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Insight into the cross-linking of synthetic polypeptide gels prepared by ring-opening polymerization using L-cystine *N*-carboxyanhydride

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ABSTRACT

Synthetic polypeptides have promising potential for various biomedical applications that often require a cross-linked network for stability against solvents or mechanical stress. Due to the natural occurrence and availability of the L-cystine α-amino acid, L-cystine *N*-carboxyanhydride (NCA) is commonly used as a cross-linker in the ring-opening (co)polymerization of α-amino acid NCAs to prepare covalently cross-linked synthetic polypeptides. In this work, we show that the L-cystine NCA tends to undergo undesired decomposition side reactions, i.e., β-elimination and formation of 2,5-diketopiperazine, which reduce the amount of the cross-linker available and make it difficult to control the final properties of the materials prepared from it. By using the analogous L-homocystine NCA instead, the decomposition side reactions can be completely avoided. We demonstrate the cross-linking performance of L-cystine NCA compared to L-homocystine NCA by studying the corresponding gels prepared by copolymerization with γ-benzyl-L-glutamate NCA.

1. Introduction

L-Cystine (Cys) is a naturally occurring dimer of two cysteine α-amino acid residues that form a disulfide bridge upon oxidation. Disulfide bridges in living systems are important in stabilizing the tertiary structure of proteins through cross-linking, and also serve as a site for redox reactions as they are cleaved in reductive environment [1]. Cys has also been recognized in materials science, where its derivatives serve as convenient building blocks for polymer synthesis [2–6], since the disulfide bonds can be cleaved and re-formed in response to various triggers such as temperature, light, radicals, and reducing agents [7]. A large difference in reduction potential between the intracellular and extracellular environments of animal cells, in addition to tumor tissues being significantly more reducing compared to normal tissues, makes Cys-based polymers valuable for the delivery of therapeutics [2]. Cys cross-linker can be prepared in the form of *N*-carboxyanhydride (NCA) for use in ring-opening polymerization (ROP) [8] to prepare synthetic polypeptides that are promising materials for biomedical applications due to their biocompatibility and (bio)degradability [9,10]. In fact, Cys NCA is by far the most commonly used difunctional NCA (di-NCA) and synthesis of only a few other di-NCA monomers has been reported [11,12]. In most cases, Cys NCA has been used to prepare drug delivery nanogels in the form of core cross-linked micelles by a one-pot polymerization approach [8]. Amino-terminated poly(ethylene glycol) (PEG) was commonly used as a macroinitiator for ROP of Cys NCA to prepare PEG-*b*-polypeptide nanogels [13,14]. Alternatively, block polypeptide nanogels were prepared by polymerizing Cys NCA from a polypeptide [15,16] or polypeptoide [17]. The properties of the cross-linked nanogel core could be adjusted by copolymerizing Cys NCA with other functional NCAs. After polymerization, the core cross-linked nanogels were prepared by precipitating, drying, and dispersing the dried polymer in aqueous solution. Cross-linking with Cys NCA increased the stability of the drug carriers and enabled their controlled degradation for targeted cargo release in a reductive environment. Besides nanogels, polypeptide network films and monoliths have also been prepared by polymerization of Cys NCA. Aminated silica nanospheres were used as initiators for copolymerization of Cys NCA and γ-benzyl-L-glutamate (BLG) NCA, and the silica was subsequently removed with hydrofluoric acid to prepare porous films [18]. In our previous work, we have prepared polypeptide scaffolds by copolymerizing Cys NCA with BLG NCA, and N_ε-carbobenzyloxy-L-lysine NCA or L-phenyalalanine NCA using a tertiary amine as a catalyst in the continuous phase of high internal phase emulsions (HIPEs) [19,20]. After deprotection, macroporous polypeptide hydrogels were obtained, which were both enzymatically and reductively degradable due to the disulfide bridges in the Cys cross-linker. All these works undoubtedly show that Cys NCA can be

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used for cross-linking during ROP. However, the focus has generally been on the cross-linked structures or their soluble counterparts after reductive degradation of the purified polymer, while the accompanying reactions of the cross-linking have not been investigated.

