Synthesis and Characterization of Multiblock Copolymers Based on Spider Dragline Silk Proteins

Chuncai Zhou,† Boxun Leng,† Jinrong Yao,† Jie Qian,† Xin Chen,† Ping Zhou,† David P. Knight,‡ and Zhengzhong Shao*†

The Key Laboratory of Molecular Engineering of Polymers and Department of Macromolecular Science, Fudan University, Shanghai 200433, P. R. China, and Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, U.K.

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Spider dragline silk with its superlative tensile properties provides an ideal system to study the relationship between morphology and mechanical properties of a structural protein. Accordingly, we synthesized two hybrid multiblock copolymers by condensing poly(alanine) [(Ala)₅] blocks of the structural proteins (spidroin MaSp1 and MaSp2) of spider dragline silk with different oligomers of isoprene (2200 and 5000 Da) having reactive end groups. The synthetic multiblock polymer displayed similar secondary structure to that of natural spidroin, the peptide segment forming a β-sheet structure. These multiblock polymers showed a significant solubility in the component solvents. Moreover, the copolymer which contains the short polyisoprene segment would aggregate into a micellar-like structure, as observed by TEM.

Introduction

During the years of evolution, human beings have learned many invaluable lessons from nature, and there is currently considerable interest in the biomimicry of biopolymers. Therefore, the natural silks, including spider silks and silkworm silk, have been extensively studied for many years. The results showed that some of them, especially spider dragline silk, are particularly promising materials with outstanding comprehensive mechanical properties superior to most of the artificial materials.

Moreover, spider silk provides an ideal system to study the relationship between protein structures and mechanical properties. The structure of silk can be described as a nanocomposite made up of a cross-linked, amorphous polymer network reinforced by well-oriented crystalline filler. Both solid-state NMR and wide-angle X-ray diffraction (WAXD) have been used to study the crystalline region, which greatly contributes to the extraordinary strength of the silk fiber. The amorphous network provides structural continuity and thus plays a major role in determining mechanical properties. A lot of evidence has shown that the structure of the silk proteins, as well as the processing conditions and consequent morphology of the silk, greatly contributes to the final properties of silk materials.

Therefore, understanding the molecular basis of silk proteins and the processing procedures involved in silk fiber formation will facilitate efforts to synthesis spider silk-like materials with predicted combinations of desirable characteristics.

The precise control of architecture exhibited by spider silk protein (spidroin) has inspired a variety of novel materials that combine elements of natural and synthetic polymers. Sogah’s group synthesized silk-like copolymers in which the glycine-rich blocks (amorphous regions) were replaced by a synthetic oligomer such as PEG, while the peptide blocks (for example, poly(alanine)) were derived from the crystalline regions of the silk. The resulting polymers showed similar secondary structure to that of natural silk proteins. Our group has explored multiblock copolymers in which GAGA and GAAAA were introduced to form the crystal regions as in certain silk proteins. However, most of the synthetic silk-like copolymers reported previously only dissolved in highly polar solvents such as hexafluoro-2-propanol (HFIP) or dichloroacetic acid, which would probably prevent further investigation and industrial development of these materials.

Here, we report the synthesis and characterization of segmented multiblock copolymers which exploit the tendency of poly(alanine) blocks to phase-separate and self-assemble into ordered β-sheet stacks in order to control nanostructure of the material. We replaced the glycine-rich blocks in spidroin with polyisoprene (PI) oligomer to improve the solubility of the synthetic copolymer. FT-IR, WAXD, and ¹³C CP/MAS NMR demonstrated the existence of β-sheet formation in the (Ala)₅ blocks in our multiblock copolymer as has been shown in previous reports. After casting in the general component solvent CHCl₃/HOCH₂CH₂Cl (chloroform and 2-chloroethanol), the micellar-like aggregation of multiblock copolymer was observed by transmission electron microscopy (TEM).

Experimental Section

Materials. Chloroform and N-methylmorpholine were distilled before use. N,N-Dimethylformamide (DMF) was used after dehydration with 4 Å molecular sieves for 2 days. Dicyclohexylcarbodiimide (DCC; from Shanghai Shesan Chemical Co., Ltd., China), N-tert-butoxycarbonyl-ω-l-alanine (BocAOH), N-hydroxysuccinimide (HOSu) (both from Yangzhou Baosheng Biochemical Co., Ltd., China), hexamethylene disiocyanate (HDI) (from Aldrich), and other reagents were used as received.

