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A Concise Approach to Site-Specific Topological Protein–Poly(amino acid) Conjugates Enabled by *in Situ*-Generated Functionalities

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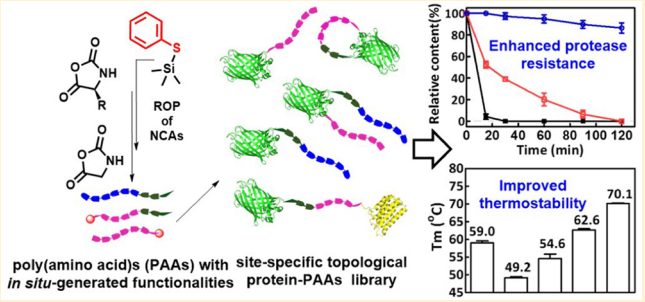
Supporting Information

ABSTRACT: Controlling the topology of polymer-modified proteins has attracted growing interest. However, one of the main challenges in this field is the lack of efficient and site-specific methods for installing multiple bioorthogonal functionalities on substrate polymers. We report here an orchestrating strategy that provides easy access to various topological protein–poly(amino acid) (PAA) conjugates in high yields. This method features the *in situ* installation of two "chemical handles", including a thioester for native chemical ligation and a polyglycine nucleophile for sortase A-mediated ligation, at both ends of substrate PAAs. As a result, neither pre-functionalization of initiator or monomer units, nor post-polymerization modification of the resultant polymers, is necessary. Site-specific topological conjugates, particularly circular conjugates, can be conveniently synthesized under mild conditions from the functionalized PAAs. The biomedical utility of our method is demonstrated by the rapid and efficient generation of several therapeutic interferon-α conjugates, which exhibit significantly enhanced protease resistance and thermostability. Given the versatility of both PAAs and proteins, the method offers a convenient approach to producing libraries of conjugates for biological applications.



INTRODUCTION

Protein–polymer conjugation (e.g., PEGylation)[1] is widely used to modulate protein functions, effect hierarchical self-assembly, and deliver therapeutic agents.[2] Recently, controlling the topology of such conjugates has become a nascent field attracting growing attention, inspired by previous findings that cyclized proteins often possess beneficial pharmaceutical and biological properties ranging from enhanced thermal stability to improved protease resistance.[3] Despite recent advances,[4] a major challenge in protein–polymer conjugates is the generation of heterogeneous populations and poor molecular weight (MW) control due to the use of nonselective chemical ligation methods. Moreover, topological conjugates, such as head-to-tail circular protein–polymer conjugates, still remain largely unexplored.[3c,d] Often, a key hurdle impeding the synthesis is not the lack of appropriate reactions to ligate the two macromolecules, but the redundant and cost-ineffective process of introducing multiple orthogonal functionalities that can be used for conjugation (e.g., producing heterotelechelic polymers). Therefore, a universal and robust synthetic platform that allows the traceless and precise generation of polymers bearing orthogonal ligation functionalities is highly desired.

Polyethylene glycol (PEG), albeit effective, is nondegradable, provides very few functionalities, and may lead to accelerated blood clearance upon repetitive injections.[5] To develop alternative materials of PEG, polypeptides, or poly(amino acid)s (PAAs), are promising candidates for their protein-like backbone, versatile side-chain functionality, and tunable degradability.[6] Indeed, the fusion of unstructured polypeptides such as XTEN or elastin-like protein to target proteins has been tested, which leads to superior pharmacological properties.[7] However, the production of such fusion proteins requires substantial genetic modification and is usually limited to the 20 canonical amino acids. In comparison, synthetic PAAs generated by the ring-opening polymerization (ROP) of α-amino acid *N*-carboxyanhydrides (NCAs)[8] are inexpensive and easily accessible in large scale, and can offer expanded chemical diversity and higher-ordered structures exploitable for numerous biomedical applications.[9] Despite these advantages, there are only a few reports on randomly labeled protein–PAA conjugates,[10] and no study has been conducted on their topological control.

