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Ring-Opening Polymerization of Amino Acid *N*-Carboxyanhydrides with Unprotected/Reactive Side Groups. I. D-Penicillamine *N*-Carboxyanhydride

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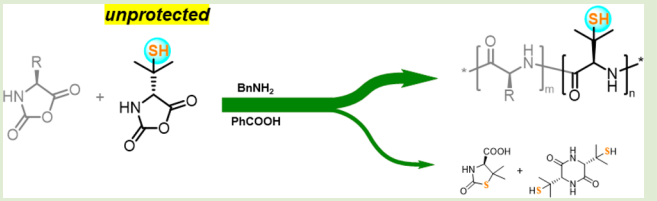
ABSTRACT: The ring-opening (co)polymerization (ROP) of *N*-carboxyanhydride (NCA) monomers bearing unprotected/reactive side groups is rare and challenging. Here, we report the ROP of a D-penicillamine NCA (Pen-NCA) monomer for the synthesis of tertiary thiol-functionalized (co)polypeptides. Through judicious selection of reaction solvents and the use of benzoic acid as an additive in the ROP, the intramolecular isomerization side reactions of Pen-NCA are suppressed, generating homo- and copolypeptides with improved yield, high molecular weight, and narrow molecular weight distributions. Successful postpolymerization modifications of the D-Pen-containing copolypeptides on the tertiary thiols are achieved with high efficiency through thiol-Michael, S_N2, and nitrosylation reactions. This work provides an efficient protection-free approach to generating functional polypeptides and creates a fundamental understanding for Pen-NCA chemistry.



Synthetic polypeptides, a.k.a. poly(amino acid)s, are biomimetic polymers with the same skeleton of natural peptides and proteins, attracting broad research interest. Thanks to their inherent biocompatibility, tunable biodegradability, and designable material properties and functions, polypeptides hold enormous application potential in the field of materials science and biomedicine.[1−4] The ring-opening polymerization (ROP) of *N*-carboxyanhydrides (NCAs) is one of the most efficient methods for the preparation of polypeptides,[1,5−7] offering excellent control over molecular weight, dispersity ($Đ$), end group fidelity, and topology of the polymerization products, albeit with no strict sequence control.

To explore the broader chemical space of polypeptides for more diverse properties and functions, a common approach is the modification of the amino acid side chains, which can be achieved through the synthesis of novel modifiable NCA monomers and/or postpolymerization modifications (PPMs).[8,9] For example, Hammond et al. reported the introduction of alkyne groups into polypeptide side chains and subsequent ROP, which allowed polypeptide derivatization via azide-alkyne cycloaddition.[10] Other functional groups, such as azide,[11] alkene,[12,13] thioether.[14,15] etc., have also been introduced into NCAs to produce the corresponding polypeptides and establish efficient side group transformations. Among those common reactive functional groups in polymer modifications, thiol (sulfhydryl) groups are particularly attractive for their rich chemistries and efficient bond-formation reactions.[16] Often, thiol-functionalized polypeptides are obtained via the homo- or copolymerization of thiol-protected L-cysteine NCAs (Cys-NCAs) followed by deprotection to regenerate the desired thiol group. For example, Heise et al. designed and synthesized Cys-NCA protected by disulfide bonds and randomly copolymerized it with monomers such as γ-benzyl L-glutamate NCA (BLG-NCA).[17] The protecting disulfide bonds of the obtained polymers were fully removed by dithiothreitol reduction, producing thiol-functionalized polypeptides. Heise et al. also demonstrated that the thiol groups on polypeptides were able to be efficiently postmodified through Michael addition and thiol-ene reactions. Jing et al. initiated the polymerization of carbobenzoxy-protected Cys-NCA with an amino-terminated polylactic acid macroinitiator, and the resulting block copolymer was deprotected and oxidatively cross-linked to give shell-cross-linked micelles.[18] Dong et al. designed a photoresponsive *S*-(*o*-nitrobenzyl)-L-cysteine NCA and initiated its ROP by amine-tethered poly(ethylene glycol) to produce micelle-forming block copolymers, whose self-assembly was effectively controlled by a photodeprotection process.[19] Moreover, in recent years, Barz et al. developed sulfonyl-protected CysNCAs.[20] By taking advantage of the good leaving ability of sulfonyl groups, disulfide-modified polycysteines were successfully synthesized by the treatment of thiol-containing small molecules. Although the aforementioned

