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Growth factor-free salt-leached silk scaffolds for differentiating endothelial cells

Liying Xiao^{a,b}, Caihong Zhu^c, Zhaozhao Ding^{a,b}, Shanshan Liu^d, Danyu Yao^{a,b}, Qiang Lu^{a,b}, and David L Kaplan^e

^aNational Engineering Laboratory for Modern Silk & Collaborative Innovation Center of Suzhou Nano Science and Technology, Soochow University, Suzhou 215123, People's Republic of China

^bKey Laboratory of Stem Cells and Biomedical Materials of Jiangsu Province and Chinese Ministry of Science and Technology, Soochow University, Suzhou 215123, People's Republic of China

^cResearch Center of Robotics and Micro System, Soochow University, Suzhou 215021, People's Republic of China

^dSchool of Medicine, Shenzhen University, Shenzhen 518060, People's Republic of China

^eDepartment of Biomedical Engineering, Tufts University, Medford, MA 02155, USA

Abstract

Recently, controllable kinetic assembly was introduced into the salt-leaching process with silk proteins to form scaffolds, which achieved improvement in tuning the micro-structural and mechanical properties. Here, more control of the kinetic assembly of silk in the process was integrated into salt-leaching process, resulting in significant mechanical modification of the scaffolds generated. Both glycerol additions and treatment to concentrate the protein were used to tune hydrophilic interactions during aqueous solution processing and to reduce beta-sheet formation during the salt-leaching process. These new scaffolds showed gradient changes in elastic modulus in the range of 0.9 to 7.9 kPa. Bone marrow mesenchymal stem cells grew well and showed endothelial differentiation behavior on the scaffolds with optimized stiffness. These results indicated that the introduction of silk kinetic assembly provides an additional option for the control of porous silk scaffold properties.

Graphical Abstract

Liying Xiao, Caihong Zhu and Zhaozhao Ding have contributed equally to this paper.

ASSOCIATED CONTENT

Supporting Information

Optical image of the scaffolds; AFM images and DLS size distribution of the silk solutions prepared through various processes; FTIR determination of secondary structures of different silk scaffolds through FSD of the amide I region.

Various kinetic factors were introduced into traditional salt-leached process to prepare silk scaffolds with tunable mechanical properties and vascularisation capacity.

Keywords

Silk; Assembly; Salt-leaching; Endothelial Differentiation; Tissue Regeneration

Introduction

Tissue engineering is emerging as a strategy to optimize biological substitutes for tissue restoration where the formation of vascular networks is critical for nutrient exchange, oxygen delivery, and long-term survival of tissues.¹ Although several strategies have been developed related to vascularization, including the incorporation of various growth factors and cell-based prevascularization to induce vascularization *in vitro* and *in vivo*, inherent limitations for these options include rapid degradation of the growth factors and time-consuming prevascularization procedures.^{2–4} More promising and simplified approaches are needed to achieve vessel formation. Recently, physical cues have been explored as effective factors to regulate cell behavior and tissue regeneration.^{5, 6} Stem cells could be differentiated into endothelial cells on growth factor-free materials with suitable stiffness,⁷ suggesting a path towards a more reliable strategy of preparing substrates or scaffolds with endothelial differentiation capacity, thus simplifying the process. These studies suggested that biomaterials with modulus in the range of 1–7 kPa could induce endothelial differentiation of stem cells without the addition of growth factors.^{5–7} Considering that the scaffolds with stiffness of near 1 kPa are matchable with nerve while that with higher modulus are suitable for skin, it is possible to develop bioactive scaffolds with endothelial differentiation capacity used in various soft tissue regenerations.

As a natural protein polymer, silk has become a useful material as a support matrix for cell culture, as scaffolds in tissue engineering and implantable devices, due to its versatile processability, unique mechanical properties, controlled biodegradability, and excellent

biocompatibility.^{8,9} Silk scaffolds with three-dimensional (3D) microenvironments have been prepared through different processes to mimic the native extracellular matrix (ECM), achieving better tissue reconstruction.^{10–12} However, the scaffolds generated often show higher stiffness than required for endothelial differentiation due to beta-sheet formation in various insolubilizing treatments. This can be overcome in part via plasticization with glycerol.^{13–16}

Recently, we described the self-assembly of silk materials in aqueous solution, suggesting the possibility of tuning nanostructures and conformations in silk scaffolds.^{17–19} Through controlling the kinetics of assembly, water insoluble silk scaffolds with vascularization capacity were formed directly via a modified lyophilization process.⁷ The study motivated us to extend the strategy to other scaffold fabrication methods. Salt-leached silk scaffolds with tunable conformations and stiffness were then developed by the introduction of controlled solution concentration and pH-adjustment processes.²⁰ However, unlike freeze-dried silk scaffolds, challenges remain to generate salt-leached scaffolds with the appropriate mechanical cues to promote cell differentiation into endothelial cells.

