Tuning the Conformation of Synthetic Co-Polypeptides of Serine and Glutamic Acid Through Control Over Polymer Composition

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Received 19 October 2016; accepted 1 March 2016; published online 29 March 2016
DOI: 10.1002/pola.28104

ABSTRACT: Ring opening polymerization (ROP) of N-carboxy anhydride (NCA) amino acids presents a rapid way to synthesize high molecular weight polypeptides with different amino acid compositions. The compositional and functional versatility of polypeptides make these materials an attractive choice for biomaterials. The functional performance of polypeptide materials is equally linked to their conformation which is determined by the amino acid sequence in the polymer chains. Here, the interplay between composition and conformation of synthetic polypeptides obtained by NCA polymerization was explored. Various copolypeptides from Glu(Bzl) and Ser(Bzl) were prepared to investigate how polypeptide composition affected the conformation of the resulting copolymer. Polymerization kinetics indicated that the copolymerization of Glu(Bzl) and Ser(Bzl) preferentially yielded alternating copolymers. Both the polydispersity and the conformation of the polypeptides were dependent on the Ser(Bzl) content in the polymer, demonstrating that polypeptide functionalities could be tuned directly by altering the relative amounts of amino acids in the chain. This work presents the first step toward an improved understanding and control over polypeptide conformation through modulating the amino acid composition of the material. Understanding this sequence-functionality relationship is essential to advancing the use of ROP as a technique to design smart polypeptide based materials with specific functions. © 2016 The Authors. Journal of Polymer Science Part A: Polymer Chemistry Published by Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. 2016, 54, 2331–2336

KEYWORDS: copolymerization kinetics; N-carboxy anhydride (NCA) polymerization; secondary structure; conformational analysis; polypeptides; structure-property relations

INTRODUCTION Synthetic polypeptides have great compositional and conformational diversity which directly contributes to their functional versatility. They have found applications as responsive materials, for example, for pH-dependent sustained drug release,1 photosensitive antibacterial devices,2,3 and enzyme responsive scaffolds in tissue engineering.4 The properties of natural and synthetic peptides are related to the peptides’ conformation which in turn depends on the type, amount, and distribution of amino acids in the peptide sequence. Understanding and manipulating these variables thus provides an attractive route to access peptide materials with a range of different properties and tailored functionalities.

Ring opening polymerization (ROP) is a versatile, low cost, and well-established technique to synthesize polymers from N-carboxy anhydrides (NCA), which has been successfully employed in the synthesis of long chain polymers.5,6 Extensive control over the polymerization has been achieved by use of low temperatures, high pressures, and catalysts to enable the elimination of unwanted side reactions permitting the synthesis of highly monodisperse polypeptides.7–13 While

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full control of the amino acid sequence is currently not possible in NCA ROPs, recent work has seen the establishment of tools to monitor NCA monomer consumption in copolymerizations and thus provided the means to exert enhanced compositional control over polypeptides by controlling the monomer feed. Understanding how monomer feed composition affects the composition of the resulting polypeptides is a first step toward enhanced control over NCA ROP derived polypeptides and several reports have used this approach to understand the underlying polymerization kinetics.

Secondary structure formation during ROP can affect the polymerization progress. Notably β-sheet adopting oligopeptides can stop chain growth completely due to steric hindrance. Therefore, an enhanced understanding over composition/conformation relationship in such systems will enable optimization for subsequent applications.

Polyglutamic acid (PGA) has been studied for applications in wound dressings, as a drug carrier and in tissue engineering. Several studies showed that PGA forms an α-helical structure under aqueous conditions at ambient temperatures. Studies reporting homo serine polymers are rare and typically use serine derivatives (e.g., phosphorylcholine side groups) instead to improve water solubility and control chain length distribution. While there are no examples in the literature of copolymers of glutamic acid and serine synthesized by ROP, a similar system, a random copolymer of L-serine and N-(4-hydroxybutyl)-glutamine, was reported to undergo a thermally induced helix-coil transition. Using circular dichroism, it was shown that serine acts as an α-helical breaker and participates in β-turns, but the effect of copolymer composition was not explored.

