Morphological Characterization and Chemical Bond Identification of Collagen-coated Native Silk Fibroin Fibers Using Chemical Method

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Abstract. Native silk fibroin (SF) fiber has been widely researched as the materials for tissue engineering scaffolds for its robust mechanical properties. Collagen is the main component of extra cellular matrix (ECM) and has remarkable biocompatibility, so it is often utilized to coat native SF scaffolds for improving the cell adhesion. However, there is lack of feasible methodology for coating collagen on the native SF fibers. In this paper we coated collagen on the native SF fibers by creating amide bonds between the carboxyls from native SF and the amino groups from collagen. Morphological characterization (including 3D surface roughness $S_q$, $S_{pq}$ and $S_{vq}$) and chemical bond identification (including amide bond and carboxyl) of the native SF and collagen-coated SF fibers were performed. The results indicated the chemical modification on the surface and collagen concentration had no significant influence on the 3D roughness. The coated and uncoated groups had high similarity in position of characteristic peaks of chemical bonds.

1. Introduction
Tissue engineering has been emerging as a potential method for tissue repair. As an essential component of tissue engineering, scaffolds can support cell adhesion, proliferation and differentiation, and affect the formation of the extra cellular matrix (ECM) [1]. This means the suitable materials of scaffolds are critical for successful tissue engineering. In recent years, collagen and silk fibroin (SF) have been widely studied as the materials for scaffolds.

Collagen is the main component of ECM, it drew early attention because of its great biocompatibility [1], but the poor mechanical properties limit its use as scaffolds. Some chemical [2, 3] and structural [4] approaches have been adopted to reinforce collagen scaffolds. However, the mechanical strength of enhanced collagen scaffolds is still less than desired.

SF is another common material for scaffolds, including native and regenerated SF. Regenerated SF is prepared by remolding SF molecules with two methods, freeze-drying [5] and electrospinning [6, 7], which will form SF molecules into regenerated SF films and fibers, respectively. The regenerated SF scaffold which is fabricated with electrospun regenerated SF fibers can suitably match ECM and has high porosity for tissue ingrowth [8]. However, the mechanical properties of regenerated SF fiber are too poor that the elastic modulus and tensile strength are only 312±193 MPa and 19.1±10.3 MPa, respectively [9]. On the contrary, the native SF fiber has robust mechanical properties with elastic modulus 16±1 GPa and tensile strength 650±40 MPa [10]. Furthermore, native SF has proper biodegradation time in vivo, which loses the majority of its mechanical strength within 1 year, and fails to be recognized within 2 years [11]. Altman, et al. [12] wove native SF fibers into a wire-rope.
scaffold to repair anterior cruciate ligament (ACL) and it had similar mechanical properties to a native human ACL. Li, et al. [13] designed 3 kinds of native SF scaffolds for ACL tissue engineering, i.e. wired, braided and straight, and tested their mechanical properties in different conditions. However, the cell adhesion on the native SF scaffolds still needs improvement.

To improve the cell adhesion of native SF, collagen is often utilized to coat native SF scaffolds. The common coating method, which is a physical method, is forming collagen microsponges among the internal space of native SF fibers by freeze-drying [14, 15]. However, the physical method is supposed to have some shortcomings. First, the weak bonding strength between the native SF and collagen might result in the unsuccessful transfer of mechanical stimuli from the scaffold to the cells. Second, the process of freeze-drying might cause the uneven distribution of collagen in the scaffold, which might result in the uneven distribution of cells and further defects in tissue engineering. Third, the physical method has not mentioned the surface roughness of the scaffold, which has significant influence on cell adhesion [16].

In order to avoid the uneven distribution of cells and the ineffective transfer of mechanical stimuli, and obtain a quantitative characterization of surface roughness, we adopted a chemical method for coating collagen on the native SF fibers, which has been applied to coating regenerate SF films with collagen [17], and we manufactured collagen-coated native SF fibers with different collagen concentrations. Morphological characterization (including 3D surface roughness $S_q$, $S_{pq}$ and $S_{vq}$) and chemical bond identification (including amide bond and carboxyl) of the native SF and collagen-coated SF fibers were performed.