Herein, we describe a one-pot, two-step polymerization process that produces heterotelechelic block PAAs leading to the facile synthesis of site-specific topological protein–PAA conjugates. Specifically, two orthogonal chemical handles, including a phenyl thioester for native chemical ligation[4d,11] (NCL, Figure 1A) and a polyglycine for sortase A-mediated ligation[12] (SML, Figure 1B), are *in situ* installed at the *C*- and *N*-

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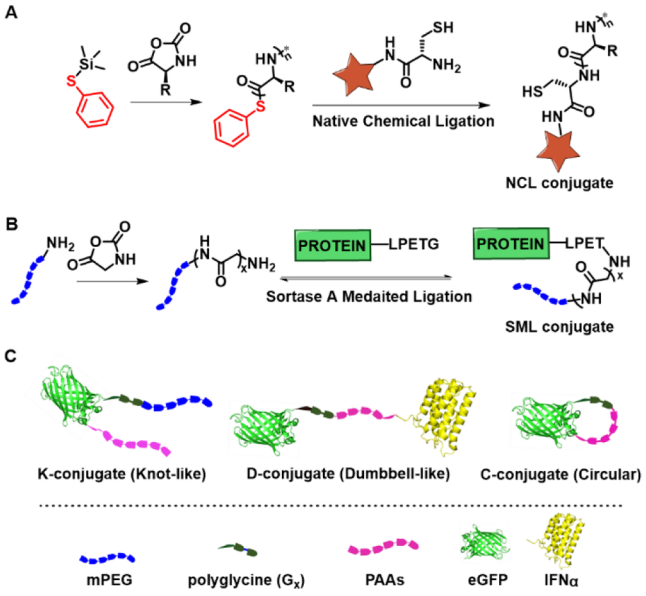

Figure 1. (A) Synthesis of poly(amino acid)s (PAAs) by phenyl trimethylsilyl sulfide (PhS-TMS)-mediated controlled ROP of NCAs. A thioester was installed to the *C*-terminus of the PAA and serves as a chemical handle for native chemical ligation (NCL). (B) A polyglycine (G_x) nucleophile was generated by the ROP of glycine NCA as a substrate for sortase A-mediated ligation (SML). (C) Illustration of various site-specific topological protein−PAA conjugates described in this work.

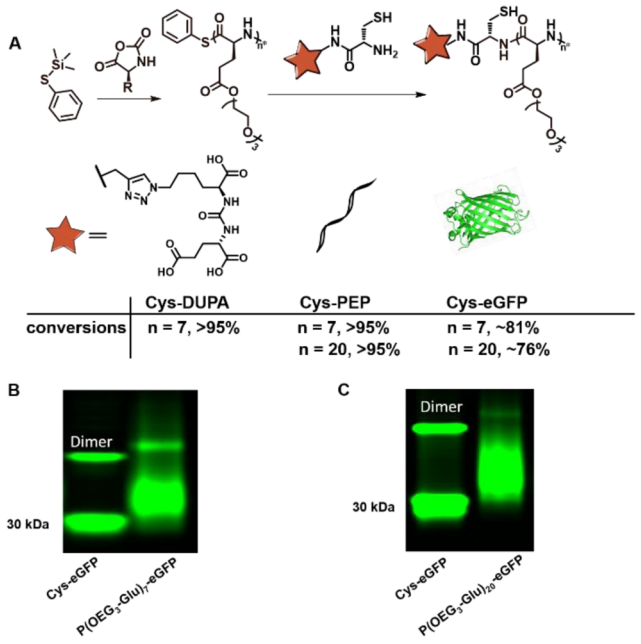

Figure 2. Synthesis and characterization of various NCL conjugates. (A) NCL of P(OEG_3-Glu)$_n$-SPh to *N*-cysteine-functionalized small molecules, peptides, and proteins. (B,C) Fluorescent native gel of P(OEG_3-Glu)$_7$-eGFP (B) and P(OEG_3-Glu)$_{20}$-eGFP (C) after purification.

termini of PAAs, respectively. As a result, neither pre-functionalization of initiator or monomer units, nor post-polymerization modification of the resultant polymers, is necessary. Combinatorial execution of NCL and SML under mild conditions enables the rapid generation of various protein−PAA conjugates with well-defined topological structures, including, in particular, cyclic conjugates (Figure 1C) that exhibit remarkable thermostability and protease resistance. Given the abundance and versatility of both PAAs and proteins, the method holds tremendous potential for biological and pharmaceutical applications.

