Concentration State Dependence of the Rheological and Structural Properties of Reconstituted Silk

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The ability to control the processing of artificial silk is key to the successful application of this important and high performance biopolymer. Understanding where our current reconstitution process can be improved will not only aid us in the creation of better materials, but will also provide insight into the natural material along the way. This study aims to understand what proportion of reconstituted silk contributes to its rheological properties and what conformational state the silk proteins are in. It shows, for the first time, that a change in rheological properties can be related to a change in silk structures present in solution and reveals a low concentration gel state for silk that may have important implications for future successful artificial processing of silk.

Introduction

In its natural form, silk possesses many attractive material properties that make it stand out amongst other fibers. But silk’s remit as a material does not end with a fiber as it can be artificially reprocessed from its originally spun form. Thus, reconstituted or reconditioned silk liquid can be turned into foams, films, gels, and powders, finding use in all manner of applications, from medicine to optics.5 Unfortunately, in spite of often comparing favorably to their synthetic petrochemical or cellulose-based rivals, reconstituted silk-based materials are yet to exploit the truly exceptional properties of natural silk (ref 6 and references wherein).

It is unquestionable that the act of reconstitution affects the silk molecules. After all, the process of reconstitution reverses the natural spinning process by returning the dehydrated proteins in the fiber back into their hydrated state prior to spinning.7 Yet this often causes collateral damage as it is usually achieved through exposure to proteolytic enzymes or chaotropic agents. While the bulk of the material is returned to a liquid state, on an individual scale, silk proteins are altered, manifesting as changes to the molecular weight distribution (MWD)8–10 and conformation10,11 of the reconstituted silk. Previously, we have shown that, in comparison with its natural silk precursor, reconstituted liquid silk exhibits rheological (flow) properties that are different in kind, not just degree.8 The molecular basis for this difference is yet to be understood but will be vital in order to fully comprehend, and improve, the reconstitution process. This study builds upon our previous investigations to view reconstituted silk’s rheological properties from a methodological and molecular perspective and is presented in a format to aid future reference as such.

Materials and Methods

Sample Preparation. Reconstituted silk was prepared using standard techniques.12 Bombyx mori cocoon silk was boiled in 0.5% NaHCO3 solution for 45 min and then rinsed with abundant water to remove the sericin. Immediately after being dissolved in 9.5 mol/L LiBr for 20 min at 45 °C to make an ∼10% solution, the liquid silk was filtered through muslin and poured into dialysis tubes with different average molecular weight cut-offs (MWCO), specifically, 1 KDa (Spectra-por), 12 KDa (Sigma Aldrich), and 50 KDa (Spectra-por). The silk then underwent dialysis at 4 °C for 3 days against three daily changes of type-II water (Elix 3-UV, Millipore, MA, U.S.A.). Once dialyzed, the silk solution was transferred to 2 mL eppendorf tubes and centrifuged to remove any large insoluble silk particles. Afterward the reconstituted silk solution was either diluted with type-II water, kept as prepared, or concentrated for either 4 or 7 h against 15% polyethylene glycol (PEG) solution (to retain silk I structure in solution13). Post experiment, remaining samples were dried to a constant mass in eppendorf tubes, and the dry weight (DW) concentration was calculated, thus, creating samples with different molecular weight cut off (1, 12, and 50 K) over a range of concentrations (0.34−24% DW).

Lyophilization. Reconstituted silk solution (50 K MWCO, concentration between 0.63−12.27% DW) concentrations were rapidly frozen to −80 °C using an ultra-low temperature freezer (MDF-U2086S, Sanyo electric biomedical Co. Ltd, Tokyo, Japan) and then kept in a vacuum freeze-dryer for 48 h at −40 °C (Micromodulyo, EC apparatus Inc., U.S.A.). The rapid freezing to −80 °C prior to lyophilization reduces any shearing force caused by ice growing, thus, best preserving the original conformation of the silk protein in solution.14−16

Rheological Analysis. All rheological tests were run on a Bohlin Gemini HR Nano 200 (Malvern Instruments, U.K.) maintained at 25 °C with environmental cuff in place to minimize sample dehydration and using a cone and plate 4° incline 40 mm diameter measuring geometry (CP 4/40).

The linear viscoelastic regions of these materials have already been established and oscillatory measurements were carried out within this region.6 Modulus values were obtained from an oscillatory sweep.
between 623 and 0.623 rad/s, target strain 0.1. Viscosity measurements were taken during a steady shear response experiment stepped between 0.1 and 4000 1/s. As these solutions appear to be gel-like, they do not possess a zero shear viscosity, as seen in native dope, hence, a low shear viscosity ($\eta_0$) was calculated from the average viscosity between 5–50 1/s for comparison between samples.

