Using Solvents with Different Molecular Sizes to Investigate the Structure of Antheraea Pernyi Silk

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ABSTRACT: The interaction between silk and polar solvents of different molecular size can be an important tool for understanding the structural features of natural silk; in particular, the disordered regions associated with the key property of mechanical toughness. In this work, we investigate the transitions induced in the tensile performance and structure of as-reeled Antheraea pernyi silks from different silkworms by a range of solvents that can only soften the protein chains in the amorphous regions. The results indicate that polar solvents with different molecular sizes affect the silk to different degrees, and silks with slightly different structures display significantly different tensile performance in the same solvent. The solvent molecular size is quantitatively correlated with the accessible volume in the amorphous regions before and after the yield point, which suggests that the volume accessible to the solvent molecules decreases as the solvent radius increases. Moreover, silks with more ordered structure (less free volume) in the amorphous regions are less sensitive to solvents than those with more disordered structures. However, silks with higher free volume have higher toughness due to the greater strain to failure.

1. INTRODUCTION

Animal silks, notably spider dragline silks and Bombyx mori (B. mori) silks, exhibit outstanding mechanical properties through an excellent balance of strength, extensibility, and toughness that surpasses most man-made fibers. 1−4 Although significant progress has been made in producing artificial silk fibers from either recombinant silk analog proteins or regenerated silk fibroins, 5−10 their mechanical properties still cannot match those of natural silks due to not reproducing their structural features. 11,12 In general, silk is regarded as a semicrystalline biopolymer, within which antiparallel β-sheet nanocrystals composed primarily of polyalanine or (GAGAGS)2 segments for spider dragline silk and B. mori silk, respectively, 13,14 are embedded in a noncrystalline matrix consisting predominantly of glycine-rich blocks. The ordered nanocrystals, which are oriented preferentially along the fiber axis, play a crucial role in determining the stiffness of silks, while the disordered noncrystalline matrix contributes to the elongation and associated toughness of the fibers. 15−17

To date, the crystalline domains have been relatively well characterized, 14,18−22 yet the noncrystalline regions are still ambiguous. The existence of a third, oriented noncrystalline phase has been suggested 14 and this third phase is depicted as the constrained chains at the interface between the crystalline domains and the amorphous matrix. 15 More generally, the amorphous chains in native silks have complex combinations of structural features and are aligned to different extents. 23 In addition, natural silks from different individuals within a species or even the same individual show significant variability, 24 which can be attributed mainly to differences in production conditions and can be correlated with the position of the glass transition temperature, Tg. 25 Therefore, a deeper insight into the amorphous regions of natural silks is crucial for understanding the relationship between structure and properties of natural silk fibers, which will guide the development of high performance biomimetic materials and be also beneficial for the fabrication of synthetic materials with improved performance.

Polar solvents can interact with polar groups of many natural proteins and synthetic polymers and consequently change their mechanical or thermal properties. The effects of a range of solvents on the structure and mechanical properties of animal silks have been studied and the results indicate that solvents with different compositions (molecular size and polarity) can affect different domains within the relatively disordered regions and further lead to distinct mechanical properties of a specific silk. 26−28 Although the effects of solvents on the mechanical properties of silks are well-known, there are no reports to date of using polar solvents with different molecular sizes to investigate the structural variability and then variability of properties in silks from different individuals of the same species. More specifically, we still lack knowledge about the details of

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how the solvent molecular size can be correlated to the free volume in the amorphous regions in the silk.

Antheraea pernyi (A. pernyi) silk is a kind of wild silkworm silk and is used to construct a cocoon, like B. mori silk. However, the amino acid sequences\(^{29}\) of A. pernyi silk are more similar to spider (major ampullate) silk than B. mori silk.\(^{30−32}\) Recent work by Fu et al. has shown that A. pernyi silk shares some comparable structure–property relationships with both B. mori silk and the major ampullate silks of a range of spiders.\(^{33}\) A better understanding of the structural features of A. pernyi silk may provide valuable insights about the structure–property relationships for the whole animal silk family. Our work presented here tries to understand the structural origin of the high toughness and high variability associated with the amorphous regions of as-reeled A. pernyi silks through using polar solvents with different molecular sizes. To this end, we first studied the effect of polar solvents (water, methanol, and ethanol) on the tensile properties and molecular structure of as-reeled A. pernyi silks from different silkworms. Then the relations between the solvent molecular size and the accessible volume in the amorphous regions before and after the yield point were quantitatively analyzed. This analysis provides a basis for an explanation for the different responses of silks from different silkworms to the same solvent.