In this work, we have studied in detail the behavior of Cys NCA during ROP by analyzing the soluble fraction of the polypeptide network. Critically, we were able to show that Cys NCA is prone to undesirable side reactions under the conditions of ROP with primary or tertiary amine. These side reactions are typical for Cys NCA and were not observed for the analogous L-homocystine (Hcys) NCA cross-linker under the same experimental conditions. In addition, we investigated the properties of polypeptide gels prepared by copolymerization of BLG NCA and Cys NCA or Hcys NCA as cross-linker to show the effects of the side reactions on the cross-linking efficiency and material performance.

2. Experimental

2.1. Materials

γ-Benzyl-L-glutamate (BLG) and Hcys were obtained from Iris Biotech GmbH. *N*,*N*'-di-(*tert*-butyloxycarbonyl)-L-cystine, triphosgene, di-*tert*-butyl dicarbonate, triethylamine (Et_3N), potassium bisulfate ($KHSO_4$), magnesium sulfate ($MgSO_4$) were purchased from Sigma Aldrich; hexylamine and trifluoroacetic acid (TFA) were purchased from Acros Organics. Dimethyl sulfoxide-d_6 (DMSO-d_6) was obtained from Eurisotop; anhydrous tetrahydrofuran (THF), anhydrous ethyl acetate, anhydrous *N*,*N*-dimethylformamide (DMF), *n*-hexane, acetonitrile, and 1,4-dioxane were purchased from Merck. All chemicals were used as received.

2.2. Synthesis of NCA monomers

BLG NCA was prepared as previously reported [19]. Briefly, BLG (5.00 g, 22.8 mmol, 1.0 eq) was suspended in anhydrous THF (60 mL) in a flame-dried Schlenk flask and heated to 55 °C. A solution of triphosgene (3.92 g, 13.2 mmol, 0.6 eq) in anhydrous THF (15 mL) was added slowly. The reaction was stirred for 1.5 h. The reaction mixture was then concentrated under vacuum and recrystallized with *n*-hexane. Recrystallization from THF/*n*-hexane was repeated twice more. BLG NCA was obtained in the form of a white powder (4.35 g, 80 % yield). The ^{1}H and ^{13}C NMR spectra with signal assignation are shown in Fig. S1.

Cys NCA was prepared as reported previously [19]. *N*,*N*'-Di-(*tert*-butyloxycarbonyl)-L-cystine (1.00 g, 2.3 mmol, 1.0 eq) was suspended in anhydrous ethyl acetate (5 mL) in a flame-dried Schlenk flask and heated to 50 °C. A solution of triphosgene (0.91 g, 3.1 mmol, 1.4 eq) in anhydrous ethyl acetate (10 mL) was added dropwise. After 16 h, the mixture was filtered through a syringe filter (Chromafil, polytetrafluoroethylene (PTFE), 0.45 μm) into 60 mL of cold *n*-hexane. The mixture was centrifuged at 8000 rpm for 4 min, and the precipitated Cys NCA was dried under vacuum. The precipitation was repeated once more. Cys NCA was obtained in the form of a white powder (0.42 g, 63 % yield). The ^{1}H and ^{13}C NMR spectra with signal assignation are shown in Fig. S2.

N,*N*'-Di-(*tert*-butyloxycarbonyl)-L-homocystine ((Boc-Hcys-OH)$_2$) was prepared by a modified procedure [21]. L-Homocystine (2.85 g, 10.6 mmol,) was suspended in aqueous K_2CO_3 solution (40 mL, 10 % m/m). A solution of di-*tert*-butyl dicarbonate (5.82 g, 26.7 mmol) in THF (35 mL) was added and the reaction was refluxed at 80 °C. After 24 h, the reaction mixture was diluted with deionized water (70 mL) and acidified with aqueous saturated $KHSO_4$ to a pH of 3–4 to precipitate the *N*,*N*'-di-(*tert*-butyloxycarbonyl)-L-homocystine. The product was extracted with chloroform (3 × 30 mL), then dried with $MgSO_4$, filtered, and the solvent removed under vacuum. The product was obtained in the form of a white powder (4.29 g, 86 % yield). The ^{1}H spectrum with signal assignation is shown in Fig. S3.

