Synthesis of Collagen-like Peptide Polymers by Native Chemical Ligation

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ABSTRACT: We report on the synthesis of high molecular weight collagen-like peptide polymers prepared by a combination of solid phase peptide synthesis, polymerization, and self-assembly. The final product is a mesh of nanofibers that maintains the characteristic circular dichroism signal for collagen triple helices and the nanofiber diameter is 10−20 nm, similar to natural collagen fibrils. This method utilizes an N-terminal cysteine and C-terminal thioester to achieve selective head to tail polymerization of peptides without the need for protecting groups and under neutral aqueous conditions in which the peptide may adopt a folded conformation. The synthesized peptide polymers were characterized by size-exclusion chromatography, circular dichroism spectroscopy, and transmission electron microscopy.

Introduction

There is a strong need for new materials that can act as scaffolds to support the adhesion, motility, growth and differentiation of cells.[1−5] These materials are critical for tissue engineering and tissue regeneration strategies. There are two groups of materials currently used in most tissue engineering approaches: those that utilize synthetic polymers such as poly-lactic acid (PLA)[6−9] and those that use materials derived from biological sources such as reconstituted collagen.[10] Although each group of materials has shown successes in particular applications,[11] neither category has all the structure and properties of the ideal material, natural extra-cellular matrix (ECM). Polymers such as PLA have the advantage of being biodegradable, biocompatible, inexpensive and easy to prepare. However, they lack the chemical information cells expect to find in the ECM and also have a molecular and nanostructure unlike this natural environment. On the other hand, reconstituted collagen has much of the chemical and structural information of the ECM but has the disadvantage of being difficult to tailor for specific tissue applications. Also, because of its biological origin, issues of purity and immune reaction are not insignificant.[3] To address these deficiencies, a number of groups are developing novel synthetic[12−16] or semisynthetic[17] scaffolds for tissue engineering. In this paper we describe a novel method for the preparation of high molecular weight polymerized peptides which mimic many of the chemical and structural characteristics of collagen.

Collagen is the major constituent of ECM and plays a central role in its organization and mechanical properties.[18] Therefore developing a method to synthesize it or a close approximation of it through chemical methods would be highly desirable. Type I collagen is 1000 amino acids in length, exceeding by a factor of 10 what can be achieved by solid phase methods, and incorporates a large quantity of the posttranslationally modified amino acid hydroxyproline. One approach to overcome the limitations of solid phase synthesis is to utilize microorganisms to synthesize larger proteins.[19−26] However, this approach has drawbacks in that both unnatural amino acids and posttranslational modifications to natural amino acids are challenging to incorporate. An alternative approach is to polymerize α-amino acid *N*-carboxyanhydride monomers.[27−29] Although this approach is excellent for a number of applications, it is limited to homopolymers, random copolymers, and block copolymers. Short peptides have also been used either by activating the C-termini of the peptide or by using diphenylphosphoryl azide reagent[30] and carrying out a head to tail polymerization, but these approaches are limited to the use of amino acids with nonreactive side chains or side chain protected peptide segments which typically have limited solubility.[31,32]

Native chemical ligation was developed to link together two synthetic peptides to yield proteins that would be difficult or impossible to prepare otherwise.[33,34] This ligation technique allows one to couple unprotected synthetic peptides in aqueous solution at neutral pHs, thus preserving their secondary or tertiary structure. Native amide bond formation at the site of ligation results from a spontaneous rearrangement of a thiol exchange product, which is chemoselectively formed between ligated peptides during a reaction of a C-terminal thioester with a thiol group of an N-terminal Cys residue (Scheme 1a). The native chemical ligation is superior to the use of water soluble carbodiimide, because the latter approach limits the range of amino acids that could be potentially incorporated into a sequence to the ones that lack amines or carboxylic acids in their side chains.

