Intrinsic fluorescence changes associated with the conformational state of silk fibroin in biomaterial matrices

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Abstract: Silk fibroin is emerging as an important biomaterial for tissue engineering applications. The ability to monitor non-invasively the structural conformation of silk matrices prior to and following cell seeding could provide important insights with regards to matrix remodeling and cell-matrix interactions that are critical for the functional development of silk-based engineered tissues. Thus, we examined the potential of intrinsic fluorescence as a tool for assessing the structural conformation of silk proteins. Specifically, we characterized the intrinsic fluorescence spectra of silk in solution, gel and scaffold configurations for excitation in the 250 to 335 nm range and emission from 265 to 600 nm. We have identified spectral components that are attributed to tyrosine, tryptophan and crosslinks based on their excitation-emission profiles. We have discovered significant spectral shifts in the emission profiles and relative contributions of these components among the silk solution, gel and scaffold samples that represent enhancements in the levels of crosslinking, hydrophobic and intermolecular interactions that are consistent with an increase in the levels of β-sheet formation and stacking. This information can be easily utilized for the development of simple, non-invasive, ratiometric methods to assess and monitor the structural conformation of silk in engineered tissues.

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References and links
1. Introduction

A variety of degradable polymeric biomaterial devices, such as collagen and poly-DL-lactic-glycolic acid (PLGA)-based sutures and sponges, are used in clinical settings and are designed to match biological, chemical and physical requirements in specific tissue restorative applications [1]. In recent years, interest in these types of biomaterial matrices has expanded into scaffold designs for cell and tissue growth *in vitro* and *in vivo* for tissue engineering [1]. With these expanding uses, it has become more important to understand the structure of the biomaterials used and their remodeling in vitro and in vivo, such that the rates and extent of remodeling can be factored into tissue integration in the form of new extracellular matrix formation and function.

The protein biomaterial silk fibroin has emerged as a useful option for these needs due to the unique mechanical properties, biocompatibility, slow degradability, absence of bioburdens, stabilization by beta sheet physical crosslinks, and versatility in processing and thus functional material properties [2]. In the present work, we focus on characterization of the endogenous fluorescence spectroscopic features of silk-based biomaterials. These degradable protein biomaterial matrices are based on the longstanding biomedical utility of silk fibroin protein from the silkworm, *Bombyx mori*, in the form of sutures [2]. The utility of this protein in a range of biomaterial matrices has recently been explored, including hydrogels, foams, ropes and films [3-7], along with the supporting utility of these systems for cell growth and tissue formation both in vitro and in vivo [8-17]. This broadened utility for cell and tissue culture applications suggests that a better understanding of how these protein matrices are remodeled in specific cell and tissue environments will provide important insight in vitro into how to optimize such material-cell interactions. This understanding will allow rates of matrix regeneration and formation into native tissues to be more predictable as the matrices degrade during remodeling and tissue regeneration. Silk biomaterials are unusual in this regard as they can be designed to degrade slowly (months to years) or rapidly (weeks to months), depending on the mode of processing [2, 16, 18]. This feature is directly related to the extent of beta sheet physical crosslinks that form during processing [16, 19]. In addition, since new extracellular matrix generated by cells growing on these matrices will be comprised primarily of nonsilk protein components such as collagen, chemical and optical distinctions may be feasible to distinguish between the starting matrix and new matrix.

Biomaterial matrix structural changes in culture are often assessed by destructive analytical methods such as X-ray, FTIR, NMR, μCT or TEM. One option for nondestructive imaging is to exploit intrinsic fluorescence spectroscopy due to the presence of crosslinks and aromatic amino acids (tryptophan, tyrosine, phenylalanine) in proteins, with the advantage of avoiding additives to the system that can generate artifacts in interpretation due to perturbation of the system.

Intrinsic fluorescence of proteins has been used in numerous basic biochemical and biophysical studies to probe protein structure and dynamics [20, 21]. Spectral signatures are influenced by the characteristics of the microenvironment and the location (chemical sequence) of the fluorophores in the protein macromolecule [20, 21]. Tryptophan has the highest quantum yield and extinction coefficient of the three aromatic amino acids, and therefore is often used to assess protein structure [22]. For example, tryptophan fluorescence spectra of actin, a cytoskeletal protein, and the contribution of each individual tryptophan was assessed using intrinsic fluorescence spectroscopy [23, 24]. Since the complete primary sequence of silk fibroin protein is known [25], and the mechanism of protein folding and assembly into functional materials has also been described [26], interpretations of spectral changes are feasible.

