Preparation and Properties of Silk Fibroin Electro Hydrogels via a Low Voltage Electrostatic Field

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Abstract. Protein hydrogels is an important biomaterial for soft tissue repair in biomedical applications. However, the most extracellular matrixes are structured and ordered, the morphology of common hydrogels are of random network structures that impeded their applications in tissue engineering. In this study, silk fibroin hydrogels with different morphologies (i.e., microspheres, regularized beads, nano/micro fibers, intertwined networks, and multi-walls) were prepared under low voltage electrostatic fields by regulating the concentration of silk fibroin solution. Additionally, their stability can be regulated with further processing routes to satisfy the tailored requirements for different applications. Fourier transform infrared spectrometer (FTIR) and X-ray diffraction (XRD) provided evidence of the stability of silk fibroin electro materials was tuned by this method effectively. Therefore, these silk fibroin electro hydrogels with various morphologies, high orientation, and stability-regulatable properties provided a promising candidate for tissue engineering.

1. Introduction
Silk fibroin (SF), as a natural polymer, has a broad range of potential applications in biomedical, due to its strong mechanical properties, good biocompatibility, and adjustable biodegradability [1]. There are many anisotropic structures in native tissues such as hearts, muscles, nerves, and tendon/ligament [2-4]. Tissue cells rely on anisotropic protein filaments to function. Count as opposed to the isotropic material, sophisticated anisotropic material provided a better environment for cell growth and proliferation [2]. Inspired by the importance of anisotropy, researchers are striving to create highly anisotropic materials as substitutes of natural organs, which may open a window for human health.

In recent years, SF hydrogels obtained a high level of attention in tissue engineering, because that provided the possibility to mimic the extracellular matrix, and form physical support for cell growth [5]. Up to now, researchers have developed various methods to prepare SF hydrogels with highly anisotropic, such as vortexing [6], sonication [7], chemical crosslinking [8], and lowering the pH [9], shearing [10]. However, traditional SF hydrogels materials clinic use is greatly limited to some problems, such as complicated processing procedures, difficulties to control the morphology, and low stability [8, 10, 11]. Therefore, we urgently need a convenient and controllable method to prepare new SF hydrogels which is highly orientation and stability-regulatable.

In this study, we prepared silk fibroin electro hydrogels (SFEG) with a unique morphological structure via low voltage electrostatic fields. FTIR and XRD spectra provided evidence that under different fabricate conditions, the structure of SFEG has changed from random structures to more stable
α-helix structures and β-sheet structures. These results revealed that SFEG had high directionality and adjustable stability, suggesting prospects for tissue engineering applications, especially for drug delivery, nerves and bone tissues.

2. Materials and Methods

2.1. Purification of aqueous SF
Bombix mori raw silk fibers (Huzhou, China) were degummed three times in 0.05wt% Na2CO3 for 30 min to remove silk sericin, rinsed thoroughly with deionized water, and then dried at 60°C in an oven overnight. Then, the degummed silk was dissolved in 9.3 M LiBr solution, by stirring at 60 ± 2 °C for 2 h. The 5wt% pure SF solution was obtained after dialysis (MWCO 9-14 kDa) in deionized water for 3 days and stored at 4°C for further use.

2.2. Preparation of SF electro materials
According to our previous researches [11, 12], different SFEGs were prepared with the process as shown in Figure 1. Thus, the SF solution diluted into three concentration levels (i.e., 0.2, 0.5, and 1.0wt%) to investigate the morphology of SFEG. For comparison, the traditional freeze-drying SF scaffold was prepared [8], at 20°C, 80°C and 196°C, respectively. Additionally, at a specific concentration, the SF materials were regulated with different procedures treated. Specifically, SFEG1 was collected on the positive electrode after 30min via a low voltage electrostatic field, then the SFEG1 was frozen at -80°C for 3h and then transferred to -4°C for 3 days for soft freezing processing to obtain SFEG2. Similarly, the SFEG3 was prepared as the same route of SFEG1 after the pH value changed to an isoelectric point with hydrogen chloride. All samples were lyophilized for about 48h to achieve dried samples for further research.