2. METHODS AND MATERIALS

2.1. Silk Fiber Preparation. Silk fibers were forcibly reeled at 8 mm/s as reported previously\(^{2,24}\) from mature A. pernyi silkworms, which previously lived on oak trees in a tussah province, China. The silks were collected and restrained on a plastic cylinder of 21 mm in diameter. Compared to the same procedure applied on the B. mori silkworm, forcibly reeling silks from the spinneret of mature A. pernyi silkworm is more convenient and controllable, which will generate nearly defect-free silks and further lead to highly repeatable mechanical properties. With the help of dividers, silk fiber (bave) with a gauge length of 9 mm was transferred and adhered onto the sample frame\(^{34}\) made from aluminum foil with double-faced tape. An extra layer of nail varnish was brushed on the adhering point to prevent water from deteriorating the adhesive effect of the glue for samples tested in water.

We tested 45 sets of silk fibers from 10 silkworms (two seasons). An adjacent set of four samples were used to test their tensile performance in air, methanol, water, and ethanol in turn (Scheme 1). The results from these 10 silkworms were then grouped into four generic types of performance and labeled arbitrarily as silkworms 1, 2, 3, and 4 mainly according to their different tensile behavior of samples in methanol and ethanol and in order of decreasing strain to break in air in order to highlight the intrinsic structural variability of animal silks. The detailed number of silkworms and samples for each type of tensile performance are displayed in Table 1.

2.2. Tensile Test with Instron. The sample frame was held unstrained in the clamps in a Netzsch DMA242 instrument, which was integrated with the custom-built accessory (Figure 1) that can be used for tensile tests either in air or in selected solvents (water, methanol, and ethanol). Before the test, the side support of the frame was cut by scissors, so that the force was transmitted through the silk fiber. An Instron S565 was used for the tensile tests at a strain rate of 0.005 s\(^{−1}\) in air (around 20−25 °C and 40−50% RH) as well as under submersion in selected solvents. The sample was given 20 min to reach equilibrium after immersion and no load was exerted on the fiber during the pretest immersion time, in order to allow for any possible contraction of the fiber (as-reeled A. pernyi silks all shrank in the three solvents used here and the shrinkage ratio was found to be about 3−5, 0.8−1.2, and 0.9−1.2% for water, methanol, and ethanol, respectively). The solvents were discharged through a hole on the base of the chamber after the test finished.

2.3. Cross-Sectional Area Measurement. The cross-sectional area of the as-reeled silk fiber was measured according to the method described in detail elsewhere.\(^{33}\) Briefly, a length of silk fiber (for area measurement in Scheme 1) was sputtered with gold for 3 min and then embedded in epoxy resin. The silk-embedded epoxy resin was fractured into six segments perpendicular to the silk fiber using custom-built fracture tools. The fracture cross section was sputtered with gold, and then observed by a Tescan 5136MM using secondary electron microscope. The silk area of the as-reeled silk fiber was measured in the direction parallel or perpendicular to the vibration direction of the laser beam. Spectra were obtained from ten acquisitions of 50 s using the 785 nm line of a semiconductor laser with energy of approximately 300 mW.

Table 1. Number of Silkworms and Samples for Each Silkworm Group

<table>
<thead>
<tr>
<th>Silkworm</th>
<th>Silkworm 1</th>
<th>Silkworm 2</th>
<th>Silkworm 3</th>
<th>Silkworm 4</th>
</tr>
</thead>
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<tr>
<td>Silkworms</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Sets of samples</td>
<td>21</td>
<td>10</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 1. Custom-built accessory used for tensile tests in solvents. The aluminum chamber was fixed on the base of the Instron S565, while the upper champ was connected to a 2.5 N load cell through a hollow aluminum pole.
Repeatable Raman results on the silk fiber samples demonstrated that there were no signs of structural damage during the measurement. All the spectra were normalized by the intensity at 1615 cm$^{-1}$ assigned to the phenyl group thought to arise mainly from the tyrosine residues, because it is insensitive to the conformation of the silk protein.  

2.5. X-ray Diffraction (XRD). The X-ray diffraction measurements were performed with a Bruker D8 ADVANCE and DAVINCLDE-SIGN X-ray powder diffractometer, using Cu K$_\alpha$ radiation ($\lambda=1.5418$ Å) with Göbel Mirror setup at 40 mA and 40 kV. The data were obtained from $5$ to $40^\circ$. The silks tested in the X-ray diffraction measurement were obtained according to the method described in detail elsewhere. Briefly, a mature *A. pernyi* silkworm was placed on a PET sheet so that it would spin a silk mat on the sheet. After drying, the silk mat was removed from the plastic sheet carefully and put on the glass sample holder of the diffractometer before and after treatment with selected solvents.

