

Cite this: *RSC Chem. Biol.*, 2022, **3**, 1276

One-step asparaginyl endopeptidase (OaAEP1)-based protein immobilization for single-molecule force spectroscopy

Xuan Ding,^{†ab} Ziyi Wang,^{†c} Bin Zheng,^{†c} Shengchao Shi,^c Yibing Deng,^c Hanyang Yu^{†a} and Peng Zheng^{†*c}

Enzymatic protein ligation has become the most powerful and widely used method for high-precision atomic force microscopy single-molecule force spectroscopy (AFM-SMFS) study of protein mechanics. However, this methodology typically requires the functionalization of the glass surface with a corresponding peptide sequence/tag for enzymatic recognition and multiple steps are needed. Thus, it is time-consuming and a high level of experience is needed for reliable results. To solve this problem, we simplified the procedure using two strategies both based on asparaginyl endopeptidase (AEP). First, we designed a heterobifunctional peptide-based crosslinker, GL-peptide-propargylglycine, which links to an N_3 -functionalized surface via the click reaction. Then, the target protein with a C-terminal NGL sequence can be immobilized via the AEP-mediated ligation. Furthermore, we took advantage of the direct ligation between primary amino in a small molecule and protein with C-terminal NGL by AEP. Thus, the target protein can be immobilized on an amino-functionalized surface via AEP in one step. Both approaches were successfully applied to the AFM-SMFS study of eGFP, showing consistent single-molecule results.

Received 30th May 2022,
Accepted 18th August 2022

DOI: 10.1039/d2cb00135g

rsc.li/rsc-chembio

^a Department of Biomedical Engineering, College of Engineering and Applied Sciences, Nanjing University, 163 Xianlin Road, Nanjing, Jiangsu 210023, P. R. China

^b State Key Laboratory of Analytical Chemistry for Life Science, Nanjing University, Nanjing, Jiangsu 210023, P. R. China

^c State Key Laboratory of Coordination Chemistry, Chemistry and Biomedicine Innovation Center (ChemBIC), School of Chemistry and Chemical Engineering, Nanjing University, Nanjing, Jiangsu 210023, P. R. China. E-mail: pengz@nju.edu.cn

† These authors contributed equally to this work.

Introduction

Protein immobilization can be critical for the application and fundamental research of proteins, such as protein detection, characterization, and single-molecule study.^{1–8} Among many methods, site-specific protein immobilization has become the most powerful method for high-precision atomic force microscopy single-molecule force spectroscopy (AFM-SMFS) study of protein mechanics. For example, the reaction between cysteine in the protein and the maleimide-coated surface is a widely used approach for protein immobilization (Fig. 1A).^{9,10} However, it suffers from an uncontrolled reaction if several cysteines are present, leading to different immobilization conditions.^{11–14} Recently, enzymatic ligation has been used, which recognizes and connects two specific peptide sequences/tags, and leads to efficient, site-specific protein immobilization. Nevertheless, it requires the two enzymatic recognition peptide sequences in the target protein and on the surface, respectively.^{15–17} Typically, the peptide-coated surface is achieved using two steps, starting from an amino-coated glass surface. First, a maleimide-coated surface is obtained using a chemical crosslinker containing an amino-reactive group N-hydroxysuccinimide (NHS) and a cysteine-reactive maleimide group (Fig. 1A, step 1). Then, a heterofunctional peptide linker with cysteine and the target peptide sequence is applied (Fig. 1B, step 3). Finally, the protein with the target peptide sequence can be immobilized



Peng Zheng

Professor Peng Zheng received his BS from Nanjing University in 2008, China. Then, he obtained his PhD from University of British Columbia, Canada, under the supervision of Prof. Hongbin Li. He did a post-doctoral fellowship at Harvard Medical School, worked with Dr Timothy. Springer. In 2015, he joined school of chemistry, Nanjing University as a full professor in the chemical biology section. His lab focuses on the studies of protein (un)folding using AFM-SMFS, in

which metal ions play a critical role. Also, he uses single-cell force spectroscopy to study protein-protein (receptor-ligand) interactions on the living cell.



(Fig. 1B, step 4).¹⁸ Here, the NHS is quite reactive, and multiple steps are needed. Thus, a high level of experience is needed for reliable results, and the whole procedure is time-consuming. Recently, we developed a click reaction-based method to avoid the use of NHS, and a much-improved success rate has been obtained (Fig. 1B, steps 1 and 2).¹⁹ However, more steps are introduced, and the maleimide group is always needed as the bridge.