RESULTS AND DISCUSSION

Synthesis of Protein−PAA NCL Conjugates. We began our study by creating protein−PAA conjugates via NCL. One of the main challenges of this approach lies in the difficulty of constructing a thioester linkage in PAAs, which in fact has been an active field of Fmoc solid-phase peptide synthesis (SPPS).[13] We have previously demonstrated that phenyl trimethylsilyl sulfide (PhS-TMS), a commercially available initiator, could mediate rapid ROP of NCAs and produce well-defined PAAs tethering an *in situ*-generated *C*-terminal phenyl thioester (PAA-SPh).[14] However, the ligation of PAA-SPh to complex substrates, especially macromolecules with rich functionalities, has yet to be established. We therefore sought to first investigate the NCL reactivity of PAA-SPh toward various cysteine-functionalized substrates ranging from small molecules, peptides to proteins. The *N*-acetyl-capped poly(γ-(2-(2-(2-methoxyethoxy)ethoxy)ethyl L-glutamate) (P(OEG_3-Glu)$_n$-SPh, n = feeding M/I ratio, Figures S1 and S2), produced by PhS-TMS-mediated ROP of γ-(2-(2-(2-methoxyethoxy)ethoxy)ethyl L-glutamate NCA(OEG_3-GluNCA),[15] was selected as a model PAA for its excellent aqueous solubility and "stealthy" PEG-like side chain (Figure 2A). Cys-DUPA (Figure 2A), a small-molecule ligand binding to the human prostate-specific membrane antigen,[16] was ligated to P(OEG_3-Glu)$_7$-SPh at a 1:1 molar ratio under room temperature, affording the product with >95% conversion (Figure S3). Similarly, NCL of P(OEG_3-Glu)$_n$-SPh (n = 7 or 20) to Cys-PEP, a 18-mer peptide (sequence: **C**GDAKGLPET-GHHHHHHK, MW = 1998 Da; Figure 2A), resulted in complete conversion at 1:1−3:1 feeding ratios (Figures S4 and S5). To generate the *N*-terminal cysteine (*N*-Cys) necessary for NCL, a short peptide tag, ENLYFQC, was fused to the *N*-termini of selected proteins and then cleaved by tobacco etch virus protease.[17] Utilizing this method, the *N*-Cys-enhanced green fluorescent protein (Cys-eGFP) was successfully produced at a yield of ∼48 mg/L. NCL of P(OEG_3-Glu)$_n$-SPh (n = 7, 20) and Cys-eGFP at room temperature for 10−12 h led to the efficient formation of well-defined conjugates with conversions ranging from ∼76% to 81% (Figure 2B,C and Figure S6). Importantly, mixing ENLYFQ-Cys-eGFP and P(OEG_3-Glu)$_n$-SPh under the same conditions did not afford any product, indicating the NCL reaction is indeed *N*-Cys selective. It is also worth noting that our ligation protocol required a significantly lower molar ratio of P(OEG_3-Glu)$_n$-SPh over Cys-eGFP (3:1 to 5:1) than what would normally be needed by conventional protein−polymer conjugation methods (typically >20:1). Moreover, unlike in standard NCL processes, additives such as 4-mercaptophenylacetic acid were not necessary, which minimized the likelihood of protein destabilization and/or precipitation. Indeed, we did not observe any significant decrease in the fluorescence of eGFP during the conjugation. Together, these results underscored the high NCL reactivity of PAA-SPhs toward various substrates, including those challenging and complex biomolecules.

Synthesis of Protein−PAA SML Conjugates. We next investigated SML for protein−PAA conjugation owing to its high efficiency and chemoselectivity (SML-conjugate, Figure 1B). Sortase A (SrtA) from *Staphylcoccus aureus* is an enzyme

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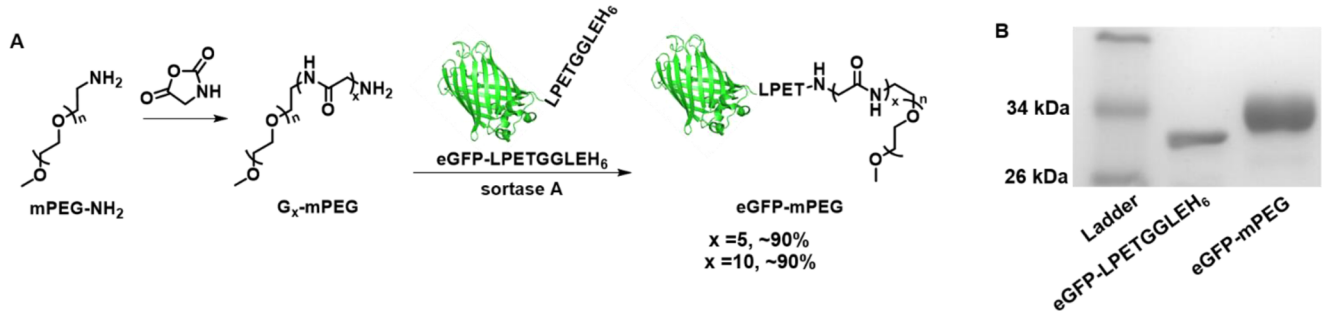

Figure 3. (A) Synthesis and (B) SDS-PAGE gel characterization of a SML-conjugate eGFP-mPEG. A polyglycine tag was generated by mPEG-NH_2-mediated ROP of GlyNCA and served as a substrate of SrtA.