Here, we report on the synthesis of high molecular weight collagen-like peptide polymers prepared by a combination of solid phase peptide synthesis, polymerization and self-assembly. For the first time, we use N-cysteine and C-thioester functionalities in the same peptide to prepare collagen-like peptide polymers with diverse sequence selection and high molecular weight. These peptide polymers are able to self-assemble into collagen-like triple helices, as observed by circular dichroism spectroscopy (CD) and size exclusion chro-

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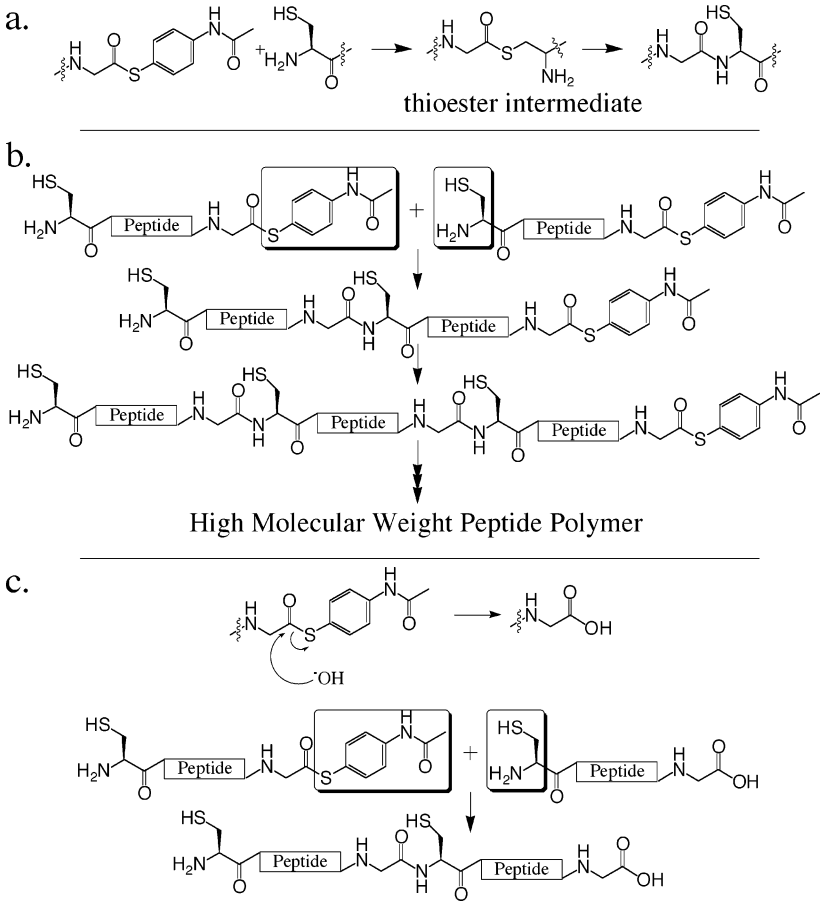
Scheme 1[a]



[a] (a) Mechanism of native chemical ligation showing the critical thioester intermediate formed by the side chain of the N-terminal cysteine. (b) Combining a C-terminal thioester and N-terminal cysteine allows for peptide polymerization through the native chemical ligation mechanism. Two reactive groups are shown in squares. (c) Hydrolysis of the chain terminal thioester group prevents the coupling of another peptide molecule to the C-terminus of the growing polymer.

Table 1. Peptides and Peptide Derivatives Prepared for This Study[a]

peptide	sequence	C-terminus
1	$(POG)_{10}$	COOH
2	$COG(POG)_9$	COS**R**
3	$COG(POG)_4EOG(POG)_4$	COS**R**
4	$COG(POG)_3PRGDOG(POG)_4$	COS**R**

[a] Amino Acid O = Hydroxyproline. C-terminal R = Para-acetamidophenyl-

matography (SEC), and into higher order fibrous aggregates, as observed by transmission electron microscopy (TEM), which are reminiscent of collagen fibrils.

Results and Discussion

We used solid phase peptide synthesis to synthesize molecules with the characteristic POG triamino acid repeat found in collagen and also incorporate amino acids with guanidinium and carboxylic acid side chains (Table 1). It has been shown that relatively short peptides synthesized with the characteristic three amino acid repeat, such as $(POG)_n$ undergo spontaneous self-assembly into well ordered triple helices.[31,35−41] However, these peptides do not undergo any further assembly into fibrils or fibers. To gain access to extremely long peptides, on the order of those found in natural collagen, with consensus sequence $(POG)_n$, we have utilized native chemical ligation[33,34,42,43] of synthesized peptides. These peptides were then used as a monomer for polymerization. We also assessed the polymerization of $(POG)_{10}$ via a simple HBTU (*O*-benzotriazole tetramethyluronium hexafluorophosphate) activated polymerization for comparison.

