Polypeptide gels incorporating the exotic functional aromatic amino acid 4-amino-L-phenylalanine

Journal:	*Polymer Chemistry*
Manuscript ID	PY-ART-03-2018-000427.R1
Article Type:	Paper
Date Submitted by the Author:	27-Apr-2018
Complete List of Authors:	Kaneko, Tatsuo; Japan Advanced Institute of Science and Technology, Ali, Mohammad Asif; Japan Advanced Institute of Sci. & Tech., Energy and Environment Area Captain, Ilya; University of California, Los Angeles (UCLA), Departments of Bioengineering, 420 Westwood plaza Perlin, Pesach ; University of California, Los Angeles (UCLA), Departments of Bioengineering Deming, Timothy; University of California, Los Angeles, Department of Bioengineering

Journal Name

ARTICLE

Polypeptide gels incorporating the exotic functional aromatic amino acid 4-amino-L-phenylalanine

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Tatsuo Kaneko[a,b,‡*], Mohammad Asif Ali[a,‡], Ilya Captain[b], Pesach Perlin[b] and Timothy J. Deming[b*]

High-molecular-weight polypeptides with functional aromatic side chains, poly(4-amino-L-phenylalanine), 4APhe, were prepared by metal-initiated polymerization of the N^{α}-carboxyanhydride of the corresponding amino acid, which is a microbial derivative of phenylalanine. The polypeptide P4APhe has good water solubility as opposed to the conventional aromatic poly(amino acid)s such as poly(L-phenylalanine) or poly(L-tyrosine). On the other hand, P4APhe shows an in-situ gelation ability upon swelling of dried samples in solvents, due to the formation of crystalline β-sheet domains that work as cross-linking junctions. Moreover, cationic copolymers of 4APhe with L-lysine showed a pH-responsive gelling behavior resulting from changes in the amphipathic balance of crystalline β-strands, α-helical structures, and hydrophilic random coils. Notably, a small amount of 4APhe units in copolypeptides with L-lysine were found to enhance adoption α-helical conformations.

1. Introduction

Polypeptides have attracted researchers' attention as biofunctional materials possessing great potential for various biomedical applications such as drug or gene delivery, bioadhesion, antibiotics, and tissue engineering.[1-4] Many recent reports on synthetic polypeptides are available in the fields of bio-related sciences and technologies due to their chemical diversity[5]. The secondary structures of polypeptides are stabilized via hydrophobic and electrostatic interactions such as hydrogen bonding and/or π-electron interactions.[3,4] One advantage of polypeptides as biomaterials is the facile controllability of secondary structure which is well defined but is inaccessible in most of the other synthetic polymeric materials.[5,6] The formation of secondary structures such as α-helix and β-sheet connects to the functionalization not only for proteins in vivo but also of polypeptide biomaterials in vitro[7-10] because the polypeptides can be self-organized into higher-ordered structures. For example, ionizable polypeptides such as poly(L-lysine) exhibit a helix-coil transition by changing pH owing to the ionization/deionization of the side chain amine groups at a critical pH around 10, which is useful to design stimuli-responsive materials.[10,11] However, there are fewer polypeptides derived from cationic amino acids than those from anionic ones, which restricts the molecular design of polypeptide materials.[12] In addition, phenylalanine containing peptides can be more efficiently assembled into crystallizable β-strands compared to non-aromatic amino acids.[13,14] However, there has been no study on water-soluble polyphenylalanine derivatives with ionizable side chains that would be expected to show pH-responsive control over conformation and crystallinity. 4-Amino-L-phenylalanine (4APhe), which exists as a component of antibiotics for *Streptomyces* sp., is a phenylalanine derivative with an ionizable side-chain group but is very exotic.[15] To improve its accessibility, we previously established a mass production route of 4APhe using genetically-manipulated *Escherichia coli*. [16-18] If 4APhe can now be used for polypeptide materials, we can open the new dimension of polypeptide design using this new building block containing a cationic aniline group.

Polypeptide chains have been produced by the ring-opening polymerization of N-carboxyanhydride amino acids (NCAs) in kilogram scale[19], which offers versatile scalability in the preparation of polypeptide structures. We have demonstrated the controlled synthesis and self-assembly of the amphiphilic block copolymers to form hydrogels by simply immersing into the water, which can lead to various applications such as tissue engineering and drug delivery.[13,20] Also worth noting, metal-initiated NCA polymerization is well-suited for preparing high molecular weight polypeptides due to the repression of side reactions and increased polymerization rates, as compared to amine-initiated methods.[21,22] High molecular weight is very important for developing the physical properties of polymer materials such as gels to impart them with high stability and excellent reliability.

Here we used metal-initiated NCA polymerization to synthesize high molecular weight polypeptides of P4APhe and its

[a] *Graduate School of Advanced Science and Technology, Energy and Environment Area, Japan Advanced Institute of Science and Technologies, 1-1 Asahidai, Nomi, Ishikawa 923-1292 Japan. Email: Kaneko@jaist.ac.jp*
[b] *Departments of Bioengineering and Chemistry and Biochemistry, University of California, Los Angeles (UCLA), 420 Westwood Plaza, Los Angeles, 90095-1600, USA. Email: demingt@seas.ucla.edu*

† Electronic Supplementary Information (ESI) available: [details syntheses of P4APhe based NCA and polymer, NMR data (Fig. S1, S3, S4, S5 and S7), IR data (Fig. S2), Copolymer (Table S1), CD data (Fig., S8 and S9), and Differential refractive index (Fig. S6), See DOI: 10.1039/x0xx00000x

‡ Equal contribution as first authors

copolypeptides with L-lysine, which are expected to have pH-responsive conformational changes. Moreover, we investigated the ability of 4APhe units in polypeptide chains to form hydrogel networks.