To solve this problem, we developed two strategies, both using *oldenlandia affinis* asparaginyl endopeptidases 1 with mutation C247A (abbreviated as *OaAEP1*, or *AEP*) for enzymatic ligation.²⁰ First, a heterofunctional peptide-based crosslinker, glycine-leucine (GL)-peptide-propargylglycine (Pra) was designed. As a result, the target GL sequence can be directly added to the N_3 -functionalized surface without using the cysteine-maleimide coupling reaction (Fig. 1C, step 2). Furthermore, a more efficient enzymatic protein immobilization was achieved by taking advantage of the AEP-mediated direct ligation between the amino group and target protein with a C-terminal NGL sequence (Fig. 1D). Thus, the target protein can be immobilized on an amino-functionalized surface in one step.²¹

We tested and applied these methods to atomic force microscopy-based single-molecule force spectroscopy. Force spectroscopy is a powerful tool that can mechanically manipulate a single molecule and study its corresponding conformational

changes or interactions.^{22–36} In the AFM-SMFS experiment of proteins, the protein of interest (POI) is immobilized and stretched from two points between the AFM tip and the protein-deposited surface, revealing a wealth of information of protein (un)folded, stability, and interactions.^{37–46} Thus, new methods generating site-specific and strong protein immobilization can be invaluable for obtaining reliable and efficient single-molecule results.^{47–52} We demonstrated these two methods by immobilizing eGFP and studied its unfolding process by AFM-SMFS, both showing ideal results.

Results and discussion

The principle and procedures of the two methods are as follows. For heterofunctional peptide method, an amino-functionalized glass surface was converted to azide first, which takes about one hour (Fig. 1C). Then, heterofunctional peptide-based crosslinker GL-peptide-Pra was added *via* the Copper-catalyzed azide-alkyne cycloaddition (CuAAC) click reaction, which takes ~8 hours catalyzed by ascorbic acid. Finally, the protein with an NGL sequence can be immobilized to the GL-coated surface *via* the *OaAEP1*-mediate enzymatic ligation for half an hour.^{18,53,54}

To demonstrate the applicability of this method for the AFM-SMFS study, a characterized protein, enhanced Green

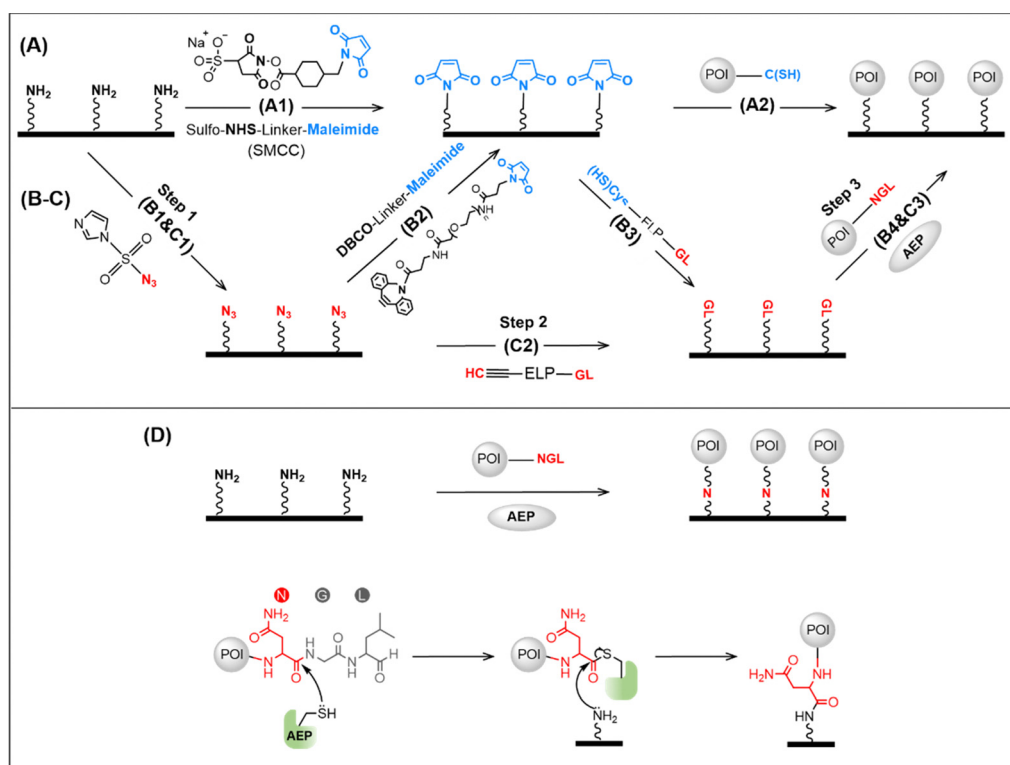


Fig. 1 Scheme of surface protein immobilization using different methods. (A) First, the surface is functionalized by maleimide using the chemical crosslinker SMCC. Then, the target protein is immobilized *via* its endogenous cysteine. (B) First, the surface is functionalized by azide, and then maleimide is added using the crosslinker DBCO-linker-Mal. Then, the GL sequence is added using a peptide Cys-ELP-GL. Finally, the protein with NGL can be immobilized enzymatically. (C) a heterofunctional peptide with an alkyne group and a GL can be used for protein immobilization. (D) Target protein with a C-terminal NGL is ligated to the amino-functionalized glass surface in one step by AEP, and its molecular mechanism is depicted at the bottom.



