Effect of Solvent on Ionic Liquid Dissolved Regenerated *Antheraea assamensis* Silk Fibroin

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ABSTRACT: We report on the dissolution of semi-domestic silk type *Antheraea assamensis* using ionic liquids. We investigated the impact of different coagulating solvents, including isopropanol and water on the structure and the morphology of the regenerated silk. We found that the water regenerated silk film showed a high β-sheet content and a native silk-like XRD pattern. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 128: 4411–4416, 2013

INTRODUCTION

Silk has been used for textile applications since ancient times due to its fineness and luster.[1] The scientific interest in silk, as an important biomaterial, has been generated by silks excellent mechanical properties and biocompatibility.[2–5] Among the different silk producing insects, Bombycidae and Saturniidae families of lepidoptera larvas have commercial significance. Silk from Saturniidae families, commonly referred to as non-mulberry silk, is produced for the textile industry and therefore offers a good source for generating silk-based biomaterials. Non-mulberry silk from Saturniidae family are generally wild or semi-domestic in nature with higher components of amino acids with polar, bulky, basic, and hydrophilic side chains when compared to the domestic mulberry silk *Bombyx mori*.[6–10] Studies suggest that the fibroin from non-mulberry silk is more bioactive than *B. mori*.[11,12]

To date, only limited work on non-mulberry silk has been reported.[13–18] Important commercial varieties of *Saturniidae* silk such as *Antheraea assamensis*, used in this study, have been largely unexplored. Common solvents used for dissolving domestic silk *B. mori* usually cannot dissolve a non-mulberry silk fiber.[19,20] The dissolution of non-mulberry silk using $Ca(NO_3)_2$ melt at $105°C$[21,22] and LiSCN at $40–55°C$ has been reported.[19,23–26] However, solubility is only partial. As such, silk biomaterials of non-mulberry silk fibroin have been mostly prepared from silk protein extracted from glands of silkworms[27–32] which has problems associated with scale up and ethical issues. Total dissolution of fibers using less toxic solvents remains a major challenge for future development of these highly biocompatible non-mulberry varieties of silk.

An important advantage of ionic liquids (ILs) is their green chemistry.[33,34] ILs are taking center stage in green chemistry applications, with one application is in the processing of biopolymers.[35,36] ILs have been shown to be considerably greener than alternative methods, which often required harsh volatile solvents and multiple steps. Apart from harmful reagents used for dissolving silk, fluorinated alcohol or formic acid is needed for the preparation of reconstituted silk dope. However, ILs can produce silk dope directly from fibers without needing any harmful chemicals or the need for a dialysis step which is time consuming.[37] Therefore, the use of ILs as the dissolution solvent simplifies this process considerably. Additionally, both degummed or undegummed fibers can be directly dissolved, and the dope can be used to prepare various silk material formats (films, powders mats, or gels, etc.). It has previously been shown that domestic silk, *B. mori*, cocoons can be directly dissolved into either 1-butyl-3-methylimidazolium chloride, BMIMCl or 1-butyl-3-methylimidazolium acetate, BMIMAC at concentrations around 10 wt %.[37–39] To the best of our knowledge no work has been reported on the use off ILs for non-mulberry silk dissolution and regeneration.

The role of the coagulating solvent is also an important consideration when regenerating silk. Often, regenerated silk prepared using conventional chemicals is more brittle than native silk. It has been shown that the processing parameters play an important role in the final properties of the regenerated silk. Recently, we showed that protic ILs could serve as new regenerating solvents resulting in regenerated silk with tuneable properties.[40] Any advantage that can be achieved by manipulating fiber

Additional Supporting Information may be found in the online version of this article.

structure to obtain tough but flexible products may have an important impact on silk biomaterial research. In this work, we have used a combination of Fourier transform infrared (FTIR), X-ray diffraction (XRD), differential scanning calorimeter (DSC), thermogravimetric analysis (TGA), and scanning electron microscope (SEM) to investigate the effect different organic solvents, as the regenerating solvent, have on the structure and morphology of IL dissolved non-mulberry silk. Additionally, we have investigated the use of water as a coagulating solvent.

EXPERIMENTAL

Preparation of Regenerated Silk

Wild-type silk cocoons were dissolved at 120°C upto a concentration of 10 wt % in ILs, BMIMCl (purchased from Sigma Aldrich, Sydney, Australia) and BMIMAc, prepared according to literature.[41] In both cases, the IL was dried overnight under vacuum at 80°C, the silk was also dried at 60°C for 2 h. The silk was added stepwise in increments of 1 wt % under a nitrogen atmosphere.

