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The influence of metal ions on native silk rheology

Andreas Koeppel, Peter R. Laity, Chris Holland*

Department of Materials Science and Engineering, University of Sheffield, Mappin Street, Sheffield S1 3JD, United Kingdom

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ABSTRACT

Whilst flow is the basis for silk fibre formation, subtle changes in a silk feedstocks' chemical environment may serve to increase both energetic efficiency and control hierarchical structure development during spinning. Despite the role of pH being largely understood, the influence of metal ions is not, only being inferred by correlative work and observations. Through a combination of rheology and microscopy, we provide a causative study of how the most abundant metal ions in the silk feedstock, Ca^{2+} and K^+, affect its flow properties and structure. Our results show that Ca^{2+} ions increase viscosity and prevent molecular alignment and aggregation, providing ideal storage conditions for unspun silk. In contrast, the addition of K^+ ions promotes molecular alignment and aggregation and therefore seems to transfer the silk feedstock into a spinning state which confirms recent 'sticky reptation' modelling hypotheses. Additionally, we characterised the influence of the ubiquitous kosmotropic agent Li^+, used to prepare regenerated silk solutions, and find that it promotes molecular alignment and prevents aggregation which may permit a range of interesting artificial silk processing techniques to be developed. In summary, our results provide a clearer picture of how metal ions co-ordinate, control and thus contribute towards silk protein self-assembly which in turn can inspire structuring approaches in other biopolymer systems.

1. Introduction

Silk spinning in Nature is a sophisticated process occurring in a benign environment, and with minimal energy input yields strong and tough fibres that can easily compete against synthetic alternatives [1–4]. The 'secret' behind such remarkable properties lies in the self-assembly of proteins into hierarchical structures under carefully controlled conditions.

It has been known for over a century [5] that the solidification of silk proteins during natural spinning is initiated by mechanical energy input via flow [4,6–9]; however, more recent work has suggested that a change of pH and ions in the silk glands of spiders [10–15] and silkworms [16–20] may also be important. Nevertheless, while a much clearer picture for native silks has recently drawn out the influence of pultrusion [8], shear [7,21], extensional flow [22,23] and pH change [6,24–26], the final processing variable, the role of metal ions, is not fully understood.

Studies on various types of silk have previously shown the influence of metal ions on the mechanical properties of a spun fibre, either through altering the interchain bonding type [27], or protein secondary structure [27–31]. However, little attention has been

given to directly link the presence of metal ions in the unspun feedstock to changes in its flow properties, despite several studies into the natural system indicating that metal ions are regulated in the spinning duct [13,16,17,19] and that genetic modification of these metal ion channels affects the spun fibre [32]. In particular, Wang et al. demonstrated that by suppressing the amount of K^+ ions, fibres were weaker and had more random coil structures, whereas overexpression of Ca^{2+} increased the amount of helical and β-sheet structures and fibre strength [32]. Hence the role of metal ions clearly has an impact on both the spinning process and fibre properties, and thus is an important factor to understand and control during any attempt at artificial silk spinning [33].

For silk solutions, the effect of metal ions on silk protein structure has been limited to studies focussing on dilute [11,34] and regenerated silk solutions [17]. However, findings appear somewhat inconsistent. For dilute spider silk solutions, it has been suggested that metal ions such as Al^{3+}, K^+, and Na^+ induce structural changes towards a more oriented structure, divalent ions such as Ca^{2+} and Mg^{2+} stabilize a disordered state, while Cs^+ and Li^+ appear not to have any significant effects [11,35]. In contrast, for dilute silkworm fibroin solutions, Ca^{2+} and Mg^{2+} ions were found to induce network formation with carboxylic groups, which are broken down when a small shear stress is applied [34]. Although spider and silkworm fibre extrusion share similar features such as a defined mechanical input via flow and changes in the chemical en-

* Corresponding author.
  E-mail address: christopher.holland@sheffield.ac.uk (C. Holland).

vironment of the gland, there are also differences in how the metal ion composition and the pH change along their respective glands. Therefore, in the following work we specifically refer to the silkworm *Bombyx mori*.

When switching focus to the natural system, it has been suggested that metal ions in *B. mori* native silk feedstock (NSF) are involved in providing optimised storage or spinning conditions for the silk proteins in the gland prior to pultrusion into a fibre [11,17,34]. Ca^{2+} and K^+ are the most abundant, naturally occurring metal ions in the silk gland and it was found that their concentrations change both along the gland <u>and</u> between different spinning stages [17,36]. Around (an estimated) 12 hours before spinning started, concentrations of around 2000 ppm Ca^{2+} and 1000 to 3000 ppm K^+ (on dry weight), were found in the middle posterior section [17]. Moreover, the amount of K^+ (along with other, less abundant ions: Mg^{2+}, Cu^{2+}, Zn^{2+}, Na^+), were found to increase from the middle posterior to middle anterior gland section, while the amount of Ca^{2+} decreased. The presence and systematic variation in concentrations of these ions along the silk gland has inspired the idea that they are important in the spinning process (i.e. not merely adventitious contaminants) and may catalyse the transition of fibroin from a random or helical conformation to β-sheets. In support of this, divalent ions such as Mg^{2+}, Cu^{2+} are also thought to induce the transition from a helical structure to β-sheets in prion formation [17,35,37–40].

In addition to the previously reported variations with position along the gland, a more recent study from our group [36] observed changes in the concentrations of several elements within the middle posterior gland section over time, as the silkworms constructed their cocoons. In particular, the concentration of Ca^{2+} increased slightly (from around 2000 to 3000 ppm, equivalent to 23 to 35 cations or 46 to 70 positive charges per chain), while the concentration of K^+ increased dramatically (from around 700 to over 4500 ppm. equivalent to 7 to over 50 cations per chain). Most intriguingly, an inverse correlation was found between the K^+ concentration and the viscosity of the NSF.