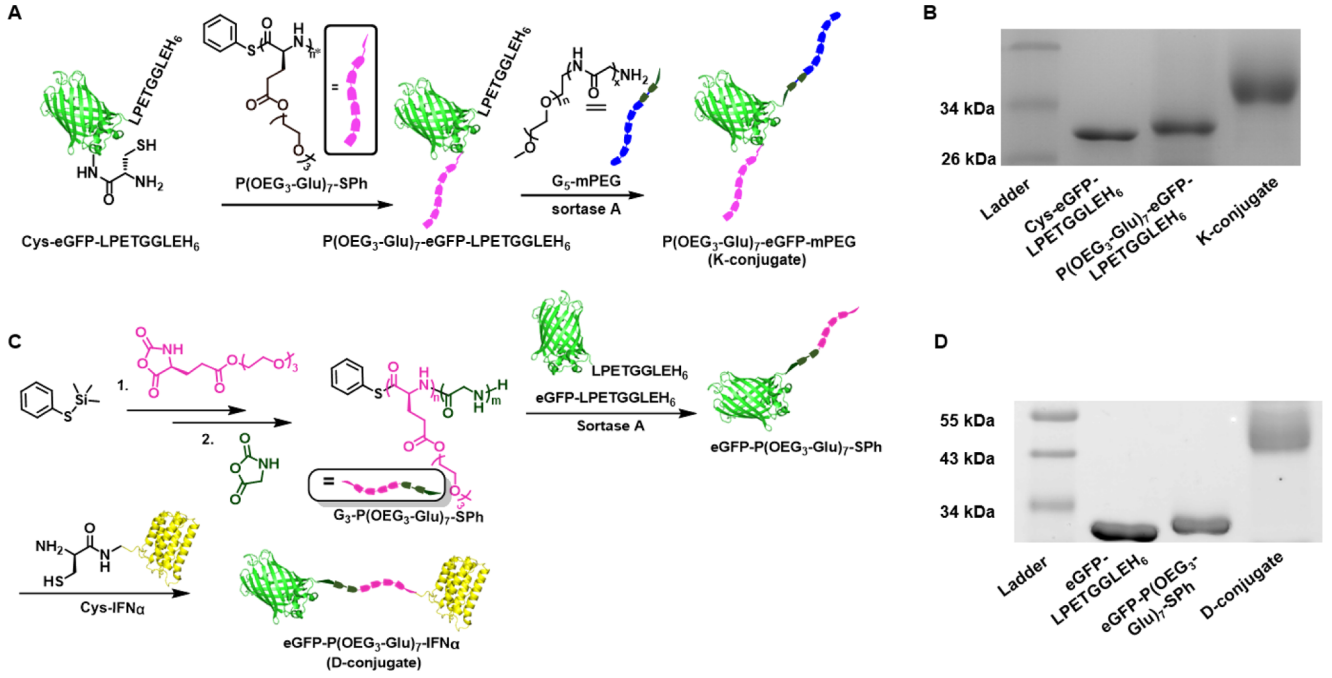

Figure 4. Synthesis and characterization of K-conjugate and D-conjugate. (A,B) Synthetic scheme (A) and SDS-PAGE gel analysis (B) of the K-conjugate P$(OEG_3$-Glu$)_7$-eGFP-mPEG. (C,D) Synthetic scheme (C) and SDS-PAGE gel analysis (D) of the D-conjugate eGFP-P$(OEG_3$-Glu$)_7$-IFNα.