To overcome this challenge, here, we developed a synergistic strategy to further control the secondary conformations of silk in salt-leached scaffolds. Both a controllable concentration step and the addition of glycerol were applied to achieve stronger hydrophilic interactions, and thus reduced beta-sheet formation in salt-leached process. By optimizing these two factors, salt-leached scaffolds with a more amorphous content in terms of secondary structure were prepared, resulting in softer mechanical properties and endothelial differentiation capacity.

Experimental

Preparation of fresh aqueous silk solution and concentrated silk solutions

Fresh aqueous silk solution was prepared as described previously.²¹ The degummed silk was obtained by boiling in 0.02 M Na₂CO₃ solution for 20 min. Then, the fresh aqueous silk solution (about 5 wt%) was prepared by dissolving the degummed silk in a 9.3 M LiBr solution at 60°C for 4 h, dialyzing in distilled water for 3 d, and removing silk aggregates by centrifugation at 9,000 r/min for 20 min.

The concentrated silk solutions were prepared by a concentration-dilution process.²⁰ The fresh silk solution was concentrated at 60°C to about 20 wt% and diluted to 5 wt% with distilled water. The silk solutions with concentrations about 20 wt% were prepared after 6 d and 2 d through covering the fresh silk solutions with two lids with different numbers of holes to control the drying rate, and termed as C6 and C2, respectively.

Preparation of silk porous scaffolds

The silk porous scaffolds were prepared as shown in Figure 1. The fresh silk solution was mixed with glycerol at silk/glycerol weight ratios of 100:20, 100:40, 100:60 and 100:80, respectively. The rapidly concentrated silk solution (C2) and slowly concentrated silk solution (C6) were mixed with glycerol at silk/glycerol ratios of 100:80 (wt/wt). The salt-leached scaffolds were prepared as reported elsewhere.²² In brief, 4 g of granular NaCl

(350–450 μm size) was placed into cylindrically shaped containers (diameter 1.5 cm) with 2 mL of silk solution (5 wt%) and placed at room temperature for 24 h. Then, the scaffolds were soaked in water for 3 d to extract the salt and glycerol, and dried for subsequent characterization. The scaffolds prepared from the fresh silk solution mixed with glycerol at silk/glycerol weight ratios of 100:20, 100:40, 100:60 and 100:80 were termed F-20%-S, F-40%-S, F-60%-S, F-80%-S, respectively. The scaffolds prepared from C2 and C6 solutions with high content of glycerol (silk/glycerol ratio of 100:80) were termed C2-80%-S and C6-80%-S, respectively. As controls, the scaffolds derived from various pure silk solutions (fresh solution, C2 and C6) were termed as F-S, C2-S and C6-S, respectively.

Morphology

The morphology of the scaffold samples was observed with SEM (Hitachi S-4800, Hitachi, Tokyo, Japan). For SEM, the samples were imaged at 3.0 kV after sputter-coated with gold.²¹ The microstructures of silk in aqueous solutions were evaluated with AFM (Nanoscope V, Veeco, NY, USA). 2 μL of the diluted samples (0.01 wt%) were dropped onto freshly cleaved mica surface and measured by AFM with a spring constant of 3 N m^{-1} in tapping mode.

Dynamic light scattering (DLS)

A DLS (Zetasizer Nano-ZS, Malvern Instrument, Worcestershire, UK) was operated with refractive indices of 1.33 for ddH_2O and 1.60 for protein at 25°C.²³ Three independent sample batches were measured.

