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The behavior of aged regenerated *Bombyx mori* silk fibroin solutions studied by ^{1}H NMR and rheology

Zainuddin [a,b,c,*], Tri T. Le [d], Yoosup Park [e,f], Traian V. Chirila [a,b,e,g], Peter J. Halley [e,f], Andrew K. Whittaker [c,e]

[a] Queensland Eye Institute, Brisbane, Qld, Australia
[b] School of Medicine, Faculty of Health Sciences, University of Queensland, Brisbane, Qld, Australia
[c] Centre for Magnetic Resonance, University of Queensland, Brisbane, Qld, Australia
[d] Department of Chemistry, University of Queensland, Brisbane, Qld, Australia
[e] Australian Institute for Bioengineering and Nanotechnology, University of Queensland, Brisbane, Qld, Australia
[f] Department of Chemical Engineering, University of Queensland, Brisbane, Qld, Australia
[g] School of Physical and Chemical Sciences, Queensland University of Technology, Brisbane, Qld, Australia

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ABSTRACT

As part of a project to utilize the regenerated silk fibroin (RSF) membranes as a supporting matrix for the attachment and growth of corneal stem/progenitor cells in the development of tissue engineered constructs for the surgical restoration of the ocular surface, the behavior of the aged RSF solutions has been investigated. The solutions were produced from domesticated silkworm (*Bombyx mori*) cocoons according to a protocol involving successive dissolution steps, filtration and dialysis. The solutions were kept at 4 °C in a refrigerator for a certain period of time until near the gelation time. The changes in molecular conformation were studied by solution-state ^{1}H NMR, while the flow of the solutions was characterized by rheological method. Upon ageing turbidity developed in solutions and the viscosity continuously decreased prior to a drastic increased near the gelation time. The ^{1}H resonances of aged solutions showed a consistent downfield shift as compared to the ^{1}H resonances of the fresh solution. Shear thinning with anomalous short recovery within a certain range of low shear rates occurred in both fresh and aged solutions. While the solutions behave as pseudo-plastic materials, the chain conformation in aged solutions adopted all secondary configurations with β-strand being predominant.

1. Introduction

It has been known that the ocular surface disorders (OSDs) caused by various chronic conditions such as thermal or chemical burns, Stevens–Johnson syndrome, cicatricial pemphigoid and chronic contact lens wear can damage the progenitor cells, leading to deficiency of limbal epithelial stem cells. A severe implication of prolonged limbal stem cell deficiency is blindness due to progressive ingrowth of fibrous tissue and opacification of the cornea [1]. Clinical studies have shown that the surgical treatment to restore OSDs either by autografts or allografts has many limitations. Autografting is restricted by the amount of tissue that can be removed from the other eye and may cause complications to the healthy eye, whilst allografting is limited by the availability of donor tissue, possible rejection and biosafety concerns. In a relatively new approach, limbal stem cells are isolated through biopsy, expanded *in vitro* on a suitable matrix and then transplanted to injured/diseased corneal surface [2–5]. Providing that the supporting material is capable of promoting cell attachment, growth and spreading, the latter technique offers a viable alternative for restoring the ocular surface.

Currently, denuded human amniotic membrane (AM) is the most widely used substrate for ocular surface repair [2,6–10]. However, as the AM is a human-derived tissue, it is a potential vector for transferring infectious disease [11]. Other drawbacks of using this biological material have also been reported [12–14]. Therefore, the search for alternative materials is increasingly important. To date, several alternative matrices have been evaluated including collagen and its derivatives, fibrin gel, Matrigel® and some synthetic polymers [4,5], but only few of them have reached animal experimentation or clinical trials and the results were unsatisfactory.

Hence, in our previous reports [15,16], we have proposed and evaluated the feasibility of the regenerated silk fibroin (RSF)

* Corresponding author Queensland Eye Institute, 41 Annerley Road, South Brisbane, Qld 4101, Australia. Tel.: +61 7 3010 3381; fax: +61 7 3010 3390.
E-mail address: z.zainuddin@qei.org.au (Zainuddin).

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membranes as a supporting matrix for cultivation of human limbal epithelial (HLE) cells. We demonstrated that the RSF membranes were able to support the growth of HLE cells at a level comparable to that on tissue culture plastic. A comprehensive study on cell behavior and its long-term differentiation is currently undergoing in our laboratories and the results will be published in due course.

It is known that the cell-adhesive properties of the RSF membranes are also influenced by the protocol for preparation of the membranes [17–22], a phenomenon which probably relates to the conformational change in solution that occurs during processing and storage [23–25] and by solvent treatment of the membranes [17–19,26–28], leading to the formation of, and an interplay between crystallizable forms known as silks I, II and III, where β-forms and α-helix structures predominate, and amorphous forms containing mainly random coil structures [29–36]. It was Coleman and Howitt who, in their seminal paper on fibroin [37], showed probably for the first time that different conformations of the silk fibroin can be reciprocally converted. They noticed that if water-soluble fibroin (“denatured”), which we know now as silk II, was dissolved in aqueous solution of copper ethylenediamine and then dialyzed against water, the resulting water-soluble fibroin (renatured) does not differ essentially from native fibroin (as found in the silk gland), which we call now silk I. However, at that time, they could not refer to silk I or silk II as these terms were to be coined a few years later by Kratky and coworkers [38,39], who were also the first to give a correct interpretation to this observation by showing that silk II can be converted into silk I after dissolution of the former in an appropriate solvent followed by dialysis against water [40,41].