(Boc-Hcys-OH)$_2$ (0.8 g, 1.7 mmol, 1.0 eq) was suspended in anhydrous ethyl acetate (4 mL) and heated to 70 °C. α-Pinene (0.8 mL, 6.8 mmol) was added, followed by a solution of triphosgene (0.68 g, 2.3 mmol, 1.3 eq) in anhydrous ethyl acetate (8 mL), which was added dropwise over the course of 2 h. After 24 h, the solution was filtered through a syringe filter (Chromafil, PTFE, 0.45 μm) into 30 mL of cold *n*-hexane. The mixture was centrifuged at 8000 rpm for 4 min and the precipitated HCys NCA was dried under vacuum. Hcys NCA was further recrystallized from ethyl acetate/*n*-hexane twice. Hcys NCA was obtained in the form of an off-white powder (0.34 g, 62 % yield). The ^{1}H and ^{13}C NMR spectra with signal assignation are shown in Fig. S4.

2.3. Di-NCA reactions

In a typical procedure, Cys NCA (18 mg, 0.06 mmol) or Hcys NCA (19 mg, 0.06 mmol) was dissolved in DMSO-d_6 (355 μL) with acetonitrile (3 μL) as an internal standard. A solution of Et_3N in DMSO-d_6 (45 μL, 0.1 M) or hexylamine (38 μL, 0.1 M) was added (Table S1). The reaction mixture was immediately aliquoted (20 μL) into separate 0.5 mL tubes and sealed. After a certain time, the aliquots were diluted and quenched with DMSO-d_6 (750 μL) and TFA (10 drops), transferred to NMR tubes and the NMR spectra were recorded immediately afterwards. The chemical composition of the soluble fraction was calculated by comparing the ^{1}H NMR signal integral of the compound of interest with the ^{1}H NMR signal integral of acetonitrile using Equation (1):

$$x(\mathrm{A}) = \frac{H(\mathrm{NCA})\cdot I_t(\mathrm{A})\cdot I_0(\mathrm{acetonitrile})}{H(\mathrm{A})\cdot I_t(\mathrm{acetonitrile})\cdot I_0(\mathrm{NCA})} \quad (1)$$

where x(A) denotes the fraction of a selected compound A, H(NCA) and H(A) denote the proton number of the integrated signal for the NCA and the selected compound, respectively (i.e. H(NCA) = 4 for the Cys NCA signal at δ 4.80 ppm). I_0(acetonitrile) and I_0(NCA) denote the initial integrals of acetonitrile (δ 2.07 ppm) and NCA (e.g. δ 4.80 ppm for Cys NCA) signals, respectively, prior to the addition of amine at $t = 0$. I_t(acetonitrile) and I_t(A) are the integrals of acetonitrile (δ 2.07 ppm) and compound A signals, respectively, at time t.

2.4. Gel preparation

Gels were prepared by dissolving BLG NCA and Cys NCA or Hcys NCA in anhydrous DMF. A solution of Et_3N catalyst in anhydrous DMF (0.1 M) was added and the solution was immediately transferred to a plastic mold. The mold was sealed with a septum cap that was pierced with a needle to allow the release of CO_2 formed during ROP. The experimental conditions for the preparation of each gel are listed in Table S2.

ROP was followed by analyzing aliquots taken at different times using FTIR. NCA conversion at time t was calculated based on the height ratio of the typical polypeptide band at a wavenumber of 1551 cm^{-1} to NCA band at a wavenumber of 1738 cm^{-1}, based on linear dependence of the ratio vs. conversion between $t = 0$ (no polypeptide) and the end of polymerization (no NCA) as confirmed by ^{1}H NMR. The onset of gelation during polymerization was determined by a vial inversion method by noting the time at which the reaction liquid ceased to flow. The gels were polymerized for 24 h at room temperature. At this point, complete conversion of the monomer was achieved, as confirmed by FTIR.

The weight of the dry gels was determined after solvent exchange with 1,4-dioxane using the Soxhlet apparatus for 24 h, followed by freeze-drying. Gel content values were calculated by dividing the weight of the dry gel by the theoretical weight of the polypeptide in the gel, assuming complete conversion of the monomers and taking into account CO_2 release, according to Equation (2).

$$\textit{Gel content}\ (\%) = \frac{m_\mathrm{d}}{m_{\mathrm{NCA1}}\cdot\left(1-\frac{M_{\mathrm{CO_2}}}{M_{\mathrm{NCA1}}}\right)+m_{\mathrm{NCA2}}\cdot\left(1-\frac{2\cdot M_{\mathrm{CO_2}}}{M_{\mathrm{NCA2}}}\right)}\cdot 100 \quad (2)$$

m_{NCA1} and m_{NCA2} denote the weights of monofunctional NCA and difunctional NCAs in the gel, respectively. M_{NCA1} and M_{NCA2} denote the molar masses of monofunctional NCA (263.25 g mol^{-1} for BLG NCA) and difunctional NCA (292.28 g mol^{-1} for Cys NCA, 320.34 g mol^{-1} for Hcys NCA) in the gel, respectively, and M_{CO2} is the molar mass of CO_2 (44.01 g mol^{-1}).