2.6. Molecular Simulations. Molecular simulations of solvent accessible volume used a combination of molecular dynamics and energy minimization in the LAMMPS program in the Scienomics MAPS suite of software with Gasteiger charge assignment and energy minimization in the LAMMPS program in the Scienomics field to give densities of 1.15 to 1.30 g/cm$^3$ in cubic boxes. For simplicity, the amorphous silk macromolecules were constructed with -GA- peptide pairs. The solvent accessible volume at each density was then analyzed with solvents of different size, as specified by their hard sphere radius: water, 1.61 Å; methanol, 2.1 Å; ethanol, 2.3 Å.

3. RESULTS AND DISCUSSION

3.1. Initial Screening of Tensile Properties in Different Media. To illustrate the underlying principle of this work, the stress–strain curves of an as-reeled *A. pernyi* silk sample tested in different media are shown in Figure 2a, and the corresponding temperature dependence of tensile storage modulus redrawn from the ref 35 with methanol effect shown schematically. Same legend for both (a) and (b).

![Figure 2](image_url)

Figure 2. (a) Typical stress–strain curves of as-reeeled *A. pernyi* silks tested in methanol, water and control sample in air. (b) Temperature dependence of tensile storage modulus redrawn from the ref 35 with methanol effect shown schematically. Same legend for both (a) and (b).

The stress–strain curve of the adjacent sample immersed in methanol is interesting. As shown in Figure 2a, the curve follows the tensile curve of the dry state in the initial linear elastic region before yield, so the modulus—temperature curve in methanol overlaps that in air before $T_g$ (Figure 2b), which indicates that methanol has hardly penetrated into the silk fiber in this stage. However, as soon as methanol penetrates into the silk fiber after yield, it reduces the $T_g$ value, so the curve deviates from the tensile curve of the dry state (Figure 2a) with the modulus decreases sharply (Figure 2b). Eventually, methanol reaches the similar plasticization as water so that the tensile curve moves across to the same curve as that of the wet state, with a large increase in plastic strain (shown schematically in Figure 2b).

Based upon this premise, water (smallest molecule), methanol (medium molecule), and ethanol (largest molecule) were chosen as softening agents that can only soften the protein chains in the amorphous regions to investigate the structural variability in the amorphous regions of as-reeeled *A. pernyi* silks from different silkworms, as described in the next section.

3.2. Tensile Performance with Solvents of Different Molecular Sizes. Figure 3 shows the tensile performance of as-reeeled *A. pernyi* silks from 4 different silkworms immersed in water, methanol, and ethanol, as well as in air for control. We see that (i) the four different silkworms show considerable variability in their conventional stress–strain response in air (see Figure S2 for a direct comparison), which suggests variability in their structure (ii) polar solvents with different molecular sizes affect the tensile performance of as-reeeled *A. pernyi* silk to different degrees and (iii) silk fibers from different silkworms show different tensile performance in the same solvent, and this becomes more significant with the increase of molecular size. Besides, it should be noted that the stress–strain curves of samples from the same silkworm are highly consistent.

The stress–strain curves in water are quite similar for all four samples, which all show a large decrease in initial elastic modulus but a noticeable increase in extensibility. Also, the moduli in the wet state are all nearly the same as the post-yield dry moduli of as-reeeled *A. pernyi* silks tested in air, as noted in previous work.

On exposure to methanol, the stress–strain curves of samples from silkworms 1, 2, and 3 all follow the tensile curves of the dry state in the initial linear elastic region, and then move across to the same curves as the wet state after yield. However, the stress–strain curve of silk fiber from silkworm 4 tested in methanol is quite different from the other three samples: although the stress–strain curve also follows the tensile curve of the dry state in the initial linear elastic region, it only partially shifts toward that for water and then runs parallel at higher stress (Figure 3d). This suggests that methanol is not able to penetrate into some regions that are accessible to water, even after yield.

