Title

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Journal

Chemistry - An Asian Journal, 13(22)

ISSN

1861-4728

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Publication Date

2018-11-16

DOI

10.1002/asia.201801031

Peer reviewed

Influence of sulfur containing di-amino acid structure on covalently crosslinked copolypeptide hydrogels

Eric D. Raftery,[b] Eric G. Gharkhanian,[b] Nicole G. Ricapito,[c] J. McNamara,[c] and Timothy J. Deming*[a]

Abstract: Biologically occurring non-canonical di-α-amino acids were converted into new di-N-carboxyanhydride (di-NCA) monomers in reasonable yields with high purity. Five different di-NCAs were separately copolymerized with *tert*-butyl-L-glutamate NCA to obtain covalently crosslinked copolypeptides capable of forming hydrogels with varying crosslinker density. Comparison of hydrogel properties with residue structure revealed that different di-α-amino acids were not equivalent in crosslink formation. Notably, L-cystine was found to produce significantly weaker hydrogels compared to L-homocystine, L-cystathionine, and L-lanthionine, suggesting that L-cystine may be a sub-optimal choice of di-α-amino acid for preparation of copolypeptide networks. The di-α-amino acid crosslinkers also provided different chemical stability, where disulfide crosslinks were readily degraded by reduction, and thioether crosslinks were stable against reduction. This difference in response may provide a means to fine tune the reduction sensitivity of polypeptide biomaterial networks.

Introduction

Novel hydrogel networks are continuously investigated for their potential to fulfill unmet needs in drug delivery, wound healing, and tissue engineering applications [1]. Hydrogels, cross-linked polymer systems that swell in aqueous environments, are useful biomedical materials that can be biocompatible and possess several tunable properties (e.g. cross-link density, pore size, degradation rate, etc.). One of the most important factors for successfully achieving a desired performance property is the rational selection of constituent molecules from which the hydrogel network is prepared. To date, a number of natural (e.g. collagen, hyaluronan, cellulose, etc.) and synthetic polymers (e.g. poly(ethylene glycol), poly(acrylamide), poly(vinyl alcohol), etc.) have been utilized in hydrogels for various purposes [2]. In the present study, we highlight the potential to synthesize new hydrogel networks with advantageous functionalities through the use of previously unexplored bio-based compounds as starting monomers for polymerization.

Molecules that are known to exist within living organisms are often multi-functional and chiral. The unique characteristics of such molecules enable polymerization into various form factors with tunable features such as 3D structure, biodegradability, and biomimetic rheological properties [2]. Additionally, the presence of multiple functional groups offers reactive sites for chemical cross-linking, drug or cell attachments, and surface property alterations. Amino acids are one such class of compounds that are frequently investigated for use in biomedical applications [3]. Natural amino acids are attractive building blocks, as they offer an array of functionalities that can be inserted into homo- and co-polymeric systems through the use of straightforward and scalable N-carboxyanhydride (NCA)-based polymerization [3].

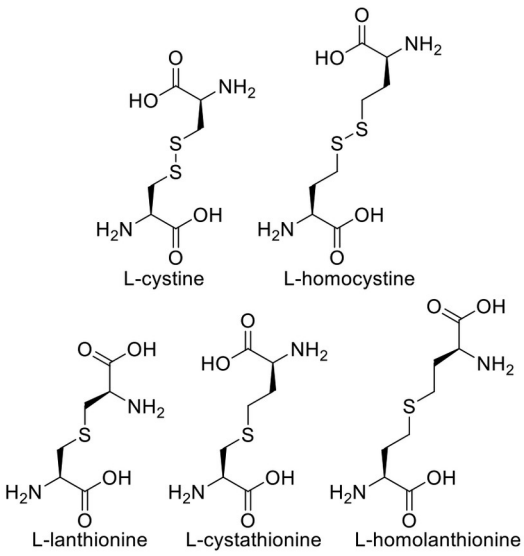

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Figure 1. Chemical structures of di-α-amino acids used to prepare di-NCA chemical crosslinkers.

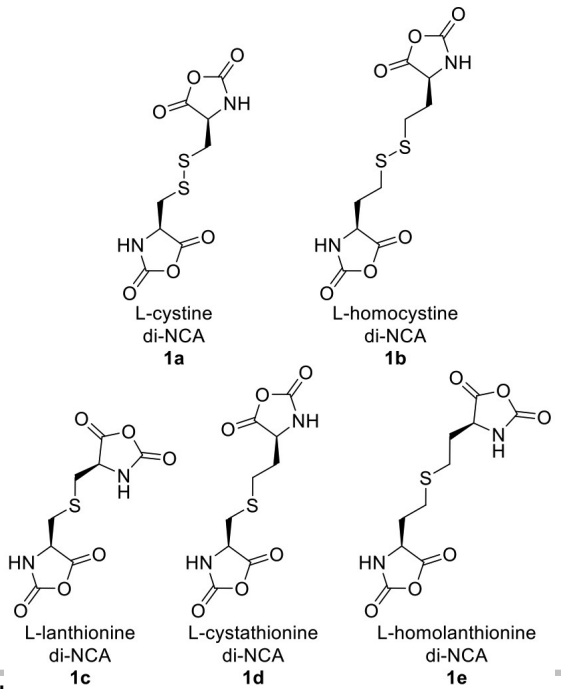
While many of the 20 standard amino acids have been utilized in biomimetic materials [3], the realm of possibilities in α-amino acid based technologies extends beyond these common moieties. A number of non-translated amino acids are known to exist in living systems and recent advances in microbial engineering strategies may allow for cost-effective production of these non-canonical amino acids in large quantities [4]. The integration of high throughput technologies, machine learning, and advanced genomics offers the potential to increase the ease with which biomolecules can be produced through fermentation in high yields, and at low costs, particularly compared to synthetic routes [5]. In the present work, we focus on the exploration of a sub-class of compounds in this category: di-α-amino acids (Figure 1). If found to be valuable for materials applications, these di-α-amino acids have potential to be practical feedstocks for downstream applications.