2.5. Characterization

NMR spectra were recorded at 25 °C in DMSO-d_6 (with a few drops of TFA) using an AVANCE NEO 400 MHz or 600 MHz NMR spectrometer (Bruker) in pulse Fourier transform mode. Chemical shifts are reported as δ, ppm, relative to tetramethylsilane (TMS, $\delta = 0$). Fourier transform infrared (FTIR) spectra were acquired using a Spectrum Two FTIR spectrometer (Perkin Elmer). The FTIR spectra were recorded in attenuated total reflectance (ATR) mode in a spectral range of 650–4000 cm^{-1}.

2.6. Mechanical testing

The compressive mechanical properties of the gel samples were measured using a DMA Q800 dynamic mechanical analyzer (TA Instruments) equipped with 40 mm diameter compression disks. A preload force of 0.05 N was used to ensure good contact between the disks and the sample. The sample was then compressed at a strain rate of 25 % min^{-1} to –50 % or until failure. The compressive modulus was determined as the slope of the stress–strain curve in the initial linear range. Measurements were performed in triplicate and results are reported as average values with standard deviation.

3. Results and discussion

NCAs are highly reactive and known to have a short shelf-life, mainly due to hydrolysis and polymerization. In the case of Cys NCA, we observed a slow formation of specific degradation products during prolonged storage, as evidenced by the appearance of additional ^{1}H NMR signals (Fig. S5). Notably, these side products are independent of the Cys NCA synthesis approach, as we observed them after the preparation of Cys NCA from *N*,*N*'-di-(*tert*-butyloxycarbonyl)-L-cystine with triphosgene as well as from *N*,*N*'-dicarbobenzyloxy-L-cystine with PBr_3. Therefore, the use of freshly prepared Cys NCA was necessary to obtain polypeptide gels with repeatable results [19,20]. In this work, we investigate the stability of Cys NCA under conditions normally used for ROP. Hexylamine served as a representative primary amine initiator commonly used in the preparation of well-defined polypeptides by the normal amine mechanism [9,22]. Et_3N was used as a basic catalyst promoting the activated monomer mechanism (AMM), which is known to give high molecular weight polypeptides in a short time, but at the expense of less control over polypeptide molecular weight characteristics [22]. After only 5 min of reaction with either hexylamine or Et_3N, Cys NCA is getting consumed, as shown by the decrease of the typical ^{1}H NMR signals of Cys NCA at δ 9.25 ppm, 4.80 ppm, 3.27 ppm, and 3.19 ppm (Fig. S6). However, rapid formation of side products is also observed as additional signals are present in the ^{1}H NMR spectra. Moreover, profile of the side products changes with time (Fig. S6). The reaction mixture remained liquid, but we found that after 24 h, up to 50 % of the sample was invisible to liquid-state NMR, using acetonitrile as internal standard, due to the formation of a covalently cross-linked nanogel. ^{1}H (Fig. S7A), ^{13}C (Fig. S7B), COSY (Fig. S8), HSQC (Fig. S9), and HMBC Fig. S10) NMR spectroscopy analyses of the reaction mixture of Cys NCA in DMSO-d_6 with 1.5 mol% Et_3N showed that the signals correspond to four different compounds. We identified the side products and interestingly found that the initial side products correspond to two side reactions taking place simultaneously and identified them as (i) formation of an intramolecular cyclic dipeptide, i.e. Cys 2,5-diketopiperazine (or 2,5-dioxopiperazine; also known as *cyclo*-L-cystine), and (ii) β-elimination in the Cys NCA side chain to form L-thiocysteine NCA and dehydroalanine NCA, later followed by the third reaction in which (iii) the dehydroalanine NCA decomposes to pyruvate and ammonia (Fig. 1). The evolution of the side products during the reaction with Et_3N over time is shown in Fig. 2.