As for ethanol, it has been reported to stiffen the *B. mori* silks by a desiccating mechanism, or by changing the structure of the disordered regions and enhance the hydrogen bonding between protein chains. Consequently, the initial elastic modulus and yield stress of silk immersed in ethanol are usually higher than those tested in air. However, after the yield point, the curves differ remarkably from one another. Samples from silkworms 3 and 4 are similar to the stress–strain curves in a dry state, but shifted to higher post-yield stress, which suggests that these two silks are immune to ethanol, even after yield. Samples from silkworm 1, nevertheless, behave in a similar way.
to those with methanol and have a post-yield approximate plateau followed by a “strain-hardening” region, which suggests that ethanol can penetrate into the silk fiber after yield. Samples from silkworm 2 have intermediate post-yield response.

We also examined the effect of immersion time on the mechanical properties of as-reeled A. pernyi silk in the three solvents, as shown in Figure 4a and b for initial elastic modulus and yield stress, respectively. When submerged in water, the stress–strain curve shows an observable yield point at very low strain (inset in Figure S3), and the elastic modulus and yield stress remain constant with increasing immersion time. With methanol (see also Figure S3), the elastic modulus and yield stress gradually decrease with increasing immersion time and remain unchanged, with immersion times longer than 15 h. Moreover, we also notice that the initial elastic modulus at long times in methanol are significantly higher than those in water, which suggests that fractions of the amorphous regions in the silk have not been affected by methanol at low strain (becoming rubberlike) but they are accessible to water. As for the stress–strain curves in ethanol (Figure S4), the initial modulus and yield stress are slightly decreased with increasing immersion time and they are still higher than those of the control sample in air.

To summarize these observations, water has the greatest effect on softening the silk, it permeates the silk very fast even at low strain, and the very low yield point suggests that all the amorphous fraction of silk has been transformed to a rubberlike state. Methanol permeates the silk more slowly than water at low strain below yield and the softening behavior of methanol is also weaker than that of water at low strain, which implies only a small fraction of the amorphous regions have been affected. Methanol has a significant softening effect through yield, and most of the amorphous fraction is usually affected. Ethanol does not normally permeate the silk without yield, and even then only occasionally does yield appear to initiate permeation of ethanol into the silk. Furthermore, observation of the surface morphology of as-reeled A. pernyi silk fiber before and after immersion in three solvents showed that the sericin coating

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**Figure 3.** Typical stress–strain curves of as-reeled A. pernyi silks from silkworms 1 (a), 2 (b), 3 (c) and 4 (d) submerged in ethanol, methanol, and water for 20 min and control sample in air. Legend is the same for (a), (b), (c), and (d).

**Figure 4.** Change of initial modulus (a) and yield stress (b) of as-reeled A. pernyi silks from silkworm 1 submerged in ethanol, methanol, and water for varying times and control sample in air. Legend is the same for both (a) and (b).
remained intact during solvent treatment, which excludes the possibility that sericin contributes to the change in mechanical properties of fibers in the experiments. This is consistent with the observation made by Fu et al.33

3.3. Structural Changes Due to Solvent Immersion. X-ray diffraction (XRD) and Raman spectroscopy were used to study the effect of solvents on the structure of A. pernyi silk. The X-ray diffraction pattern (Figure 5a) of native A. pernyi silk nonwoven mat after immersion in water for 72 h shows that the diffraction peak at \(2\theta = 11.95^\circ\) (lattice spacing, \(d = 0.740 \text{ nm}\)) assigned to \(\alpha\)-helix structure39 (included in the amorphous regions) almost disappears. This is consistent with the Raman spectra results (Figure 5b) that the characteristic peaks of \(\alpha\)-helix structure40 at 1658 and 1105 cm\(^{-1}\) also nearly disappear after water treatment. The \(\alpha\)-helix structure in the as-reeled A. pernyi silk fiber has been considered to be the remnant part of those originally existing in the spinning dope and failed to be converted to \(\beta\)-sheet structure during quick drying of the silk fiber in air.\(^{33}\) Thus, we confirm that water can penetrate into \(\alpha\)-helix domains in the amorphous regions of as-reeled A. pernyi silk.

Conversely, the XRD patterns (Figure 5a) of native A. pernyi silk nonwoven mat and the Raman spectra (Figure 5c and 5d) of as-reeled A. pernyi silk after immersion in methanol and ethanol for 72 h are quite similar to those of the control sample, which indicates that methanol and ethanol are hardly able to penetrate into the \(\alpha\)-helix domains without strain. However, the \(\beta\)-sheet structure\(^{33}\) of as-reeled A. pernyi silk remains unchanged after all solvents treatment. These results agree with previous results that solvent primarily penetrates into relatively disordered regions, while the well-defined crystalline domains remain rigid and intact although the orientation of the crystallites may be influenced in some cases.\(^{41-45}\)

3.4. Correlations between Solvent Molecular Size and Accessible Volume in the Silk. As mentioned above, the polar solvents used in this investigation have significantly different effects on the tensile performance and structure of as-reeled A. pernyi silk. Moreover, the solvents used in our work only affect the protein chains in the amorphous regions, rather than those in the well-defined crystalline domains. Therefore, using the conventional theory that molecular diffusion is controlled by the free volume in the polymer and molecular size of the diffusing molecules,\(^{46}\) it is interesting to quantitatively relate solvent molecular size with the accessible volume in the amorphous regions before and after the yield point.

