EXTRUSION PRINTED SILK FIBROIN SCAFFOLDS WITH POST-MINERALIZED CALCIUM PHOSPHATE AS A BONE STRUCTURAL MATERIAL

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Abstract: Artificial bone materials are of high demand due to the frequent occurrence of bone damage from trauma, disease, and ageing. Three-dimensional (3D) printing can tailor-make structures and implants based on biomaterial inks, rendering personalized bone medicine possible. Herein, we extrusion-printed 3D silk fibroin (SF) scaffolds using mixed inks from SF and sodium alginate (SA), and post-mineralized various calcium phosphates to make hybrid SF scaffolds. The effects of printing conditions and mineralization conditions on the mechanical properties of SF scaffolds were investigated. The SF scaffolds from ~10 wt% SF ink exhibited a compressive modulus of 240 kPa, which was elevated to ~1600 kPa after mineralization, showing a significant reinforcement effect. Importantly, the mineralized SF 3D scaffolds exhibited excellent MC3T3-E1 cell viability and promoted osteogenesis. The work demonstrates a convenient strategy to fabricate SF-based hybrid 3D scaffolds with bone-mimetic components and desirable mechanical properties for bone tissue engineering.

Keywords: 3D-printing; Structural biomaterial; Mineralization; Hybrid material

1. Introduction

The incidence rate of bone injury and defect is increasing due to the aging of the global population and the ever-increasing rate of cancer and tumor1,2. Despite being the clinical gold standard, traditional bone implant materials based on titanium alloys have disadvantages of low induction, high incidence of interfacial detachment, and inability to degrade in vivo. Therefore, there has been continued and concerted efforts in developing implants based on biopolymer and biominerals that exhibit improved biocompatibility and body-absorption properties3,4.

Three-dimensional (3D) printing provides a method to process materials into devices through computer-aided design5,6. The large design freedom in the composition and structure of materials across multiple scales as well as the production efficiency make the technology highly popular in biomedical engineering, for example, 3D-printed scaffolds for bone and cartilage repair7,8. Among different techniques, the extrusion-based printing is the most widely used technique with high “ink” designability and printing efficiency9,10. Ideal bio-inks for 3D printing should have good mechanical properties, rheological properties,
Silk fibroin (SF) is a natural protein extracted from silks, which can be processed into a variety of morphologies with tunable molecular structures and mechanical properties, ranging from soft hydrogels to stiff thermoplastics \cite{16-18}. SF has been approved by the U.S. Food and Drug Administration for use as biomaterials for decades. Many researchers have advanced the field of using regenerated SF as biomaterials especially in tissue engineering \cite{19-21}. These studies have indicated that SF has excellent biocompatibility with many tissues including bone, low immunogenicity, and inflammation compared with other synthetic and natural polysaccharides. Moreover, SF possesses excellent properties for stimulating bone repair, because the fibrous structure of SF is mostly similar to collagen I (Col I) resulting in accelerated bone healing by improving local blood supply and collagen synthesis. When supplemented with growth factors, SF could enhance mineralization, among which the effect from the functional amino acids with amide groups and hydroxyl groups and the conformation effect from β-sheets were believed to be the most significant in regulating mineral nucleation and formation \cite{22-24}. The challenge for printing SF solution is its low viscosity and poor shape fidelity. Sodium alginate (SA) produces viscous aqueous solutions and exhibits shear thinning characteristics, which is desirable for 3D printing. Mixture inks from SF and SA or alginic acid (ALG) have been studied to fabricate scaffolds with good cell compatibility \cite{25,26}. The results also suggested although SF was expected to enhance the stiffness and strength of hydrogel scaffolds. However, the lack of control over the conformational structure of SF could limit the reinforcement effect. The comprehensive properties of 3D-printed SF scaffolds could be conveniently tuned through changing the porosity, pore size in the printing design, to match that of specific tissues. At present, SF scaffolds can be fabricated to suffice most soft-tissue application requirements \cite{27,28}.

Bones are the stiffest among all the human anatomical tissues, which is attributed to the high mineral content and a sophisticated collagen-hydroxyapatite (HA) hybrid morphology \cite{29}. Therefore, minerals, particularly various forms of calcium phosphates including HA \cite{30}, tricalcium phosphate \cite{31} (TCP), and monetite (DCP) \cite{32}, are often introduced to reinforce the 3D-printed bone scaffolds. HA is the natural mineral crystal that exhibits a satisfying bone induction effect. Both brushite (DCPD, CaHPO$_4·2$H$_2$O) and monetite (DCP/DCPA, CaHPO$_4$$_x$) can be converted into HA under physiological conditions \cite{33,34}. DCPD can be further converted into DCP in aqueous solutions or at high temperatures \cite{35,36}. In contrast to HA, DCPD and DCP have unique advantages in terms of better resorption and regeneration of bone under physiological circumstances \cite{37}. In addition, the introduction of minerals can improve cell adhesion and promote cell proliferation \cite{38}.