2. Experimental

Materials.

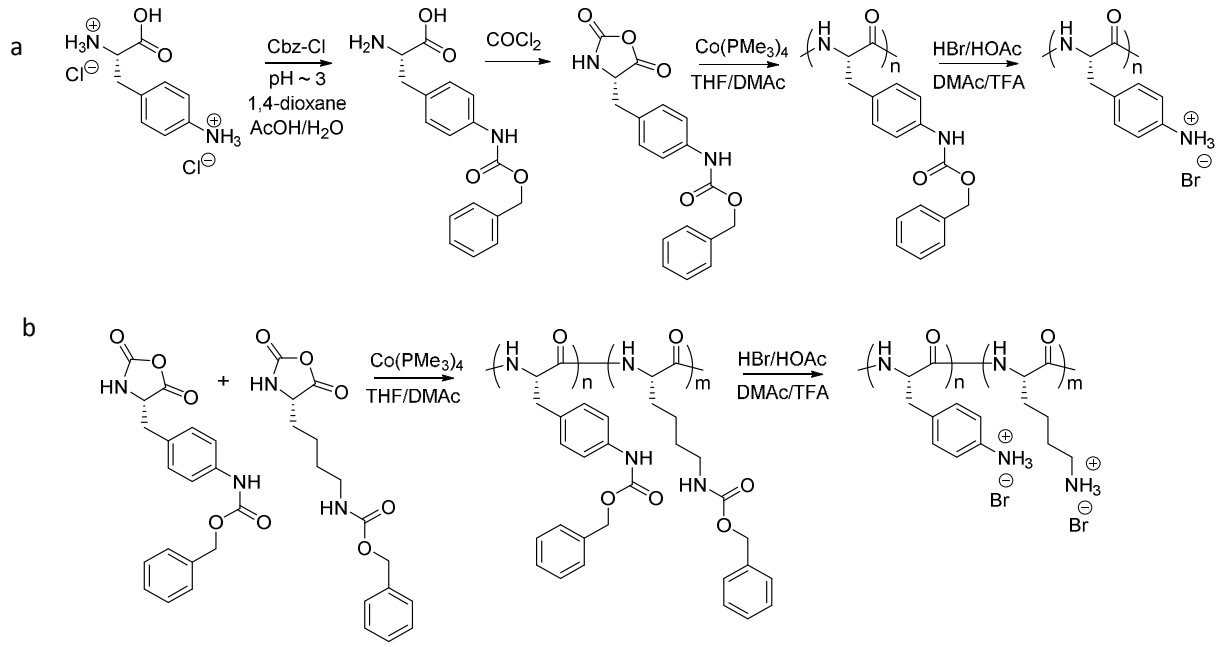
The initiator[23] $Co(PMe_3)_4$ was synthesized according to literature procedures[24] and was used after sublimation purification. 4-Amino-L-phenylalanine (4APhe) HCl salt and N^{ε}-benzyloxycarbonyl-L-lysine used as precursors for NCA monomers were purchased from WAKO and Sigma-Aldrich, respectively, and used as received without further purification. Hexane, tetrahydrofuran (THF), and *N,N*-dimethylformamide (DMF) were all purchased from Sigma-Aldrich and were thoroughly dried by first purging with dry nitrogen, followed by passage through columns of activated alumina (or 4Å molecular sieves for DMF). *N,N*-Dimethylacetamide (DMAc) was purchased from Sigma-Aldrich and was used after distillation from calcium hydride. 1,4-Dioxane, acetic acid, and benzyloxycarbonylchloride (Cbz-Cl) were all purchased from Sigma-Aldrich and used without purification. Trifluoroacetic acid (TFA) and hydrogen bromide in acetic acid solution (30 %) were used without further purification. Phosgene (20 %) in toluene solution purchased from Sigma-Aldrich and was used without further purification.

Synthesis of 4-carbobenzyloxyamino-L-phenylalanine, Cbz-4APhe

4-carbobenzyloxyamino-L-phenylalanine was synthesized by the following procedure. 1,4-Dioxane solution (180 ml) of CbzCl (3.82 g, 0.0224 mol) was added to a solution of 4-aminophenylalanine (5.40 g, 0.0213 mol) in 10 % AcOH aq (180 ml). pH was adjusted to 3-4 by adding NaOH aq (5 M), where precipitate appeared if pH was too high. The mixture was stirred overnight at room temperature. The pH of the aqueous phase was gradually increased by dropping 5 M NaOH resulting in precipitation. The precipitates were gathered by filtration and then dried in vacuo to obtain the product of 4-carbobenzyloxyamino-L-phenylalanine (Z4APhe) as a white solid (yield: 92 %), as shown in Scheme 1. The product was used directly for NCA monomer synthesis without further purification. (Figure S1 of supporting information) ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 2.9-3.1 (dd, 2H, -CH_2), 3.8 (t, 1H, -CH), 5.15 (s, 2H, 2H), 7.18 (d, 2H, -ArH), 7.40 (m, 7H, -ArH), 9.72 (s, 1H, -NH).

