Silk fibroin/sericin 3D sponges: The effect of sericin on structural and biological properties of fibroin

Abdollah Zakeri Siavashani^{1,2}, Javad Mohammadi^{1,*}, Markus Rottmar², Berna Senturk², Jhamak Nourmohammadi¹, Behnam Sadeghi³, Lukas Huber⁴, Katharina Maniura-Weber^{2,*}

¹ Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran.

² Empa, Swiss Federal Laboratories for Materials Science and Technology, Biointerfaces, St.Gallen, Switzerland.

³ Translational Cell therapy Research (TCR), Department of CLINTEC, Karolinska Institutet, Stockholm, Sweden.

⁴ Empa, Swiss Federal Laboratories for Materials Science and Technology, Laboratory for Building Energy Materials and Components, Dübendorf, Switzerland.

Corresponding Author

* E-mail: Katharina.Maniura@empa.ch

* E-mail: javad.mohammadi@ut.ac.ir

Abstract

Among naturally occurring polymers, silk fibroin and sericin have attracted much attention in the field of tissue engineering; however, clinical application of silk fibroin/sericin scaffolds in a combined form is mainly questioned due to the possible pro-inflammatory reaction against native silk and fibroin/sericin 3D constructs. The objective of this study was to fabricate 3D spongy fibroin/sericin scaffolds and to explore the structural, biological and immunological properties of different ratios of fibroin and sericin. Structural characterization revealed a highly porous structure (>91%) with large surface area and water uptake capacity for all fibroin/sericin

This document is the accepted manuscript version of the following article:
Siavashani, A. Z., Mohammadi, J., Rottmar, M., Senturk, B., Nourmohammadi, J., Sadeghi, B., ... Maniura-Weber, K. (2020). Silk fibroin/sericin 3D sponges: the effect of sericin on structural and biological properties of fibroin. *International Journal of Biological Macromolecules*. <https://doi.org/10.1016/j.ijbiomac.2020.02.316>

scaffolds. Notably, the scaffolds showed enhanced mechanical properties and a higher degradation rate with increasing sericin content. Excellent cell attachment and no significant cytotoxicity was observed in all scaffold types 7 days after seeding of osteoblast-like MG63 cells. Gene expression of pro-inflammatory markers TNF- α , CXCL10 and CD197 as well as TNF- α secretion by THP-1-derived macrophages revealed no significant immune response to all fibroin/sericin scaffold types compared to sericin-free F1:S0 samples and TCP (M ϕ) control group. These results demonstrate that spongy fibroin/sericin scaffolds are able to support the growth of osteoblast-like cells without eliciting a pro-inflammatory response, thus being a promising material for bone tissue engineering.

Key-words: silk, fibroin/sericin scaffolds, immune response, 3D scaffold

1. Introduction

Natural polymers find increasing application in tissue engineering and regenerative medicine due to their biochemical and structural resemblance of extracellular matrix [1-3]. Among these polymers, silk proteins derived from *Bombyx mori* silkworm have played a prominent role due to ease of their processing, providing reactive functional groups for surface modification, and the possibility of fabricating scaffolds from low cost resources [4].

Fibroin is the major protein in silk and has been investigated in a variety of applications such as bone tissue scaffolds, vascular grafts, skin substitutes, and nerve tissue engineering [5-8]. In native silk, fibroin makes up the core of the fibers, providing the structural support with its remarkable mechanical properties and very slow degradation rate [9]. In contrast to other protein-based biomaterials, the process of isolation and purification of silk fibroin is neither

expensive nor complicated. Furthermore, it can be processed into a broad range of formats including sponges, particles, hydrogels, fibers, and films [10-12]. Fibroin has further been used in combination with different natural and synthetic polymers in bone tissue engineering. It has been found that combining fibroin with chitosan and collagen scaffolds can significantly improve their mechanical properties as bone tissue engineering constructs [13, 14]. Moreover, fibroin has been shown to increase cell affinity and overcome the lack of available amine and carboxyl functional groups of polycaprolactone (PCL) in 3D fibroin/PCL composites, and enhance cell-scaffold interaction by providing more cell recognition sites [15]. Silk fibroin β -sheet crystals can also provide required sites for nucleation and deposition of hydroxyapatite nanocrystals, which has been shown to up-regulate osteoblast differentiation markers [16-18].

Sericin, on the other hand, is the second major protein in silk with many promising applications for regeneration of various tissues [19, 20]. Sericin is a glue-like protein which coats fibroin fibers and facilitates adhesion between fibers. In contrast to fibroin, sericin shows brittleness and, due to its higher hydrophilicity, degrades more rapidly in aqueous solution. It was shown that sericin not only has anti-oxidant and anti-tyrosinase activity, but also it can promote collagen production in L929 murine fibroblast cells [21-23] and, if used as a surface coating, the osseointegration and osteoconduction potential of titanium implants [24, 25]. Despite these promising reports, to date only few studies have investigated the use of sericin in bone and cartilage tissue engineering scaffolds [26, 27].

In natural *Bombyx mori* silkworm cocoons, fibers are made up of 70-80% fibroin and 20-30% sericin and fibroin together with sericin form one of the strongest fibers in nature. Hence, inspired by nature, combination of fibroin and sericin for preparation of bone tissue engineering scaffolds, with the possibility to specifically tune their ratio, potentially allows combine the

virtues of both proteins and improve the shortcomings of each component. While fibroin should provide the flexible and stable basis of the scaffold, adding sericin should not only improve its mechanical properties due to adhesive characteristics of sericin but can also increase the degradation rate of the scaffolds due to higher hydrophilicity of sericin. Moreover, combining silk sericin with fibroin is known to improve weak structural properties of sericin as a fragile protein [28, 29]. Since the composition of native silk highly depends on the cocoon source and geographical and seasonal variations, it is very difficult to prepare scaffolds with exactly the same fibroin/sericin composition from native silk. Moreover, there are some other biomolecules in native silk such as seroin and fibrohexamerin which might influence the final characteristics of the scaffolds as impurities. Therefore, extracting fibroin and sericin from silk fibers and mixing them after purification offers the potential to fabricate fibroin/sericin scaffolds with more reproducible properties. For instance, Rao *et al.* could successfully fabricate PLGA-coated multi-walled fibroin/sericin nerve conduits for peripheral nerve regeneration by combining extracted fibroin and sericin [28].

The mechanisms behind the adverse immune response to native silk have been a matter of debate in the field of tissue engineering [30, 31]. Although for many years it was believed that sericin causes allergic reactions and should be removed from natural silk for immunological reasons through the process of degumming, a number of studies have shown anti-inflammatory activities for solubilized sericin when added to cells in culture [32-34]. However, in a recent study proposing the application of silk mats in bone regeneration, Jo *et al.* have claimed that released sericin from silk mats up-regulates TNF- α expression, which in turn counteracts bone regeneration [35]. On the contrary, other studies attributed the immunogenicity of some silk based materials to fibroin rather than sericin. Kurosaki *et al.* reported that soluble factors

released from fibroin induce secretion of inflammatory cytokines such as TNF- α [33]. Moreover, formation of granuloma and foreign body giant cells around fibroin was reported by Kweon *et al* [36, 37]. It has also been claimed that the physical association of fibroin and sericin is triggering immunologic reactions [38]. Hence, for designing 3D spongy fibroin/sericin scaffolds, it is necessary to consider possible immune responses and evaluate the induction of pro-inflammatory cytokines and markers by inflammatory cells.

Considering the positive and negative characteristics of both fibroin and sericin, the aim of this study was the fabrication of 3D spongy fibroin/sericin scaffolds with different weight ratios to evaluate the optimized combination of fibroin/sericin to make improved scaffold for bone tissue engineering. For this, 3D fibroin/sericin sponges were fabricated by adding sericin powder into a fixed concentration of fibroin, followed by freeze-drying and cross-linked using glutaraldehyde vapor with subsequent characterization of structural features, and evaluation of biological activity and macrophage response towards the different type of fibroin/sericin scaffolds.

