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Research paper

Biocompatibility and osteoconduction of macroporous silk fibroin implants in cortical defects in sheep

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Dedicated to Hans Peter Merkle on the occasion of his 70th birthday

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ABSTRACT

The goal of the presented study was to compare the biocompatibility and cellular responses to porous silk fibroin (SF) scaffolds produced in a water-based (UPW) or a solvent based process (HFIP) using two different SF sources. For that reason, four different SF scaffolds were implanted ($n = 6$) into drill hole defects in the cancellous bone of the sheep tibia and humerus. The scaffolds were evaluated histologically for biocompatibility, cell–material interaction, and cellular ingrowth. New bone formation was observed macroscopically and histologically at 8 weeks after implantation. For semiquantitative evaluation, the investigated parameters were scored and statistically analyzed (factorial ANOVA). All implants showed good biocompatibility as evident by low infiltration of inflammatory cells and the absent encapsulation of the scaffolds in connective tissue. Multinuclear foreign body giant cells (MFGCs) and macrophages were present in all parts of the scaffold at the material surface and actively degrading the SF material. Cell ingrowth and vascularization were uniform across the scaffold. However, in HFIP scaffolds, local regions of void pores were present throughout the scaffold, probably due to the low pore interconnectivity in this scaffold type in contrast to UPW scaffolds. The amount of newly formed bone was very low in both scaffold types but was more abundant in the periphery than in the center of the scaffolds and for HFIP scaffolds mainly restricted to single pores.

1. Introduction

Bone grafts or bone substitutes are indicated when the physiologic healing process fails or is delayed, e.g., due to complicated fractures, age of the patient, long-term use of nicotine, in the presence of diabetes or autoimmune diseases, or in case of non-bridging defects after surgical removal of large masses of bone [1,2]. For orthopedic applications, 500,000–600,000 interventions involving bone grafting are conducted every year in the US alone [3], including allografts and artificial porous bones. Despite the numerous advantages, synthetic bone graft substitutes – mostly ceramics and polyesters – currently represent only 10% of the bone substitutes on the market [4,8]. Disadvantages of ceramic implants include high brittleness, variable rates of resorption, and potentially adverse effects on normal bone remodeling [3]. In new generations of substitutes, biopolymers such as protein or polysaccharides are the major components of the extracellular matrix (ECM) and comprise of the chemical properties to provide an ideal microenvironment, wherein cells can strive and foster the natural regeneration process. However, collagen-based bone substitutes, the most abundant ECM protein in bone, exert only low mechanical strength and undergo rapid degradation. Therefore, collagens perform poorly when it comes to provide the necessary mechanical support and spatial guidance during bone regeneration. Accordingly, their application potential is limited [5].

Silk fibroin (SF) can be harvested in relevant amounts from cocoons of the silkworm *Bombyx mori* and represents one of nature's toughest fibers, able to absorb more energy before break than synthetic fibers such as Kevlar [6]. Previously reported challenges regarding the biocompatibility of this material could be resolved by separating the immunogenic glycoprotein sericin from the pure SF proteins [7,8]. Various scaffolding technologies have been developed to produce 3-dimensional porous SF constructs from either organic solvent or water-based systems, benefiting from its versatile processability [9–12]. When implanted in rodents, SF revealed only moderate inflammatory potential, comparable or lower than that observed with collagen or biodegradable polyesters [5–8,13–16]. SF has been used as a carrier for growth factors, fabricated either by an organic solvent based process using hexafluoro-isopropanol

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(HFIP) such as with BMP-2 loaded SF scaffolds [17], or through an ultra purified water (UPW) based freeze-drying protocol comprising IGF-I and aqueous SF solutions [18]. Both approaches showed promising results for future applications of SF as bioactive implant material. Despite the promising record of SF for biocompatibility, the final performance of an SF implant under physiological conditions *in vivo* still awaits testing. In particular, the influence of the silk source as well as different scaffolding techniques, e.g., in terms of process parameters and residual solvents on the *in vivo* performance and biocompatibility calls for further experimental confirmation.

In the present work, we hypothesize that the source of SF is of little relevance when following an established protocol for its purification, and therefore, standard industrial silk cocoons represent an abundant and cheap raw material for biomedical implants. Moreover, we speculate that a UPW based process that omits the use of solvents which are not generally recognized as safe and produces scaffolds with well defined pore structure and pore interconnectivity will improve the biocompatibility of the material and bone ingrowth into the scaffold structure.

In order to evaluate the effect of the proposed processing parameters on the performance and biocompatibility of SF scaffolds in one single experiment, the sheep drill hole model was used, representing a relevant *in vivo* animal model that allows for the reduction in animal numbers but still satisfying statistical requirements [19–22].

2. Materials and methods

2.1. Materials and instruments

In addition to routine surgical instruments, Weitlaner- and Gelpi-retractors, a periosteal elevator, a pneumatic drill (Synthes, Waldenburg, Switzerland) with an 8 mm, slightly modified drill bit (KaVo INTrASurg 500®, KaVoDental AG Biberach, Germany, modified by Synthes, Waldenburg, Switzerland) and a corresponding drill guide were used. The drill bit was modified with a special depth-regulating device, and the tip was flattened while still having good cutting characteristics. *n*-Hexane for paraffin extraction and gas chromatography was from Scharlau-Chemie S.A. (Sentmenat, Barcelona, Spain), and gelatine (Golddruck, plus alle Angaben) was purchased from Hänseler AG (Herisau, Switzerland). Paraffin with a melting temperature of 54–56 °C, hexafluoro-isopropanol (HFIP), and lithium bromide were from Sigma–Aldrich (Fluka, St. Louis, MO).