To begin to map tissue remodeling in the context of silk biomaterials and cell interactions, a baseline understanding of silk protein matrix structure and changes in this structure in vitro or in vivo is critical. Toward this goal, detailed fluorescence spectroscopic assessments of silk fibroin protein matrices prepared in solution, gel and solid state forms (3D scaffolds) are described in the present work. The results provide an initial window into options to track the
silk fibroin matrix structure and remodeling using nondestructive optical imaging and spectroscopy tools.

2. Materials and methods

2.1 Preparation of silk biomaterials

White Japanese raw Bombyx mori silkworm cocoons were boiled for 20 minutes in an aqueous solution of 0.02 M Na₂CO₃ and rinsed with cold de-ionized water to remove the glue-like sericin proteins using methods we have previously reported [27]. The silk fibroin fibers were dissolved in 9 M LiBr solution at 60°C for 4 hours. After complete dissolution, the concentration of the silk solution was about 20 w/v%. The solution was subsequently dialyzed for 2 days (Pierce, MWCO 3500) and after dialysis the concentration was about 8 w/v%. This solution was diluted to 1 w/v% for spectroscopic measurements. All solutions were stored at 7°C to avoid premature gelation. The 8 w/v% silk fibroin solution gelled after about 1 month at 7°C. The preparation of the solid state 3D porous silk fibroin scaffolds has also been previously reported [28]. Briefly, granular NaCl about 500 μm in diameter (4 g) was added to 2 ml of ~8% silk aqueous solution in disk-shaped vials. The vials were covered at room temperature for 24 hours and then submerged in de-ionized water for 2 days at room temperature to remove the NaCl. Detailed structural and morphological features of the above materials have been previously reported [7, 10].

2.2 Preparation of pure component solutions

Pure L-tryptophan and L-tyrosine were purchased from Sigma (St. Louis, MO) and diluted in PBS at a concentration of 0.0186 and 1 mM, respectively. Di-tyrosine was prepared from L-tyrosine as described previously [29] and further purified by high performance liquid chromatography. The fluorescent fractions monitored with excitation and emission wavelengths set to 320nm and 400nm, respectively, were collected, pooled, and lyophilized to dryness. Fluorescence EEMs were acquired from a 0.28 mM sample of di-tyrosine dissolved in water.

2.3 Intrinsic fluorescence spectroscopy of silk solution, gel and scaffold samples

A Hitachi fluorescence spectrophotometer (F4500, 450 W Xenon arc lamp; San Jose, CA) was used to characterize the fluorescence properties of silk solutions, gels and scaffolds. Spectra were collected in the front-face geometry to reduce the effects of scattering, especially in the case of the gels and scaffolds. Samples in solution were placed in a 1 cm path length quartz cuvette; while silk gels were sandwiched between two quartz slides. Since silk scaffolds are a spongy solid, no supporting plates were used for these measurements. Before the start of each experiment, a fluorescence excitation-emission matrix of a rhodamine standard (2.14 μM in ethylene glycol) and a diffuser were acquired and used to account for day to day variations and the spectral response of the instrument. Water was employed to check sensitivity of the instrument, as specified by the manufacturer. Typically, the signal to noise ratio was greater than 100 and drift was less than 1%. Fluorescence excitation-emission matrices were measured incrementally for excitation wavelengths in the 250-335 nm range and corresponding emission in the 250 – 600 nm range in 5 nm increments. The scan speed was 1200 nm/min with excitation and emission slits set to yield a 2.5 nm resolution. The photomultiplier tube detector gain was set to 700 V. These wavelength ranges allowed for intrinsic fluorescence measurements of tyrosine, tryptophan and other endogenous fluorophores in the UV-VIS range.