2. Materials and methods

2.1. Sericin extraction

In order to extract sericin, Bombyx mori cocoons were cut into small pieces and boiled in a solution of 0.02 M sodium carbonate (Na_2CO_3 , Merck) in deionized water, based on previous reports [39, 40]. After heating for 1 h at 90 °C fibroin fibers were removed and the solution was centrifuged for 20 min at 14000 x g. Next, the sericin solution was dialyzed against deionized water using a 12000-14000 Da cellulose membrane for 48 h. Finally, the dialysate was mixed with 96% ethanol (75:25), stirred at room temperature for 1 h and finally lyophilized for 3 days using a freeze dryer (OPERON, FDB-5503).

2.2 Fibroin extraction

Silk fibroin was extracted from silk cocoons according to an established procedure with some modifications.[34] Briefly, fibers obtained from boiled cocoons in 0.02 M Na₂CO₃ were washed three times in distilled water and then dried at room temperature. Thereafter, 20 mg lithium bromide (LiBr, Sigma) was dissolved in 20 mL deionized water and added to 2.5 g fibroin fibers and incubated at 50 °C for 1 h. The obtained solution was dialyzed against deionized water using a 12000-14000 Da cellulose membrane for 3 days. After centrifugation at 12000 x g the dialysate was used as fibroin solution (approx. 4.7 wt%).

2.3. Preparation of fibroin/sericin scaffolds

Five different scaffold types of different fibroin/sericin weight ratios were prepared by dissolving different amounts of sericin powder (0 wt%, 1.175 wt%, 2.35 wt%, 3.525 wt%, 4.7 wt%) in a fixed concentration of fibroin (~4.7 wt% final) and stirring for 5 min in an ice bath.

The solution was poured into 48-well plates, with single wells as template and transferred to -80 °C for 24 h. Finally, solutions were lyophilized for 72 h to obtain fibroin/sericin scaffolds. With increasing the concentration of sericin in the fibroin solution, the scaffolds were termed based on the fibroin/sericin weight ratios = F1:S0, F1:S0.25, F1:S0.5, F1:S0.75 and F1:S1 (Table 1).

Table 1. Composition of fibroin/sericin scaffolds.

sample code	fibroin (wt%)	sericin (wt%)
F1:S0	4.7	0
F1:S0.25	4.7	1.175
F1:S0.5	4.7	2.35

F1:S0.75	4.7	3.525
F1:S1	4.7	4.7

For chemical cross-linking, the scaffolds were exposed to glutaraldehyde vapor at 50 °C for 18 h. Cross-linked scaffolds were subsequently immersed into 99% ethanol for 3 min to induce structural transition from random coil to β -sheet conformation with superior stability and mechanical properties. Then, cross-linked scaffolds were immersed in 8 wt% L-glutamic acid solution and shaken for 3 days to deactivate unreacted glutaraldehyde [41]. Afterwards, the scaffolds were washed thoroughly with deionized water and dried at room temperature.

2.4. Morphological characterization

Scanning electron microscopy was employed to observe the microstructure of the scaffolds before and after cross-linking. After cross-sectioning the scaffolds, they were sputter-coated with a 5 nm gold layer, and SEM images were obtained using 15 kV operating voltage (AIS2100, Seron Technologies). ImageJ software was used to measure the average pore size. For each scaffold, cross-sectional images from 3 independent samples were analyzed.

2.5. Mechanical properties

To assess compressive strength and modulus of the scaffolds, specimens of 6 mm in diameter and 7 mm height were pre-wetted in PBS for 24 h and then examined using a compression instrument (Zwick/Roell Z050, Germany). The stress-strain curve was recorded at a compressive speed of 1 mm/min and scaffolds were compressed to 50% of their initial height.

2.6. Water uptake

In order to evaluate water uptake of the samples, dry cross-linked scaffolds were weighed (W_i) and immersed in deionized water. After 24 h wet samples were placed on filter papers to remove excess water and weighed again (W_w). Following equation was used to evaluate water uptake of the scaffolds:

$$\text{Water uptake (\%)} = \left[\frac{W_w - W_i}{W_i} \right] \times 100$$

2.7. In vitro degradation

After weighing dried scaffolds (W_i), they were immersed in 5 mL PBS buffer (10 mM, pH 7.2) and incubated at 37 °C for 3, 7, 14, 28 and 60 days. Four separate sets of scaffolds were employed for each time interval. After the predetermined times, buffer was removed and scaffolds were washed thoroughly to remove precipitated salts. The weight of the scaffolds was measured after drying in an oven at 40 °C for 24 h [42], and *in vitro* degradation was calculated as follows:

$$\text{Weight loss (\%)} = \left[\frac{W_i - W_d}{W_i} \right] \times 100$$

2.8. Surface area measurement

Surface area of the scaffolds was measured by applying the Brunauer–Emmet–Teller (BET) method [43]. Kr adsorption-desorption isotherms of the samples at 77 °K were recorded using a Micromeritics 3Flex Surface area and Porosity Analyzer. Approximately 100 mg of each scaffold was degassed at 105 °C for 20 h at a pressure of 1.3×10^{-2} mbar before the measurement. The relative pressure (P/P_0) range was set between 0.02 and 0.62 and both adsorption and desorption branches were collected. Each sample was measured three times in order to get reproducible results.

2.9. Density and porosity measurement

The envelope density (ρ) of the scaffolds was measured using a Geopyc 1360 (Micromeritics). In this characterization method, the sample was placed in a bed of DryFlo[®] granular medium, which was agitated and carefully consolidated around the scaffold using a piston. The sample was getting consolidated by agitation and rotation of the cylindrical chamber since the piston was gradually pushed into the chamber until the consolidation force was reached, followed by retraction and recompression [44]. For each measurement, 10 cycles were carried out using a 12.7 mm diameter chamber, a conversion factor of 0.1246 and a consolidation force of 4 N. The skeleton density ($\rho_{skeleton}$) of the scaffolds was determined with an AccuPyc II 1340 helium pycnometer (Micromeritics, helium purity of 99.999%) equipped with a 1 cm³ chamber. The skeleton volume of the sample was measured by detecting the change in pressure owing to the volume of helium that was displaced by the scaffold within the sealed and pressure-equilibrated chamber. Each measurement consisted of 20 purge cycles and 20 analysis cycles. The samples were dried for 12 h at 50 °C prior to all density measurements. The porosity (Φ) was determined from the envelope density (ρ) and the skeleton density ($\rho_{skeleton}$) using the following equation:

$$\Phi = 1 - \left(\frac{\rho}{\rho_{skeleton}} \right)$$

The pore volume (V_{pore}) was calculated as follows:

$$V_{pore} = \left(\frac{1}{\rho} \right) - \left(\frac{1}{\rho_{skeleton}} \right)$$

2.10. Cytotoxicity and cell attachment

Prior to seeding, each side of the scaffolds was exposed to UV light for 20 min under sterile conditions and the scaffolds incubated for 4 h at 37 °C in complete Minimal Essential Medium Eagle (MEM, Sigma) medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin/neomycin to allow proteins from FBS to adsorb to the surface of the scaffolds and to remove air bubbles. Afterwards, the pre-wetted scaffolds were placed in 48-well plates and a 6 mm silicone ring was placed around each sample to prevent cells from falling off the scaffolds onto the bottom of the culture plate. Then, a small concentrated volume of cell suspension (100 μ L, 88×10^3 cells) was pipetted directly onto the top of each scaffold to ensure a higher fraction of cells to adhere to the surface, incubated for 10 min without agitation, and 900 μ L complete medium was added slowly to reach 1 mL medium. Finally, the scaffolds were incubated for 7 days prior to analysis at 37 °C in a 5% CO₂ atmosphere. Cell culture medium was replaced with fresh medium every 3 days.