Many works have explored the composites/hybrids from SF and calcium phosphate or precursors for bone regeneration. In most recent research, SF-based hybrid scaffolds were fabricated by freeze-drying. Not many works focused on the mineralization on 3D printed SF scaffolds with highly porous morphologies and high β-sheet conformation content \cite{22,38,39}.

In this study, we prepared SF scaffolds through extrusion-based printing with sodium alginate (SA) as a thickener; then introduced various calcium phosphates for hybrid scaffolds through post-mineralization. The relationship between structure and morphology and the mechanical performance SF and hybrid scaffolds was studied. Furthermore, a series of in vitro biological experiments, including CCK-8 cytotoxicity test, by q-PCR alkaline phosphatase (ALP) expression test, and osteogenesis gene (Runx2, OPN, OCN, OSX and Col1a) expression test, were conducted to prove the excellent osteogenesis effect of these mineralized 3D printed SF scaffolds. The work would provide a convenient strategy to engineer mechanically robust hybrid scaffolds for bone tissue engineering.

2. Materials and methods

2.1. Experimental materials

Ethanol (99.7%), methanol (99.5%), and ammonia solution (25 – 28%) were purchased from Modern Oriental Technology Development Co., Ltd. (Beijing, China). Glacial acetic acid (99.5%) and sodium bicarbonate (analytically pure) were purchased from Beijing Chemical Works (Beijing, China). Lithium bromide was purchased from Sigma-Aldrich (St. Louis, MO). Calcium acetate (99%), diammonium hydrogen phosphate (99.9%), and polyethylene glycol (PEG) were obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). SA (chemically pure) was purchased from Xilong Chemical Co., Ltd. (Guangdong, China).

2.2. Preparation of SF and SA mixture inks

The preparation of SF aqueous solution was carried out in accordance with the protocol in ref.\cite{40}. 10 g Bombyx mori cocoon silk (Fuxiang Pharmaceutical Technology Co., Ltd., Shanghai, China) was boiled in 0.5% NaHCO$_3$ solution for 45 min and rinsed with distilled water to wash off the residual sericin before drying at 45°C for 24 h. Then, 14 g dried silk fiber was dissolved in 100 mL 9.3 M lithium bromide solution at 40°C for 3 h, and dialyzed in deionized water for 3 days using a dialysis bag (8 – 14 kDa). Subsequently, the SF solution was dialyzed in 15% PEG
(Macklin Biochemical Co., Ltd., Shanghai, China) solution to reach the final concentration of 15.0 - 16.0 wt% and stored at 4°C refrigerator for further use.

0.3 g SA was dissolved in 9.7 g deionized water at 45°C for 30 min to obtain the 3.0 wt% SA solution. To prepare mixture solutions, the SF solution and the SA solution were mixed in different mass ratios of 1.5:1, 1.8:1, and 2.0:1, which were named Ink 1, Ink 2, and Ink 3, respectively, as shown in Figure 1B. The solid content of the mixed solutions was 10.2 – 11.0%. The mixed solution was stirred at room temperature for 1 h using magnetic stirring, then centrifuged at 3500 rpm/min for 10 min, and the final mixture solutions were stored in a refrigerator at 4°C.

2.3. Extrusion printing of SF scaffolds

Fisnar liquid dispenser (F4200N.2) with a –20°C RT platform was used to print scaffolds. The ink was stored in a syringe connected to a needle with a diameter of 210

Figure 1. Fabrication of silk fibroin hybrid scaffolds with post-mineralized calcium phosphate. (A) The extrusion printing of mixture inks of silk fibroin and sodium alginate followed by the stabilization treatment using a methanol-based solution and the removal of sodium alginate using citric acid solution. (B) Ink formulations for extrusion printing of silk fibroin scaffolds and the viscosity-shear rate curves of silk fibroin (15 wt%), sodium alginate (3 wt%) and the mixed inks. (C) The post-mineralization of the SF scaffolds under conditions of varied pH and temperatures.
μm. The extrusion pressure was 1.2 – 1.5 bar. A typical 3D intersecting grid structure was designed. The three mixture inks were extrusion-printed to fabricate scaffolds at the printing speeds of 6 – 10 mm/s. As shown in Figure 1A, the scaffolds were frozen at various platform temperatures (−10°C, −13°C and −15°C). Then, the frozen scaffolds were carefully transferred to a solution containing 80% methanol, 10% deionized water, 5% ethanol, and 5% calcium chloride for 24 h to complete deformation transition of SF and reach stabilization. SA was dissolved and removed by putting the scaffolds in 1 M citric acid solution, which were then soaked and washed with deionized water.

