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Multiple silk coatings on biphasic calcium phosphate scaffolds: Effect on physical and mechanical properties, and *in vitro* osteogenic response of human mesenchymal stem cells

Jiao Jiao Li^{1,2}, Eun Seok Gil¹, Rebecca S. Hayden¹, Chunmei Li¹, Seyed-Iman Roohani-Esfahani², David L. Kaplan¹, and Hala Zreiqat^{2,*}

¹Department of Biomedical Engineering, Tufts University, Medford, MA 02155, USA

²Biomaterials and Tissue Engineering Research Unit, School of AMME, University of Sydney, Sydney, NSW 2006, Australia

Abstract

Ceramic scaffolds such as biphasic calcium phosphate (BCP) have been widely studied and used for bone regeneration, but their brittleness and low mechanical strength are major drawbacks. We report the first systematic study on the effect of silk coating in improving the mechanical and biological properties of BCP scaffolds, including 1) optimisation of the silk coating process by investigating multiple coatings, and 2) *in vitro* evaluation of the osteogenic response of human mesenchymal stem cells (hMSCs) on the coated scaffolds. Our results show that multiple silk coatings on BCP ceramic scaffolds can achieve a significant coating effect to approach the mechanical properties of native bone tissue and positively influence osteogenesis by hMSCs over an extended period. The silk coating method developed in this study represents a simple yet effective means of reinforcement that can be applied to other types of ceramic scaffolds with similar microstructure to improve osteogenic outcomes.

Keywords

Silk; Scaffold; Coating; Human mesenchymal stem cells (hMSCs); Osteogenic

1. Introduction

Annually, over 2.2 million bone grafting procedures are performed worldwide for the treatment of bone defects, making bone the second most common tissue for transplantation.¹ However, due to the limitations of conventional bone graft treatments, the successful regeneration of critical-sized bone defects remains a clinical challenge. Autografts, the gold standard for bone replacement, face problems of limited availability and donor site complications, while allografts are associated with reduced bioactivity and the potential for disease transmission and immunogenicity.² Bone tissue engineering is emerging as a novel solution that can address the demand for alternative therapies. This approach relies on the use of scaffolds that are biocompatible and bioactive, biodegradable, have sufficient mechanical integrity for implantation in load-bearing defects, and have highly porous and interconnected architecture for bone and vascular ingrowth.³ Ceramic materials including hydroxyapatite, beta-tricalcium phosphate, biphasic calcium phosphate, bioactive glass and calcium silicate have been widely studied as bioactive scaffolds for bone regeneration, some formulations of which have been employed clinically as bone graft substitutes.^{1,4–13} These

*Correspondence to: A/Prof Hala Zreiqat, Email: hala.zreiqat@sydney.edu.au.

materials represent competitive choices for bone scaffolds due to their inherent bioactivity, which derives from their chemical similarity to the mineral component of bone, as well as bioactive ion release and substitution mechanisms which enhance bone formation.⁵ Nevertheless, the use of ceramic scaffolds in load-bearing applications is limited by their low mechanical strength and high brittleness,^{14,15} which is exacerbated at the high porosities (>80%) and pore sizes (200–500µm) required for bone regeneration.^{14,16,17} A well-known example is biphasic calcium phosphate (BCP), an established bone scaffold material that is in clinical use as bone graft substitutes.^{6,9} Owing to their brittleness and weak mechanical properties, BCP scaffolds have seen limited success in the regeneration of load-bearing bone defects.^{18,19}

Much research effort has been directed at coating ceramic scaffolds with polymeric materials to produce composite scaffolds with improved properties for bone regeneration. The polymer can fill existing cracks in the ceramic microstructure, thereby reducing brittleness while increasing strength and toughness of the scaffold by lowering the chance of crack propagation under load.²⁰ Different types of ceramic scaffolds have been coated with biocompatible and biodegradable polymers, including poly(lactic-co-glycolic acid) (PLGA),^{21,22} poly(D,L-lactic acid) (PDLA),^{23–26} polycaprolactone (PCL),^{27–29} and poly(3-hydroxybutyrate) (PHB).³⁰ Significant improvements in mechanical properties were generally observed, particularly in terms of strength and toughness. However, these polymeric coatings require the use of organic solvents in the fabrication process, the residuals of which may be harmful to transplanted cells or host tissues. They are also weakly osteoconductive at best and may mask bioactivity of the underlying ceramic substrate. Several studies have attempted to address this problem by incorporating an additional ceramic component into the coating, including hydroxyapatite powder,^{26,27} calcium phosphate deposition,²⁹ bioactive glass powder,²² and bioactive glass nanoparticles.²⁸ While these strategies are effective at imparting bioactivity and osteoconductivity to the coated surface, they do not circumvent the use of organic solvents during fabrication and also introduce additional processing complexity.