2. Materials and Methods

2.1. Preparation of Collagen-coated Native SF Fibers

Raw silk fibers (Bombyx mori) were purchased from a sericulturist in Shijiazhuang, China. First, the native SF fibers were obtained by degumming raw silk fibers in 0.02 mol/L Na$_2$CO$_3$ (Beijing Chemical Works, Beijing, China) at 100 °C for 60 min [1]. To activate the carboxyls from aspartic and glutamic acid residues, the native SF fibers were immersed in 0.1 mol/L 2-(N-morpholino) ethanesulfonic acid buffer (MES pH=6.0, Coolaber, Beijing, China) with 0.5 mg/ml N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC·HCL, Sigma, St. Louis, USA) and 0.7 mg/ml N-hydroxysuccinimide (NHS, Sigma, St. Louis, USA) at 4 °C for 15 min [17, 18] then phosphate buffered saline (PBS, Biosharp, Hefei, China) at 4 °C for 1 min. After that, the activated native SF fibers were moved into 0.1 mol/L acetic acid solution with different collagen type I (purified from pig skin, Chengdu Ke Le Biotechnology Co., Ltd., Chengdu, China) concentrations at 4 °C for 5h, and rinsed with distilled water three times to remove the free collagen. The activated carboxyl from native SF could react with the amino group from collagen and an amide bond was found. Four collagen concentrations (0, 1, 2, and 3 mg/ml) were adopted in the preparation of the coated SF samples, corresponding to groups SC0, SC1, SC2 and SC3, respectively. In total, five groups were compared in this paper where SF was the native SF fiber, SC0 was the chemically modified (carboxyl activation and soaking in acetic acid solution) SF fiber without collagen coated, and groups SC1–SC3 were the collagen-coated native SF fibers under different collagen concentrations.

The integrity of collagen coating was confirmed by fluorescein. First, collagen was labeled with fluorescein Alexa Fluor 647 (AF647, Thermo scientific, Waltham, USA). Then the native SF fibers were coated with the 1 mg/ml AF647-labeled collagen solution, rinsed with distilled water three times to remove free AF647-labeled collagen, and observed with laser scanning confocal microscope (LSM780, Zeiss, Oberkochen, Germany) under 633 nm. By controlling the concentration of fluorescein, in one collagen molecule, only two amino groups were labeled with fluorescein molecules, other amino groups were used to react with activated SF.

2.2. Morphological Characteristics

Before quantifying the 3D roughness, groups SF–SC3 were glued to slides using neutral balsam (BL704A, Biosharp, Hefei, China). The original surfaces at more than three sites along the axial
direction of the samples were scanned by an atomic force microscope (AFM) (ICON, Bruker, Billerica, USA) with a scan size 1.5 \( \mu m \times 1.5 \mu m \). The original surface could be uncoupled into waviness surface and roughness surface (figure 1) by two-dimensional Gaussian filter (ISO 16610-61) with a cut-off wavelength 0.25 \( \mu m \) (ISO 4287). Due to the distortion of the surface edge caused by the two-dimensional Gaussian filter, half of the cut-off wavelength was cut away around each side of the areas. So only the central 1.25 \( \mu m \times 1.25 \mu m \) of the roughness surface was actually used in calculating 3D roughness \( S_q \), \( S_{pq} \) and \( S_{vq} \) (table 1, ISO 25178, \( n=8-12 \) for each group).

Figure 1. The original surface (a) was uncoupled into waviness surface (b) and roughness surface (c).

Table 1. Definition of \( S_q \), \( S_{pq} \) and \( S_{vq} \). \( f(x, y) \) is the height value of the roughness surface. \( M = 256 \) and \( N = 512 \) are the number of points on each row and column of scanning image, respectively. \( S_{m(e)} \) is the ratio of the material area at a height \( c \) to the evaluation area.

| Name                        | Description                  | Calculation                                                                 |
|-----------------------------|------------------------------|-----------------------------------------------------------------------------|
| Root mean square height \( S_q \) | Similar to \( R_q \) in two dimension | \( S_q = \left[ \frac{1}{MN} \sum_{i=1}^{M} \sum_{j=1}^{N} f^2(x_i, y_j) \right]^{1/2} \) |
| Root mean square height at peaks \( S_{pq} \) | \( S_q \) at peaks | \( 0 \leq S_{m(e)} \leq 10\% \) |
| Root mean square height at valleys \( S_{vq} \) | \( S_q \) at valleys | \( 80 \leq S_{m(e)} \leq 100\% \) |
2.3. Chemical Bond Identification
For discovering the changes in chemical bonds before and after collagen coating, the infrared spectrums of collagen and SF–SC3 were acquired using Fourier infrared spectroscopy (FTIR) (Thermo scientific, Nicolet 6700, Waltham, USA) with ATR mode. The wave numbers ranged from 600 to 4000 cm\(^{-1}\).

2.4 Statistical Analysis
One-way analysis of variance (ANOVA) was used in calculate statistically significant differences in the results of different groups. Bonferroni (if homogeneous variance) and Tamhane (if heterogeneous variance) were selected as the method of post-hoc. Significance was considered as p<0.05.

3. Results
3.1. Integrity of Collagen Coating
The results of laser scanning confocal microscope indicated that native SF fiber and original collagen had few autofluorescence under 633 nm while the fluorescein-labeled collagen emitted strong red fluorescence. Figure 2 is the fluorescence image of coated-group SC1. Every native SF fiber has been coated by ‘red’ collagen, meaning that the chemical coating was integral and successful.

