Dissolution of Biopolymers Using Ionic Liquids

Robert A. Mantz[a], Douglas M. Fox[b], J. Marshall Green III[c], Paul A. Fylstra[c], Hugh C. De Long[d], and Paul C. Trulove[c]

[a] Army Research Office, Durham, NC 27703, USA
[b] American University, Washington DC 20016, USA
[c] U.S. Naval Academy Department of Chemistry, Annapolis, MD 21402, USA
[d] Air Force Office of Scientific Research, Chemistry and Life Sciences Directorate, Arlington, VA 22203, USA

Reprint requests to Dr. R. A. M.; E-mail: robert.a.mantz@us.army.mil

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Ionic liquids represent a unique class of solvents that offer unprecedented versatility and tunability. Nature has developed a wide variety of materials based upon both proteins and polysaccharides. Many of these materials have unique properties that are a function not only of the material identity but are also largely dictated by processing conditions. Recent work has shown the potential of ionic liquids as solvents for the dissolution and processing of biopolymers. In this research we have expanded upon the limited data available to date using several biopolymers including: silk, chitin, collagen and elastin.

Key words: Ionic Liquids; Silk; Biopolymers; Solubility; Chitin; Collagen; Elastin.

1. Introduction

Nature has developed a wide variety of materials based upon both proteins and polysaccharides including silks, chitin, cellulose, and elastin, to name a few. These materials have unique properties that have not been duplicated with currently available synthetic polymers. Natural silks have mechanical properties that compete with the best synthetic polymers [1]. In order to tailor silk for particular applications, alternative processing methodologies are desirable. The actual dissolution of silkworm silk is a formidable task [2, 3]. Many of the process limitations result from our inability to prepare stable silk solutions.

Ionic liquids represent a unique class of solvents that offer unprecedented versatility and tunability [4, 5]. Recent work has clearly shown the potential of ionic liquids as solvents for the dissolution and processing of biopolymers [6 – 9].

In this work we present continued studies of the solubilization of *Bombyx mori* silk, as well as the solubilization of chitin, collagen and elastin. We have also explored processing conditions to reconstitute biopolymers from ionic liquids.

2. Experimental

2.1. Materials

The preparation and purification of all ionic liquids and $AlCl_3$ were performed as described in [10, 11]. Collagen type I from bovine Achilles tendon, elastin powder from bovine neck ligament, 20 μm powdered microcrystalline cellulose, and crab shell chitin were used as received from Sigma-Aldrich Co.

The cocoon silk used in these experiments originated from two sources: 1) silkworms grown on a diet of Silkworm Chow (Mulberry Farms, Falbrook, CA); the pupae were extracted from the cocoons intact 2 – 7 d after spinning formation by cutting open the cocoons; 2) silk purchased from Rudolph Desco.

Sericin is a water-soluble biopolymer that is co-extruded with the silk fiber by silk worms. For the purposes of this study it was viewed simply as an impurity. Sericin removal procedures varied depending on the ionic liquid studied. For the $BMIm^+Br^-$, $BMIm^+I^-$, and $BMIm^+BF_4{}^-$ experiments, the sericin remained on the cocoons which were vacuum-dried. For the $BMIm^+Cl^-$ and $BMMIm^+Cl^-$ experiments, the sericin was removed from the silk fibroin in a

0.05 M Na_2CO_3 and 0.5 M Na_2EDTA solution with 9% (w/w) cocoons at 65 °C for 24 h. In all the $EMIm^+$ experiments, the sericin was removed in a 0.2 M Na_2CO_3 solution by boiling for 2 h. These cocoons were rinsed thoroughly and dried at 100 °C in a vacuum oven overnight prior to solubility testing.