Fluorescent Protein (eGFP), was chosen as a model protein.⁵⁵ First, a seven-amino acid (aa) length peptide GL-GSGSG-Pra was designed as the heterofunctional crosslinker, which reacted with the N_3 -functionalized coverslip (Fig. 2A). Then, a fused protein Cohesion (Coh)-eGFP-NGL containing eGFP was designed for subsequent high-precision AFM measurement. It was ligated to the immobilized peptide. Consequently, it can be probed by an ELP_n-GB1-Xmodule-dockerin (XDoc) functionalized AFM tip for the protein unfolding experiment (Fig. 2A). Here, protein domain GB1 with a known ΔLc of 18 nm was used as a single-molecule fingerprint.^{56–58} ELP is a flexible yet mechanically labile elastin-like polypeptides functioning as a spacer to eliminate the non-specific interaction.⁵⁹ XDoc and Coh form a reversible [Coh:XDoc] protein–protein interaction with a high rupture force of ~ 500 pN for efficient single-molecule pick-up.⁶⁰

Upon stretching, the fused protein unfolded sequentially as observed from force-extension curves (Fig. 2B). Besides the 18 nm-length unfolding peak from GB1 (colored in blue), peak(s) from one-step (traces 1 and 2, in green) and two-step unfolding (trace 3, in gray) of eGFP with a (summed) ΔLc of ~ 80 nm were observed. This value agrees well with the theoretical ΔLc of eGFP unfolding in which 227 aa were extended ($227 \text{ aa} \times 0.36 \text{ nm aa}^{-1} - 2.4 \text{ nm} = 79.3 \text{ nm}$). Moreover, the multiple unfolding pathways (Fig. 2C, $n = 247$) are similar to

previous single-molecule results of GFP.⁶¹ A long featureless curve ($81.9 \pm 13.8 \text{ nm}$, in red) appeared before the first peak, which is from the initial extension of the mechanically labile peptide in the protein system (the seven aa-length peptide crosslinker plus the 145 aa-length ELP_n and other 28 aa-length peptide in the fused protein).

We chose the β domain of metallothionein (β MT), which binds to zinc, as another model protein. As a small size protein domain with only 37 amino acids, β MT contains 11 endogenous cysteines (Fig. 3A).⁶² It has been studied by AFM-SMFS using a non-specific immobilization method before.^{63,64} Thus, it serves as an excellent example of this cysteine/maleimide-free method for AFM measurement. A fused protein Coh-GB1- β MT-NGL was constructed and immobilized into the system using the seven aa-length peptide crosslinker accordingly. And the same ELP_n-GB1-XDoc functionalized AFM tip was used for probing (Fig. 3B). Upon stretching, two GB1 unfolding peaks were observed as expected (Fig. 3C). Moreover, peak(s) from one-step (traces 1 and 2, in orange) and two-step unfolding (traces 3 and 4, in pink) of β MT with a (summed) ΔLc of $\sim 12 \text{ nm}$ were observed ($n = 205$, Fig. 3D). This value agrees with the theoretical ΔLc of β MT unfolding in which 37 aa were extended. Moreover, this small protein domain's previously observed multiple unfolding pathways were detected and verified using our new method.^{63,64}

Although the heterofunctional peptide method is one step less, the procedure is still tedious and time-consuming. A recent protein labeling work showed that OaAEP1(C247A) could directly catalyze the attachment of a small molecule with a primary amine to a protein *via* its C-terminal NGL sequence.²¹ Thus, we hypothesize that a surface covered with primary amino may also react with properly designed target protein catalyzed by AEP, and a much more convenient one-step protein immobilization may be achieved.

We then pipetted 50 μL of protein mixture containing 6 mg mL^{-1} Coh-eGFP-NGL and 0.2 mg mL^{-1} AEP on an amino-functionalized coverslip, which was incubated under 25 $^\circ\text{C}$ for half an hour. After washing with high-salt Tris buffer (1 M NaCl, 100 Tris, pH = 7.4) several times, the coverslip was used for AFM-SMFS measurement directly (Fig. 4A). As expected, eGFP was immobilized as the characteristic eGFP unfolding peak with ΔLc of $\sim 80 \text{ nm}$, including both one-step and two-step unfolding pathways were observed (Fig. 4B). Moreover, we analyzed the mechanical stability of immobilized eGFP in this new method. The unfolding force was $93.6 \pm 27.1 \text{ pN}$ ($n = 78$), consistent with previous experiments (Fig. 4C). Therefore, this one-step enzymatic protein immobilization is well-suited for AFM measurement. Moreover, with purified proteins and amino-functionalized coverslip, this one-step method only needs less than an hour for the whole protein immobilization procedure and saves time.