ATR-FTIR Measurements. Spectra of the lyophilized silk samples were collected using a single bounce diamond attenuated total reflectance (ATR) module on a Fourier-transform infrared (FTIR) spectrometer (Nicolet Nexus 6700) equipped with a liquid Nitrogen cooled mercury–cadmium–telluride (MCT) detector. To eliminate the spectral contribution due to atmospheric water vapor and CO$_2$, the instrument was continuously purged with dry air (Peak Scientific air dryer, flow rate 45 CFM). The resolution of each spectra was 4 cm$^{-1}$, and 128 interferograms were coadded in the range of 500–4000 cm$^{-1}$. Measurements for each sample were repeated at least three times. The spectra were analyzed using PeakFit software (SPSS Inc. Version 4). Linear baseline correction was applied to the amide I region cm$^{-1}$ before the band was deconvolved by Gauss Amplitude function. For the curve fitting procedure, the initial band positions were fixed, allowing their widths and heights to vary. The best fit was sorted according to how the residual values were normally distributed. We assume that the extinction coefficient for the C=O stretch vibration is the same for the different structural components, thus, the band area could represent the relative content of each secondary structural component.

SEM. Scanning electron microscopy (JSM-5510, JEOL, Japan) was performed using a 15 kV voltage on the lyophilized silks, which were gold-coated for 2 min in a sputter coater (E 5000) at 0.07 Torr.

Results and Discussion

Rheological Properties. Comparing solutions of reconstituted silk over similar concentrations yet with different MWCO allows us to assess the effective pore size of silk molecules, which might contribute the most to the rheology of the solution/suspension. All cutoff weights had very similar rheological properties (Figure 1, 1 K MWCO; data not shown, as no comparable concentration was obtained). Additionally, varying the concentration of the sample in the 9.5 M LiBr prior to dialysis made no difference to the rheology (1% 12 K and 5% 12 K). Consequently, our data support the conclusion that standard reconstituted silk’s rheology appears to be primarily influenced by the concentration of silk molecules with an effective pore size greater than 50 KDa.

However, the observed concentration dependence was unexpected for a typical polymer. Surprisingly, the 1.3 and 1.4% DW samples appeared to be gelled, with $G' > G''$ and displaying a viscosity greater than samples three times more concentrated (Figure 1). Low concentration gelation has been observed previously in native silk solutions, however, viscosity and modulus both had a positive correlation with concentration. From Figure 1 it seems that there is a negative correlation between concentration and modulus/viscosity in standard reconstituted silks.

To better understand this low concentration gelation, all data were pooled to compare concentration effects against rheological properties. Low shear viscosity ($\eta_0$) was chosen over oscillatory readings because of greater machine sensitivity when studying such dilute samples (Figure 2).

From Figure 2, the critical concentration ($C_p$) at which gelation occurs is approximately 2.5% DW. Below $C_p$, $\eta_0$ increases over an order of magnitude and is equal to a silk solution 10× more concentrated ($\approx 0.4$ Pa·s, line c) with a corresponding 1000-fold increase in modulus was also observed (data not shown). In a few tests, samples occupied a lower plateau ($\approx 0.01$ Pa·s, line b), which may indicate an unstable state prior to the formation of a gel. Above 2.5% DW, reconstituted silk follows an approximate $\eta \propto C^2$ relationship akin to dilute polymers (line a, $\eta \propto C^{2.4}$, $R^2 = 0.96, n = 22$) and tends toward an upper plateau at around 15% DW.

