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Communication

Cryopolymerization of 1,2-Dithiolanes for the Facile and Reversible Grafting-from Synthesis of Protein−Polydisulfide Conjugates

Jianhua Lu, Hao Wang, Ziyou Tian, Yingqin Hou, and Hua Lu*

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ABSTRACT: Grafting-from (GF) is an important yet underdeveloped strategy toward protein−polymer conjugates. Here, we report a simple cryopolymerization method that enables highly efficient GF synthesis of cell-penetrating protein−polydisulfide conjugates. Rapid and controlled ring-opening polymerization of 1,2-dithiolanes under cryo-conditions can be initiated by proteins bearing a reactive cysteine, owing to both favored thermodynamics and augmented kinetics arising from frozen-induced high local concentration of substrates. This method is applicable to various wild-type or genetically engineered proteins without the need of chemical installation of an initiation group. The resulting conjugates can be reversibly degrafted under mild conditions to regenerate functional "native" proteins in a traceless fashion. These unique features make such conjugates highly useful in applications such as a dynamic switch of protein functions, cytosolic delivery of protein therapeutics, and protein purification. The method is also potentially useful for the in situ growth of other types of polymers from protein surface.

Protein−polymer conjugates (PPCs) are hybrid molecules with great application potential as biotherapeutics, high-stability industrial catalysts, and novel self-assembling materials.[1−6] Current strategies for synthesizing PPCs generally involve bioconjugation of a protein-of-interest (POI) with a preformed polymer ("grafting-to", GT)[7−10] or in situ polymerization from a POI surface-tethered initiator ("grafting-from"; GF).[11−13] Although the GF approach is generally more efficient due to simpler product purification and reduced steric hindrance during monomer addition, its synthetic application has been hampered by the limited choices of polymerization methods that can be used. To date, only controlled radical polymerizations have been commonly applied for the GF approach,[14−19] with ring-opening metathesis polymerization as the only other exception recently.[20] Even then, all methods require sophisticated synthetic and biochemical knowledge to ensure successful installation of suitable initiating groups on the POI and introduce only nonbiodegradable moieties. Therefore, simple and efficient GF polymerization strategies that can grow biodegradable polymers from native proteins are highly desirable.

As illustrated in Figure 1A, we envision that ring-opening polymerization (ROP) of 1,2-dithiolanes[21−24] can be initiated by a thiol group of a native or genetically introduced cysteine (Cys), providing easy access to protein−polydisulfide (protein−PDS) conjugates via the GF route. This strategy is theoretically applicable to any protein with an accessible Cys and allows native protein to be released in a traceless fashion. Previously, Matile pioneered small molecular thiol-initiated ROP of 1,2-dithiolanes for the synthesis of cell-penetrating PDS.[25−28] Combining this approach with GT, Yao et al. later successfully prepared protein−PDS conjugates for highly efficient cytosolic delivery.[29−31] Until now, there has not been any literature report on the in situ growing of PDS on a protein surface via the GF method.

We began our current study by first testing the ROP of neutralized **M1** in D_2O at different temperatures with sodium 2-mercaptoethanesulfonate, a small molecular thiol, as initiator. The reaction showed a relatively high equilibrium monomer concentration ($[M]_{eq}$) of 262 mM at room temperature. We next proceeded to evaluate the ROP of various 1,2-dithiolanes (**M1−3**, Figures S1−S3) at room temperature initiated by TEV-Cys-EGFP,[32] an enhanced green fluorescence protein containing a genetically incorporated reactive Cys near its N-terminus. Unfortunately, our initial attempts at room temperature did not lead to any significant product formation even at an initial monomer concentration ($[M]_0$) of 300 mM, as evidenced by nonreducing SDS-PAGE gel analysis (Figure S4). Moreover, such a high concentration of monomers could also cause undesirable side reactions for certain monomers and/or loss of protein function. To circumvent these problems, we turned our attention to protein-initiated cryopolymerization of 1,2-dithiolanes at a biorelavant lower $[M]_0$, based on the rationale that reducing the reaction temperature would attenuate the entropy penalty and thus boost the equilibrium constant (K_{eq}) based on the Van't Hoff plot (Figure 1B and see SI). Density function theory (DFT) calculations of all possible transition states implied that the energy barrier of the ROP (Figures 1C and S5 for secondary thiolate and Figure S6 for primary thiolate) was low enough to ensure a satisfactory reaction rate even at reduced temperatures. Most importantly, there is substantial evidence demonstrating that reactions are often, counterintuitively, accelerated in frozen systems due to the fact that solutes are concentrated through their expulsion