2.4. Post-mineralization of SF scaffolds

SF scaffolds from Ink 3 with a SF concentration of 10.0 wt% were chosen to fabricate hybrid SF scaffolds. As shown in Figure 1C, for the post-mineralization of SF scaffolds, calcium acetate and diammonium phosphate solutions were first prepared with three pH values (4 – 5, 7 – 8, and 10 – 11) and two solvent systems, aqueous-based and methanol-based, respectively. About 25% ammonia and 25% acetic acid solution were used to adjust the pH. The scaffolds were cut into cuboid shapes with 4 mm × 4 mm at the bottom side, and then dipped in calcium acetate solution and diammonium phosphate solution alternately, each time for 30 s. The excess solution on the scaffolds was gently absorbed by filter paper after taking out from the first solution and before immersing in the next solution. The procedure was repeated for 5, 10, and 15 times. For each mineralization group, more than five samples were prepared.

2.5. Rheological test

Rheology tests of the inks were performed on a Rheometer (DHR-2, TA Instruments, New Castle, DE, USA). For all the experiments, 25 mm 2° cone plate was used. The viscosity-shear rate curves were obtained under the steady flow mode with the shear rate range of 0.1 – 1000 s⁻¹. Strain sweep tests were conducted to obtain the linear viscoelastic region and the limit of the elastic shear strain.

2.6. Scanning electron microscopy (SEM)

The microstructure and morphology of freeze-dried scaffolds were observed on a SEM (Quanta 250 FEG) at an accelerating voltage of 20 kV. The wet SF scaffolds were first cut and frozen at −40°C for 12 h before transferring to a freeze-dryer at −50°C for 24 h. The dry scaffolds were sputter-coated with gold. The external and internal cross-sectional morphologies of the scaffolds were observed.

2.7. Fourier transformed infrared spectroscopy (FTIR)

The conformation structure of SF scaffolds was analyzed using FTIR. The scaffolds were ground into powder flakes and mixed with potassium bromide at a mass ratio of 1:100, we then pressed the mixture to obtain a transparent film. The spectra were recorded in the wavenumber range of 650–4000 cm⁻¹ through the accumulation of 32 scans at 25°C. PeakFit (Version 4.12) was used to perform the peak deconvolution of Amide I region (1600–1700 cm⁻¹) and to estimate the conformation contents. For peak deconvolution, set parameters are Gaussian type, the peak width at half height (full width at half peak) of 5 cm⁻¹, 11 peaks in total, and the peak position specified according to ref.[41].

2.8. X-ray diffraction (XRD)

Crystalline structure of the SF scaffolds and the hybrid scaffolds was analyzed using an X-ray diffractometer (D/Max2200PC, Rigaku, Beijing, China) with Cu Kα radiation (wavelength of 0.1542 nm) in a step-scan mode in the 2θ range of 10–70° at a scanning speed of 6°/min. The wet scaffolds were firstly cut into 1–2 mm thick slices, which were then compressed for the test.

2.9. Porosity measurement and calculation

For metallic or inorganic porous materials, the Archimedes drainage method is convenient to measure the porosity of the scaffolds. However, for polymer materials water could go in not only the voids, but also the cell wall/polymer structure, causing swelling. Therefore, the measurement of porosity can be inaccurate. Here, we used the Archimedes drainage method to measure the porosity ($P_1$) of the SF scaffolds using Equation 1. It was repeated 5 times to obtain the average porosity for each scaffold.

$$P_1 = \frac{(M_2 - M_1)}{(\rho \times V_1)}$$

$M_1$ is the mass of the dry scaffold; $M_2$ is the mass of the scaffold saturated with water; $\rho$ is the density of water; $V_1$ is the apparent volume of the scaffold.

In addition, the theoretical porosity was also calculated using Equation 2 and taking an average density value of 1.3 g/cm³ for SF solids.

$$P_2 = 1 - \frac{M_1}{(\rho_2 \times V_1)}$$

$M_1$ is the mass of the dry scaffold; $\rho_2$ is density of SF; $V_1$ is the apparent volume of the scaffold.