2.2. Biopolymer Dissolution

The solubility experiments were conducted under an inert atmosphere of N_2 or He. To quantify the solubility of each biopolymer, small amounts of biopolymer were sequentially added and mechanically stirred until they are completely dissolved. The resultant solutions containing silk and cellulose were clear with an amber color, while those containing chitin, elastin, and collagen were translucent with a yellow color. The solutions were quite viscous and very cohesive when the biopolymer content was above 10 wt%. The temperature of the mixtures during dissolution was maintained with a temperature-controlled oil bath at 100 °C for the $BMIm^+$ ionic liquids and for $BMMIm^+Cl^-$. The temperature of the $EMIm^+$ ionic liquids were maintained with a hot plate and monitored with a NIST calibrated thermometer.

2.3. Silk Regeneration

A 9.5 wt% silk in $BMIm^+Cl^-$ solution at 100 °C was used to cast films on both silicon wafers and glass slides. The $BMIm^+Cl^-$ was removed by rinsing the slides in either acetonitrile or anhydrous methanol. Attempts to rinse the ionic liquid with de-ionized water resulted in dissolution of the silk fibroin.

A 10.4 wt% silk in $EMIm^+Cl^-$ solution was used to generate silk fibroin fibers. 7 wt% de-ionized water was added to the solution to lower its melting point to ca. 50 °C and the resultant solution was transferred to a warmed glass syringe fitted with a pipetting needle. The silk solution was injected into a solvent bath and allowed to soak for 24 h. Methanol, ethanol, 0.10 M citric acid/0.10 M sodium dihydrogen citrate buffer, 0.10 M sodium dihydrogen citrate/0.10 M sodium hydrogen citrate, 0.14 M sodium dihydrogen citrate/0.060 M sodium hydrogen citrate, and de-ionized water were used as reconstituting solvents.

A 5 wt% silk in $BMIm^+Cl^-$ solution was used to produce silk fibers using an electrospinning apparatus. The blend was electrospun at 9 kV with a bath to needle distance of 7 cm (no use of the roller). A glass slide was introduced in the bath and some fibers were recovered for confocal imaging. Other fibers were washed and filtered with methanol for DSC and TGA analysis.

2.4. WAXS

Powder X-ray diffraction experiments were performed on a Phillips Powder diffractometer. The d-spacing was calculated from the peak positions using $Cu(K_\alpha)$ radiation ($\lambda = 0.15418$ nm) and Bragg's law. Silk samples were prepared by taping to a 2 cm · 1.6 cm mold and subtracting the tape background signal. Standard X-ray measurements were performed using a step size of 0.02° and 3 s dwell time.

2.5. Reflectance Optical Microscopy

Digital pictures of the silk fibers were obtained using a Bodelin ProScope instrument with a 30× nonreflective lens.

2.6. Thermal Analysis

Thermal stabilities were measured using a TA Instruments Q-500 thermogravimetric analyzer. (5.0 ± 0.2) mg samples were placed in open platinum pans and heated at a scan rate of 10 °C/min while purged with 100 mL/min N_2 or compressed air. The mean of typically three replicate measurements was reported. The temperature of both the onset (5% mass fraction loss) and peak mass loss rate have an uncertainty of ±2 °C. All samples were held at 90 °C for 1 h prior to each scan to remove any residual water and, in the case of the thermal stability in nitrogen, to remove any residual oxygen from the furnace. Melting and freezing points were determined using a TA Instruments DSC2910 differential scanning calorimeter connected to a refrigerated cooling system. 3.0 mg to 5.0 mg samples were hermetically sealed in aluminum pans and were heated and cooled at a scan rate of 5 °C/min while purged with 100 mL/min N_2. Data were collected during the second consecutive scans.

3. Results

3.1. Solubility of Biopolymers in Ionic Liquids

Bombyx mori silk was dissolved in a series of ionic liquids to determine the effects of the cation alkyl

Table 1. Solubility of silk fibroin in ionic liquids.