In this work, we demonstrated the application of AEP to efficient protein immobilization for high-precision AFM measurement of protein mechanics. Thanks to its efficient and site-specific ligation, several enzymes have been used for this purpose, including sortase, sfp, haloalkane dehydrogenase, formyl glycine generating enzyme, *etc.*^{17,48,59,65} However, all these

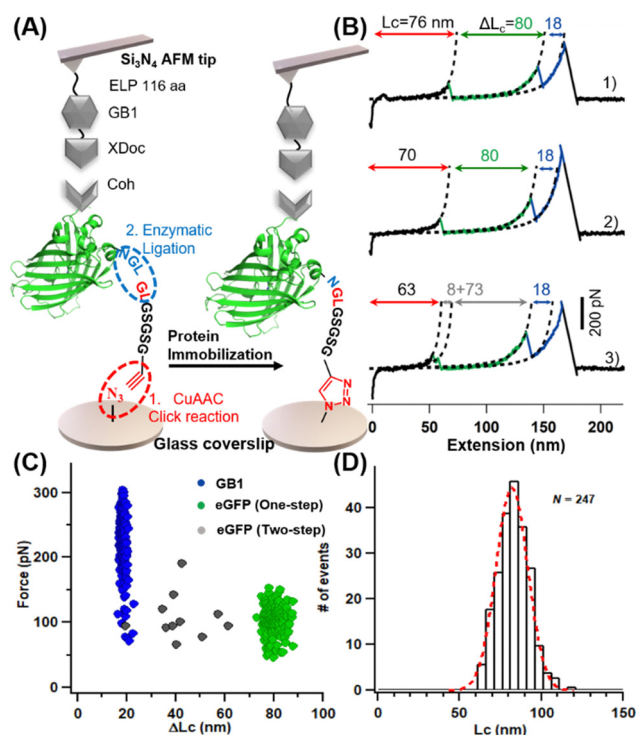


Fig. 2 AFM-SMFS studies of immobilized eGFP. (A) Schemes show the heterofunctional peptide-based GFP immobilization for AFM-SMFS measurement. (B) Typical traces represent different unfolding pathways of GFP: one step (traces 1 and 2) and two steps (traces 3). (C) Scatter plots of $F-\Delta Lc$ of the fused protein shows expected unfolding results for eGFP and GB1. (D) The histogram of the contour length of the construct shows a value of 82 nm.



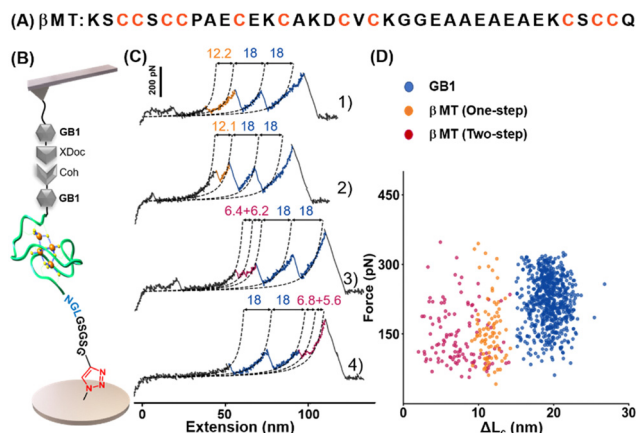


Fig. 3 (A) The amino acid sequence of β MT shows eleven endogenous cysteines. (B) AFM-SMFS setup for β MT unfolding studies. (C and D) Representative force-extension curves and scatter plots of F - ΔL_c of fused protein showed expected unfolding scenarios for each protein (domain).

enzymes, including AEP, require surface functionalization with the corresponding peptide/tag, which leads to multiple steps and lengthy procedures. Here, we optimized the AEP-based protein immobilization from two directions. The first strategy uses a heterofunctional peptide-based crosslinker, connecting the enzymatic recognition site and click reaction group in a single molecule.^{44,48,65} As a result, one step is removed.

Compared to cysteine-engineered protein coupling or immobilization, this method avoids disadvantages of the cysteine-maleimide or cysteine-cysteine reaction. For example, previous work using cysteine engineering/coupling to study GFP cannot stretch the protein from N and C terminus, possibly due to intramolecular disulfide bond formation. Our enzymatic method avoids it and makes this stretching direction possible. In addition, the previous cysteine coupling-based immobilization method cannot prepare a suitable metallothionein sample for single molecule studies, as there were so many endogenous cysteines and formation of intramolecular disulfide bond messes up the measurement.