Cytotoxicity of the scaffolds was assessed by measuring the release of lactate dehydrogenase (LDH) from the cells on days 1 and 7 using a CytoTox 96 kit (Promega). In brief, to prepare the maximum LDH release positive control, 45 min prior starting the experiment at day 1 and 7, 50 μ L lysis solution were added into triplicate wells of cells on tissue culture plate (TCP) containing 500 μ L medium. For LDH measurement of cells on the scaffolds, 50 μ L supernatant of the samples were transferred to 96-well plates with 3 replicates and incubated with 50 μ L CytoTox96 Reagent for 30 min in the dark at room temperature. Finally, 50 μ L stop solution were added and the absorbance was measured at 490 nm using a Mithras² LB 943 Multimode Microplate Reader (Berthold Technologies, Germany)

To observe cell attachment on each scaffold type on day 7, the scaffolds were washed with PBS and cells were fixed using a 4% paraformaldehyde (PFA) solution for 30 min. Fixed cells were

permeabilized by adding 0.1% Triton-X 100 solution for 30 min. Then, the solution was replaced with 1% bovine serum albumin (BSA) solution and samples blocked for 30 min. Finally, the staining solution containing phalloidin 633 (Alexa Fluor 633 phalloidin, Molecular Probes) and DAPI (Sigma) in 1% BSA was added to the scaffolds, samples were gently shaken for 2 h at room temperature in the dark and subsequently imaged using a confocal laser scanning microscope (Carl Zeiss LSM780 laser-scanning confocal microscopy system).

2.11. Cultivation of THP-1 cells and gene expression analysis

Human monocytic leukemia cells (THP-1) were used for evaluation of the immune response. To differentiate THP-1 monocytic cells into macrophages, phorbol 12-myristate 13-acetate (PMA, Sigma) was used^[45]. Prior to the experiment, the samples were sterilized using UV radiation and were pre-wetted in 200 μ L RPMI-1640 medium supplemented with 10% FBS, 1% PSN, 1% L-glutamine overnight. THP-1 cells were seeded in cell culture flasks using RPMI-1640 medium (Sigma, Life Science) supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin/neomycin, and 100 nM PMA. After 3 days, PMA-containing medium was removed and replaced with fresh PMA-free complete medium and incubated for 24h. Differentiated naïve macrophages (M ϕ) were detached from the flasks using TripLETM dissociation agent (Gibco Life Technologies) and 4×10^5 cells were directly seeded onto the surface of pre-wetted scaffolds of 5 mm in diameter and 2 mm in height in 48-well plates in a volume of 100 μ L of complete RPMI-1640 medium. After 10 min, 900 μ L medium was slowly added to the samples to get the total volume of 1 mL and incubated for 24 h. For control purposes, macrophages were polarized into a M1-like phenotype by adding bacterial

lipopolysaccharide (LPS, 100 ng/mL; Sigma-Aldrich) and purified recombinant human interferon γ 1b (IFN- γ , 20 ng/mL; Miltenyi Biotec) for 24 h ^[46].”

After cultivating macrophages for 24 h on the scaffolds, RNA from all samples was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The concentration and quality of RNA was determined by measuring the absorbance at 260/280 nm. RNA samples with A260/A280 ratio of 1.9–2.1 were considered as pure RNA. In order to obtain cDNA, RNA was reverse transcribed using the iScript cDNA synthesis Kit (BioRad). The reverse transcription reaction was started in a total reaction volume of 20 μ L containing 15 μ L RNA (150 ng/ μ L) and 5 μ L reaction mix (5x iScript Biorad reaction mix and 1x iScript reverse transcriptase) at 25 °C for 5 min, 42 °C for 30 min to reverse transcribe and 85 °C for 5 min to inactivate the reverse transcriptase.

RT-PCR analysis was performed with the iQTM SYBR Green Supermix kit (BioRad) in a 12 μ L final volume containing 5 μ L diluted cDNA with dH₂O (1:5) and 7 μ L reaction mix from a master mix (6 μ L iQ SYBR Green Supermix, 0.24 μ L forward primer of a 10 μ M stock solution, 0.24 μ L reverse primer of a 10 μ M stock solution and 0.52 μ L ultrapure water) according to the manufacturer's instructions.

The cycling conditions were as follows: an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s (denaturation) and 57 °C for 30 s (annealing). Then, a melting curve was monitored by heating from 65 °C to 95 °C in temperature steps of 0.5 °C. The reverse transcription and RT-PCR were performed using a C1000TM Thermal Cycler. The forward and reverse primers used for RT-PCR are shown in Table 2. Fold changes in gene expression were calculated via the $2^{-\Delta\Delta C_t}$ method with RPL37a as housekeeping gene.

Table 2. Sequences of primers used in RT-PCR.

Gene	Abbreviation	PCR primers (5'-3')
Tumor necrosis factor α	TNF- α	Fw. CTT TGG AGT GAT CGG CCC C Rv. GGT TAT CTC TCA GCT CCA CGC
C-X-C motif chemokine 10	CXCL10	Fw. CAGTCTCAGCACCATGAATCAA Rv. CAGTTCTAGAGAGAGGTACTCCTTG
C-C chemokine receptor type 7	CD197	Fw. GTGGTTTTTACCGCCCAGAGA Rv. CACTGTGGTGTTGTCTCCGA
Ribosomal protein L37a	RPL37a	Fw. ATTGAAATCAGCCAGCACGC Rv. AGGAACCACAGTGCCAGATCC

2.12. DNA quantification and cytokine secretion

Quantification of total cell DNA was performed using a Hoechst assay. One day after seeding macrophages onto the scaffolds, the supernatant was removed for the measurement of cytokine secretion by ELISA and 250 μ L deionized water was added to the cells remaining on the scaffolds. The samples were frozen at -20°C . After 24 h, they were thawed and vigorously shaken at RT for 3 h. Freeze-thaw cycles were repeated for another 2 times. bisBenzimide dye (Hoechst 33258, Sigma-Aldrich) in TNE buffer (10 mM Tris; 2 M NaCl; 1 mM EDTA; pH 7.4) was used for DNA quantification and calf thymus DNA (Sigma, D-3664) was used for preparation of a standard curve by serial dilution from 30 μ L/mL. Then, 100 μ L of samples and standards were mixed with 100 μ L of Hoechst working solution. After shaking for 1 h at RT in the dark, the fluorescence measurement was performed at 350/460 nm excitation/emission wavelengths.

The amount of secreted TNF- α in the supernatant was evaluated using a TNF- α Human ELISA Kit (Invitrogen) following the manufacturer's protocol. All samples were prepared in 3 replicates

and cytokine measurement was performed for 3 sets of experiments. The obtained results were normalized to the DNA content of the samples.

2.13. Statistics

Statistical analysis was performed using Graph Pad Prism software (GraphPad, CA USA). Data obtained from weight loss of the scaffolds and LDH assay were analyzed using a two-way analysis of variance [47] with Tukey's multiple comparisons test. For all other data, one-way ANOVA with Tukey's multiple comparisons test was used to determine significant differences of the experiments. Results are presented as mean $\pm$ standard deviation. Values of $p < 0.05$ were considered as statistically significant.

3. Results and discussion

3.1. Structural characterization of the scaffolds

3.1.1 Morphology and porosity

Fabrication steps of 3D spongy fibroin/sericin scaffolds by freeze-drying and subsequent cross-linking with glutaraldehyde vapor is shown in Figure 1A. The microstructure of the scaffolds was evaluated by scanning electron microscopy [48], Brunauer–Emmet–Teller (BET) and porosity measurement (Figure 1B, Table 3, Supplementary Figure S1). Furthermore, the presence of sericin in the scaffolds was evidenced through attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR) (Supplementary Figure S4).

SEM images revealed the formation of a highly inter-connected porous structure after the lyophilization process for all formulations, which was also confirmed by the porosity measurements. The obtained structural data showed porosity values of 91–94%, BET surface

area of 0.54–1.74 m²/g, V_{pore} of 8.74–14.76 cm³/g, and an apparent density of 1.06–1.18 g/cm³. The pore size distribution of different scaffold types before and after crosslinking is displayed in Table S1. Following the crosslinking procedure, although pore diameter of the scaffolds decreased from 143 ± 35, 119 ± 24, 120 ± 25, 87 ± 18, and 84 ± 13 μm for samples F1:S0, F1:S0.25, F1:S0.5, F1:S0.75 and F1:S1, respectively before crosslinking to 109 ± 21, 102 ± 23, 102 ± 21, 83 ± 16, and 72 ± 14 μm after crosslinking, the microporous structure was still well conserved. Moreover, with increasing sericin content a decrease in the pore size, porosity (of approximately 3 %), and V_{pore} (of approximately 13-40%) of the scaffolds was achieved, which was also evident from SEM images. However, no significant structural change in pore morphology was observed. The effect of sericin in pore size is consistent with literature on sponge studies, where with increasing sericin concentration pores become smaller [49]. The range of pore sizes in the interconnected network of trabeculae of cancellous bone is about 75–200 μm and the minimum pore size for bone growth is considered to be around 75–100 μm [50]. Therefore, all fibroin/sericin scaffold types meet the minimum required pore size for supporting tissue growth.