The use of di-α-amino acids for the synthesis of covalently crosslinked polypeptides dates back to the early 1950s, when the di-N-carboxyanhydride, di-NCA, of L-cystine was first prepared and subsequently homopolymerized to produce an intractable solid that could be solubilized by reduction with 2-mercaptoethanol [6]. Copolymerization of D/L-cystine di-NCA with NCAs of L-lysine and L-glutamic acid was also reported, which yielded water insoluble, crosslinked solids presumably containing both covalent and ionic crosslinks [7]. These materials were found to reversibly swell in either acidic (pH < 5) or basic media (pH > 10), which would be expected to disrupt ionic crosslinks via neutralization of glutamate or lysine side-chains, respectively.

In more recent usage, L-cystine di-NCA has been incorporated into covalently crosslinked polypeptide containing nanogels or star particles. In these systems, linear polymer chains, typically either PEG [8], poly-L-lysine [9], or poly-L-glutamate [10] with free amine groups on their chain ends were reacted with L-cystine di-NCA with or without additional NCA monomers to give covalently crosslinked nanoparticles. The resulting water soluble particles offer attractive properties for drug delivery since they can be either swollen or dissolved by reduction of the cystine disulfide bond crosslinks [8-10]. The ability to degrade a cross-linked system in a controlled manner through exposure to a trigger, such as a reducing agent, is an area of high interest in the development of stimuli-responsive materials. For example, disulfide based technologies are often investigated for the targeted intracellular release of therapeutics owing to increased levels of the reducing agent glutathione in the cytosol compared to the bloodstream [8-10].

An important observation from previous polymerizations of L-cystine di-NCA was that consumption of this monomer during polymerization was typically incomplete, stopping around 70 to 80% conversion [8-10]. This result may be due to the inability of polymer bound NCA groups from single additions of L-cystine di-NCA to find and react with growing chain ends once networks are formed. In the nanoparticle systems described above, the unreacted cystine NCA groups were beneficially utilized for attachment of functional amines to tune nanoparticle properties [8-10]. However, lack of complete polymerization hints that L-cystine di-NCA may not fully incorporate during copolypeptide synthesis.

Here, we sought to explore the potential for preparation of covalently crosslinked copolypeptide hydrogels via copolymerization of di-NCA monomers and a monofunctional NCA. In particular, due to potential issues observed with incomplete incorporation of L-cystine di-NCA during copolymerizations, as well as the limited availability of alternative di-NCA molecules to evaluate structure-property relationships, we sought to develop and incorporate other natural di-α-amino acids as comonomers for hydrogel preparation. More specifically, we aimed to expand the palette of di-NCA monomers toward the goal of tuning hydrogel properties including their sensitivity to reduction and oxidation. We selected the set of di-α-amino acids shown in Figure 1 since they allow evaluation of copolymerization and hydrogel formation with different linker segment lengths, and also utilize both reducible (i.e. disulfide) and non-reducible (i.e. thioether) tethers. In this study, we prepared di-NCA monomers for all the di-α-amino acids shown in Figure 1 (Figure 2), and individually investigated their copolymerization with *tert*-butyl-L-glutamate NCA (Bu-Glu NCA) to prepare covalently crosslinked copolypeptide hydrogels. Subsequent comparison of hydrogel properties with residue structure

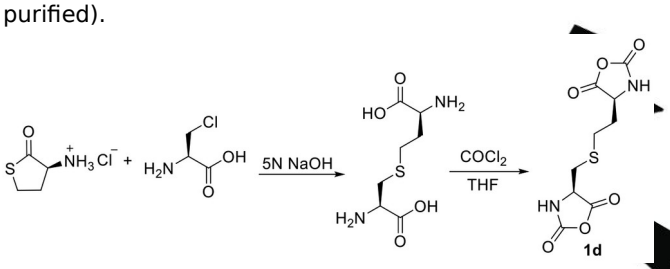


was used to evaluate the potential of each di-α-amino acid to act as a crosslinker. The ability of each hydrogel to respond to chemical reduction stimulus was also evaluated.

Figure 2. Structures of di-NCAs **1a-1e**.