2.4 Data analysis

To quantify and understand the biochemical origins of the fluorescence EEMs acquired from the different silk samples, we used the alternating least squares (ALS) algorithm of the Matlab PLS Toolbox (Mathworks Inc, Natick MA). The ALS algorithm was developed to extract the spectral line shapes and corresponding concentrations of the components that describe a set of
spectra [30]. Inputs to the algorithm include the data set to be analyzed, the number of components that are expected to describe the spectral features of the data and an initial guess for either the components’ concentrations for each sample or their spectral line shape. To ensure that physiologically meaningful parameters would be extracted from the fits, we imposed non-negativity constraints on both the concentration and spectral profiles of the components. Our goal was to identify the smallest number of components that could be used to describe the data for the range of excitation and emission wavelengths that we examined.

The overall procedure that we used to analyze each set of spectra from the silk solution, gel and scaffold samples is depicted in Fig. 1. We noticed that the emission spectra collected for excitation at 325-335 nm had identical features. Thus, we assumed that a single spectral component can be used to describe the fluorescence emission spectra for 325-335 nm excitation. Using the ALS algorithm, we extracted the basis spectrum of this component and its fluorescence excitation efficiency at each wavelength for each of the silk samples. We also measured the emission spectrum from a tyrosine solution sample and we assumed that tyrosine emission from the silk residues would be the same, since it is known that tyrosine emission is not dependent on its environment and remains fairly constant [31]. Thus, we used the measured tyrosine spectrum and the basis spectrum (attributed most likely to crosslinks) extracted from analysis of the spectra at 325-335 nm excitation as two fixed spectral components that can potentially contribute to emission excited between 250 and 300 nm. The ALS algorithm was used to identify the spectrum of a third component for each of these excitation wavelengths that would be required to describe the remaining features of the measured silk spectra. We then used ALS to identify three spectral components that would optimally describe the spectral variations of this third component for 250-300 nm excitation from each of the samples. These components are attributed to tryptophan residues that experience different microenvironments. We used the resulting tryptophan, tyrosine and crosslink spectral components to fit all the spectra from 250 to 320 nm excitation and to assess the contribution of each chromophore to fluorescence emission at each excitation wavelength and to the overall fluorescence excitation-emission matrix.

Fig. 1. Schematic flow of data analysis steps for silk solution and gel samples (left panel). The slightly modified approach to extract the spectral components contributing to the silk scaffold fluorescence is shown on the right panel.
3. Results and discussion

3.1 Silk fibroin protein composition and structure

The various processing modes and primary sequence of the silk fibroin protein are shown in Fig. 2. The elementary unit of B. mori silk fibroin consists of six sets of a disulfide linked heavy chain-light chain heterodimers and a glycoprotein. Depending on the physical state of silk, there are at least four different types of secondary structures in silk: random coil, $\alpha$-helix, silk I [32], and silk II [33]. Silk fibroin in solution consists of a combination of random-coil, silk I and $\alpha$-helical conformations, corresponding to the non-crystalline region of the protein. A small percentage of $\beta$-sheet silk II secondary structure is also present in the crystalline region as not all $\beta$-sheet structure disappears in solution. However, in the solid form of a gel or a scaffold an increasingly larger percentage of the random coil, silk I, and $\alpha$-helix conformations, is converted into the highly insoluble $\beta$-sheet silk II conformation. The illustrative diagram of Fig. 2 (left column) shows the increased organization of the protein via the formation of $\beta$-sheet cross links as the protein moves from solution to gel and then 3D matrix state. These changes reflect decreased water content to promote increased hydrophobic interactions via the dominating hydrophobic sequences in the protein as evidenced by the glycine and alanine repeats that run throughout the high molecular weight chain of fibroin (Fig. 2, right panel; [25]). The predominance of tyrosines throughout the sequence is evident, while a few tryptophans are also available. The formation of $\beta$-sheet crosslinks implicates highly organized crystalline domains in the material, which are more predominant as the process moves from the solution to the solid state [34]. Furthermore, the presence of extensive crosslinking enhances the probability that tyrosine and tryptophan chromophores are in close proximity.