Synthesis of 4-carbobenzyloxyamino-L-phenylalanine NCA, Cbz-4APhe NCA

The amino acid Z4APhe (5.1 g) was dried under vacuum for 1.5 hrs in a Schlenk flask, and then anhydrous THF (100 ml) was added at room temperature to obtain a suspension. Two equivalents of phosgene in toluene solution (17.1 ml, *d* 0.94) was added to the Z4APhe suspension under nitrogen. The temperature was elevated gradually up to 50 ^{o}C and the reaction magnetically agitated for 2 hrs to obtain a clear solution. Toluene and THF solvents were removed under vacuum to produce a white solid which was then dried under vacuum overnight. The solids were transferred to a glove box and were recrystallized 3 times through layer crystallization using anhydrous THF/hexane (3/10, v/v), to yield white crystals of the NCA product. We also synthesized other NCA monomers such as Cbz-protected L-lysine by a similar procedure. FTIR spectra of the NCA product showed the characteristic stretching bands of the mixed anhydride at 1854 cm^{-1}, 1779 cm^{-1}, and the carbonate side-chain group at 1702 cm^{-1} (Figure S2 of supporting information). Additionally, 1500-1600 cm^{-1} stretches were assigned to the aromatic group of 4APhe. ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 2.9 (dd, 2H, -CH_2), 4.7 (t, 1H, -CH), 5.1 (s, 1H, -CH), 7.1 (d, 2H, -ArH), 7.2-7.4 (m, 7H, -ArH), 9.1 (s, 1H, -NH), 9.8 (s, 1H, -NH) (Figure S3 of supporting information).



Scheme 1. a) Synthesis of poly(4-amino-L-phenylalanine), P4APhe, via the ring-opening polymerization of Cbz-protected 4APhe NCA monomer. b) Synthesis of copolymers using Cbz-4APhe and Cbz-L-lysine NCAs.

Synthesis of poly(4-carbobenzyloxyamino-L-phenylalanine)

The initiator $Co(PMe_3)_4$ was added to anhydrous THF in a dried tube under nitrogen, to prepare the initiator solution with a concentration of 10 mg/mL. The NCA of Z4APhe was separately dissolved in anhydrous THF to prepare a monomer solution with a predetermined concentration and then the initiator solution was added. The solution was mixed and was left to react for a week at room temperature in the glove box. The completion of polymerization was confirmed by FTIR analysis of a reaction aliquot: no NCA stretches were observed, and strong amide bands at 1650 cm^{-1} and 1540 cm^{-1} were observed. Next, the polymerization solution was poured into MeOH/HCl (1 mM) to precipitate the polypeptide, PZ4APhe, which was isolated by centrifugation and then dried. The structure of the isolated PZ4APhe was confirmed by FTIR (Figure S2) and NMR spectroscopy (Figure S4). DMAc and DMF were also used as polymerization solvents to compare different conditions. Isolated yields ranged between 92-98 %. FTIR spectrum showed bands at 1706 and 1636 cm^{-1} representing the Cbz group and amide bonds, respectively. The aromatic stretches at 3000-3100 cm^{-1} and 1500-1600 cm^{-1} (overlapping with amide II) were also present. ^{1}H NMR (400 MHz, DMSO-*d*6, δ ppm): 2.9 (d, 2H, -CH_2), 4.5 (br, 1H, -CH), 5.1 (s, 2H, -CH_2), 7.1 (br, 2H, -ArH), 7.3 (br, 7H, -ArH) 8 (br, 1H, -NH), 9.5 (br, 1H, -NH).

Synthesis of poly(4-amino-L-phenylalanine)

P4APhe was synthesized by deportation of PZ4APhe as follows. PZ4APhe was dissolved in TFA (2 mL). 5 equiv of HBr in acetic acid solution (33 wt %) was then added to the polypeptide solution in an ice bath. The solution was stirred for 1h, and the polypeptide was then precipitated by addition of diethyl ether, and then dried under a flow of nitrogen. The crude polymer was dissolved in water/methanol (1/4, v/v), and then reprecipitated by adding to diethyl ether. After drying, the polypeptide was dissolved in 1M LiBr aqueous solution and purified by dialysis against water for 2 days until pH around 6-7. Lyophilization then gave the polypeptide as a powder, whose structure and composition were confirmed using FTIR (Figure S2) and ^{1}H NMR spectroscopy (Figure S5)[24]. In the FTIR spectra, the peak at ~1650-1620 cm^{-1} and ~1540-1520 corresponds to the carbonyl (C=O) stretch which confirmed the presence of the specific bond. The aromatic stretches at 3000-3100 cm^{-1} and 1500-1600 cm^{-1} (overlapping with amide II) were also present. Isolated yields of the deprotected copolymer ranged between 75% and 90%. ^{1}H NMR in D_2O indicated over 99% removals of benzyloxycarbonyl groups from 4APhe residues. ^{1}H NMR (400 MHz, DMSO-*d*6, δ ppm): 2.9 (d, 2H, -CH_2), 4.5 (br, 1H, -CH), 5.1 (s, 2H, -CH_2), 7.1-7.4 (m, 4H, -ArH), 8.3 (br, 1H, -NH).

