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Synthesis and viscoelastic characterization of microstructurally aligned Silk fibroin sponges

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ABSTRACT

Silk fibroin (SF) is a model candidate for use in tissue engineering and regenerative medicine owing to its biocompatible mechanochemical properties. Despite numerous advances made in the fabrication of various biomimetic substrates using SF, relatively few clinical applications have been designed, primarily due to the lack of complete understanding of its constitutive properties. Here we fabricate microstructurally aligned SF sponge using the unidirectional freezing technique wherein a novel solvent-processing technique involving Acetic acid is employed, which obviates the post-treatment of the sponges to induce their water-stability. Subsequently, we quantify the anisotropic, viscoelastic response of the bulk SF sponge samples by performing a series of mechanical tests under uniaxial compression over a wide range of strain rates. Results for these uniaxial compression tests in the finite strain regime through ramp strain and ramp-relaxation loading histories applied over two orders of strain rate magnitude show that microstructural anisotropy is directly manifested in the bulk viscoelastic solid-like response. Furthermore, the experiments reveal a high degree of volume compressibility of the sponges during deformation, and also evince for their remarkable strain recovery capacity under large compressive strains during strain recovery tests. Finally, in order to predict the bulk viscoelastic material properties of the fabricated and pre-characterized SF sponges, a finite strain kinematics-based, nonlinear, continuum model developed within a thermodynamically-consistent framework in a parallel investigation, was successfully employed to capture the viscoelastic solid-like, transversely isotropic, and compressible response of the sponges macroscopically.

1. Introduction

In the field of tissue engineering and regenerative medicine, a major challenge is to mimic the tissue-specific mechanoresponsive architecture and biochemical milieu (Brown, 2013). A widely prevalent tissue architecture exhibited within human body is that of a 3-dimensional (3D) matrix with significant microstructural anisotropy, characterized by intrinsic fibrous structure and pore alignment. Anisotropy is widely prevalent in vivo functional tissues such as cardiac muscles, as well as dysfunctional pathology such as the extracellular matrix (ECM) present around the periphery of tumors (Provenzano et al., 2006).

In order to replicate such anisotropic features microstructurally while controlling material parameters like stiffness, surface chemistry, etc.,it is necessary to have a material with tunable microstructure. Several biocompatible materials have been studied till now, including gelatin (Oryan et al., 2016), elastin (Annabi et al., 2016), PLGA (Astete and Sabliov, 2006), titanium alloys such as Ti6Al4V (Murr et al., 2009), etc. In this context, Silk fibroin (SF) is a mass-reproducible, naturally derived, protein polymer with desirable thermomechanical properties. It is relatively hassle-free to obtain, without needing any regulatory clearances, from *Bombyx Mori* silk cocoons. Its composition has been precisely logged (Cook, 1984; Hakimi et al., 2007) to contain only sericin and fibroin, thus making it an ideal candidate for biomimetic applications (Kim et al., 2004; Shao and Vollrath, 2002; Holland et al., 2012). Several techniques have been developed for controlling the microstructure and geometry of scaffolds and artificially bioengineered tissues made from SF, namely, sponges (Nazarov et al., 2004; Tamada, 2005), hydrogels (Yucel et al., 2009; Wang et al., 2008), micro- and nano-fibers (Rockwood et al., 2008; Wittmer et al., 2011), micro- and nanospheres (Wang et al., 2010b, 2007), tubes (Lovett et al., 2007, 2008), etc. Notably amongst these material formats, SF based sponges have found a variety of applications in bone and cartilage tissue engineering (Kim et al., 2007; Hofmann et al., 2006), disease models for breast cancer (Wang et al., 2010a), implant devices (Meinel et al., 2006), soft tissue repair (Mauney et al., 2007), and in vitro 3-dimensional (3D) culture systems (Liu et al., 2012), amongst others.

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Moreover, SF sponges with anisotropic microstructure have been produced through techniques such as unidirectional freezing (Davidenko et al., 2012; Mandal et al., 2013) or introduction of linear wire arrays (Wray et al., 2012).

In contrast to significant advancements made in the design and fabrication of anisotropic SF sponges, mechanical characterization of these sponges has been primarily limited to single load ramp strain tests (at strain rates approximately in the range of 0.1–1.2 min^{-1}) (Asuncion et al., 2016; Yao et al., 2012; Oliveira et al., 2012; Mandal et al., 2013). This severely limits the usage of SF sponges in engineering biomimetic tissues and implants as these investigations were primarily aimed at quantifying only the elastic component of the mechanical response of SF sponges, and that too for a limited range of strain rates and strains pertaining to small deformation, through quantification of Young's modulus and yield stress. But in view of the load-bearing or high-stretch conditions necessary for implants, material characterization under finite compressive or tensile strains and within a wide range of strain rates is necessitated. Additionally, under physiologically hydrated conditions, the spongy polymeric material subsumes a significant amount of hydrating fluid, rendering a strong viscous component to the response of the SF sponge. Together, these two considerations necessitate for thorough biomechanical studies on aligned SF sponges to quantify the macroscopic viscoelastic response of the sponges over multiple orders of deformation. Furthermore, a direct quantitative assessment of the volumetric compliance of the sponges is needed to account for the high degree of volumetric compression that the sponges are expected to undergo under high compressive strains due to their porous microstructure.

