Correlation between microstructure and microrheological parameters of various silk fibres

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The changes in microcrystalline parameters of raw wild varieties of silk fibres, like tasar, muga and eri, have been studied using wide angle X-ray scattering technique and a line profile analysis. A method involving an exponential distribution has been used to compute the microstructural parameters for the crystallite. In addition, a home-built open microscope set-up is also used for determining the microrheological parameters for all the three silk varieties in solution form. A comparative study reveals interesting correlations in the relative strengths of the varieties of silk fibres in both crystalline form and in solution. Further, the findings also reveal that muga is stiffer than the other non-mulberry silk varieties and this is observed in both the forms.

Keywords: Microcrystalline parameters, Microrheology, Silk fibroin, Wide angle X-ray scattering

1 Introduction
Silk is semi crystalline protein polymer that is spun into fibre by some Lepidoptera larvae, such as silk worms, spiders, scorpions, mites and flies. There are two classes of silks, viz. mulberry (Bombyx mori) and non-mulberry (tasar, muga and eri). These are commercial varieties of silk fibres produced in India and are extensively used in the Indian textile industry. A knowledge of the microstructural differences between these fibres helps in making a comparative assessment of the strength and properties of the fibres. Macrostructural studies such as measurements of cross-section of silk fibres and densities of the Indian silk varieties have been reported by Sen and Murugesh through the analyses of silk fibroin amino acid contents. Rajkhowa et al. have studied the tensile stress-strain relation with XRD. Kothari et al. have observed that the stress relaxation is significantly greater in non-mulberry silk than in mulberry silk and that the differences among non-mulberry silk fibres are relatively small.

The mechanical properties of regenerated silk fibroin (RSF) polymer solutions of Bombyx mori silk, using microrheological techniques have been studied earlier. The unique mechanical properties of the RSF make it especially interesting for many different applications, apart from its wide use in the textile industry, as mentioned earlier. A recent study on the effect of shear field (spinning rate) and temperature on rheological properties of native silkworm and native spider dope has shown that both dopes behave like polymer melts. These findings provide a better understanding of silk formation from the silk worm, and have tremendous potential in enabling industrial production of silk that matches the qualities of natural silk.

In the present work, wide angle X-ray scattering (WAXS) technique has been used to study the microcrystalline parameters, such as crystal size and lattice disorder by employing Fourier method. The study also reports the line profile analysis of Bragg reflections observed in X-ray patterns of the three Indian silk fibres. Crystal imperfection parameters are computed with exponential distribution functions for each of the samples, after adopting robust refinement procedures. Crystallite shape ellipses were obtained by plotting the crystallite size along two orthogonal directions of

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the crystal for all the silk fibres. The areas of these ellipses are indicators of the differences in stiffness between the silk fibres.

Video microscopic technique has been used to study the micro rheological properties of the RSF solutions. The position of an embedded tracer polystyrene bead is recorded in silk fibroin solution of the same concentration for all the three varieties in order to measure the storage and loss moduli and thus to characterize the viscoelastic properties of these media. The computed micro rheological parameters from such an analysis are compared with microstructural parameters of the silk fibres.

This study further reports the analysis of the relative stiffness of two different forms of the silk, one in crystallite form and the other in polymer solution form. The studies reveal a correlation in the relative strengths of the forms, for all the silk varieties. For example, the Muga silk variety that shows the largest crystallite shape ellipse area, and hence greater stiffness, also exhibits in solution form. The studies reveal a correlation in the relative stiffness of two different forms of the silk, one in crystallite form and the other in polymer solution. For a paracrystalline material, $A_d(n)$ can be obtained using the following Gaussian strain distribution:

$$A_d(n) = \exp \left( -2\pi^2 n^2 g^2 \right)$$

where $m$ is the order of the reflection; and $g = \Delta d/d$, the lattice strain. Normally, one also defines the mean square strain $\langle \epsilon_i \rangle$, which is given by $\sum g^2/n$. This mean square strain is dependent on $n$, whereas $g$ is independent of $n$ (refs 11, 13). For a probability distribution of column lengths $P(i)$, following relationship is obtained:

$$A_i(n) = 1 - \frac{nd}{D} \frac{d}{D} \int_0^n P(n) \, dn - \int_0^n P(i) \, di$$