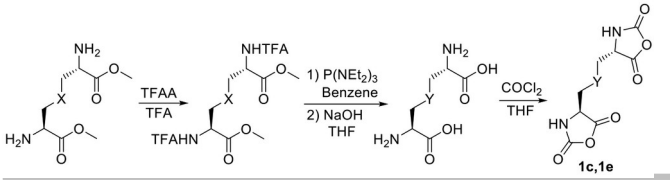
Results and Discussion

In order to study the potential of di-α-amino acids in Figure 1 for formation of crosslinked copolypeptide hydrogels, many di-α-amino acids and new di-NCAs needed to be prepared. Of the molecules in Figure 2, only L-cystine di-NCA has been previously reported [6]. Since L-cystine is readily available, it can be converted to the di-NCA via direct reaction with phosgene or a phosgene equivalent such as triphosgene. Due to the low solubility of L-cystine in the reaction mixture, low yields of L-cystine di-NCA (**1a**) have typically been reported (*ca*. 20-25%, isolated and purified) [6,8]. Higher yields have been reported with use of a more soluble precursor for this reaction, such as N,N'-bis(benzyloxycarbonyl)-L-cystine, at the expense of reduced atom economy [9,10]. Since L-homocystine is also commercially available, we prepared its corresponding di-NCA (**1b**) by direct reaction with triphosgene. Due to the greater solubility of L-homocystine versus L-cystine in THF, a reasonable yield of new di-NCA **1b** was obtained (*ca*. 42%, isolated and purified).



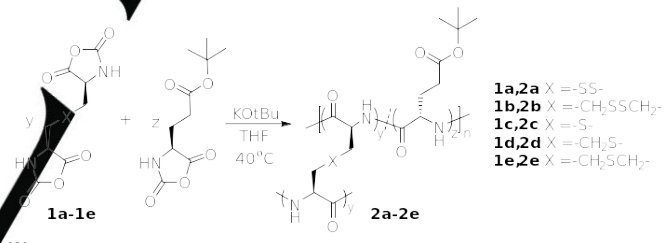
Scheme 1. Preparation of di-NCA monomer **1d**.

Due to their limited commercial availability and high cost, the remaining di-α-amino acids in Figure 1 were prepared using chemical synthesis. L-Cystathionine was prepared from L-homocysteine thiolactone and L-chloroalanine via a modified literature procedure as shown in Scheme 1 [11]. This product was subsequently phosgenated to obtain the new L-cystathionine di-NCA (**1d**) in reasonable yield (*ca*. 54%, isolated and purified). L-Lanthionine and L-homolanthionine were prepared from protected L-cystine and L-homocystine derivatives, respectively, via desulfurization using a modified literature procedure as shown in Scheme 2 [12]. Their corresponding new di-NCAs (**1c**, **1e**) were obtained by phosgenation. While **1c** was obtained in reasonable yield (*ca*. 59%, isolated and purified), poor solubility of L-homolanthionine resulted in a low yield of **1e** (*ca*. 12%, isolated and purified).



Scheme 2. Preparation of di-NCA monomers **1c** (X= -SS-, Y= -S-) and **1e** (X= -CH_2SSCH_2-, Y= -CH_2SCH_2-).

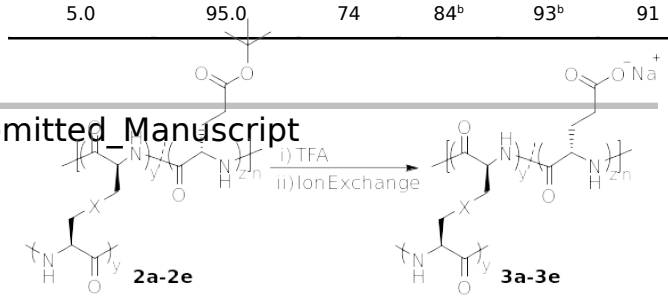
With di-NCAs **1a-1e** in hand, we prepared copolypeptides of each of these with Bu-Glu NCA toward the goal of preparing covalently crosslinked networks [13]. *tert*-Butyl-L-glutamate was chosen as a suitable residue to form soluble, linear polypeptide segments between the di-α-amino acid crosslinks [14]. After removal of *tert*-butyl protecting groups, L-glutamate residues also provide water solubility at neutral pH, which should enable copolypeptide network swelling in water to form hydrogels. Copolypeptides of Bu-Glu NCA with each different di-NCA were prepared in THF using KOtBu initiator (Scheme 3, Table 1). While other initiators, e.g. $Co(PMe_3)_4$, could be used to obtain living NCA copolymerizations [15], we deemed this unnecessary here since all chains were ultimately expected to be crosslinked into networks. Strongly basic KOtBu initiator was chosen since it favors formation of high molecular weight copolymers [16], which was advantageous since long chain lengths were expected to maximize the number of crosslinks per chain. The mole percentage of di-NCA in copolymerizations was varied from 1.0 to 5.0 % of total monomer content, and under these conditions most copolymerizations went to complete consumption of all NCA monomers. Notably, the copolymerizations using L-homolanthionine di-NCA were exceptions, since they were found to stop with considerable amounts of monomer remaining (*ca*. 30%). Also, at higher di-NCA fractions, small amounts of copolypeptide precipitation was observed in some samples, which also likely limited monomer consumption.



Scheme 3. Copolymerization of di-NCAs (**1a-1e**) with Bu-Glu NCA to form copolypeptide networks. y,z = mole percent of each comonomer. n = degree of polymerization.