2.10. Mechanical tests

Quasi-static compression tests were conducted on a dynamic mechanical analyzer/DMA Q800 (TA Instruments, Waters Ltd.). The scaffolds were firstly
submerged in phosphate-buffered saline (PBS) buffer solutions at room temperature for 12 h to equilibrate. The strain rate was 10% s⁻¹ and the maximum compressive strain was 70%. The preload force was set as 0.01 N. The compressive modulus was calculated from the initial strain range 1–3% and a linear fit. Five samples were repeated for each group.

Tensile tests of printed monofilaments were conducted on a Zwick mechanical testing machine (Zwick Line 0.5, ZwickRoll, Germany). The extension rate was 2 mm·min⁻¹. The tensile modulus was calculated from the initial strain range 1–3% and a linear fit. Fifteen samples were repeated for each group.

2.11. Water retention test

PBS solutions were used to measure the water retention behavior of the scaffolds. After soaking the scaffolds in PBS for 1–24 h, the excess water on the outer surface of the hydrated scaffold was absorbed carefully using filter paper and the mass was measured. The hydration degree H of the scaffold was calculated by the Equation 3.

\[ H = \frac{M_w - M_d}{M_d} \]  

Equation 3

Where, \( M_w \) is the weight of the scaffold before testing and \( M_d \) is the weight of the hydrated scaffold.

In vitro degradation test, the degradation behavior of scaffolds in vitro was evaluated using enzyme Protease XIV from Streptomyces griseus following a procedure reported in ref[42]. Protease XIV (3.5 U/mg) was dissolved in PBS solution at 2 U/mL. Dry samples were weighed and immersed in 1 mL protease XIV/PBS solution at 37°C. The enzyme/PBS solution was changed every 3 days. Samples were taken out after 7, 14, 21, and 28 days, rinsed with deionized water, freeze-dried, and weighed for analysis. Three repeats were recorded for each time point. The weight loss rate was calculated using Equation 4.

\[ L(\%) = \frac{M_i - M_2}{M_i} \times 100\% \]  

Equation 4

Where, \( M_i \) is the initial mass of the scaffold and \( M_2 \) is the remaining mass of the scaffold after varied degradation time.

2.12. Cell culture

Mouse derived pre-osteoblasts (MC3T3-E1) were purchased from American Type Culture Collection. Cell were cultured by Dulbeccos Modified Eagle Medium (DMEM, GIBCO, US) containing 10% fetal bovine serum (FBS, GIBCO, US) and 1% penicillin streptomycin (PS, GIBCO, US) under an incubator containing 5% CO₂ at 37°C.

2.13. CCK-8 experiment

3D printed scaffolds (SF scaffold 3 and SF scaffold 3-CaP10) were placed in 48-well culture plates. MC3T3-E1 cells at a density of 5 × 10⁴ cells/mL were inoculated on 3D printed scaffolds. After 2 h, 400 mL medium was added and cultured for 1, 3, and 5 days. At the predetermined time point, 20 μL Counting Kit-8 (CCK-8) reagent (Dojindo Kagaku, Japan) was added to each group. After further incubation for 2 h, 100 μL mixed medium was transferred to 96-well plate and the absorbance of the solution was measured at 450 nm using a microplate reader (TECAN unlimited F50, Switzerland).

2.14. Detection of Alkaline Phosphatase (ALP) activity

MC3T3-E1 cells at a density of 5 × 10⁴ cells/mL were inoculated on 3D printed scaffolds which placed in a 48-well culture plate. After 2 h, 400 mL medium was added and cultured for 1, 4, 7, and 10 days. The complete DMEM medium containing osteogenic induction fluid (OIF) was added and replaced every other day (OIF: 50 μg/mL ascorbic acid, 10 mM β-Glycerol phosphate, and 10 μg/mL and 10⁻⁸ M dexamethasone). At the predetermined time point, cells were lysed with 100 μL RIPA lysis buffer, and the cell supernatant was collected into 96-well plates. After the substrate was incubated with p-nitrophenol at 37°C for 30 min, ALP activity was measured at 405 nm wavelength by ALP detection kit (beyotime, China). Meanwhile, bicinchonic acid protein analysis kit (beyotime, China) was used to determine the total protein content at 560 nm wavelength. Finally, the ALP level was standardized according to the protein content of each group, and the ratio of OD405/OD560 was calculated to determine the ALP level of each group.