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Fig. 2. Conformational transitions of silk fibroin protein from solution to gel to 3D scaffold; and relationship between the folding of a single chain of B. mori silkworm silk heavy chain fibroin and its primary sequence. The tyrosine (y) and tryptophan (w) residues are highlighted.
3.2 Intrinsic fluorescence spectra of silk solution, gel and scaffold samples

A representative fluorescence excitation-emission matrix from a silk solution sample is shown in Fig. 3(A) as an iso-intensity contour plot (i.e. each line represents a set of excitation-emission wavelengths with equal fluorescence emission intensities, with blue and red hues representing low and high intensities, respectively). The excitation-emission maxima for the three components that describe this EEM and are attributed to tyrosine, tryptophan and crosslinks along with the high intensity peaks representing the scattered excitation light are indicated with arrows. Representative peak normalized fluorescence emission spectra acquired from silk solution, gel and scaffold samples are shown for 265 nm [Fig. 3(B)] and 310 nm [Fig. 3(C)] excitation. These spectra illustrate some of the characteristic spectral line shape differences that we observe for silk samples representing these three distinct structural conformations. Note that the solution and scaffold spectra exhibit significant differences in both cases, while the gel spectra resemble more the corresponding solution or scaffold spectra depending on the excitation wavelength.

Fig. 3. (A) Fluorescence excitation-emission matrix of silk in solution. Arrows indicate major contributions from tyrosine, tryptophan and cross-links. Representative fluorescence emission spectra acquired at 265 nm (panel B) and 310 nm (panel C) from silk in solution, gel and scaffold configurations are shown as solid lines. The corresponding fits achieved using the ALS scheme outlined in Fig. 1 are shown as dashed lines.

3.3 Spectral decomposition of silk solution, gel and scaffold samples

To model and understand the origins of the spectral differences depicted in Fig. 3, we used an alternating least squares (ALS) fitting algorithm to extract the spectral line shapes and relative intensity contributions of the minimum number of components that could describe the recorded fluorescence excitation-emission matrices (Fig. 1). This analysis identified five spectral components that were needed to describe the silk solution, gel and scaffold spectra. The corresponding fits that we achieved for each one of the spectra in Figs. 3(B) and 3(C) are shown as dotted lines and demonstrate excellent agreement between the analysis model and the data. More detailed information with respect to the range and the mean values for the root mean square error (RMSE) and the % of unmodeled variance for each type of silk sample is included in Table 1.

Table 1. Measures of fit quality of silk spectra using the ALS-based algorithm

<table>
<thead>
<tr>
<th></th>
<th>RMSE range</th>
<th>RMSE mean</th>
<th>% unmodeled variance range</th>
<th>% unmodeled variance mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silk Solution</td>
<td>0.003-0.07</td>
<td>0.036</td>
<td>0.003-0.15</td>
<td>0.0467</td>
</tr>
<tr>
<td>Silk Gel</td>
<td>0.002-0.018</td>
<td>0.014</td>
<td>0.027-0.37</td>
<td>0.1147</td>
</tr>
<tr>
<td>Silk Scaffold</td>
<td>0.008-0.27</td>
<td>0.12</td>
<td>0.008-0.275</td>
<td>0.0925</td>
</tr>
</tbody>
</table>

Figures 4 and 5 show the fluorescence emission spectra of each one of the components extracted from ALS analysis of the data and used to achieve the fits of Fig. 3. To describe the fluorescence recorded from silk solution, gel and scaffold samples for 250-335 nm excitation,
we needed to include five distinct spectral components. The wavelength of maximum emission \( \lambda_{\text{max}} \), the corresponding full width at half maximum (FWHM) for each component and its relative contribution to the overall fluorescence from each sample type are included in Table 2.