The intramolecular Cys 2,5-diketopiperazine formed in the reaction mixture is identified by the ^{1}H NMR signals at δ 8.45 ppm (–NH–), 4.41 ppm (–CH–), 3.73 ppm (–CH–CHH–S–), 3.48 ppm (–CH–CHH–S–), which is consistent with the previously reported values of intramolecular Cys 2,5-diketopiperazine synthesized from Boc-cysteine (Acm)-cysteine(Acm)-OMe dipeptide [23]. Intermolecular 2,5-diketopiperazines of various α-amino acids are known to be possible side-products formed from the α-amino acids themselves [24] or during the synthesis and ROP of NCAs [25–27]. The amount of Cys 2,5-diketopiperazine formed during the reaction increased with time until the Cys di-NCA was completely consumed (Fig. 2), and we did not observe any further reactions of the Cys 2,5-diketopiperazine.

We assign the signals at δ 9.27 ppm (–NH–), 4.85 ppm (–CH–) and 3.37 ppm (–CH$_2$–S–) to the L-thiocysteine NCA, as they partially overlap and very closely resemble those of the Cys NCA. The signals at δ 11.45 ppm (–NH–), 5.32 ppm (–C=CHH) and 5.07 ppm (–C=CHH) belong to dehydroalanine NCA [28]. L-thiocysteine and dehydroalanine are known products of the β-elimination of Cys, which can occur in di-(α-amino acid) or in Cys-containing polypeptides under basic conditions and/or at elevated temperatures [24,29]. While β-elimination results in equimolar amounts of L-thiocysteine and dehydroalanine residues, their ratio in the reaction mixture deviates from 1:1 as depletion of both NCAs is observed (Fig. 2). The L-thiocysteine residues can decompose into L-cysteine and H_2S, react with free thiols to form corresponding disulfide and H_2S [30], or trigger reshuffling of the remaining disulfide bonds [24]. We do not observe the L-thiocysteine NCA or its side products in the soluble fraction of the reaction mixture after time, indicating that they become part of the insoluble fraction. On the other hand, when the dehydroalanine NCA ring opens, it does not readily polymerize [28]. Instead, its unstable enamine structure tautomerizes to the corresponding imine, which can further react to pyruvate and ammonia [24,28]. The decomposition of dehydroalanine in the Cys NCA reaction mixture is evident from the appearance of ^{1}H NMR signals for pyruvate (δ 2.32, –CH$_3$) and ammonia (δ 7.18 ppm, 7.10 ppm, 7.01 ppm) over time. In addition, the –NH– ^{1}H NMR signal of dehydroalanine NCA is at a higher frequency compared to most NCAs (resulting in > 1.0 ppm difference), indicating its significantly higher acidity, which may have a negative effect on polymerization if present in sufficient concentration.

The elimination reaction is less likely to occur with the analogous L-homocystine (Hcys), which contains an additional methylene group on each side of the disulfide. The longer linker between the NCA rings probably also prevents the formation of intramolecular Hcys 2,5-diketopiperazine, since in this case a less favorable dicyclic compound of 6- and 8-membered rings should form. We synthesized Hcys NCA in a similar way to Cys NCA. Unlike Cys NCA, the Hcys NCA solution remained transparent when reacted with either hexylamine (1.3 mol%) or Et_3N (1.5 mol%) in DMSO-d_6, and no side products were observed in the soluble part of the reaction mixtures (Fig. S11). The absence of an elimination reaction in the homo-analogue was also suggested in the case of poly(2-bromoethylbenzylphospho-L-homoserine), while poly(2-bromoethylbenzylphospho-L-serine) underwent elimination of phosphate groups under basic conditions [31].