This led to the hypothesis that Ca^{2+} (or, potentially, other polyvalent metal) cations can form transient 'salt bridges' between carboxylate-substituted amino acids (a combined total of 77 Asp and Glu in fibroin, based on the published sequences: https://www.uniprot.org/uniprot/P05790 and https://www.uniprot.org/uniprot/P21828). These ionic bridges resist the chain motion and diffusion along the tube defined by adjacent chains (reptation), inspiring the concept of 'sticky reptation' [41,42]. This retards the relaxation of flow-stress and increases the viscosity relative to a similar system without stickers. In this respect, it may be noted that the effects of relatively small numbers of ionic or other transient interactions on the flow properties of synthetic polymers has been widely reported [43–47,48]. Thus, it is plausible that salt bridges could affect protein conformation and other characteristics (such as crystallisation), in addition to the changes in solution flow properties [16,17,34,40,49].

Conversely, K^+ (and other monovalent) cations are expected to break or weaken the salt bridges by competing for the carboxylate groups or through ionic shielding. It may be noted that other studies have shown that K^+ ions accelerate the dissolution of the native silk feedstock [17], suggesting that they help to break down transient interactions within the protein network. Thus, these findings are in good agreement with the recent observation that increased K^+ ions in the silk feedstock correlate with a decrease in viscosity [36].

This picture of NSF as a natural example of sticky reptation, with the viscosity controlled by the concentrations of Ca^{2+} and K^+ cations, has recently been tested by modelling. The work by Schaefer et al. [30] demonstrated that sticky reptation provides a good description of silk feedstock rheology (at least for NSF from

B. mori). It was suggested that the fibroin chains in NSF form fairly constant numbers of physical entanglements (6 – 12) and 'ionic stickers' (Ca^{2+} bridges, 4 – 5) per chain. The potential for stickers is defined by the presence of carboxylate-substituted (aspartate and glutamate) amino acids along the fibroin sequence, which can interact with polyvalent cations (Ca^{2+} being the most abundant in *B. mori* NSF) to form ionic bridges between fibroin chains. Thus, the number of stickers predicted by modelling the rheology fits quite well with the expected distribution of carboxylate side-chains within 12 or 13 patches along the fibroin chain. Moreover, the magnitude of the viscosity increase (relative to non-sticky reptation) is controlled by the lifetime of the stickers, which depends on the electrostatic environment (i.e. the ionic strength). Thus, increasing the concentration of monovalent cations (K^+ being the most abundant in NSF) moderated the viscosity by shortening the lifetime (i.e. increasing the exchange rate) of the stickers.

Therefore, based on this body of previously correlative work and observations, we conducted a causative study of how metal ions affect the flow properties of NSF. One major challenge is that direct mixing of metal ions into the silk solution is not possible due to its high viscosity and shear sensitivity [50,51]. Therefore, we developed a means by which the NSF could be diluted and reconcentrated after the metal ions were added. Importantly, we show that reconcentration does not change the flow properties of native silk and thus we were able to investigate the effects of several metal ions on native *B. mori* silk feedstock. We find that Ca^{2+} ions not only increase the viscosity of the silk feedstock, suggesting salt bridge formation, but also make the proteins less sensitive to shear alignment and aggregation, providing ideal storage conditions for silk. In contrast, K^+ ions prepare silk feedstocks for spinning, presumably breaking down the salt bridge network and allowing the proteins to rearrange, align and aggregate.

Finally, we investigated the effect of Li cations on the flow properties of silk. Li^+ does not naturally occur in the silkworm but plays a crucial role in dissolving already spun silk in a process called regeneration/reconstitution [52]. We found that just by changing the cation, from KCl to LiCl, silk feedstocks can still align under flow but do not aggregate. Thus, we also provide helpful insight how Li cations can affect the properties of regenerated/reconstituted silk feedstocks.

2. Experimental Methods

2.1. Silk sample preparation

Native silk proteins were obtained from the middle-posterior gland section of fifth instar *B. mori* silkworms during 'early' or 'late' stages of cocoon construction, based on the amounts of fibre spun. Dissection was performed as previously reported [53]. Mixing silk with metal ions cannot be achieved in its native state due to its high viscosity and shear sensitivity; consequently, the native silk proteins obtained from multiple glands were dissolved in Type I water (ultrapure water with a resistivity higher than 18 MΩ.cm) to prepare a 2 wt.% stock solution. Based on the rheology of the initial NSF samples, the metal ion content of this starting stock solution is expected to lie within the natural range of around 25 to 40 Ca^{2+} and 11 to 34 K^+ ions per chain, as determined in our previous study [36].

The concentration of protein in the diluted NSF solution (predominantly fibroin) was determined gravimetrically and the molar concentration was calculated, based the published sequences for the heavy and light chain components, (Fib-H, https://www.uniprot.org/uniprot/P05790 and Fib-L, https://www.uniprot.org/uniprot/P21828). These published sequences also indicated that the combined fibroin (Fib-H+Fib-L) chain contains 77 carboxylate-substituted amino acids (Asp and Glu), which are expected to be

dissociated at the pH of the samples used and could form ionic interactions with cations. (Phenolic Tyr side-groups are more abundant in the fibroin, but are expected to remain associated below pH = 9, therefore, unavailable to participate in ionic interactions.)

