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High-strength silk fibroin scaffolds with anisotropic mechanical properties

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ABSTRACT

In contrast to isotropic morphologies of synthetic hydrogels, many biological tissues possess anisotropic hierarchical morphologies leading to extraordinary mechanical properties that cannot be mimicked by synthetic materials. Here, we report preparation of anisotropic silk fibroin cryogels and scaffolds exhibiting a Young's modulus in the range of MPa that sustain up to 20 MPa compressive stresses. The cryogels were prepared by a combined directional freezing – cryogelation process starting from an aqueous 4.2 wt% fibroin solution containing butanediol diglycidyl ether cross-linker and N,N,N′,N′-tetramethylethylenediamine. In the first step, the reactor containing the aqueous solution of fibroin, cross-linker, and TEMED was immersed into liquid nitrogen at a controlled rate to create a directionally frozen ice template. In the second step, cryogelation reactions were conducted in this frozen solution at −18 °C whereby the cryo-concentrated fibroin in the unfrozen microzones of the reaction system forms a 3D fibroin network. The scaffolds exhibit anisotropic microstructure and hence anisotropic mechanical properties, e.g., the Young's modulus is 3.4 ± 0.5 MPa and 0.8 ± 0.3 MPa when measured along the directions parallel and vertical to the freezing direction, respectively. All the cryogels could completely be compressed due to squeezing out of water from their pores. Upon removal of the load, the compressed cryogels immediately recover their original dimensions and mechanical properties by absorbing the released water into their pores.

1. Introduction

Hydrogels are chemically or physically cross-linked polymers absorbing large quantities of water without dissolving [1]. Softness, smartness, high water sorption capacity, and similarity to biological tissues make hydrogels unique soft materials. However, there is still a clear distinction between synthetic hydrogels and biological tissues with regard to the microstructure and structure-related mechanical properties. In contrast to the isotropic morphologies of synthetic hydrogels, many tissues including muscles [2], tendon [3], cartilage [4], intervertebral disc [5], and cornea [6] possess anisotropic hierarchical morphologies leading to extraordinary mechanical properties that cannot be mimicked by synthetic materials. Biocompatible anisotropic hydrogels with a good mechanical performance have a broad range of potential applications in tissue engineering, bioseparation, microfluidics, and organic electronics [7].

To prepare gels with anisotropic properties, several strategies have been presented in the past years including directional freezing [7,8], strain-induced reorientation [9–14], self-assembly [15–17], dielectrophoresis [18], micropatterning [19], and 3D printing [20]. Directional freezing is a simple and promising approach to the preparation of aligned porous materials [7,8]. By this technique, the growth of solvent crystals during freezing of a polymer solution is controlled in one direction, e.g., by immersing the reactor containing the solution into a cold bath at a controlled rate. Uniaxial freezing of the solutions of synthetic and natural polymers such as polyvinyl alcohol (PVA) [21], agar [22], agarose [23], chitosan [24], alginate [25,26], collagen [27], gelatin [28], soy proteins [29], silk fibroin [30,31], or colloidal solutions of polymers and nanoparticles [8], such as PVA and silica in a cold bath followed by removing oriented solvent crystals via freeze-drying produce aligned porous materials with various microstructures. However, such materials generally dissolve in good solvents and exhibit poor mechanical properties due to the absence of chemical cross-links interconnecting the polymer chains or the particles, limiting their application areas. For instance, anisotropic scaffolds based on

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chitosan/alginate produced by directional freezing exhibit a Young's modulus of around 5 kPa [26], while gelatin scaffolds rupture under 0.23 MPa compressive stresses [28]. However, anisotropic scaffolds suitable for tissue engineering applications should sustain high stresses in the order of MPa to protect their integrity.

An alternative strategy is to combine the directional freezing with the cryogelation technique to prepare mechanically strong anisotropic gels. Cryogelation is a simple route to produce 3D highly porous polymer networks of high toughness and superfast responsivity [32,33]. Cryogelation reactions are conducted below the freezing point of the reaction solution, during which the solvent crystals are in equilibrium with the unfrozen liquid microchannels containing cryo-concentrated reactants [32]. Thus, the reactions only proceed in the unfrozen microchannels leading to the formation of macroporous gels with thick pore walls. The combined directional freezing – cryogelation approach was first reported by our group in 2008 to produce butyl rubber (BR) organogels with an aligned porous structure [34]. BR solution in cyclohexane was first directionally frozen below the melting point of the solution and then BR chains are intermolecularly cross-linked in the frozen solution using sulfur monochloride as a cross-linker. The anisotropic BR organogels are very tough and can be compressed up to about 99% strain without any crack development [34]. Several research groups have recently reported the combination of the directional freezing technique with redox-, gamma-, or UV-initiated cross-linking cryopolymerization to prepare hydrogels based on chemically cross-linked poly (meth)acrylates with anisotropic morphologies and mechanical properties [35–40]. For instance, poly (ethylene glycol) diacrylate scaffolds produced by directional freezing – cryopolymerization technique exhibit a Young's modulus of 8 kPa and 80 kPa during compressions along the directions perpendicular and parallel to the freezing direction, respectively [36].