Ionic liquid	Solubility (wt%) Silk fibroin	Ionic liquid	Solubility (wt%) Silk fibroin
$EMIm^+Cl^-$	23.3	$EMIm^+BF_4^-$	0.0
$EMIm^+SCN^-$	0.0	$EMIm^+NO_3^-$	0.0
$EMIm^+Tf^-$	0.0	$BMIm^+Cl^-$	13.2
$BMIm^+Br^-$	0.7	$BMIm^+I^-$	0.2
$BMIm^+BF_4^-$	0.0	$BMMIm^+Cl^-$	8.3
MIm^+Cl^-	0.0	$MMIm^+NO_3^-$	0.0
$EtNH_3^+MeSO_4^-$	0.0		

length, the counter anion, and C-2 hydrogen substitution on the solubility of silk. The temperature was maintained just above the melting temperature of $EMIm^+Cl^-$ ($T_{mp} = 87$ °C) to minimize any potential protein degradation. After identifying ionic liquids that were suited to dissolve silk we tested the solubility of other biopolymers in these solvents. Once the most effective ionic liquids were determined, the solubility of several other biopolymers was measured. A summary of the solubility data is shown in Table 1. In addition to the results in Table 1, other biopolymer solubilities were determined in $EMIm^+Cl^-$ including: collagen 1.3%, elastin 6.0%, chitosan < 1%, and chitin 10%. Chitin was also determined to be insoluble in $EMIm^+Br^-$, $EMIm^+BF_4^-$, and $EMIm^+Tf^-$.

The presence of the chloride ion has previously been identified as important in the dissolution of biopolymers [8]. However, the ionic liquids in Table 1 all melt at elevated temperatures which could lead to biopolymer degradation. To further reduce the melting point of the ionic liquid while retaining a high concentration of chloride ions, we prepared several ionic liquid melts: a basic chloroaluminate melt (1.0 : 0.7 $EMIm^+Cl^-/AlCl_4^-$), a saturated tetrafluoroborate melt (0.48 : 0.52 $EMIm^+Cl^-/BF_4^-$), and 2 equimolar chloride imidazolium melts (50 : 50 $EMIm^+/BMIm^+Cl^-$ and 50 : 50 $EMIm^+/BMMIm^+Cl^-$). Neither silk fibroin, collagen, elastin, chitosan, nor chitin showed any solubility in the 1.0 : 0.7 $EMIm^+Cl^-/AlCl_4^-$ melt. The silk fibroin was soluble in: 0.48 : 0.52 $EMIm^+Cl^-/BF_4^-$ 0.7%, 50 : 50 $EMIm^+/BMIm^+Cl^-$ 7.43%, and 50 : 50 $EMIm^+/BMMIm^+Cl^-$ 10.3%.

We also have used water as a cosolvent in the $EMIm^+Cl^-$ ionic liquids as a method to reduce the viscosity of the solution. We have found that the order of addition has a pronounced effect on the silk solubility. In one case we added 8% water prior to silk addition. This mixture was a liquid at 25 °C, and the silk began to dissolve at 65 °C. However, the maximum silk solubility was only 5%. In the second case we added 8% water to an 8% silk solution. There was no sign of precipitation, and the solution appeared to be stable even upon cooling to room temperature.

Table 2. Solubility of silk fibroin in amino acid-based ionic liquids.

Ionic liquid	Solubility (wt%)	Ionic liquid	Solubility (wt%)
$EMIm^+Gly^-$	26.3	$EMIm^+Ala^-$	> 20
$EMIm^+Ser^-$	> 20	$(n\text{-}Bu)_4N^+Gly^-$	0.0

Table 3. Effect of the reconstituting solvent for $EMIm^+Cl^-$ silk solutions.

Solvent	pH calcd.	pH measured	Precipitate	Single fiber
Methanol			Y	Y
Ethanol			Y	N
De-ionized water	7.00	5.98	N	N
0.10 M H_3Ct – 0.10 M NaH_2Ct	3.13	2.96	N	N
0.10 M NaH_2Ct – 0.10 M Na_2HCt	4.77	4.29	Y	N
0.14 M NaH_2Ct – 0.060 M Na_2HCt	4.40	4.05	Y	N

Fig. 1. ProScope image (50X) of silk regenerated from a 0.10 M H_3Ct – 0.10 M NaH_2Ct aqueous buffer solution.