Silk fibroin has gained increasing popularity in recent years as a candidate material for bone tissue engineering due to its biocompatibility, slow degradability and outstanding mechanical properties.³¹ Silk harvested from the domesticated silkworm, *Bombyx mori*, is known for its remarkable strength and toughness exceeding that of most other polymeric materials employed for bone regeneration.^{31,32} Silk can be processed into an aqueous solution for preparation of different material morphologies without involving harmful organic solvents, with the additional advantage of versatility of sterilisation options including autoclaving.³² Silk degrades predictably over time frames that can be tuned by processing method. For example, silk scaffolds can be made to retain more than 50% of their mechanical properties after two months of *in vivo* implantation and completely degrade within one year.^{33,34} The biocompatibility of silk is demonstrated by its minimal immunogenic potential.^{35,36} Silk fibroin in various forms (films, fibres, yarns, meshes, hydrogel and porous sponges) has been reported to support the adhesion, proliferation, and osteogenic differentiation of stem cells.^{31,37} The use of silk scaffolds for bone regeneration has been extensively reported in literature.^{38–44} Most studies have demonstrated the ability of silk scaffolds to promote osteogenesis from human mesenchymal stem cells (hMSCs) *in vitro*, as well as evidence for bone formation *in vivo* and reasonable defect bridging. However, a major drawback is that the mechanical properties of porous silk scaffolds are significantly lower than those of ceramic scaffolds with similar physical characteristics, and are therefore not matched to cancellous bone. Recently, silk particles⁴⁵ and silk fibres⁴⁶ have been incorporated into porous silk matrices to form silk-silk composite structures with significantly improved mechanical properties. Nevertheless, the mechanical properties of

these reinforced silk-silk matrices are generally still lower than the aforementioned polymer-coated ceramic scaffolds.

Limited studies have investigated the efficacy of silk-coated ceramic scaffolds in bone regeneration.^{47,48} The process of silk coating deposition to the ceramic scaffold has not been optimised, leading to relatively poor mechanical properties after coating, or the need to incorporate other polymers to increase coating adhesion that require the use of organic solvents. Furthermore, there have been no long-term studies investigating the biological behaviour of polymer-coated ceramic scaffolds. Many studies did not perform *in vitro* testing on the developed scaffolds, while others used human bone-derived cells, osteoblast-like cell lines or hMSCs to investigate short-term cellular responses to the scaffolds for up to 7 days. Compared to other cell sources, the use of hMSCs for *in vitro* testing of scaffolds intended for bone regeneration is more relevant both biologically and also from a translational perspective,⁴⁹ and a minimum culture period of 5–6 weeks is usually required to allow sufficient time for cell proliferation and differentiation in order to derive meaningful biological data.^{41,42,50}

The purpose of the present study was to investigate the use of silk coatings to improve the properties of ceramic scaffolds for bone regeneration. BCP was chosen as the ceramic substrate due to its extensive use as a bone scaffold material. Furthermore, BCP scaffolds have low-density struts with many micropores and defects, which exemplify many other types of crystalline ceramic scaffolds. In this study, we show that coating BCP scaffolds with multiple layers of silk can address the brittleness of ceramic scaffolds and substantially improve their mechanical properties, while enhancing their bioactivity and preserving their cancellous bone-like architecture to favour *in vitro* osteogenesis. We also report for the first time 1) optimisation of the silk coating process (including method of coating deposition and effect of multiple coating layers), and 2) *in vitro* evaluation of the effect of silk coatings on a ceramic scaffold substrate (using hMSCs over a 6 week culture period).

2. Materials and Methods

2.1 Preparation of BCP ceramic scaffolds

Calcium phosphate-deficient apatite powder was prepared via an aqueous precipitation reaction (reagents from Sigma-Aldrich, USA) as previously described.⁴⁸ The precipitated powder was thermally treated at 600°C for 1 hour. The powder was crushed using a mortar and pestle and classified using stainless steel sieves to give particles of <75µm size for scaffold fabrication. The polymer sponge method was used for scaffold fabrication. Fully reticulated polyurethane foam (The Foam Booth, Sydney, Australia) was cut to appropriate dimensions and used as sacrificial templates for scaffold replication. The ceramic slurry was prepared by adding the ceramic powder to 0.01 M polyvinyl alcohol (PVA) binder solution to make a 30 wt% suspension. Foam templates were immersed in the ceramic slurry and compressed gently a few times to facilitate slurry penetration, and excess slurry was squeezed out. After drying, the ceramic-coated foams were sintered in air in an electric furnace using a five-stage schedule: (i) heating from 25°C to 600°C at a heating rate of 1°C min⁻¹, (ii) holding the temperature at 600°C for 1 hour, (iii) heating from 600°C to 1200°C at 2°C min⁻¹, (iv) holding at 1200°C for 2 hours, and (v) cooling to 25°C at a cooling rate of 5°C min⁻¹. The resulting BCP scaffolds were composed of 40% hydroxyapatite and 60% β-tricalcium phosphate, with dimensions of 7mm diameter and 4.5 mm height which were used for all subsequent experiments.

