). We noted that the reaction reaches to saturation in 30 mins at pH 7.5 buffer, and the thioester is stable in absence of polyglycine (Figure S2). Next is the preparation of the substrate for the 2nd installation of protein-unit. We treated the monolayer with TP for 2 h for complete removal of the 6xHis-tag and exposed the amine group of N-terminal polyglycine for next layer of ES (Figure 1). After each step of ES and EC, we monitored the extent of the reaction using immunohistochemistry (IHC) with Total Internal Reflection Fluorescence Microscope (TIRFM) (Figure 2A and 2B), and SMFS using Atomic Force Microscopy (AFM) (Figure 2C and 2D). For IHC, we probed the 6xHis-site by incubating the surface with fluorescently labelled anti-His antibody. Since each protein-unit contains 6xHis at the N-terminus just before TP cut site, we expected bright fluorescence upon IHC after ES. Similarly, upon

removal of 6xHis by EC, the substrate is expected to be dark where the darkness quantifies the extent of EC. As expected, we observed bright fluorescent spots after every ES, and substantial drop in intensity after every EC (Figure 2B). To quantify the growth of the polyprotein, we estimated the absolute fluorescence intensity after each ES and each EC (Figure 2B). For ES, we subtracted the fluorescence contributions from scattering, non-specific attachment of dyes, and incomplete EC reaction for each ES. For EC we subtracted only the scattering and non-specific contributions. Our results indicate that the variations in fluorescent intensities after each steps of ES remained nearly unaltered, suggesting no detectable differences in the extent of reactions in successive steps. Similar trend is observed for EC as well, supporting for complete cleavage after each steps. These results qualitatively validate a high monodispersity of the surface with respect to the length of the polyproteins. For further validation, we monitored the polyprotein-growth using SMFS after each batch of ES and EC, where the protein constructs (I27_n-ES; n=2,4,6,8) remain covalently attached to surface; however, cantilever picks molecules non-specifically from any residue of the polyprotein.

We thereafter estimated the contributions from each new layer by monitoring the frequency of force-extension events showing unfolding of domains from the new layer. For example, we estimated the frequency of events showing more than two unfolding features in single force-extension curves after completion of the second layer, containing two sets of I27₂-ES on the surface. Similarly, after 3rd (3 consecutive (I27)₂) and 4th (4 consecutive (I27)₂) batches of combined ES and EC, we estimated the frequency of events showing more than four and six unfolding features respectively in single force-extension curves (Figure 2C and 2D). We observed monotonous decrease in the number of events with higher numbers of domain unfolding which could be due to the stochastic binding of cantilever to the domains, however, the frequency of unfolding for all polyproteins of different domain numbers overlay with each other. We also performed the experiment with I27₈ obtained from DC method, and compared the frequency of events with polyproteins obtained from ES (Figure 2C). Both sets of data overlay with each other confirming again the homogeneous growth of the polyprotein in ES, thus maintaining the monodispersity. Finally, we compared the single molecule stretching behavior of the polyproteins constructed by ES with the I27-octamer I27₈ obtained from DC method²⁴ (Figure 3). For both cases, the force-distance curves featured the WLC behavior with identical parameters. The L_C of the I27₈ polyprotein made of ES is 26.9 ± 3.2 nm, comparable to value obtained for I27₈ made of DC (26.3 ± 1.03 nm).

However, we measured significantly low unfolding forces for I27₈ made of successive ES and EC, ($F_{mp} = 58.6 \pm 6.6$ pN, Figure S3A) in comparison to the unfolding of I27₈ obtained from DC method ($F_{mp} = 192 \pm 1.7$ pN, Figure S3A). This indicates more elastomeric nature of our tailored polyproteins. To address this, we first nullified the experimental artefacts often arising from the surfaces or from cantilevers, by maintaining same treatment to the coverslips and using same cantilevers for both the measurements. However, we noted a striking difference in the length of the inter-domain linkers between two octamer constructs (Figure S3B). The linker for the construct obtained from ES and EC contains eight residues (-LPETGGGG-) in comparison to just two residues (-RS-) in the DC construct. Interestingly, the longer linker is also enriched with residues like P & G (>60%) that are identified as elastic motifs in natural elastomeric proteins (e.g., elastin²⁷, abductin²⁸, Byssus²⁹ etc.),

expected to impart higher flexibility in the ES construct. Higher flexibility in the inter-domain region of the stapled polyprotein construct facilitates domain-domain interactions which are known to contribute in the elasticity of the proteins.³⁰