2.3. Morphology observation
To observe the surface morphology of SFEG, all samples were cut into sheets and glued to the electron microscope sample table with conductive adhesive for further observation. Prior to imaging, specimens sputtered with a gold layer of 20~30 nm thickness. Then, the morphology of all SFEG was observed by using a Scanning Electron Microscopy (SEM, IT-300, JEOL, Japan) at an operating voltage of 20kV.

2.4. Structure Analysis of the SFEG
FTIR analysis of the SFEG was performed with a VERTEX 70 spectrometer. For each measurement, the spectra were obtained in the range of 400-4000 cm⁻¹ with an accumulation of 64 scans and a resolution of 4 cm⁻¹. To identify the secondary structures, the peak position of the amide I region (1595-1705 cm⁻¹) absorption from Fourier self-deconvolution performed by Opus 5.0 software (Bruker, Germany), as previously described [13]. XRD is used to determine the secondary crystal structure of SFEG. XRD was performed by X’Pert-Pro MPD diffractometer and CuKα radiation with a wavelength of 1.5406 Å, the diffraction intensity curves with 20 from 5° to 45° were obtained with scanning speed was 2°/min.

3. Results and Discussions
3.1 Morphology of SFEG

Here, with different concentrations, SFEG with different morphologies were prepared via low voltage electrostatic fields (Figure 2). As the concentration increased, the SFEG showed better orientation, dimensional hierarchical structures, and stability-regulatable properties. When the SF concentration reached 0.2%, SF appeared with massive relatively regular and uniform microspheres (Figure 2 (A-A2)). When the SF concentration reached 0.5%, SF existed mainly as beads with a certain orientation and partly as microspheres with minor size (Figure 2 (B-B2)). Concentrations of SF reached 1%, the SF exhibited as nano/micro-fibers with higher orientation and hierarchy and more obvious hole wall structure (Figure 2 (C-C2)).

![Figure 2. SEM (A-C2) micrographs of SFEG. (A-A1), (B-B2), (C-C2) were derived from 0.2, 0.5, 1.0 (wt%) SF concentration, respectively, via low voltage electrostatic fields. Scale bars: (A, A1, B, C2) 10μm, (A2, B1) 5μm, (B2) 1μm, (C) 100μm, (C1) 50μm.](image)

At 20°C, 80°C and 196°C, compared to fragment and disorder SF hydrogels prepared at low temperatures (Figure 3 (D-F)), SFEGs produced via low voltage electrostatic fields (Figure 3 (A-C)), showed highly consistent regular and orientation without affected by temperature, with bands structures parallel to the direction of the field applied. Under low voltage electrostatic fields, the negatively charged silk nanofibers migrated toward the anode and began to gels on the positive electrode. The process is based on local pH changes as a result of water electrolysis generating H⁺ and OH⁻ ions at the (+) and (-) electrodes, respectively [14]. Influenced by electrostatic interactions, an external force acts on the fibroin molecules, lead to that fibroin dipoles feel a torque moment and tend to continuously align along the electric field direction [15], force the negatively charged silk nanofibers to align regularly [16], enable intermolecular self-assembly. At increasing SF concentration, molecules chains tend to get aggregate and their mobility reduces, and long-range dipole-dipole cooperatively interactions increase the stability of the structure [15]. These realities provided evidence that the existence of low voltage electrostatic fields can effectively regulate the orientation and stability of the SFEG, and the higher concentration of SF solution, the better orientation, as shown in Figure 2.
Figure 3. SEM(A-F) micrographs were derived from 3.0wt% SF concentration. (A-C) were produced via low voltage electrostatic fields at 20°C, 80°C and 196°C, respectively. (D-F) were produced at 20°C, 80°C and 196°C, respectively. Scale bar: (C) 1mm, others are 500μm.