2.2 Silk coating of BCP scaffolds

Silk fibroin aqueous solution was prepared from *B. mori* cocoons as previously described,⁵¹ with a final concentration of *ca.* 7.8 wt%. Silk-coated BCP scaffolds were prepared by

immersing the ceramic scaffold in silk solution and pipetting the solution through the scaffold to ensure uniform infiltration. Excess silk solution was removed from the scaffold by absorbing with tissue before vacuum drying at 80°C for 30 min. The coating was stabilised by immersing the coated scaffold in methanol for 5 min to induce β -sheet formation, followed by vacuum drying at 80°C for 10 min. This coating procedure was repeated 1, 5 and 7 times to give silk-coated BCP scaffolds with different coating thicknesses. A total of 4 groups were prepared for subsequent characterisation, which were denoted as BCP control, BCP-1x, BCP-5x and BCP-7x (Table 1).

2.3 Physical properties of the scaffolds

Scaffolds were fractured in liquid nitrogen using a razor blade and sputter coated with Pt/Pd. Pore morphology and microstructure of the scaffolds were examined via field emission scanning electron microscopy (FE-SEM) using Zeiss Ultra 55 (Carl Zeiss, Germany). The silk-coated scaffolds were weighed before and after coating, and the percentage weight increase after coating was calculated. Porosity of the scaffolds was measured according to Archimedes' principle, using hexane as the liquid medium since hexane is a non-solvent of silk and can permeate easily through the silk-coated scaffolds without causing swelling or shrinkage. Scaffold porosity was calculated as a percentage according to the following formula: % Porosity = $(W_2 - W_1)/(W_2 - W_3) \times 100\%$, where W_1 is the dry weight of the scaffold in air, W_2 is the weight of the scaffold impregnated with hexane in air, and W_3 is the weight of the scaffold while it is suspended in hexane.

2.4 Mechanical properties of the scaffolds

Mechanical properties of the scaffolds were determined via unconfined compression testing on an Instron 3366 (Norwood, MA, USA) testing frame equipped with a 100N capacity load cell. Scaffolds with flat surfaces were selected as test specimens, and were incubated in phosphate buffered saline (PBS) at room temperature for 24 hours prior to testing. Tests were conducted at 37°C in a temperature controlled 0.1M PBS bath, using a displacement control mode with crosshead displacement rate of 1mm min⁻¹. The highest peak in the stress-strain curve within 5–30% compressive strain was used to determine the compressive strength and modulus. Compressive strength was measured as the maximum stress at the top of the peak, and compressive modulus was calculated as the slope of the curve leading up to the peak. The energy required to deform 1mm of the scaffold height per unit surface area (in J m⁻²) was also calculated by integrating the area under the stress-strain curve corresponding to 0.2–1.2mm compressive extension (approximately 5–30% compressive strain).

2.5 Cell culture

All reagents were purchased from Invitrogen (Carlsbad, CA, USA) unless otherwise stated. Human mesenchymal stem cells (hMSCs) were extracted from a single donor from commercially obtained fresh human bone marrow aspirate (Lonza, Gaithersburg, MD, USA). Aspirate donors were male, under 25 years of age and free of HIV, hepatitis B and hepatitis C. The aspirate was diluted 10-fold with expansion medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics-antimycotics (100U mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin, 0.25 μ g mL⁻¹ fungizone), 0.1mM non-essential amino acids, and 1ng mL⁻¹ basic fibroblast growth factor (bFGF). The diluted aspirate was plated in T-185 flasks at an average seeding density of 3.5×10^5 bone marrow mononuclear cells per cm². Cells were cultured for 10 days at 37°C in 5% CO₂, after which the non-adherent cells (haematopoietic cells) were removed and adherent cells (hMSCs) were kept in expansion medium to reach confluence. No sorting of the cells was performed and cells were tested for osteogenic and adipogenic differentiation by monolayer and micromass culture. Osteogenic differentiation was

confirmed at 21 days by the accumulation of mineralised calcium phosphate stained with Alizarin red (Fig. 1A). Adipogenic differentiation was confirmed at 21 days by intracellular accumulation of lipid-rich vacuoles stained with Oil Red O (Fig. 1B). Osteogenic medium consisted of α -minimum essential medium (α MEM) supplemented with 10% FBS, antibiotics-antimycotics (100U mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin, 0.25 μ g mL⁻¹ fungizone), 0.1mM non-essential amino acids, 10mM β -glycerol-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 100nM dexamethasone (Sigma-Aldrich), 0.05mM L-ascorbic acid (Sigma-Aldrich), and 100ng mL⁻¹ human recombinant BMP-2 (Wyeth, Cambridge, MA, USA). Adipogenic medium consisted of Dulbecco's modified eagle medium (DMEM) high glucose with L-glutamine supplemented with 10% FBS, 1% penicillin-streptomycin, 0.1mM non-essential amino acids, 50 μ M indometacin (Sigma-Aldrich), 0.5 μ M 3-isobutyl-1-methylxanthin (IBMX, Sigma-Aldrich), and 1 μ M dexamethasone (Sigma-Aldrich).