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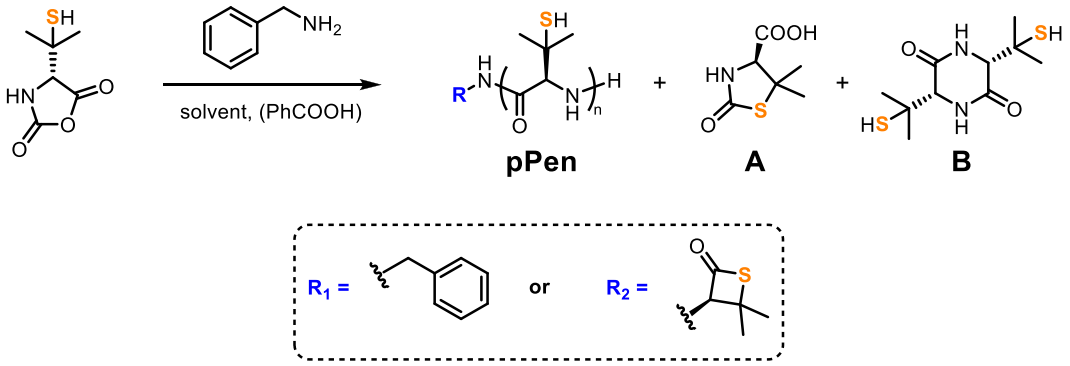
Table 1. Homopolymerization of Pen-NCA under Different Conditions[a]



entry	$[M]_0/[I]_0$	solvent	$[BA]_0$[b]	time (h)	pPen[c] (%)	A[c] (%)	B[c] (%)	DP[c] (NMR)
1	50/1	$CHCl_3$	-	50	58	28	14	12
2	50/1	DCM	-	95	48	38	14	18
3	50/1	THF	-	21	11	84	5	4
4	50/1	$CHCl_3$	0.01 M	40	60	26	14	12
5	50/1	$CHCl_3$	0.1 M	21	74	14	12	16
6	50/1	DCM	0.1 M	40	65	25	10	15

[a]Polymerizations were performed atroom temperature (~25 °C) and under air atmosphere, $[M]_0$ = 0.25 M. Conversions of monomer were monitored by IR spectroscopy and were all above 95%. [b]Concentration of benzoic acid (BA). [c]Determined with ^{1}H NMR spectra of the reaction system.

cysteine-based protection−deprotection strategies are fairly robust, the protection of amino acids and the deprotection of the polymer products are time-consuming and laborious, leading to longer synthetic routes and reduced atom efficiency. On the other hand, since the primary thiol group of cysteine is relatively sensitive to oxidative conditions and thus prone to spontaneous cross-linking, there is also a need for the development of polypeptides bearing secondary or tertiary thiols.

D-Penicillamine (D-Pen), the metabolic product of penicillin, is a natural amino acid bearing a tertiary thiol and is being used as an oral drug for the treatment of Wilson disease,[21−23] cystinuria,[24,25] and rheumatoid arthritis.[26,27] Compared with common primary thiols, tertiary thiols are resistant to oxidization and considerably more sterically hindered to participate in nucleophilic reactions.[22,28−31] Consequently, the *S*-modified D-Pen derivatives are usually kinetically or/and thermodynamically more stable. For example, *S*-nitroso D-Pen derivatives are well-known nitric oxide (NO) donors, with its stability significantly higher than that of the primary thiol analogues.[32−34] Moreover, disulfide bonds formed by Pen are also found to be more stable than Cys,[35,36] potentially useful in peptide folding.[37−40] Despite these unique properties, studies regarding D-Pen-based polypeptides have been sparse.[41] This is, perhaps, because the steric hindrance raised by the β-C geminal dimethyl group and the *S*-protection group would render the resulting NCA monomers low polymerizability.[42] To this end, we propose that if the less hindered *S*-unprotected D-Pen NCA monomer could be directly (co)polymerized, the synthesis of D-Pen-based polypeptides not only would be made possible but also with a notably improved efficiency by eliminating tedious (de)protection steps. The challenge, however, lies at the side reactions posed by the unprotected thiol groups.