Polymerization Using HBTU as a Coupling Reagent. Peptide **1** was prepared to test a simple HBTU activated polymerization because this peptide contains only the relatively unreactive secondary hydroxyl group to compete with head to tail polymerization. The peptide was dissolved in *N*,*N*-dimethylformamide (DMF) and treated with HBTU and *N*,*N*-diisopropylethylamine (DiEA) to initiate covalent polymerization. A precipitation was immediately observed, collected by centrifugation and suspended in 10 mM phosphate buffer, pH 7, by sonication. The material was not fully soluble. However, briefly boiling the material dissolved the majority of the suspension. Slowly cooling the suspension over 30 min induced the self-assembly of peptide triple helices as indicated by CD. The supernatant was collected and examined by SEC which revealed a polymeric material with $M_w = 28\,000$, $M_n = 12\,000$, PDI = 2.3, comparable to earlier work.[31]

Polymerization through Native Chemical Ligation. To gain access to high molecular weight peptides that contain reactive side chains, such as those found in the cell adhesion ligand RGD, a different approach had to be employed. Native chemical ligation can be done in aqueous conditions under which peptides and proteins may adopt their native fold. The rate of the

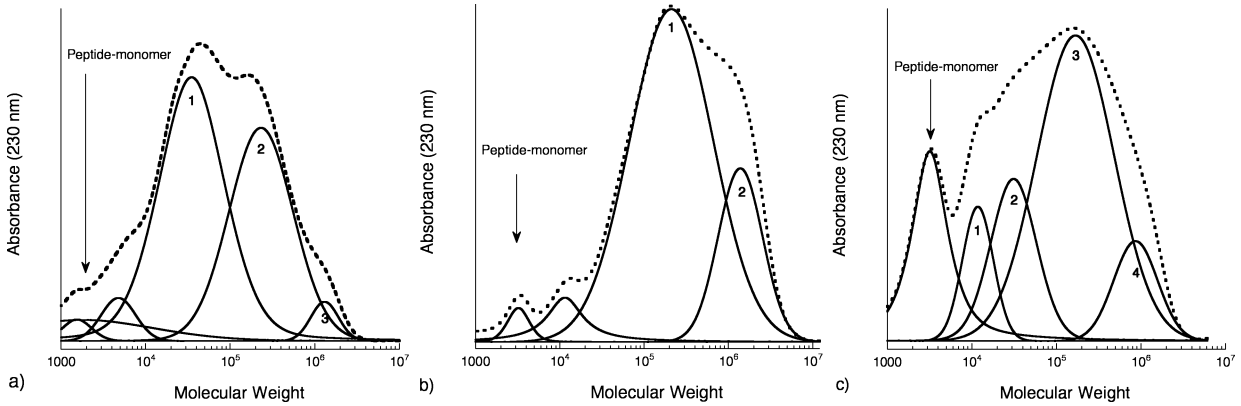


Figure 1. SEC of peptide polymers formed by native chemical ligation: (a) poly-**2**; (b) poly-**3**; (c) poly-**4**. Observed SEC traces are shown with dashed lines. Each chromatogram was deconvoluted using a mixed Gaussian−Lorentzian fitting algorithm and corresponding peaks are shown with solid lines.

reaction greatly depends on the pH, being the fastest near neutral pH (7−8) and significantly slower at acidic pH, allowing easy purification of the peptide−thioesters in TFA containing water/acetonitrile gradients by RP-HPLC. The reaction does not take place in the organic solvents if water is excluded from the system, which also permits a wide range of solvent mixtures for purification. The ligation reaction is catalyzed by the nucleophilic side chain of cysteine in a cross thioesterification reaction. The new thioester can then readily be attacked by the adjacent N-terminus of the peptide (Scheme 1a). This reaction has been shown to run effectively in the presence of any of the 20 natural amino acids.[42] Peptides **2**−**4** were prepared with both an N-terminal cysteine and a C-terminal thioester to allow for the possibility of peptide polymerization (Scheme 1b). The peptide−thioesters were prepared using an Fmoc protection strategy coupled with the use of an acid labile 2-chlorotrityl resin as described by Beck-Sickinger and Beyermann.[44] The aromatic peptide−thioester formed by this approach was found to be active enough in native chemical ligation to eliminate the need for activating thiols, which are typically utilized.[34] This is advantageous for polymer workup and subsequent use in cell culture experiments.