The decrease in mean pore size with increasing sericin content might be due to the increase of total fibroin/sericin mass per unit volume, which is in line with the results of Mandal *et al.* who related the reduced pore size of gelatin/sericin sponges with a fixed amount of gelatin to the addition of sericin and subsequent increase in mass per unit volume [51]. Furthermore, since the porosity of the scaffolds directly depends on viscosity and ice-crystal formation during the freezing process [52], the increasing viscosity of the fibroin/sericin solution with addition of sericin potentially resulted in smaller ice-crystals being formed. In this regard, Kurland *et al.* showed that despite low molecular weight of sericin, it can increase the viscosity of fibroin

solution, and attributed this phenomenon to glue-like and granular characteristics of sericin arising from its ability to entangle with neighboring molecules [53, 54].

Besides pore size, surface area and porosity play a critical role in the function of biomaterials as two important structural parameters. The presence of a highly interconnected porous structure can support the initial cell attachment and influence on various cellular activities such as cell proliferation and cell survival. High porosity and surface area can also facilitate the transport of nutrients and waste. It was reported previously that scaffolds with high porosity are able to increase the proliferation rate of the cells [55]. Based on Table 3, the porosity of all scaffold types was higher than 91%, and even though an increasing sericin content lead to a decrease in surface area of the scaffolds, this surface area is still higher than some commercially available bone substitutes and high enough for effective cell growth applications [56].

The obtained results regarding scaffold density showed a very similar apparent density of the scaffolds to that of normal human bone which makes them suitable candidates for bone tissue engineering. For instance, Misch *et al.* have reported a bone density around 1.18 g/cm^3 for mandibular trabecular bones with bone marrow in situ and Seong *et al.* have reported an average bone density of 1.18 g/cm^3 in cortical bone and trabecular mandible and of 0.67 g/cm^3 in maxilla bone [57, 58].

These data suggest that all types of fibroin/sericin scaffolds support the basic structural requirements to be considered for tissue engineering approaches, e.g. bone tissue engineering.

Figure 1. Preparation and morphological characterization of fibroin/sericin scaffolds. (A) Schematic diagram of fabrication process of fibroin/sericin scaffolds. (B) SEM micrographs of cross sections of the different scaffold types. Scale bars = 100 μm .

Table 3. BET surface area, density, porosity and pore volume of the scaffolds.

Sample	BET surface area (m^2/g)	envelope density (g/cm^3)	skeleton density (g/cm^3)	porosity (%)	V_{pore} (cm^3/g)
F1:S0	1.74	0.064	1.068	94	14.76
F1:S0.25	0.99	0.074	1.122	93.4	12.71
F1:S0.5	0.72	0.080	1.090	92.7	11.58
F1:S0.75	0.77	0.104	1.176	91.2	8.76
F1:S1	0.54	0.104	1.162	91	8.74

3.1.2 Mechanical properties

The compressive strength and Young's modulus of the scaffolds were measured by compression tests in wet state. Figure 2 indicates that the mechanical behavior of the scaffolds is highly affected by sericin content. Silk sericin degrades under alkaline condition into small polypeptides with lower molecular weight (~250 kDa- 10kDa) compared to its native state in the silkworm gland [59, 60], and this phenomenon was also observed within this study (data not shown). The obtained results from compressive strength showed an increase in the Young's modulus and elasticity from 57.8 ± 4.2 kPa (sample F1:S0) to 119.5 ± 3.3 kPa (sample F1:S1) with increasing sericin content, without being negatively influenced by sericin degradation.

Moreover, the required stress for compressing the scaffolds to 50% of the initial height has increased from 22.9 kPa for F1:S0 scaffolds to 32.1 kPa for the F1:S1 scaffold type. The scaffolds also showed viscoelasticity in response to compressive force, which is in agreement with previous studies of fibroin scaffolds [61, 62]. Since stiffness and compressive strength of 3D spongy scaffolds were enhanced with decreasing pore size and overall porosity, due to reducing scaffold void volume and thickening pore walls, the enhancement of compressive strength and Young's modulus of fibroin/sericin scaffolds can be attributed to the reduced pore size and V_{pore} as shown by SEM and porosity measurements, which is in agreement with the literature [63, 64]. Although a phase separation between fibroin and sericin blends has been observed previously, and phase separation of polymers could weaken mechanical properties of the scaffolds due to very weak interactions between two polymers, it is suggested that strong interaction at the boundary of fibroin and sericin proteins in fibroin-sericin blends occurs, which restricts the molecular movement of the silk fibroin^[65, 66]. Furthermore, dominant structural effects of sericin on the fibroin construct, probably due to formation of hydrogen bonds between

fibroin and sericin as well as conformational transition of sericin after ethanol treatment to a β -sheet conformation, potentially help to enhance the overall mechanical properties of the scaffolds [65, 67-70]. Moreover, since sericin is also generally considered stiff with high elastic modulus, the increase in elastic area of the samples might correspond to intrinsic brittleness and poor plasticity of sericin, as shown previously [71, 72].

In recent years, the importance of the scaffold stiffness and compressive elastic modulus in determining cell fate and behavior has been reported by different researchers [73, 74]. Kong and colleagues demonstrated that both proliferation and osteogenic differentiation of pre-osteoblasts (MC3T3-E1) was enhanced with increasing elastic modulus from 20 to 110 kPa [75], and in line with this they found an increasing number of focal adhesions. Similarly, it has been reported that stiffer substrates in the range of ~ 0.1 to ~ 100 kPa up-regulate markers of osteogenic differentiation in both human mesenchymal stem cells (hMSCs) and MC3T3-E1 cells compared with cells on softer matrices. This cell behavior has been attributed to mimicking the elastic modulus of the bone collagenous osteoid precursors (~ 100 kPa) and that of cancellous bone (~ 134 kPa) [73, 76, 77]. These reports provide evidence that all fibroin/sericin scaffold sample types are within an appropriate range of elastic modulus for supporting osteogenesis, and that they exhibit superior mechanical properties with increasing sericin content compared with fibroin (sample F1:S0), indicating that an increasing sericin weight percentage may play a role in bone formation.

Figure 2. Mechanical properties of the fibroin/sericin scaffolds. (A) Young's modulus. (B) Stress/strain curve of the scaffolds under compressive force ($\pm$ SD, $n = 3$). *** $p \leq 0.001$, ** $p \leq 0.01$.

3.1.3. Water uptake and weight loss

Results of water uptake and degradability assessment of the samples are indicated in Figure 3. The water uptake was assessed by immersing the scaffolds in deionized water and after 24 h, all scaffold types showed high water uptake capacity (~650–850%). A slight increase in water uptake was observed with increasing sericin content, however, there was no significant difference compared to sample type F1:S0 (Figure 3A).

Figure 3B shows the degradation rate of all sample types over 60 days. For evaluating the degradability of the scaffolds, they were incubated in PBS for up to 60 days. While sample type F1:S0 showed almost no mass loss after 60 days, scaffolds F1:S0.25, F1:S0.5, F1:S0.75 and F1:S1 showed 6%, 11%, 13% and 19% weight loss by 14 days and 7.6%, 14.7%, 24% and 38.3%, respectively by 60 days. These results indicate that with increasing sericin content, a higher weight loss with more rapid degradation rate has occurred as follows: $F1:S1 > F1:S0.75 > F1:S0.5 > F1:S0.25 > F1:S0$. Hence, it is apparent that the increasing

degradation rate is dominated by the sericin content. Moreover, all scaffolds except sample type F1:S0 lost weight with increasing immersion time.

High water uptake without negative structural change of fibroin-based scaffolds has also been reported by Riberio *et al.*, a combination of properties that can be considered advantageous of fibroin based scaffolds over some other natural polymers [78-80]. The high water uptake of the presented fibroin/sericin scaffolds can be attributed to the characteristics related to the porosity and hydrophilicity of the scaffolds [39, 81, 82].