Measurements

^{1}H NMR spectra were recorded on a Bruker spectrometer (at 500 MHz) with DMSO-d_6 or $CHCl_3$-*d* as solvents and were reported relative to deuterated solvent signals. Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz) and integration. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and br, broad. ^{13}C NMR spectra were recorded on Bruker spectrometers as with ^{1}H NMR. Data for ^{13}C NMR spectra are reported in terms of chemical shift. All Fourier Transform Infrared (FTIR) samples were prepared as thin films on NaCl plates and spectra were recorded on a Perkin Elmer RX1 FTIR spectrometer and are reported in terms of frequency of absorption (cm^{-1}). Molecular weights of the protected polymers were determined using tandem gel permeation chromatography/light scattering (GPC/LS) which was performed on a SSI Accuflow Series III liquid chromatograph pump equipped with a Wyatt DAWN EOS light scattering (LS) and Optilab rEX refractive index (RI) detectors. Separations were affected by 10^5, 10^4, and 10^3 Å Phenomenex 5 micron columns using 0.1 M LiBr in DMF eluent at 60 °C. All GPC/LS samples were prepared at concentrations of 5 mg/mL. The value of d*n*/d*c* for PZ4APhe was determined from the inclination of absolute refractive indices plots against polymer concentration of 1.82 x 10^{-4}, 3.33 x 10^{-4}, 5.71 x 10^{-4}, 9.09 x 10^{-4}, 20.0 x 10^{-4} g/ml, and was found to be 0.393 + 0.0130 ml/g as shown in Figure S6. Circular dichroism spectra were recorded on an OLIS RSM spectrophotometer running in conventional scanning mode, and 1 nm bandwidth. Peptides were dissolved to a final concentration of 2.5 mg/ml in water. The spectra were plotted as mean residue ellipticity versus wavelength. Circular dichroism spectra (200-250 nm) were recorded in a quartz cuvette of 0.1 cm path length. All spectra were recorded as an average of 3 scans. The spectra are reported in units of molar ellipticity [θ] (deg·cm^2·$dmol^{-1}$). The formula used for calculating molar ellipticity, [θ], was [θ] = (α x 100 x M_W)/(*c* x *l*) where α is the experimental ellipticity, M_W is the average molecular weight of a residue in g/dmol, *c* is the peptide concentration in g/cm^3; and *l* is the cuvette path length in cm.

3. Results and discussion

4-amino-L-phenylalanine homopolymer

P4APhe previously prepared by amine-initiated NCA polymerization had low molecular weight and was not reported to form hydrogels.[25] Here, we used $Co(PMe_3)_4$ initiated NCA polymerization to obtain high molecular weight polymers.[23] PZ4APhe was synthesized in three different solvents of THF, DMF, or DMAc at a constant monomer concentration of 20 mg/ml. The polymerization reaction solution in THF was more viscous than those prepared in other two solvents. The polymerization was monitored with molecular weight measurement by GPC-MALLS in DMF containing 0.1 M LiBr at 25 °C showing a structureless single peak (inset of Figure 1). The results shown in Figure 1 indicate that molecular weights initially increased with increasing M/I ratio, but then eventually plateau at M/I ratios of ca. 100. The plateau in molecular weight observed may be due to small amounts of impurities in the NCA monomers that become significant at high M/I ratios. The highest polypeptide molecular weights obtained in DMAc, DMF, and THF were found to be ca. 4.1 kg/mol, 5.9 kg/mol, and 84 kg/mol, respectively. THF was the most efficient solvent of the three for obtaining high molecular weight polypeptide. However, PZ4APhe was found to occasionally precipitate during polymerization in THF, while it had good solubility in DMAc and DMF. We then tested the solvent effect on molecular weight using different mixed compositions of DMAc and THF. At a constant M/I ratio of 80 to 1, PZ4APhe molecular weight was found to increase by the addition of a small amount of DMAc to THF solvent, but then decreased as the fraction of DMAc increased. According to the results shown in Figure 2, 10/90 vol/vol of DMAc/THF was found to be optimal for increasing the molecular weight of PZ4Phe while also maintaining good polymer solubility. Thus, we developed polymerization conditions to produce PZ4APhe with molecular weight over 80 kg/mol, which is high enough to process the polymer into tough cast films as shown in the inset picture of Figure 1. PZ4APhe was then deprotected by an HBr in acetic acid solution, to obtain

P4APhe, which was purified to remove all residual impurities such as cobalt ions by extensive dialysis against deionized water.

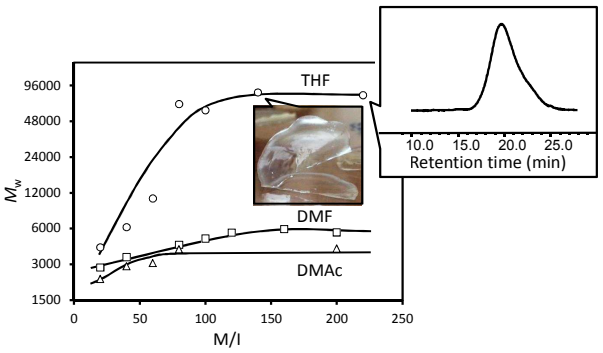


Figure 1. Effects of solvent on M_w (weight-average molecular weight) of poly(4-carbobenzyloxyamino-L-phenylalanine) polymerized at a different monomer to initiator ratios (M/I) at monomer concentrations of 20 mg/ml at room temperature. Inset chromatogram is representative GPC data of the polymer prepared with M/I = 220 in THF. The inset picture is a representative film cast from the polymerization solution with M/I = 140 in THF. All polymerizations went to completion of NCA consumption, which was monitored using FTIR. Polymerization time was ca. 6 days for M/I < 100, while 11 days was necessary for M/I > 100.