Recently, our group reported a robust approach enabling the moisture-tolerant synthesis of various challenging unprotected NCA (UP-NCAs) monomers bearing reactive functional groups (e.g., hydroxy, thiol, and carboxylic acid) in the side chain.[43] Among them, thiol-bearing monomers such as L-cysteine NCA (Cys-NCA) and D-penicillamine NCA (Pen-NCA) were particularly intriguing. We envision that detailed polymerization studies on these challenging monomers will generate new knowledges distinctively different from existing NCA chemistries and in the meantime create functional polypeptides with unprecedented structures. For example, our previous study showed that Cys-NCA is an inimer in that the side primary thiol group is nucleophilic enough to open NCA rings under certain conditions. As a result, the copolymerization of Cys-NCA with other NCAs produced hyperbranched polypeptides bearing thioester branching sites and rich amine and thiol functionalities.[43,44] In this work, we aim to investigate the (co)polymerization of Pen-NCA in detail and explore the feasibility of synthesizing tertiary thiol-functionalized polypeptides with high efficiency.

To begin with, the stability of Pen-NCA in different (deuterated) solvents was studied, which indicated that the monomer was kept intact in nonpolar/low-polar solvents such as dichloromethane (DCM) and chloroform for 48 h but would completely isomerize to thiocarbamate A[45−47] (Table 1) within 30 min in polar solvents such as, *N*,*N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), and dimethylsulfoxide (DMSO) (Figures S1~4). The stability of Pen-NCA in tetrahydrofuran (THF) was found to be intermediate (Figure S5). The rate of isomerization appeared to be related to the solvent polarity in general and was significantly accelerated by the addition of an organic base such as triethylamine (Figures S6 and S7). Therefore, we screened the solvent of the homopolymerization of Pen-NCA in those nonpolar or less polar solvents (entries 1−3, Table 1), with benzylamine as initiators (room temperature, air atmosphere, monomer initiator ratio $[M]_0$:$[I]_0$ is 50:1). The ^{1}H NMR spectra of the reaction systems (Figures S8 and S9) showed that the yields of the ROP of Pen-NCA in chloroform and DCM were moderate, 58% and 48%, respectively (entries 1 and 2, Table

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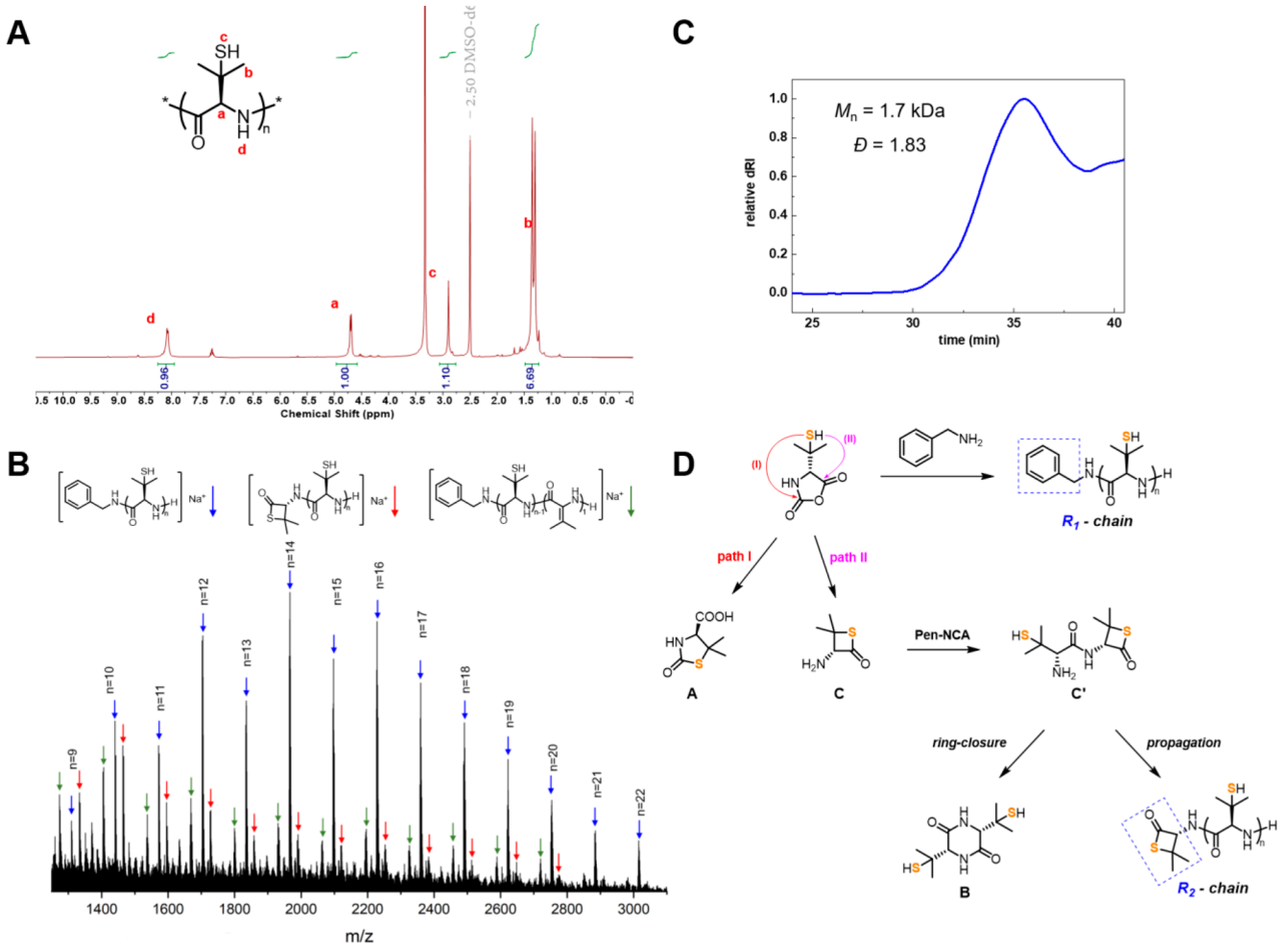