Here, we systematically explored the effect of different compositions on the conformation of copolymers obtained through the ROP of NCA derivatives of side chain protected glutamic acid and serine which are of interest for future applications as enzyme responsive materials. Our work presents the first report of the effect of different amounts of the α-helical breaker serine in a Glu(Bzl)/Ser(Bzl) copolymer on the degree of α-helix formation and demonstrates how control over the copolymer composition can be used to obtain polypeptides with specific secondary structure contents.

**EXPERIMENTAL**

**Materials**
The benzyl protected amino acids, H-Ser(Bzl)-OH and H-Glu(Bzl)-OH were purchased from Bachem. Dry ethyl acetate, dry DMF, and trifluoroacetic acid (TFA) were purchased from Acros. Ethyl acetate, anhydrous diethyl ether; acetoniitrile, hydrochloric acid, and petroleum ether were purchased from Fisher Scientific. Triphosgene was purchased from Alfa Aesar. Heptylamine 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) and x-pinene were purchased from Sigma-Aldrich.

**NCA Synthesis**
NCA synthesis from benzyl protected amino acids was performed according to experimental procedures reported in the literature, adapted for serine and glutamic acid. Detailed procedures can be found in the Supporting Information.

**NCA Polymerization**
The procedure was adapted from the literature. Random copolypeptides of NCA-Glu(Bzl) (E) and NCA-Ser(Bzl) (S) were prepared with monomer feed ratios (S:E) of 0:1, 1:2, 1.5:2.5, 1:1, 2:1.3, 2:1, 4:1, and 1:0. A total monomer concentration of 0.5 M (in dry DMF) and 0.1 mmol heptylamine (2 mol%) were used in all polymerizations to obtain a theoretical chain length of 50 amino acids in each experiment. Polymerizations were performed under nitrogen and kept at 1 °C-3 °C using a metal cooling block whose temperature was regulated by a cryostat. Completion of the polymerization was confirmed by FTIR by following the reduction of the C=O signal of the NCA at 1760–1800 cm⁻¹. On completion of the reaction, products were precipitated and washed (20 mL x 3) using diethyl ether and isolated by vacuum filtration. Resultant product was dried in a vacuum oven overnight at 40 °C.

**Copolymerization Kinetics**
The copolymerization parameters for the copolymerization of E and S were determined following a previously established procedure. About 5 mmol solutions of NCA-Glu(Bzl) and NCA-Ser(Bzl) were prepared at ratios (S:E) 1.5:1, 2.5:1, 3:1, 4:1, 4.5:1, and 6.5:1 in 500 µL of DMF, placed under N₂ and polymerizations were initiated with heptylamine (2%). Reactions were stopped after 20 minutes (~10% monomer conversion) and reaction mixture was added into a quenching solution (1 mL) of composition; CH₂CN:H₂O:HCl (1 M), (40:40:15) to deactivate unreacted NCA monomers. The polymers were precipitated and washed in diethyl ether (2 and 10 mL, respectively). Isolation was performed by vacuum filtration and the polymers were dried in a vacuum oven at 50 °C overnight.

**Characterization FTIR**
An IR Agilent technologies Cary 630 FTIR instrument equipped with an attenuated transmission reflection (ATR) was used for FTIR analysis. About 32 scans were taken for each sample. Peak areas for signals at 1787 cm⁻¹ (COO stretching of NCA) were obtained to monitor reaction progress. For conformational studies spectra of solid samples (n = 3) were taken using 128 scans, peak areas for Amide I (1600–1700 cm⁻¹), were used for component peak fitting with Casa XPS software. Spectra were normalized and peaks fitted using linear base until sum of peak areas followed total peak area (new components <5% of peak area, and residual error of peaks fitted <0.01).
kinetic analysis was achieved using Mestrenova software. Spectra were baseline corrected using Whittaker smoother. Peaks were fitted to the CH regions using manual peak fitter and the software automatically fit the added peaks to the spectrum, until an optimal fit was achieved.

**Gel Permeation Chromatography (GPC)**

Relative molecular weights and polydispersities of the polymers were determined using GPC. The instrument was equipped with a Waters 1515 Isocratic HPLC pump, a Waters 2414 refractive index detector (40 °C), a Waters 2707 autosampler, a PSS PFG guard column and subsequently with two PFG-linear-XL columns (8 × 300 mm, 7 micron) in series (at 40 °C). The eluent (flow rate: 0.8 mL/min) used was HFIP (Biosolve) containing potassium trifluoroacetate (3 g/L). Polymethyl methacrylate standards with $M_p$ of 580 Da up to 7100 kDa were used as a reference (Polymer Laboratories).