The spectral profiles of each of these components required for the fits of silk in solution, gel and scaffold conformation were similar but not identical, as shown in Figs 4 and 5 and Table 2. For example, the spectrum of the dominant tryptophan component, identified as Tryptophan 1, extracted from analysis of the gel and scaffold data becomes progressively narrower and blue-shifted, when compared to the corresponding spectrum extracted from the silk solution samples [Fig. 5(A)]. The latter spectrum is in turn blue shifted with respect to the pure tryptophan solution spectrum [Fig. 5(A)]. These spectral changes reflect an increasingly hydrophobic milieu for the tryptophan residues [20, 21, 35], consistent with the protein forming more extensive \( \beta \)-sheets and excluding water in the process (Fig. 2; [26]). However, the location of the emission maximum of the tryptophan spectrum at 345 nm is characteristic of tryptophan residues that are exposed to water [20, 21] and it is consistent with the sequence data shown in Fig. 2 indicating that tryptophan is present along the hydrophilic chains of the silk protein. Emission from the third tryptophan component is also consistent with tryptophan present in a highly hydrophilic environment that is similar for all types of silk samples.

Table 2. Summary of spectral components of the components used to describe silk samples

<table>
<thead>
<tr>
<th>Silk Solution</th>
<th>Silk Gel</th>
<th>Silk Scaffold</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda_{\text{max}} )</td>
<td>FWHM</td>
<td>Contribution</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>305nm 34 nm</td>
<td>32 %</td>
</tr>
<tr>
<td>Tryptophan 1</td>
<td>345nm 66 nm</td>
<td>39 %</td>
</tr>
<tr>
<td>Tryptophan 2</td>
<td>310nm 37 nm</td>
<td>12%</td>
</tr>
<tr>
<td>Tryptophan 3</td>
<td>345nm 57 nm</td>
<td>15%</td>
</tr>
<tr>
<td>Crosslinks</td>
<td>395nm 82 nm</td>
<td>2 %</td>
</tr>
</tbody>
</table>

Table 2. Summary of spectral components of the components used to describe silk samples

Fig. 4. Fluorescence emission spectra of the components extracted from ALS analysis of silk in solution (A), gel (B) and scaffold (C) configuration.

The tryptophan component identified as tryptophan 2 is significantly blue-shifted with respect to the dominant tryptophan component with the emission peak at 310 nm. The spectrum of Trp2 from the silk solution samples includes a prominent feature in its tail region, indicating that it may be actually a composite spectrum from two chemical species. The spectrum of Trp2 is broader for the silk gel and scaffold samples than the silk solution samples, may be as a result of a change in the relative contributions of these two components. Unfortunately, we were not able to identify two distinct spectral features in this wavelength regime using the ALS algorithm. Tryptophan emission exhibiting a maximum at 308 nm is consistent with tryptophan residues that do not form hydrogen-bound complexes in the excited state [20, 21, 35]. Peak emission in the 316-332 nm region is typically associated with buried tryptophan residues that do form hydrogen-bound complexes [20, 21, 35]. Thus, this component likely represents emission from a subset of tryptophan residues that have limited

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access to water as silk β-sheets acquire an increasingly stacked conformation within the scaffold structure. This hypothesis is also supported by the fact that the contribution of this component to the overall silk sample fluorescence, increases gradually from 12% to 23% and to 32% for the silk solution, gel and scaffold samples, respectively. Another possibility for a species contributing to fluorescence emission in the 320-330 nm region is tyrosinate. However, tyrosinate is highly unstable and would likely appear as crosslinked products [31]. Since crosslinking in silk has not been found to any large degree to date, despite the high content of tyrosine, tyrosinate is not expected to be a major contributor to the observed spectra.

![Fig. 5. Comparison of the spectral features of the components attributed to (A) tryptophan 1, (B) tryptophan 2, (C) tryptophan 3, and (D) crosslinks from each type of silk sample. Measured spectra from a tryptophan (A), tyrosine and dityrosine (D) solution are also included.](image)

The spectrum that we attribute to crosslink formation is significantly broader and red-shifted for the scaffold compared to the silk solution and gel spectra [Fig. 5(D)]. This suggests that the nature of the crosslinks may be different or that there may be more than one type of crosslink involved as the β-sheets acquire an increasingly stacked conformation. The origins of the fluorescence emission that we attribute to crosslinks are not well defined. Oxidized tryptophan is one possible contributor. Di-tyrosine crosslinks are also known to emit fluorescence in the 400 nm region [36] and we include an emission spectrum of such crosslinks for comparison in Fig. 5(D). However, it is believed that the cross-links that are present in silk samples are through hydrogen bonding interactions and do not involve covalent bonds. For this reason, we also examined emission in this region of the spectrum from a pure tyrosine solution to examine whether non-covalent interactions between tyrosine molecules could also yield similar emission. Indeed, we have found that for excitation between 295 and 335 nm there is fluorescence emission that is very similar to that of di-tyrosine [Fig. 5(D)]. Thus, we presume that non-covalent interactions between tyrosine, tryptophan, phenylalanine and potentially other protein components that are enhanced with β-sheet formation and stacking contribute to the silk fluorescence emission in the visible region of the spectrum.