Next, we demonstrate how the difference in side reactions of the di-NCAs affect their role as cross-linkers. We prepared organogels by copolymerizing BLG NCA with Cys NCA or Hcys NCA in DMF to prepare poly(BLG-*co*-Cys) (Cys-polypeptide) and poly(BLG-*co*-Hcys) (Hcys-polypeptide), respectively (Fig. 3). When the polypeptides were prepared by NAM using hexylamine, the Cys-polypeptide with a targeted degree of polymerization (DP) of 80 (1.00 M) remained liquid, and the Hcys-polypeptide formed only a soft gel under the same experimental

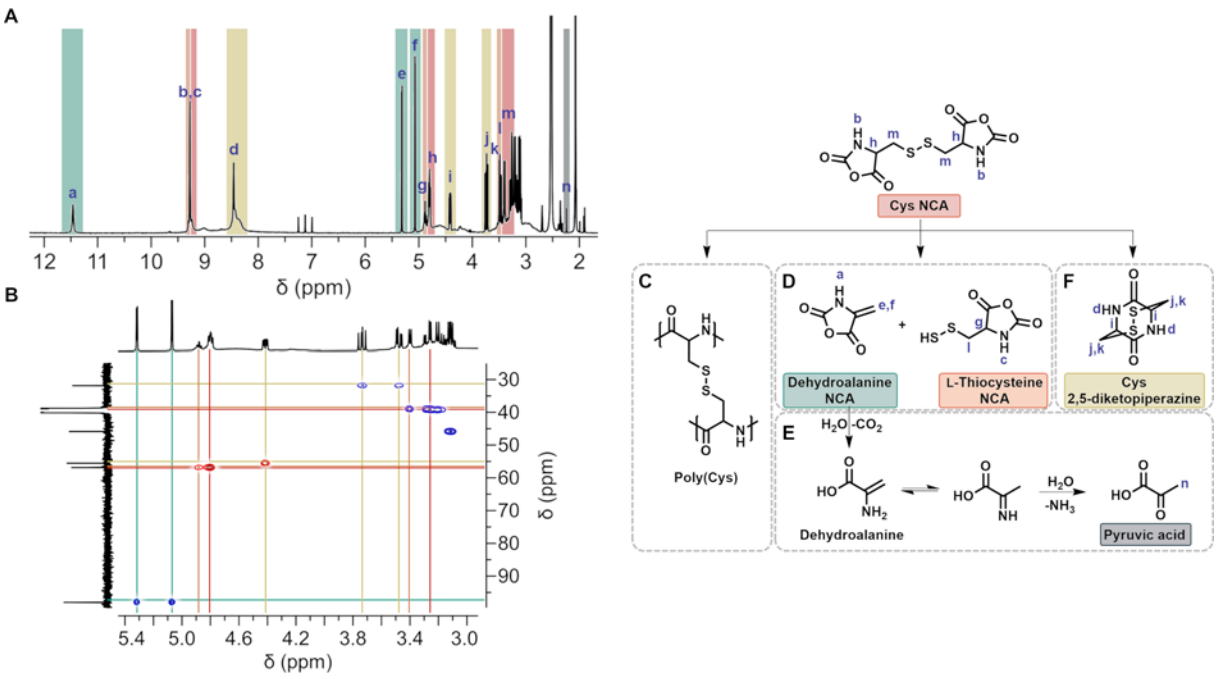

Fig. 1. (A) ^{1}H NMR spectrum and (B) HSQC NMR spectrum of the reaction mixture of Cys NCA in DMSO-d_6 with Et_3N and proposed reactions of Cys NCA in the presence of amines (Et_3N or hexylamine) or after prolonged storage: (C) ROP of Cys NCA, (D) β-elimination reaction, (E) decomposition of dehydroalanine NCA, (F) formation of Cys 2,5-diketopiperazine.

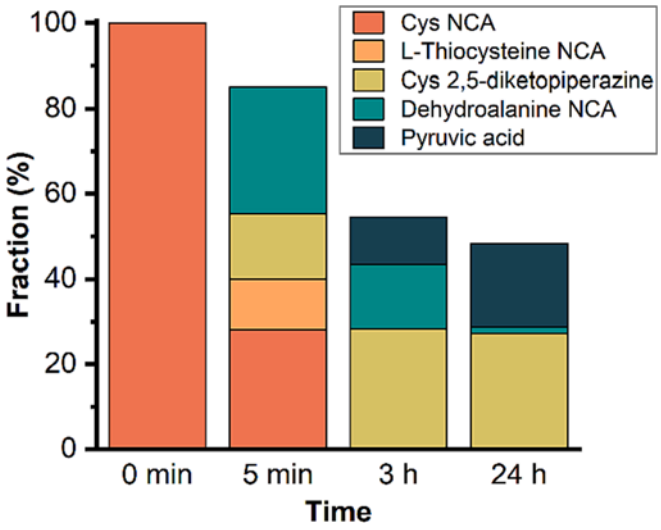

Fig. 2. Composition of the soluble part of the reaction mixture of Cys NCA in DMSO-d_6 with Et_3N over time, evaluated from the intensity of the ^{1}H NMR signals compared to acetonitrile as internal standard. The remaining difference to 100 % is presumed to be in the form of an insoluble, covalently cross-linked nanogel.