Structural characterization

Fourier transform infrared spectroscopy (FTIR, Nicolet 5700, Thermo Scientific, FL, America) and X-ray diffraction (XRD, Nano ZS90, Malvern instruments, Malvern, UK) were used to analyze the structural features of the silk scaffolds. FTIR measurements were performed with the wavenumber range from 400 to 4000 cm^{-1} and 64 scans were co-added with a resolution of 4 cm^{-1} .²³ XRD was measured under 30 mA and 40 kV with a scanning speed of 6°/min.²¹

Mechanical properties

An Instron 3365 testing frame (Instron, Norwood, MA) with a 100 N loading cell was used to measure the compressive properties of the scaffolds in hydrated conditions. After the scaffolds (14 mm in diameter and 20 mm in height) were hydrated in water for 4 h, measurements were carried out at 25°C with a cross head speed of 2 mm/min until the cylinder were compressed by more than 30% of its original height. The modulus was acquired by measuring the slope of the stress-strain curve in the elastic region.²⁰ Five samples were measured for each group.

Degradation properties

Samples were cultured with PBS solution at a sample/water weight of 1:99. The samples were placed at 37°C for different days, and then dried at 60°C and weighed. Since the degraded small particles were gradually separated from the scaffolds after 7 days and

dispersed in PBS solution, it is impossible to collect the particles effectively from the solution, making the failure of quantitative evaluation of degradation property. Only qualitative information was obtained.

***In vitro* cytocompatibility of scaffolds**

All *in vitro* studies followed the ethical guidelines of the experimental animals approved by Institutional Animal Care and Use Committee, Soochow University. Bone marrow mesenchymal stem cells (BMSCs) derived from male Sprague-Dawley (SD) rats were cultured to assess the *in vitro* cytocompatibility of the scaffolds. BMSCs (1.0×10^5 cell) were cultured on the sterilized (^{60}Co γ -irradiation at the dose of 25 kGy) scaffolds disks (diameter 8 mm and height 2 mm) in Dulbecco's modified Eagle medium (DMEM, low glucose) supplemented with 1 % IU ml^{-1} streptomycin-penicillin (Invitrogen, Carlsbad, CA) and 10 % fetal bovine serum (FBS).

Cell morphology was evaluated by confocal microscopy (Olympus FV10 inverted microscope, Nagano, Japan). After cells were cultured for 1 and 12 days, the samples washed three times with PBS were fixed in 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO) for 30 min and followed by further washing. After permeabilization with 0.1% Triton X-100 for 5 min, the cells were incubated with Alexa Fluor® 488-phalloidin (Invitrogen, Carlsbad, CA) for 20 min, followed by washing with PBS. Finally, the cell-seeded scaffolds were stained with DAPI (Sigma-Aldrich, St. Louis, MO) for 10 min. Representative fluorescence images of the stained samples were obtained with excitation/emission at 358/462 nm and 494/518 nm.

To study cell proliferation in the scaffolds, samples (day 1 to 12) were digested with proteinase K overnight at 56°C. Deoxyribonucleic acid (DNA) content was measured using PicoGreen DNA assay, following the protocol of the manufacturer (Invitrogen, Carlsbad, CA, USA). Samples ($n=5$) were measured with excitation/emission at 480/530nm, using a BioTeK Synergy 4 spectrofluorometer (BioTeK, Winooski, VT). The amount of DNA was calculated based on a standard curve prepared with λ -phage DNA in 10×10^{-3} M Tris-HCl (pH 7.4), 5×10^{-3} M NaCl, and 0.1×10^{-3} M EDTA over a range of concentrations.

Cell differentiation on the scaffolds

In vitro cell differentiation in the scaffolds was evaluated via immunofluorescence staining and Western Blot analysis.⁷ For immunofluorescence staining, CD31 (endothelial cell marker) was used to characterize endothelial differentiation of the BMSCs. The samples were fixed with 4 % paraformaldehyde in PBS for 30 min, permeabilized in PBS containing 1 % Triton X-100 for 10 min, followed by washing three times with PBS, and blocked with 3 % BSA in PBS for 1 h. Then, the samples were incubated with anti-CD31 primary antibodies (Abcam, Cambridge, MA, USA), diluted in blocking buffer for 1 h, rinsed three times with PBS containing 0.1 % Tween-20, and incubated with secondary antibodies. DAPI was used to stain DNA and silk scaffolds. F-actin was identified by staining with FITC-phalloidin. Representative fluorescence images of stained samples were obtained by confocal laser scanning microscopy.