Scaffolds were sterilised by autoclaving, placed in 12-well plates, incubated overnight in expansion medium and aspirated dry prior to cell seeding. All hMSCs used for *in vitro* experiments were at passage 2. After the cells reached 80–90% confluence, they were trypsinised and subsequently suspended in expansion medium. For the attachment and proliferation experiments, hMSCs were seeded onto the scaffolds at a seeding density of 2×10^5 cells per scaffold. The seeded scaffolds were incubated for 2 hours at 37°C to allow cell attachment, after which 2.5mL of expansion medium was added to each well. Expansion medium was replaced at 3 days for the proliferation experiment. Cell attachment was evaluated after 2 and 24 hours of culture, while proliferation was evaluated at 3 and 7 days. For all other *in vitro* experiments, hMSCs were seeded onto the scaffolds at a seeding density of 1×10^6 cells per scaffold. The seeded scaffolds were incubated for 2 hours at 37°C to allow cell attachment, after which 2.5mL of expansion medium was added to each well. After 24 hours, the expansion medium was replaced with 2.5mL of osteogenic medium. 1.5mL of osteogenic medium was replaced twice per week. The scaffolds were collected for analysis after 3 and 6 weeks. All cultures were maintained at 37°C in 5% CO₂.

2.6 Attachment and proliferation

Cultured scaffolds were analysed for the attachment and proliferation of hMSCs. For attachment, cultured scaffolds were harvested at each time point and fixed in 4% PBS buffered paraformaldehyde for at least 24 hours. The scaffolds were rinsed in PBS and dehydrated through graded ethanol, dried in hexamethyldisilazane for 3 min and then desiccated overnight. The scaffolds were sputter coated with gold prior to SEM examination. For proliferation, metabolic activity of hMSCs on the cultured scaffolds was measured using Alamar Blue (Life Technologies, Grand Island, NY, USA). The scaffolds were transferred to a new sterile well plate for each Alamar Blue assay to ensure that only the metabolic activity of cells growing on the scaffolds was measured. Light exposure was minimised during all working steps. The Alamar Blue working solution was prepared by mixing the calculated volume of expansion medium with 10% Alamar Blue. 500 μ L of the freshly prepared solution was added to each well, and the scaffolds were incubated at 37°C for 90 min (day 3) or 60 min (day 7). Fluorescence was measured for 100 μ L of the incubated solution with excitation at 560nm and emission at 590nm. Background fluorescence from an empty scaffold incubated in Alamar Blue solution was subtracted.

2.7 Gene expression

Quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) was used to evaluate osteogenic gene expression of hMSCs on the cultured scaffolds. At each time point, scaffolds were rinsed in PBS and stored in Trizol (Life Technologies, Grand Island, NY, USA) at –80°C. For analysis, the scaffolds were thawed and chopped using

microscissors. RNA was isolated using the single step acid-phenol guanidinium method, and purified using the RNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. Primer sequences from Assays-on-Demand (Life Technologies) were used for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905_m1), as well as for collagen type I (COL1A1, Hs00164004_m1), alkaline phosphatase (ALPL, Hs01029144_m1), and bone sialoprotein (IBSP, Hs00173720_m1). Expression levels were quantified using a Stratagene Mx3000P QPCR System (Stratagene, La Jolla, CA, USA) and normalised to GAPDH using the comparative Ct (2^{-C_t}) method. A list of the genes tested and their abbreviations is shown in Table 2.

2.8 Biochemical analysis

Cultured scaffolds were analysed for DNA content and alkaline phosphatase (ALP) activity of hMSCs. At each time point, scaffolds were rinsed in PBS and stored at -20°C . For analysis, the scaffolds were thawed and chopped using microscissors in 200 μL of 0.2% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) with 5mM magnesium chloride (Sigma-Aldrich). DNA content was measured using a PicoGreen assay (Invitrogen) and detected on a fluorescent plate reader with excitation at 480nm and emission at 528nm. ALP activity was measured and normalised to DNA content. Samples were centrifuged at 12,000 rpm for 10 min at 4°C , after which the supernatant was collected and kept on ice, and subsequently diluted to appropriate concentration. 80 μL of the diluted sample was combined with 20 μL of 0.75M 2-amino 2-methyl 1-propanol (Sigma-Aldrich) in water and 100 μL of 10mM *p*-nitrophenol phosphate substrate (Sigma-Aldrich). Dilutions of 1mM *p*-nitrophenol were used to generate a standard curve. The sample mixtures were incubated in a 96-well plate at 37°C for 1 hour until colour developed. 100 μL of 0.2M NaOH was added to stop the reaction, and the absorbance was read at 405nm.