2.15. Quantitative real-time PCR (q-PCR)

Cells at a density of 10⁵ cells/ml were seeded on the 3D printed scaffold that was placed into the 6-well plates for 2 h, and then ALP detection assay was performed. After 10 days, total RNA in each group was extracted for 2 h, 400 mL medium was added and cultured for 1, 3, and 5 days. The following were described according to our previous study[43]. The Runx2, OPN, OCN, OSX, and Colla mRNA levels were normalized to that of GAPDH which was used as reference. The corresponding primers were synthesized by Sangon Biotech Crop (Shanghai, China) and the corresponding primer sequences are listed in Table 1.

2.16. Statistical analysis

All experimental data were measured on \( n \geq 3 \) samples. All data are presented as mean ± standard deviation (M ± SD). For \( n \geq 5 \) measurements, one-way analysis of variance (ANOVA) was performed to determine the statistical differences between groups. The statistical significance was designated as * for \( P < 0.05 \), ** for \( 0.01 < P < 0.05 \), and *** for \( 0.001 < P < 0.01 \).
Table 1. Primer sequences of Runx2, OPN, OCN, OSX, and COL1A1.

| Gene   | Primer sequences                                      |
|--------|-------------------------------------------------------|
| Runx2  | Forward: 5'-ATGCTTCATTGCGCTCACAAA-3'                  |
|        | Reverse: 5'-GCACCTCAGTCACGCAGTTTG-3'                 |
| OPN    | Forward: 5'-AGCAAGAAACTTCTCAGCAGAA-3'                |
|        | Reverse: 5'-GTAGAGTTAGTTGAGTCAGATCA-3'               |
| OCN    | Forward: 5'-CGCTCTGTCTCTCTGACCTC-3'                  |
|        | Reverse: 5'-CACTACCTATTGCGGCTTGC-3'                  |
| OSX    | Forward: 5'-ATGGCGTCCTCTCTGTTG-3'                    |
|        | Reverse: 5'-TGAAAGGTCAGCGATGGCCT-3'                  |
| COL1A1 | Forward: 5'-GCTCCTTCTTAGGGCCACT-3'                   |
|        | Reverse: 5'-CCACGTCTCACTATTGGGG-3'                   |

3. Results and discussion

3.1. Viscosity characterization of SF-based inks

As mentioned earlier, SF aqueous solutions exhibit low viscosity compared with other natural polymers, especially SA solution. Thus, SA was introduced to thicken the SF-based inks. Three formulations were prepared by carefully mixing the two solutions of varied weights, pure SF solution (15 wt%) and SA solution (3 wt%). The three mixture inks have slightly varied solid contents ranging from 10.2 wt% to 11.0 wt% and SF content from 9.0 wt% to 10.0 wt%. The viscosity-shear rate curves of SF solution, SA solution, and the three inks from steady-flow tests are shown in Figure 1B. SA solution shows stable viscosity of 6.5 Pa·s at low shear rate and an obvious shear-thinning behavior at shear rate >10 s⁻¹. In contrast, SF solution shows initial shear-thinning at low shear rate <1 s⁻¹, which may suggest a disassociation or disentanglement process of SF chains under shear. The viscosity of SF solution was ~0.1 Pa·s across the 1–10⁴ s⁻¹ shear rate range, a typical Newtonian liquid. The mixed inks exhibit intermediate viscosities between pure SF and SA solutions and also a shear-thinning behavior at both low and high shear rates, combining the characteristics of the two solutions. The viscosity of mixture inks falls in a printable range, and the three inks were applied in the extrusion printing for fabricating scaffolds. The properties of ink in oscillation mode as shown in Figure S1 reveal that the relationships between the storage modulus ($G'$) and loss modulus ($G''$) with frequency for SA and SF inks are similar, but the printable frequency range of inks gradually narrows with increasing content of SF.

3.2. Morphology and structure of SF scaffolds with or without mineralization

Figure 2A shows the micromorphology of the extrusion-printed 3D SF scaffolds from the three mixture inks. The details in the surface and the cross-section of a single filament can be seen. The needle with an inner diameter of 200 μm was used. However, the printed monofilaments showed a significant Barus “swelling” effect after extrusion. The diameter of the filament in SF scaffolds decreased from 386 ± 48 μm for Scaffold 1 to 293 ± 32 μm for Scaffold 3. It suggests that increasing the SF content can suppress the swelling effect due to its less elastic or retractable conformations compared with SA. The monofilament exhibits a lamellar structure, which could be attributed to the freezing process and similar morphologies were reported in the literature. The thickness of the lamella appeared to increase from Scaffold 1 to Scaffold 3.