Table 1. Copolymerization data for varying comonomer feed ratios.

di-NCA (mol %)	Bu-Glu NCA (mol %)	Yield (%)[a]			
		3a	3b	3c	3d
1.0	99.0	81	99	89	97
1.5	98.5	99	83	99	99
2.5	97.5	97	91	98	90
5.0	95.0	74	84[b]	93[b]	91



[a] Isolated yield of deprotected copolypeptides. [b] Incomplete monomer consumption observed in copolymerization.

Scheme 4. Deprotection of copolymers to yield samples capable of hydrogel formation. y,z = mole percent of each comonomer. n = degree of polymerization. X = same as in Scheme 3.

All the copolypeptide samples (**2a-2e**) prepared in Scheme 3 were found to form self-supporting organogels in THF, with **2a-2d** shown in Figure 3. These organogels were stable against dilution confirming the formation of crosslinked networks in these samples. By visual inspection, organogel stiffness for all samples was generally observed to increase with increasing mole fraction of di-NCA, as would be expected for increased crosslink density. After removal of THF from these samples under vacuum, the *tert*-butyl groups were removed from L-glutamate residues by addition of TFA, followed by neutralization with $NaHCO_3$ and exhaustive dialysis against DI water to give the deprotected copolypeptides as sodium salts (**3a-3e**) (Scheme 4).

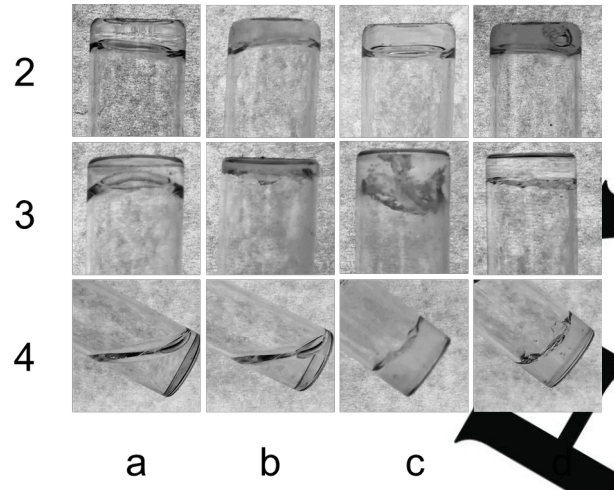


Figure 3. Sample images. **2a-2d**) 4.0 wt% organogels formed during copolymerization in THF. **3a-3d**) Hydrogels formed from copolymers at 1.0 wt% in DI H_2O. **4a-4d**) Network degradation after exposure of copolymer networks (1.0 wt%) to TCEP for 48 hrs.

Addition of DI water to samples **3a**-**3e** resulted in swelling of the copolypeptide networks and hydrogel formation. The samples from L-homolanthionine formed only weak hydrogels (**3e**, Figures S1 and S2). Since the compositions of these samples (**3e**) were not known due to incomplete monomer consumption, more detailed analysis of their properties was not pursued. With sufficient water added to give 1.0 wt% sample concentrations, all of the remaining copolypeptides prepared with 2.5 mole percent di-NCA gave self-supporting hydrogels (Figure 3, Figure S3). Equilibrium swelling studies of samples in excess DI water also showed that hydrogel swelling ratio was inversely correlated to crosslinker content, as would be expected since crosslink density should increase with crosslinker content (Figure S4, Table S1). An important observation from this initial set of samples was that L-cystine was the least effective crosslinker among the different di-amino acids tested, as it formed the most fragile hydrogels (**3a**) via visual inspection (*vide infra*). In contrast, samples prepared using all the other di-amino acids formed hydrogels with much greater stiffness.

To better quantify differences in hydrogel properties among different samples, we chose to compare samples **3a-3d**, all containing 2.5 mole percent di-α-amino acid crosslinker. Rheological analysis revealed that all four samples behaved as elastic hydrogels, as evidenced by storage moduli (G') being greater than loss moduli (G") over a range of frequency (Figure 4A). The hydrogels were found to be somewhat brittle, as they began to break down at strain amplitudes of *ca*. 10 (Figure 4B). The most important result from the rheology measurements was how hydrogel stiffness, as measured by G', differed as a function of di-α-amino acid crosslinker. As expected from visual observations discussed above, the L-cystine crosslinked sample **3a** formed the weakest hydrogel (lowest G'), which was substantially weaker than all the others tested. The L-homocystine **3b** and L-lanthionine **3c** samples gave hydrogels with nearly an order of magnitude greater G' compared to the L-cystine hydrogel (Figure 4A). The sample containing L-cystathionine **3d** gave the stiffest hydrogel, which was more than twice as rigid as the **3b** and **3c** hydrogels. In terms of efficiency of network formation, these data show that L-cystine is the least efficient, and L-cystathionine is most efficient, in forming covalent copolypeptide hydrogel networks. While additional studies are needed to analyze these systems in more detail, it is clear that small changes in di-NCA tether length and the nature of the tether itself (i.e. thioether vs. disulfide) greatly impact the ability of the di-NCA monomers to form interchain crosslinks during copolymerization.