Measurements. For FTIR analysis, the chloroform solution of the polymer was dropped onto a KBr window and allowed to dry. Spectra were obtained on a Nicolet Magna-550 spectrometer (4 cm⁻¹ resolution; 32 scans). ¹H NMR spectra were obtained using the Bruker AVANCE EM-500 spectrometer, using tetramethylsilane (TMS) as an internal...
protected intermediate products were purified by column chromatography (silica gel (200–300 mesh)) eluted with a 1:1 (v/v/v) mixture of chloroform, methanol, and acetic acid. The removal of protective group (Boc) with hydrochloride acetic acid solution and purification by Soxhlet extraction with chloroform afforded compound III, a light yellow powder. The total yield was about 37%. FT-IR spectra showed peaks or shoulders at (cm⁻¹) 3281, 3070, 2983, 2935, 1690, 1655, 1632, 1545, 1448, 1375, 1245, and 117, while ¹H NMR (500 MHz, DMSO) gave resonances (ppm) at 1.16–1.37 (m, 4H, CH₂ of CH₂CH₂CH₂, CH of Ala), 2.95–3.05 (m, 4H, CH₂ of CH₂CH₂CH₂, CH of Ala), 3.84–3.94 (m, 10H, CH of Ala), 7.63–8.63 (m, NH of Ala, NH of (CH₂)₇NH, NH of NH₃⁺ of end group).

Functional Isoprene Oligomers (HO—PI—OH). While continuously stirring a mixture of 10 mL isoprene, 100 mL cyclohexane, and 5 mL tetrahydrofuran (THF) under dry argon, lithium naphthalene THF solution (initiator) was injected via syringe. The yellow solution was stirred for 1 h at room temperature. The solution lost its color immediately when excess dried ethylene oxide (4 times molar excess over the lithium naphthalene) was injected to add the functional group at both ends of the oligomer. After stirring at room temperature for 4 h, 10 mL of methanol was added to terminate the reaction. The product was obtained by precipitation in methanol, washed with both water and methanol several times, and dried under vacuum for 2 days. The functional isoprene oligomers with hydroxyl at both ends (HO—PI—OH) were obtained. The molecular weight of the HO—PI—OH was determined by GPC as described above, e.g., M₆ = 2200 (d = 1.11) and M₅ = 5000 (d = 1.09).

OCN—PI—NCO (Scheme 2). First, HO—PI—OH was dried by azotropic distillation. Under anhydrous condition, a solution of 0.001 mol of this compound in 30 mL of chloroform was added slowly dropwise to 0.504 g of hexamethylene diisocyanate (HDI) (three times the molar quantity of the PI) and 0.05 mL of dibutyltin dilaurate over 2 h. The mixture was stirred for 1 h at room temperature. The final solution (H) should in theory contain equimolar quantities of OCN—PI—NCO and HDI. GPC results confirmed the absence of PI linked by oligomer themselves in the final product.

Multiblock Copolymer (polyolpeptide-co-polysiprene, PEP—PI). The compound III (0.002 mol) was swollen in 15 mL of DMF for 10 h, and then, 0.023 mol N-methylmorpholine (excess) and 120 mL chloroform were added. The solution H was then slowly dripped in within 1 h under dry nitrogen. During the addition of H, the opaque solution gradually cleared. The mixture was allowed to react at room temperature for 48 h and then at 60 °C for 4 h. At the end of the reaction, the solvents were removed from the gel-like product. The crude product was washed successively with deionized water, methanol, and chloroform and vacuum-dried for 2 days at room temperature to give a faintly yellow powder.