catalyzing the transpeptidation between an LPXTG motif (X = any canonical amino acid) and the *N*-terminal amino group of a polyglycine tag. Previously, it has been noted that StrA can accommodate polyglycine substrates of various lengths (G_x, x = 1−5),[12d] which prompted us to test whether the enzyme could also recognize the polyglycine generated by the ROP of glycine NCA (Gly-NCA). To this end, two block copolymers denoted as G_x-mPEG (x = 5 or 10, Figure S7) were prepared by mPEG-amine (MW = 2000 Da) initiated Gly-NCA polymerization (Figure 3A). After optimizing reaction conditions (Figure S8), SML of G_x-mPEG (5 equiv) to eGFP bearing a *C*-terminal $LPETGGLEH_6$ tag (eGFP-$LPETGGLEH_6$) proceeded smoothly within 30 min in the presence of 0.1 equiv of SrtA and afforded the desired products in ∼90% conversion (Figure 3B). It should be emphasized that, although G_x is accessible by SPPS or genetic engineering, our method, with comparable SML efficiency,[3c,12d] is simpler and more scalable, and can be readily applied to non-peptidic substrates.

Synthesis of Protein−PAA K- and D-Conjugates. The combinatorial application of NCL and SML was demonstrated in the synthesis of a wide range of challenging multidomain[18] and circular protein−PAA conjugates. Sequential attachment of P$(OEG_3$-Glu$)_n$-SPh via NCL and G_x-mPEG via SML to Cys-eGFP-$LPETGGLEH_6$ (Figure 4B and Figure S9) furnished a knot-like polymer−protein−polymer conjugate (K-conjugate, Figure 4A). In another example, a dumbbell-like heterodimeric protein−polymer−protein conjugate (D-conjugate) was synthesized from a heterotelechelic block PAA G_3-P$(OEG_3$-Glu$)_7$-SPh (Figure S10), which was generated by one-pot, two-step ROP of OEG_3-GluNCA and Gly-NCA (Figure 4C) without additional post-polymerization modifications. SML of G_3-P$(OEG_3$-Glu$)_7$-SPh with eGFP-$LPETGGLEH_6$, followed by NCL of the resultant product with Cys-IFNα, led to the efficient generation of the desired D-conjugate (Figure 4D and Figure S11).

Synthesis of Peptide/Protein−PAA Circular Conjugates. Our ligation method can also be used to prepare head-to-tail cyclic protein−PAA conjugates (C-conjugate, Figure 5A), which have remained synthetically inaccessible. In a model reaction, NCL of heterotelechelic G_3-P$(OEG_3$-Glu$)_7$-SPh and bifunctional Cys-PEP (sequence: **C**GDAKG**LPETG**HHHHHHK) was conducted to afford the linear intermediate, which then readily underwent SML-mediated cyclization to generate

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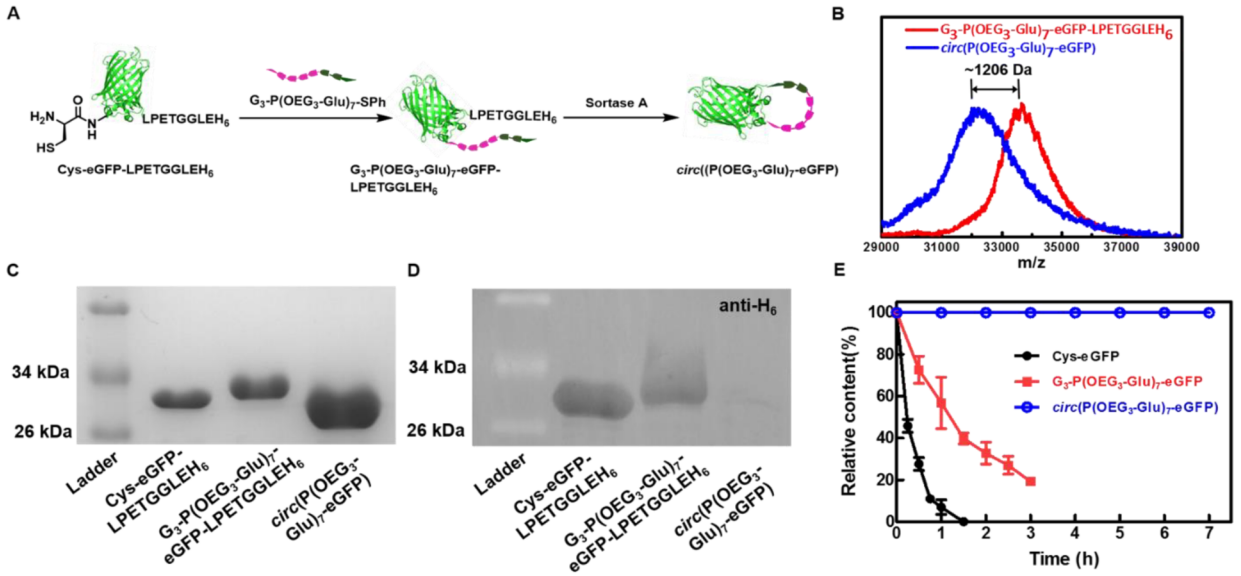