Most importantly, we then demonstrated that AEP could immobilize protein with a C-terminal NGL sequence on an amino-functionalized surface in one step. This method only needs an incubation of half an hour for the ligation as the only step, which significantly saves time and simplifies the protein immobilization procedure. In addition, only a C-N covalent bond forms between the target protein and the surface as the linkage using this method. This bond is similar to the peptide backbone of proteins with a much higher mechanical stability compared with other linkages such maleimide-thiol adduct (~ 1100 pN after hydrolysis) and 1,2,3-triazole (< 1800 pN).¹⁹ Thus, we believe this new method not only simplifies the enzymatic protein immobilization but also broadens the systems that can be studied by AFM-SMFS.

Experimental material and procedure

Materials

All reagents were purchased from commercial suppliers and used accordingly. Peptide GL-ELP_n-Propargylglycine and C-ELP₂₀-NGL were purchased from GenScript. (3-Aminopropyl) triethoxysilane (APTES, Sigma-Aldrich), Imidazole-1-sulfonyl azide hydrochloride (ImSO₂N₃-HCl; Abydos Scientific, China), and DBCO-(polyethylene glycol)₄-Mal (DBCO-PEG₄-Mal; Biocone, China) were stored and used in the dark. Vitamin C, K₂CO₃, and CuSO₄ were from Aladdin. Solvents, including toluene, ethanol, and DMSO were purchased from Sinopharm Chemical Reagent Co. Ltd. Aqueous solutions were configured with Milli-Q water (18.2 M Ω cm⁻¹, 0.22 μ m filter). Microporous membrane filters (0.22 and 0.45 μ m) were used for further purification (Jet Biofil, China). For the culture of *E. coli*, Luria-Bertani (LB) medium and agar plates (Sangon Biotech) were used. Plasma cleaner was used to activate the glass surface. Protein concentrations were routinely determined by Nanodrop 2000.

Surface preparation

The glass coverslip (Sail Brand) and silicon nitride-based MLCT-BIO-DC AFM cantilever (Bruker Corp.) were first cleaned

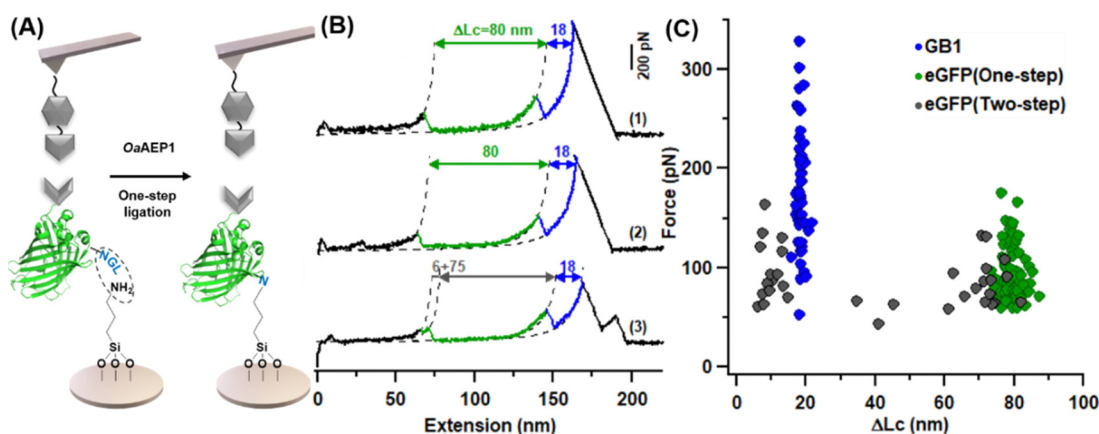


Fig. 4 One-step immobilization of eGFP for AFM-SMFS study. (A) Scheme of AEP-mediated eGFP immobilization on the amino-functionalized glass surface. (B) Representative force-extension curves showing different unfolding pathways of GFP: one-step (traces 1 and 2) and two-step (trace 3). (C) Scatter plots of F - ΔL_c of fused protein showed expected unfolding results for eGFP.



by plasma for 10 min. Then, they were amino-silanized and azide-functionalized according to published protocols.¹⁹ The peptide C-ELP₂₀-NGL was immobilized on the AFM cantilever according to literature too.

Different from the cantilever, coverslips were modified using the heterofunctional peptide-based crosslinker. A ~30 μL solution of GL-ELP_n-Pra (mixed aqueous solution of 2 mM GL-ELP_n-Pra, 1 mM CuSO₄, 100 mM Vitamin C, and 0.5 × PBS) was sandwiched between two azide-functionalized glass coverslip surfaces, and incubated for 8 hours at room temperature in the dark.