The regenerated silk films were prepared by pouring the IL/silk solution into a Teflon cast. Submerging the Teflon cast in the chosen coagulation solvent, the film was then placed in a bath of the same coagulation solvent for 5 min. The coagulation liquid was replaced several times within the 5 min immersion.

Characterization

Infrared spectra were recorded with an attenuated total reflectance FTIR spectrophotometer (Vertex 70, Bruker Biosciences Pty, Australia). Each spectrum was obtained in absorbance mode in the range of 4000–600 cm^{-1}. To measure different conformations, an average of three spectra in the amide I mode (1595–1705 cm^{-1}) were deconvoluted, and curve was fitted using OPUS 5.5 software adapting the procedure used by Hu et al.[42] with some modifications. Deconvolution was carried out adapting a Lorentzian model using a bandwidth of 25 cm^{-1} and a noise reduction factor of 0.3. A straight base line correction of FSD spectrum was performed followed by curve fitting using a Gaussian model. Band positions were determined according to the second derivatives of the original spectra, and were automatically adjusted by the autofit program using Local Least Square followed by Levenberg–Marquardt algorithms. Finally, each individual spectrum was area normalized to obtain percentage conformations within the amide I region. The procedure was repeated three times for each spectrum. Results were statistically analyzed using two tailed Student's t-tests at 95% confidence level to compare the sample means wherever applicable. A statistically significant difference was considered if $P < 0.05$.

DSC results were obtained using a TA Q200 at a scanning rate of 10°C/min. TGA results were obtained using a Netzsch DSC/TGA at a rate of 10°C/min.

Wide angle X-ray scattering (WAXS) of films was performed on a diffractometer (X'Pert PRO from PANalytical) with Cu Kα radiation (λ = 0.154 nm), operated at 40 kV and 30 mA. Scanning rate was 0.02°/min. Background scattering was removed using Spectra Xpert High Score plus software.

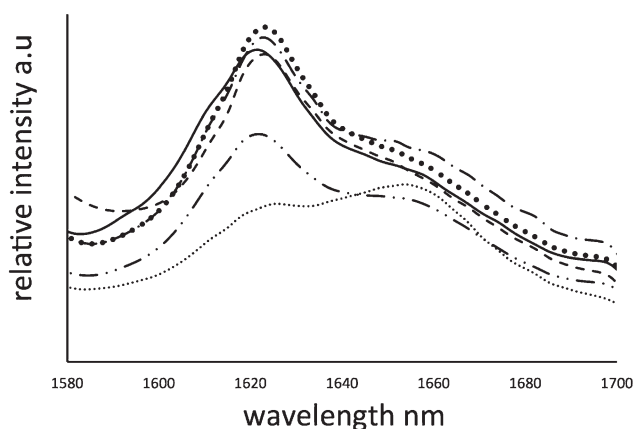Scanning electron microscope was performed using a Jeol 1100. Films were sputter gold coated.

Figure 1. Amide 1 region of silk films regenerated using isopropanol (solid black line), methanol (line and 1 dash), ethanol (dots, small), water (dash), 50% isopropanol : 50% methanol (line and 2 dash) and 50% isopropanol : 50% ethanol (dots, large).

RESULTS AND DISCUSSION

The dissolution of *A. assamensis* silk was examined using both BMIMCl and BMIMAc at 120°C. It was found that 12.2 wt % of the native fiber could be dissolved in BMIMCl. While 10.14 wt % of the native fiber could be dissolved in BMIMAc. Due to the lower melting point of BMIMAc the solution containing 10 wt % dissolved *A. assamensis* native fiber was considerably less viscous and easier to handle at room temperature when compared to the same wt % dissolved in BMIMCl. Therefore, to investigate the impact of the coagulating solvent on the regenerated films structure and morphology, we decided to cast films using BMIMAc at 10 wt % dissolved *A. assamensis* native fiber.

Figure 1 shows the amide I region for the silk films regenerated using the different coagulating solvents used in this study. The coagulating solvents used include water, methanol, ethanol, isopropanol, and mixtures thereof. The amide I region identifies different secondary structures of the silk according to the following assignments, 1605–1618 cm^{-1} is tyrosine side chain, 1619–1635 cm^{-1}, 1693–1700 cm^{-1} are for β-sheet, 1635–1654 cm^{-1} is random coil, 1655–1664 cm^{-1} is α-helix and 1665–1692 cm^{-1} is β-turns.[42,43] Methanol is routinely used as a coagulating solvent and is known to induce β-sheet crystallinity for films prepared from water-based or organic-based *B. mori* silk dope solution.[44] It can be seen from Figure 1 that indeed the IL-based silk film coagulated from methanol has a peak at 1620 cm^{-1} indicating β-sheet, however from the deconvolution (Table I) considerable helical content is also present. Ethanol has also been used in the past as a stabilizing solvent particularly for non-mulberry silk. Ethanol is known to promote β-sheet at prolonged soaking times and usually at an ethanol concentration of 70 wt % for *B. mori* silk dope. From Figure 1 it can be seen that the silk film regenerated from 100% ethanol, under the conditions used in this study, results in a film with both β-sheet and α-helix content as supported by the clear peak at 1660 cm^{-1} which is indicative of α-helix. Isopropanol as a coagulating solvent for silk has not been previously reported, from Figure 1 it can be seen that isopropanol promotes β-sheet