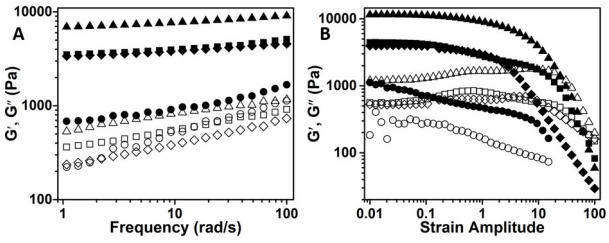
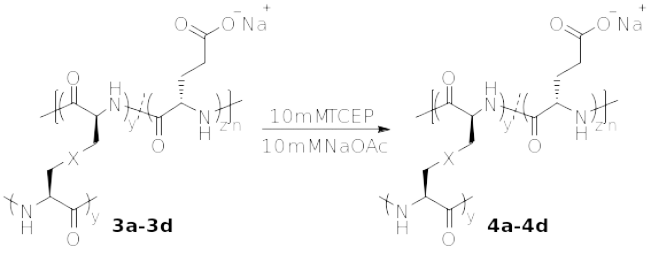


Figure 4. Rheology data for hydrogels **3a** (●), **3b** (♦), **3c** (■), and **3d** (▲) at 1.0 wt% in DI water. A) Frequency sweep experiments at constant strain amplitude of 0.1. B) Strain sweep experiments at constant frequency of 10 rad/s. G′ = solid symbols; G″ = open symbols.



Scheme 5. Reduction of hydrogel networks with TCEP. y,z = mole percent of each comonomer. n = degree of polymerization.

Beyond their utility for formation of copolypeptide hydrogel networks, the di-α-amino acids of this study also provide chemical functionality that can impart different stimulus responsive properties to the hydrogels. As a proof of concept, hydrogels **3a-3d** were subjected to chemical reduction using tris-carboxyethylphosphine (TCEP) in aqueous media (Scheme 5). TCEP is well known to readily reduce disulfide linkages to thiols, but should not reduce thioether linkages. Upon treatment with TCEP, we observed that disulfide crosslink containing samples **4a** and **4b** became free-flowing liquids (Figure 3), which confirmed that the di-α-amino acids were acting as the network crosslinks in the hydrogels and that these were broken upon reduction. As expected, the thioether crosslink containing hydrogels **4c** and **4d** were unaffected by treatment with TCEP, which confirmed that these linkages were resistant to cleavage by reduction. These different responses to chemical reduction provide a means to tune hydrogel properties by choice of crosslinker.

Conclusions

We have shown that biologically occurring non-canonical di-α-amino acids can be converted into di-NCA monomers in reasonable yields with high purity. While di-α-amino acids are present in biology, the new di-NCA monomers reported here have never before been used in the chemical synthesis of polypeptide biomaterials. In order to evaluate structure-property effects of different di-α-amino acid tethers on network properties, each di-NCA was copolymerized with a monofunctional NCA to obtain covalently crosslinked copolypeptides capable of forming hydrogels. Notably, use of widely studied L-cystine di-NCA resulted in significantly weaker hydrogels compared to the di-NCAs of L-homocystine, L-cystathionine, and L-lanthionine, suggesting that L-cystine is likely not the best choice of di-α-amino acid for preparation of copolypeptide networks. The di-α-amino acid crosslinkers also provided different chemical stability, where disulfide crosslinks were readily degraded by reduction, and thioether crosslinks were stable against reduction. This difference in reduction response may provide a means to fine tune the reduction sensitivity of polypeptide biomaterial networks.

This work exemplifies that while readily available bio-based compounds are attractive targets for biomaterials, there exists a greater palette of naturally occurring molecules to be explored. Such compounds can offer enhanced performance properties and may be produced through advanced microbial engineering technologies. More specifically, we have shown that biological di-α-amino acid feedstocks can be converted into monomers for preparation of covalently crosslinked copolypeptide networks with attractive biomaterial properties. These new building blocks thus expand the design possibilities of novel functional biomaterials for applications such as drug delivery, wound healing, and tissue engineering.

Experimental Section

Materials and Methods

TEA (Fisher) was distilled from CaH_2 under N_2 and stored over 4 Å molecular sieves. TMSCl (Sigma-Aldrich) was purified by distillation under N_2. The following chemicals were used as received from the vendor: trifluoroacetic acid (Oakwood), L-cystine dimethyl ester and L-homocystine dimethyl ester (Chem-Impex Intl.), tris(diethylamino)-phosphine (Sigma-Aldrich), triphosgene and 15% phosgene in toluene (Sigma-Aldrich), tris(carboxyethyl)phosphine 0.5 M in H_2O (Sigma-Aldrich). H_2O was purified by reverse osmosis. L-Homocysteine thiolactone hydrochloride [17], 3-chloro-L-alanine [18], and 2 kDa PEG-isocyanate [19] were prepared by previously reported methods. NCA purification [20] and polymerizations [15] were performed in an N_2 filled glove box using established techniques. Reactions at elevated temperature were controlled using a Corning PC 420D thermostated hotplate equipped with a thermocouple probe. Room temperature reactions were performed at *ca*. 22 ºC ambient temperature. All reactions were performed under N_2 and unless otherwise described at 22 ºC. THF and hexanes were degassed by sparging with nitrogen and dried by passage through columns of dried alumina. Thin-layer chromatography was performed with EMD gel 60 F254 plates (0.25 mm thickness) and visualized using a UV lamp or permanganate stain. Column chromatography was performed using Silicycle Siliaflash G60 silica (60-200 µm). ESI-MS spectra were recorded on a Waters LCT Premier spectrometer or a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. ATIR spectroscopy was performed on a PerkinElmer Spectrum RX spectrometer or a JASCO FT/IR-4100 spectrometer. NMR spectroscopy was performed on a Bruker AV400 spectrometer. Abbreviations: acetic acid (AcOH), attenuated total reflectance infrared spectroscopy (ATR-IR), chlorotrimethylsilane (TMSCl), circular dichroism (CD), methoxy polyethylene glycol (PEG), molar equivalent (eq), molecular weight cutoff (MWCO), triethylamine (TEA), trifluoroacetic acid (TFA), trifluoroacetic anhydride (TFAA), trimethylsilyl (TMS), tetrahydrofuran (THF), tris(carboxyethyl)phosphine (TCEP)