In the last decades, there have been considerable efforts to understand the transformation of the viscous silk I in the middle section of domesticated silkworm’s glands (about 25% of concentration) to crystalline fibrous silk II spun through the spinneret [42–47]. As a result, many factors have been identified to affect the transition of silk I to silk II, including pH, removal of calcium ions and water molecules from the ducts and the presence of external forces during the spinning process [28,42–47]. Based on the experiments developed to mimic the natural spinning process of silkworm silk it was revealed that the external force played an important role in the transition of silk I to silk II [42–47]. Within this context, the rheometer has also been utilized to generate a similar structural transformation by applying shear force to the silk fibroin solutions [42–55]. Although limited studies on the rheology of the RSF aqueous solutions have been reported [47–50,53,55], it appeared that shear thinning dominated the process and the formation of aggregates was occasionally observed at considerably high shear rate [47,53,55].

Another factor which also contributes to the transformation of silk I to silk II is the structural nature of the silk fibroin. It is well known that the silk fibroin is constituted by highly repeated hydrophobic and crystallisable molecules with the primary sequences of amino acid residues of Gly–Ala–Gly–Ala–Gly–Ser, $[Gly–Ala]_n$–Gly–Tyr and $[Gly–Val]_n$–Gly–Ala ($n = 1–8$) separated by 11 amorphous region of mainly Gly–Ala–Gly–Ser and Gly–Ala–Gly–Ala–Gly–Ser sequences [51]. Therefore, upon storage, the RSF solution tends to undergo self-transformation to adopt a more stable conformation by forming intra- and/or intermolecular hydrogen bonds [23,56–58].

In this study, to shed more light on the conformational change during storage and to determine the useful life time of the RSF solutions prior to gelation, the behavior of RSF solutions was monitored by using rheological and proton NMR methods. This knowledge is essential, particularly when the RSF membranes are intended to be used as substrates for tissue engineering applications.

2. Materials and methods

2.1. Materials

The *Bombyx mori* cocoons were purchased from Tajima Shoji Co. Ltd. (Yokohama, Japan). Sodium carbonate (Na_2CO_3) and lithium bromide (LiBr) were obtained from Sigma-Aldrich. Methanol and deionized water (18.2 MΩcm) were used as solvents.

2.2. Preparation and ageing of RSF aqueous solutions

The *Bombyx mori* cocoons were cut in smaller pieces, vacuum dried, weighed, and placed in 1 L boiling solution of Na_2CO_3 (0.02 M) for 1 h to remove sericins. Subsequently the silk fibers were rinsed three times in 1 L hot water (~70 °C) for 20 min each and then let to dry in a fume hood overnight. After sericin-free silk fibers were dried under vacuum for few hours, they were dissolved in an aqueous lithium bromide solution (9.3 M) at 60 °C for 4 h to obtain a silk concentration of about 10%. The solution was pre-filtered through a syringe filter with a pore size of 0.8 μm (Minisart®-GF, Sartorius) and followed by a 0.20-μm pore size filter (Minisart®High-Flow, Sartorius). About 10 mL of the filtrate was injected into a 3–12 mL dialysis cassette with a molecular weight cut-off of 3500 (Slide-A-Lyzer®, Pierce) to be dialysed against water. Following six changes of water within 3 days of dialysis, the resulting solution was collected and filtered through a 0.20-μm pore size filter (Minisart®High-Flow). The resulting aqueous solution with a concentration in silk fibroin of about 3.8% was kept at 4 °C for 1 to 4 months.

2.3. ^1H NMR measurements

The samples were diluted to four times of the initial volume of the RSF aqueous solutions with deuterated water (D_2O). ^1H NMR spectra were obtained on a 500 MHz Avance Bruker spectrometer operating at 500.13 MHz with a 5 mm probe. Water (HDO) resonance was used as an internal standard for determination the ^1H chemical shifts. The water signal was suppressed by a thousand fold or more using watergate pulse sequence with gradient double echo. Phasing and baseline corrections were completed manually. The software used for these procedures was TOPSPIN version 1.3.

2.4. Rheological measurements

The viscosity of the RSF aqueous solutions was measured using an AR-G2 Rheometers (TA Instrument Ltd., USA). The shear viscosity was acquired by linearly increasing the shear rate without oscillation from 0.05 to 500 s^{-1}. Meanwhile, the measurement of storage (G') and loss (G'') modulus as a function of frequency (ω) was performed within the linear viscoelastic region using 40-mm diameter cone plate with 57 μm gap. All rheological measurements were carried out at room temperature (25 ± 0.5 °C). A solvent trap was used throughout the experiment to avoid the possible loss of water.

3. Results and discussion

3.1. ^1H NMR analysis

Though the proton (^1H) resonances of many proteins and peptides in the solution-state have been well documented, little data are available on the solutions of silk fibrous proteins, including the regenerated *Bombyx mori* silk fibroin [59]. The fact that the silk fibroin does not readily dissolve in most organic solvents and it is structurally unstable in aqueous environment due to conformational change. Our observation on the effect of ageing on the behavior of the aqueous silk fibroin solution, however, has prompted us to carry out the ^1H NMR measurement in aqueous system. Under this condition we are aware that the interference of the water ^1H resonance is imminent. However, because all of the samples contain the same amount of water and this study was to compare the ^1H resonances of the aged and fresh silk fibroin solutions, the same magnitude of interference would apply to all samples, thus the changes in the molecular structure due to ageing effect would directly correspond to the changes in chemical shift and line shape. Fig. 1 shows the ^1H NMR spectra of the fresh and 4 month old silk fibroin solutions with and without water peak suppression. As expected, the major peaks were mainly contributed by the primary structure of the silk fibroin (i.e. alanine, glycine, serine, tyrosine and valine). Close inspection of the spectra (see Fig. 2a–f) reveals that while the global chemical shift of the aged solutions consistently moved downfield (relative to the fresh silk