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Silk as a biocohesive sacrificial binder in the fabrication of hydroxyapatite load bearing scaffolds

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ABSTRACT

Limitations of current clinical methods for bone repair continue to fuel the demand for a high strength, bioactive bone replacement material. Recent attempts to produce porous scaffolds for bone regeneration have been limited by the intrinsic weakness associated with high porosity materials. In this study, ceramic scaffold fabrication techniques for potential use in load-bearing bone repairs have been developed using naturally derived silk from *Bombyx mori*. Silk was first employed for ceramic grain consolidation during green body formation, and later as a sacrificial polymer to impart porosity during sintering. These techniques allowed preparation of hydroxyapatite (HA) scaffolds that exhibited a wide range of mechanical and porosity profiles, with some displaying unusually high compressive strength up to 152.4 ± 9.1 MPa. Results showed that the scaffolds exhibited a wide range of compressive strengths and moduli (8.7 ± 2.7 MPa to 152.4 ± 9.1 MPa and 0.3 ± 0.1 GPa to 8.6 ± 0.3 GPa) with total porosities of up to 62.9 ± 2.7% depending on the parameters used for fabrication. Moreover, HA-silk scaffolds could be molded into large, complex shapes, and further machined post-sinter to generate specific three-dimensional geometries. Scaffolds supported bone marrow-derived mesenchymal stem cell attachment and proliferation, with no signs of cytotoxicity. Therefore, silk-fabricated HA scaffolds show promise for load bearing bone repair and regeneration needs.

1. Introduction

It is estimated that by the year 2020 approximately 6.6 million orthopedic surgeries will be performed annually [1]. Many of these operations will aim to repair “critical-sized” bone defects that result from traumatic fracture, osteosarcoma, or congenital malformation, and have lost regenerative capacity [2]. Growing concerns over the complications of autografting (e.g. donor site morbidity, infection) and allografting (e.g. graft rejection), as well as the limited availability of these tissue repair options has prompted the development of porous scaffolds for bone defect repair [3–5].

Calcium phosphates (CaP), particularly hydroxyapatite (HA), have drawn attention for orthopedic applications due to their close semblance to the mineral phase and crystalline structure of bone [6,7]. Owing to their biocompatibility and ability to be resorbed by the body, advancement in CaP scaffolding has accelerated over the past decade. Attempts at generating resorbable scaffolds for bone replacement have focused on achieving high porosity to promote cell infiltration and maintain oxygen and nutrient diffusion throughout the graft. Despite encouraging results, these efforts have been limited by the inherent weakness associated with increased porosity, which remains the fundamental limitation of bone tissue engineering [8]. Typical compressive strengths of porous ceramic scaffolds range from 5 to 40 MPa, which surpass the strength of human trabecular bone (2–10 MPa) but cannot match that of cortical bone (170–200 MPa), thereby restricting these scaffolds to non-load bearing bone repairs [7,9–12]. Thus, there is an urgent need in the field of bone tissue engineering for ceramics fabrication techniques that can produce grafts to match load-bearing requirements. Ideally, fabrication methods should have flexible processing parameters to control mechanical and porosity profiles of the scaffold, and allow for the production of high strength grafts with complex geometry for patient-specific needs.

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Recent advances in ceramics fabrication have employed a variety of synthetic (e.g. poly(lactide-co-glycolide), poly(methyl methacrylate), polycaprolactone) and natural (e.g. chitosan, alginate, starch, cellulose) binders and porogens to generate CaP scaffolds by methods such as foam casting, direct foaming, porogen burnout, freeze drying, slip casting, and gel casting [8,13–20]. While each of these methods has unique benefits, significant disadvantages include the use of toxic initiators in solvent-based gel casting, low ceramic density in direct foaming or foam casting, or the use of harsh chemicals and organic solvents in fabricating synthetic porogens [18]. After molding, many of these techniques involve high-temperature sintering of the green body, the ceramic structure prior to sintering, to harden and densify the finished scaffold. Although sintering naturally decreases total pore volume, porosity can be maintained in a controlled manner with the introduction of a sacrificial polymer in the green body that burns off during sintering [21].

Over the past decade, the incorporation of polymeric additives in ceramic formulations has received much attention due to the fine control that porogens afford over the final scaffold properties [22]. Since total porosity and strength of a ceramic scaffold is a function of both macroporosity, from the addition of porogens, as well as microporosity, from the spaces between adjacent ceramic grains, optimal ceramic fabrication should involve ceramic grain consolidation during green body formation [9,23]. This allows for greater contact between ceramic grains and improved grain fusion during sintering, resulting in high scaffold density in regions between macropores [24]. Since relative density is a critical determinant of mechanical performance in ceramics, grain consolidation during green body formation is imperative for the creation of high strength, load-bearing scaffolds [25,26].