Figure 6 shows the predicted correlations between accessible volume in the silk fiber and solvent radius, where we see how the volume accessible to the solvent molecules decreases as the solvent radius increases. Four values of density of a model amorphous cell of silk were selected as being representative of...
those to be expected in silk with different degrees of hydrogen-bonded packing. In particular, the density 1.30 g/cm$^3$ is taken to be that of an undeformed silk, and 1.25 g/cm$^3$ is approximately that of a silk at its yield point. The lower values of density are for high strain values and some highly disordered structures.

Figure 6 indicates that water, as the smallest molecule used here, can insinuate itself into the silk structure quickly under all conditions. Methanol has intermediate size and is just at the point of having solvent accessible volume available. In particular, volume becomes available quickly at lower densities above yield or in more disordered structures. Ethanol does not normally permeate the silk without significant strain above yield or highly disordered structures (more typical of regenerated silk structures than native silks), but permeation is still possible under more extreme conditions.

Considering the different responses of A. pernyi silk fibers from different silkworms to the same solvent, especially in methanol or ethanol, we can infer that the free volume in the amorphous regions varies from silkworm to silkworm, which may arise from a distribution of different degrees of disorder and secondary structure forms of molecular chains in the amorphous regions. The molecular chains in the amorphous regions of silk fibers from silkworm 1 may be more disordered (a lower limit), which leads to larger free volume in the amorphous regions, so solvents with larger molecular size (ethanol) become accessible after yield, but it still cannot permeate the silk fiber without yield. The molecular chains of silk fibers from silkworm 4 may be more ordered (a higher limit), methanol becomes partially accessible after yield and ethanol cannot permeate these silk fibers; even they are stretched after yield. However, the other silk fiber samples show intermediate degrees of disorder of molecular chains in the amorphous regions.

It is important to note that the mechanical properties of the silk in the conventional dry state stress-strain experiments are linked to the free volume effects shown in the solvent tests. The higher strain to failure at approximately constant stress to failure with higher free volume gives considerably higher toughness (energy to failure) in silk worm 1 (222 ± 27 MJ m$^{-3}$) that decreases down to silkworm 4 (138 ± 21 MJ m$^{-3}$) with lower free volume, which is a key property of natural silks.

The experimental observations presented here on solvent effects upon mechanical properties, the rather simplified model analysis of solvent accessibility, and previous quantitative models for the effect of water upon silk thermomechanical properties suggest that a more quantitative molecular level model for solvent activity may be formulated. This will allow details about secondary structure variability and distributions in different silks to be deduced from experimental measurement of the kind presented here. In particular, the free volume associated with secondary structural types such as different helical forms ($\alpha$, $\beta$-sheet, $\beta$-helix, for example) and random coil will need to be tested, along with their relative change in length through extension, which may help explain the different strain to failure values.

## CONCLUSIONS

We have investigated the effect of polar solvents with different molecular sizes on the tensile properties and structure of as-reeled A. pernyi silk. Polar solvent with smaller molecular size is more invasive to silk compared with those with larger molecular size. The link between solvent molecular size and the accessible volume in the amorphous regions before and after the yield point has been simulated quantitatively, which indicates that the volume accessible to the solvent molecules decreases as the solvent radius increases. In this study, silks from different silkworms are used to highlight structural variability possible in as-reeled A. pernyi silks. Silks with more ordered structure in the amorphous regions are less sensitive to solvents than those with more disordered structure in the amorphous regions. Silks with higher free volume have higher strain to failure and the key property of toughness. The interaction between polar solvent and silk is a powerful approach that can be used to identify slight differences in structures of natural silks. This work provides valuable information that using polar solvents with appropriate molecular size can unravel the microstructural feature of natural proteins and synthetic polymers.

## ASSOCIATED CONTENT

### Supporting Information

The change of stress-strain curves with immersion in methanol or ethanol for different times and control samples tested in air and water. This material is available free of charge via the Internet at http://pubs.acs.org.

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