**Circular Dichroism (CD)**

Measurements were taken on a JASCO J8108 spectrometer. Samples were prepared at 0.2 mg/mL in HFIP and transferred into a 250 μL quartz cuvette. Spectra were taken from 260 to 195 nm, CD and absorbance were measured. Spectral bandwidth used was 1 nm, response time 4 seconds and 2 scans were accumulated per sample. Data was normalized and molar ellipticity was calculated using the following equation: 

$$[\theta] = 100 \times \theta/(C \times L)$$

Concentration was calculated using polymer molecular weights as determined from NMR spectra.

**RESULTS AND DISCUSSION**

**Synthesis and Characterization of Copolypeptides with Systematically Changing Composition**

To prepare a series of serine and glutamic acid copolymers with a systematic change in amino acid composition via NCA polymerization (Scheme 1), polymerizations were carried out with varying monomer feed ratios (Table 1) but constant total monomer (0.5 M) and initiator concentrations (2 mol %). NCAs of the benzyl protected monomers were synthesized according to literature procedures and characterized by NMR and mass spectrometry (Supporting Information Figs. S1–S4).

The copolypeptides obtained after polymerization were characterized to determine polymer composition, molecular weight and polydispersity. The NMR spectra (Supporting Information Fig. S5) showed that polymerization was successful and allowed experimental determination of the numbers of glutamic acid and serine residues present in each polymer (Supporting Information Fig. S6, Table 1). The degree of polymerization obtained for the copolymers was very close to theoretical predictions, achieving 90% conversion. In contrast, homopolymers did not reach full conversion. In the case of the serine homopolymerization, this can be rationalized by the poor solubility of the serine-NCA in DMF. Poor serine-NCA solubility may also be responsible for the lower chain lengths observed in the serine homopolymer and the 2:1 copolymer when compared with the other copolymers. The low degree of polymerization for the Glu(Bzl) NCA obtained here was repeatable and consistent for three separate reactions but stands in contrast to the literature which reported between 80% and 100% conversion.16,25 Different reaction conditions and analysis methods (NMR vs. GPC) may account for the discrepancy between our results and the literature.

**SCHEME 1** Preparation of copolypeptides of Glu(Bzl) and Ser(Bzl) via NCA polymerization. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**TABLE 1** Characteristics of Copolymers Prepared from Ser(Bzl)-NCA (S) and Glu(Bzl)-NCA (E) at Different Monomer Ratios as Determined by NMR

| Feed Ratio (S:E) | Degree of Polymerization (Theoretical) | Degree of Polymerization (Experimental, $n=3$) | Mole Fraction of Serine (Theoretical) | Mole Fraction of Serine (Experimental, $n=3$) |
|------------------|----------------------------------------|---------------------------------------------|-------------------------------------|---------------------------------------------|
| 0:1              | 50                                     | 34 ± 1                                      | 0                                   | 0                                           |
| 1:2              | 50                                     | 48 ± 1                                      | 0.33                                | 0.32 ± 0.01                                 |
| 1:1              | 50                                     | 47 ± 2                                      | 0.5                                 | 0.47 ± 0.02                                 |
| 2:1              | 50                                     | 42 ± 7                                      | 0.67                                | 0.61 ± 0.03                                 |
| 1:0              | 50                                     | 33 ± 4                                      | 1                                   | 1                                           |
To investigate the unprotected polypeptides alongside the protected materials, we attempted deprotection of the polypeptides in TFA/HBr (3:1) for 16 h, triisopropylsilane (TIS)/H₂O/TFA (95:2.5:2.5) and by hydrogenation with Pd/C under H₂. Residual signals of the aromatic compounds of the protection groups in the NMR spectrum (data not shown) indicated that acid catalyzed deprotection did not proceed to completion. The polypeptides were not stable under hydrogenation conditions, a fact that was previously reported for other polypeptides, as well.²⁶,²⁷ Thus, subsequent analysis was carried out on benzyl protected polymers only.