The peptides were polymerized in phosphate buffered water (pH = 7.3) in the presence of DTT (dithiothreitol) to ensure that the cysteines remained in their reduced form. Though the buffer is not essential to run the native chemical ligation, generally it is better to control the pH during the reaction due to the formation of rather acidic side products, which can slow the rate of reaction. The peptides remain soluble during the course of the reaction, unlike the HBTU polymerization and SEC revealed the appearance of high molecular weight peptide polymers for all synthesized peptides (Figure 1).

Molecular weights reported here are all relative molecular weights and the actual molecular weights may be lower than observed here due to the expected rigid and nonrandom coil nature of synthesized collagen-like peptide polymers. Peptides **2**−**4** displayed a second peak at a mass approximately 4 times that of the parent peak. We believe this can be explained by the presence of the self-assembled, triple-helical form of the peptide. The reason for the deviation from the expected 3 times mass is probably due to the increased rigidity and rodlike shape of the triple helix and its effect on the retention time within the SEC column. It was also noticed that a small peak corresponding to peptide monomer appeared in SEC, indicating that the polymerization reaction did not go to completion. Varying time conditions from 24 to 48 h did not significantly improve the results. An increase of the concentration led to precipitate formation, which complicated further SEC and CD analysis because of the concentration changes. MALDI-MS analysis of the reaction mixture indicated the presence of peptide−acid in the solutions, which can be attributed to hydrolysis of the peptide−thioester monomer in water solution at pH 7−8. The same side reaction may happen to the peptide polymer thioester end group itself, terminating further chain growth (Scheme 1c). This is also in agreement with the fact that samples of peptide−thioesters slowly hydrolyze during storage. The traces of peptide−acids found during polymerization will reduce the final MW of the polymer but do not prevent the polymerization itself. On the basis of the fact that only traces of peptide−thioester monomer can be observed in the MALDI-MS of all polymerization mixtures after 24 h, the peptide−thioester monomer is virtually entirely reacted during the polymerization reaction.

Determination of Triple-Helical Conformation in the Solution. Circular dichroism (CD) spectroscopy was carried out to determine the triple-helical character of synthesized peptide polymers. Peptide polymer solutions were equilibrated overnight at 5 °C in the presence of 3 mg/mL of DTT to prevent the remaining free cysteine thiol side chains from oxidizing and forming cross-links. Collagen exhibits a unique CD spectrum with a small positive peak between 220 and 225 nm and a large negative peak at 197 nm.[35,37,39,45,46] The CD spectra of the polymerized peptides were in good agreement with the native collagen spectrum and showed a strong positive peak at 225 nm and a large negative peak at 190 nm (Figure 2a).

Thermal transition curves monitored by optical rotation measurements were also used to confirm the presence of triple-helical conformation of peptide polymers in the solution. Thermal denaturation studies indicate that unfolding starts at approximately 40 °C for **1**, 50 °C for **4**, and 60 °C for **2** and **3** (Figure 2b). The difference in the unfolding profile can be attributed to the different sequence of peptide polymers as well as different MW and polydispersity of the samples. As expected, the polydisperse nature of peptide polymer solutions gave rise to a broad transition in the CD melting diagram. Due to the presence of very high