2.9 Histology

At each time point, cultured scaffolds were harvested for histological analysis and fixed in 4% PBS buffered paraformaldehyde for at least 24 hours. The scaffolds were plastic embedded according to previously published procedures,⁵² which facilitate histological analysis of hard tissue specimens. Briefly, the scaffolds were dehydrated through graded ethanol, then infiltrated with and embedded in polymethyl-methacrylate resin. Upon polymerisation, longitudinal sections were cut from the embedded scaffolds using an Exakt 300 band saw (Norderstedt, Germany) and subsequently polished using a Struers Dap-7 grinder/polisher (Denmark). Sections which included the centre of the scaffolds were used for histological analysis. After surface etching with 2% (v/v) acid ethanol, the sections were surface stained with toluidine blue, as well as von Kossa for calcium deposition. The sections were mounted to slides and imaged on an Olympus BX51 optical microscope (Japan) equipped with an Olympus DP71 camera.

2.10 Statistical analysis

Data for all experiments were obtained from four independent samples. All data were expressed as mean $\pm$ standard deviation (SD) and analysed using one-way ANOVA. Differences were considered as statistically significant for $p < 0.05$.

3. Results and Discussion

3.1 Morphology and microstructure of the scaffolds

SEM examination showed that the BCP control had highly porous architecture with fully interconnected pores and pore sizes of 400–500 μm (Fig. 2A). Many cracks and defects were

present in the struts of the scaffold, and the microstructure contained large amounts of micropores (Fig. 2B). These imperfections allow easy crack propagation under load, accounting for the brittle nature of BCP ceramic scaffolds and their weak mechanical properties. The BCP-1x scaffold had pore morphology and microstructure similar to the control (Fig. 2C, D). There was no visible silk coating on the surface of the scaffold, likely because the silk diffused through the scaffold micropores. After 5 times silk coating, open porosity was maintained in the BCP-5x scaffold (Fig. 2E). The silk not only formed a uniform coating layer on the surface of the scaffold, but also infiltrated the micropores and filled the voids in the interior of the scaffold struts (Fig. 2F), thereby making the scaffold a composite on both macrostructural and microstructural levels. The uniform silk coating had an average thickness of 3 μ m (Fig. 2G). Closer examination of the coating interface showed that the silk was interwoven with the BCP microstructure, where the silk filled the micropores and formed mechanical interlocking with the BCP substrate that strengthens the durability of the coating (Fig. 2H). Some blocked pores were present in the BCP-7x scaffold towards the periphery due to the thick silk coating (Fig. 2I). The strut morphology and microstructure were quite similar to the BCP-5x scaffold, however the silk coating was not uniform and had variable thickness (Fig. 2J, K). With increasing number of silk coatings, porosity reduction was quite linear while weight increase was exponential (Table 1). All silk-coated BCP scaffolds had porosity suitable for bone regeneration, with the BCP-5x scaffold having a porosity of 80%.

From the three groups of silk-coated BCP scaffolds with different coating thicknesses, the BCP-5x possessed an optimal balance of characteristics suitable for bone regeneration, where a substantial coating thickness was achieved while maintaining an open and interconnected pore structure that bears resemblance to cancellous bone, which is known to have porosity of 50–90%.⁷ The process of silk coating deposition has been optimised in this study to address the common drawbacks of other scaffold coating techniques, including clogging of pores and weak coating adhesion to substrate.¹⁷

3.2 Mechanical properties of the scaffolds

Compression testing was performed on the control and silk-coated BCP scaffolds. The silk-coated scaffolds with multiple silk coatings showed significant improvements in mechanical properties compared to the control. The stress-strain curve of the BCP control reflects its brittle and weak nature, and the curve of the BCP-1x displays a similar trend (Fig. 3). In contrast, the stress-strain curves of the BCP-5x and BCP-7x show notable transition to elastic behaviour, with a defined peak that corresponds to compressive strength. The smooth curves bear resemblance to those of pure silk scaffolds,⁵³ indicating that these composite scaffolds with multiple silk coatings no longer undergo brittle fracture due to crack propagation under load. The multiple silk coatings led to a significant 6-fold increase in compressive strength of the BCP-5x and BCP-7x compared to the control (from 0.05MPa to 0.3MPa) (Fig. 4A). Furthermore, corresponding to the large increase in area under the stress-strain curve, there was a 12-fold increase in energy required to deform 1mm of the scaffold height per unit area for the BCP-5x and BCP-7x compared to the control, indicating dramatic improvements in scaffold toughness (Fig. 4C). There were no significant differences in mechanical properties between the BCP control and BCP-1x, or between the BCP-5x and BCP-7x, and compressive modulus was not significantly different across all groups (Fig. 4B). The lack of considerable improvements in mechanical properties after single silk coating in the BCP-1x is in agreement with previous results,⁴⁸ and is likely because there is insufficient silk in one coating to infiltrate and fill the micropores in the BCP microstructure (Fig. 2D). The transition to silk-like behaviour in the BCP-5x and BCP-7x can be explained by the >50% weight increase after silk coating (Table 1). After multiple silk coatings, the originally weak and brittle BCP ceramic struts were saturated

with silk, thereby compensating for their low density and forming silk-ceramic composite struts with much higher strength and toughness (Fig. 2F). As the silk infiltrated into and coated the ceramic struts, it bridged the small defects present in the BCP microstructure, thereby reducing the chance of crack propagation along these defects under load. The BCP-5x and BCP-7x scaffolds resembled an interpenetrating ceramic-polymer composite, where the strengthening and toughening effect of the silk could be attributed to a micron-scale crack-bridging mechanism.⁵⁴