Western blot analysis was carried out as described previously.⁷ Samples were lysed with 10 $\mu\text{g mL}^{-1}$ leupeptin, 10 $\mu\text{g mL}^{-1}$ aprotinin, and 1 mM PMSF in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % SDS, 1 % NP-40, and 0.5 % sodium deoxycholate). Equal amounts of lysates were electrophoresed in 12% SDS-polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane. Membranes were blocked with 5% defatted milk and probed with anti-CD31 antibodies, then incubated with a horseradish-peroxidase conjugated secondary antibody. The ECL Western blot analysis system was used to detect the substrates.

Statistical methods

A one-way ANOVA was used to compare the mean values of the data sets. Measures are presented as means $\pm$ standard deviation, unless otherwise specified. * $P < 0.05$ was considered significant and ** $P < 0.01$ was very significant.

Results and discussion

As a typical fabrication method, salt-leaching is often used for preparing silk scaffolds.^{20, 24–27} Silk materials usually transform from the amorphous to beta-sheet structure in the salt leaching process, resulting in high stiffness. A self-assembly mechanism was introduced to the salt leaching process for tuning the conformations and nanostructures in the scaffolds.^{20, 22} Although concentration of silk protein treatments and pH adjustments were used to facilitate stronger hydrophilic interactions of silk in aqueous solutions, limiting beta-sheet formation remains a challenge for these scaffolds to achieve reduced stiffness required for vascularization.²² Therefore, further treatments were studied here to tune hydrophilic interactions between the silk molecules to control beta-sheet transformation.

Besides the concentration treatment and pH adjustment, the addition of glycerol is also effective in improving hydrophilic interactions among silk molecules.^{13–16} Water insoluble silk films with lower beta-sheet content and tunable hydrophilic interactions were prepared by adding glycerol into silk solutions.^{14, 28, 29} More aggregates with larger sizes appeared in the silk-glycerol solutions with a higher content of glycerol (Fig S1), suggesting stronger hydrophilic interactions.²² The silk aggregates were maintained after the salt-leaching process, which resulted in similar macroscopical morphology (Fig S2) but different hierarchical microspheres-microporous structures (Fig 2). Similar to glycerol-silk films, these hydrophilic interactions limited beta-sheet formation during the salt-leached process. Compared to salt-leached scaffolds from the same silk solutions without glycerol, higher amorphous content appeared in the scaffolds containing glycerol and further increased in the scaffolds with higher glycerol contents (Table S1). The tunable secondary conformations significantly influenced the mechanical properties of the scaffolds, achieving stiffness in the range of 4.0 to 7.9 kPa.

The concentration treatment and the addition of glycerol were synergistically used to further regulate the properties of the salt-leached scaffolds (Figure 3). Here, 80% of the glycerol was added to the concentrated silk solutions for stronger hydrophilic interactions (Fig S3). Compared with the scaffolds from the same concentrated solutions without glycerol, more random conformations appeared in the scaffolds containing 80% of glycerol (Figure 3, Table

S2), resulting in lower stiffness. As shown in Figure 3, the stiffness of the scaffolds further decreased gradually in the range of 0.9 to 3.5 kPa. The degradation behaviours of the various scaffolds were evaluated in PBS solution. Although we failed to obtain quantitative degradation rates since the small degraded particles of the scaffolds can't be collected effectively, it was found that the silk scaffolds with lower stiffness degraded more quickly due to their different secondary conformations, which might be suitable for various soft tissue regenerations. Therefore, by controlling the self-assembly of silk and adding glycerol in the salt-leaching process, tunable mechanical properties could be achieved. If all the scaffolds could achieve endothelial differentiation capacity, the scaffolds with stiffness of above 1 kPa would be better matrices for skin regeneration while that with stiffness near 1 kPa would be facilitate nerve repair.

Matrix stiffness is a critical cue to regulate cell behavior.^{5, 30, 31} Several studies suggested that an elastic modulus of 1–7 KPa could induce the differentiation of stem cells into endothelial cells.^{6, 7, 21} In order to evaluate the influence of stiffness on the endothelial differentiation capacity and the feasibility of the scaffolds as matrices of various soft tissues, three salt-leached scaffolds with modulus of 7.9 kPa, 4.0 kPa and 0.9 kPa, respectively (F-S, F-80%-S, C6-80%-S), were chosen to culture BMSCs. BMSC attachment and proliferation were used to assess cell compatibility.^{32–34} All of the scaffolds showed desirable cell compatibility (Fig 4). The cells adhered and spread on the scaffolds at day 1 and then occupied all of the spaces in the scaffolds by 12 days. DNA content indicated various cell proliferation behaviors on the scaffolds.²⁰ Although F-S and F-80%-S scaffolds showed similar cell proliferation, the BMSCs grew significantly better on the C6-80%-S scaffolds than on the F-S and F-80%-S scaffolds. The results are coincident with previous studies in which the scaffolds with reduced beta-sheet content usually support more rapid cell replication.^{35–38}