Recently, Ohno's research group has prepared ionic liquids with amino acid-based ions. 80% of the protein sequence comprising silk are the three amino acids, glycine, serine, and alanine [12]. To help facilitate the formation of amorphous regions within the reconstituted silk and to minimize the presence of inorganic anions in the reconstituted biopolymer, we investigated the use of ionic liquids with the anions of glycine, serine, and alanine to dissolve and reconstitute the biopolymers. The solubility results for silk in these amino acid-based ionic liquids are shown in Table 2.

3.2. Regeneration and Characterization of Silk

The silk fibers regenerated from $EMIm^+Cl^-$ solutions exhibited different structures depending on the reconstituting solvent bath. Table 3 summarizes the usefulness of the reconstituting solvent on extracting silk from the ionic liquid solution. An image of

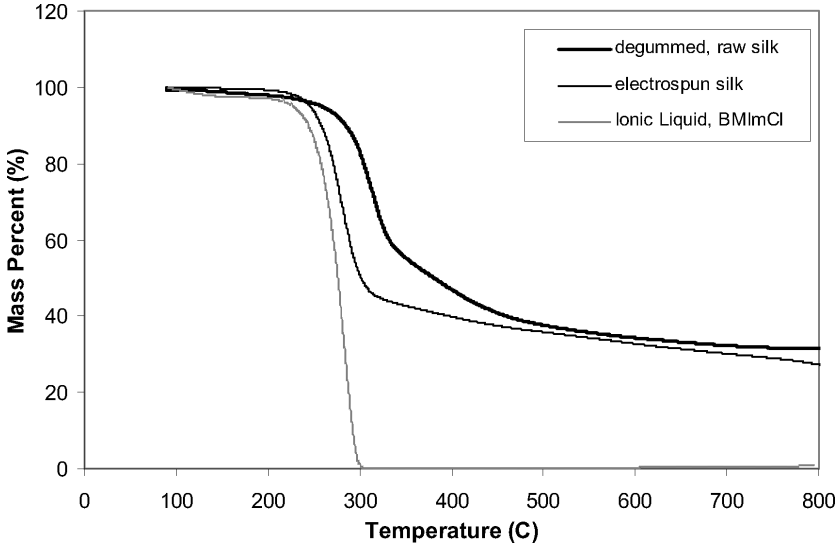

Fig. 2. Thermal stability of native silk and regenerated electrospun silk at 10 °C/min under nitrogen.

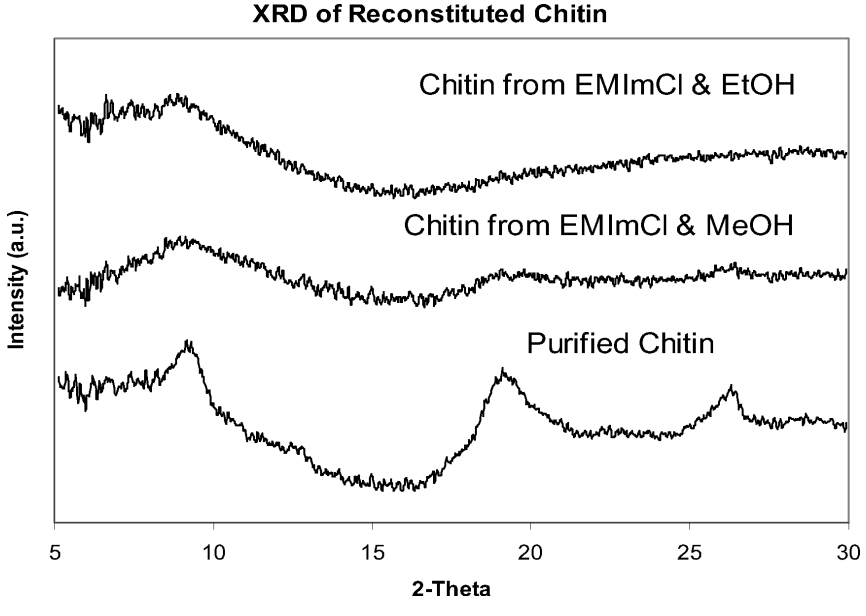

Fig. 3. XRD pattern of native chitin and chitin reconstituted from methanol and ethanol. The chitin reconstituted from methanol exhibits crystallinity more similar to the native material.

the fibrous precipitate from the citric acid/sodium dihydrogen citrate buffer solution is pictured in Figure 1.