where $D = \langle N \rangle d_{\text{std}}$ is the crystallite size; and $i$, the number of unit cells in a column. In the presence of two orders of reflections from the same set of Bragg planes, Warren$^{11}$ has demonstrated a method of obtaining the crystal size $\langle N \rangle$ and lattice strain ($g$ in %). But in polymers it is very rare to find multiple reflections. In order to determine the finer details of microstructure, the size profile was approximated by a simple analytical function for $P(i)$ by considering exponential functions. Another advantage of this method is that the distribution function differs along different directions$^{14,15}$. Here, it is emphasized that the Fourier method of profile analysis (single order method used here) is quite a reliable one, according to the recent survey and the results of Round Robin test conducted by IUCr$^{16}$. In fact, for further refinement of microstructural parameters, the effect of the background was also corrected by introducing a parameter$^{17}$.

2 Theoretical Considerations

2.1 Line Profile Analysis Theory

Micro structural parameters such as crystal size ($\langle N \rangle$) and lattice strain ($g$ in %) are usually determined by employing Fourier method of Warren$^{13}$. The intensity of a profile ($I(s)$) in the direction joining the origin to the center of the reflection can be expanded in terms of the Fourier cosine series, as shown below:

$$I(s) = \sum_{n=-\infty}^{\infty} A(n) \cos \left( 2\pi nd \left( s - s_0 \right) \right)$$

The coefficients of the harmonics $A(n)$ are functions of the size of the crystallite and the disorder of the lattice. Here, $s$ is the $\sin(\theta)/\lambda$; $s_0$, being the value of $s$ at the peak of a profile; $n$, the harmonic order of coefficient; and $d$, the lattice spacing. The Fourier coefficients can be expressed as

$$A(n) = A_s(n) A_d(n)$$
\[ P(i) = \begin{cases} 
0 & \text{if } i < p \\
\alpha_w \exp\left(-\alpha_w(i-p)\right) & \text{if } i \geq p 
\end{cases} \quad \text{(5)} \]

where \( \alpha_w = 1/N - p \) is the width of the distribution function. On substituting this in Eq. (4), following relationship is obtained:

\[ A_n(n) = \begin{cases} 
A(0)(1-n/(N)) & \text{if } n < p \\
A(0)\exp\left(-\alpha_w(n-p)\right)/(\alpha_wN) & \text{if } n \geq p 
\end{cases} \quad \text{(6)} \]

Here \( \langle N \rangle \) is the number of unit cells counted in a direction perpendicular to the \((hkl)\) Bragg plane.

The procedure adopted for the computation of the parameters is as follows: initial values of \( g \) and \( N \) were obtained using the method as reported by Nandi et al.\(^{19}\). Using these values in Eqs (1)-(6), the corresponding value for the width of the distribution is obtained. These are only rough estimates, so the refinement procedure must be sufficiently robust to start with such values. Here, standard deviation for the microstructural parameters is computed by the following relationship:

\[ \Delta^2 = \frac{I_{\text{cal}} - (I_{\text{exp}} + BG)^2}{npt} \quad \text{(7)} \]

where \( BG \) represents the error in the background estimation; \( npt \), the number of data points in a profile; \( I_{\text{cal}} \), the intensity calculated using Eqs (1)-(6); and \( I_{\text{exp}} \), the experimental intensity. The values of \( \Delta \) were divided by half the maximum value of intensity, so that it is expressed relative to the mean value of intensities, and then minimized.

### 2.3 Microrheology

The position information of a bead of radius \( a \) embedded in the given medium at a temperature \( T \) is used to obtain its time dependent mean squared displacement (MSD=\( \langle \Delta r^2(t) \rangle \)) in that medium\(^{20}\), as given below:

\[ \langle \Delta r^2(t) \rangle = \langle (r(t+\tau) - r(t))^2 \rangle \quad \text{(8)} \]

Here \( \tau \) is the lag time; and \( \langle \rangle \) denotes (i) the average being calculated for all starting times \( t \) for a single bead and also (ii) the ensemble average of MSD for a group of beads. The complex shear modulus \( G'(\omega) \) is calculated from MSD\(^{21,22}\) using the following equation,

\[ G'(\omega) = \frac{k_B T}{\pi a \langle \Delta r^2(1/\omega) \rangle \Gamma\left[1+\alpha(\omega)\right]\left(1+\beta(\omega)/2\right)} \quad \text{(9)} \]

Here \( \alpha(\omega) \) and \( \beta(\omega) \) are the first and second order logarithmic time derivatives of the MSD; \( \Gamma \), the \( \Gamma \) - function; and \( k_B T \), the thermal energy of the RSF medium.