conditions, which disintegrated when removed from the mold (Fig. S12A). When Et_3N was used as catalyst instead, both polypeptides formed self-supporting gels (Fig. S12B). The Hcys-polypeptide formed a cross-linked network much faster compared to the Cys-polypeptide, as shown by the much shorter gel times at the same conditions. As CO_2 is formed during ROP of NCAs, the escaping gas may get trapped within the gel and cause bubbling of the sample (Fig. S12C). This is affected by polymerization kinetics and can therefore be avoided by appropriately adjusting the amount of the catalyst [19]. Indeed, to obtain Hcys-polypeptide gels without bubble formation, lower amounts of Et_3N were required (Table S2). Cys-polypeptides would not gel at such a low

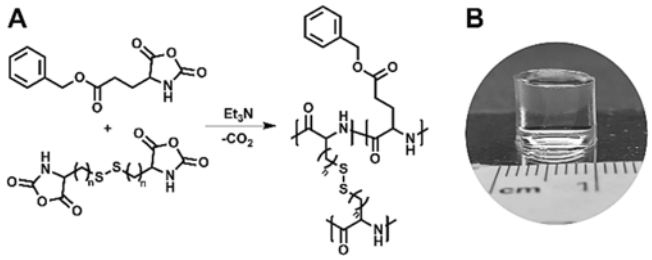

Fig. 3. (A) Preparation of poly(BLG-*co*-Cys) (n = 1) and poly(BLG-*co*-Hcys) (n = 2) gels and (B) a photograph of a representative gel (P(BLG-co-Cys) prepared with 0.75 M, 5.0 % Cys and 0.2 % Et_3N is shown).

amount of Et_3N, indicating that side reactions precede the polymerization. Under the conditions where the Cys-polypeptide gels (e.g. at 7.5 mol% Cys NCA and 0.2 mol% Et_3N), the ^{1}H NMR spectrum of the soluble part of the gel also shows the presence of the side products, indicating that the cross-linker degrades during copolymerization as well (Fig. S13).

At a high NCA concentration (≥ 0.75 M), 2.5 mol% di-NCA (either Cys or Hcys) was sufficient for gelation (Fig. 4). As the NCA concentration decreased, P(BLG-*co*-Cys) at a concentration of 0.63 M required 5.0 % Cys NCA for gel formation and did not form a gel when the concentration was further decreased to 0.50 M. Hcys NCA, on the other hand, exhibited a much wider window for gel formation, as even a 0.50 M solution with 2.5 % HCys formed a stable gel. The Cys-polypeptides behave as having a lower cross-linker amount compared to the feed, which is due to the partial degradation of Cys NCA, resulting in a lower concentration of available cross-linker. However, adding a higher amount of Cys NCA to the reaction mixture to compensate for the partial degradation of the cross-linker is not necessarily a viable solution, as this leads to a higher amount of the side products, which in turn can interfere with ROP and network formation. The L-thiocysteine NCA can presumably copolymerize into the growing polypeptide network and thus reduce the actual degree of cross-linking. In addition, nucleophilic