Table I. Deconvolution of the FTIR Data (Mean ± Standard Deviation)

	Side chain	β-sheet	Random coil	Alpha	Turns
Methanol	3.74 ± 0.01	41.47 ± 0.04	8.82 ± 0.01	33.69 ± 0.07	12.29 ± 0.03
Isopropanol–ethanol	6.06 ± 0.06	55.31 ± 0.09	7.11 ± 0.02	19.34 ± 0.04	12.18 ± 0.01
Isopropanol–methanol	10.33 ± 0.05	50.372 ± 0.12	11.01 ± 0.07	14.24 ± 0.03	14.04 ± 0.05
Isopropanol	9.70 ± 0.08	51.14 ± 0.21	9.90 ± 0.03	11.63 ± 0.01	17.62 ± 0.08
Ethanol	6.78 ± 0.04	42.693 ± 0.23	15.14 ± 0.09	18.34 ± 0.10	17.04 ± 0.02
Water	3.52 ± 0.01	50.87 ± 0.07	11.09 ± 0.08	31.67 ± 0.13	2.84 ± 0.01
Native	0.82 ± 0.02	58.17 ± 0.21	19.7 ± 0.12	12.2 ± 0.06	9.07 ± 0.07

formation with a clear peak at 1620 cm^{-1} observed, and according to the deconvolution, the β-sheet content is 51%. We also investigated water as a coagulating solvent and again from Figure 1, it can be seen that water regenerated silk has clear β-sheet structure. The deconvolution of the water regenerated peak indicates a β-sheet content of 50.8%. Native silk fiber has a β-sheet content of 58%. It has previously been reported that water vapor[45] induces β-sheet in regenerated *B. mori* silk, this is the first report of directly using water as a coagulating solvent to regenerate silk. The use of mixtures containing either 50 : 50 isopropanol : methanol or isopropanol : ethanol as the coagulating solvents both showed predominately β-sheet structure. The deconvolution for all films is shown in Table I. Differences in β-sheet contents between the films stabilized by isopropanol, isopropanol–methanol, and water are not significant based on pair wise Students's t-test ($P > 0.05$). Other differences in β-sheet contents are statistically significant. Films regenerated by different solvents and their combinations also vary significantly from each other in their random coil, α-helix, and turns based on Student' t-tests ($P < 0.05$). Usually all non β-sheet contents are grouped under non-crystalline domains and often the distinction between random coil, α-helix, and β-turns are more complicated and there is no clear agreement between the reported studies.[42,46] However, according to the assignments used here a notable difference is that the low amount of side chain contribution in the native silk is found to be 0.82. In contrast, the regenerated films all have side chain contributions ranging from 3.5% for the water regenerated silk film to 10.3% for the isopropanol/methanol regenerated silk film. This suggests that the regenerated films have a reduced amount of ordered β-sheet crystals when compared to native silk fibers.

Figure 2(a,b) shows the DSC and TGA traces, respectively, of the regenerated films using the different coagulating solvents. The DSC trace of all films shows two endothermic peaks occurring at 300°C and 360°C. The absence of any exothermic peak implies that the films regenerated are in the crystalline state. It has been reported[22] that the 300°C peak corresponds to the melting/degradation of disorientated β-sheet while the higher 360°C peaks corresponds to the melting/degradation of β-sheet crystals. The film regenerated with isopropanol has a clear peak at 360°C when compared to the other films. The film regenerated with methanol shows no clear peak at 360°C, suggesting that little to no ordered β-sheet crystals are present. The film regenerated with the methanol/isopropanol has a small peak at

360°C suggesting that some ordered β-sheets are present in the methanol/isopropanol coagulated silk film. This indicates that isopropanol is important in inducing ordered β-sheet. Figure 2(b) shows the TGA traces for each film. It can be seen that no appreciable difference is observed with all films showing the commencement of decomposition at 230°C, this temperature is lower than the melting/degradation temperature detected by the DSC.