### 3 Materials and Methods

#### 3.1 Sample Preparation for WAXS

Silk cocoons of various wild varieties were collected from the germplasm stock of the Central Sericulture Training and Research Institute (CSTRI), Bangalore and then reeled following the standard procedures. First, the cocoons were cooked in boiling water for 2 min to soften the sericin and later transferred to water bath at 65ºC for 2 min. Then, the cocoons were reeled in warm water with the help of reeling equipment known as Epprouvite.

#### 3.2 Sample Preparation for Microrheology

Raw silk samples of all three varieties were first cleaned with distilled water and then degummed to remove sericin following the standard methods\(^4,7\). A 75:25 (weight ratio) mixture of Ca (NO\(_3\))\(_2\) 4H\(_2\)O and absolute methanol (CH\(_3\)OH), which has the strongest dissolving capacity for the silk fibroin, was used to dissolve silk fibroin\(^6,7\). 200mg of silk was dissolved in 20 mL of solvent to obtain 1.00% (w/v) regenerated silk fibroin (RSF) solution for all the three varieties\(^{22}\). Polystyrene beads (Cat. No. 07310-15, 0.989 ± 0.02 µm, Polysciences Inc., USA) were added to these samples at low concentrations (5 µL in 1 mL of RSF) to avoid interbead interactions. These beads are used as probes to characterize the samples. About 40 µL of sample was taken in a well constructed by an ‘O’ ring (diameter 10 mm and thickness 1 mm) on a coverslip (number 1 grade) for each trial. The sample was agitated gently by a micropip to achieve uniform mixing of beads in solution for about a min and then covered with another small coverslip. The sample was finally allowed to relax for 30 min, before each measurement.
3.3 X-Ray Diffraction Pattern

The XRD diffractograms of the polymer samples were recorded using a X’Pert Pro X-ray diffractometer with Ni filtered, CuKα radiation of wavelength λ = 1.5406 Å, with a graphite monochromator. The scattered beam was focused on a detector. The specifications used for the recordings were 40 kV, 30 mA. The samples were scanned in the 2θ range 12º-100º with a scanning step size of 0.017º. Figure 1 shows the X-ray diffraction patterns obtained for tasar, muga and eri silk fibres, which are essentially silk II modification.

3.4 X-ray Profile Analysis

For the analysis, X-ray diffraction data have been used in Eqs (1)-(7) to simulate the intensity profile by varying the necessary parameters till one gets a good fit with the experimental profile. For this purpose, a multi-dimensional algorithm ‘SIMPLEX’ was used for minimization\(^23\). The computed crystal imperfection parameters are given in Table 1 for exponential distribution functions for each of the samples.

3.5 Video Microscopy

Brownian motion of the beads in 1.00% RSF solutions of three silk varieties was recorded using our open microscope\(^24\). Nearly 1000 images were acquired for each measurement at a rate of 10 fps, after focusing the microscope well above (about 30 µm) the cover slip to avoid bead surface hydrodynamic interactions. The on-screen magnification of the imaging system was determined by camera pixel density of 1280×1024 (Electrim, USA) and optical magnification of ×100 (1.25 NA objective, Olympus)\(^25,26\). This is 63 nm/pixel for our system. Through multi-particle tracking, 5-8 beads were tracked simultaneously for each field of view, and this helps in saving time while obtaining good statistics of the data from only a few movies. A feature finding algorithm, originally by Crocker and Grier in IDLVM software\(^25\) is used for tracking the beads in non-deinterlaced fields of the movie. The tracking measurements are based on a centroid localization algorithm performed on sequenced image frames, which can detect displacements of a bead with a precision of a few tenths of a nanometer\(^25,26\). The final resolution achievable depends not only on the choice of algorithm but also on signal-to-noise ratio of the raw data.