Results and Discussion

Copolymerization and Physical Properties of the Silk-Like Polymers. The compound III, based on the poly(alanine) blocks of the crystalline regions of spidroin I and II (MaSp1 and MaSp2) was synthesized by the traditional liquid method (Scheme 1), while the isoprene oligomer containing two hydroxyl groups was synthesized by anionic polymerization. The silk-like copolymer was prepared by step-growth polymerization of the compound III and HO—PI—OH with disiocyanate.
as the chain extender in CHCl₃/DMF and in the presence of N-methylmorpholine (Scheme 2). The silk-like copolymer we synthesized did not dissolve in general polar or nonpolar solvents, even in highly polar solvents including HFIP and HFA, probably as a result of the great contrast in the polarity between the (Ala)₅ block and PI block, as well as the strong hydrogen bonding between the (Ala)₅ blocks (see the discussion below). Instead, we were obliged to try complex solvent systems able to compete with the hydrogen bonds to break down the interactions between the (Ala)₅ blocks yet able to separate the aggregation of PI block. We were successful with a mixture of CHCl₃/CICH₂CH₂OH. The inherent viscosity (ηinh) measurements of the copolymers dissolved in CHCl₃/CICH₂CH₂OH (1:1 or 2:1, depending on the molecular weight of the PI block) were used to estimate polymer molecular weights. To study the effect of the length of the oligomer on the structure of the poly(alanine) blocks, isoprene oligomers with molecular weights of 2200 and 5000 Da (whose molecular weight was confirmed by GPC) were used to synthesize PEP–PI2200 and PEP–PI5000, respectively. The ηinh values for PEP–PI2200 and PEP–PI5000 were 0.98 dL/g and 0.76 dL/g respectively, much larger than those of compound III (0.08 dL/g) and PI 2200 (0.11 dL/g). Moreover the copolymers began to decompose around 220 °C (PEP–PI2200) and 210 °C (PEP–PI5000) without melting (see Table 1). PEP–PI2200 and PEP–PI5000 displayed apparent glass transition temperatures (Tg) at 12 °C and 15 °C respectively, probably arising from the mobile PI blocks. All of these results confirm that condensation between isoprene oligomer and the peptide had occurred as predicted. The higher Tg of the poly(alanine)-containing copolymer compared to that of isoprene oligomer (−15 °C) probably resulted from a reduction in the mobility of PI blocks in the copolymer caused by multiple hydrogen bonds between the (Ala)₅ blocks.

Table 1. Physical Properties of Compound III, Isoprene Oligomer (PI2200), and Multiblock Polymers (PEP–PI2200, PEP–PI5000).

<table>
<thead>
<tr>
<th>Sample</th>
<th>ηinh (dL/g)</th>
<th>Tg (°C, TGA)</th>
<th>Tg (°C, DSC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>compound III</td>
<td>0.08a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PI2200</td>
<td>0.11a</td>
<td>302</td>
<td>-15</td>
</tr>
<tr>
<td>PEP–PI2200</td>
<td>0.38a</td>
<td>221</td>
<td>12</td>
</tr>
<tr>
<td>PEP–PI5000</td>
<td>0.76b</td>
<td>210</td>
<td>15</td>
</tr>
</tbody>
</table>

a Measured at 25.0 °C in the mixture of CHCl₃/CICH₂CH₂OH (v/v = 1:1) and the concentration of 0.5 g/dL. b Measured in the mixture of CHCl₃/ CICH₂CH₂OH (v/v = 2:1).

The synthetic poly(Alanine)-containing copolymer compared to that of isoprene oligomer (−15 °C) probably resulted from a reduction in the mobility of PI blocks in the copolymer caused by multiple hydrogen bonds between the (Ala)₅ blocks.

Evidence for the Presence of β-Sheet Structure in the Synthetic Poly(Alanine)-Containing Copolymer. Solid-State ¹³C NMR. Solid-state ¹³C NMR is widely used to demonstrate the formation of β-sheet in polypeptides and proteins. Figure 1 shows ¹³C CP/MAS NMR spectra of the copolymers PEP–PI2200 and PEP–PI5000. Because the resonance of Ala-Cβ (at about 18 ppm) was overlapped by that of Cβ in the isoprene segment, we only discuss the shifts of Ala-Cα and Ala-C=O in the spectra. The resonance at 48 ppm (Ala-Cα) and 171 ppm (Ala-C=O) in both copolymers was attributed to β-sheet aggregates. However, only in PEP–PI5000 was there a slight shoulder peak at 51–52 ppm and 176 ppm, suggesting the coexistence of other conformations for Ala-Cα and Ala-C=O in this copolymer.