Figure 3 shows the XRD spectrum for the IL dissolved *A. assamensis* silk films stabilized using isopropanol, methanol, ethanol, and water. Included in this figure is the XRD pattern for native *A. assamensis* type fiber. The spectrum for native silk fiber has two characteristic peaks at 17.5° and 20°. The films
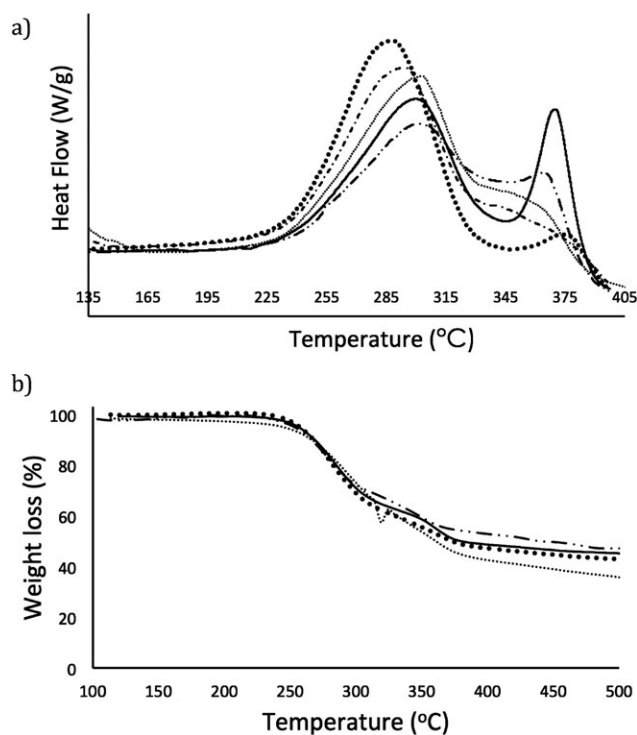

Figure 2. (a) DSC traces and (b)TGA traces of silk films regenerated using isopropanol (solid black line), methanol (line and 1 dash), ethanol (dots, small), 50% isopropanol : 50% methanol (line and 2 dash), and 50% isopropanol : 50% ethanol (dots, large).
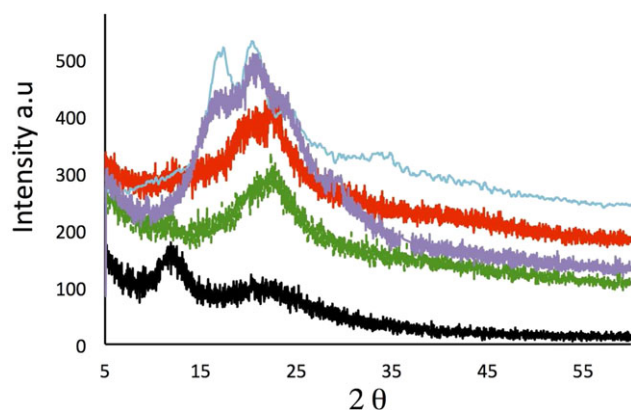
Figure 3. XRD pattern of silk films regenerated using isopropanol (black line), methanol (red line), ethanol (green line), and water (purple). The blue curve is native *A. Assamensis*. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

regenerated using methanol show two peaks, however these peaks are shifted to 20° and 22°. A possible explanation for this shift could be the presence of some residual IL within the film. The film regenerated with ethanol shows a similar spectrum to the methanol film. Interestingly, the film with isopropanol shows a clear sharp peak at 11.75°, with a broader peak at 21°. The peak at 11.75° corresponds to helical structures and is often

difficult to detect using powder XRD on films so the detection of this peak for the isopropanol film was a surprising outcome. Given the evidence of the helical peak in the isopropanol film, we tested the film to see whether it was water soluble. We found that the film was not water soluble, which indicates that sufficient β-sheet is present to render the film water insoluble. All films were found to be water insoluble including the film regenerated using water. The water regenerated silk film shows an XRD pattern most similar to the native pattern, with two peaks occurring at 16.75° and 20°. This is likely due to complete removal of the BMIMAc, since this IL is water soluble. This may have allowed the films to self-assemble in a manner that enhanced the formation of ordered β-sheet, certainty from the FTIR deconvolution the water regenerated film showed the least side chain contribution.