Table 1—Microstructural parameters of tasar, muga and eri silk fibres by using exponential distribution functions

<table>
<thead>
<tr>
<th>Fibre</th>
<th>Profile No.</th>
<th>2θ, deg</th>
<th>g, %</th>
<th>delta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tasar</td>
<td>1</td>
<td>16.62</td>
<td>0.5</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20.08</td>
<td>0.5</td>
<td>0.05</td>
</tr>
<tr>
<td>Muga</td>
<td>1</td>
<td>17.04</td>
<td>0.3</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20.08</td>
<td>0.1</td>
<td>0.05</td>
</tr>
<tr>
<td>Eri</td>
<td>1</td>
<td>16.76</td>
<td>0.5</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20.08</td>
<td>1.0</td>
<td>0.04</td>
</tr>
</tbody>
</table>
4 Results and Discussion

The simulated profile was obtained using the Eqs (1)-(6) with appropriate model parameters. Goodness of the fit between the simulated and experimental profiles was around 50% (not shown here). The computed microcrystalline parameters, such as crystallite size (number of unit cells) $\langle N \rangle$, lattice strain (g in %), and the standard deviation are given in Table 1. It is observed that the lattice strain in raw wild variety of silk fibre is small in muga and reasonably large in tasar fibre. Here, the standard deviation in all the cases for the microstructural parameters are taken as delta (Table 1).

A graphical part of the crystallite shape ellipse was obtained by taking the crystal size value corresponding to $2\theta = 16.5^\circ$ (020) direction along the x-axis and the other parameter corresponding to $2\theta = 20^\circ$ (210) direction along the y-axis from the Fig. 1 data. The angle between (020) and (210) planes is around 90°. These crystallite shape ellipse for the three silk varieties is shown in Fig. 2. The strength of silk fibre is normally proportional to crystalline area which is equal to ellipse area determined by microstructural parameters. It is evident that the crystallite shape ellipse area in muga is greater compared to eri and tasar, while the value of the crystallite shape area of eri lies in between muga and tasar. Sen and Murugesh\textsuperscript{3} found that muga exhibits the highest density, followed by tasar and eri among the non mulberry silk varieties. This suggests that the degree of crystallinity and crystallite orientation are more in muga as compared to other varieties. The WAXS studies suggest that the eri have highest intensity (> 400; Fig. 1) for 110 planes than muga (> 350) and tasar (> 300) silk, indicating the high degree of crystallinity for eri silk, although its absolute measurement is difficult.

The video microscopy set-up for rheological studies has been calibrated using Newtonian liquids such as pure water and pure glycerol. The details of this calibration procedure can be found elsewhere\textsuperscript{6}. Briefly, the measured viscosities for water and glycerol are found to be in agreement with the standard values at 22°C. This microrheological technique is suitable for characterizing mechanical properties of liquids. With this set-up, one can probe materials having shear moduli values lying between $5 \times 10^{-4}$ Pa and 1 Pa (ref. 6). For these reasons, concentrations of silk sample are chosen so as to lie within the measurement range of set-up.

In video microscopy, the static error $\bar{\varepsilon}_2$ in tracking the embedded beads was estimated by analyzing the positions of $2a = 0.989$ µm beads stuck to the coverslip in 1000 frames maintaining nearly the same environment used for the analysis of RSF samples. This gives the value of spatial resolution $\bar{\varepsilon}_2 = 18$ nm from the MSD via the relationship $\bar{\varepsilon}_2 = \sqrt{\left\langle (\Delta r^2(\tau)) / 2 \right\rangle}$ at short lag times for which statistical accuracy is the best\textsuperscript{27}. At longer lag times (above 60 s), the static error for stuck beads in our set-up, using feature tracking, is increased to a value of 33 nm due to the statistical inaccuracy in obtaining MSD with only few data points. It was also found that, at even longer lag times this static error further increases (though marginal) with lag time. The reason for this monotonous increase at high lag times is yet to be understood. Further, dynamic error also contributes to spatial error during particle tracking in microrheological studies. It has been found that the spatial error follows the same temporal distribution as the pixel intensity noise in the movie. The dynamic error depends on the exposure time $\sigma$ of the camera\textsuperscript{27}. With these spatial errors, the measured MSD could be given as\textsuperscript{27}

$$\left\langle \Delta r^2(\tau, \sigma) \right\rangle_{\text{measured}} = 2D(\tau - \sigma / 3) + 2\bar{\varepsilon}_2^2 \quad \ldots \quad (10)$$

Fig. 2—Variation in crystallite shape ellipse for raw wild varieties of silk fibres.
where \( D = k_B T / 6\pi \eta a \) is the bead’s diffusion coefficient in a medium of viscosity \( \eta \). By using \( \sigma \) value to 1/10th of lag time (i.e. 0.01s), it has been ensured that the contribution of dynamic error to MSD is minimum. Super diffusion in the MSD values at very short lag times (between 0.1 s and 0.4 s) has been observed due to the dynamic error in the set-up. For this reason, the data up to 0.4 s has been removed from the MSD values.