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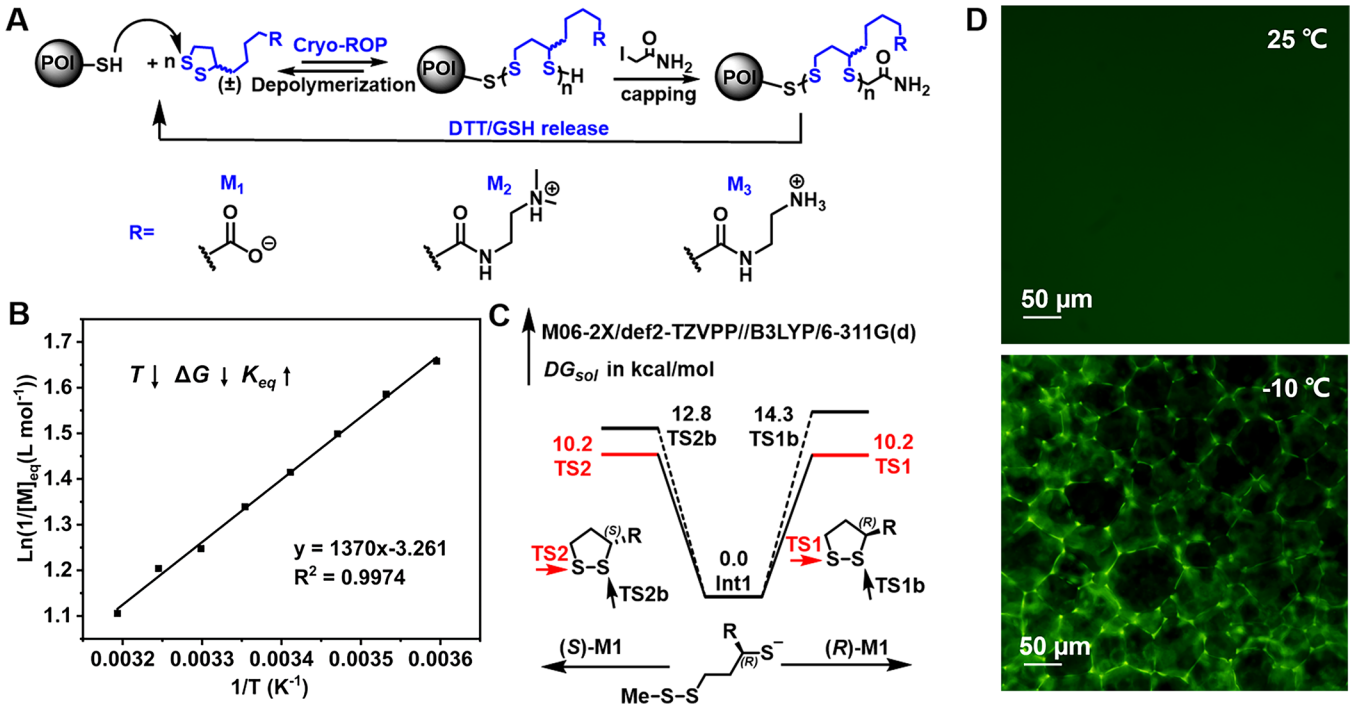

Figure 1. (A) Scheme of protein-initiated cryopolymerization of various 1,2-dithiolanes (**M1**–**M3**) for the GF synthesis and reversible degrafting of protein–PDS conjugates. (B) Van't Hoff plot of the ROP of **M1**. (C) DFT calculation of the energy barrier for four possible ROP transition states (TS); arrows point to the sulfur atoms being attacked in each TS (available in Figure S5). (D) Fluorescence microscopy of the reaction mixture of TEV-Cys-EGFP and **M1** at 25 and −10 °C.