Printing speed is one critical parameter for extrusion printing. As shown in Figure 2B, the increase in the printing speed from 6 mm/s to 10 mm/s did not appear to change the mean size of the monofilaments for the Scaffold 3, but the shape fidelity of the cross-sections became poor with the increasing speed. Although the micromorphology of the SF scaffolds was not affected by the printing speed in this range, the mechanical tests in Figure S2 revealed that increasing the printing speed led to dramatically reduced compressive modulus of the scaffolds, suggesting changes in the microstructure or conformation structure of the scaffolds. Thus, the lowest speed 6 mm/s was chosen. In addition, lowering the substrate temperatures from −10°C to −15°C did not appear to affect the filament morphology (Figure 2C). Although the diameters of the filaments were more consistent at −15°C, indicating a faster freezing process and shape fixation, we chose −10°C for easier temperature control in an open-air printing environment.

After a stabilization treatment of the frozen scaffolds in the methanol solution and rinse out of the SA using citric acid, the 3D SF scaffolds were further processed with post-mineralization. Methanol and ethanol can instantly induce the β-sheet conformation transition in SF and led to a robust physically cross-linked morphology.[45,46] Moreover, the calcium ions can coordinate with carboxylic groups in SA chains and could result in a crosslinking structure in SA. The content of mineralized calcium phosphate in the scaffold can be controlled by the number of deposition cycles as
shown in Figure 1C. The morphology of mineralized hybrid SF scaffolds from Ink 3 after 15 deposition cycles is shown in Figure 2D. At pH 4 – 5, both the organic solvent and aqueous environments promoted the formation of calcium phosphate on the SF filaments. With increasing pH, the minerals intended to form in the space of the scaffold, reducing the porosity of the scaffold. Overall, the mineralization can successfully introduce a calcium phosphate “shell” for the filaments and conveniently produced the hybrid scaffold. Although the micromorphology of the hybrid SF scaffold did not change much under varied pH conditions, the mineral crystalline phase and the morphology of crystals changed as shown in Table S1.

Figure 3A compares the conformation and crystalline structure of SF scaffolds prepared in various conditions. ATR-FTIR was used to characterize the conformation structure of various SF scaffolds and the mineralized scaffolds from Ink 3. The characteristic absorption peaks centered at 1624 cm\(^{-1}\), 1526 cm\(^{-1}\), and 1233 cm\(^{-1}\) correspond to Amide I, Amide II, and Amide III bands of SF. Peaks at 3279 cm\(^{-1}\), 1590 cm\(^{-1}\), 1409 cm\(^{-1}\), and 1030 cm\(^{-1}\) are characteristic of SA. Although the majority of SA was dissolved by citric acid wash, residual SA was detected in the scaffolds. The conformation contents of SF were further analyzed based on the Amide I peak deconvolution (Figure S3) using the method in ref.\(^\text{[41]}\). With increasing SF content, the Silk II or β-sheet
conformation gradually increased from 38.7% for Ink 1 to 52.1% for Ink 2. The β-sheet content of mineralized scaffolds was maintained, suggesting that no changes were induced in the conformation of SF scaffolds during the post-mineralization. The peak at 1030 cm$^{-1}$ for the mineralized SF scaffold was characteristic of PO$_4^{3-}$, which further proved the formation of calcium phosphate in the scaffold.

Figure 3B compares the XRD patterns of three mineralized SF scaffolds under aqueous conditions. The wide peak at 20.96° denotes the crystalline phase of SF$^{[49,50]}$, which further proved the formation of calcium phosphate in the scaffold.

At pH 10–11, an amorphous calcium phosphate phase (ACP) of calcium phosphate also appeared. Figure 3D reveals that the morphology of CaHPO$_4$ (monetite or DCP) prepared at two temperatures was similarly irregular particles, and Figure 3E confirms that both were the same DCP crystalline phase compared to the DCPD phase at room temperature. Table S2 summarizes the results from the XRD of SF scaffolds under various mineralization conditions. For the aqueous environment, at pH 4 – 5, DCPD was the only calcium phosphate phase; at pH 7, it changed to a combination of DCPD and DCP; at pH 10–11, it reverted to a completely different amorphous phase of ACP. Such mineralization results agreed well with previously reports without using...
templates\textsuperscript{[34]}, suggesting that the SF scaffold does not have much template effect on the crystalline formation of calcium phosphate under general thermodynamics. Thus, the crystalline phase of calcium phosphate on SF scaffolds can be designed and controlled using common mineralization parameters and conditions.