In order to study the chain conformation of P4APhe, CD measurements were carried out at a concentration of 2.5 mg/ml at different pH in water at room temperature. CD spectra of P4APhe were found to change as a function of pH, especially in the positive region at around 220 nm, Figure 3A. When molecular ellipticity [θ] at 220 nm was plotted versus pH, Figure 3B, it was found that [θ] decreased with increasing pH where it was below zero above pH 3.4, which is higher than pK_a of the 4APhe side groups. [2,14] The positive Cotton effects around 220 nm likely signify a disordered or random-coil conformation when protonated at low pH, but the transition into a negative band indicates that P4APhe likely adopts an ordered conformation is adopted at increased pH, that might be α-helix or β-strands. [14] Above pH 4, polypeptide precipitates also appeared in the sample, suggesting that precipitation may occur as a result of polypeptide assembly, perhaps due to β-strand formation. Slow precipitation of PZ4APhe also occurred when solutions were kept for long periods at pH 3.01. These precipitates were isolated and their crystallinity checked by XRD (Figure 4A). Three broad X-ray diffractions appeared at diffraction angles, 2θ, of 11.6 °, 23.9 °, and 35.1 °, corresponding to *d*-spacing's of 0.76 nm, 0.38 nm, and 0.25 nm. The XRD peaks are too broad to analyze in detail, but main peaks can be derived from the electron-rich functional groups such as phenylenes. Generally fully extended α-polypeptide chains show a 0.37-0.38 nm periodicity between peptide units, suggesting the P4Phe chains adopted fully-extended conformations. When the side chains alternately stick out from different sides of the main chain as in a β-sheet conformation, as shown in Figures 4A and 4B, periodicity with a 0.75 nm interval is expected. Thus the data suggest that the β-sheet conformation drawn in Figure 4B may have been adopted by the precipitated P4Phe chains. Unfortunately, no distinct diffraction peaks were detected at smaller angles. On the other hand, another sheet structure can be available based on the interaction of amine group at the side chains end with carbonyls of peptide backbones, which also have the abovementioned periodicity but the interchain distance was different from the data of Figure 4A. Also, derivatives of P4APhe such as poly-L-tyrosine and poly-L-phenylalanine are known to adopt β-strand [7]

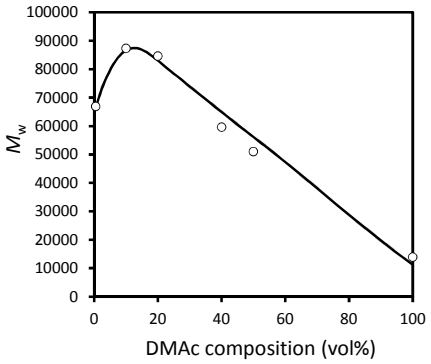


Figure 2. Effects of solvent composition in mixtures of DMAc and THF on the *M*w of poly(4-carbobenzyloxyamino-L-phenylalanine) at constant M/I = 80, and [NCA] = 40 mg/ml. All polymerizations went to completion of NCA consumption. DMAc/THF = 10/90 was found to be most effective in obtaining high M_w polymer.

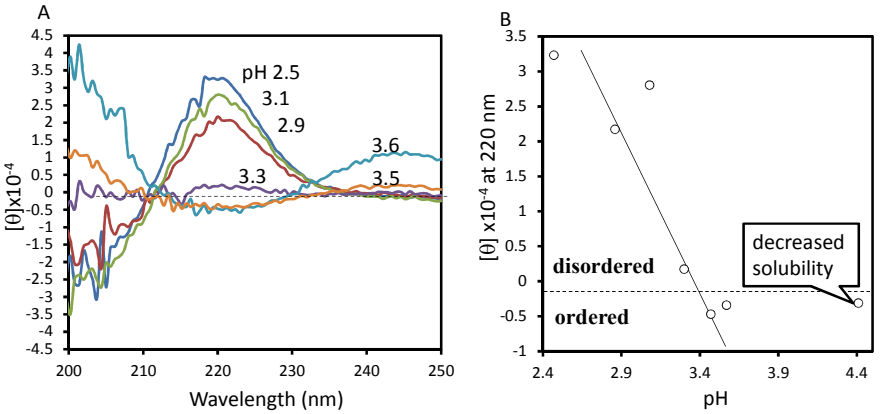


Figure 3. A) CD spectra at different pH for aqueous solutions of P4APhe at concentrations of 2.5 mg/ml. B) Molar ellipticity [θ] at 220 nm for samples in panel A as a function of pH. [θ] was found to decrease as pH increased, and then plateaued above pH = 3.5, likely due to a change in chain conformation.