The silk fibers electrospun from $BMIm^+Cl^-$ solutions using a methanol reconstitution bath were analyzed using thermal gravimetric analysis (TGA) and differential scanning calorimetry (DSC). In Fig. 2 the TGA curves of native silk, electrospun silk fibers, and silk fibers reconstituted from $BMIm^+Cl^-$ are compared.

3.3. Regeneration and Characterization of Chitin

Chitin dissolution differed from the other biopolymers that were studied in that the kinetics of dissolution was very slow, i. e. generally silk dissolves in a few hours while chitin can take days. At this time we have no explanation for the slow dissolution kinetics. Future studies will address these differences. Similarly to silk fibroin, $EMIm^+Cl^-$ was the most effective solvent and methanol is an excellent coagulation bath. Unlike silk

fibroin, water functions as a coagulation bath for chitin. In Fig. 3 the XRD pattern of the native and chitin samples reconstituted from methanol and ethanol are compared.

4. Discussion

4.1. Dissolution of Biopolymers

The solubility of silk fibroin in ionic liquids is dependent upon the ability of the ions (both cations and anions) to disrupt in hydrogen bonding, with the anion having a much more significant effect. Previously the solution of silk/$BMIm^+Cl^-$ has been shown to be amorphous, lacking β-sheet structure [8]. In addition, the absence of peaks associated with $BMIm^+Cl^-$ crystallinity suggests that there are strong interactions between the $BMIm^+Cl^-$ and silk molecules. Furthermore, there is virtually no solubility in ionic liquids with weaker H-bonding anions and cations. These data suggest that the solubilization is dependent upon the ionic liquid's ability to disrupt the hydrogen bonding present in the β-sheet structure of the silk.

We chose to use $EMIm^+Cl^-$ to explore the solubility of other biopolymers because it has the greatest H-bonding ability of all the ionic liquids used in this study. The solubility of microcrystalline cellulose in $EMIm^+Cl^-$ is comparable to the solubility in $BMIm^+Cl^-$, suggesting that H-bonding of the cation plays a less important role in solubilizing cellulose than silk. The complete lack of silk solubility in Br^- and SCN^- ionic liquids supports this. Chitin has a structure similar to cellulose, so its solubility in $EMIm^+Cl^-$ should be comparable to its solubility in other short chain dialkylimidazolium chlorides. Chitin is like cellulose, except that there is an acetylated amino group instead of a hydroxy group at the C-2 position. The reduction in solubility upon the reduction in the number of hydroxy groups of the biopolymer supports the presumption that cellulose is solubilized through the hydrogen bonding of the hydroxy groups to the anions of the ionic liquids [6].

Collagen is rich in the amino acid glycine and forms supermolecular helixes like silk. Thus, it is likely that cation H-bonding will play a more important role in its solubility. The insolubility of collagen is largely due to the extensive covalent cross-linking between the three polypeptide chains that form the superhelical cable structure of collagen. The polypeptide chains are stabilized by H-bonding between the NH groups of glycine residues and the peptide CO groups of residues on the other chains as well as between the OH groups of hydroxyproline residues and water molecules. The solubility of collagen, and its lack thereof, in ionic liquids is presumably a function of the ability of the ionic liquid to disrupt these H-bonds. The limited solubility suggests that the H-bonds along the entire polypeptide chain must be disrupted.