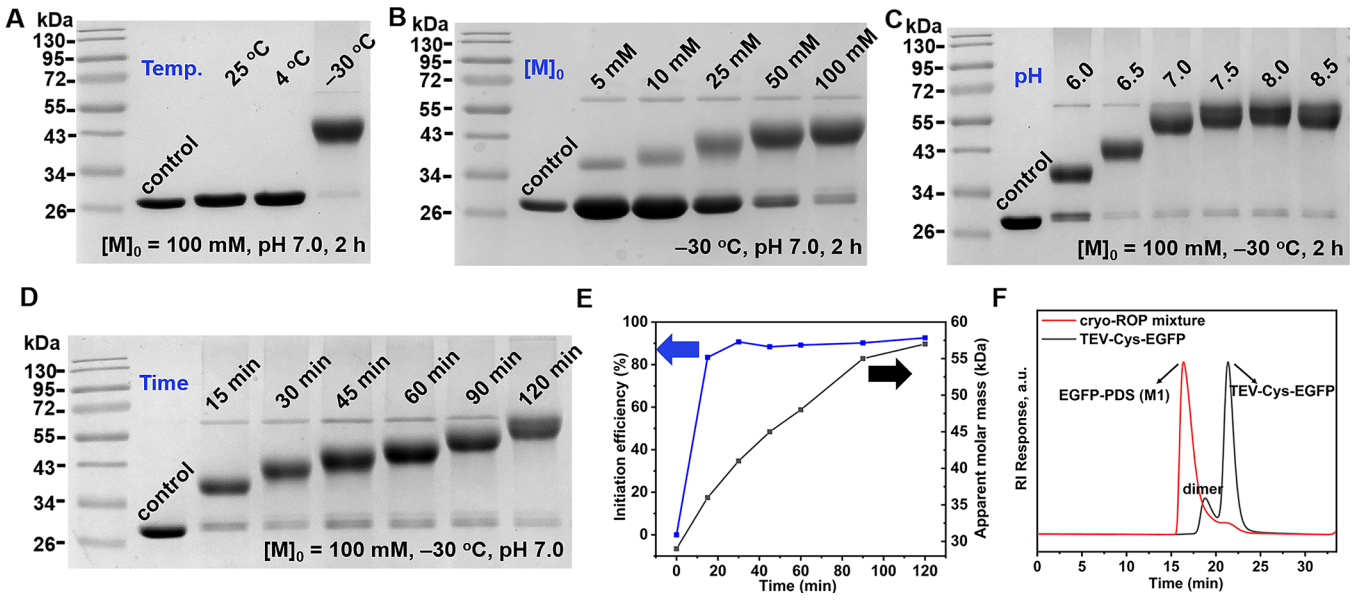

Figure 2. Optimization of the TEV-Cys-EGFP (2.0 mg/mL)-mediated cryo-ROP of **M1**. (A–D) Nonreducing SDS PAGE gel of the ROP at various (A) temperatures, (B) $[M]_0$, (C) pH, and (D) reaction time. (E) Plots of the initiation efficiency and apparent M_n as a function of incubation time. (F) SEC of TEV-Cys-EGFP (black) and the mixture (red) of TEV-Cys-EGFP-mediated cryo-ROP of **M1** (pH 7.0, $[M]_0$ = 100 mM; −30 °C for 2 h).

from ice crystals.[33,34] Indeed, fluorescent microscopic studies clearly indicated a significantly augmented local concentration of the protein substrate in the nonfrozen liquid phase at −10 °C (Figure 1D). In contrast, the EGFP fluorescence was homogeneously distributed at a much lower signal intensity in the same mixture at room temperature.

To test the hypothesis, we performed TEV-Cys-EGFP-mediated ROP of **M1** at −30 °C ($[M]_0$ = 100 mM, Figure 2A) to produce the conjugate denoted as **EGFP-PDS(M1)**. As expected, the frozen reactions afforded a plausible product band with high initiation efficiency (IE, defined as the percentage of protein participating in the ROP) in nonreducing SDS-PAGE gel analysis (Figure 2A). Careful investigation of the cryo-ROP from 0 to −30 °C suggested the one at −30 °C gave the highest IE and molar mass (M_n), possibly due to the greater K_{eq} at lower temperatures (Figure

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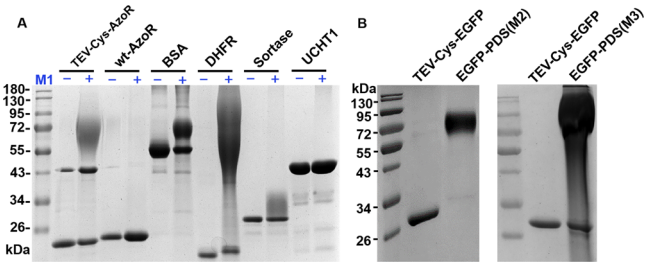

Figure 3. Nonreducing SDS PAGE gel of (A) various protein-mediated cryopolymerizations of **M1** and (B) purified **EGFP-PSD(M2)** and **EGFP-PSD(M3)** conjugates. Typical conditions: pH 7.0, protein concentration 2.0 mg/mL, $[M]_0$ = 100 mM, −30 °C, 2 h; gel was stained with Coomassie brilliant blue R-250.