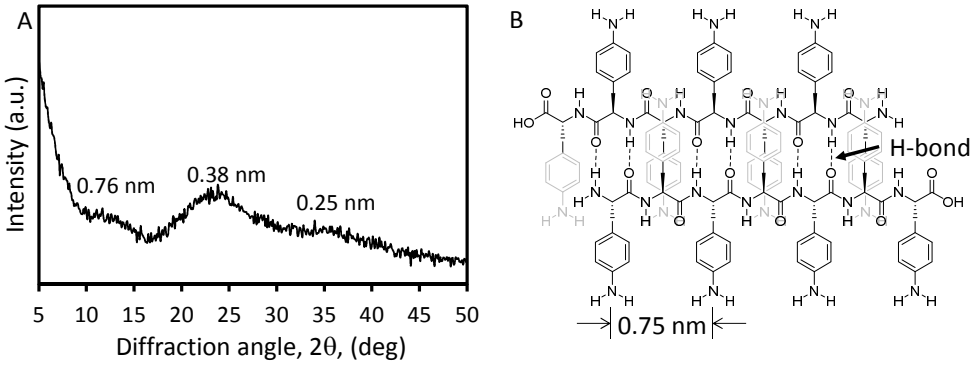


Figure 4. A) XRD diagram of a P4APhe film cast from aqueous solution at pH > 3.5. B) Diagram showing proposed chain packing of P4APhe based on formation of β-sheet structures.

4-Amino-L-phenylalanine copolymers with L-lysine.

In order to investigate copolymerization ability of 4APhe with L-lysine, we utilized the cobalt initiated polymerization method to obtain high molecular weights and readily tune compositions from 0 to 80 mol% lysine. The resulting copolymer compositions were confirmed by ^{1}H NMR (representative: Figure S7) by comparison of integral value ratios of α-methine protons of each repeat unit around 4.2-4.5 ppm. Table S1 shows the copolymer compositions, which were close to monomer feed ratios, and their molecular weights as determined by SEC-MALLS. The molecular weights are high enough to adopt order chain conformations. CD spectra of deprotected 4APhe copolymers with L-lysine at various compositions were measured in an acidic aqueous solution of pH 2.0 (representative: Figure S8). Double minima at 208 and 222 nm were seen only in the copolymer with 20 mol% of 4APhe. When the 4APhe content increased, the local minimum at 208 nm disappeared, and the negative peak at 222 nm became less distinct. It is likely that absorption of the aromatic side-chains in these copolypeptides strongly influences their CD spectra, such that interpretation is not straightforward. An interesting observation was made when the dried copolypeptide samples were immersed with different pH 2 water. ATR FTIR spectra of Amide I bands around 1600-1650 cm^{-1} were also recorded for the freeze-dried copolymers. Results are summarized in Figure 5A. The Amide I bands shifted gradually toward higher wavenumber with increased L-lysine content. Amide II bands around 1550 cm^{-1} changed their structures. These spectra changes indicate increased chain disorder as L-lysine content increased, due to the copolymerization effects. Opaque elastic hydrogels were observed to form with degrees of swelling that depended on their composition. By contrast, the homopolymer poly(L-lysine) simply dissolved to give a clear solution under these conditions. As shown in Figure 5B, degrees of hydrogel swelling relative to dry weight showed a maximum value of ca. 300 times for the sample with 20 mol% lysine, compared to a degree of hydrogel swelling of only 65 times for 4APhe homopolymer. The remarkable increase in swelling ratio upon incorporation of lysine may be due to the breaking of β-sheet packing of P4APhe segments. However, as lysine content was increased beyond 40 mol%, the degree of swelling was found to decrease again. The copolymer with a lysine/4APhe composition of 80/20 gave a hydrogel that was robust enough to be easily handled (inset picture of Figure 5B), which allowed investigation of the pH-responsiveness of this material. Changes in conformation were analyzed by ATR FTIR spectroscopy as a function of pH (Figure 6). Figure 6A shows IR

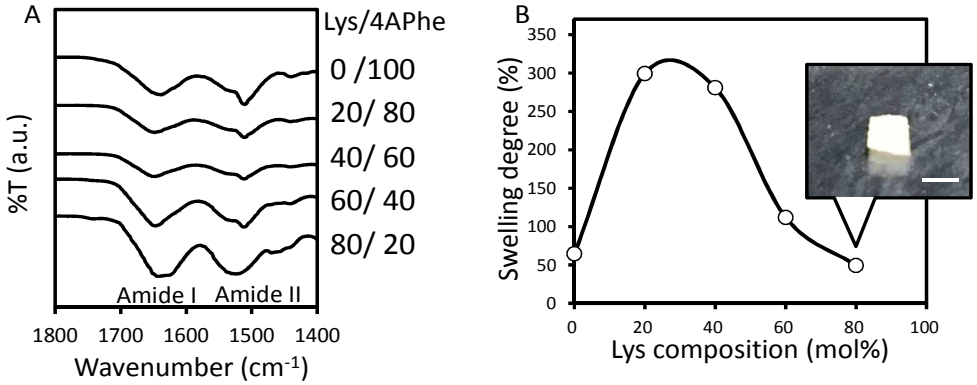


Figure 5. A) Partial ATR-FTIR spectra of statistical copolymers of 4APhe and L-lysine showing amide region. Samples were all freeze-dried from pH = 2.0 aqueous solutions. B) Degree of swelling of 4APhe/lysine copolymer films in pH = 2.0 water as a function of composition. Inset picture shows the swollen network formed from the 80 mol% lysine copolymer in pH 2.0 water. Bar = 2 mm.