Synthetic Procedures

TFA-L-cystine-OMe and TFA-L-homocystine-OMe were prepared from commercially available L-cystine-OMe and L-homocystine-OMe according to a literature procedure [12]. TFA-L-lanthionine-OMe was prepared from TFA-L-cystine-OMe according to a literature procedure [12].

TFA-L-homolanthionine-OMe

TFA-L-homocystine-OMe (0.60 g, 1.2 mmol, 1.0 eq) was dissolved in anhydrous benzene. Tris(diethylamino)phosphine (0.35 mL, 1.3 mmol, 1.05 eq) was added dropwise. The reaction was heated to 30 ºC and allowed to react for 16 hrs [20]. The reaction was

cooled to room temperature and solvent was removed *in vacuo*. The residue was purified via column chromatography (9:1 Hex:EtOAc to 3:1 Hex:EtOAc) to yield TFA-L-homolanthionine-OMe as a white solid. (0.52 g. 94% yield).

L-Lanthionine [12]

TFA-L-lanthionine-OMe (1.0 g, 2.3 mmol, 1.0 eq), was dissolved in THF (12 mL) and cooled to 0 ºC. 2N NaOH (25 mL) was added dropwise and the reaction was allowed to stir for 1 hr at 0 ºC. 1N HCl (12 mL) was added and the mixture was adjusted to pH 6 with 1N HCl. The solvent was removed *in vacuo*. The residue was suspended in water and filtered to give L-lanthionine as white crystals (0.27 g, 55% yield). ^{1}H NMR (400 MHz, TFA-D, 25 ºC): δ 4.69 (s, 2 H), 3.63-3.53 (m, 2H), 3.5-3.4 (m, 2H). ^{13}C NMR (100 MHz, TFA-D, 25 ºC): δ 170.3, 53.0, 31.2.

L-Homolanthionine [21]

TFA-L-homolanthionine-OMe (0.56 g, 1.2 mmol, 1.0 eq), was dissolved in THF (8 mL) and cooled to 0 ºC. 2N NaOH (12 mL) was added dropwise and the reaction was allowed to stir for 1 hr at 0 ºC. 1N HCl (6 mL) was added and the mixture was adjusted to pH 6 with 1N HCl. The solvent was removed *in vacuo*. The residue was dissolved in water (5 mL) and precipitated into absolute ethanol (45 mL) and placed in the freezer overnight. The reaction was centrifuged and the supernatant was removed. Excess ethanol was removed under high vacuum to yield white crystals (0.24 g, 81% yield). ^{1}H NMR (400 MHz, TFA-D, 25 ºC): δ 4.70 (q, J=6.0 Hz, 2 H), 3.00 (t, J=6.7 Hz, 2H), 2.67-2.45 (m, 4H). ^{13}C NMR (100 MHz, TFA-D, 25 ºC): δ 172.7, 53.0, 28.3, 26.8.

L-Cystathionine [11]

L-Homocystine thiolactone-HCl (0.60 g, 3.9 mmol, 1.0 eq) and 3-chloro-L-alanine (0.63 g, 5.1 mmol, 1.3 eq) were added to a flask and flushed with N_2. Degassed 5 N NaOH (3.5 mL, 18 mmol, 4.5 eq) was added. The mixture was stirred for 64 h. The pH was adjusted to 10.5-11.0 with conc. $HCl_{(aq)}$. The mixture was cooled to 4 ºC and allowed to stand for 16 h. The precipitate was collected by vacuum filtration. The amino acid was recovered as a colorless solid (470 mg, 54% yield). ^{1}H NMR (400 MHz, D_2O, 25 ºC): δ 4.20 (dd, *J* = 7.3, 4.5 Hz, 1 H), 4.20 (t, *J* = 6.5 Hz, 1 H), 3.25 (dd, *J* = 15.2, 4.6 Hz, 1 H), 3.15 (dd, *J* = 15.1, 7.2 Hz, 1 H), 2.80 (m, *J* = 7.6 Hz, 2 H), 2.30 (m, 1 H), 2.21 (m, 1 H).