Finally, we investigated the effect the coagulating solvent had on the morphology of the regenerated silk films. Figure 4(a–d) shows the SEM images of the IL dissolved *A. assamensis* type silk films regenerated using methanol, ethanol, isopropanol, and water. The morphology appears to be dependent on solvent and time. The methanol and ethanol regenerated films [Figure 4(a,b)] have a rough morphology with voids present. Whereas, the isopropanol and water regenerated film [Figure 4(c)] have a smooth flat void-free surface. This maybe dominated by the diffusion of the IL from the silk film, as we saw a time dependency, will all films showing a higher degree of porosity at
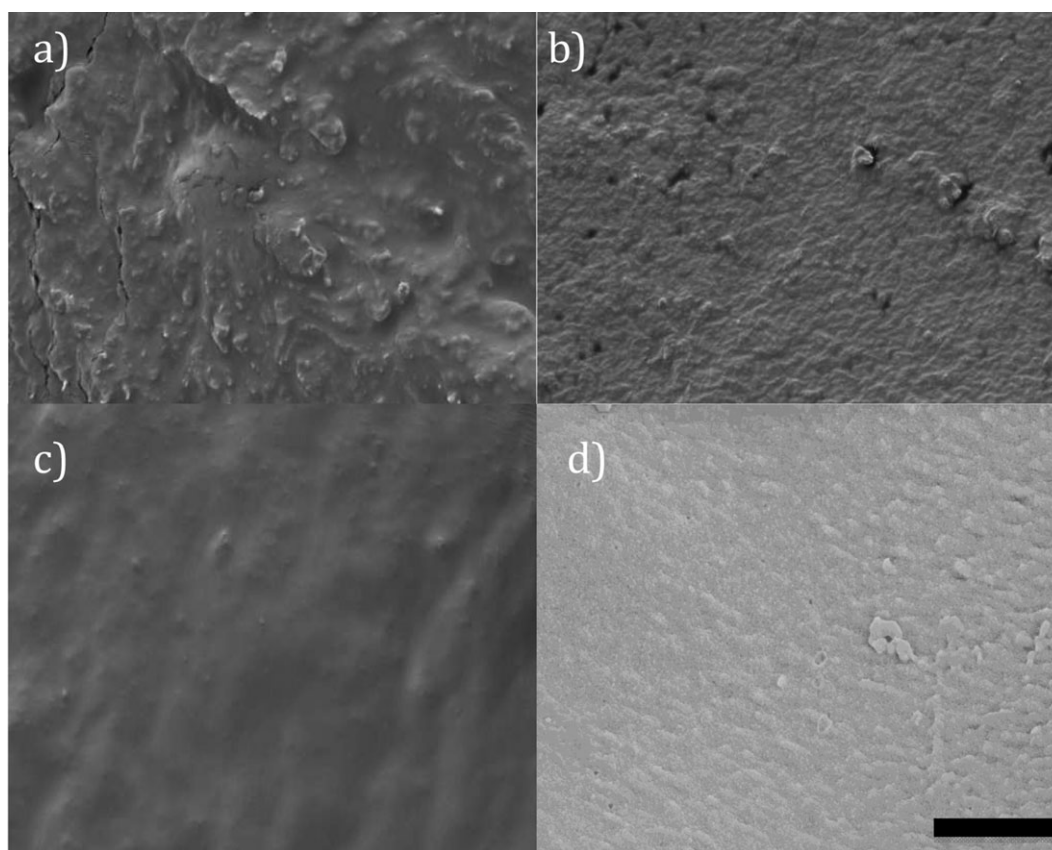

Figure 4. SEM images of IL dissolved silk, regenerated using (a) methanol, (b) ethanol, and (c) isopropanol, and (d) water. Scale 10 μM.

shorter immersions times (Supporting Information Figure 1). BMIMAc is a polar solvent[47] and may have different solubilities in the organic solvents used here.[48] This may impact the porosity of the regenerated films, however, the biggest impact on film porosity was seen to be immersion time.

CONCLUSIONS

We report for the first time on the dissolution of non mulberry *A. assamensis* type silk using ILs. We then investigated the impact the coagulating solvent had on the structure, morphology, and the thermal properties of the regenerated silk films. We reported on the use of isopropanol and water as new solvent for silk film regeneration. We showed that both water and isopropanol stabilized β-sheet. The use of water as a coagulating solvent for IL dissolved silk suggests that the dissolved state is different to the traditional dissolution methods, since traditionally prepared silk dope is water soluble.

Interestingly, the water regenerated silk film showed an XRD pattern most native like suggesting that water is a very good coagulating solvent for IL dissolved silk. The ability to use water as a coagulating solvent over organic solvents is an important finding and presents an environmentally friendly route for the creation of silk-based biomaterials. Future work will be aimed at investigating differences in the traditionally prepared silk dope and the IL prepared silk dope.

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