Ideally, degradation of the scaffolds should not take place before sufficient tissue in-growth. On the other hand, the scaffolds should provide extra space for tissue growth through degradation over time. It was found here that despite a decrease in the pore size with increasing sericin content, the scaffolds with higher sericin concentration have higher degradation rates. This phenomenon can be attributed to the amorphous and highly hydrophilic nature of the sericin molecule [81, 83]. In this regard, Mandel and colleagues showed that an increase in sericin content of gelatin/sericin scaffolds, with a constant gelatin concentration, increases weight loss in spite of the decrease in pore size and water uptake [51]. They attributed this phenomenon to remaining uncross-linked sericin molecules and found that the effect of sericin content on degradability dominates the effect of the reduced pore size. A decrease in degradation rate of the scaffolds with increasing immersion time (after 30 days) can also be observed, which has been reported also for other polymeric scaffolds [84, 85].

As a result, blending sericin with fibroin increases the degradation rate of the scaffolds and thus potentially provides optimal environment for new regenerated tissue.

Figure 3. Water uptake and weight loss of fibroin/sericin scaffolds. (A) Water uptake of the scaffolds ($\pm$ SD, $n = 4$). (B) Weight loss of all sample types ($\pm$ SD, $n = 4$). *** $p \leq 0.001$, ** $p \leq 0.01$.

3.2. Cell cytotoxicity and attachment on the scaffolds

To evaluate the effect of the scaffolds on cytocompatibility, cytotoxicity and cell attachment were assessed using LDH assay (1 and 7 days post-seeding) and actin/DAPI staining (7 days post-seeding) (Figure 4). To assess the cytotoxicity of the scaffolds, the LDH assay was performed to measure the leaked LDH through damaged cell membranes in the culture medium (Figure 4A). In all samples low LDH release (approximately 5–8.5%) was observed after 1 day incubation, showing no significant difference compared to the TCP control group. After 7 days, an increase of approximately 12–17% of LDH level was observed for all scaffold types, which can be related to the increase in the total number of cells and possible cell death due to higher cell confluency. As the LDH level of all sample types is similar to the TCP control group, it can be concluded that all fibroin/sericin scaffold types have no adverse effect on osteoblastic-like MG63 cells.

A)

B)

Figure 4. MG63 cell response to fibroin/sericin scaffolds. (A) Cytotoxicity after 1 and 7 days determined by the LDH assay ($\pm$ SD, $n = 3$). (B) Maximum intensity projections of confocal z-stack images of MG63 cells seeded on fibroin/sericin scaffolds after 7 days of culture. (green and blue fluorescence represent F-actin filaments and cell nuclei, respectively, scale bars = 50 μ m, inset scale bars = 10 μ m). TCP represents cells on tissue culture polystyrene as control.

Figure 4B and Supplementary Figure S2 show fluorescent images of osteoblastic-like MG63 cells with nuclei (DAPI) and phalloidin 633 staining after 7 days incubation on fibroin/sericin scaffolds. These images demonstrate that the osteoblast-like MG63 cells were attached to the walls of the scaffold which had previously been washed with L-glutamic acid and that cell numbers were comparable on all scaffold types. Notably, confocal z-stack images showed that the cells were able to grow both vertically and horizontally within the porous structure of the scaffolds with well-developed actin filaments. These finding demonstrate that scaffolds support cell growth by serving as physical support structure that mimics native ECM and show that adding different concentrations of sericin to fibroin scaffolds does not negatively affect cell

attachment and proliferation. In addition, distribution of the cells within the pores of all scaffold types showed good cell-scaffold and cell-cell interaction after 7 days. These data are in good agreement with previous reports on fibroin based scaffolds and sericin-containing materials, and support the here described cytocompatibility of different fibroin/sericin scaffold types [6, 86, 87].

3.3. Macrophage response to the scaffolds

Since the success or failure of implanted biomaterials highly depends on the extent of initial macrophage response and release of pro-inflammatory cytokines [88, 89], to understand how different scaffold compositions affect human THP-1-driven macrophages, cells cultivated on different scaffold types were stained for actin and nuclei, and gene expression of pro-inflammatory markers was evaluated by RT-PCR after 24 h culture on the samples (Figure 5). The genes of interest (TNF- α , CXCL10 and CD197) were chosen according to the literature to monitor inflammatory response towards scaffolds of different fibroin/sericin ratios [90].

Confocal microscopy indicated the attachment of macrophages on the surface of the scaffolds after 24 h (Figure 5A). The attached macrophages showed mostly an irregular hemispherical morphology on all samples, indicating that the chemical composition and porous structure of the scaffolds do not induce any apparent change in macrophage morphology and attachment in response to the different composition of the scaffold surfaces.

Analysis of gene expression of pro-inflammatory markers by RT-PCR showed very low expression levels of TNF- α , CXCL10 and CD197, being similar for all scaffold types (Figure 5B). In fact, with increasing sericin content, no significant difference in levels of pro-inflammatory markers on all sample types (with or without sericin) was seen.

Figure 5. Attachment of macrophages onto fibroin/sericin scaffolds and relative gene expression of pro-inflammatory markers after 24 h. (A) Fluorescent microscopy images of macrophages seeded on the scaffolds. (40x, green and blue fluorescence represent F-actin filaments and cell nuclei, respectively, scale bars = 50 μm, inset scale bars = 10 μm). (B) Relative expression of TNF-α, CXCL10, CD197 as pro-inflammatory markers. Expression levels $\pm$ SD were normalized to macrophages seeded on F1:S0 scaffolds. RPL37a was used as a housekeeping gene. n=3 (no statistical difference was observed between F1:S0 and other scaffolds).

Release of TNF-α cytokine was measured by ELISA after 24 h of culturing macrophages on the scaffolds and its levels were normalized to DNA content of cells on the samples (Supplementary Figure S3). The concentration of TNF-α secreted from macrophages on TCP (Mφ), F1:S0, F1:S0.25, F1:S0.5, F1:S0.75, and F1:S1 was 0.45 ± 0.2 pg/mL, 0.60 ± 0.2 pg/mL, 1.48 ± 1.1 pg/mL, 0.98 ± 0.6 pg/mL, 1.01 ± 0.5 pg/mL, and 5.51 ± 2.2 pg/mL, respectively. The low level of TNF-α secretion supported the results of gene expression for all scaffold types and showed the same TNF-α production level compared to sericin-free F1:S0 sample and TCP (Mφ) control

group. Moreover, with regard to the LPS and IFN- γ stimulated control group (TCP (M1)) (178.54 ± 46.3 pg/mL), all scaffold types showed much lower cytokine secretion which indicates very low inflammatory reaction towards the scaffolds. This result is in agreement with Rao *et al.*, who showed a low immunogenic response from PLGA-coated fibroin/sericin multiwall tubes (with 1 mm in outer diameter and 10 mm in length) as nerve conduits with similar structure to nerve fasciculi, as well as adhesion of Schwann cells with neuritis differentiation along located micro-pores on different channels surfaces [28]. These results indicate that 3D spongy fibroin/sericin scaffolds are a promising candidate in tissue engineering without elucidating significant inflammatory immune responses.

Figure 6. TNF- α secretion from THP-1-derived macrophages seeded on fibroin/sericin scaffolds after 24 h. ($\pm$ SD, n = 3) (no significant difference was observed between F1:S0 and other scaffolds). TCP represents tissue culture polystyrene as control using M ϕ (TCP (M ϕ)) and M1 (TCP (M1)) macrophage medium.