Figure 1. Homopolymerization of Pen-NCA. (A) ^{1}H NMR spectrum of the purified pPen in DMSO-d_6. (B) MALDI-TOF MS spectrum of pPen. (C) SEC chromatogram of pPen. (D) Proposed mechanism of side reactions in the homopolymerization of Pen-NCA.

1), higher than the 11% yield in THF (entry 3, Table 1). Different degrees of side reactions occurred in all three polymerizations, generating small molecular byproducts in the reaction system according to ^{1}H NMR analysis (Figures S8 and S9). Being aware that bases would promote the isomerization side reaction of Pen-NCA, we attempted to use different amounts of benzoic acid (BA) as an additive to lower the basicity and suppress related side reactions (entries 4−6, Table 1).[48,49] Interestingly, we found that the addition of 0.1 M benzoic acid not only increased the yield of poly(D-penicillamine) (pPen) but also the rate of the polymerization. For example, 0.1 M benzoic acid in chloroform shortened the reaction time from 50 to 21 h, and the NMR yield of pPen was increased from 58% to 74% (entry 5, Table 1). Albeit counter-intuitively, this acid-promoted polymerization was to some extent similar to what was previously observed for the ROP of *N*-thiocarboxyanhydride (NTA), in which acids were proposed to facilitate the transfer of proton and elimination of COS and accelerate the polymerization.[48,49] In the ROP of Pen-NCA, it was likely that a carboxylic acid additive promoted the proton transfer and decarboxylation process as well as hampered base-induced side reactions. The detailed mechanism behind this intriguing phenomenon will be studied in our follow-up studies.

To identify the structures of byproducts in the polymerization system, we conducted a large batch of ROP in chloroform and isolated two small molecular byproducts from the polymerization system, which were later confirmed as the rearranged thiocarbamate (compound **A**, Table 1) and diketopiperazine (compound **B**, Table 1) with the help of ^{1}H and ^{13}C NMR, high-resolution mass spectrometry (HRMS), and infrared spectroscopy (IR) (Figures S10∼15). The polymeric product (Table 1, entry 5), purified by precipitation from the reaction mixture, was characterized with ^{1}H NMR (Figure 1A), ^{13}C NMR (Figure S16), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Figure 1B), which collectively confirmed that the polymer had the repeating unit of penicillamine but with two different end groups. Specifically, the major and minor products in MS were attributed to pPen bearing the benzylamine end group (denoted as R_1) and the penicillamine thiolactone end group[50,51] (denoted as R_2), respectively (Figure S17). Freshly purified pPen-5 is soluble in DMSO, DMF, or hexafluoroisopropanol (HFIP) but insoluble or only partly soluble in chloroform, DCM, or THF. pPen-5 showed a broad unimodal distribution in DMF/LiBr size exclusion chromatography (SEC), with the number-average molecular weight (M_n) of 1.7 × 10^3 g/mol and *Đ* of 1.83 (Figure 1C). A branched structure was tentatively excluded in the product pPen-5 as there were no significant changes in molecular weight after the treatment of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and