Like collagen and silk, elastin is rich in the amino acid glycine. It is also rich in non-polar aliphatic residues and contains extensive cross-links, rendering it highly insoluble. The hydrophobic domains in elastin occur in tandem repeat domains and form a series of β-sheet and α-turns [13 – 15]. This structure resembles that proposed for spider silk and suggests that it is responsible for the self-aggregation of elastin monomers and the aligning of the polypeptide chains for cross-linking. The increased solubility of elastin in $EMIm^+Cl^-$ over the collagen type I [which consists of two a1(I) chains and one α(I) chain] suggests that $EMIm^+Cl^-$ is more effective in disrupting hydrogen bonds between β-sheets than between α-helixes.

4.2. Characterization of Reconstituted Silk

Previously the crystallinity of silk films cast on silicon wafers as a function of rinse solvent has been reported [8]. Our results show that dry methanol and dry ethanol are effective solvents for removing the ionic liquid and reconstituting the silk fibroin fibers. Using the methanol bath, fibers extruded from the syringe remained intact as a single fiber. All other solvents either produced a matte-like precipitate consisting of multiple fibers or completely dissolved the silk. The use of dry methanol, however, has been shown to produce very brittle regenerated fibers [16, 17]. Wang et al. have shown that a water rinse can significantly improve the mechanical properties of the regenerated silk [16]. Furthermore, Dicko et al. have shown that, although deionized water induces α-helix structures, acidic water and the presence of certain cations, such as Al^{3+} and Na^+, induce the β-sheet formation of silk [18]. The regeneration of silk in acidic sodium citrate buffers indicates that it is indeed feasible to use acidic water solutions to remove the ionic liquids from the ionic liquid/silk mixtures. The transition to an all β-sheet structure does take several hours to complete, using the sodium citrate salts, but may be increased up by the addition of Al^{3+} cations. The results shown in Table 3 indicate that the use of water as the regenerating solvent does

require a significant acidic environment to be effective. We are currently attempting to draw this fibers in the solvent bath in an attempt to produce single fibers with better mechanical strength. In addition, we are conducting WAXS and Raman spectroscopy on the acidic water washed samples to compare their structure to that of native silk.

The regenerated silk, prepared by electrospinning, exhibits inferior thermal properties than native silk. As shown in Fig. 2, the thermal stability of the regenerated silk was by about 20 °C lower than that of native silk. The mass loss of the regenerated fibers suggests that not all of the $BMIm^+Cl^-$ was removed by the MeOH bath. It is possible that excess Cl^- ions in the biopolymer matrix initiate decomposition at lower temperatures via a nucleophilic attack.

5. Conclusions

Imidazolium chlorides are viable solvents for the dissolution of biopolymers due to their ability to disrupt intramolecular hydrogen bonds. The ability of the cation to disrupt hydrogen bonds improves the dissolution of proteins, but not polysaccharides. Hence, the solubility of cellulose in $EMIm^+Cl^-$ is comparable to that in $BMIm^+Cl^-$, while the solubility of silk increases in the order $BMMIm^+Cl^- > BMIm^+Cl^- > EMIm^+Cl^-$. $EMIm^+Cl^-$ is also a viable solvent for the dissolution of chitin, elastin, and collagen, though there is a limited solubility of collagen in the ionic liquid. Water can be used as a cosolvent to tune the solution viscosity, however, the order of addition between silk and water can have an effect on the solubility limit of the silk. Further studies are underway to understand these interactions.

The structure of the films and fibers cast from silk in ionic liquids is highly dependent on the rinse treatment. Acetonitrile yields a convoluted surface structure with little crystallinity, methanol yields a transparent film with a high degree of crystallinity, neutral water dissolves the silk fibroin, and acidic water yields a fibrous matte when used as regeneration bath. In addition, the complete removal of the ionic liquid has shown to be dependent upon the rinse time in the regenerating solvent. TGA and DSC of regenerated electrospun silk fibers indicate that the presence of excess ionic liquid has a detrimental effect on the thermal stability. Our results demonstrate that ionic liquids are viable solvent systems for the dissolution and regeneration of many biopolymers, including silk, cellulose, chitin, collagen, and elastin.

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