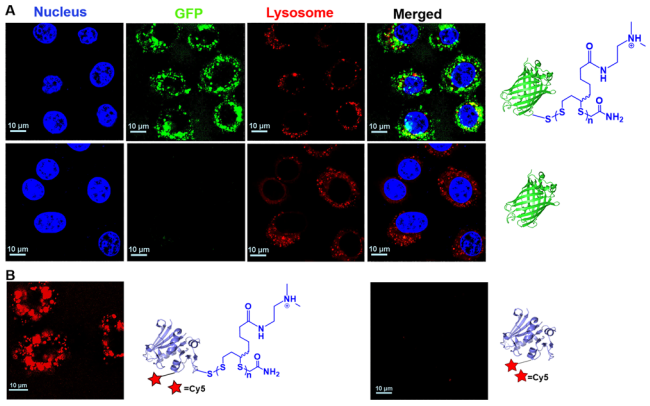

Figure 5. Cellular uptake of **protein-PDS(M2)**. (A) CLSM images of HeLa cells incubated with GFP or **GFP-PDS(M2)** (100 nM) at pH 6.8 for 1 h. (B) CLSM images of HeLa cells incubated with Cy5-labeled DHFR and **DHFR-PDS(M2)** (20 nM) at pH 6.8 for 1 h.

S7). **M1** was found to increase the IE and M_n of the resultant conjugates in a dose-dependent manner at −30 °C (Figure 2B). Of note, product formation could be detected even at a low $[M]_0$ of 5 mM. Furthermore, optimization of buffer pH allowed us to achieve an IE from ∼70% to >95% when the pH was raised from 6.0 to 6.5 or above ($[M]_0$ = 100 mM, Figure 2C; $[M]_0$ = 50 mM, Figure S8). The apparent M_n of the conjugates gradually increased with pH, but plateaued in the pH range of 7.5−8.5 (Figure 2C), attributable to the pK_a, and thus reactivity, of thiol. Kinetic study indicated an 80% IE within the first 15 min at −30 °C, which plateaued at ∼90% within 30 min (Figure 2D and Figures S9 and S10). The apparent M_n of the conjugates exhibited a linear increase from 29 kDa to ∼55 kDa during the first 90 min ($[M]_0$ = 100 mM, Figure 2E), which corresponded to ∼8.2% **M1** consumption. Given this, [M] can be considered constant and the linear growth of M_n with reaction time (zero-order kinetics) thus indicates a living chain growth. Moreover, size exclusion chromatography (SEC) analysis of the reaction mixture showed a monomonal sharp peak of the product and a small shoulder peak of the unreacted TEV-Cys-EGFP, further confirming the high IE and narrow dispersity of the conjugates (Figure 2F). Based on these results, we conducted most of the