Finally, both cantilevers and coverslips were washed with 50 mL high-salt buffer (100 mM Tris, 1 M NaCl, pH 7.4), rinsed by H₂O, and then dried by N₂ stream or filter paper. The ELP-coated glass coverslips and cantilevers were immediately used or kept at -20 °C.

AFM-SMFS experiment

A 50 μL mixed solution of 30 μM fusion protein Coh-eGFP-NGL and 1 μM enzyme AEP was pipetted on the GL-functionalized coverslips, and the NGL-functionalized cantilevers with ELP₂₀ as linker were incubated with 50 μL mixed solution of 60 μM GL-GB1-XDoc and 1 μM AEP in the measurement buffer (100 mM Tris, 100 mM NaCl, pH 7.4). The OaAEP1-mediated protein ligation was performed in the measurement buffer, forming a covalent NGL linkage between the C-terminal NGL and N-terminal GL or an amino-functionalized glass surface (Fig. 1).

We used a Nanowizard 4 atomic force microscope to perform the AFM-SMFS experiment. After calibration, the AFM tip was pushed onto the surface, and the target protein was captured through the specific [Coh:XDoc] protein-protein interaction. Then, moving the tip up at a constant velocity (1000 nm s⁻¹), the fused protein was stretched and unfolded. Finally, the data was filtered using data processing software (JPK) and analyzed by Igor Pro 6.12 (Wavemetrics).

Funding

This work was funded by Natural Science Foundation of Jiangsu Province (No. BK20200058), the National Natural Science Foundation of China (Grant no. 21771103 and 21977047) and Fundamental Research Funds for the Central Universities (Grant no. 14380259).

Conflicts of interest

The authors declare no competing interests.

References

- 1 C. Garcia-Galan, Á. Berenguer-Murcia, R. Fernandez-Lafuente and R. C. Rodrigues, *Adv. Synth. Catal.*, 2011, **353**, 2885–2904.

- 2 T. D. Becke, S. Ness, S. Sudhop, H. E. Gaub, M. Hilleringmann, A. F. Schilling and H. Clausen-Schaumann, *JoVE*, 2018, e58167.
- 3 P. López-García, A. D. de Araujo, A. E. Bergues-Pupo, I. Tunn, D. P. Fairlie and K. G. Blank, *Angew. Chem., Int. Ed.*, 2021, **60**, 232–236.
- 4 L. F. Milles, K. Schulten, H. E. Gaub and R. C. Bernardi, *Science*, 2018, **359**, 1527–1533.
- 5 H. Fu, Y. Jiang, D. Yang, F. Scheiflinger, W. P. Wong and T. A. Springer, *Nat. Commun.*, 2017, **8**, 324.
- 6 Y. Zhang, Z. Wang, T. B. Kouznetsova, Y. Sha, E. Xu, L. Shannahan, M. Fermen-Coker, Y. Lin, C. Tang and S. L. Craig, *Nat. Chem.*, 2021, **13**, 56–62.
- 7 M. Leitner, J. Brummeir, G. O. Plaimer, I. Kefer, A. Poturnayova, T. Hianik and A. Ebner, *Methods*, 2022, **197**, 54–62.
- 8 H. E. Morgan, W. B. Turnbull and M. E. Webb, *Chem. Soc. Rev.*, 2022, **51**, 4121–4145.
- 9 J. L. Zimmermann, T. Nicolaus, G. Neuert and K. Blank, *Nat. Protoc.*, 2010, **5**, 975–985.
- 10 D. Sluysmans, N. Willet, J. Thevenot, S. Lecommandoux and A.-S. Duwez, *Nanoscale Horiz.*, 2020, **5**, 671–678.
- 11 M. Synakewicz, D. Bauer, M. Rief and L. S. Itzhaki, *Sci. Rep.*, 2019, **9**, 13820–13830.
- 12 R. Barattin and N. Voyer, *Chem. Commun.*, 2008, 1513–1532.
- 13 B. P. Duckworth, J. Xu, T. A. Taton, A. Guo and M. D. Distefano, *Bioconjugate Chem.*, 2006, **17**, 967–974.
- 14 W. Zhang and X. Zhang, *Prog. Polym. Sci.*, 2003, **28**, 1271–1295.
- 15 W. Ott, E. Durner and H. E. Gaub, *Angew. Chem., Int. Ed.*, 2018, **57**, 12666–12669.
- 16 Y. Deng, B. Zheng, Y. Liu, S. Shi, J. Nie, T. Wu and P. Zheng, *JoVE*, 2020, e60774.
- 17 S. Garg, G. S. Singaraju, S. Yengkhom and S. Rakshit, *Bioconjugate Chem.*, 2018, **29**, 1714–1719.
- 18 Y. Deng, T. Wu, M. Wang, S. Shi, G. Yuan, X. Li, H. Chong, B. Wu and P. Zheng, *Nat. Commun.*, 2019, **10**, 2775–2785.
- 19 S. Shi, Z. Wang, Y. Deng, F. Tian, Q. Wu and P. Zheng, *CCS Chem.*, 2022, **4**, 598–604.
- 20 R. Yang, Y. H. Wong, G. K. T. Nguyen, J. P. Tam, J. Lescar and B. Wu, *J. Am. Chem. Soc.*, 2017, **139**, 5351–5358.
- 21 F. B. H. Rehm, T. J. Tyler, K. Yap, S. J. de Veer, D. J. Craik and T. Durek, *J. Am. Chem. Soc.*, 2021, **143**, 19498–19504.
- 22 J. Oroz, A. Galera-Prat, R. Hervas, A. Valbuena, D. Fernandez-Bravo and M. Carrion-Vazquez, *Sci. Rep.*, 2019, **9**, 13306.
- 23 T. Hoffmann, K. M. Tych, M. L. Hughes, D. J. Brockwell and L. Dougan, *Phys. Chem. Chem. Phys.*, 2013, **15**, 15767–15780.
- 24 M. Mora, A. Stannard and S. Garcia-Manyes, *Chem. Soc. Rev.*, 2020, **49**, 6816–6832.
- 25 R. Petrosyan, S. Patra, N. Rezajooei, C. R. Garen and M. T. Woodside, *Proc. Natl. Acad. Sci. U. S. A.*, 2021, **118**, e2010213118.
- 26 M. Yu, Z. Zhao, Z. Chen, S. Le and J. Yan, *Nat. Commun.*, 2020, **11**, 4476.
- 27 X. Ma, M. Zhu, J. Liu, X. Li, L. Qu, L. Liang, W. Huang, J. Wang, N. Li and J.-H. Chen, *et al.*, *Bioconjugate Chem.*, 2019, **30**, 2998–3006.