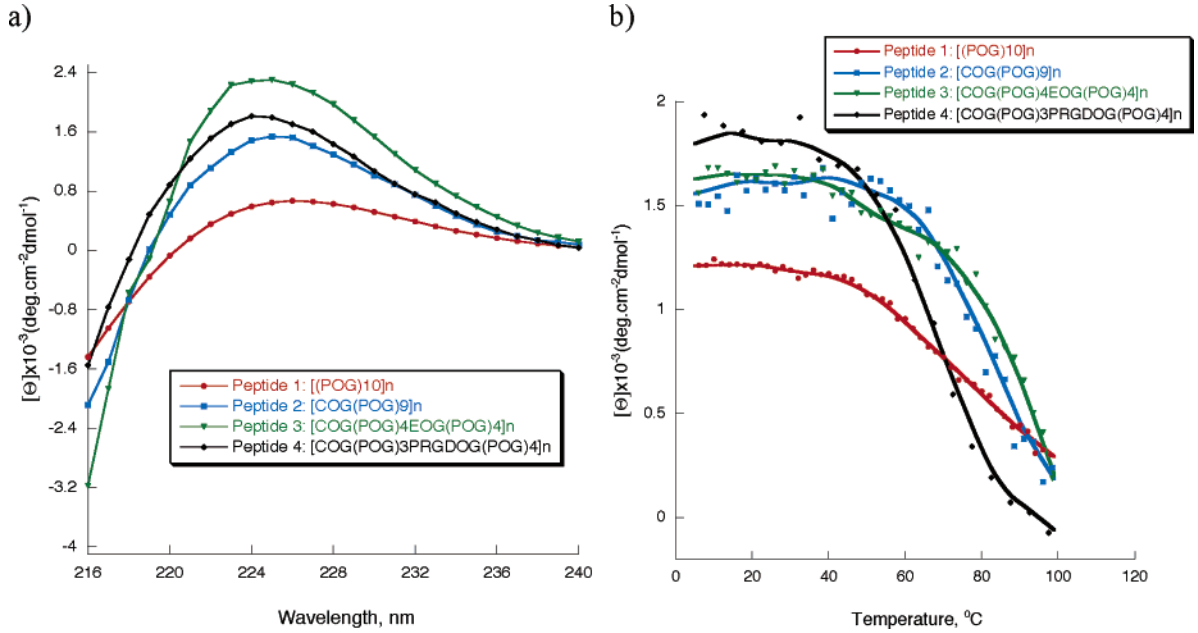


Figure 2. CD (a) wavelength and (b) temperature studies of synthesized peptide polymers.

molecular weight fractions the transition curves in most cases do not level off and continue to decrease even at temperatures close to boiling. Under these circumstances, the complete unfolding of the peptide polymers lies beyond the temperatures that can be achieved in water solution. Nonetheless, the nonlinear shape of a transition curve is indicative of cooperative unfolding of three single polypeptide chains and suggests the triple-helical character of the synthesized peptide polymers. Lower values of mean residue ellipticity observed as compared to the literature[39,45,46] can be attributed to the existence of partially unfolded domains in the peptide polymers and the imperfections in their tertiary structure that arise during the polymerization. The polydisperse nature of the peptide polymer molecule can lead to the formation of the peptide regions where one peptide chain may stick out of the triple helix and either interact with the adjacent molecules of a different polymer molecule or stay unfolded in water solution.

TEM Microstructure Studies. To elucidate the microstructure, the samples were extensively studied by negative stain TEM. CD of unpolymerized $(POG)_{10}$ showed a wavelength scan and thermal denaturation curve characteristic of a triple helix, but TEM did not show any signs of higher order self-assembly. In contrast, TEM images of the peptide polymers reveal a dense network of fibers 10−20 nm in diameter and microns in length (Figure 3). The diameter of the fibers is about 10 times higher than the diameter of a single triple-helical molecule, which indicates that the single triple-helical molecules interact with one another and form hierarchical aggregates similar to those of native collagen fibrils. This supports the idea that bundle formation in native collagen occurs through the weak noncovalent interactions between triple helices and may also include the formation of water bridges between self-assembled fibrils. The synthetic polymer diameter is similar to that of natural collagen fibrils; however, the characteristic banding pattern observed in type I collagen is not present. This is due to the polydisperse nature of the sample, which prevents alignment of the ends of the triple helices and more closely resembles type VII[47] and other collagens[48] that do not display banding patterns. Some amorphous aggregates that give dark contrast in the TEM images can also be observed. These aggregates can be attributed to a random coil peptide polymer that is not folded in the triple-helical conformation. This is also in agreement with lower absolute values of mean residue ellipticity that are observed in the CD spectra of peptide polymers, compared to the literature.