The stock solution was divided into portions (10 ml), which were mixed with suitable aliquots of aqueous salt (KCl, $CaCl_2$, $LiBr$ and $LiCl$) solution to give the desired 100 or 3000 additional positive charges per chain, based on the calculated molar concentration of fibroin. A further portion was retained without added salt, as a control.

Rather than attempting to remove the naturally occuring K^+ and Ca^{2+} cations (e.g. by dialysis, which could also remove other naturally occurring metallic and non-metallic cations, anions and other low molecular weight components in the NSF), it was decided that examining the effects of additional ions would be more informative. Hence, added salt concentrations equivalent to a slight excess of 100 positive charges per chain were deliberately chosen in order to explore the effects of approximately physiological ion concentrations, allowing that some may be sequestered by other free anions in the feedstock. Higher additions of salt, equivalent to a large excess of 3000 +ve charges per chain, ensured maximal interaction (i.e. saturation) with all carboxylate groups present.

It may be noted, however, that this did not guarantee equivalent ionic strengths for each solution. The ionic strength achieved by adding salts depends on the dissociation equilibrium, which depends on concentration and the nature of the anions present.

Afterwards, each of these solutions (the control and with added salts) was re-concentrated to around 20 wt % in a petri dish via unforced evaporation of water under standard lab conditions (23 ± 1 °C, 50 ± 5% RH) on a lab shaker to realize a very gentle shaking. As a further set of controls, native silk samples from an early and late cocoon stage were taken directly from the silk gland and characterised. The natural pH of all silk samples (i.e. not adjusted via a buffer solution) was measured using a glass-free micro-electrode pH sensor (CupFET 3200-010 pH probe) attached to a SI600 pH meter (Sentron, The Netherlands).

2.2. Rheological characterisation

Rheological characterisation was performed on a Discovery Hybrid Rheometer (DHR 2, TA Instruments, USA) equipped with an 8 mm parallel plate geometry. A modular microscope accessory with a 1x magnification objective (Nikon, Japan) and polarizers were attached to the rheometer, which allowed for flow visualisation under polarized light. A gap setting of 500 μm was used, giving an optical path-length of 1 mm. To prevent the samples from drying out, water droplets were placed around the geometry (not touching the sample) and a loose cover was placed over it. Flow experiments were conducted in three steps: first, the sample was sheared at a constant rate of 1 s^{-1} for 100 seconds to remove any residual stresses that occur due to sample handling and loading as well as to evenly distribute the sample between the plates; then, oscillatory measurements were performed from 100 to 0.1 rad s^{-1} at a shear strain of 0.02 which is within the materials linear viscoelastic region; lastly, all samples were subjected to a flow sweep test from 0.1 s^{-1} to 100 s^{-1} over 150 s, and back from 100 to 0.1 s^{-1} (also over 150 s). (It should be noted, however, these values of shear or shear rate were nominal and varied across the sample, due to the use of parallel plate geometry). All experiments were carried out under standard lab conditions (23 ± 1 °C, 50 ± 5% RH) and a total of 10 repeats have been measured for each sample. The pixel brightness value was determined from the micrographs via Image J. Firstly, the brightness of the pictures was normalized for a gap height of 500 μm and a background image of the loaded sample prior to shearing was subtracted. The images were then all converted to greyscale with the same threshold setting before a

false colouring look-up table was applied for presentation in this paper. It should be noted that the parallel plate geometry cannot exactly mimic the flow profiles in the native silk gland (i.e. shear vs extension). However, the focus of this work is on characterising the influence of metal ions on the intrinsic bulk rheological properties which in turn will practically affect any extrinsic response to different flow profiles.

2.3. Dynamic Light Scattering (DLS) measurements

An aliquot (1 ml) of the native 2% w/w silk stock solution was added into a standard cuvette (10 mm path length) and measured using a Zetasizer Nano ZS (Malvern Panalytical Ltd., UK) at 25°C after a 60 s equilibration time. First, the effect of dilution was checked, by adding aliquots of distilled water. This revealed a small but significant decrease in the (apparent) hydrodynamic diameter (d_H) from 41 ± 5 nm at 2% to 30 nm at 1.27% silk protein concentration. Note: this apparent reduction in size may actually indicate a decrease in the interactions between protein chains; for a polymer with a formula weight of 419.3 kDa (based on the published amino acid sequence) and a true diameter of 41 nm, chain overlap would be expected above 2% concentration. Subsequently, identical samples of the silk protein solutions were measured after adding salt solutions to give 100 or 3000 positive charges per fibroin chain. This allowed a comparison of both levels of salt concentrations using the same initial silk solution.

2.4. Infrared spectroscopy

Specimens of silk protein solution were cast into films which were then dried at under vacuum at 70°C. Spectra were recorded at ambient temperature (23°C) in attenuated total reflection (ATR) mode using a Fourier transform infrared (FTIR) spectrometer (Nicolet 380, Thermo-Electron Corp. Madison, WI, USA) fitted with a deuterated triglycine sulphate (DTGS) detector and 'Golden gate' attenuated total reflectance (ATR, SpecAc, UK). The optical path and specimen area were purged with dried, purified air to avoid interference from atmospheric carbon dioxide and water vapour. Spectra were collected at 4 cm^{-1} resolution by co-adding 64 scans. In order to investigate changes in the amide I and II bands (centered around 1640 and 1525 cm^{-1}, respectively), first and second derivative spectra were computed by numerical differentiation over a window of 7 spectral points (resulting in the 2nd derivative being obtained from 13 spectral points, equivalent to 23 cm^{-1}). It should be noted that this method appeared to offer the best compromise between introducing false peaks due to noise (if too narrow a window was used) and excessive smoothing (if too wide a window was used).