Conclusion

Due to some concerns regarding a negative immune response to silk biomaterials, the application of fibroin and sericin in a combined form has remained largely unexplored and there has been no report regarding their possible immunogenicity when combined in 3D spongy scaffolds. Hence, in this study novel fibroin/sericin 3D spongy scaffolds were fabricated and their structural, biological and immunological characteristics were evaluated. Morphological and structural results indicated with increasing sericin content not only the Young's modulus and compressive strength of fibroin enhanced but also it accelerated degradation rate of fibroin scaffold, so that F1:S1 samples with highest sericin content of 4.7 wt% showed the highest mechanical properties and degradation rate. Moreover, despite decrease in pore size and surface area with increasing sericin content, the scaffolds keep their high porosity and meet the basic structural requirements for supporting cell growth. Through *in vitro* evaluation of MG63, it was observed that all of the scaffolds support the attachment and viability of the cells with no sign of toxicity regardless of sericin content. Furthermore, due to low expression level of pro-inflammatory markers, it can be concluded that macrophage-scaffold interaction does not lead to any change in macrophage polarization towards pro-inflammatory M1-like macrophages. Accordingly, none of the scaffolds showed any advantage over other compositions regarding biological activities. This research provides new insights into possible application of 3D spongy fibroin/sericin scaffolds in bone tissue engineering. Further studies should be performed in animal models to fully address the biological characteristics and consequences in order to explore potential applications of fibroin/sericin scaffolds.

Conflicts of interest

There are no conflicts to declare.

Acknowledgement

The authors would like to thank Yvonne Elbs-Glatz and Ursina Tobler for their valuable technical support. This research was partially supported by the Iran National Science Foundation (INSF) Grant No. 96000394.

Journal Pre-proof

References

- [1] W.W. Thein-Han, J. Saikhun, C. Pholpramoo, R.D. Misra, Y. Kitiyanant, Chitosan–gelatin scaffolds for tissue engineering: Physico-chemical properties and biological response of buffalo embryonic stem cells and transfectant of GFP–buffalo embryonic stem cells, *Acta Biomater* 5 (2009) 3453-3466.
- [2] J. Melke, S. Midha, S. Ghosh, K. Ito, S. Hofmann, Silk fibroin as biomaterial for bone tissue engineering, *Acta Biomater* 31 (2016) 1-16.
- [3] P. Gupta, K.K. Nayak, Optimization of keratin/alginate scaffold using RSM and its characterization for tissue engineering, *Int J Biol Macromol* 85 (2016) 141-149.
- [4] S. Kapoor, S.C. Kundu, Silk protein-based hydrogels: Promising advanced materials for biomedical applications, *Acta Biomater* 31 (2016) 17-32.
- [5] X. Tang, C. Xue, Y. Wang, F. Ding, Y. Yang, X. Gu, Bridging peripheral nerve defects with a tissue engineered nerve graft composed of an in vitro cultured nerve equivalent and a silk fibroin-based scaffold, *Biomaterials* 33 (2012) 3860-3867.
- [6] M. Zhu, K. Wang, J. Mei, C. Li, J. Zhang, W. Zheng, D. An, N. Xiao, Q. Zhao, D. Kong, L. Wang, Fabrication of highly interconnected porous silk fibroin scaffolds for potential use as vascular grafts, *Acta Biomater* 10 (2014) 2014-2023.
- [7] H.W. Ju, O.J. Lee, J.M. Lee, B.M. Moon, H.J. Park, Y.R. Park, M.C. Lee, S.H. Kim, J.R. Chao, C.S. Ki, C.H. Park, Wound healing effect of electrospun silk fibroin nanomatrix in burn-model, *Int J Biol Macromol* 85 (2016) 29-39.
- [8] L. Tozzi, P.A. Laurent, C.A. Di Buduo, X. Mu, A. Massaro, R. Bretherton, W. Stoppel, D.L. Kaplan, A. Balduini, Multi-channel silk sponge mimicking bone marrow vascular niche for platelet production, *Biomaterials* 178 (2018) 122-133.
- [9] Q. Lu, B. Zhang, M. Li, B. Zuo, D.L. Kaplan, Y. Huang, H. Zhu, Degradation mechanism and control of silk fibroin, *Biomacromolecules* 12 (2011) 1080-1086.
- [10] A.J. Meinel, K.E. Kubow, E. Klotzsch, M. Garcia-Fuentes, M.L. Smith, V. Vogel, H.P. Merkle, L. Meinel, Optimization strategies for electrospun silk fibroin tissue engineering scaffolds, *Biomaterials* 30 (2009) 3058-3067.
- [11] X. Wang, T. Yucel, Q. Lu, X. Hu, D.L. Kaplan, Silk nanospheres and microspheres from silk/pva blend films for drug delivery, *Biomaterials* 31 (2010) 1025-1035.
- [12] S. Hofmann, C.T. Foo, F. Rossetti, M. Textor, G. Vunjak-Novakovic, D.L. Kaplan, H.P. Merkle, L. Meinel, Silk fibroin as an organic polymer for controlled drug delivery, *J Control Release* 111 (2006) 219-227.
- [13] H. Lee, G.H. Yang, M. Kim, J. Lee, J. Huh, G. Kim, Fabrication of micro/nanoporous collagen/dECM/silk-fibroin biocomposite scaffolds using a low temperature 3D printing process for bone tissue regeneration, *Mater Sci Eng C Mater Biol Appl* 84 (2018) 140-147.
- [14] G.J. Lai, K.T. Shalumon, S.H. Chen, J.P. Chen, Composite chitosan/silk fibroin nanofibers for modulation of osteogenic differentiation and proliferation of human mesenchymal stem cells, *Carbohydr Polym* 111 (2014) 288-297.
- [15] J. Luo, H. Zhang, J. Zhu, X. Cui, J. Gao, X. Wang, J. Xiong, 3-D mineralized silk fibroin/polycaprolactone composite scaffold modified with polyglutamate conjugated with BMP-2 peptide for bone tissue engineering, *Colloids Surf B* 163 (2018) 369-378.
- [16] B. Marelli, C.E. Ghezzi, A. Alessandrino, J.E. Barralet, G. Freddi, S.N. Nazhat, Silk fibroin derived polypeptide-induced biomineralization of collagen, *Biomaterials* 33 (2012) 102-108.
- [17] J.R. Vetsch, S.J. Paulsen, R. Müller, S. Hofmann, Effect of fetal bovine serum on mineralization in silk fibroin scaffolds, *Acta Biomater* 13 (2015) 277-285.
- [18] F. Han, Y. Hu, J. Li, J. Gong, Q. Guo, C. Zhu, X. Zhu, H. Yang, B. Li, In situ silk fibroin-mediated crystal formation of octacalcium phosphate and its application in bone repair, *Mater Sci Eng C Mater Biol Appl* 95 (2019) 1-10.
- [19] C. Qi, J. Liu, Y. Jin, L. Xu, G. Wang, Z. Wang, L. Wang, Photo-crosslinkable, injectable sericin hydrogel as 3D biomimetic extracellular matrix for minimally invasive repairing cartilage, *Biomaterials* 163 (2018) 89-104.
- [20] S. Gilotra, D. Chouhan, N. Bhardwaj, S.K. Nandi, B.B. Mandal, Potential of silk sericin based nanofibrous mats for wound dressing applications, *Mater Sci Eng C* 90 (2018) 420-432.
- [21] P. Aramwit, S. Damrongsakkul, S. Kanokpanont, T. Srichana, Properties and antityrosinase activity of sericin from various extraction methods, *Biotechnol Appl Biochem* 55 (2010) 91-98.
- [22] P. Aramwit, S. Kanokpanont, T. Nakpheng, T. Srichana, The effect of sericin from various extraction methods on cell viability and collagen production, *Int J Mol Sci* 11 (2010) 2200-2211.
- [23] J.P. Kumar, B.B. Mandal, Antioxidant potential of mulberry and non-mulberry silk sericin and its implications in biomedicine, *Free Radic Biol Med* 108 (2017) 803-818.
- [24] S. Nayak, T. Dey, D. Naskar, S.C. Kundu, The promotion of osseointegration of titanium surfaces by coating with silk protein sericin, *Biomaterials* 34 (2013) 2855-2864.