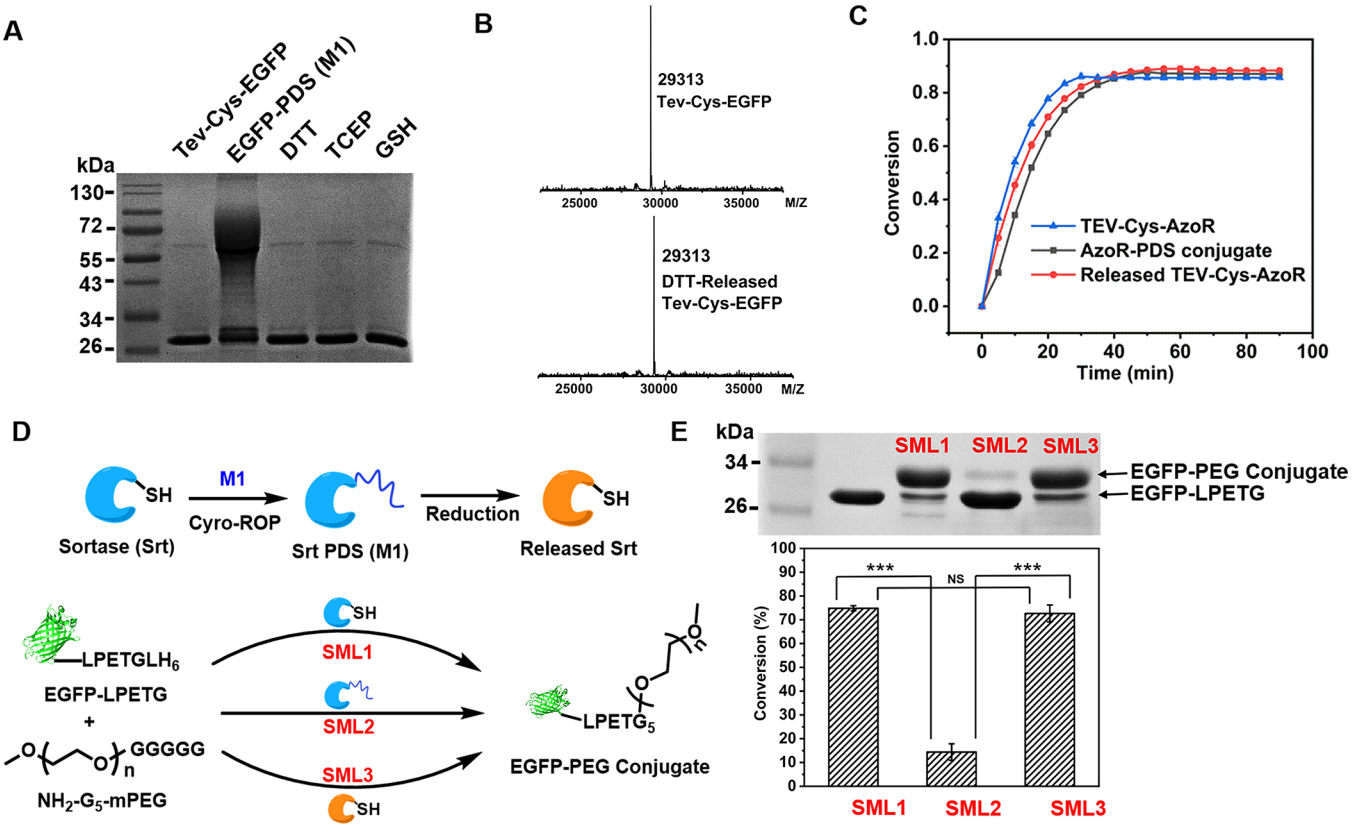

Figure 4. Traceless release of native protein from protein−PDS conjugates. (A) SDS PAGE gel of **EGFP-PDS(M1)** treated with various reducing agents at 37 °C for 10 min. (B) ESI-MS spectrometry of pristine (top) and released (bottom) TEV-Cys-EGFP after DTT treatment (2 mM, 10 min, 37 °C). (C) Enzymatic assay of pristine TEV-Cys-AzoR, purified **AzoR-PDS(M1)**, and the released TEV-Cys-AzoR at room temperature. (D) Cartoon illustration and (E) SDS-PAGE gel analysis of various Srt-mediated ligation of EGFP-LPETG and NH_2-G_5-mPEG.

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subsequent studies at pH 7.0 and −30 °C, with $[M]_0$ of 50/100 mM and 2 h of incubation.

Next, we examined the scope and selectivity of Cryo-ROP of 1,2-dithiolanes by using a variety of proteins and monomers. TEV-Cys-AzoR, a mutant enzyme containing a reactive Cys, was found to be amenable to Cryo-ROP, whereas wt-AzoR, containing no accessible Cys, was completely unreactive ($[M]_0$ = 100 mM, Figure 3A; $[M]_0$ = 50 mM, Figure S11). Native proteins bearing a reactive Cys, such as bovine serum albumin (BSA), sortase A (Srt), and dihydrofolate reductase (DHFR), were all suitable macroinitiators achieving 60−80% IE (Figure 3A). Moreover, incubation of **M1** with UCHT1, a human anti-CD3 fragment of antigen binding (FAB) antibody bearing two disulfide bonds between its heavy and light chains, at −30 °C gave no detectable changes of the antibody during non-reducing SDS-PAGE gel electrophoresis (Figure 3A). These results collectively demonstrated that the cryopolymerization of 1,2-dithiolanes requires a reactive Cys in the POI and is not affected by the presence of native disulfide bonds. The generality of 1,2-dithiolanes was also tested by using cationic monomers such as **M2** and **M3** (Figure 1A). Both monomers gave satisfactory ROPs, affording polycationic **EGFP-PDS-(M2)** and **EGFP-PDS(M3)** conjugates successfully (Figure 3B).