2.5. Statistical analysis

The data in this work was analysed with Microsoft Excel and OriginLab Pro. The rheological measurements were performed with at least 5 repeats while the pH and concentration measurements were repeated 10 times. The DLS measurements for Fig. 4 were performed in triplicate (n=3). Error bars represent the standard deviation.

3. Results and discussion

3.1. Influence of silk preparation and natural variation comparison

Before the influence of individual ions are considered, as an important control, the flow behaviours of the native and reconcentrated silk solutions were compared to investigate whether our preparation process significantly altered the rheological properties.
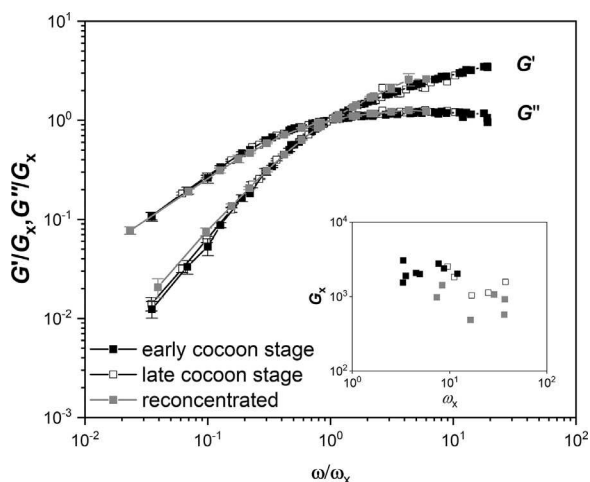
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Fig. 1. Oscillatory test for early and late cocoon as well as reconcentrated silk. The storage (G') and loss (G'') modulus for all three samples against angular frequency (ω) show the same behaviour when normalised to the cross over point (denoted as G_x and ω_x) Inset graph: Reconcentrated and late cocoon silk show a higher cross-over frequency and lower dynamic modulus compared to early cocoon silk, which indicates less molecular interaction between the molecular chains. Error bars represents the standard deviation based on at least 5 repeats.

Furthermore, to ensure the entire natural range of silk's rheological properties were accounted for, NSF samples were extracted directly from *B. mori* silkworms, at different stages during cocoon construction [36]. In order to compare oscillatory data from the different samples, the values of storage and loss moduli (G', G'') and angular frequency were normalised by their cross-over values (Fig. 1). Although the cross-over points varied extensively and systematically with the flow properties (Fig. 1 inset), this normalisation removed the majority of the variation between different feedstock samples, whether taken directly from the silkworms, or after reconcentration, as noted previously [54]. Close scrutiny revealed small differences in G'/G_x between the samples at low frequencies; however, these were not considered to be significant, in view of the larger effects of adding metal ions. Hence these results indicate that dilution and reconcentration on its own did not significantly alter silk's rheological properties.

However, differences were observed when comparing the flow curves of all three feedstock samples (Fig. 2). Higher viscosities were observed from samples obtained during the early stages of cocoon construction compared with later samples (Fig. 2). This phenomenon has been previously observed and discussed by our group [36] and led to the hypothesis that Ca^{2+} ions form inter-protein salt bridges and thus increase feedstock viscosity in the early cocoon stage. Later, ionic screening through an increase of K^+ ions in the feedstock causes shorter lifetimes (i.e. faster exchange) of the salt bridges, thereby reducing the viscosity [36]. This hypothesis has recently been tested and refined, based on predictions from a 'sticky reptation' model of silk rheological behaviour [55].

It should be noted that these observations were all made using samples from middle posterior gland sections, in order to avoid the significant addition of sericin to the fibroin solution that happens further along the middle section [53]. Consequently, our observations do not imply that silkworms spin fibres from NSF of significantly varying viscosity, however; we think it more likely that the silkworm controls the ion content of the feedstock closer to the spinneret, to provide a consistent viscosity. This would be consistent with the studies from Zhou et al. [17] and Wang et al. [18] who have already shown that the Ca^{2+} concentration decreases (-500%) from the MP to the anterior gland section, while the K^+ concentration increases (+300%).

Moving beyond pseudo-static studies, when sheared, it appeared that the initially high viscosity of the 'early cocoon' sample decreased rapidly, in a way not shown by the other samples (Fig. 2). This may indicate that flow stress caused breakdown of the network formed by the salt bridging Ca^{2+} ions in the early cocoon stage samples, resulting in the sharp drop in viscosity not seen in the other samples. Moreover, it also appeared that over the timescale of this experiment (5 min), this network breakdown was not able to recover, as the viscosity only regained around 10% of its initial value on returning to low shear rates (right-hand side of the plot in Fig. 2).

In addition to differences in viscosity, polarised light microscopy revealed that the feedstocks' sensitivity for molecular alignment and aggregation also changed between different cocoon stages (Fig. 2). In the early cocoon stage there appeared to be little molecular alignment under flow, as indicated by the absence of birefringence during shearing. In contrast, silk from a late cocoon stage showed strong birefringence, which ultimately led to aggregation and silk gelation. These differences in flow-induced orientation were also corroborated by first normal stress measurements (not shown), which increased more rapidly at lower shear rates, for the later stage samples. From our previous work where we employed rheo-FTIR to study silk under flow [56,57] we know that molecular alignment in silk is correlated with orientation of the protein backbone in the flow direction while aggregated samples show an increase in β-sheet content. While the rheo-microscopy setup used in this work prevented similar measurements, an increase in axial force associated with aggregation was seen on both the rheo-FTIR and rheo-microscopy experiments, reassuring us that the material was responding to flow in the same manner.