To systematically quantify both the short-term and long-term viscoelastic mechanical behavior of the anisotropic sponges under different loading scenarios, a constitutive model that defines and quantifies a set of material properties that individually account for the intrinsic time and stress/strain dependencies of the mechanical response of the material is needed. However, a lacuna exists as no such analytical work has been reported for Silk fibroin sponges to the best of the authors' knowledge.

In the present work, we first fabricated anisotropic SF sponges as a tunable biomimetic material using a novel solvent processing methodology. Next, unconfined uniaxial compression experiments were performed to (1) characterize the time-dependent, viscoelastic response of hydrated, aligned SF sponges for strain levels of up to 75%; (2) determine the mechanical response of the hydrated sponges anisotropically over two orders of strain rate magnitude (approx. 0.001–0.1 s^{-1}) in the finite strain regime; (3) quantify the volume change of the SF sponges under compressive strains in the large deformation regime. Finally, a large strain kinematics-based, nonlinear, anisotropic, macroscopic viscoelastic model developed in a parallel study (Panda et al., 2017) for aligned SF sponges is employed here directly to determine the viscoelastic material properties of the pre-characterized anisotropic SF sponges for a given SF concentration. Since thermodynamic consistency was implicitly incorporated in this macroscopic model, the predictions of the essential features of the viscoelastic response for the sponges are physically realistic in nature.

2. Materials and methods

2.1. Preparation of Silk fibroin solution

Silk fibroin solution was extracted from untreated silk yarn (M. Jiju Silk Mills, Bengaluru, India) using previously established protocol (Rockwood et al., 2011). Briefly, silk yarn was mixed with 0.02 M Na_2CO_3 solution and boiled for 30 min, followed by drying overnight and subsequent dissolution in 9.3 M LiBr solution at 60 °C for 4 h. The solution was subsequently dialysed in de-ionized water for 3 days using Snakeskin pleated dialysis membrane (35000 K MWCO, Thermo Scientific) and then twice centrifuged at 5000 rpm for 20 min to collect the purified Silk fibroin solution as the supernatant liquid (henceforth referred to as the regenerated Silk fibroin (RSF) solution). The RSF solution was then diluted with glacial acetic acid to attain a fibroin concentration of 1 w/v% and an acid concentration of 1 w/w% in the final solution.

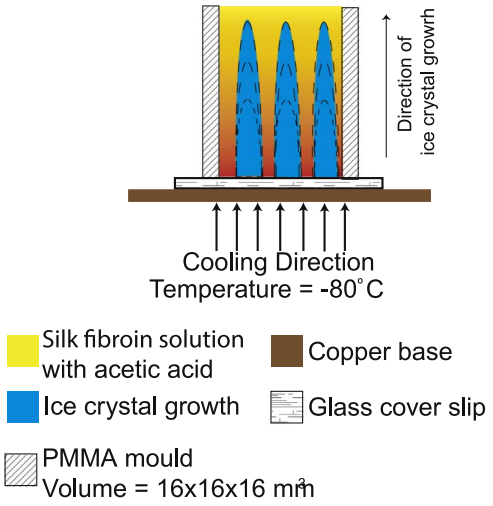

Fig. 1. A schematic diagram representing the experimental setup to create Silk fibroin sponges with unidirectionally aligned porous microstructure using the unidirectional freezing technique.

2.2. Preparation of SF sponge samples with aligned, porous structure

To prepare SF sponge samples with aligned, porous microarchitecture, Polymethyl methacrylate (PMMA) molds of height 16 mm, with a single square cross-section hole of edge length 16 mm, were fabricated. These PMMA molds (containing Silk fibroin solution mixed with Acetic acid) with glass base, were then placed on a copper surface that was cooled by liquid nitrogen contained in a container (refer to Fig. 1). Once frozen unidirectionally, the samples were stored at −60 °C for least 24 h, and subsequently freeze-dried at −42 °C and 0.1 mbar for 48 h in a lyophilizer (Sub Zero Lab Instruments, Chennai, India). To perform experiments with hydrated samples, these dry sponges were soaked in Phosphate-buffered saline (PBS) for 5 h at room temperature prior to mechanical testing.

2.3. Microstructural characterization studies on RSF solution and SF sponge

The RSF solution was incubated at 37 °C for 60 h till it underwent complete gelation. This SF hydrogel was then frozen and lyophilized to obtain the gel in its freeze-dried form for performing its subsequent microstructural characterization studies along with that of the SF sponge.

2.3.1. Fourier transform infrared spectroscopy (FTIR)

The structural conformation of the SF sponge, fabricated by the aforementioned technique, and the SF hydrogel were studied using FTIR (Platinum-ATR, Bruker Alpha, CA) technique. For the latter, the RSF solution was incubated at 37 °C for 60 h till it underwent complete gelation. The SF hydrogel was then frozen and lyophilized to obtain the gel in its freeze-dried form. The spectrum data was analyzed in the range of 675–4000 cm^{-1}, with a resolution of 4 cm^{-1}. The IR spectra (Fig. 2a) of both the SF hydrogel as well as the SF sponge revealed sharp drops in transmittance at the proximity of the wave numbers 1624 cm^{-1}, 1524 cm^{-1} and 1234 cm^{-1}, thereby confirming the presence Amide I, Amide II absorption bands corresponding to Silk II structural conformation, and some remnant silk I conformation, respectively, as reported previously (Lu et al., 2010; Hu et al., 2006; Srisa-Ard and Baimark, 2013).