The micron-length fibers should give rise to a molecular weight far in excess to that observed by SEC. This can be explained by taking into account two facts. First, the polymers may initially self-assemble into triple helices but the polydispersity of the sample leaves significant portions of the peptide in a monomeric or dimeric coil to which further peptides may bind, extending the fibers by a supramolecular polymerization/self-assembly. This is similar to the "sticky ended" assembly observed in DNA and in some coiled-coil peptides.[49] Second, SEC characterizes the properties of rather dilute solutions as oppose to TEM, which estimates fiber dimensions in dried films. It is likely that triple-helical molecules will aggregate into larger fibers upon increasing the concentration.

Conclusions

We have described a novel system for preparing high molecular weight peptide polymers that can contain arbitrary sequence and reactive side chain functionalities. By utilizing native chemical ligation, peptides can be polymerized under conditions that allow the material to take on its folded conformation and help to retain good solubility in an aqueous environment. The peptide polymers prepared by this method recapitulate the basic features of natural collagen including the POG repeats, triple-helical character, and nanofibrous structure. This synthetic collagen mimic material is an important step in the preparation of novel synthetic scaffolds that imitate the chemistry and nanostructure of extracellular matrix and more generally illustrates a novel chemical approach to high molecular weight peptide polymers with diverse sequence selection.

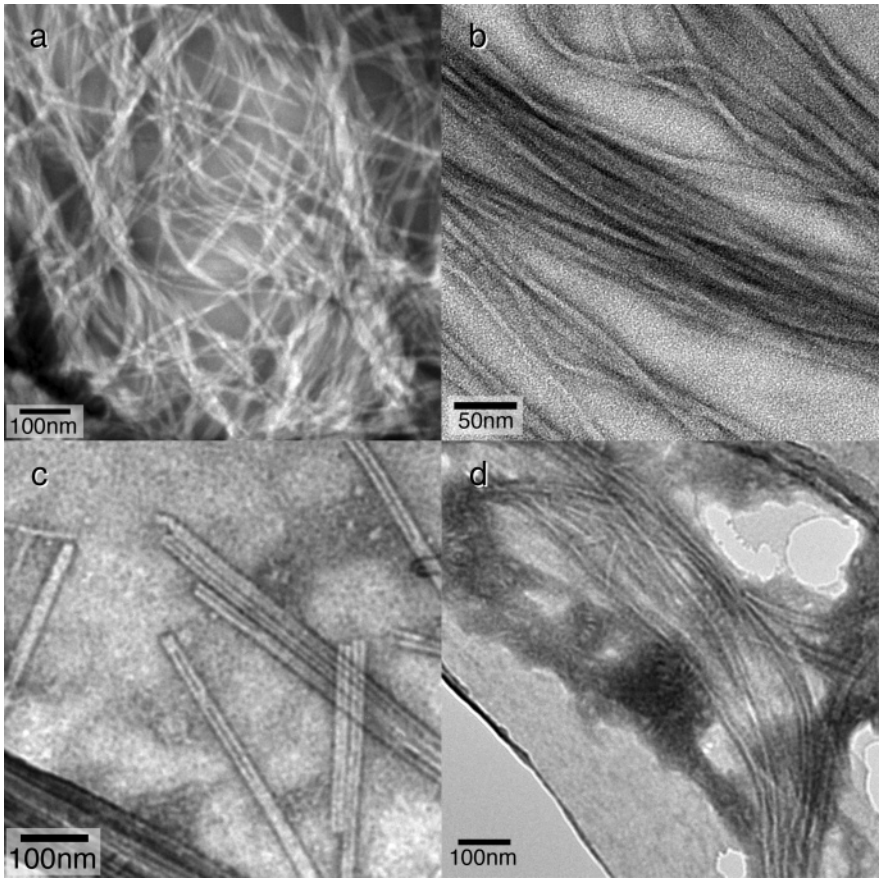


Figure 3. TEM images (a)−(d) of fibers prepared from polymerized peptides **1**−**4**, respectively.