The tyrosine emission spectrum that we employed for analysis of all silk samples was fixed to the measured tyrosine solution spectrum, since tyrosine emission is not very sensitive to its local environment [31]. While the relative concentration of tyrosine to tryptophan doesn’t change as the silk changes conformations from its silk to its gel and scaffold conformations, we observe a significant decrease in relative contribution of tyrosine fluorescence to the overall sample fluorescence. Specifically, while tyrosine contributes significantly (32%) to the overall fluorescence emission from silk in solution, it comprises
only 12% and 1% of the silk gel and scaffold samples, respectively. This is likely the result of enhanced resonance energy transfer (RET) between tyrosine and tryptophan [31]. Specifically, as the tyrosine and tryptophan molecules are found in closer proximity to each other when the silk acquires its two- and three-dimensional conformation, it becomes more probable for non-radiative energy transfer to occur between an excited tyrosine and one of the tryptophan molecules, which then fluoresces as it decays to its ground state.

Thus, from examination of the differences in the emission spectral properties of the fluorescent components that yield the measured excitation-emission matrices of different silk samples we can acquire insight on the structural and conformational changes that characterize the state of the silk protein in solution, gels and scaffolds. In addition, this spectral decomposition yields information about the contribution of each one of the components to the measured fluorescence intensity. For example, we can see in Fig. 6(A) that the ratio of tryptophan to tyrosine fluorescence at 275 nm excitation increases gradually as we compare the solution, gel and scaffold samples. As mentioned above, this increase represents the gradually increasing levels of RET that occur as the protein acquires a tighter three-dimensional conformation that brings the tyrosine and tryptophan molecules closer together.

Fig. 6. (A). The ratio of tryptophan to tyrosine fluorescence detected at 275 nm excitation and (B) the level of fluorescence attributed to crosslinks relevant to the overall amino acid (tyr and trp) fluorescence increase as silk achieves increasing levels of β-sheet conformation in its solution, gel and scaffold configurations.

Contributions from different chromophores at different excitation wavelengths can also be combined to represent quantitative changes. For example, in Fig. 6(B) we show that the ratio of the fluorescence attributed to crosslinks at 330 nm excitation to that of tyrosine and tryptophan at 275 nm excitation increases significantly as the silk acquires its gel configuration and even more as it forms scaffolds. Thus, this type of ratiometric measurements that can be implemented with spectral filtering in imaging configurations may provide a simple method for quantitative assessment of the structural conformation of silk.

4. Summary

In summary, we present a detailed study of the intrinsic fluorescence properties of silk proteins in solution, gel and scaffold structures when excited in the 250-335 nm range. We demonstrate that quantitative analysis and modeling of the recorded spectra as a sum of fluorescing biochemical components reveals useful information with regards to the conformation and structure of the protein. Specifically, we find that the fluorescence and excitation emission spectra attributed to tryptophan are highly characteristic of the structural conformation of the protein and they report a decrease in the water content of the tryptophan milieu and the distance with neighboring tyrosine molecules. In addition, we also observe that the level of fluorescence attributed to crosslinks increases significantly as the silk protein acquires its gel and scaffold conformations. Both of these properties are in excellent agreement with previous studies that indicate that the silk protein undergoes intra- and interchain folding leading to organized beta sheets dominated by hydrophobic amino acids, as the silk fibroin solution progresses from solution to gel and then solid 3D scaffold states [26]. Thus, this type of quantitative spectral analysis of endogenous optical signals provides a direct means of assessing the structural conformation of the silk protein and may prove a powerful
tool for monitoring non-invasively the integrity and/or modification of silk matrices within tissue engineering constructs.

Acknowledgments

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