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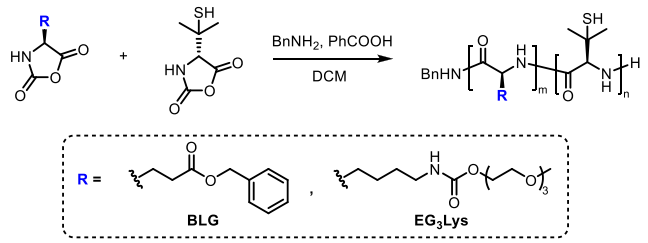
Table 2. Copolymerization of Pen-NCA with Different Monomers.



entry[a]	X-NCA	$[X]_0/[Pen]_0/[I]_0$	$[X]_0/[Pen]_0$	time (h)	Pen in polymer[b] (%)	A[b] (%)	B[b] (%)	X/Pen in polymer[c]	M_n (kDa)[d]	$Đ$[d]
1	BLG	25/13/1	2/1	16	70	24	6	2.8/1	5.4	1.28
2	BLG	50/25/1	2/1	25	62	32	7	3.2/1	9.6	1.25
3	BLG	100/50/1	2/1	41	60	34	6	3.1/1	14.2	1.27
4	BLG	25/8/1	3/1	16	78	20	2	3.7/1	5.4	1.21
5	BLG	50/17/1	3/1	25	69	26	5	3.9/1	10.1	1.20
6	BLG	100/33/1	3/1	41	65	31	4	4.3/1	16.4	1.22
7	EG_3Lys	25/8/1	3/1	15	-	-	-	3.8/1	9.2	1.19
8	EG_3Lys	50/17/1	3/1	21	-	-	-	4.5/1	16.2	1.27

[a]Polymerizations were performed atroom temperature (~25 °C) and under air atmosphere, $[X\text{-NCA}]_0$ = 0.1 M, [BA] = 0.1 M, solvent = DCM. Conversions of monomer were monitored by IR spectroscopy and were all above 95%. [b]Determined with ^{1}H NMR spectra of the reaction system. [c]Residue ratios are calculated from ^{1}H NMR spectra of products. [d]Determined by SEC in DMF (with 0.1 M LiBr) relative to polystyrene standards.