3.2 Structural analysis of SFEG

XRD and FTIR were essential to examine the change in the crystalline and conformation structure of SF. According to previous studies [17], the main diffraction peaks of the silk I crystal structure occurs at 12.2°(d=7.25 Å, medium strong), 19.7°(d=4.5Å, strong), 24.7°(d=3.60 Å, medium), and 28.2°(d=3.16Å, medium), and the silk II occurs at 9.1°(d=9.7 Å, medium strong), 18.9°(d=4.69 Å, medium strong) and 20.7°(d=4.30 Å, very strong). XRD showed the crystalline structure changes of SFEG (Figure 4(A)). An arc-shaped diffraction peak was observed at 2θ=20.7° for SFEG1 and SFEG3, indicated that the condense structure of SFEG1 and SFEG3 was crystalline and amorphous coexistence. At the meantime, SFEG3 had minor peaks at 2θ=9.8°, indicated that SFEG prepared by adjusting pH had a relatively obvious crystal structure. SFEG2 exhibited a strong diffraction peak at 19.7°, a weak diffraction at around 12.0°, which suggested Silk I was the main crystalline structure in SFEG2.

For FITR Spectra, the characteristic absorption bands at around 1650-1655cm⁻¹ (amide I) and 1525-1540 cm⁻¹ (amide II) and 1266 cm⁻¹ (amide III), are characteristic of α-helix structure, and 1620-1635 cm⁻¹(amide I), 1531 cm⁻¹ (amide II), 1230~1235 cm⁻¹ (amide III) are indicated of β-sheet structure, and 1655-1660cm⁻¹ (amide I), 1535~1545 cm⁻¹ (amide II), 1235 cm⁻¹ (amide III), represented of random coil structure [18, 19]. As shown in Figure 4(B), FTIR analysis was performed to determine the secondary conformational changes of SFEG. For SFEG1 and SFEG3, a strong peak at 1652cm⁻¹, corresponding to the α-helix structure, with another peak at 1540 cm⁻¹, indicating the existence of α-helix structure and random coils structure. However, the SFEG2 shows a strong absorbance wave at 1521cm⁻¹, assigning to locate between α-helix structure and β-sheet structure. For SFEG2, the soft freezing process triggers the SF protein from a random structure to an ordered structure [20]. In fact, under a certain external force such as low temperature or electric field action, the SF molecular chain tightly wound, lead to that the SF macromolecules in the SF solution spontaneously change from a random coils to α-helix structure and β-sheet structure [21], then, the α- helix structure may continue to transform, and eventually exist as more stable β-sheet structure.

Figure 4. Structural analysis of SFEG1, SFEG2, and SFEG3. (A) FTIR spectra, (B) XRD patterns, and (C) FTIR peak splitting.
To further study the structural transformation of SFEG, determined FTIR peak splitting at amide I is indispensable. As shown in Figure 4 (C), there is no significant difference in the content of various secondary structures of SFEG1 and SFEG2, indicating that the effect of soft freezing treatment on the silk fibroin molecular chain was relatively slight, and the SFEG could maintain certain stability at low temperatures. However, compared to SFEG1 and SFEG2, SFEG3 showed an obvious decrease of β-sheet structure, only 23.9%, and a slight increase in α-helix structure. The result was consistent with the XRD demonstration, suggesting that SFEG3 possess conformational changes from random coils to α-helix structure due to the synergistic effect of pH regulation and low voltage electric fields [22]. Especially, the adjustment of the PH value accelerated the gelation process, resulting in that SF macromolecules quickly assemble into large pieces, without enough time to expand and adjust the β-sheet structure, and form silk II crystals [11]. Generally, in this situation, the content of the β-sheet structure still enough to build a relatively high strength [11]. These results indicated that after further processing, the SF molecules transformed from random structures to more stable α-helix structures and β-sheet structures, which strongly proves that these methods for adjusting the stability of SFEG are feasible.

4. Conclusions
In this study, SFEGs with different morphological characteristics (i.e., microspheres, regularized beads, nano/micro fibers, intertwined networks, and multi-walls) were prepared by controlling the concentration of SF solution, via low voltage electrostatic fields. And the FTIR and XRD of SFEGs revealed the stability of SF electro materials can be regulated with further processing routes. These findings provide a new window to prepare SF hydrogels with high orientation and stability-regulatable and can be used as an alternative substitution for various tissue engineering, especially for drug delivery, nerves, and bone tissues.

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