This gelation appears to be a consequence of physical necessity. The intercept of the concentration-dependent phase
occurs at a silk concentration of approximately 1
influence on viscosity.20 Therefore, the material must change
molecules have no interaction with water and, hence, no
a low concentration state cannot exist, as it implies that the silk
suggesting an efficiency of rehydration of this reconstitution method
approximately one-third of the noncentrifuged samples concentration,
though further study is required to support this.
silk proteins lower critical solution temperature (LCST), al-
 solvent. This in turn may be a phenomenological result of the
increase in viscosity was attributable to previously condensed
thus could be further analyzed using these techniques.14
information, Figure s2).23 When a Gaussian band shape was
selected for curve fitting, the maximum positions were fixed at
1620, 1640, 1660, and 1691 cm
played two peaks at 1620 cm
R
1720 cm
C), we
amplitude of the amide I area (Figure 4) with
at each concentration were obtained by calculating each band
or success, between batches of reconstituted silk.
important method to quickly assess the percentage rehydration,
or success, between batches of reconstituted silk.
Due to the sensitivity of the rheometer it was not possible to
obtain readings for the noncentrifuged samples below 4% DW,
however this limit is lowered to <1% DW for centrifuged
samples. If we now superimpose noncentrifuged data onto the
centrifuged silk curve, no concentration-dependent gelation is
observed. This may be because the suspended insoluble particles
do not permit the formation of an extended gel network. It may
also explain the few outliers/unstable samples seen in Figure 2
along line b.
From this simple retrospective comparison we may infer two
important points. First, centrifugation has little effect on shear
history of reconstituted silk. Second, the efficiency of this
method of reconstitution for rehydrating silk molecules is
approximately 33%.
Finally, in some of the reconstituted silk samples, white flocs
appeared post-shearing (Supporting Information, Figure s1),
which is not a novel observation.22 However, in our study, these
flocs were present only in the gelled, low concentration, samples.
ATR-FTIR analysis of the flocs revealed a high degree of
ordered structures, similar to that of spun silk (Supporting
Information, Figure s2), and is in agreement with previous
observations.22 The presence of a transient extended gel network
in a dilute solution may briefly enable the silk molecules to
reach an energy density sufficient to initiate phase transition
and a conformational flexibility to aggregate. In contrast, a more
concentrated or “dirty” (noncentrifuged) environment is unable
to offer the flexibility required to form the network in the first
place.
Concentration Effects on Silk Conformation and Mor-
phology. Clearly the rheological properties of reconstituted silk
are dependent on concentration and, in part, preparation, but
how does this translate to the molecular structure of the silk
proteins and aggregation morphology? FTIR and SEM were
used to examine the structural properties of silk solutions across
a similar range of concentrations. Through lyophilization of
reconstituted silk solutions (rapidly freezing to −80 °C),
we assume that the silk structures in solution are immobilized
and thus could be further analyzed using these techniques.14–16
FTIR has been widely used to characterize the secondary
structure of silk proteins.23–25 In the FTIR-ATR spectra of the
lyophilized silk, the amide I band (1580–1720 cm
was analyzed to
determine to conformation of fibroin. This band represents primarily
the C=O stretching vibration of the amide group,
thus, it is indicative of protein secondary structures.12
In general, the absorbance associated with various conformationes
are as follows; α-helix, helical occurs at 1650–1660 cm
–1,
random coil at 1640–1650 cm
–1, and β-sheet at 1620–1640
cm
–1.26–28
For low concentration samples, a broad band centered at 1640
cm
–1 was noticed, indicating primarily random coil and/or
α-helix conformations present. Post-shear, these samples
displayed two peaks at 1620 cm
–1 and 1691 cm
–1, which
are assigned to β-sheet and β-turns, respectively (Supporting
Information, Figure s2).23 When a Gaussian band shape was
selected for curve fitting, the maximum positions were fixed at
1620, 1640, 1660, and 1691 cm
–1 for a series of spectra,
allowing the band widths to be automatically adjusted by the
software (Supporting Information, Figure s3).29
Estimates of the proportion of secondary structures present
at each concentration were obtained by calculating each band
area as a percentage of the total amide I area (Figure 4) with
original IR spectra displayed in Supporting Information, Figure

Figure 3. Concentration dependence of nongelled reconstituted silk
viscosity for centrifuged (orange diamonds) and noncentrifuged
samples (cyan left facing triangles from ref 6). Arrow represents effect
of centrifugation. Note that, for the same ηc, centrifuged samples are
approximately one-third of the noncentrifuged samples concentration,
suggesting an efficiency of rehydration of this reconstitution method
of approximately 33%. Also, while the overall concentration-dependent viscosity curves are qualitatively similar on a log–log scale, their
actual fits differ slightly. This may be explained through the presence
of insoluble silk particles or the quality of the solvent (centrifuged ηc
α c−4, R = 0.96, n = 22; noncentrifuged ηc α c−3, R = 0.96, n = 17).

(line a) with the viscosity of the solvent (water, 10
−3 Pa·s) occurs at a silk concentration of approximately 1–2% DW. Such
a low concentration state cannot exist, as it implies that the silk
molecules have no interaction with water and, hence, no
influence on viscosity.20 Therefore, the material must change
its rheological behavior before reaching the viscosity of the
solvent. This in turn may be a phenomenological result of the
silk proteins lower critical solution temperature (LCST), al-
though further study is required to support this.
A similar behavior has been seen by Hossain et al. in
reconstituted solutions of silk containing 6 M LiBr.21 Here the
rheology was likened to polyelectrolytes in aqueous solutions,
although this critical concentration was found to be much lower
(~0.07% DW). It was hypothesized by Hossain et al. that this
increase in viscosity was attributable to previously condensed
counterions disassociating from the silk proteins, causing chain
expansion and a gel network to form. However, the authors
admit this may not be applicable due to the high concentration
of LiBr present. Attempts were made to investigate this at the
time of their study using pure water (supposedly type-I),
although this caused the silk proteins to precipitate instantly
(and by our definition denature20). Our study represents the
middle ground of this spectra as it uses type-II water, indicating
that this hypothesis may still be valid.
By comparing the reconstitution methods used here with those
used in a previous study,6 we see differences in silk rheology
at the same concentration. This may be attributed to only a slight
change in the means of preparation. Post dialysis, the current
study used an additional centrifugation step to remove large
insoluble pieces of silk, whereas previously this was not
performed because of native silk’s shear history dependence.17
By direct comparison of these two preparations, Figure 3
demonstrates that centrifuged samples have the same viscosity
as noncentrifuged samples three times more concentrated. From
this we may interpret that approximately two-thirds of the silk
molecules present in the noncentrifuged samples did not contribute to the rheology (i.e., were not in solution). Conse-
sequently, we propose that rheological analysis may be an
important method to quickly assess the percentage rehydration,
forms intramolecular associations (bonds. At higher concentrations reconstituted silk preferentially nature) by forming either intra- or intermolecular hydrogen associations (d) %DW (scale bars: a, 40 µm; b–d 50 µm). Also shown is (e) the 0.63 %DW sample under higher magnification (scale bar 10 µm).