- [25] F. Zhang, Z. Zhang, X. Zhu, E.T. Kang, K.G. Neoh, Silk-functionalized titanium surfaces for enhancing osteoblast functions and reducing bacterial adhesion, *Biomaterials* 29 (2008) 4751-4759.
- [26] L. Chen, J. Hu, J. Ran, X. CShen, H. Tong, A novel nanocomposite for bone tissue engineering based on chitosan-silk sericin/hydroxyapatite: biomimetic synthesis and its cytocompatibility *RSC Adv* 5 (2015) 56410-56422.
- [27] S. Dinescu, B. Gălățeanu, M. Albu, A. Lungu, E. Radu, A. Hermenean, M. Costache, Biocompatibility assessment of novel collagen-sericin scaffolds improved with hyaluronic acid and chondroitin sulfate for cartilage regeneration, *Biomed Res Int* (2013) 598056.
- [28] J. Rao, Y. Cheng, Y. Liu, Z. Ye, B. Zhan, D. Quan, Y. Xu, A multi-walled silk fibroin/silk sericin nerve conduit coated with poly(lactic-co-glycolic acid) sheath for peripheral nerve regeneration *Mater Sci Eng C* 73 (2017) 319-332.
- [29] B. Kundu, R. Rajkhowa, S.C. Kundu, X. Wang, Silk fibroin biomaterials for tissue regenerations, *Adv Drug Deliv Rev* 65 (2013) 457-470.
- [30] A.E. Thurber, F.G. Omenetto, D.L. Kaplan, In vivo bioresponses to silk proteins., *Biomaterials* 71 (2015) 145-157.
- [31] C. Holland, K. Numata, J. Rnjak-Kovacina, F.P. Seib, The biomedical use of silk: past, present, future, *Adv Healthcare Mater* 8 (2018) 1800465.
- [32] M. Dewair, X. Baur, K. Ziegler, Use of immunoblot technique for detection of human IgE and IgG antibodies to individual silk proteins, *J Allergy Clin Immunol* 76 (1985) 537-542.
- [33] J.C. Celedón, L.J. Palmer, X. Xu, B. Wang, Z. Fang, S.T. Weiss, Sensitization to silk and childhood asthma in rural China, *Pediatrics* 107 (2001) E80.
- [34] D.N. Rockwood, R.C. Preda, T. Yücel, X. Wang, M.L. Lovett, D.L. Kaplan, Materials fabrication from Bombyx mori silk fibroin, *Nat Protoc* 6 (2011) 1612-1631.
- [35] Y.Y. Jo, H.Y. Kweon, D.W. Kim, K. Baek, M.K. Kim, S.G. Kim, W.S. Chae, J.Y. Choi, H. Rotaru, Bone regeneration is associated with the concentration of tumour necrosis factor- α induced by sericin released from a silk mat, *Sci Rep* 7 (2017) 15589.
- [36] S. Kurosaki, H. Otsuka, M. Kunitomo, M. Koyama, R. Pawankar, K. Matumoto, Fibroin allergy: IgE mediated hypersensitivity to silk suture materials, *Nihon Ika Daigaku Zasshi* 66 (1999) 41-44.
- [37] H. Kweon, S.G. Kim, J.Y. Choi, Inhibition of foreign body giant cell formation by 4-hexylresorcinol through suppression of diacylglycerol kinase delta gene expression, *Biomaterials* 35 (2014) 8576-8584.
- [38] B. Panilaitis, G.H. Altman, J. Chen, H.J. Jin, V. Karageorgiou, D.L. Kaplan, Macrophage responses to silk, *Biomaterials* 24 (2003) 3079-3085.
- [39] B. Kundu, S.C. Kundu, Silk sericin/polyacrylamide in situ forming hydrogels for dermal reconstruction, *Biomaterials* 33 (2012) 7456-7467.
- [40] H. Massoumi, J. Nourmohammadi, M.S. Marvi, F. Moztarzadeh, Comparative study of the properties of sericin-gelatin nanofibrous wound dressing containing halloysite nanotubes loaded with zinc and copper ions, *Int J Polym Mater* (2018) 1-12.
- [41] J.E. Gough, C.A. Scotchford, S. Downes, Cytotoxicity of glutaraldehyde crosslinked collagen/poly(vinyl alcohol) films is by the mechanism of apoptosis, *J Biomed Mater Res* 61 (2002) 121-130.
- [42] S. Dash, P.N. Murthy, L. Nath, P. Chowdhury, Kinetic modeling on drug release from controlled drug delivery systems, *Acta Pol Pharm* 67 (2010) 217-223.
- [43] S. Brunauer, P.H. Emmett, E. Teller, Adsorption of gases in multimolecular layers, *J Am Chem Soc* 60 (1938) 309-319.
- [44] C.E. Brewer, V.J. Chuang, C.A. Masiello, H. Gonnermann, X. Gao, B. Dugan, L.E. Driver, P. Panzacchi, K. Zygourakis, C.A. Davies, New approaches to measuring biochar density and porosity, *Biomass Bioenerg* 66 (2014) 176-185.
- [45] W. Chanput, J.J. Mes, H.J. Wichers, THP-1 cell line: An in vitro cell model for immune modulation approach, *Int Immunopharmacol* 23 (2014) 37-45.
- [46] A. Kerschenmeyer, Ø. Arlov, V. Malheiro, M. Steinwachs, M. Rottmar, K. Maniura-Weber, G. Palazzolo, M. Zenobi-Wong, Anti-oxidant and immune-modulatory properties of sulfated alginate derivatives on human chondrocytes and macrophages, *Biomater Sci* 5 (2017) 1756-1765.
- [47] V. Carvalho, P. Castanheira, T.Q. Faria, C. Goncalves, P. Madureira, C. Faro, L. Domingues, R.M. Brito, M. Vilanova, M. Gama, Biological activity of heterologous murine interleukin-10 and preliminary studies on the use of a dextrin nanogel as a delivery system, *Int J Pharm* 400 (2010) 234-242.
- [48] H. Headon, A. Kasem, K. Mokbel, Capsular contracture after breast augmentation: An update for clinical practice, *Arch Plast Surg* 42 (2015) 532-543.
- [49] M.J. Jang, I.C. Um, Effect of sericin concentration and ethanol content on gelation behavior, rheological properties, and sponge characteristics of silk sericin, *Eur Polym J* 93 (2017) 761-774.
- [50] L. Polo-Corrales, M. Latorre-Esteves, J.E. Ramirez-Vick, Scaffold design for bone regeneration, *J Nanosci Nanotechnol* 14 (2014) 15-56.