The results presented in Sections 3.1 and 3.2 collectively indicate that, from the three groups of silk-coated BCP scaffolds with different coating thicknesses, the BCP-5x possessed an optimal combination of structural and mechanical properties for bone regeneration. The BCP-5x was therefore selected as the test group for subsequent biological characterisation, and will hereon be denoted as BCP-silk. Although the mechanical properties of BCP-silk did not match those of cancellous bone, which is known to have compressive strength of 2–12MPa and modulus of 0.1–5GPa,⁷ it represented a significant improvement from the unmodified BCP ceramic scaffold and exhibited favourable elastic behaviour with greatly enhanced toughness. The BCP-silk also provided a better match of mechanical properties to native bone tissue than other example systems involving pure silk, silk-silk composite and silk-ceramic composite scaffolds.^{45,47,50,51}

3.3 Attachment and Proliferation

Attachment and morphology of hMSCs cultured on BCP control and BCP-silk scaffolds were assessed by SEM. At both 2 and 24 hours, cells were well attached on the surface of the BCP control and mostly exhibited an elongated morphology (Fig. 5A, B). The cells had a flattened appearance and were visible as dark imprints on the scaffold surface. Cells on the BCP-silk appeared flattened and well spread out after 2 hours with a more star-shaped morphology (Fig. 5C). The cells could be seen to have formed extended filopodia contacting the scaffold surface and also adjacent cells. By 24 hours, the cells had established extensive cell-cell contacts and formed a continuous sheet covering parts of the scaffold surface in the BCP-silk (Fig. 5D). The cells were moulded to the contour of the scaffold surface and were generally indistinguishable except in areas where the cells were slightly lifted from the surface. The SEM images demonstrated that BCP-silk is a favourable substrate for the attachment and spreading of hMSCs.

Metabolic activity of hMSCs cultured on BCP control and BCP-silk scaffolds in expansion medium was measured over a 7 day period as indication of cell proliferation (Fig. 6). Cell numbers increased significantly from 3 to 7 days for both groups, but were significantly lower in the BCP-silk compared to the control at both time points. Lower cell numbers in the BCP-silk over short-term culture in expansion medium is likely the result of lower porosity compared to the control, which translates to reduced surface area for cell growth. Lower porosity is known to stimulate osteogenesis *in vitro* by suppressing cell proliferation and forcing cell aggregation.¹⁴ Reduced cell proliferation in scaffolds with lower porosity was also observed in other studies,^{55,56} with concurrent enhancement of osteogenic differentiation.⁵⁵ The multiple silk coatings in the BCP-silk scaffold may therefore render it more effective at inducing osteogenic differentiation, by suppressing cell proliferation while maintaining porosity that is similar to cancellous bone. A significant increase in cell proliferation was also observed from 3 to 7 days which, taken together with the attachment results, demonstrates the biocompatibility of the BCP-silk scaffold and the absence of adverse effects on cells.

3.4 Gene expression

The BCP control and BCP-silk scaffolds were analysed for osteogenic gene expression, and data for all genes tested were expressed as fold increase from the 3 week BCP control. Collagen type I expression was quite stable for the BCP control between 3 and 6 weeks, while for the BCP-silk it was noticeably higher than the control at 3 weeks with a marginally significant difference ($p = 0.09$), and reduced to the same level as the control by 6 weeks (Fig. 7A). Alkaline phosphatase expression displayed a similar trend, with the BCP-silk showing a near significant increase from the BCP control at 3 weeks ($p = 0.06$) (Fig. 7B). There was also significant downregulation of alkaline phosphatase expression in the BCP-silk from 3 to 6 weeks. Bone sialoprotein expression was significantly upregulated in the BCP control from 3 to 6 weeks, while levels remained quite stable for the BCP-silk (Fig. 7C). Notably at 3 weeks, bone sialoprotein expression in the BCP-silk exhibited a substantial 11-fold increase from the BCP control. Overall, the gene expression data indicate that multiple silk coatings on the BCP ceramic scaffold promoted the *in vitro* osteogenic differentiation of hMSCs. Previous studies have demonstrated that the development of the osteoblast phenotype by osteogenic cells in culture exhibits a temporal sequence of gene expression, which is defined by three distinct periods separated by two transition points.^{57,58} Initially, there is a period of active proliferation during which cell growth-related genes are expressed, accompanied by maximum levels of collagen type I expression. This is followed by the downregulation of proliferation and a subsequent period of matrix maturation, which is characterised by a peak in alkaline phosphatase expression. Matrix mineralisation forms the final period of the osteoblast developmental sequence, and is associated with strong induction in the expression of mineral-binding proteins such as bone sialoprotein. Two transition points between the developmental periods have been established experimentally, the first of which occurs with completion of the proliferative period, and the second is reached at the onset of matrix mineralisation. The gene expression data of the BCP control and BCP-silk scaffolds suggest that the 3 week time point fell around the first transition point, while the 6 week time point fell within the matrix mineralisation period. At the first time point, the BCP-silk showed higher transcript levels of collagen type I and alkaline phosphatase compared to the control, demonstrating its ability to promote the growth of hMSCs and their commitment to osteogenic differentiation. At the second time point, the BCP-silk showed downregulation of these early markers, indicating that it could encourage the hMSCs to progress through the osteoblast developmental sequence. The significant and early upregulation of bone sialoprotein in the BCP-silk at 3 weeks also suggests that the composite scaffold might induce earlier progression to matrix mineralisation in the hMSCs and result in earlier bone formation. The positive effects of BCP-silk in promoting osteogenic differentiation of hMSCs can be attributed to the multiple silk coatings, and may be the result of several factors including improved mechanical properties, lower biodegradability, and possible formation of a nanofibrous structure. The differentiation of hMSCs is known to be influenced by substrate mechanical properties, which contribute to modulating cell shape through cell-matrix interactions.⁵⁹ The addition of silk to the BCP ceramic scaffold also lowers the biodegradability of the composite scaffold, as silk undergoes slower biodegradation *in vitro*³³ than BCP.⁶⁰ It has been shown that the osteogenic activity of hMSCs is improved on more slowly degrading scaffolds.⁶¹ Furthermore, it is possible that a nanofibrous structure was formed in the process of silk coating deposition to the BCP ceramic substrate,⁴⁸ which imitates the nanostructure of native bone matrix. There is evidence to suggest that osteogenic cells show earlier and enhanced expression of the osteoblast phenotype when cultured on nanofibrous scaffolds.^{62,63}