Figures 3C and D show the details of the crystalline morphology under varied mineralization conditions. The crystals from the organic solvent environment were more uniform and in slightly greater sizes than those from the aqueous solution. Most crystalline morphologies were plate-like and the exception was the amorphous phase under pH 10–11 and aqueous environment. It is worth noting that the amorphous phase of calcium phosphate was believed to be better resorbed compared with other forms, thus providing a better calcium source, and exerting a better effect of bone-induction\textsuperscript{[50]}. This mineralization condition deserves further exploration.

3.3. Water retention and in vitro degradation behavior of SF scaffolds

Water retention of scaffold is a basic characteristic of porous scaffolds, reflecting the porosity and the hydrophilicity of scaffold materials. Figure S4A shows the mass change of scaffolds after water absorption for 24 h. For SF scaffolds, the equilibrium water contents are in the range of 860%–880%. The first 1 h was the fastest water absorption period, followed by a slow-increase period. The scaffold reached saturation of water absorption after only 1 h, which is appropriate for the scaffold to be applied in tissue engineering.

Porosity is also an important parameter of scaffolds. High porosity promotes the transportation of nutrients, oxygen, metabolites, etc. The drainage method was used to estimate the porosity of the scaffold. According to Equation 1, despite the increase of SF in the scaffolds, the porosity values \( P_1 \) of 68 ± 5%, 67 ± 7% and 70 ± 2% did not show obvious difference. According to Equation 2, the porosity \( P_2 \) of the scaffolds can be calculated as 91 ± 1%, 87 ± 1% and 90 ± 1%. For the drainage method, water may not enter the closed pores or small pores of the scaffold, resulting in smaller amount of absorbed water. On the other hand, the density used to calculate \( P_2 \) may be greater than the actual density in the pore walls of the scaffolds, leading to greater porosity values. Therefore, the actual porosity of the scaffolds should be between \( P_1 \) and \( P_2 \). There was no significant difference among the scaffolds of varied SF contents.

The degradation behavior of the scaffold must also be considered in tissue engineering. Figure S4B shows the mass change of the scaffolds during the in vitro degradation in the solution of protease XIV and PBS. The scaffolds degraded the fastest in the 1\textsuperscript{st} week, and reached a slower degradation rate in the later 3 weeks. The residual mass of scaffolds at the end of 4 weeks was 30.0 ± 8.5%, 41.0 ± 10.0%, and 43.0 ± 9.8%, respectively. Because protease XIV acts on the hydrophilic region of SF, it easily attacked the hydrophilic sites that were more exposed to water and led to the initial fast degradation. Obviously, the silk II structure that was much less hydrophilic was difficult to degrade in SF. Thus, the SF scaffold with more SF and hence more silk II structure appeared the slowest to degrade and maintained the greatest mass at a similar time point. In contrast with pure SF scaffolds, the mineralized scaffolds showed much slower degradation rate in the 1\textsuperscript{st} week and exhibited a slight mass increase in the later 3 weeks. It is proposed that the mass increase may be the dissolution and recrystallization of DCPD crystals into HA crystals, which was observed in another research\textsuperscript{[22]}. The mineralized scaffolds with the minerals/ crystals covering the SF scaffolds were more stable under in vitro degradation, which may provide longer support as an implant material.

3.4. Mechanical properties

Before evaluating the mechanical performance of the scaffolds, we extrusion-printed the monofilaments from three inks and tested their tensile properties. Figure 4A shows the tensile stress-strain curves of various filaments. It is interesting to note that the tensile breaking stress of the monofilament increased from 0.24 MPa for Ink 1 to 0.94 MPa for Ink 3 (Figure 4B). Similarly, the tensile modulus showed a marked increase from 0.82 ± 0.17 MPa to 2.82 ± 0.43 MPa. With only 1% increase in the SF content, both tensile strength and modulus were elevated by ~3 times, confirming that the conformation structure in Ink 3-printed scaffolds was different and contained a significantly greater content of Silk II structure.

The compressive stress-strain curves of the SF scaffolds are compared in Figure 4C. The compressive modulus derived from the initial linear region between 1% and 3% strain in Figure 4D are 166 ± 16 kPa, 242 ± 36 kPa, and 241 ± 16 kPa for Ink 1, Ink 2, and Ink 3-based scaffolds, respectively. The compressive properties of the scaffolds were consistent with that of monofilaments. Our SF scaffolds were significantly stiffer than previously reported SF/SA scaffolds based on inks with lower SF concentration, for example, ~5%\textsuperscript{[51]}. After mineralization, the compressive modulus of mineralized SF scaffolds increased to 698 ± 62 kPa, 1611 ± 273 kPa, and 1188 ± 347 kPa after 15 deposition cycles for the three inks, exhibiting a five-fold increase as shown in Figure 4G. These results agreed with Morris’s “eggshell” theory, suggesting the hard and stiff mineral deposit can markedly enhance the modulus of the mineralized SF scaffolds.