Figure 5. Synthesis and characterization of C-conjugate *circ*(P(OEG$_3$-Glu)$_7$-eGFP). (A) Synthetic scheme for *circ*(P(OEG$_3$-Glu)$_7$-eGFP). (B,C,D) Successful generation of *circ*(P(OEG3-Glu)7-eGFP) was confirmed by MALDI-TOF mass spectrum (B), SDS-PAGE gel (C), and anti-H$_6$ tag Western blot (D) of *circ*(P(OEG$_3$-Glu)$_7$-eGFP). (E) Degradation kinetics of Cys-eGFP, G$_3$-P(OEG$_3$-Glu)$_7$-eGFP-LPETGGLEH$_6$, and *circ*(P(OEG$_3$-Glu)$_7$-eGFP) incubated with 80 μg/mL carboxypeptidase C; the experiments were repeated as triplicates and error bars represent standard deviations.

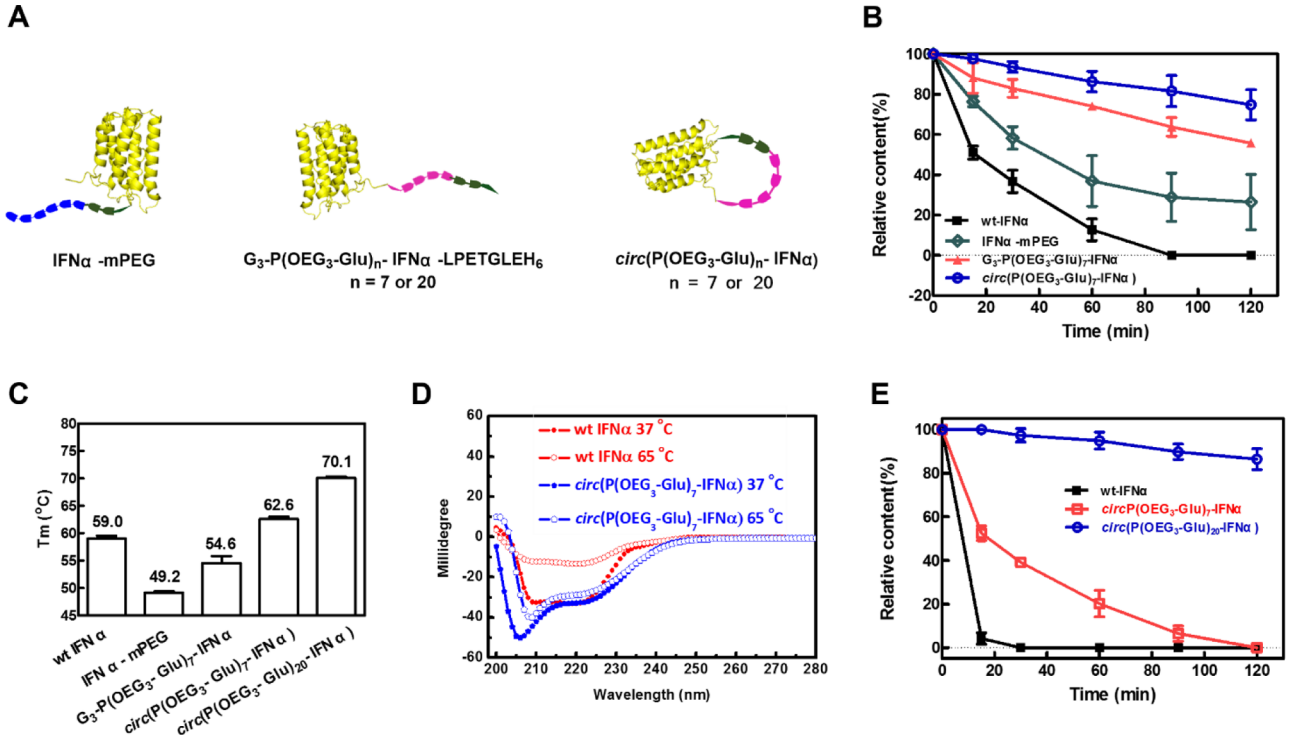

Figure 6. Protease resistance and thermostability of wt- and polymer-conjugated IFNα. (A) Cartoon illustration of IFNα conjugates prepared in this study. (B) Degradation kinetics of wt-IFNα, G$_3$-P(OEG$_3$-Glu)$_7$-IFNα and *circ*(P(OEG$_3$-Glu)$_7$-IFNα) by trypsin (0.5 μg/mL). (C) Melting temperatures (T_m) of wt-IFNα, IFNα-mPEG, G$_3$-P(OEG$_3$-Glu)$_7$-IFNα and *circ*(P(OEG$_3$-Glu)$_n$-IFNα) (n = 7 and 20) measured by thermofluor assay. (D) CD spectra of wt-IFNα and *circ*(P(OEG$_3$-Glu)$_7$-IFNα) at 37 and 65 °C. (E) Degradation kinetics of wt-IFNα, *circ*(P(OEG$_3$-Glu)$_7$-IFNα) and *circ*(P(OEG$_3$-Glu)$_{20}$-IFNα) by trypsin(2.5 μg/mL). All experiments were repeated as triplicates, and error bars represent standard deviations.

the desired C-conjugate in 61% overall yield. The cyclization product was unambiguously confirmed by MALDI-TOF mass spectrometry (Figure S12). Next, we used Cys-eGFP-LPETGGLEH$_6$ as the cyclization partner with G$_3$-P(OEG$_3$-Glu)$_7$-SPh. Again, the two substrates were first ligated via NCL into the linear intermediate and then cyclized by SML in a diluted solution (~1.0−1.5 mg/mL). This conjugation sequence resulted in the cleavage of a GGLEH$_6$ tag (1160 Da) from the cyclization product, which allowed the reaction to be readily monitored by both MALDI-TOF mass spectrometry and Western blotting, and enabled convenient purification by passing the resultant mixture through a NiNTA column and collecting the flow-through. Indeed, the formation of the C-conjugate *circ*(P(OEG$_3$-Glu)$_7$-eGFP) was confirmed by