Silk fibroin gels are important materials due to their attractive properties such as a high mechanical strength, biocompatibility, and controlled degradability [41–43]. Gelation of silk fibroin in aqueous solutions mainly occurs by self-assembly of fibroin molecules via intermolecular β-sheet crystallites acting as physical cross-links. The formation of β-sheets and hence fibroin gelation can be induced by several triggers such as pH [44], temperature [45], fibroin concentration [46], cations [47], diepoxide cross-linkers [48], vortexing [49], and electrical field [50]. In tissue engineering applications, a high mechanical strength and an interconnected open pore structure with micrometer-sized pores are essential considerations in the development of silk fibroin gels and scaffolds. In addition, an anisotropic microstructure and anisotropic mechanical properties are also required in fibroin scaffolds to mimic the biological tissues. To our knowledge, there is only one report on the preparation of silk fibroin scaffolds with anisotropic mechanical properties [30]. These scaffolds were prepared by uniaxial freezing of aqueous fibroin solutions followed by freeze-drying to fix the anisotropic microstructure formed due to the ice template. Because the scaffolds are soluble in aqueous environment, they were finally treated with methanol to induce the formation of β-sheets [30]. The scaffolds thus produced exhibit a low mechanical strength, e.g., their Young's modulus is below 4 kPa [30]. This is expected due to the fact that the cross-linking occurs after forming the pore walls of the scaffold leading to the formation of weak intermolecular bonds.

The aim of this study was to prepare high-strength biocompatible scaffolds with anisotropic mechanical properties. Here, we describe preparation of anisotropic silk fibroin cryogels and scaffolds exhibiting a Young's modulus in the range of MPa that sustain up to 20 MPa compressive stresses. The cryogels were prepared by a combined directional freezing – cryogelation process starting from an aqueous 4.2 wt% fibroin solution containing butanediol diglycidyl ether cross-linker and N,N,N′,N′-tetramethylethylenediamine (TEMED). In the first step, the reactor containing the aqueous solution of fibroin, cross-linker, and TEMED was immersed into liquid nitrogen at a controlled rate to create a directionally frozen ice template. In the second step, the cryogelation reactions were conducted in this frozen solution at −18 °C whereby the cryo-concentrated fibroin in the unfrozen microzones of the reaction system forms a 3D fibroin network. The scaffolds exhibit anisotropic microstructure and hence anisotropic mechanical properties, e.g., the Young's modulus is 3.4 ± 0.5 MPa and 0.8 ± 0.3 MPa when measured along the directions parallel and vertical to the freezing direction, respectively. As will be seen below, all the cryogels could completely be compressed due to squeezing out of water from their pores. Upon removal of the load, the compressed cryogels immediately recover their original dimensions and mechanical properties by absorbing the released water into their pores.

2. Experimental section

2.1. Materials

The cross-linker butanediol diglycidyl ether (BDDE, Sigma-Aldrich), N,N,N′,N′-tetramethylethylenediamine (TEMED, Sigma-Aldrich), Na_2CO_3 (Merck), and LiBr (Merck) were used as received. *Bombyx mori* cocoons were purchased from Bursa Association of Agricultural Sales Cooperatives for Silk Cocoons (Kozabirlik, Turkey). Silk fibroin was separated from cocoons by boiling them for 1 h in aqueous solution of 0.02 M Na_2CO_3 to remove the sericin proteins followed by washing the remaining fibroin three times with distilled water at 70 °C, for 20 min each [44]. Silk fibroin was dissolved in aqueous 9.3 M LiBr at 60 °C for ~2 h and then dialyzed using dialysis tubing (10000 MWCO, Snake Skin, Pierce) for 3 days against water that was changed three times a day [44]. After centrifugation, the final concentration of silk fibroin in aqueous solution was about 5 w/w%, which was determined by weighing the remaining solid after drying.

2.2. Preparation of fibroin cryogels

Anisotropic fibroin cryogels were prepared by directional freezing of aqueous 4.2 wt% fibroin solution containing BDDE cross-linker and TEMED (0.25 v/v%) in liquid nitrogen followed by conducting the cryogelation reactions at −18 °C for 24 h. BDDE concentration in the fibroin solution was set to 20 mmol epoxy per gram of fibroin [48]. Typically, 5 mL of 5 wt% fibroin solution were mixed with BDDE (0.50 mL), TEMED (15 μL), and water to make the final volume 6 mL. The homogeneous fibroin solution was then transferred into several 1 mL plastic syringes of 4 mm internal diameter. Each plastic syringe containing the gelation solution was then connected to the upper clamp of the Zwick-Roell test machine, which was moved downward at a constant rate R into a cold bath containing liquid nitrogen. Experimental apparatus for directional freezing of aqueous 4.2 wt% fibroin containing BDDE and TEMED is shown in Fig. 1a. The immersion rate R of the syringes into liquid nitrogen controlled by the software of the test machine was varied between 2.5 and 35 mm min^{-1}. The syringes were then placed in a cryostat at −18 °C to conduct the cryogelation reactions for 1 day. Control experiments were also carried out as described above, except that the directional freezing step was not applied to the fibroin solutions.