Immunofluorescent staining of CD31 was used to reveal endothelial differentiation behavior of the BMSCs on the scaffolds.⁷ As expected, the highest endothelial differentiation appeared with the BMSCs cultured on F-80%-S scaffolds with a stiffness of about 4.0 KPa. Although both F-S and C6-80%-S scaffolds had elastic modulus in the range of 1 to 7 kPa, little CD31 staining was found for the cells cultured on the stiff F-S scaffolds but not on soft C6-80%-S scaffolds (Fig 5A). This result coincide with our recent finding that other amorphous silk scaffolds with softer mechanical properties (1–3.5 kPa) also failed to facilitate endothelial differentiation of stem cells.³⁹ Western blotting confirmed the endothelial differentiation capacities of the scaffolds, indicating that the salt-leached scaffolds with endothelial differentiation capacity could be fabricated without the addition of growth factors (Fig 5B). Previous studies indicated that stiffness in the range of 1–7 KPa could provide physical cues to induce the differentiation of stem cells into endothelial cells.^{6, 7, 21} Since the mechanical property of the scaffolds with endothelial differentiation capacity matches with skin tissues, the scaffolds would be suitable matrices for skin regeneration. For the softer scaffolds with stiffness of near 1 kPa, more cues such as growth factors are still necessary to achieve vascularization capacity when they are used in nerve repair. Finally, our present results combine with our recent finding³⁹ implied that endothelial differentiation capacity could be optimized through tuning the stiffness elaborately, which would be realized in our future study.

Salt-leaching is a traditional method of fabricating silk-based porous scaffolds.^{40, 41} A new strategy was developed to control the assembly processes of silk.²⁰ Limited control of hierarchical microstructures and mechanical cues has been achieved through various modified salt-leaching processes, suggesting the feasibility of the strategy. Here, factors controlling silk assembly (glycerol and concentration treatment) were introduced to salt-leaching, resulting in regulation of mechanical properties and thus biological outcomes. Besides salt-leached scaffolds with myogenic differentiation capacity,²⁰ scaffolds with endothelial differentiation capacity were prepared without the need to add growth factors.

Conclusions

A synergistic strategy was developed to tune silk assembly in the salt-leaching process. Glycerol and concentration treatments were used to regulate the transformation of silk, achieving subtle adjustments of scaffold stiffness in the range of 0.9 to 7.9 kPa. After optimization of mechanical cues, the scaffolds showed endothelial differentiation capacity without the addition of growth factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Notes and references

1. Lovett M, Lee K, Edwards A, Kaplan DL. *Tissue Eng Part B Rev.* 2009; 15:353–370. [PubMed: 19496677]
2. Sun W, Motta A, Shi Y, Seekamp A, Schmidt H, Gorb SN, Migliaresi C, Fuchs S. *Biomed Mater.* 2016; 11:035009. [PubMed: 27271291]
3. Samal J, Weinandy S, Weinandy A, Helmedag M, Rongen L, Hermanns-Sachweh B, Kundu SC, Jockenhoevel S. *Macromol Biosci.* 2015; 15:1433–1446. [PubMed: 26097223]
4. Zhang WJ, Wray LS, Rnjak-Kovacina J, Xu L, Zou DH, Wang SY, Zhang ML, Dong JC, Li GL, Kaplan DL. *Biomaterials.* 2015; 56:68–77. [PubMed: 25934280]
5. Engler AJ, Sen S, Sweeney HL, Discher DE. *Cell.* 2006; 126:677–689. [PubMed: 16923388]
6. Wingate K, Bonani W, Tan Y, Bryant SJ, Tan W. *Acta Biomater.* 2012; 8:1440–1449. [PubMed: 22266031]
7. Han HY, Ning HY, Liu SS, Lu Q, Fan ZH, Lu HJ, Lu GZ, Kaplan DL. *Adv Funct Mater.* 2016; 26:421–432. [PubMed: 27293388]
8. Kundu B, Rajkhowa R, Kundu SC, Wang XG. *Adv Drug Delivery Rev.* 2013; 65:457–470.
9. Thurber AE, Omenetto FG, Kaplan DL. *Biomaterials.* 2015; 71:145–157. [PubMed: 26322725]
10. Maghdouri-White Y, Bowlin GL, Lemmon CA, Dréau D. *Mater Sci Eng C.* 2016; 59:1168–1180.
11. Wang YY, Wang XY, Shi J, Zhu R, Zhang JH, Zhang ZR, Ma DW, Hou YJ, Lin F, Yang J, Mizuno M. *Sci Rep.* 2016; 6:39477–39490. [PubMed: 27996001]
12. Chaturvedi V, Naskar D, Kinnear BF, Grenik E, Dye DE, Grounds MD, Kundu SC, Coombe DR. *J Tissue Eng Regen Med.* 2016; doi: 10.1002/term.2227
13. Pei YZ, Liu X, Liu SS, Lu Q, Liu J, Kaplan DL, Zhu HS. *Acta Biomater.* 2015; 13:168–176. [PubMed: 25463497]