From the measured value of \( \bar{\varepsilon}_2 \) for the entire range, one can remove static error by using Eq. (10), to obtain static error free MSD (\( \langle \Delta r^2(\tau, \sigma) \rangle_{s\text{free}} \)) data by modeling it as

\[
\langle \Delta r^2(\tau, \sigma) \rangle_{s\text{free}} = \langle \Delta r^2(\tau, \sigma) \rangle_{\text{measured}} - 2\bar{\varepsilon}_2^2 \quad \ldots (11)
\]

Though the dynamic error still persists in the data, this method of removing static error is valid whenever one estimates averaged properties, such as MSD\(^2\).

This calibrated set-up was used for recording the Brownian motions of beads in all the three varieties of silk at a room temperature of 23.5±0.5°C and then analyzed offline. The ensemble averaged MSD of several (80-100) beads in 1.00% RSF of eri, muga and tasar as a function of lag time was calculated using Eq. (8). The static error corrected MSD data were obtained using Eq. (11) (Fig. 3a). All the curves in this plot have varying slope between 0 and 1, a characteristic of viscoelastic materials. At small lag times, the material shows solid like behavior due to the presence of the polymer network of RSF. At higher lag times, RSF sample shows liquid like behavior, as the bead diffuses freely in the sample. The MSD of embedded beads in tasar RSF is greatest and that of muga is lowest at all frequencies, while the MSD of embedded beads in eri lies in the middle. Note that the MSD of beads in pure solvent is much higher than that in the sample for the entire frequency range\(^6\).

The shear moduli of the three samples were calculated using Eq. (9) (Fig. 3b). The shear modulus of tasar RSF is lower and that of muga and eri is higher at all the frequencies. This demonstrates that muga and eri are stiffer than the tasar. In addition, higher mechanical strength suggests that muga has higher molecular weight than other RSF samples. Intrinsic viscosities of 0.76, 0.71 and 0.69 dL/g have been reported respectively for muga, tasar and eri\(^3\) and this trend is borne out in our measurements of shear moduli of the varieties, except in the relative values of viscosities for eri and tasar. Possible reason for this difference may be due to difference in the varieties of silk fibres used in this study as compared to that reported earlier\(^3\).

The relative strengths of the fibres measured in terms of their shear moduli using microrheological techniques, and the fibre stiffness characterized through crystallite shape ellipses computed from WAXS data reveals an interesting correlation between fibre strength and corresponding shear moduli of the same fibre in solution form. This investigation reveals that the silk polymer chain that has the highest (lowest) relative stiffness, measured by WAXS analysis, also gives rise to the highest (lowest) shear modulus in solution form at all frequencies. This interesting correlation seems to be based on the possibility that the entangled network of the fibres with greater stiffness, offers greater resistance to the tracer bead’s movement when it is embedded within a mesh formed by the entangled structure resulting in greater shear modulus of the polymer solution. Although more detailed experiments in future are needed to systematically understand this correlation and possible limits to it, these studies pave the way for further work on the micro structure-rheological property correspondence.

![Fig. 3](image-url)
5 Conclusions
The studies on comparison of the microstructure and microrheological parameters of three wild varieties of Indian silks (muga, eri, and tasar) shows that muga has greater mechanical strength as compared to eri and tasar silk fibres at all the measured frequencies, a result that correlates with the calculations of crystallite shape ellipses computed using line profile analysis of WAXS data recorded from these fibres. The shape ellipses do reveal information about the crystallite region of the fibers in muga silk variety that contributes to making these fibres stiffer than the other two varieties. This demonstrates the correlations between the techniques in the measurement of fibre stiffness.

Industrial Importance: As these findings reveal that muga has greater stiffness than the other non-mulberry silk varieties as manifested in both forms studied, muga silk is recommended in industrial production of silk based materials wherein greater stiffness of the fibre is required.

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