**Figure 1.** Ala Cα, Cβ, and carbonyl regions in solid-state ¹³C CP/MAS NMR spectra of PEP–PI2200 and PEP–PI5000.

**Figure 2.** FT-IR spectra (amide I) of PEP–PI2200 (solid line) and PEP–PI5000 (dash line).
of poly(alanine) blocks whether in our synthetic copolymer or spider dragline silk is similar.

The FT-IR spectrum of PEP–PI5000 (Figure 2) showed bands 1630 and 1689 cm\(^{-1}\) attributable to antiparallel \(\beta\)-sheet conformation. However, other peaks, such as 1655, 1663, 1675, and 1643 cm\(^{-1}\) indicate that other conformations may coexist in this copolymer. These observations suggest that the peptide in the copolymer with the smaller molecular weight PI showed much more selective assembly into the antiparallel \(\beta\)-sheet conformation than the peptide with the larger PI.

Wide-Angle X-ray Diffraction. Figure 3 shows the WAXD patterns of copolymers PEP–PI2200 and PEP–PI5000 and oligomer PI5000, respectively. Two diffraction peaks with \(d\) spacing of 5.29 Å (2\(\theta\) = 16.9°) and 4.39 Å (2\(\theta\) = 20.4°) could be detected in both multiblock copolymers, similar to the values reported for the antiparallel \(\beta\)-sheet of \(N.\) clavipes dragline silk (5.3 Å)\(^{22}\) and other synthetic silk-like copolymers containing poly(alanine) (5.28 Å, 4.4 Å).\(^{15,18,33}\) These peaks were particularly prominent in PEP–PI2200 but were undetectable in the oligomer PI5000. Thus, powder X-ray diffraction analysis confirmed the existence of the antiparallel \(\beta\)-sheet in both our synthetic silk-like copolymers. However, a larger and broader amorphous scattering peak with a maximum at about 2\(\theta\) = 13° was prominent in PEP–PI5000 and in the oligomer PI5000. This suggested that the poly(alanine) segments form much less crystalline domains in PEP–PI5000, probably as a result of the predominance of the longer PI block.

TEM Observations. The multiblock copolymer of PEP–PI has two kinds of segments with different polarity. Thus, it form micelles in the selective solvent as common amphiphilic block copolymers and be detected by TEM. Figure 4 is the TEM image of PEP–PI2200 that shows similar micellar-like structures as in poly(ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide).\(^{34}\) In the case of our experimental condition, electron-dense material was arranged in a remarkably regular pattern (“plum blossom”) consisting of circular patches with a fairly constant diameter (80–110 nm) containing a variable number of dense granules (10–20 nm in diameter). The circular patches were rather uniformly distributed with a center-to-center separation of 10–20 nm. A few small, dense granules were also seen between the circular patches. We may suggest that they are micelles because of the circular profile and remarkably uniform diameter. On the other hand, such pattern was not seen in PI–PEPS000 (image not shown), probably because of the long PI segments. Furthermore, the Congo red was applied to stain the samples, as it was thought to be capable of intercalating between \(\beta\)-strands in \(\beta\)-sheet crystalites in proteins.\(^{20}\) However, the image of Congo red stained samples did not have any difference from unstained one. This may imply that the electron-dense part in the images contain both (Ala)\(_5\) and PI segments.

Conclusions

By taking advantage of the high reactivity of diisocyanates and by copolymerizing bis(oligopeptides) and isoprene oligomers, we have synthesized spidroin-like multiblock copolymers containing (Ala)\(_5\) blocks derived from the crystalline region of spider dragline silk. We have used FT-IR and solid-state \(^{13}\)C CP/MAS NMR spectroscopy to demonstrate that the \(\beta\)-sheet conformation predominates in our synthetic polymers. Our X-ray data provides conclusive evidence for the formation of antiparallel \(\beta\)-sheet crystalline domains. Of the two copolymers we synthesized, the one with the shorter isoprene blocks formed micellar-like aggregation under certain conditions. Thus, our synthetic copolymers show not only similar structure and behavior to that of spider dragline silk protein, but also tunable assembly, for example, by varying the length of the soft block. Thus, it may eventually prove possible by conventional chemical routes to synthesize block copolymers capable of directed assembly into tough nanofibrillar composites resembling spider silk.

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References and Notes