The polydispersity (PDI) of the homo- and copolypeptides was determined by GPC (Table 2). No data could be obtained for the Serine homopolymer due to the low solubility of the polypeptide. The PDI of the polymers increased with increasing Ser(Bzl) content, indicating that Ser(Bzl)-NCA has a negative effect on chain length homogeneity. The GPC chromatograms show that high relative amounts of Ser(Bzl) causes a bimodal distribution of the polymer (Supporting Information Table S1). It is possible that the low solubility of poly(Ser(Bzl)) and copolymers with high Ser(Bzl) content causes these polymers to precipitate from the reaction mixture. This is supported by the observation of precipitation early during the polymerization which would reduce the likelihood of chain propagation for these materials. More soluble copolymers with higher Glu(Bzl) content subsequently grow faster, giving rise to the unimodal distribution observed in the GPC. Consequently, our data suggests that greater control over polymerization can be achieved with copolymers where glutamic acid predominates in the initial monomer feed. As GPC measures the hydrodynamic radius of the polypeptides, this would differ to that of the PMMA standard. Likewise the variation in the chemical composition of the different polypeptides, can be expected to affect the GPC derived molecular weights, preventing any further comparison between the polymers. This may also explain the higher than expected Mn for GPC derived measurements. Molecular weights derived from the NMR spectra were calculated based on the number of amino acid residues present in each polymer. While reaction mixtures with low S:E feed ratios result in significantly higher molecular weights, the molecular weights do not consistently increase with increasing Glu(Bzl) content and the trends observed for the GPC and the NMR based data are consistent with the theoretical Mn. Attempts to further characterize the polymers with MALDI TOF MS were unsuccessful, due to poor solubility of the polymers.

**Copolymerization Kinetics**

Polymerization kinetics can provide insight into the probable distribution of the monomers in the polymer chain. We, therefore, monitored the initial rate of consumption of the amino acid NCAs to determine the copolymerization parameters for Glu(Bzl)-NCA and Ser(Bzl)-NCA (rSer and rGlu). Reaction kinetics for the copolymerization were determined from polymer samples isolated at low conversion (~10%) to eliminate the effects of compositional drift. Seven different monomer feed ratios were used, and the copolymer composition was determined via NMR analysis (Supporting Information Table S1).

Two different methods, the Finemann–Ross (FR) and the Kelen–Tudos (KT) method (see Supporting Information for details), were used to calculate the copolymerization parameters rSer and rGlu (Table 3). A one way ANOVA test was used to determine that there is no significant difference between rSer and rGlu for the two methods, suggesting that the values are good approximations of the monomer reactivities. Both rSer and rGlu are closer to 0 than 1, indicating that Ser(Bzl) and Glu(Bzl) preferentially form alternating copolymers. This is visually demonstrated in Figure 1, which shows that the feed and monomer compositions closely match each other. Only a small drift is observed when the feed concentrations of the two NCAs approach equimolarity. We can therefore conclude that polymerization kinetics dictate a uniform polymer composition and favors alternating copolymers. Hence, only copolymers with significantly different monomer starting concentrations will be expected to display a compositional drift toward the end of the polymer chain. Based on the kinetics data, the structure of our copolymers is expected to be alternating for the 1:1 copolymer and alternating with a block-like end for the 1:2 and 2:1 copolymers.

**TABLE 2** Number (Mn) and Weight (Mw) Average Molecular Weight and PDI of Copolymers Prepared from Ser (Bzl)-NCA and Glu (Bzl)-NCA at Different Ratios as Determined by NMR and GPC

| Feed Ratio S:E | Mn (g/mol) Theoretical | Mn (g/mol)ub (n = 3) | Mw (g/mol)ub | Mn (g/mol)ub | PDIub |
|---------------|------------------------|----------------------|--------------|--------------|--------|
| 0:1           | 11,077                 | 7,600 ± 200          | 8,000        | 7,000        | 1.1    |
| 1:2           | 10,377                 | 10,000 ± 200         | 14,000       | 10,000       | 1.36   |
| 1:1           | 10,026                 | 10,800 ± 1,100       | 17,000       | 11,000       | 1.5    |
| 2:1           | 9,676                  | 8,300 ± 1,200        | 12,000       | 7,000        | 1.79   |
| 1:0           | 8,975                  | 7,600 ± 400          | –            | –            | –      |

a As determined from NMR. b As determined from GPC.