Figure 5. SEM micrographs of lyophilized reconstituted silk solutions at 0.63 (a), 1.25 (b), 5.05 (c), and 12.27 (d) %DW (scale bars: a, 40 µm; b–d 50 µm). Also shown is (e) the 0.63 %DW sample under higher magnification (scale bar 10 µm).

Figure 4. Secondary structure estimates of lyophilized silk solutions vs concentration. Symbols are as follows: random coil (cyan, squares), α-helix (blue, circles), β-sheet (navy, triangles). The sum of all values per concentration is 100%; error bars are ±1 standard error.

s4. The results show that, as concentration increases, there is a decrease in random-coil structures and an increase of β-sheet structures. The point at which this changeover occurs is approximately 2.5% DW, which is in perfect agreement with our rheological findings for Cg.

We believe this phenomena can be explained by the reconstituted silk proteins overall solubility, which is tuned (as in nature) by forming either intra- or intermolecular hydrogen bonds. At higher concentrations reconstituted silk preferentially forms intramolecular associations (β-sheet folded structures), while at lower concentrations such packing is lost and intermolecular associations are more thermodynamically stable. Therefore, below Cg, the reconstituted silk proteins tend toward a partial unfolding into random coil structures, leading to increased associations with water. This weak hydrogen bonding forms an extended gel network, which is responsible for the increased modulus and viscosity of the material, as observed by the rheology. However, it is important to note that this type of gel network formation is not the same as previously observed in silk hydrogels or indeed the natural system in which the hydrogen-bonded network is based on β-sheet structures, not random coil.

SEM studies on lyophilized silk samples revealed gross morphological features that support our rheological and spectroscopic observations (representative samples may be seen in Figure 5). The SEM photos of lyophilized diluted silk are typical of a loose structure with voids and the presence of band-like structures (Figure 5a,b,e). In contrast, the lyophilized silk solution above 2.5% DW is made up of condensed layers. This may be explained as follows: Below Cg, the high surface area of bands (rather than layers) indicates an increased interaction between the reconstituted silk proteins and water, a result of increased solubility of proteins at a low concentration. Above Cg, molecules are compact due to hydrophobic effects and thicker, more condensed, layering arises. Finally, assuming the band-like structure is representative of an extended state of silk proteins, this would facilitate better alignment under shear and support our observations of β-sheet rich precipitates akin to the natural fiber in some of our low concentration rheology samples (Supporting Information, Figure s1).

Conclusions

Understanding the mechanisms behind reconstitution is vital if we are ever to truly capitalize upon silk’s superior mechanical properties. We believe that our study has made some progress regarding preparation and processing of silk reconstitution and reconditioning, namely, (i) silk molecules with an effective pore size larger than 50 kDa contribute the most toward bulk rheological properties, (ii) sample dilution history has little effect on rheology, (iii) centrifugation improves the quality of the silk solution, and finally, (iv) silk protein concentration is the governing rheological factor.

Building upon the observations made with respect to concentration dependence, this work is part of one of the first examples of a change in silk structure being related to mechanical properties in solution (summarized in Figure 6). The
existence of a low-concentration extended gel network can be quantitatively linked to a change in the secondary protein structure in the silk proteins and SEM studies have linked this to differences in gross morphological structure. This preponderance for reconstituted silk molecules to form either inter- or intramolecular bonds, depending on their concentration is critically important for us to comprehend for use in future applications of silk and potentially in understanding the mechanisms used by the real masters of silk processing, the silkworm, and spider.

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Supporting Information Available. Photographs of white flocs (silk aggregates), ATR-FTIR spectra of white flocs, a representative spectrum of lyophilized reconstituted silk with curve fitting and original IR spectra used for Figure s4. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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