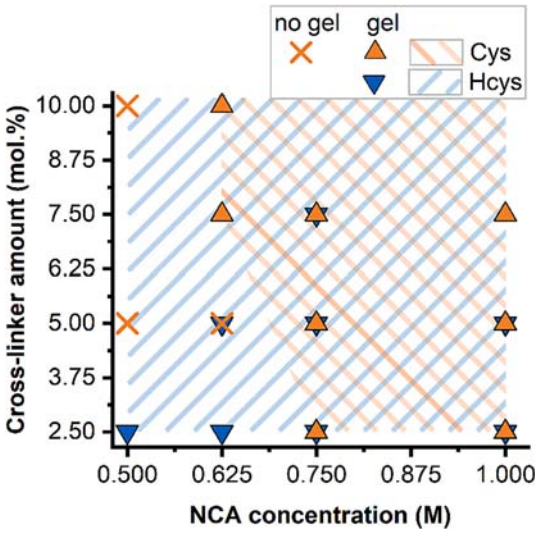

Fig. 4. Total NCA monomer concentration and NCA cross-linker fraction at which the reaction mixtures of P(BLG-*co*-Cys) (▲) and P(BLG-*co*-Hcys) (▼) gel, and P(BLG-*co*-Cys) (×) do not gel. The striped areas indicate the gelation windows of P(BLG-co-Cys) (\, orange) and P(BLG-co-Hcys) (/, blue). The crossing stripes show that both polypeptides gel in this area.

species, such as thiols, may serve as initiation sites [32] and the quality of the cross-linking network may also be affected by the disulfide reshuffling reactions.

We further analyzed the gel series obtained at a concentration of 0.75 M NCA. The ROP was followed by FTIR (Fig. S14) and the onset of gelation was determined by the vial inversion method. Based on these results, the monomer conversion at the gel point was determined using FTIR (Fig. S15A). A higher amount of either cross-linker leads to gelation at a lower monomer conversion. It is noteworthy that in the case of the Hcys-polypeptides, a lower monomer conversion is required to form a gel, which is consistent with the degradation of the Cys cross-linker. This is also confirmed by the fact that Cys-polypeptides have a lower gel content compared to Hcys counterparts (Fig. S15B). Further difference between Cys- and Hcys-polypeptide gels is evident in their mechanical properties (Fig. 5). Namely, Cys NCA forms a softer gel than Hcys NCA even at the same amount of the cross-linker, with compressive moduli of 7.2 ± 2.3 kPa and 14.4 ± 2.7 kPa for Cys- and Hcys-gel, respectively. Lower compressive moduli of polypeptide cross-linked with Cys NCA were also observed for poly((L-glutamate)–*co*-Cys) hydrogels compared to their Hcys, L-lanthionine, L-cystathionine, and L-homolanthionine counterparts [12]. While the di-NCAs differ slightly in tether length, which may affect the interchain cross-linking ability, we believe that this large deviation in the properties of the Cys-based gels is primarily caused by the 2,5-diketopiperazine formation and β-elimination reaction of the Cys NCA.

4. Conclusion

While Cys NCA is an obvious choice for cross-linking synthetic polypeptides during ring-opening copolymerization, we have shown that it has the disadvantage of undergoing the undesirable decomposition side reactions of 2,5-diketopiperazine formation and β-elimination into L-thiocysteine NCA and dehydroalanine NCA. The decomposition of Cys NCA directly results in a lower amount of available cross-linker for the preparation of the polypeptide gels. In addition, the degradation products of Cys NCA can also affect ROP and network formation. All this leads to lower cross-linking efficiency and poorer performance of the Cys-polypeptide gel compared to the analogous Hcys-polypeptide gel, where these side reactions are not observed. Therefore, the final properties of synthetic polypeptide gels can be better controlled by shifting the focus from Cys NCA to Hcys NCA or other di-NCAs as alternative cross-linkers.

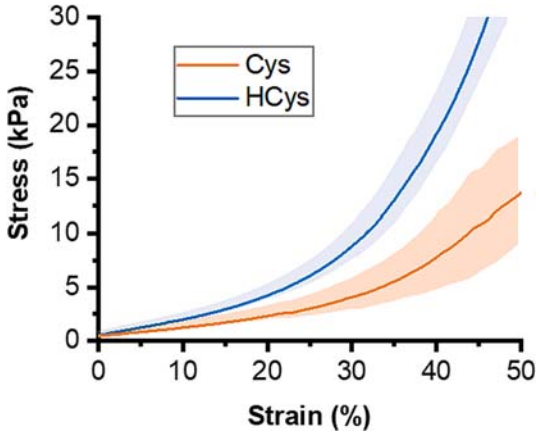

Fig. 5. Compressive stress–strain curves of P(BLG-*co*-Cys) (orange) and P(BLG-*co*-Hcys) (blue) polypeptide gels with 5.0 mol% of cross-linker. The curve lines represent the average of three measurements, enclosed by the standard deviations.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eurpolymj.2023.112707.

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