We next investigated whether the native POI could be released from the PDS conjugate in a traceless fashion under reducing conditions (Figure 4A). Both SDS-PAGE gel (Figure 4A) and electrospray ionization mass spectrometry (Figure 4B and Figures S12 and S13) found that treatment of **EGFP-PDS(M1)** with tris(2-carboxyethyl) phosphine (TCEP), DL-dithiothreitol (DTT), or glutathione (GSH) at 37 °C for 10 min all produced the cleaved TEV-Cys-EGFP identical to the pristine protein. We then evaluated whether the activities of two model proteins, Srt and TEV-Cys-AzoR, were affected by polymer conjugation. The catalytic activity of Srt is dependent on the same Cys that would participate as initiator in the ROP of **M1**. Therefore, we expected the resultant conjugate to be nonfunctional in sortase-mediated ligation (SML) due to the occupation of its catalytic thiol by the PDS. Indeed, while wt-Srt was shown to be able to promote the ligation between EGFP-LPETG and NH_2-G_5-mPEG2K,[32] purified **Srt-PDS-(M1)** almost completely lost its SML activity (SML1-2, Figure 4D,E). In contrast, Srt that was freshly detached from **Srt-PDS(M1)** (DTT treated) regained its ligation function with comparable yield to pristine Srt (SML3, Figure 4D,E). On the other hand, the catalytic function of TEV-Cys-AzoR is independent of Cys. Correspondingly, purified **AzoR-PDS-(M1)** conjugate and the released TEV-Cys-AzoR both showed unaffected enzymatic activity relative to the pristine AzoR (Figure 4C). Taken together, the cryo-ROP-produced protein−PDS conjugates can release native proteins without compromised biological functions.

Next, we tested whether the protein−PDS conjugates exhibited similar cell-penetrating behaviors to those previously reported. To this end, **M2** with a tertiary amine side chain was grafted to two model proteins, TEV-Cys-EGFP and Cy5-labeled DHFR, via cryopolymerization. The purified conjugates (see SI) were incubated with HeLa cells in varying concentrations. Confocal laser scanning microscopy (CLSM) detected pervasive and intense fluorescence inside the treated cells when 100 nM **EGFP-PDS(M2)** (Figure 5A) or 20 nM Cy5-labeled **DHFR-PDS(M2)** was used (Figure 5B). Importantly, the fluorescence signals were shown to have only minor overlap with the lysosomes (Figure 5A). On the contrary, cell treatment with neither free protein produced any significant intracellular fluorescence.

In conclusion, we developed a simple and general cryo-ROP strategy that enabled the efficient synthesis of cell-penetrating protein−PDS conjugates. The polymerization is highly controlled and selective for a broad scope of proteins that bear a reactive Cys. The resultant conjugates can be degrafted under mild conditions to regenerate fully functional proteins in a traceless manner. These unique features, coupled with the functional versatility of PDS,[35,36] make such conjugates highly useful in a broad range of applications, such as dynamic switch of protein functions, cytosolic delivery of protein therapeutics, and protein purification. The method is also potentially useful for the in situ growth of other types of polymers from protein surfaces.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.9b12937.

Materials and methods, experimental protocols, ^{1}H NMR spectra of **M2** and **M3**, SDS-PAGE gel electrophoresis, DFT calculation, and coordination parts (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Hua Lu − *Peking University, Beijing, People's Republic of China*; orcid.org/0000-0003-2180-3091; Email: chemhualu@pku.edu.cn

Other Authors

Jianhua Lu − *Peking University, Beijing, People's Republic of China*

Hao Wang − *Peking University, Beijing, People's Republic of China*

Ziyou Tian − *Peking University, Beijing, People's Republic of China*

Yingqin Hou − *Peking University, Beijing, People's Republic of China*

Complete contact information is available at: https://pubs.acs.org/10.1021/jacs.9b12937

Notes

The authors declare no competing financial interest.

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