14. Lu SZ, Wang XQ, Lu Q, Zhang XH, Kluge JA, Uppal N, Omenetto F, Kaplan DL. *Biomacromolecules*. 2010; 11:143–150. [PubMed: 19919091]
15. Rodriguez MJ, Brown J, Giordano J, Lin SJ, Omenetto FG, Kaplan DL. *Biomaterials*. 2017; 117:105–115. [PubMed: 27940389]
16. Brown JE, Moreau JE, Berman AM, McSherry HJ, Coburn JM, Schmidt DF, Kaplan DL. *Adv Healthcare Mater*. 2017; 6:1600762.
17. Lu Q, Zhu H, Zhang C, Zhang F, Zhang B, Kaplan DL. *Biomacromolecules*. 2012; 13:826–832. [PubMed: 22320432]
18. Bai SM, Liu SS, Zhang CC, Xu WA, Lu Q, Han HY, Kaplan DL, Zhu HS. *Acta Biomater*. 2013; 9:7806–7813. [PubMed: 23628774]
19. Zhang Y, Zhang CC, Liu LJ, Kaplan DL, Zhu HS, Lu Q. *Front Mater Sci*. 2015; 9:382–391.
20. Xiao LY, Liu SS, Yao DY, Ding ZZ, Fan ZH, Lu Q, Kaplan DL. *Biomacromolecules*. 2017; 18:2073–2079. [PubMed: 28574695]
21. Ding ZZ, Ma J, He W, Ge ZL, Lu Q, Kaplan DL. *J Mater Chem B*. 2017; 5:4789–4796. [PubMed: 29098078]
22. Yao DY, Dong S, Lu Q, Hu X, Kaplan DL, Zhang BB, Zhu HS. *Biomacromolecules*. 2012; 13:3723–3729. [PubMed: 23016499]
23. Xiao LY, Lu GZ, Lu Q, Kaplan DL. *ACS Biomater Sci Eng*. 2:2050–2057.
24. Nazarov R, Jin HJ, Kaplan DL. *Biomacromolecules*. 2004; 5:718–726. [PubMed: 15132652]
25. Kim UJ, Park J, Kim HJ, Wada M, Kaplan DL. *Biomaterials*. 2005; 26:2775–2785. [PubMed: 15585282]
26. Lu Q, Wang X, Hu X, Cebe P, Omenetto F, Kaplan DL. *Macromol Biosci*. 2010; 10:359–368. [PubMed: 20217856]
27. Agostini E, Winter G, Engert J. *J Control Release*. 2015; 213:134–141. [PubMed: 26100366]
28. Kim JH, Sheikh FA, Ju HW, Park HJ, Moon BM, Lee OJ, Park CH. *Int J Biol Macromol*. 2014; 68:158–168. [PubMed: 24794196]
29. Ribeiro VP, da Silva Morais A, Maia FR, Canadas RF, Costa JB, Oliveira AL, Oliveira JM, Reis RL. *Acta Biomater*. 2018; doi: 10.1016/j.actbio.2018.03.047
30. Pek YS, Wan ACA, Ying YJ. *Biomaterials*. 2010; 31:385–391. [PubMed: 19811817]
31. Park JS, Chu JS, Tsou AD, Diop R, Tang Z, Wang A, Li S. *Biomaterials*. 2011; 32:3921–3930. [PubMed: 21397942]
32. Guan D, Chen Z, Huang C, Lin Y. *Appl Surf Sci*. 2008; 255:324–327.
33. Wang X, Yu T, Chen G, Zou J, Li J, Yan J. *Tissue Eng Part C Methods*. 2017; 23:169–179. [PubMed: 28142371]
34. Tang YL, Xu Y, Xiao ZF, Zhao YN, Li J, Han SF, Chen L, Dai B, Wang L, Chen B, Wang H. *Sci Rep*. 2017; 7:1–15. [PubMed: 28127051]
35. Foroughi MR, Karbasi S, Ebrahimi-Kahrizsangi R. *J Porous Mater*. 2012; 19:667–675.
36. Hu X, Park SH, Gil ES, Xia XX, Weiss AS, Kaplan DL. *Biomaterials*. 2011; 32:8979–8989. [PubMed: 21872326]
37. Zhu J, Zhang Y, Shao H, Hu X. *Polymer*. 2008; 49:2880–2885.
38. Zhu MF, Wang K, Mei JG, Li C, Zhang JM, Zheng WT, An D, Xiao NN, Zhao Q, Kong DL, Wang LY. *Acta Biomater*. 2014; 10:2014–2023. [PubMed: 24486642]
39. Sang YH, Li M, Liu J, Yao YL, Ding ZZ, Wang LL, Xiao LY, Lu Q, Fu XB, Kaplan DL. *ACS Appl Mater Interfaces*. 2018; 10:9290–9300. [PubMed: 29485270]
40. Wang YZ, Kim UJ, Blasioli DJ, Kim HJ, Kaplan DL. *Biomaterials*. 2005; 26:7082–7094. [PubMed: 15985292]
41. Kim HJ, Kim UJ, Kim HS, Li CM, Wada M, Leisk GG, Kaplan DL. *Bone*. 2008; 42:1226–1234. [PubMed: 18387349]