We hypothesise that the strong tendency towards alignment and aggregation was caused by the increased amount of K^+ ions in the silk feedstock at the late cocoon stage, weakening the network and allowing protein coils to move, deform, rearrange, align and interact with each other more easily, producing a higher susceptibility for registration and subsequent aggregation [4,36]. Of particular note is that the flow behaviour of our reconcentrated silk fell between these two extremes, thus making it a reassuringly representative native silk sample.

3.2. Effect of adding various metal ions on the properties of reconcentrated native silk

After adding metal ions to the dilute silk solutions in different amounts (100 and 3000 +ve charges per fibroin chain), the samples were reconcentrated to natural silk concentrations (~21% protein dry weight [53], see Table S1). Much like the control experiments, the addition of salts did not seem to have a measureable influence on the linear oscillatory behaviour of silk (data not shown); however, when subjected to a non-linear deformation, the flow curves showed distinct differences (Fig. 3).

Adding 100 +ve charges of K^+ and Ca^{2+} ions per fibroin chain, equivalent to that found in the native silk feedstock [17,36], did not significantly change the viscosity profile. However, for both K^+ and Ca^{2+} ions, some birefringence was visible, suggesting molecular alignment, and samples aggregated towards the end of shearing, as indicated by their increasing viscosity. Whilst a more significant response was expected, it may be that during sample preparation, the addition of the salts to a dilute solution favoured intramolecular salt bridge formation, which constrained the protein conformation into a tighter coil and reduced the opportunities for new intermolecular associations to occur when the sample was reconcentrated.

To probe this, an excess of 3000 +ve charges per fibroin chain were added. Firstly, $CaCl_2$ samples with 3000 +ve charges per
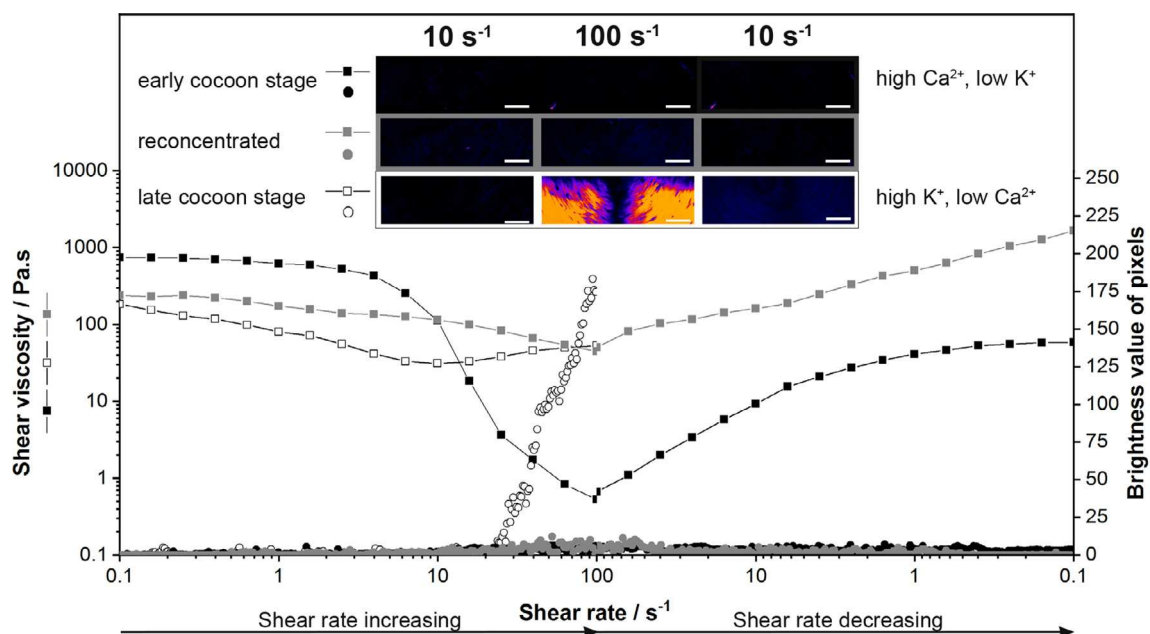
Fig. 2. The shear viscosity and pixel brightness values are plotted against shear rate from $0.1 s^{-1}$ to $100 \ s^{-1}$ and back to $0.1 s^{-1}$ for early and late cocoon as well as reconcentrated silk. The inset pictures show micrographs at $10 \ s^{-1}$, $100 \ s^{-1}$ and again $10 \ s^{-1}$. For better visualisation, each micrograph shows only half of the area of the 8 mm parallel plate. The scale bar is 1 mm and the flow direction is clockwise. Higher resolution images of the inset pictures can be found in Figure S1 in the Supplementary Information.
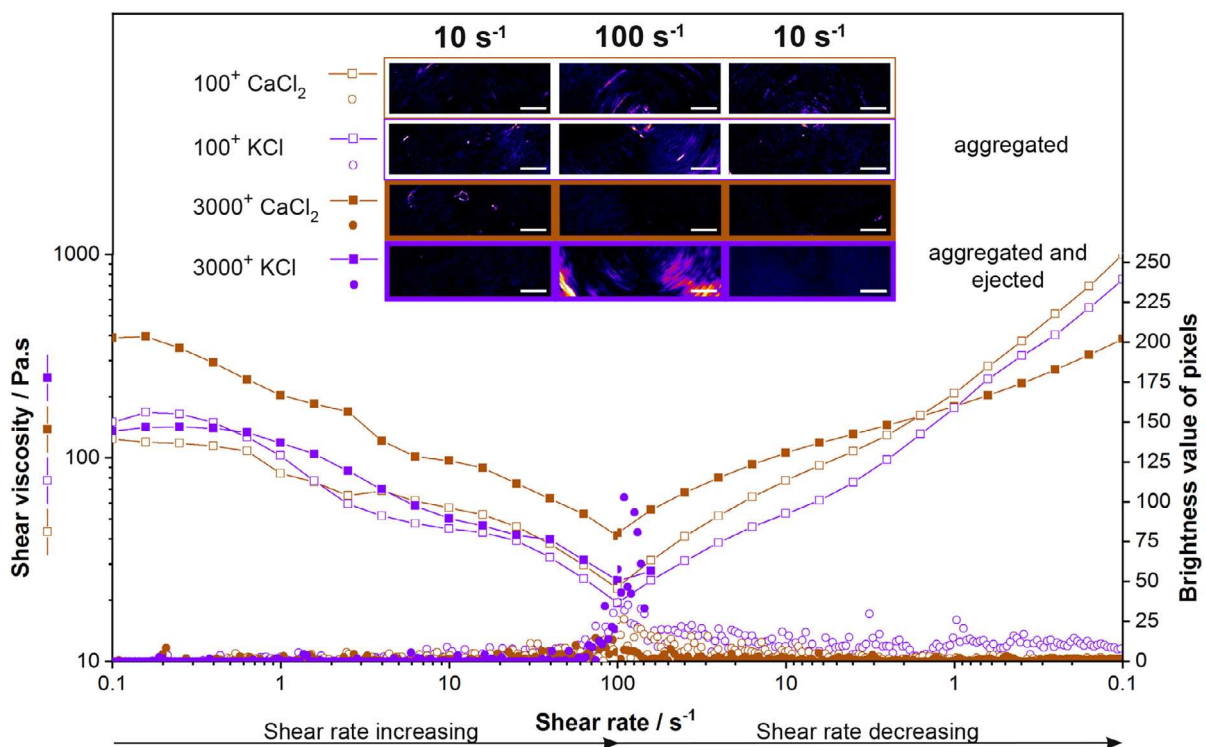