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MALDI-TOF analysis that indicated a MW decrease of ~1200 Da (Figure 5B), a shift of the eGFP protein band toward the lower MW range (Figure 5C), and the disappearance of the C-conjugate band in the anti-H_6 Western blot (Figure 5D). In contrast, P(OEG$_3$-Glu)$_7$-eGFP-LPETGGLEH$_6$, which contained no *N*-terminal polyglycine, did not cyclize in the presence of StrA under similar conditions. A ~42% purified yield was obtained for *circ*(P(OEG$_3$-Glu)$_7$-eGFP) with ~95% purity and less than 5% higher MW byproduct (Figure S13B–D). In all cases, the SML method that we developed required only 0.05–0.1 equiv of SrtA and 30 min of reaction time, whereas previously published SrtA-based protein/peptide cyclization examples were considerably less efficient in comparison (typically 0.33–1 equiv of SrtA and 16–88 h of reaction time).[3c,19] Moreover, no proteolytic degradation of *circ*(P(OEG$_3$-Glu)$_7$-eGFP) was observed up to 7 h when it was incubated with carboxypeptidase C, a *C*-terminal protease with broad amino acid specificity. This result offered additional evidence for cyclization, which was consistent with the unavailability of the *C*-terminal carboxylic acid due to the successful cyclization. In contrast, when subjected to the same treatment, ~80% of the linear precursor G$_3$-P(OEG$_3$-Glu)$_7$-eGFP-LPETGGLEH$_6$ and almost 100% of Cys-eGFP were degraded in ~3 and ~1.5 h, respectively (Figure 5E and Figure S14A–C). Notably, a higher substrate concentration (e.g., 6.0 mg/mL) led to a significantly lower cyclization yield (~13%) due to precipitation of protein in SML buffer. To examine the efficiency of PAAs with greater MWs, we subjected G$_3$-P(OEG$_3$-Glu)$_{30}$-SPh (MW ≈ 8500 Da) to protein cyclization under SML condition. The cyclized product was obtained at a ~15% yield after purification (data not shown).