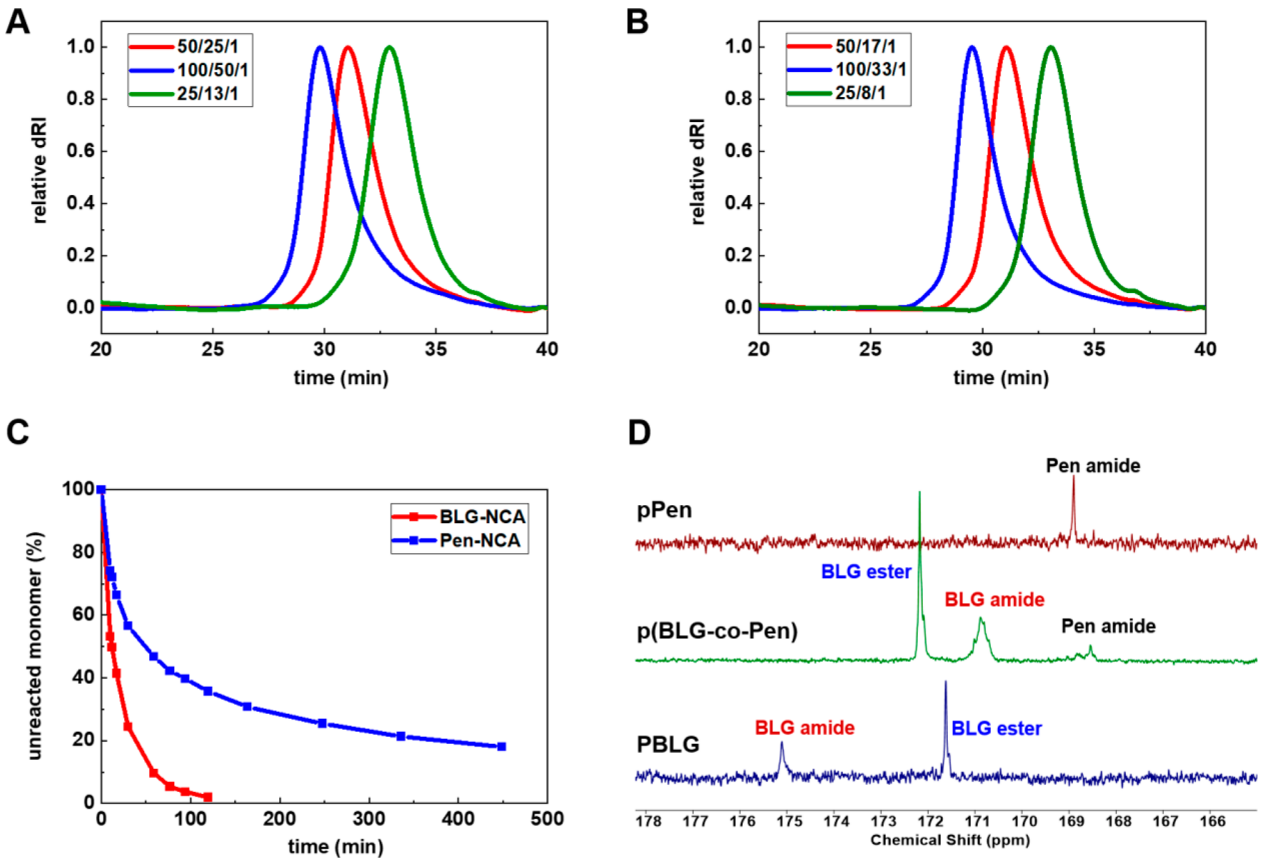


Figure 2. Copolymerization of Pen-NCA and BLG-NCA. (A) SEC chromatogram of p(BLG-*co*-Pen) (Table 2, entries 1−3). (B) SEC chromatogram of p(BLG-*co*-Pen) (Table 2, entries 4−6). (C) Copolymerization kinetics characterized by ^{1}H NMR spectroscopy in situ. (D) Overlay of ^{13}C NMR spectra of p(BLG-*co*-Pen), pPen, and PBLG.

benzyl mercaptan in DMF (Figure S18), which was known to promote thiol−thioester exchange.[52] This result suggested that, unlike the ROP of Cys-NCA that generated hyperbranched polymers, the homopolymerization of Pen-NCA produced pPen with a linear topology.

Based on the above experimental results, we herein propose a full picture of Pen-NCA homopolymerization (Figure 1D). In DCM or chloroform, the thiol group of Pen-NCA was a relatively weak nucleophile to attack NCA rings either intramolecularly or intermolecularly. However, with basic

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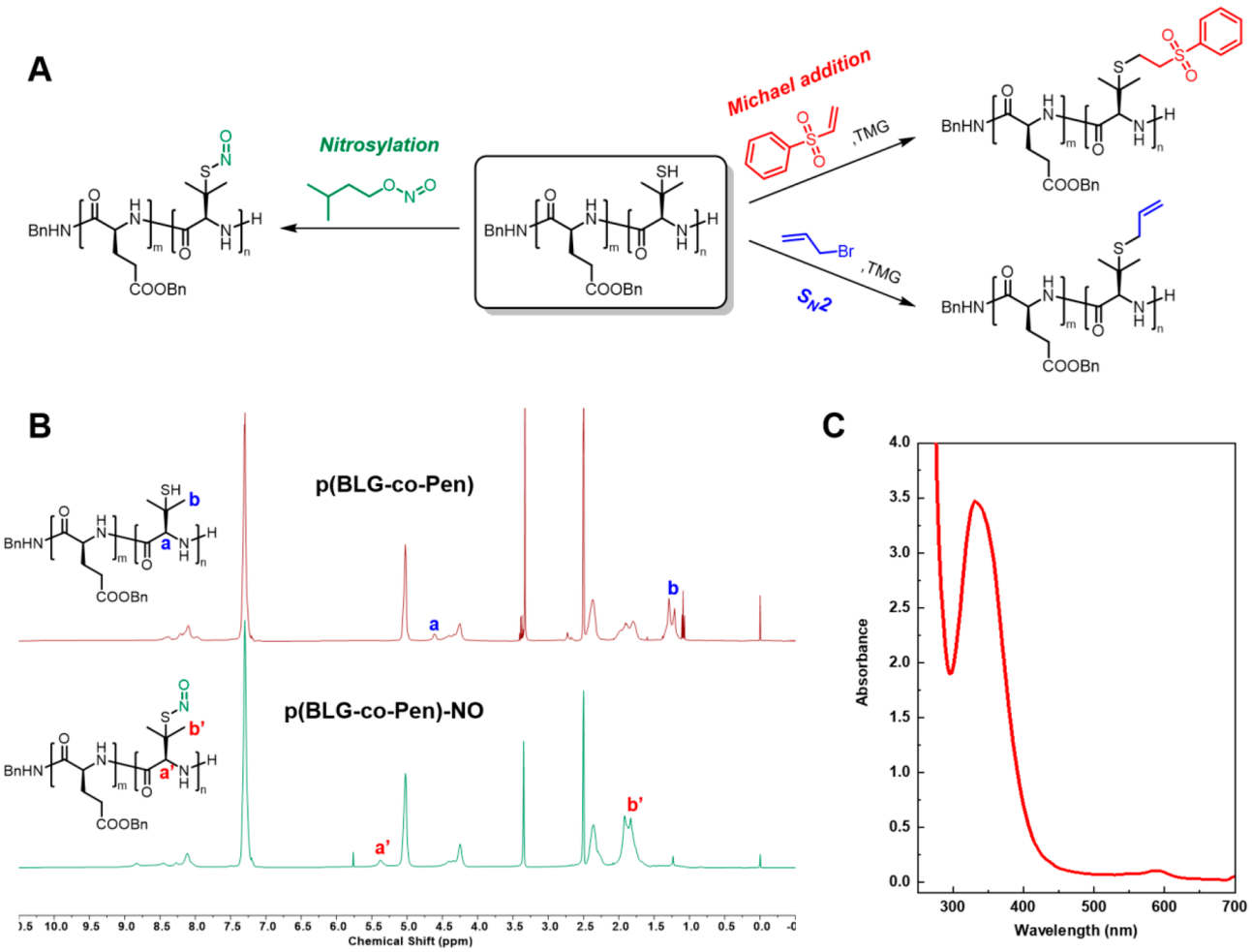