Fig. 3. The shear viscosity and pixel brightness values are plotted against shear rate from $0.1 s^{-1}$ to $100 \ s^{-1}$ and back for KCl and $CaCl_2$ with 100 positive charges per fibroin chain and 3000 positive charges per fibroin chain. The inset pictures show the micrographs at $10 \ s^{-1}$, $100 \ s^{-1}$ and again $10 s^{-1}$. For better visualisation, each micrograph shows only half of the area of the 8 mm parallel plate. The scale bar is 1 mm and the flow direction is clockwise. The sample with 3000 K^+ per chain aggregated so much that it was ejected from the plate/plate geometry. Higher resolution images of the inset pictures can be found in Figure S1 in the Supplementary Information.

chain exhibited a much higher initial viscosity than with 100 +ve charges per chain, consistent with salt bridge formation and the sticky reptation hypothesis. The alignment and aggregation behaviour became very similar to native silk from an early cocoon stage (Fig. 1), suggesting intermolecular salt bridging was occurring. In this case, however, no birefringence was detectable, sug-

gesting that molecular alignment did not occur in the samples with 3000 additional +ve charges per chain, using $CaCl_2$.

In contrast, the 3000 KCl samples displayed alignment and aggregation similar to native silk from a late cocoon stage. This provides clear evidence that the differences in the flow and aggregation behaviour of early and late cocoon native silk feedstocks are

dependent on the Ca^{2+} and K^+ ions present, proving our previous hypothesis [36] and confirming recent modelling predictions [55]. Although the pH decreased slightly upon addition of the Ca^{2+} and K^+ salts, the tendency towards aggregation was much higher at 3000 +ve charges which had a higher pH compared to 100 +ve charges. Therefore, the aggregation behaviour can be attributed to the effect of the metal ions rather than the drop in pH.

It may be noted, however, that the sample preparation method used did not guarantee equivalent ionic strengths for each solution. The ionic strength produced by adding salts depends on the dissociation equilibrium, which depends on concentration. In the present context, it is expected that KCl would be more dissociated than $CaCl_2$. Moreover, it should be noted that the degree of dissociation (particularly of the Ca^{2+} salt) is likely to be higher under more dilute conditions. A level of 100 +ve charges per chain in 21 % w/w fibroin solution corresponds to 0.05 M for KCl or 0.025 M for $CaCl_2$. Under these fairly dilute conditions, essentially complete dissociation can be expected for salts with 'strong' anions such as Cl^-; hence, the ionic content would relate directly to the concentration. The higher level of 3000 +ve charges per chain corresponds to around 1.5 M for the monovalent cations or 0.75 M for the Ca^{2+} salt. Under these conditions, complete dissociation cannot be assumed. Moreover, as the degree of dissociation also depends on the anions, carboxylate salts (including those with Asp or Glu side-groups) are expected to be less dissociated than the added halides, with the consequence that saturation of ionic bridge formation can be expected for Ca^{2+} at 3000 +ve charges per chain.

It may also be noted that, in previous work [7], we have suggested the importance of the hydration shell around fibroin in solution, and its destabilisation as a precursor to gelation. Thus, it is possible that the viscosity or a predisposition to aggregation may be affected through competition for the hydration shell of the fibroin – particularly at higher salt levels.