L-Cystine di-NCA (1a)

L-Cystine (1.5 g, 6.2 mmol, 1.0 eq) was suspended in THF (50 mL). Triphosgene (2.5 g, 8.3 mmol, 1.3 eq) was added in one portion. The mixture was stirred at 50 ºC for 24 h. The turbid mixture was concentrated and the crude product was purified by column chromatography (50:50 THF:hexanes) under inert atmosphere. After concentration, the material was diluted with THF and precipitated into hexanes. This provided a pale yellow solid (0.42 g, 23% yield). The IR and ^{1}H NMR for this material were in accordance with those previously reported [8-10].

L-Homocystine di-NCA (1b)

L-Homocystine (0.4 g, 1.5 mmol, 1.0 eq) was suspended in THF (30 mL). Triphosgene (0.59 g, 2.0 mmol, 1.3 eq) was added in one portion. The mixture was stirred at 50 ºC for 24 h. The turbid mixture was concentrated and the crude product was purified by column chromatography (60:40 THF:hexanes) under inert atmosphere. After concentration, the material was diluted with THF and precipitated into hexanes. This provided a colorless solid (0.20 g, 42% yield). ^{1}H NMR (400 MHz, CD_3CN, 25 ºC): δ 6.87 (br s, 2 H), 4.47 (ddd, *J* = 6.9, 5.4, 1.3 Hz, 2 H), 2.80 (t, *J* = 7.2 Hz, 4 H), 2.19 (m, 4 H). ^{13}C NMR (100 MHz, CD_3CN, 25 ºC): δ 172.1, 153.2, 57.5, 34.2, 31.8. FTIR (thin film) 1860, 1792 cm^{-1} ESI-MS *m/z* = 319.0063 $[M+H]^-$ (calcd 319.0059 for $C_{10}H_{11}N_2O_6S_2$).

L-Lanthionine di-NCA (1c)

L-Lanthionine (0.27 g, 1.3 mmol, 1.0 eq) was suspended in THF (30 mL). 15% phosgene solution in toluene (3.6 mL, 5.1 mmol, 4 eq) was added. The mixture was stirred at 45 ºC for 18 h and then concentrated. The crude product was purified by column chromatography (60:40 THF:hexanes) under inert atmosphere. After concentration, the material was diluted with THF and precipitated into hexanes. This provided the product as a white solid (0.20 g, 59% yield). ^{1}H NMR (400 MHz, CD_3CN, 25 ºC): δ 6.84 (br s, 2 H), 4.62-4.58 (m, 2 H), 3.12-3.07 (m, *J* = 4.0, 14.5 Hz, 2 H), 3.01-2.95 (m, 2H). ^{13}C NMR (100 MHz, CD_3CN, 25 ºC): δ 169.51, 151.67, 58.1, 34.0. FTIR (thin film) 1860, 1790 cm^{-1}. ESI-MS *m/z* = 259.0031 $[M-H]^-$ (calcd 259.0025 for $C_8H_7N_2O_6S$).

L-Cystathionine di-NCA (1d)

L-Cystathionine (0.30 g, 1.3 mmol, 1.0 eq) was suspended in THF (20 mL). 15% phosgene solution (3.9 mL, 5.4 mmol, 4 eq) was added. The mixture was stirred at 45 ºC for 18 h and then concentrated. The crude product was purified by column chromatography (60:40 THF:hexanes) under inert atmosphere. After concentration, the material was diluted with THF and precipitated into hexanes. This provided a colorless solid (0.20 g, 54% yield). ^{1}H NMR (400 MHz, CD_3CN, 25 ºC): δ 6.86 (br m, 2 H), 4.62 (dd, *J* = 4.2, 1.3 Hz, 1 H), 4.44 (dd, *J* = 1.4, 5.6 Hz, 1 H), 3.04 (dd, *J* = 14.5, 4.0 Hz, 1 H), 2.92 (dd, *J* = 14.5, 5.2 Hz, 1 H), 2.71 (t, *J* = 7.4 Hz, 2 H), 2.06 (m, 2 H). ^{13}C NMR (100 MHz, CD_3CN, 25 ºC): δ 170.9, 169.7, 151.8, 151.8, 58.2, 56.1, 32.4, 30.8, 28.0. FTIR (thin film) 1860, 1792 cm^{-1}. ESI-MS *m/z* = 273.0204 $[M-H]^-$ (calcd 273.0181 for $C_9H_9N_2O_6S_2$).

L-Homolanthionine di-NCA (1e)

L-Homolanthionine (0.23 g, 1.0 mmol, 1.0 eq) was suspended in THF (30 mL). A solution of 15% phosgene in toluene (2.8 mL, 4.0 mmol, 4 eq) was added. The mixture was stirred at 45 ºC for 24 h. The turbid mixture was concentrated and the crude product was purified by column chromatography (60:40 THF:hexanes) under inert atmosphere. After concentration, the material was diluted with THF and precipitated into hexanes. This provided a sticky white solid (0.03 g, 12% yield). ^{1}H NMR (400 MHz, CD_3CN, 25 ºC): δ 6.87 (br s, 2 H), 4.47 (ddd, *J* = 6.9, 5.4, 1.3 Hz, 2 H), 2.80 (t, *J* = 7.2 Hz, 4 H), 2.19 (m, 4 H). ^{13}C NMR (100 MHz, CD_3CN, 25 ºC): δ 171.0, 151.8, 56.2, 30.5, 26.3. FTIR (thin film) 1856, 1788 cm^{-1}. ESI-MS *m/z* = 287.0337 $[M-H]^-$ (calcd 287.0338 for $C_{10}H_{11}N_2O_6S$).