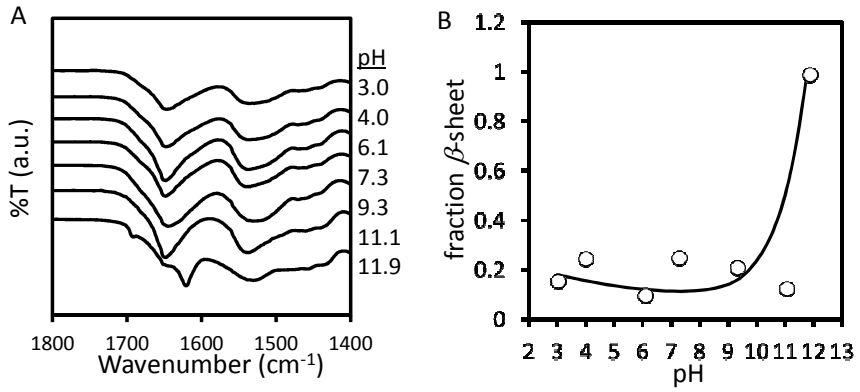


Figure 6. A) Partial ATR-FTIR spectra of the 80 mol% lysine statistical copolymer of 4APhe and L-lysine at different pH. Samples were all freeze-dried from aqueous solutions, where pH was adjusted by addition of NaOH. B) The fraction of β-sheet content in the 80 mol% lysine copolymer in solid samples from solutions of different pH, as determined by decomposition of the Amide I bands between 1620 and 1690 cm^{-1} in the IR spectra.

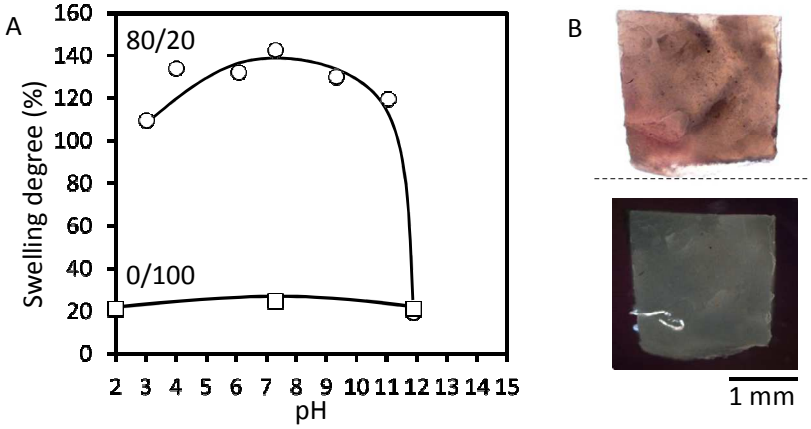


Figure 7. A) Degree of swelling of 0 and 80 mol% lysine 4APhe/lysine copolymer films in water as a function of solution pH. B) Images of the swollen network formed from the 80 mol% lysine copolymer in pH 3.01 water. Bar = 1 mm. Upper image = bright field. Lower image = with crossed-nicol polarizers.

spectra of the lysine/4APhe 80/20 composition copolymer, where specimens were dried at different pH. From the decomposition analysis of the Amide I bands around 1650 cm^{-1}, fractions of β-sheet content were determined as a function of pH (Figure 6B). The copolypeptide showed a minimum β-sheet fraction of 0.18, 0.22 at pH 3.01 and 4.0, but this fraction was decreased by increasing pH, presumably due to chain disordering based on the different ionization behavior between 4APhe and Lysine. On the other hand, at pH 11.9 means at the highest pH, β-sheet fraction was remarkably increased due to complete deionization of amine groups for both 4APhe and lysine units. Figure 7A shows a degree of hydrogel swelling for the 80/20 composition copolymer at different pH compared to P4APhe homopolymer. While the degree of hydrogel swelling for the 80/20 copolymer at pH 3.01 was around 109 times, an increase in pH resulted in a substantial increase in swelling degree that only diminished above pH 11. This pH-dependent trend correlates well with the fractional β-sheet content at different pH shown in Figure 6B. In the 80/20 composition copolymers, decreased β-sheet content results in fewer interchain crosslinks, leading to an increase the swelling degree. The observed pH-dependent changes in swelling degree for this copolymer hydrogel were found to be fully reversible as pH was increased then decreased. On the other hand, the P4APhe homopolymer hydrogel showed a negligible change of swelling degree regardless of pH, likely due to strong aggregation and packing of the P4APhe side chains. Figure 7B shows a microscopic image of the 80/20 composition copolymer hydrogel film hydrated at pH 3.01. The bright field image (upper) reveals the low transparency of the hydrogels, and the image tale under cross-nicol polarizers (lower) reveals some birefringence (light transmission), due to some ordering from small crystals in the hydrogel, where β-strands can form the cross-linking junctions.