FTIR measurements were performed to investigate conformational changes induced by the addition of the metal ions (Figure S2). Yet, for our samples, the direct spectra and the corresponding second derivatives only indicated slight changes in the amide I peak (around 1640 cm^{-1}, predominantly due to the $>C=O$ stretching vibration), suggesting that the added salts did not significantly affect the overall chain conformations in the dried film specimens examined. A band centered at 1640 cm^{-1} has previously been ascribed to a random coil configuration [58–60] which is consistent with expectations for fibroin in solution. Moreover, rather unexpectedly, larger changes were indicated in the amide II band (around 1525 cm^{-1}, due to a combination of C-N stretching and N-H bending vibrations). As these changes may indicate complexation between the peptide group and the added salts, this also raises uncertainty in the interpretation of the amide I peak. Consequently, this is an important issue that merits investigation; however, a more detailed analysis is beyond the scope of the present work.

3.3. Effect of adding various metal ions on the aggregation of dilute fibroin

The difference in the aggregation behaviour of silk due to the presence of Ca^{2+} and K^+ ions was also observed by DLS in the dilute solutions before reconcentration (Fig. 4). Addition of $CaCl_2$ at 100 or 3000 +ve charges per chain produced considerable increases in the hydrodynamic diameter (d_H), corresponding to a decrease in diffusion and suggesting stronger interactions between the silk protein chains or aggregate formation. By contrast, addition of KCl at 100+ charges per chain produced no observable effect, while the effect at 3000+ charges per chain was significantly less than that produced by Ca^{2+} at 100+ charges per chain.
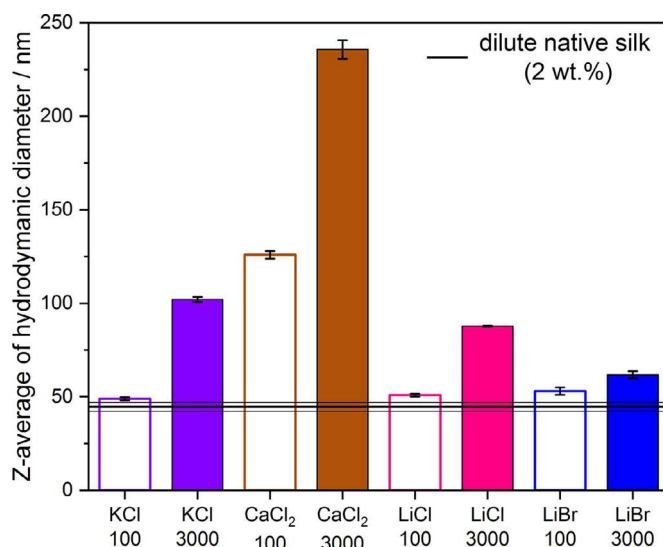

Fig. 4. The Z-average of the hydrodynamic diameter determined by DLS measurements for dilute silk solutions (2 wt.%) when different metal ions are added (numbers underneath bar labels refer to charges per chain). The horizontal line indicates the value obtained for diluted NSF. Error bars represents the standard deviation based on 3 repeats.

Although these observations are consistent with the suggested roles of Ca^{2+} and K^+ in the sticky reptation hypothesis, they are still only circumstantial. The field remains open for more direct evidence, which may arise from further DLS and rheology, or other methods (e.g. circular dichroism, conductivity or small-angle scattering measurements). This is beyond the present study, but will be addressed in further work.

To probe the effect of other ions on silk feedstock's rheological properties, LiCl and LiBr were tested, as both salts are key components of well-known solvents for spun silk fibres [33,61]. Addition of either salt at a level of 100 positive charges per chain produced only very small increases in d_H, while the effects at 3000+ charges were considerably smaller than that produced by the same concentration of KCl.

In addition to changes in the average values of d_H, polydispersity data from DLS was examined. It may be expected that aggregation would cause broadening of the size distribution and polymodality; however, no systematic changes in polydispersity due to added salts were observable in the present data. Instead, it appeared that all the protein was affected equally, which may be explained by the expected dynamic nature of the salt bridges over the timescale of the DLS measurements.

Unsurprisingly, almost no aggregation in silk solutions with added LiCl or LiBr was observable during flow, although the samples at the lower level of 100+ charges had aggregated when the shearing was stopped. Moreover, alignment developed as shear rate increased, but it diminished again as the shear rate decreased (Fig. 5). We suggest that for 100+ charge samples the high shear rate allowed the chains to come closer together, which facilitated registration and subsequent aggregation. However, while the alignment of the chains at 3000+ charges was much more pronounced, no aggregation occurred, suggesting that the alignment was completely reversible under these conditions, which may be of relevance for future liquid crystal inspired artificial spinning strategies [62].