γ-tert-Butyl L-glutamate NCA (Bu-Glu NCA)

γ-tert-Butyl L-glutamic acid (2.0 g, 9.8 mmol, 1.0 eq) was suspended in THF (60 mL). Triethylamine (2.75 mL, 19.7 mmol, 2.0 eq) and TMSCl (2.5 mL, 19.7 mmol, 2.0 eq) were added and the turbid mixture was stirred for 1hr at room temperature. Phosgene (15% in toluene) (10.2 mL, 14.7 mmol, 1.5 eq) was added and the mixture was heated to 45 ºC and allowed to react for 2 hrs. The turbid mixture was concentrated and the crude product was purified by column chromatography (30:70 THF:Hexanes to 50:50 THF:Hexanes) under inert atmosphere. After concentration, the material was crystallized twice from 3:1 Hex:THF to provide a white fluffy solid (1.75 g, 68%). The IR and ^{1}H NMR for this material were in accordance with those previously reported [14].

Binary copolymerizations of di-NCAs with Bu-Glu NCA

Separate stock solutions of each di-NCA (**1a-1e**) and Bu-Glu NCA were prepared in THF (note that 3:1 THF:DMF was required to dissolve **1c**) at concentrations of 50 mg/mL each. These were mixed in different proportions to obtain the desired comonomer feed ratios. The resultant solutions were treated with a defined volume of initiator (20 mg/mL solution of KO*t*Bu in THF) to obtain the desired monomer to initiator ratio. The mixtures were then heated at 40 ºC for 24 h, whereupon all samples formed organogels. For each copolymerization, an aliquot of the polymerization mixture was analyzed by FTIR spectroscopy to determine if consumption of NCA monomers was complete. The copolymerizations were then concentrated under a gentle stream of air followed by high vacuum. The obtained solids were then carried through to deprotection without further purification.

Copolypeptide deprotection

Protected copolypeptides (**2a-2e**) were each treated with TFA (40 µL per mg of sample) and allowed to stand for 24 h. The mixtures were then evaporated under a stream of air to provide solvent-swollen solids. Volatiles were then removed under high vacuum. The crude copolymer networks were each transferred to 2 kDa MWCO dialysis bags and dialyzed against aqueous 50 mM NaCl and 50 mM $NaHCO_3$ (8h, 2 solvent changes) followed by H_2O (16h, 3 solvent changes). The retentates were then lyophilized to provide the deprotected copolypeptide networks.

Preparation of Hydrogels

Deprotected copolypeptide networks (**3a-3e**) were swollen at 1 wt% concentration in DI H_2O, sealed under N_2, and centrifuged for 30 seconds to obtain hydrogel samples.

Reduction of Hydrogels

Deprotected copolypeptide networks (**3a-3d**) were swollen at 1 wt% concentration in a 10mM NaOAc/10 mM TCEP aqueous solution, sealed under N_2, and then centrifuged to obtain hydrogel samples, which were let stand for 12 h. Degradation of hydrogel networks was determined via inversion of the resulting samples (**4a-4d**).

Rheology

An Anton Paar MCR 102 rheometer with a 25 mm cone and plate geometry and aqueous solvent trap was used for all measurements. Frequency sweeps were measured at a constant strain amplitude of 0.1. Strain sweeps were measured at a constant frequency of 10 rad/s.

Equilibrium Hydrogel Swelling Experiments

Each copolypeptide network sample (**3a-3d**) (~5mg) was swollen in excess (3 mL) DI H_2O for 24 hours at room temperature. Excess water was wicked away and the mass of the maximally swollen hydrogel was measured.

Acknowledgements

This research was developed with funding from the Defense Advanced Research Projects Agency's (DARPA) Living Foundries program. The views, opinions, and/or findings expressed are those of the author and should not be interpreted as representing the official views or policies of the Department of Defense or the U.S. Government. The mass spectrometry instrumentation used in this study was supported by the National Institutes of Health under instrumentation grant no. 1S10OD016387-01.

Keywords: hydrogel • crosslink • polypeptide • di-amino acid • stimuli responsive

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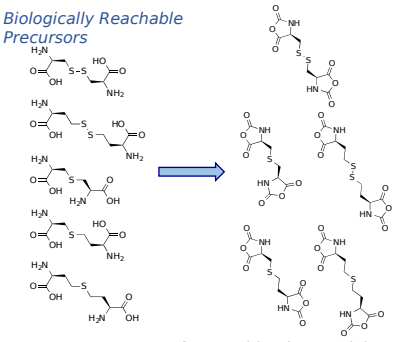
Entry for the Table of Contents (Please choose one layout)

Layout 1:

FULL PAPER

Biologically occurring non-canonical di-α-amino acids were converted into new di-N-carboxyanhydride, di-NCA, monomers in reasonable yields with high purity. These new building blocks enabled synthesis of covalent copolypeptide biomaterial hydrogels with tunable properties.



Components for Tunable Biomaterial Networks

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Influence of sulfur containing di-amino acid structure on covalently crosslinked copolypeptide hydrogels