As discussed above, with increasing pH, the crystalline phase from mineralization would change from DCPD at acidic pH to DCP and HA at basic pH. The DCPD...
phase was proven stiffer and stronger than DCP\(^{[52]}\). Thus, as shown in Figure 4E and F, mineralization at pH 10 – 11 resulted in hybrid SF scaffolds with significantly reduced compression modulus and strength.

Figure 4. Tensile and compression mechanical properties of dry SF scaffolds. (A) Stress-strain curves of different monofilaments. (B) Tensile modulus of different monofilaments. (C) Compressive stress-strain curves of various scaffolds. (D) Compressive modulus of various scaffolds. (E) Compressive stress-strain curves of mineralized SF scaffolds from Ink 3 at various pH conditions. (F) Derived compressive modulus from the initial linear region 1% to 3% of the curves in (E). (G) Relationship between compressive modulus and mineralization times of scaffolds mineralized in methanol solution. Significant differences are denoted as: * for \(P<0.05\); ** for 0.01<\(P<0.05\); *** for 0.001<\(P<0.01\).
Interestingly, the Ink 2-based SF scaffolds exhibited the greatest modulus. The acidic residues and hydrophilic groups can interact better with Ca\(^{2+}\), thus leading to more calcium mineral[53,54]. β-sheet conformation tended to induce the formation of calcium minerals. Nevertheless, we propose multiple reasons that resulted in the high modulus of the SF scaffolds, and the establishment of structure-morphology-mechanical property correlations requires future investigations.

3.5. 3D printed SF scaffold 3-CaP10 promotes cell proliferation and osteogenesis

According to the results of our CCK-8 experiment, on the 1st day, there was no significant difference of OD value between the control, SF scaffold 3, and SF scaffold 3-CaP10 groups (Figure 5A). On the 3rd and 5th days, the OD value in each group significantly increased, and SF scaffold 3-CaP10 showed significantly greater cell density compared with the control and SF scaffold 3 groups, but there was no significant difference between the control and SF scaffold 3 groups (Figure 5A). On day 5, the number and morphology of cells were in good shapes under the light microscope (Figure S5). These findings show that 3D printed SF scaffold 3 and SF scaffold 3-CaP10 have no obvious toxicity to the normal MC3T3-E1 cells and show a trend of promoting cell growth. From the results of the ALP assay (Figure 5B), we can see that with the extension of culture time, the intracellular ALP level of OIC, OIC + SF scaffold 3, and OIC + SF scaffold 3-CaP10 groups showed an upward trend compared with the control group. On days 4 and 7, the intracellular ALP levels of OIC, OIC + SF scaffold 3, and OIC + SF scaffold 3-CaP10 groups showed significant differences compared with the control group. On day 10, we found that OIC + SF scaffold 3-CaP10 showed the highest ALP level.

In the meantime, we detected the expression of osteogenic genes in each group on day 10 (Figure 5C). The results showed that there were significant differences in osteogenesis-related genes (Runx2, OPN, OCN, OSX, and Col1a1) between OIC + SF scaffold 3-CaP10 and the rest of groups including control, OIC, and OIC + SF scaffold 3. Notably, there were no significant differences between OIC and OIC + SF scaffold 3 groups, which was consistent with the results of ALP assay. The above results prove that the mineralized SF scaffold (SF scaffold 3-CaP10) has the potential to promote cell osteogenesis.

Conclusion

In this study, SF-based inks with SA as a “thickener” were extrusion-printed to prepare 3D scaffolds, and various printing parameters including extrusion speed and substrate temperature were investigated. Post-mineralization was applied subsequently to prepare mineralized SF scaffolds, and various mineralization conditions were compared. The study provides a facile way to fabricate “egg-shelled” scaffolds with tuned mineral phases and mechanical properties. Most importantly, in vitro cell experiments proved the mineralized SF scaffolds exhibit low cell toxicity and promote cell osteogenesis. We propose that such mechanically robust and osteocyte-compatible scaffolds could be potential candidates for structural materials in bone tissue engineering.

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Conflict of interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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