4. Conclusions

We investigated the role of exotic amino acid with functional aromatic side chains, 4APhe, which exists in nature but very rarely, in the formation of polypeptide hydrogel materials. 4APhe was shown to be incorporated into high molecular weight homo- and copolypeptides via the N-carboxyanhydride monomer. In addition, data and properties showed that this residue favoured formation of

β-strands in the polypeptides that result in physical crosslinking of dried films. Rehydration of these films gave stable polypeptide hydrogels, which in copolymers with lysine were found to exhibit strong and reversible pH-dependent changes in swelling behavior in water. These properties of 4APhe can lead to its usage as a new building block for soft polypeptide materials, providing a general platform for stimuli-responsive bio-related materials.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The studies were partly by a financial support of JST programs of Advanced Low Carbon Technology Research and Development Program (5100270) and from a COI project "Construction of next-generation infrastructure using innovative materials".

References

1 S. Wong, M. S. Shimb, Y. J. Kwon, *J. Mater. Chem. B*, 2014, **2**, 595-614.
2 K. Bauri, M. Nandi, P. De, *Polym*., Chem.10.1039/C7PY02014G.
3 N. Hadjichristidis, H. Latrou, M. Pitsikalis, G. Sakellariou, *Chem. Rev.*, 2009, **109**, 5528-5578.
4 K. Inoue, H. Sakai, S. Ochi, T. Itaya, T. Tanigaki, *J. Am. Chem. Soc*., 1994, **116**, 10783-10784.
5 D. B. Khadka, M. C. Cross, D. T. Haynie, *ACS Appl. Mater. Interfaces*, 2011, **3**, 2994-3001.
6 M. G. Ryadnov, D. N. Woolfson, *J. Am. Chem. Soc*., 2007, **129**, 14074-14081.
7 K. Pagel, S. C. Wagner, K. Samedov, H. V. Berlepsch, C. Böttcher, B. Koksch, *J. Am. Chem. Soc*., 2006, **128**, 2196-2197.
8 a) M. Torrent, D. Mansour, E. P. Day, K. Morokuma, *J. Phys. Chem. A*, 2001, **105**, 4546-4557. b) D. E. Clarke, E.T. Pashuck, S. Bertazzo, J. V. M. Weaver, M. M. Stevans, *J. Am. Chem. Soc.*, 2017, **139**, 7250-7255.
9 A. Acharya, B. Ramanujam, A. Mitra, C. P. Rao, *ACS Nano*, 2010, **4**, 4061-4073.
10 E. G. Bellomo, M. D. Wyrsta, L. Pakstis, D. J. Pochan, T. J. Deming, *Nat. Mater*., 2004, **3**, 244-248.
11 S. F. Burke, C. J. Barrett, *Biomacromolecules*, 2003, **4**, 1773-1783.
12 M. Obst, A. S. Steinbüchel, *Biomacromolecules*, 2004, **5**, 1166-1176.
13 X. Mu, K. M. Eckes, M. M. Nguyen, L. J. Suggs, P. Ren, *Biomacromolecules*, 2012, **13**, 3562-3571.
14 a) C. Solanas, B. G. D. L. Torre, M. F. Reyes, C. M. Santiveri, M. A. Jiménez, L. Rivas, A. I. Jiménez, D. Andreu, C. Cativiela, *J Med Chem*., 2010, **53**, 4119-4129; b) J. O. Kim, H.S. Oberoi, S. Desale, A. V. Kavanov, T. K. Bronich, *J Drug Target*., 2013, **21**, 981-993; c) K. R. Brundent, Y. Uratani , W. A. Cramer, J. *Biol. Chem.,* 1984, **259**, 7682-7687;.d) M. Goodman, E. Peggion, *Biochemistry*, 1967, **6**, 1533-1540.
15 S. Masuo, S. Zhou, T. Kaneko, N. Takaya, *Sci. Rep*., 2016, **25764**, 1-8.
16 P. Suvannasara, S. Tateyama, A. Miyasato, K. Matsumura, T. Shimoda, T. Ito, Y. Yamagata, T. Fujita, N. Takaya, T. Kaneko, *Macromolecules,* 2014, **47**, 1586-1593.
17 A. Kumar, S. Tateyama, K. Yasaki, M. A. Ali, N. Tataya, R. Singh, T. Kaneko, *Polymer*, 2016, **83**, 182-189.
18 H. Shin, S. Wang, S. Tateyama, D. Kaneko, T. Kaneko, *Ind. Eng. Chem. Res*., 2016, **55**, 8761-8766.
19 T. J. Deming, *Chem. Rev*., 2016, **116**, 786-808.
20 V. Singh, R. K. Rai, A. Arora, N. Sinha, A. K. Thakur, *Sci. Rep*. 2014, **3875**, 1-8.
21 *T. J. Deming*, *Soft Matter*, *2005*, **1**, *28-35.*
22 J. R. Karmer, T. J. Deming, *J. Am. Chem. Soc*., 2010, **132**, 15068-15071.
23 H. F. Klein, H. H. Karsch, *Inorg. Chem*., 1975, **108**, 944-955.
24 T. J. Deming, *Macromolecules*, 1999, **32**, 4500-4502.
25 M. Sela, E. Katchalski, *J. Am. Chem. Soc*., 1954, **76**, 129-133.
26 R. Cerpa, F. E. Cohen, I. D. Kuntz, *Fold Des*., 1996, **1**, 91-101.
27 A. Dasgupta, J. H. Mondal, D. Das, *RSC Adv*., 2013, **3**, 9117-9149.

TOC