Experimental Section

Materials. Amino acids, SPPS resins, HBTU, HOBt, PyBOP were purchased from Novabiochem. DiEA, DMF, TFA, diethyl ether, acetic acid, DCM, acetonitrile were purchased from Fisher. DTT, p-acetamidothiophenol, triisopropylsilane were purchased from Aldrich Chem. Co. EDT was purchased from Fluka. Reagents were used as provided.

Synthesis of Peptide 1, H_2N-[$(POG)_{10}$]-COOH. The peptide was prepared on an Advanced ChemTech 396 multipeptide automated synthesizer. The scale of the synthesis was 0.3 mmol. A preloaded Fmoc-Gly-Wang resin was used. Amino acid coupling cycles were 45 min in length with the following proportions of reagents: 4 equiv of amino acid, 4 equiv of *O*-benzotriazole *N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 4 equiv of 1-hydroxybenzotriazole hydrate (HOBt), 6 equiv of *N*,*N*,-diisopropylethylamine (DiEA) with *N*,*N*,-dimethylformamide (DMF) as a solvent. All proline residues were double coupled. Fmoc was deprotected with two 7 min treatments of 25% (by volume) piperidine in DMF. Cleavage of the peptide was accomplished by treatment of the resin with 20 mL of trifluoroacetic acid (TFA)/triisopropylsilane/H_2O (18:1:1 by volume) for 3 h at room temperature. The solution was collected by filtration followed by rinsing the resin twice with 10 mL of neat TFA. All washings were combined and rotoevaporated to a thick solution of approximately 5 mL. The peptide was triturated by addition of 50 mL of cold diethyl ether. The precipitate was collected by centrifugation and the pellet washed two times with cold ether. The pellet was then dried under vacuum overnight and redissolved in deionized water for purification by HPLC (see below). MALDI-TOF analysis was obtained using a 3,5-dimethoxy-4-hydroxycinnamic acid matrix: $[M + H]^+$ obs 2690.3; $[M + H]^+$ calc 2690.2.

Synthesis of Peptides 2−4, [COG$(POG)_9$]-COSR, H_2N-[COG$(POG)_4$EOG$(POG)_4$]-COSR, H_2N-[COG$(POG)_3$PRGDOG$(POG)_4$]-COSR; R = *p*-Acetamidophenyl. Peptides were prepared as described above with the following modifications. Peptides were synthesized on a prederivatized Fmoc-Gly-2-chlorotrityl resin (Novabiochem). Each amino acid residue following Hyp was double coupled. To protect the N-terminus of the peptide during the thioester formation, Boc-Cys(Trt)-OH was used on the final coupling step. Cleavage of the resin bound peptide was accomplished by treatment with acetic acid/trifluoroethanol/DCM (1:1:8 by volume) for 2 h at room temperature. The excess of acetic acid was removed by repeated washes with *n*-hexane and rotoevaporation. The residue was redissolved in dioxane and lyophilized.

The thioester was formed as follows: 60 mg of the crude peptide was dissolved in 20 mL of DCM to which 10 equiv of benzotriazole-1-yl-*N*-oxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), 50 equiv of *p*-acetamidothiophenol, and 1 mL of DiEA were added. Because C-terminal glycine is not subject to racemization, a large excess of base during thioester formation was found to improve yield. The solution was allowed to react for 3 h, followed by rotoevaporation to dryness. Deprotection of the side chains and N-terminal Boc protecting group was accomplished by treatment with 20 mL of TFA/ethanedithiol (EDT)/H_2O/triisopropylsilane (94:2.5:2.5:1 by volume) for 2 h at room temperature. The reaction mixture was rotoevaporated to a thick solution that was triturated with 50 mL of cold diethyl ether. The pellet was collected by centrifugation and washed two times with cold ether. The pellet was then dried under vacuum for 30 min and redissolved in 37% acetonitrile in water and purified by HPLC (see below). MALDI-TOF analysis was obtained using a 3,5-dimethoxy-4-hydroxycinnamic acid matrix: **2** $[M + H]^+$ obs 2846.9, $[M + H]^+$ calc 2847.1; **3** $[M + H]^+$ obs 2879.3, $[M + H]^+$ calc 2879.1; **4** $[M + H]^+$ obs 2907.1, $[M + H]^+$ calc 2908.2.