- 28 M. Muddassir, B. Manna, P. Singh, S. Singh, R. Kumar, A. Ghosh and D. Sharma, *Chem. Commun.*, 2018, **54**, 9635–9638.
- 29 R. X. Yao, X. Li, N. Xiao, W. G. Weng and W. K. Zhang, *Nano Res.*, 2021, **14**, 2654–2658.
- 30 P. Sonar, L. Bellucci, A. Mossa, P. O. Heidarsson, B. B. Kragelund and C. Cecconi, *Biophys. J.*, 2020, **119**, 1821–1832.
- 31 P. Zhao, C.-Q. Xu, C. Sun, J. Xia, L. Sun, J. Li and H. Xu, *Polym. Chem.*, 2020, **11**, 7087–7093.
- 32 M. Zhao and M. T. Woodside, *Nat. Chem. Biol.*, 2021, **17**, 975–981.
- 33 S. Zhang, H.-j. Qian, Z. Liu, H. Ju, Z.-y. Lu, H. Zhang, L. Chi and S. Cui, *Angew. Chem., Int. Ed.*, 2019, **58**, 1659–1663.
- 34 H. Xing, Z. Li, W. Wang, P. Liu, J. Liu, Y. Song, Z. L. Wu, W. Zhang and F. Huang, *CCS Chem.*, 2020, **2**, 513–523.
- 35 Z. L. Guo, H. Y. Hong, G. Yuan, H. Qian, B. Li, Y. Cao, W. Wang, C. X. Wu and H. Chen, *Phys. Rev. Lett.*, 2020, **125**, 198101.
- 36 E. M. Mulhall, A. Ward, D. Yang, M. A. Koussa, D. P. Corey and W. P. Wong, *Nat. Commun.*, 2021, **12**, 849.
- 37 M.-A. LeBlanc, M. R. Fink, T. T. Perkins and M. C. Sousa, *Proc. Natl. Acad. Sci. U. S. A.*, 2021, **118**, e2019566118.
- 38 J. Perales-Calvo, D. Giganti, G. Stirnemann and S. Garcia-Manyes, *Sci. Adv.*, 2018, **4**, eaaq0243.
- 39 A. Alonso-Caballero, J. Schonfelder, S. Poly, F. Corsetti, D. De Sancho, E. Artacho and R. Perez-Jimenez, *Nat. Commun.*, 2018, **9**, 2758.
- 40 C. Suay-Corredera, M. R. Pricolo, D. Velazquez-Carreras, D. Pathak, N. Nandwani, C. Pimenta-Lopes, D. Sanchez-Ortiz, I. Urrutia-Irazabal, S. Vilches and F. Dominguez, *et al.*, *ACS Nano*, 2021, **15**, 10203–10216.
- 41 Z. Wang, J. Nie, S. Shi, G. Li and P. Zheng, *Chem. Commun.*, 2021, **57**, 11489–11492.
- 42 F. Tian, G. Li, B. Zheng, Y. Liu, S. Shi, Y. Deng and P. Zheng, *Chem. Commun.*, 2020, **56**, 3943–3946.
- 43 W. Cao, C. Dong, S. Kim, D. Hou, W. Tai, L. Du, W. Im and X. F. Zhang, *Biophys. J.*, 2021, **120**, 1011–1019.
- 44 J. Y. Nie, F. Tian, B. Zheng, Z. Y. Wang and P. Zheng, *Chem. Lett.*, 2021, **50**, 1667–1675.
- 45 F. Rico, A. Russek, L. Gonzalez, H. Grubmuller and S. Scheuring, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 6594–6601.