Conclusion

Use of a polyprotein in SMFS has become a standard protocol for studying the protein folding-dynamics in 3-dimensional architecture. Limitation is in the ease of making polyproteins of the POI or inserting the POI in a polyprotein constructs. The recombinant DNA based technology of making polyproteins faces many challenges including the design of the DNA construct, expression, folding and purification of the large polyprotein and many more. The process becomes even more tedious with the intrinsically disordered proteins. Use of chemical bonds other than peptide linkage, i.e., cysteine-based contacts, limit the use of buffer for experiments, lacks control over monodispersity, and the orientations of the subunits in the polyprotein construct. We demonstrated a method of connecting protein units through peptide bonds, and synthesize the polyprotein in steps to avoid nearly all existing demerits. Apart from making polymeric constructs with single protein units, our method introduces the flexibility of inserting the POI at any position in a chimeric construct, in any preferred directions. With the ease of making chimeric polyproteins using our method, we can even compare the mechanical stability of two proteins, e.g., WT and mutant, in a single experiment in a single force-extension curve.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematics of the synthesis of polyprotein on a solid surface using two enzymes of orthogonal functionalities.

A) Protein of interest (POI) which is specifically designed for this enzymatic stapling is having N-terminal 6xHis-tag for affinity purification, oligoglycine(3G's), protected by TEV protease cut site and C-terminal is having LPETGSS (SrtA recognition site). **B)** During first step of the reaction, POI and SrtA is allowed to react for 30min to form the thioester intermediate. This mixture is incubated on polyglycine exposed glass coverslip for 45min for the transpeptidation reaction between polyglycine and POI, this step is called Enzymatic stapling (ES). In the second step, surface is incubated with TEV protease (TP) for enzymatic cut (EC) for 2h to expose the polyglycine of POI. This polyglycine-exposed surface becomes ready to react with thioester intermediate to facilitate the next ES. We continue this alternative ES and EC till we get a polyprotein having desired number of protein units.

Figure 2. The process of polyprotein construction, and validation using TIRF and SMFS
A) Schematics of the IHC, we executed to quantify the extent of ES and EC at every step using TIRF microscope. After 1st step of ES, the substrate is incubated with Anti-His antibody (rabbit) for 45min, followed by incubation with fluorescently (Alexa-488) labeled secondary antibody for 30min. Next TEV protease is employed for EC for the exposure of polyG. EC also removes 6xHis, thus the loss of fluorescence signal from the substrate. After completion of one cycle of ES and EC, the process is repeated for next batch of ES and EC respectively. The extent of reaction is monitored using IHC after every step.

B) Fluorescence images indicate the stepwise growth of polyprotein. In Step (I), bright fluorescence signal is observed corresponding to 1st ES and then, there is steep decrease in intensity as expected in Step (II) as EC removes all 6xHis. Similar pattern in fluorescence intensity is observed after every step of ES and EC reaction. We have monitored the reaction for four ES and three EC to get an octamer from I27₂ unit. **C)** Frequency of events showing 2,3,4,5,6,7 and 8 unfolding events are plotted for I27₄ (black), I27₆ (red) and I27₈ (pink) obtained using ES, and compared with I27₈ (olive green) made using DC. **D)** Representative force-distance curves showing two (I27₂-ES), three & four (I27₄-ES), and six (I27₆-ES) unfolding. The red solid lines represent the fitting of WLC model to the respective stretching.

Figure 3. Comparison of the single molecule stretching of I27₈-ES and I27₈-DC

A) Force distance curve with seven unfolding and their fitting to WLC model are shown for I27₈-ES. B) The distribution of ΔLc as shown in Figure A are measured for the difference of two successive WLC fit to stretching curves and plotted as histogram. The black solid line represents the Gaussian fit to the histogram estimating peak maxima at 26.9 ± 3.2 nm C) Force distance curve showing seven unfolding and their fitting to WLC model of I27₈-DC D) Histogram of ΔLc for I27₈ of DC and the corresponding Gaussian fit is shown. The most probable ΔLc obtained for the fit is 26.3 ± 1.03 nm.