Figure 3. Post-polymerization modifications of Pen-containing copolypeptides. (A) Scheme of the three reactions attempted for the modification of p(BLG-*co*-Pen). (B) Overlay of ^{1}H NMR spectra of p(BLG-*co*-Pen) before and after nitrosylation. (C) UV−vis spectrum of the nitrosylation product p(BLG-*co*-Pen)-NO in DMSO-d_6 solution.

species such as the primary amine initiator or chain propagation center presented in the polymerization system, the nucleophilicity of the tertiary thiol could be activated to evoke rearrangement side reactions through two possible routes to give two byproducts, **A** (path I) and **C** (path II), respectively. While the thiocarbamate **A** was an inert species, by contrast, compound **C** had a primary amine to further react with Pen-NCA to give the intermediate **C′**, which either underwent intramolecular ring closure to generate the stable six-membered ring byproduct **B** or reacted with more Pen-NCA monomers to achieve chain propagation, giving pPen bearing the R_2 end group. Because of the above side reactions, the homopolymerization of Pen-NCA finally produced pPen with lower molecular weight than expected and two sets of end groups.

Next, the copolymerizations of Pen-NCA with a less sterically hindered NCA monomer were investigated, for which we expected a higher polymerization selectivity of Pen-NCA over side reactions as compared to the homopolymerization. BLG-NCA was selected as the model monomer, and the copolymer of Pen-NCA and BLG-NCA was expected to have higher molecular weight and improved solubility in organic solvents. Following the optimized conditions of homopolymerization, we conducted the copolymerization reactions with the additive benzoic acid and at different monomer-to-initiator ($[BLG]_0/[Pen]_0/[I]_0$) feeding ratios (Table 2, entries 1−6). To our delight, IR monitoring indicated complete conversions of monomers in 16−41 h. SEC characterizations of the copolymer products all showed unimodal narrow MWDs ($Đ$ < 1.3), and the M_n of copolymers grew with increasing $[BLG]_0/[Pen]_0/[I]_0$ ratios (Table 2, Figure 2A and B). ^{1}H NMR spectra of the copolymerization reaction mixture (Figure S19) and copolymer products (Figures S20 and S21) revealed that, although there were still 22∼40% Pen-NCA isomerized into the byproducts **A** and **B** (Table 2), the formation of the thiolactone end group (path II, Figure 1D) was successfully suppressed. No R_2 end group was observed in the purified copolymers, which gave higher end group fidelity and relatively controlled $Đ$. To further investigate the microstructure of the copolymer, we characterized the copolymerization kinetics in situ with ^{1}H NMR, which revealed simultaneous decline of the two monomers in the first 2 h of copolymerization (Figures 2C and S22). Due to the relatively higher reactivity of BLG-NCA over Pen-NCA, the former was consumed more and used up earlier than the latter, suggesting a gradient or tapered sequence. After the complete conversion of BLG-NCA, further consumption of Pen-NCA became much slower. ^{13}C NMR characterization indicated that the carbonyl carbon signals of BLG and Pen residues in the copolymer were significantly different from those of the two homopolymers, poly(γ-benzyl