3.5 Biochemical analysis

DNA content of the BCP control and BCP-silk scaffolds was measured to quantify cell proliferation over 6 weeks in osteogenic medium (Fig. 8A). Cell numbers as represented by DNA content increased significantly from 3 to 6 weeks for both groups, with the BCP-silk exhibiting a greater increase ($p < 0.001$) than the BCP control ($p = 0.01$). At both 3 and 6 weeks, cell numbers were significantly lower in the BCP-silk compared to the control. The trends observed in cell proliferation confirmed the gene expression results in that BCP-silk promoted earlier and more enhanced osteogenic differentiation of hMSCs. The reciprocal relationship between cell proliferation and differentiation during bone formation was demonstrated in a study where osteogenic cells were experimentally induced to proceed with osteogenic differentiation by supplementing the culture medium with ascorbic acid.⁵⁸ Cells cultured in the absence of ascorbic acid continued to grow during the 28 day culture period and reached the highest cell density, while cells cultured in the presence of 50 μ g/mL ascorbic acid reached a plateau in cell number with the lowest cell density. Markers of osteogenic differentiation including collagen accumulation, ALP activity and calcium deposition were shown to be greatly elevated for cells cultured in the presence of ascorbic acid, but remained at baseline levels for proliferating cells in the absence of ascorbic acid. Comparable with the results of this study, the lower cell numbers in the BCP-silk at both time points is indicative of a plateau in cell number due to earlier downregulation of proliferation which marks the transition to differentiation along the osteogenic lineage. The BCP-silk also showed a greater increase in cell number from 3 to 6 weeks compared to the control, suggesting that it not only encourages earlier commitment to osteogenic differentiation in hMSCs, but also enhances growth of the committed cells.

ALP activity of the BCP control and BCP-silk scaffolds was evaluated (Fig. 8B). A significant reduction in ALP activity was observed in the BCP-silk from 3 to 6 weeks, but there were no significant differences between groups at each of the two time points. The reduction in ALP activity over time in the BCP-silk verifies that the hMSCs were progressing down the path of osteogenic differentiation, and reached the matrix mineralisation stage by 6 weeks. The trends observed in ALP activity were quite consistent with the gene expression results (Fig. 7B).

3.6 Histology

Histological analysis showed that the hMSCs infiltrated the BCP control and BCP-silk scaffolds (Fig. 9). At both time points, the cells were homogeneously distributed throughout the BCP control, while the BCP-silk showed more cell aggregations and mineralised masses towards the periphery. This was likely due to the static culture conditions coupled with reduced porosity of the BCP-silk scaffold, causing the cells to preferentially grow towards the periphery and cells on the periphery to be more active than those in the centre. The inhomogeneous cellular activity observed in the BCP-silk is also seen in other studies involving long-term static culture of hMSCs in silk-based scaffolds.^{38,45} Bioreactor culture can overcome the diffusional limitations experienced in mass transport under static conditions and will warrant further investigation. As silk has slow biodegradation, the BCP-silk scaffold in this study is likely able to withstand the medium flow in most bioreactors with little loss in mechanical integrity over the culture period.