- 46 D. J. Müller, A. C. Dumitru, C. Lo Giudice, H. E. Gaub, P. Hinterdorfer, G. Hummer, J. J. De Yoreo, Y. F. Dufrene and D. Alsteens, *Chem. Rev.*, 2021, **121**, 11701–11725.
- 47 G. Song, F. Tian, H. Liu, G. Li and P. Zheng, *J. Phys. Chem. Lett.*, 2021, **12**, 3860–3867.
- 48 B. Yang, Z. Liu, H. Liu and M. A. Nash, *Front. Mol. Biosci.*, 2020, **7**, 85.
- 49 Y. Liu, F. Tian, S. Shi, Y. Deng and P. Zheng, *J. Phys. Chem. Lett.*, 2021, **12**, 10914–10919.
- 50 Q. Li, D. Apostolidou and P. E. Marszalek, *Methods*, 2022, **197**, 39–53.
- 51 D. R. Jacobson and T. T. Perkins, *Proc. Natl. Acad. Sci. U. S. A.*, 2021, **118**, e2020083118.
- 52 J. Nie, Y. Deng, F. Tian, S. Shi and P. Zheng, *Nano Res.*, 2022, **15**, 4251–4257.
- 53 F. B. H. Rehm, T. J. Tyler, J. Xie, K. Yap, T. Durek and D. J. Craik, *ChemBioChem*, 2021, **22**, 2079–2086.
- 54 T. J. Harmand, N. Pishesha, F. B. H. Rehm, W. Ma, W. B. Pinney, Y. J. Xie and H. L. Ploegh, *ACS Chem. Bio.*, 2021, **16**, 1201–1207.
- 55 Z. Ganim and M. Rief, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**, 11052–11056.
- 56 Y. Cao, C. Lam, M. Wang and H. Li, *Angew. Chem., Int. Ed.*, 2006, **45**, 642–645.
- 57 P. Zheng, S. J. Takayama, A. G. Mauk and H. Li, *J. Am. Chem. Soc.*, 2012, **134**, 4124–4131.
- 58 P. Zheng and H. Li, *J. Am. Chem. Soc.*, 2011, **133**, 6791–6798.
- 59 W. Ott, M. A. Jobst, M. S. Bauer, E. Durner, L. F. Milles, M. A. Nash and H. E. Gaub, *ACS Nano*, 2017, **11**, 6346–6354.
- 60 S. W. Stahl, M. A. Nash, D. B. Fried, M. Slutzki, Y. Barak, E. A. Bayer and H. E. Gaub, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 20431–20436.
- 61 H. Dietz, M. Bertz, M. Schlierf, F. Berkemeier, T. Bornschlogl, J. P. Junker and M. Rief, *Nat. Protoc.*, 2006, **1**, 80–84.
- 62 M. D. Peris-Diaz, R. Guran, C. Domene, V. de los Rios, O. Zitka, V. Adam and A. Krezel, *J. Am. Chem. Soc.*, 2021, **143**, 16486–16501.
- 63 G. Yuan, Q. Ma, T. Wu, M. Wang, X. Li, J. Zuo and P. Zheng, *Sci. Rep.*, 2019, **9**, 10518.
- 64 G. Yuan, F. Curtolo, Y. Deng, T. Wu, F. Tian, Q. Ma, Y. Liu, J. Zuo, G. M. Arantes and P. Zheng, *Research*, 2021, **2021**, 9756945.
- 65 I. Popa, R. Berkovich, J. Alegre-Cebollada, C. L. Badilla, J. A. Rivas-Pardo, Y. Taniguchi, M. Kawakami and J. M. Fernandez, *J. Am. Chem. Soc.*, 2013, **135**, 12762–12771.

