



Initial cell pre-cultivation can maximize ECM mineralization by human mesenchymal stem cells on silk fibroin scaffolds

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ABSTRACT

Fast remineralization of bone defects by means of tissue engineering is one of many targets in orthopedic regeneration. This study investigated the influence of a range of pre-culture durations for human bone marrow derived mesenchymal stem cells (hMSC) before inducing differentiation into osteoblast-like cells. The aim was to find the conditions that lead to maximal extracellular matrix (ECM) mineralization, in terms of both amount and best distribution. Additionally, the influence of silk fibroin scaffold pore size on mineralization was assessed. The formation of mineralized ECM by hMSCs cultured in osteogenic medium on silk fibroin scaffolds was monitored and quantified for up to 72 days in culture using non-invasive time-lapse micro-computed tomography (micro-CT). ECM mineralization increased linearly 3 weeks after the beginning of the experiment with addition of differentiation medium. Biochemical end-point assays measured the amount of DNA, calcium deposits, alkaline phosphatase activity and cell metabolic activity to corroborate the hypothesis that an initial pre-culture period of hMSCs on silk fibroin scaffolds can accelerate mineralized ECM formation. According to the micro-CT analysis mineralization on silk fibroin scaffolds with pores of 112–224 μm diameter was most efficient with an initial cell pre-culture period of 9 days, showing $6.87 \pm 0.81 \times$ higher mineralization values during the whole cultivation period than without an initial cell pre-culture period.

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1. Introduction

Bone tissue engineering is becoming increasingly of interest as a regeneration strategy in clinical orthopedics [1–3]. Research debates about whether optimal scaffold pore diameters exist at all and whether they are similar for all kinds of materials are ongoing [4]. Tissue engineers around the world are nowadays able to induce cells to mineralize their extracellular matrix (ECM) in vitro, but the amount and distribution of this mineralized matrix is still poor and far from the connected structures that would mimic native bone morphologies.

Strategies to engineer bone have focused on the use of natural or synthetic materials as scaffolds for cell transplantation or as conduits to guide new bone growth. It has been shown that the success of scaffolds for tissue engineering is dependent on the material properties, which ideally should be biocompatible, osteoconductive, easy to sterilize and tunable for degradation into products that can be metabolized or excreted [5].

Silk fibroin is a fibrous protein derived from the cocoon of the silkworm *Bombyx mori*. For decades it has been used for clinical sutures [6]. Different methods are available to generate three-dimen-

sional (3D) scaffolds from silk fibroin that can be tailored to different sizes, shapes, crystallinities and mechanical properties [7,8]. Scaffolds prepared from silk fibroin have shown promising results as substrates for the in vitro and in vivo culture of bone-like tissue [7,8]. They demonstrated an equivalent or better biocompatibility than collagen scaffolds and defined the mechanical strength and mechanical integrity compared with other commonly used degradable polymeric biomaterials [6]. Furthermore, silk fibroin scaffolds degrade over a variable timeframe depending on proteolytic degradation, losing their tensile strength within 1 year and being resorbed within 2 years in vivo [6,9]. Studies have shown that silk fibroin matrices could be successfully used for bone tissue engineering with various cell types, such as osteoblasts, fibroblasts and mesenchymal stem cells, as well as other cell types [2,7,8,10].

Human mesenchymal stem cells (hMSCs) derived from human bone marrow are a well-known cell source to engineer bone-like tissue. In contrast to osteoblasts, they can be expanded to large cell numbers in an undifferentiated state and, when exposed to the appropriate extrinsic signals, selectively differentiate along mesenchymal lineages, including bone [11]. Previous studies with silk fibroin postulated that the structure of tissue engineered bone from hMSCs may be controlled by silk fibroin scaffold geometry [12], emphasizing the influence of the 3D environment on cell behavior.

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The current standard for seeding hMSCs on scaffolds is to pipette hMSCs on top of the scaffolds and cultivate them under osteogenic conditions from the day of cell seeding [7,13]. A higher mineral deposit efficiency would be desirable to enhance scaffold toughness, as well as to reduce the *in vitro* cultivation time for the final implantable tissue engineered product. Although ECM mineralization can be enhanced by extrinsic factors (e.g. bone morphogenic protein-2) to a certain extent *in vitro*, constructs resembling bone with interconnected trabecular structures and bone-like toughness have not been engineered so far. Our hypothesis was that a higher number of cells will be achieved by initial cellular proliferation directly on silk fibroin scaffolds prior to cell differentiation and will augment mineral deposits in the course of osteogenic cell differentiation. Pipetting cells on top of silk fibroin scaffolds by static seeding may not allow all cells to immediately adhere to the surface due to initial medium exchanges soon after seeding and a possible saturated cell density on the outer scaffold surfaces [13]. Therefore, a cell pre-culture of up to 9 days may allow adherent cells to spread and grow into the scaffold to produce a better distributed ECM. Additionally, we hypothesized that cellular in-growth into scaffolds will be dependent on scaffold pore size and interconnectivity between pores. We therefore extended the examination of our hypothesis to scaffolds with different pore sizes.

The aim of this study was to examine (i) whether different hMSC pre-culture durations on silk fibroin scaffolds have an impact on the amount and structure of the produced mineralized ECM and (ii) if scaffold pore size has an influence in this regard.

Undifferentiated hMSCs were seeded by pipetting on silk fibroin scaffolds. For each individual pore size four groups consisting of five cell-seeded scaffolds each were subjected to pre-culture durations of either 0, 3, 6 or 9 days. This was then followed by cultivation in differentiation medium for 72 days. Formation of bone-like tissue was studied over 9 weeks *in vitro*. In house designed bioreactors were used to non-invasively monitor the mineralization of the ECM on each scaffold using micro-CT [7]. At the end of the study destructive biochemical assays were performed to compare cell metabolic activity, alkaline phosphatase (ALP) activity, the calcium content in the ECM and the amount of DNA.