The histological images show that the BCP control contained mostly spindle-shaped fibroblast-like cells at both 3 and 6 weeks (Fig. 9A, B, I J), with a few cuboidal osteoblast-like cells distributed randomly through the pores only at 6 weeks. Little mineralisation was present in the BCP control at 3 and 6 weeks as shown by von Kossa staining (Fig. 9C, D, K, L). In contrast, aggregations of cuboidal osteoblast-like cells were already evident in the BCP-silk at 3 weeks amongst other fibroblast-like cells (Fig. 9E, F), and pericellular

mineralisation was also detected (Fig. 9G, H). At 6 weeks, masses of connective tissue were observed in the BCP-silk, consisting of cuboidal osteoblast-like cells contacting each other via short processes, surrounded by extracellular matrix containing some collagen-like fibres and many dense spots of mineralisation dispersed throughout the matrix (Fig. 9M, N). The extracellular matrix was shown to be mineralised with calcium phosphate by von Kossa staining (Fig. 9O, P). The significant and early commitment of hMSCs to mineralisation in the BCP-silk as shown by the histological images paralleled the trends observed in bone sialoprotein expression (Fig. 7C). The enhanced osteogenic differentiation of hMSCs on the BCP-silk as illustrated by the gene expression results was also demonstrated histologically by the development of quite advanced osteogenic tissue in the BCP-silk at 6 weeks compared to the control. The histology results confirm that multiple silk coatings on the BCP ceramic scaffold have positive effects on encouraging the *in vitro* osteogenic differentiation of hMSCs.

4. Conclusion

The effect of silk coatings on a common ceramic scaffold for use in bone regeneration was systematically investigated in this study. The silk coating process was optimised to address the common drawbacks of polymer-coated ceramic scaffolds. The BCP-silk composite scaffold developed in this study showed a notable coating effect, where the addition of multiple silk coating layers to the low-density BCP ceramic struts resulted in significantly improved mechanical properties and enhanced osteogenic response of hMSCs *in vitro*. A similar coating effect can be anticipated for other types of crystalline ceramic scaffolds with comparable microstructure. The use of multiple silk coatings to improve the properties of ceramic scaffolds for bone regeneration represents a simple and effective method of reinforcement and holds promise for clinical translation, and future work will focus on bioreactor culture and *in vivo* integration.

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Figure 1.
(A) Alizarin red and (B) Oil Red O staining of isolated hMSCs.

Figure 2. Pore morphology and microstructure of (A, B) BCP control, (C, D) BCP-1x, (E, F, G, H) BCP-5x, and (I, J, K) BCP-7x scaffolds. Arrows indicate silk which has infiltrated the micropores and filled the voids in the BCP microstructure.

Figure 3.
Typical stress-strain curves of BCP control, BCP-1x, BCP-5x, and BCP-7x scaffolds.

Figure 4.

Mechanical properties of BCP control, BCP-1x, BCP-5x, and BCP-7x scaffolds in compression under hydrated conditions. (A) Compressive strength, (B) compressive modulus, and (C) toughness expressed as energy required to deform 1mm of the scaffold height per unit area. * $p < 0.05$; ** $p < 0.0001$.

Figure 5. Attachment and morphology of hMSCs cultured on (A, B) BCP control and (C, D) BCP-silk scaffolds after 2 and 24 hours. Arrows indicate attached cells on the scaffold surface.

Figure 6. Proliferation of hMSCs cultured on BCP control and BCP-silk scaffolds over 7 days in expansion medium. * $p < 0.05$ between groups; # $p < 0.05$ between time points within same group.

Figure 7.

Osteogenic gene expression of hMSCs cultured on BCP control and BCP-silk scaffolds over 6 weeks, expressed as fold increase from 3 week BCP control. (A) Collagen type I, (B) alkaline phosphatase, and (C) bone sialoprotein. * $p < 0.05$ between groups; # $p < 0.05$ between time points within same group.

Figure 8. (A) DNA content and (B) alkaline phosphatase activity of hMSCs cultured on BCP control and BCP-silk scaffolds over 6 weeks. * $p < 0.05$ between groups; # $p < 0.05$ between time points within same group.

Figure 9. Histology of BCP control and BCP-silk scaffolds with toluidine blue (left panel) and von Kossa (right panel) staining after 3 (top panel) and 6 (bottom panel) weeks. Asterisk = scaffold; scale bar = 100µm (low magnification) and 50µm (high magnification).

Table 1

Scaffold groups showing composition, porosity and weight increase after coating.

Scaffold Groups	Scaffold Composition		Porosity (%)	Weight increase after coating (%)
	Ceramic Substrate	Number of Silk Coatings (7.8 wt%)		
BCP Control	BCP	0	92.8 ± 0.9	-
BCP-1x	BCP	1	92.4 ± 0.4	8 ± 3
BCP-5x	BCP	5	80.4 ± 5.8	49 ± 21
BCP-7x	BCP	7	80.0 ± 6.2	117 ± 54

Table 2

Genes tested by qRT-PCR and their abbreviations.

Gene	Abbreviation
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Collagen type I	COL1
Alkaline phosphatase	ALP
Bone sialoprotein	BSP