3.2. Morphological Characteristics
Figure 3 shows the results of quantitative assessment of the 3D surface roughness. The root mean square height \(S_q\) of SF–SC3 were 6.60±2.37, 6.79±1.92, 8.51±2.83, 7.41±2.59 and 7.21±2.27 nm, respectively (Mean±SD, each group n=8–12, similarly hereinafter). \(S_{pq}\) is \(S_q\) at peaks, the \(S_{pq}\) of SF–SC3 were 5.81±3.09, 6.28±3.46, 7.07±2.17, 6.02±2.34 and 5.96±3.73 nm, respectively. \(S_{vq}\) is \(S_q\) at valleys, the \(S_{vq}\) of SF–SC3 were 7.90±1.69, 7.02±1.30, 9.52±3.70, 8.93±2.99 and 7.94±2.60 nm, respectively. Furthermore, no significance was found in the comparisons of \(S_q\), \(S_{pq}\) and \(S_{vq}\) in all the groups.

![Figure 2](image1.png)
**Figure 2.** Fluorescence image of SC1, SF was successfully coated with ‘red’ collagen.

![Figure 3](image2.png)
**Figure 3.** 3D surface roughness \(S_q\), \(S_{pq}\) and \(S_{vq}\) of SF–SC3, each group n=8–12.
3.3. Chemical Bond Identification
The infrared spectrums of collagen and SF–SC3 are shown in figure 4. The chemical bonds represented by the position of characteristic peaks are listed in table 2. Two chemical bonds are identified in this paper, i.e. amide bond and carboxyl. The amide bond has five characteristic peaks, amide A, amide B, amide I, amide II and amide III. Amide A and amide B represent the N–H stretching vibration. Amide I represents the C=O stretching vibration. Amide II represents a vibration of major N–H bending coupled with a little C–N stretching. Amide III represents a vibration of major C–N stretching coupled with a little N–H bending [17]. Carboxyl has one characteristic peaks, the C=O stretching vibration.

|                | Amide A | Amide B | C=O stretching of carboxyl | Amide I | Amide II | Amide III |
|----------------|---------|---------|---------------------------|---------|----------|-----------|
| Collagen       | 3302    | 3072    | 1651                      | 1630    | 1545     | 1235      |
| SF             | 3276    | 3075    | 1697                      | 1620    | 1510     | 1227      |
| SC0            | 3278    | 3066    | 1696                      | 1620    | 1513     | 1227      |
| SC1            | 3278    | 3074    | 1695                      | 1620    | 1514     | 1228      |
| SC2            | 3277    | 3074    | 1697                      | 1619    | 1511     | 1228      |
| SC3            | 3279    | 3067    | 1698                      | 1621    | 1514     | 1228      |

4. Discussion
The fluorescent results of the collagen-coated SC1 demonstrated that the chemical method could successfully coat collagen on the native SF fibers. The native SF was the core of the composite fibers to provide robust mechanical properties while the collagen was the shell to provide remarkable biocompatibility. Comparing with the physical coating method, our chemical method is advantageous for avoiding the uneven distribution of cells and the ineffective transfer of mechanical stimuli, and offering quantitative characterization of roughness.

Figure 4. The infrared spectrums of collagen and SF–SC3.
It is known that the roughness of scaffold surface has significant influence on cell adhesion. In general, neither too rough nor too smooth surface is satisfactory [16]. In this paper, the roughness of SF–SC3 was quantified by AFM. However, no significance was found in comparing the $S_q$, $S_{pq}$ and $S_{vq}$ results of all the groups, revealing that the chemical modification and collagen concentration had no significant influence on roughness. To SF–SC3, the coefficient of determination ($R^2$) between $S_q$ and $S_{pq}$, $S_q$ and $S_{vq}$ were 0.27 and 0.77, respectively, which indicated that the $S_q$ had weak linear correlation with $S_{pq}$ while strong linear correlation with $S_{vq}$.

The infrared spectrums were adopted to find the position changes in characteristic peaks before and after collagen coating. However, there was a high similarity in position of peaks between the uncoated groups (SF and SC0) and coated groups (SC1, SC2 and SC3). Two reasons possibly lead to this result. First, collagen and native SF are both proteins essentially and the positions of peaks are very close. Second, the quantity of collagen on native SF is not large enough for FITR to distinguish. Hence compared with infrared spectrums, fluorescein is an optimal method to recognize collagen on native SF fibers for its high sensitivity.

5. Conclusion
The chemical method used in this paper could integrally coat collagen on the native SF fibers. The chemical modification on the surface and collagen concentration had no significant influence on the 3D roughness $S_q$, $S_{pq}$ and $S_{vq}$. The coated and uncoated groups had high similarity in position of characteristic peaks of chemical bonds.

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7. References
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