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L-glutamate) (PBLG) and pPen (Figure 2D). The above results collectively supported the notion of copolymerization, generating random copolymers rather than separate homopolymerizations.

To make water-soluble polypeptides bearing the tertiary thiols, we copolymerized Pen-NCA with a lysine-based NCA tethered with an ε-monomethoxyl triethylene glycol side group (N^{ε}-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)carbonyl-L-lysine NCA, EG_3Lys-NCA, Table 2, Figures S23 and S25). As shown in Table 2 (entries 7 and 8), p(EG_3Lys-*co*-Pen)s were obtained with narrow $Đ$ less than 1.3 and M_n up to 16.2 kDa (Figure S26). The proportion of Pen in the copolymer product was slightly lower than the feeding ratio, likely a result of the rearrangement side reaction (Figure S27). Both copolymers showed good water solubility (greater than 10 mg/mL), which might be useful for biomedical applications in the future.

With the Pen-containing copolypeptides in hand, we next investigated the PPM reactions by taking advantage of the tertiary thiols (Figure 3A). p(BLG-*co*-Pen)-2 (Table 2, entry 2) was selected as the model polymer. Thiol-Michael reaction with phenyl vinyl sulfone and nucleophilic substitution reaction with allyl bromide (S_N2) were conducted, and both reactions reached a high modification efficiency of ∼90% under the catalysis of tetramethylguanidine (Figures S28 and S29). Less reactive alkyl bromide, *n*-hexylbromide, however, resulted in a lower conversion of 37% under the same conditions (Figure S30). These results indicated that, though sterically hindered, the tertiary thiol group of Pen was modifiable and useful for PPM under optimized conditions. Moreover, recent NO delivery studies showed an uprising interest in polymeric NO delivery platforms for cancer therapy and antimicrobial therapeutics.[53,54] In order to investigate the NO loading capacity of Pen polypeptides, we conducted the nitrosylation reaction of p(BLG-*co*-Pen)-2 with isopentyl nitrite.[55] The reaction finally produced a solid product with a characteristic green color.[32] The successful generation of the desired SNO species was confirmed by the signal shift of the α-H and *gem*-dimethyl groups of Pen after nitrosylation according to ^{1}H NMR spectroscopy, from which the modification efficiency was calculated to be ∼90% (Figure 3B). Moreover, UV−vis spectroscopy results echoed the conclusion by showing the characteristic absorptions of tertiary nitrosothiols (R-SNOs) at ∼350 nm (strong) and ∼590 nm (weak) (Figure 3C).[33,56] IR spectra also supported the formation of nitrosothiols (Figure S31).[57]

In summary, we explored and screened the conditions for the homopolymerization and copolymerization of the *S*-unprotected Pen-NCA. Through judicious solvent selection and the presence of benzoic acid, rearrangement side reactions of the monomer were partially suppressed, and the selectivity for polymerization was improved to generate tertiary thiol-functionalized (co)polypeptides in a one-pot and protection-free fashion. After condition optimization, the yields of polypeptide products were significantly increased. Although the homopolymer pPen was limited by its low M_n, high-molecular-weight Pen-containing copolypeptides were successfully synthesized with narrow MWD. Various postpolymerization modifications of Pen-containing copolypeptides were demonstrated through thiol-Michael, S_N2, and nitrosylation reactions. The high efficiency of PPMs underscored the biomedical application potentials of these polymers in hydrogels and polymeric NO donors. Our lab is currently studying the polymerization of various NCAs with unprotected side groups, which will substantially enrich the paradigm of NCA chemistry with new understandings on the interplay of side chain reactive functional groups with the chain propagation. These studies will also open up new opportunities by offering unprecedented polypeptide materials.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmacrolett.3c00065.

Materials, instrumentation, experimental methods, and supplementary figures (PDF)

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Notes

The authors declare no competing financial interest.

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