**TABLE 3** Copolymerization Parameters for Serine (rSer) and Glutamic Acid (rGlu)

| Method        | rSer ± 0.01 | rGlu ± 0.01 | rSer * rGlu ± 0.02 |
|---------------|-------------|-------------|---------------------|
| Finemann–Ross | 0.25 ± 0.01 | 0.13 ± 0.09 | 0.03 ± 0.02         |
| Kelen–Tudos   | 0.24 ± 0.01 | –0.03 ± 0.06| 0.01 ± 0.02         |
Copolymer Conformation

The prevalence of secondary structure motifs in the copolypeptides was measured to study the effect of changing polymer composition on polypeptide conformation. The presence of secondary structures in five polymers at ratios (S:E) 0:1, 1:2, 1:1, 2:1, and 1:0 was measured with CD for solutions and FTIR for solid samples.

As applications of the polypeptide materials are likely to be in a solvated environment, we started by investigating the conformation of the polymers in solution. All samples follow a similar trace with negative bands at \( \theta_{222} \) and \( \theta_{208} \) characteristic of \( \alpha \)-helical secondary structures (Supporting Information Fig. S9). Due to poor solubility of the polymers, HFIP was the only suitable solvent; it is, however, known as a strong \( \alpha \)-helical inducer, and the presence of \( \alpha \)-helices are therefore not necessarily indicative of the native peptide conformation.28 We, therefore, also studied the secondary structure of the copolypeptides in their solid state using FTIR.

The amide I band (1600–1700 cm\(^{-1}\)) is indicative of C=O stretching and composed of overlapping bands corresponding to different secondary structure types (i.e., \( \alpha \)-helices, \( \beta \)-sheets, unordered, and random), enabling quantification of the prevalence of specific secondary structure motifs.29 The deconvoluted spectra of the amide I band can be found in Supporting Information (Supporting Information Fig. S10) and the numeric data along with the relative amount of Ser(Bzl) in the polymers that was determined by NMR are provided in Supporting Information Table S2. It was found that the polymers formed predominantly \( \alpha \)-helical and \( \beta \)-sheet structures. The change of the relative amount of these two secondary structural motifs with changing Ser(Bzl) content in the copolypeptides is plotted in Figure 2.

Homopolymers of glutamic acid adopted \( \alpha \)-helical conformations and (in contrast to the CD results) serine homopolymers showed \( \beta \)-sheet structures. Conformations adopted by both homopolymers are consistent with previous studies in the literature.30,31 By altering the monomer ratio in a systematic manner, we have highlighted that an increase in the serine content of a Glu(Bzl)/Ser(Bzl) copolypeptide directly correlates with an increasing presence of \( \beta \)-sheet motifs and a decrease in the \( \alpha \)-helix content in the polymer. The latter is consistent with previous reports identifying serine as an \( \alpha \)-helix breaker.22 Serine forms hydrogen bonds with the backbone of the \( \alpha \)-helix, disrupting the helical structure. Here we have shown that this effect is tunable by altering the composition of the copolypeptides which can be readily accomplished through controlling the monomer feed ratio.

CONCLUSIONS

To fabricate well-defined polypeptides using ROP it is essential to understand and to be able to modulate the properties of these polypeptides in a controlled manner. This is not only limited to sequence control but also includes the conformation of the polypeptides. Here we have for the first time studied systematically how the composition of a Glu(Bzl)/Ser(Bzl) copolypeptide affects its conformation. The homogeneity of the polypeptides was directly affected by the Ser(Bzl) to Glu(Bzl) ratio with polymers of good polydispersity being obtained with high Glu(Bzl) feed ratios. The kinetic analysis indicates that Ser(Bzl) and Glu(Bzl) monomers preferentially co-polymerize in an alternating sequence. The ability to exert control over polypeptide conformation by modulating the monomer feed ratio provides a facile way to adjust the properties of these materials and is essential for the further development of polypeptides.

ACKNOWLEDGMENTS

We are grateful for Eleonora Cerasoli for assistance with the Circular Dichroism. Financial support from the EPSRC (EP/F500491/1) for AC and the Tor Vergata university exchange program for AP is gratefully acknowledged.
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