We hypothesise that the increased sensitivity for alignment for both LiBr and LiCl was conferred by Li^+, a kosmotrope which strongly binds water molecules due to its high charge density [63]. Previously it has also been suggested that Li^+ interacts strongly with peptide groups [64,65]; either the peptide carbonyl interacts directly with the Li^+ cations or via water molecules of the lithium
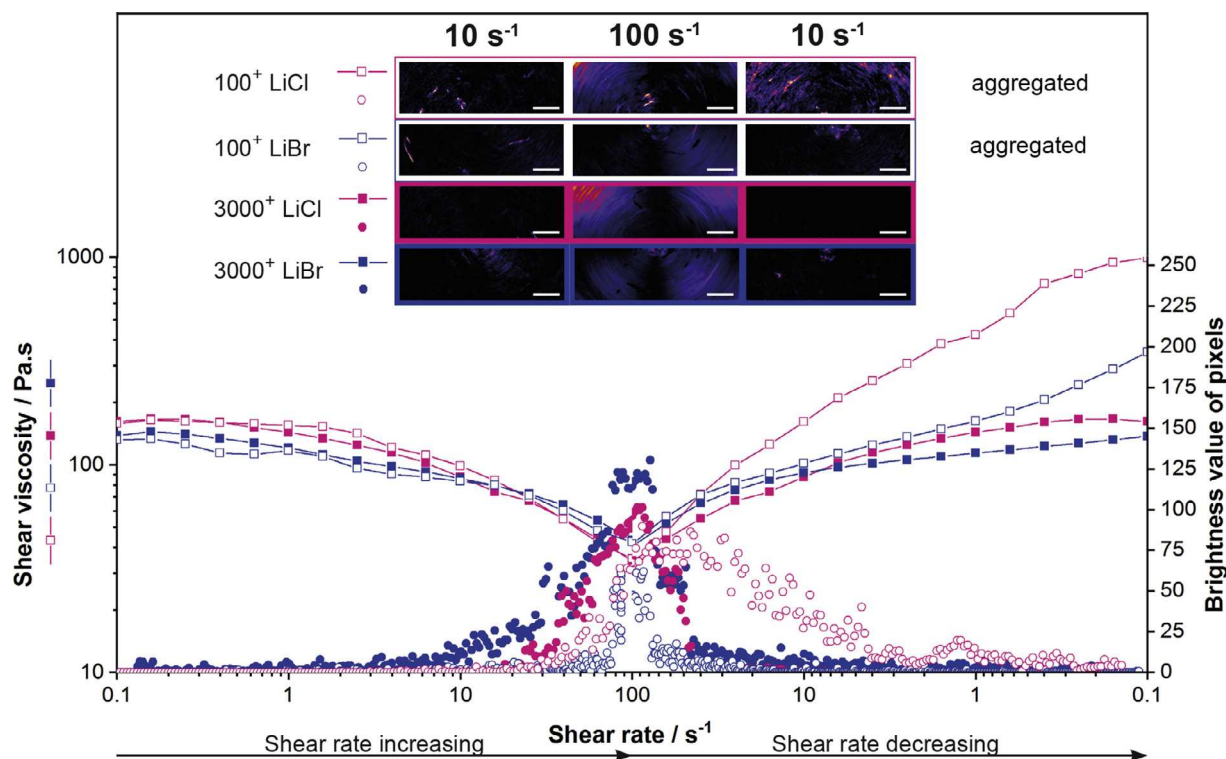
Fig. 5. The shear viscosity and pixel brightness values are plotted against shear rate from $0.1 s^{-1}$ to 100 s^{-1} and back for LiBr and LiCl with 100 positive charges per fibroin chain and 3000 positive charges per fibroin chain. The inset pictures show the micrographs at 10 s^{-1}, 100 s^{-1} and again 10 s^{-1}. For better visualisation, each micrograph shows only half of the area of the 8 mm parallel plate. The scale bar is 1 mm and the flow direction is clockwise. Higher resolution images of the inset pictures can be found in Figure S1 in the Supplementary Information.

Table 1
Qualitative illustration of how the combination of different cations and anions changes the sensitivity of the silk feedstock for molecular alignment and aggregation. The check marks show the tendency towards aggregation and alignment from low (one check mark), medium (two check marks) to high (three check marks). The red "x" illustrates that no alignment or aggregation occurs.

Cation	Anion	100⁺ per fibroin chain		3000⁺ per fibroin chain	
		Alignment	Aggregation	Alignment	Aggregation
K^+	Cl^-	✓	✓	✓✓✓	✓✓✓
Ca^{2+}	Cl^-	✓	✓	✗	✓
Li^+	Cl^-	✓✓✓	✓✓	✓✓✓	✗
Li^+	Br^-	✓✓	✓✓	✓✓	✗

hydration sphere, thereby reducing intermolecular hydrogen bonding between protein chains. Thus, at high concentrations (3000⁺ charges) lithium salts are able to prevent formation of larger aggregates compared to other salt solutions [64], which is supported by our DLS data in Fig. 4.

4. Conclusions

In this study we provide rheological evidence that metal ions can prepare silk proteins for either storage or spinning by altering their propensity to orient and aggregate under flow. We show that the addition of Ca^{2+} ions induces a storage stable feedstock by increasing viscosity, which we attribute to an increase in intra- and interchain interactions, presumably by salt bridge formation between charged side groups. In contrast, K^+ ions prime silk for spinning by lowering feedstock viscosity and increasing protein sensitivity for molecular alignment, registration and aggregation. However, there is a limit to suppression of salt bridge formation; by substituting K^+ for Li^+, a common component of solvents for silk regeneration, we find that Li^+ reduces molecular interactions to the extreme and ultimately prevents aggregation (see overview in Table 1).

In summary, from an evolutionary perspective, the ability to control the bulk rheological properties of silk feedstocks through the simple addition of metal ions appears to be a very elegant and practical way to maintain NSF in a stable storage condition, then activate the proteins when required for spinning. From an industrial perspective, not only does this work provide another process variable in bioinspired silk production, but silk feedstocks themselves may provide insights into the design of biomaterials that are able to activate complex, hierarchical self-assembly processes on demand [66].

4.1. Statement of Significance

Silk fibres exhibit an impressive combination of strength and toughness that cannot be replicated in synthetic materials. To achieve this, Nature has developed a range of elegant means to control the mechanical properties of silk as it is spun from gel into fibre, which we investigate here. By rheo-optical measurements we find Ca^{2+} and K^+ control the aggregation and alignment of silk proteins in solution and thus serve to condition them for either storage or spinning. We believe our experimental approach and results provide new insights into how metal ions can promote or suppress self-assembly of silks, validating both recent flow-models and with potential application to other hierarchical biomaterials in general (i.e. mucus, alginates).

Declaration of Competing Interest

None.

Acknowledgements

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2020.09.045.

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