Peptide Purification. Preparative HPLC was performed on a Varian PrepStar220 using a reversed phase semipreparative C_{18} column at a flow rate of 10 mL/min. The elution gradient was 5−95% B in 45 min where A was 0.05% TFA in H_2O and B was 0.05% TFA in acetonitrile. Detection was at

Table 2. Calculated M_n, M_w, and Polydispersity of Native Chemical Ligation Polymerization

peptide	peak	M_w	M_n	PDI
poly-**2**	1	61 100	20 700	2.95
	2	342 000	155 000	2.21
	3	1 400 000	1 220 000	1.15
poly-**3**	1	402 000	110 000	3.65
	2	1 660 000	1 150 000	1.45
poly-**4**	1	12 900	10 800	1.19
	2	39 300	24 700	1.60
	3	290 000	98 100	2.96
	4	1 030 000	721 000	1.43

230 nm. Analytical RP-HPLC was performed on the same system using analytical C_{18} column at a flow rate of 1 mL/min with the same elution gradient.

HBTU Polymerization. A 3 mg sample of $(POG)_{10}$, peptide **1**, was dissolved in 1 mL DMF to which 0.4 mg HBTU (1 equiv) and 15 μL of DiEA (90 equiv) were added. A precipitation was immediately observed. After stirring for 30 min, the precipitate was collected by centrifugation, washed with DMF and DCM, dried under vacuum, and suspended in 10 mM phosphate buffer, pH 7, by sonication. The material was not fully soluble. Briefly boiling the material dissolved the majority of the suspension. The suspension was slowly cooled over 30 min to induce self-assembly of triple helices.

Native Chemical Ligation Polymerization. Peptide thioesters **2**−**4** were individually dissolved at a concentration of 3 mg/mL in a 10 mM phosphate buffer, pH 7.3, containing 3 mg/mL of dithiothreitol (DTT). The ligation mixture was stirred at room temperature for 10 h and monitored by size-exclusion chromatography.

Circular Dicroism and Thermal Transition Curve Measurements. Measurements were performed on a JASCO J-810 spectropolarimeter. Samples were prepared at a concentration 1.7 mg/mL at pH 7 (10 mM phosphate buffer). Solutions were equilibrated at 5 °C overnight before recording the spectra. The cell path length was 0.1 cm. The spectra were recorded from 240 to 180 nm with a scan speed of 1 nm/s, and the signal was averaged over 10 scans. The thermal transition curve was obtained following the CD signal at 225 nm. The temperature was increased at 10 °C/h using JASCO PTC-423S temperature controller. The mDeg of rotation were converted to mean residue ellipticity.

Size Exclusion Chromatography. Polymerized samples were analyzed on a Ultrahydrogel Linear size exclusion column (Waters Corporation) with mass range 10^3 to 10^6. The column was calibrated using poly(ethyleneoxide) standards. Elution was carried out in 10 mM phosphate buffer (pH 7). The elution pattern was followed by UV absorption of polymers at 230 nm. Data obtained were baseline corrected and then deconvoluted using a mixed Gaussian−Lorentzian fitting algorithm. M_n, M_w, and polydispersity values were calculated from the deconvoluted peaks (Table 2).

TEM. A 7 μL aliquot of an aqueous solution of peptide at a concentration of between 1 and 3 mg/mL was deposited on a holey carbon coated copper grid (Quantifoil R 1.2/1.3) and allowed to sit undisturbed for 3 min. The excess solution was blotted off and the grid allowed to air-dry. Next, 7 μL of a 2 wt % solution of phosphotungstic acid, which had been adjusted to a pH of 4, was deposited on the grid and allowed to sit undisturbed for 3 min after which the excess was blotted off. The grid was then allowed to air-dry. Specimens were observed on a JEOL 2010 TEM equipped with a Gatan 2 × 2K CCD camera using an accelerating voltage of 200kV.

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