2.2. Methods

2.2.1. Silk preparation

Silk fibroin solution was prepared as described previously [11]. Briefly, cocoons from *Bombyx mori* were obtained from either M. Tsukada, Institute of Sericulture, Tsukuba, Japan (silk source 1) or Trudel, Zurich, Switzerland (silk source 2) and boiled in 0.02 M Na_2CO_3 solution, rinsed and dissolved in 9 M LiBr for 3 h at 58 °C to generate a 10% (w/v) solution. The solution was dialyzed (Pierce, Rockford, IL; molecular weight cutoff 3500 g/M) in 1.5 L of ultra purified water (UPW; NANOpure Diamond, Barnstead International, IA, USA). UPW was exchanged 5 times in 72 h resulting in a purified silk fibroin concentration of 3% (w/v).

2.2.2. HFIP scaffolding protocol

HFIP type SF scaffolds were prepared as previously described [9]. Dialyzed silk fibroin (3% w/w) was lyophilized and resolved in hexafluoro-2-propanol (HFIP, 17% w/v). A given amount of granular NaCl (200–300 μm) as porogen was accurately weighed in a Teflon container, and silk solution was added at a ratio of 20:1 (NaCl/silk). HFIP was allowed to evaporate for 2 days, and for hardening, NaCl/silk blocks were immersed in 90% (v/v) methanol for 30 min. Blocks were removed and dried, and NaCl was washed out with water for 2 days. Porous scaffolds were cut into cylinders of 8 mm diameter and packed in aluminum foil before they were steam autoclaved at 121 °C for 20 min.

2.2.3. UPW scaffolding protocol

Instead of using granular NaCl, paraffin spheres served as porogen. Their preparation followed an emulsion process using water, gelatin, and solid paraffin as described before [11]. Solidified paraffin spheres were harvested and washed with ultra purified water. The spheres were filtered through 12–25 μm pore filter paper (Schleicher & Schuell, Switzerland), dried at room temperature, and classified with standard sieves (Retsch, Haan, Germany). Fractions of >500 μm, 400–500 μm, 300–400 μm, 200–300 μm, and 100–200 μm were collected and stored at 4 °C. For scaffolding, heat induced sintering of paraffin globules (200–300 μm) in a cylindrical mold was performed as described previously [11]. Briefly, 1.2 g of paraffin porogen was loaded into 20 ml falcon syringes (BD Bioscience, Maryland, MD). The surface was evened by the use of a pistil, followed by controlled sintering at 37 °C to assure pore interconnectivity (Thermocenter, Salvis OAKTON Instruments, Vernon Hills, NY) for 50 min. After sintering, the tubular mold was left to cool to room temperature and stored for later use. Freshly prepared silk solution in UPW was transferred to a dialysis chamber and further dialyzed against a 13.3% (w/v) PEG 6000 solution in UPW overnight resulting in a 20% (w/v) silk fibroin solution. One ml of this solution was soaked into the prepared tubular mold (d = 8 mm, height = 20 mm) consisting of sintered paraffin spheres, so that silk fibroin solution filled all pores throughout the mold. The mold was then shock frozen in liquid nitrogen for 1 min and freeze dried at −30 °C under temperature control for 48 h (Lyovac GT2, Finn-Aqua, Hurth, Germany). For hardening, the scaffolds featuring highly interconnective pores were immersed in 90% (v/v) methanol for 30 min and then air-dried. As validated, exhaustive extraction of paraffin was achieved by complete exchange of hexane every 12 h for a total of 4 exchanges (see below). The porous silk scaffolds were air-dried and then placed in a vacuum-dryer for 24 h. The dried scaffolds were packed in aluminum foil and autoclaved at 121 °C for 20 min.

2.2.4. Gravimetric and gas chromatographically analysis of paraffin leaching procedure

Extraction of paraffin spheres from scaffolds was by leaching with *n*-hexane (n = 4) at a ratio of 27 ml *n*-hexane per g of paraffin. Hexane was completely replaced every 12 h until a total of 6 changes. The paraffin content in one ml of solvent was assessed, and the percentage of leached paraffin was calculated. Batches 4–6 were analyzed by gas chromatography for residual paraffin in the hexane using a Trace MC (Thermo Electron corporation, US) equipped with a quadrupole mass spectrometer and a Factor-four Vf-5MS column (Variant, US). Helium flow rate was set to 6 ml/min, inlet temperature set at 180 °C, and split-less time was 1 min with a split ratio of 1:6. The oven temperature was increased from 40 to 45 °C at 2 °C/min and from 45 to 250 °C at 30 °C/min.

2.2.5. Sheep

Three adult Swiss Alpine sheep, all castrated male of 2 years of age, with a body weight ranging from 53–70 kg were used for the study, which was approved by the local Ethical Committee and veterinary authorities (application number 188/2004). The sheep were examined for their state of health both clinically and hematologically. Tetanus vaccine and an anthelmintic were administered. The animals were acclimatized to their new housing facilities for 2 weeks before surgery.