2. Materials and methods

2.1. Materials

Silkworm cocoons from *B. mori* were kindly supplied by Trudel Inc. (Zürich, Switzerland). Sodium carbonate (Na_2CO_3), lithium bromide (LiBr), sodium chloride (NaCl), and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) were from Fluka (Buchs, Switzerland). Methanol was provided by EGT Chemie AG (Trägerig, Switzerland), while ethanol was from Alcosuisse (Bern, Switzerland). Three different cell media were applied in this study: control medium, comprising Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), and 1% antibiotic/antimycotic solution; proliferation medium, comprising control medium with 1 ng ml^{-1} basic fibroblast growth factor (bFGF) and 1% non-essential amino acids (NEAA) (consisting of 8.9 mg l^{-1} L-alanine, 13.2 mg l^{-1} L-asparagine, 13.3 mg l^{-1} L-aspartic acid, 14.7 mg l^{-1} L-glutamic acid, 7.5 mg l^{-1} glycine, 11.5 mg l^{-1} L-proline, 10.5 mg l^{-1} L-serine); osteogenic differentiation medium, comprising control medium with $50 \text{ } \mu\text{g ml}^{-1}$ L-ascorbic acid 2-phosphate (AA), 100 nM dexamethasone, 10 mM β -glycerolphosphate (β -GP). DMEM, FBS, antibiotic/antimycotic, bFGF, NEAA and 0.25% trypsin-EDTA were obtained from Invitrogen (Basel, Switzerland). AA, dexamethasone and β -GP were from Sigma-Aldrich (Buchs, Switzerland). All other substances were of analytical or pharmaceutical grade and were

obtained from Sigma-Aldrich (Buchs, Switzerland). Bone marrow was obtained from Lonza Group Ltd. (Basel, Switzerland).

2.2. Silk fibroin scaffold preparation

Silk fibroin scaffolds were prepared as previously described [14,15]. Briefly, silk fibroin cocoons from *B. mori* were boiled for $2 \times 1 \text{ h}$ in an aqueous solution of 0.02 M Na_2CO_3 and rinsed with ultrapure water to extract the coating protein sericin. Purified silk fibroin was solubilized in 9 M LiBr solution and dialyzed against water (3500 molecular weight cut-off, Pierce, Woburn, MA) with five water exchanges within 3 days. The dialyzed aqueous silk fibroin solution was frozen at -80°C , lyophilized (Christ, Osterode, Germany) and redissolved in HFIP to obtain a 17% (w/v) silk fibroin solution. NaCl crystals were sieved (Retsch, Arlesheim, Switzerland) and crystals in the ranges 112–224 (small pores, S), 315–400 (medium pores, M), or 500–600 μm (large pores, L) were weighed in a Teflon container and silk fibroin/HFIP solution was added at a ratio of 20:1 (NaCl:silk fibroin). The HFIP evaporated within 3 days. The NaCl/silk fibroin blocks were immersed in 90 vol.% methanol for 30 min to induce a conformational change of the water-soluble silk fibroin to the water-insoluble crystalline β -sheet structure [10]. NaCl was leached by incubation in ultrapure water with five water changes in 2 days. Blocks were cut into disks with a height of 2 mm and punched out (dermal punch, Miltey, Lake Success, NY) to form scaffolds with a diameter of 5 mm and autoclaved.

2.3. Scanning electron microscopy (SEM)

Unseeded scaffolds were visualized for their topography by means of SEM. Dried scaffolds were sputtered with gold and observed by SEM at a working distance of 35 mm, an accelerating voltage of 20 keV and a magnification of $85\times$ (Zeiss Leo Gemini 1530, Cambridge, UK).

2.4. Mechanical properties of silk fibroin scaffolds

Unseeded silk fibroin scaffolds prewetted in phosphate-buffered saline (PBS) ($n = 5$ per group) of all three different pore sizes were mechanically tested for scaffold stiffness and stress relaxation properties. The disk-shaped silk fibroin scaffolds were tested by stepwise stress relaxation indentation (diameter 5 mm) in PBS using a mechanical testing system (Zwick 1456, Ulm, Germany) equipped with a calibrated 10 N load cell, built-in displacement control, and a cylindrical, plane-ended, stainless steel indenter tip with a diameter of 1.2 mm. To detect the sample position and measure sample thickness a preload of 20 mN was applied and held for 5 min. Stepwise stress relaxation indentation was then carried out in three strain steps of 5%, 10%, and 15% of the measured sample thickness at a strain rate of 5 mm min^{-1} . After each indentation step the specimens were left to relax for 45 min to reach equilibrium. Force, displacement, and time data were recorded throughout the whole test period. The equilibrium Young's modulus was determined from the equilibrium stress-strain curve.

2.5. Micro-computed tomography (micro-CT) of silk fibroin scaffolds

For silk fibroin scaffold morphometrical analysis ($n = 5$ per group) a micro-computed tomography system (micro-CT 40, Scanco Medical, Brüttisellen, Switzerland) equipped with a $5 \text{ } \mu\text{m}$ focal spot X-ray tube as source was used. A two-dimensional charged-coupled device camera coupled to a thin scintillator as a detector permitted acquisition of about 100 tomographic images in parallel. An isotropic nominal resolution of $6 \text{ } \mu\text{m}$ (high resolution mode) was chosen. Dry scaffolds had to be used for measurement in air

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