- [51] B.B. Mandal, A.S. Priya, S.C. Kundu, Novel silk sericin/gelatin 3-D scaffolds and 2-D films: Fabrication and characterization for potential tissue engineering applications, *Acta Biomater* 5 (2009) 3007-3020.
- [52] M.G. Haugh, C.M. Murphy, F.J. O'Brien, Novel freeze-drying methods to produce a range of collagen-glycosaminoglycan scaffolds with tailored mean pore sizes, *Tissue Eng Part C* 16 (2010) 887-894.
- [53] N.E. Kurland, J. Kundu, S. Pal, S.C. Kundu, V.K. Yadavalli, Self-assembly mechanisms of silk protein nanostructures on two-dimensional surfaces *Soft Matter* 8 (2012) 4952-4959.
- [54] J.S. Ko, K. Yoon, C.S. Ki, H.J. Kim, D.G. Bae, K.H. Lee, Y.H. Park, I.C. Um, Effect of degumming condition on the solution properties and electrospinnability of regenerated silk solution, *Int J Biol Macromol* 55 (2012) 161-168.
- [55] Y. Takahashi, Y. Tabata, Effect of the fiber diameter and porosity of non-woven PET fabrics on the osteogenic differentiation of mesenchymal stem cells, *J Biomater Sci* 15 (2004) 41-57.
- [56] M. Figueiredo, J. Henriques, G. Martins, F. Guerra, F. Judas, H. Figueiredo, Physicochemical characterization of biomaterials commonly used in dentistry as bone substitutes: comparison with human bone, *J Biomed Mater Res B* 92 (2010) 409-419.
- [57] C.E. Misch, Z. Qu, M.W. Bidez, Mechanical properties of trabecular bone in the human mandible: implications for dental implant treatment planning and surgical placement, *J Oral Maxillofac Surg* 57 (1999) 700-706.
- [58] W.J. Seong, U.K. Kim, J.Q. Swift, Y.C. Heo, J.S. Hodges, ., C.C. Ko, Elastic properties and apparent density of human edentulous maxilla and mandible, *Int J Oral Maxillofac Surg* 38 (2009) 1088-1093.
- [59] L.S. Wray, X. Hu, J. Gallego, I. Georgakoudi, F.G. Omenetto, D. Schmidt, D.L. Kaplan, Effect of processing on silk-based biomaterials: reproducibility and biocompatibility., *J Biomed Mater Res B Appl Biomater* 99 (2011) 89-101.
- [60] T.V. Chirila, S. Suzuki, L.J. Bray, N.L. Barnett, D.G. Harkin, Evaluation of silk sericin as a biomaterial: in vitro growth of human corneal limbal epithelial cells on Bombyx mori sericin membranes, *Prog Biomater* 2 (2013) 14.
- [61] D. Panda, S. Konar, S.K. Bajpai, A. Arockiarajan, Synthesis and viscoelastic characterization of microstructurally aligned Silk fibroin sponges, *J Mech Behav Biomed Mater* 71 (2017) 362-371.
- [62] W.H. Elliott, W. Bonani, D. Maniglio, A. Motta, W. Tan, C. Migliaresi, Silk hydrogels of tunable structure and viscoelastic properties using different chronological orders of genipin and physical cross-linking, *ACS Appl Mater Interfaces* 7 (2015) 12099-12108.
- [63] S.L. Levengood, M. Zhang, Chitosan-based scaffolds for bone tissue engineering, *J Mater Chem B Mater Biol Med* 2 (2014) 3161-3184.
- [64] N. Annabi, J.W. Nichol, X. Zhong, C. Ji, S. Koshy, A. Khademhosseini, F. Dehghani, Controlling the porosity and microarchitecture of hydrogels for tissue engineering, *Tissue Eng Part B* 16 (2010) 371-383.
- [65] H.W. Kwak, J.E. Ju, M. Shin, C. Holland, K.H. Lee, Sericin promotes fibroin silk I stabilization across a phase-separation, *Biomacromolecules* 18 (2017) 2343-2349.
- [66] K.H. Lee, Silk sericin retards the crystallization of silk fibroin, *Macromol* 25 (2004) 1792 - 1796.
- [67] Y. Hang, Y. Zhang, Y. Jin, H. Shao, X. Hu, Preparation of regenerated silk fibroin/silk sericin fibers by coaxial electrospinning, *Int J Biol Macromol* 51 (2012) 980-986.
- [68] M. Yang, Y. Shuai, G. Zhou, N. Mandal, L. Zhu, C. Mao, Tuning molecular weights of Bombyx mori (B. mori) silk sericin to modify its assembly structures and materials formation, *ACS Appl Mater Interfaces* 6 (2014) 13782-13789.
- [69] H.P. Zhang, X.Y. Wang, S.J. Min, M. Mandal, M.Y., M.Y. Yang, L.J. Zhu, Hydroxyapatite/sericin composite film prepared through mineralization of flexible ethanol-treated sericin film with simulated body fluids, *Ceram Int* 40 (2014) 985-991.
- [70] T.K. H. Teramoto, Y. Tamada, ., B.B.a. Biochemistry, Preparation of gel film from Bombyx mori silk sericin and its characterization as a wound dressing, *Biosci Biotechnol Biochem* 72 (2008) 3189-3196.
- [71] A. Farsi, A.D. Pullen, J.P. Latham, J. Bowen, M. Carlsson, E.H. Stitt, M. Marigo, Full deflection profile calculation and Young's modulus optimisation for engineered high performance materials, *Sci Rep* 7 (2017) 46190.
- [72] H. Yun, M.K. Kim, H.K. Kwak, J.Y. Lee, M.H. Kim, E.H. Kim, K.H. Lee, Preparation and characterization of silk sericin/glycerol/graphene oxide nanocomposite film, *Fibers Polym* 14 (2013) 2111-2116.
- [73] G. Chen, C. Dong, L. Yang, Y. Lv, 3D scaffolds with different stiffness but the same microstructure for bone tissue engineering, *ACS Appl Mater Interfaces* 7 (2015) 15790-15802.
- [74] N. Huebsch, P.R. Arany, A.S. Mao, D. Shvartsman, O.A. Ali, S.A. Bencherif, J. Rivera-Feliciano, D.J. Mooney, Harnessing traction-mediated manipulation of the cell-matrix interface to control stem cell fate, *Nat Mater* 9 (2010) 518-526.
- [75] H. Kong, T.R. Polte, E. Alsberg, D.J. Mooney, FRET measurements of cell-traction forces and nano-scale clustering of adhesion ligands varied by substrate stiffness, *Proc Natl Acad Sci USA* 102 (2005) 4300-4305.
- [76] A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher, Matrix elasticity directs stem cell lineage specification, *Cell* 126 (2006) 677-689.

- [77] T. Zhang, S. Lin, Shao X1, Shi S1, Zhang Q1, Xue C1, Lin Y1, Zhu B2, C. X, Regulating osteogenesis and adipogenesis in adipose-derived stem cells by controlling underlying substrate stiffness, *J Cell Physiol* 233 (2018) 3418-3428.
- [78] M. Ribeiro, M.A. de Moraes, M.M. Beppu, M.P. Garcia, M.H. Fernandes, F.J. Monteiro, M.P. Ferraz, Development of silk fibroin/nanohydroxyapatite composite hydrogels for bone tissue engineering, *Eur Polym J* 67 (2015) 66-77.
- [79] Y.K. Lin, D.C. Liu, Studies of novel hyaluronic acid-collagen sponge materials composed of two different species of type I collagen, *J Biomater Appl* 21 (2007) 265-281.
- [80] A. Tripathi, J.S. Melo, Preparation of a sponge-like biocomposite agarose–chitosan scaffold with primary hepatocytes for establishing an in vitro 3D liver tissue model, *RSC Adv* 5 (2015) 30701-30710.
- [81] R.I. Kunz, R.M. Brancalhão, L.F. Ribeiro, M.R. Natali, Silkorm sericin: properties and biomedical applications, *Biomed Res Int* 2016 (2016) 8175701.
- [82] N. Naseri, J.M. Poirier, L. Girandon, M. Fröhlich, K. Oksmana, A.P. Mathew, 3-Dimensional porous nanocomposite scaffolds based on cellulose nanofibers for cartilage tissue engineering: tailoring of porosity and mechanical performance, *RSC Adv* 6 (2016) 5999-6007.
- [83] H. Teramoto, A. Kakazu, K. Yamauchi, T. Asakura, Role of hydroxyl side chains in Bombyx mori silk sericin in stabilizing its solid structure, *Macromolecules* 40 (2007) 1562-1569.
- [84] Y. Wang, X. Wang, J. Shi, R. Zhu, J. Zhang, Z. Zhang, D. Ma, Y. Hou, J. Yang, M. Mizuno, A biomimetic silk fibroin/sodium alginate composite scaffold for soft tissue engineering, *Sci Rep* 6 (2016) 39477.
- [85] Z.S. Shen, X. Cui, R.X. Hou, Q. Li, H.X. Deng, J. Fu, Tough biodegradable chitosan–gelatin hydrogels via in situ precipitation for potential cartilage tissue engineering, *RSC Adv* 5 (2015) 55640-55647.
- [86] A. Varkey, E. Venugopal, P. Sugumaran, G. Janarthanan, M.M. Pillai, S. Rajendran, A. Bhattacharyya, Impact of silk fibroin-based scaffold structures on human osteoblast MG63 cell attachment and proliferation, *Int J Nanomedicine* 10 (2015) 43-51.
- [87] V. Mitran, M.G. Albu, E. Vasile, A. Cimpean, M. Costache, Dose-related effects of sericin on preadipocyte behavior within collagen/sericin hybrid scaffolds, *Pro Nat Sci Mater* 25 (2015) 122-130.
- [88] P.C. Bota, A.M. Collie, P. Puolakkainen, R.B. Vernon, E.H. Sage, B.D. Ratner, P.S. Stayton, Biomaterial topography alters healing in vivo and monocyte/macrophage activation in vitro, *J Biomed Mater Res* 95 (2017) 649-657.
- [89] A. Crupi, A. Costa, A. Tarnok, S. Melzer, L. Teodori, Inflammation in tissue engineering: The Janus between engraftment and rejection, *Eur J Immunol* 45 (2015) 3222-3236.
- [90] R.M. Boehler, J.G. Graham, L.D. Shea, Tissue engineering tools for modulation of the immune response, *Biotechniques* 51 (2011) 239-passim.

Graphical abstract