Figure 1.
The preparation processes of silk porous scaffolds.

Figure 2.

Characterization of the scaffolds prepared from the fresh silk solution (F-S) and the blended silk solutions with glycerol at silk/glycerol weight ratios of 100:20 (F-20%-S), 100:40 (F-40%-S), 100:60 (F-60%-S) and 100:80 (F-80%-S): (A) SEM images, (B) FTIR spectra, (C) XRD spectra and (D) compressive modulus in wet conditions. * $P < 0.05$ indicated statistically significant and ** $P < 0.01$ indicated very significant.

Figure 3.

Characterization of the scaffolds derived from silk solutions treated with concentration-dilution processes: (A) morphology images, (B) FTIR spectra, (C) XRD spectra and (D) compressive modulus in wet conditions. The samples were as follows: C2-S, silk scaffolds prepared from the fast concentrated silk solution; C2-80%-S, scaffolds derived from the fast concentrated silk solution containing glycerol at silk/glycerol weight ratios of 100:80; C6-S, scaffolds prepared from the slowly concentrated silk solution; C6-80%-S, scaffolds derived from the slowly concentrated silk solution containing glycerol at silk/glycerol weight ratios of 100:80. * $P < 0.05$ indicated statistically significant.

Figure 4.

Proliferation behavior of BMSCs on silk scaffolds: (A) Fluorescence microscopy images at days 1 and 12 with silk scaffolds and nuclei in blue and F-actin in green. (B) DNA analysis ($n = 3$, * $P < 0.05$ statistically significant). The samples were as follows: F-S, silk scaffolds prepared by the salt-leaching process; F-80%-S, silk scaffolds derived from the fresh silk solution containing glycerol at silk/glycerol weight ratios of 100:80; C6-80%-S, scaffolds derived from the slowly concentrated silk solution containing glycerol at silk/glycerol weight ratios of 100:80.

Figure 5.

BMSC differentiation behavior on silk scaffolds at day 28: (A) Expression of CD31 (red) by confocal microscopy with silk scaffolds and cell nuclei in blue and F-actin in green. (B) CD31 expression as detected by Western blot. The samples were as follows: F-S, silk scaffolds prepared by the salt-leaching process; F-80%-S, silk scaffolds derived from the fresh silk solution containing glycerol at silk/glycerol weight ratios of 100:80; C6-80%-S, scaffolds derived from the slowly concentrated silk solution containing glycerol at silk/glycerol weight ratios of 100:80.