Protease Resistance and Thermostability of Various Interferon-α PAA Conjugates. Wild-type interferon-α[20] (wt-IFNα) is an important therapeutic protein that often requires polymer modification in clinical applications due to its suboptimal *in vitro* and *in vivo* stability. To demonstrate the benefits of PAA modification, we prepared several IFNα conjugates with ~62–80% purified yields, including a NCL conjugate G$_3$-P(OEG$_3$-Glu)$_7$-IFNα, a SML conjugate IFNα-mPEG, and a C-conjugate *circ*(P(OEG$_3$-Glu)$_7$-IFNα), using the ligation protocol described earlier (Figure 6A and Figures S15–S17). All three substrate polymers used in conjugation have near-identical MWs (~2000 Da) to minimize the impact of mass differences. Trypsin digestion assay indicated that both *circ*(P(OEG$_3$-Glu)$_7$-IFNα) and G$_3$-P(OEG$_3$-Glu)7-IFNα were significantly more protease-resistant than IFNα-PEG and wt-IFNα (Figure 6B and Figures S18 and S19), which were rapidly degraded. Next, we examined the thermal stability of the free and conjugated IFNαs by measuring their melting temperatures (T_m) in a thermofluor assay (Figure 6C and Figure S20).[3c] Interestingly, both IFNα-PEG (49.2 °C) and G$_3$-P(OEG$_3$-Glu)$_7$-IFNα (54.6 °C) showed lower T_m's than wt-IFNα (59.0 °C); in contrast, the T_m of *circ*(P(OEG$_3$-Glu)$_7$-IFNα increased to 62.6 °C, which was comparable to the reported value of another cyclized IFNα.[3c] To further verify *circ*(P(OEG$_3$-Glu)$_7$-IFNα)'s enhanced thermostability, the conjugate and wt-IFNα were separately subjected to a heating ramp from 37 to 65 °C over a course of 90 min. Circular dichroism (CD) spectroscopy detected no obvious loss of helicity in *circ*(P(OEG$_3$-Glu)$_7$-IFNα) after the heat shock, whereas wt-IFNα became denatured and precipitated at ~65 °C (Figure 6D). Similarly, *circ*(G$_3$-P(OEG$_3$-Glu)$_7$-IFNα) undergoing lyophilization showed no change in its CD pattern, suggesting its ability to withstand freeze-drying without denaturation (Figure S21). It should be emphasized that our *circ*(P(OEG$_3$-Glu)$_7$-IFNα) showed comparable *in vitro* anti-proliferation activity to wt-IFNα in Daudi cells, indicating that the cyclization did not affect their biological activities (Figure S22). Together, these results confirmed that a circular topology can protect protein conjugates against various environmental stresses including protease degradation and heat shock.

To examine how the MW of PAAs affects the protease and thermal stability of C-conjugates, we synthesized the much bulkier *circ*(P(OEG$_3$-Glu)$_{20}$-IFNα) and incubated it with trypsin at a concentration 5 times higher than what was used in Figure 6B. Compared to the smaller *circ*(P(OEG$_3$-Glu)$_7$-IFNα), which showed slightly enhanced resistance over wt-IFNα under this condition, almost no degradation of *circ*(P(OEG$_3$-Glu)$_{20}$-IFNα) was detected up to 2 h of trypsin treatment (Figure 6E and Figure S23). In thermofluor assay, *circ*(P(OEG$_3$-Glu)$_{20}$-IFNα) showed a T_m value of 70.1 °C, which was ~11.1 °C higher than that of wt-IFNα (Figure 6C and Figure S20) and arguably the highest value among all reported IFNα variants so far. Overall, these results indicated that C-conjugates with higher MWs tend to be more protease-resistant and thermostable.

■ CONCLUSIONS

In conclusion, we have developed a concise and orchestrating strategy for the one-pot production of heterotelechelic PAAs, which led to rapid and efficient generation of well-defined topological protein–PAA conjugates. Particularly, the current study provided the first synthesis of head-to-tail circular protein–PAA conjugates to our best knowledge and demonstrated the effectiveness of cyclization on improving the thermostability and protease resistance of proteins such as eGFP and IFNα. We envision that our conjugation method would enable convenient access to a wide range of well-defined protein–PAA conjugates for numerous biological and medical applications. We are currently investigating the *in vivo* anticancer efficacies of various therapeutic protein conjugates including IFNα.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b05413.

Materials and methods, instrumentations, and ^{1}H NMR, UPLC-ESIMS, MALDI-TOF MS, and SDS-PAGE data, including Figures S1–S23 and Tables S1 and S2 (PDF)

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Author Contributions

†Y. Hou and J. Yuan contributed equally to this work.

Notes

The authors declare no competing financial interest.

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