In this study we investigated the potential of silk as a biocohesive agent and a porogen in the fabrication of hydroxyapatite ceramic scaffolds. The aim of this work was to leverage the adhesive nature of the silk protein [27], its exceptional thermal stability [28,29], and ability to form irreversible beta-sheet conformations in response to localized dehydration [30] to produce robust ceramic structures. We further assessed the potential of silk to generate hydroxyapatite scaffolds with a wide range of porosity and mechanical profiles.

2. Materials and methods

2.1. Materials

Silkworm cocoons from *Bombyx mori* were obtained from Tajima Shoji Co., LTD (Yokohama, Japan). Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), penicillin and streptomycin (Pen–Strep), Fungizone, nonessential amino acids (NEAA, consisting of 8.9 mg/l L-alanine, 13.21 mg/l L-asparagine, 13.3 mg/l L-aspartic acid, 14.7 mg/l L-glutamic acid, 7.5 mg/l glycine, 11.5 mg/l L-proline, 10.5 mg/l L-serine), and trypsin were from Gibco (Grand Island, NY, USA). All other chemicals of pharmaceutical grade were obtained from Sigma.

2.2. Preparation of B. mori silk fibroin

B. mori silk fibroin solution was prepared as previously described [31]. Briefly, five grams of silk cocoons were boiled in two liters of an aqueous solution of 0.02 M sodium carbonate for either 20 or 60 min, rinsed with deionized water, and dried. The dry silk fibers were dissolved in a 9.3 M lithium bromide solution (25% wt/v) at 60 °C for 4–6 h, and the resulting solution was dialyzed against deionized water using 3500 Da molecular weight cut off dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA) to remove the lithium bromide. The final concentration of the aqueous silk solution after dialysis was 6–8% wt/v, which was determined by massing the remaining silk solid after drying a known volume.

2.3. Preparation of concentrated silk solution, soluble silk powder, and silk macroporogens

Concentrated silk solution was prepared by dehydrating the 8% wt/v aqueous silk solution from 20-min boiled silk fibroin in Slide-a-Lyzer dialysis cassettes (MWCO 3500) (Thermo Fisher, Rockford, IL) by air-drying for 4–6 h with mixing by inversion of the cassette. The final concentration of the concentrated silk solution was 15% wt/v. The concentrated solution was stored at 4 °C until further use. Soluble silk powder was prepared by freezing the 8% wt/v aqueous silk solution from 60-min boiled silk fibroin for 24 h at −20 °C, and lyophilizing (Labconco, Kansas City, MO) at a pressure of 0.020 Torr for 24–48 h. After lyophilization, the resulting silk foams were ground and blended on high setting for two minutes in a conventional kitchen blender (Model KSB560, KitchenAid, Inc., St. Joseph, MI) with a cup size of approximately 1 L and stored at ambient conditions until further use. Silk macroporogens were prepared in the same manner as the soluble silk powder, except that, after lyophilization, the resulting silk foams were blended for only 30 s in a conventional kitchen blender to obtain larger silk particles. The particles were then placed in an open stage, closed-top sealed desiccator and solvent annealed by vapor from a separate 100% methanol source (approximately 200 mL) beneath the open stage for 24 h. The resulting insoluble silk macroporogens (SMPs) were separated according to size using stainless steel particle sieves with mesh sizes of 800 μm and 300 μm (Fisher Scientific, Pittsburg, PA) to generate a small particle fraction (less than 300 μm) and a large particle fraction (300 μm–800 μm). SMPs were stored at ambient conditions until further use.

2.4. Preparation of hydroxyapatite scaffolds

2.4.1. Silk solvent (SS) method

Silk solution (15% wt/v) was mixed with HA powder in HA/silk mass ratios of 99/1, 90/10, and 80/20. Additional deionized (DI) water (approximately 1 mL per gram of HA-silk material) was added to the mixture to obtain a moldable HA-silk paste. The HA-silk mixture was kneaded by hand into a homogenous paste and subsequently molded into silicone molds (Dragon Skin, Smooth-On, Inc., Easton, PA) of any shape or geometry. For materials evaluation, small cylinders ($ø = 10$ mm, $h = 10$ mm) were formed. Molded green bodies were incubated at 60 °C for 24 h to render the silk insoluble in aqueous environments by inducing beta sheet formation. After 24 h, the HA-silk green bodies were sintered in a Lindberg Blue-M Tube furnace (Thermo Scientific, Waltham, MA) at 1300 °C or 1400 °C for 3 h at maximum temperature with linear heating and cooling rates of 8 °C per minute. Sintered scaffolds were stored at ambient conditions until testing.

2.4.2. Silk powder (SP) method

Soluble silk powder was mixed with HA powder in HA/silk mass ratios of 99/1, 90/10, and 80/20. Additional DI water (approximately 1 mL per gram of HA-silk material) was added to the mixture to obtain a moldable HA-silk paste. The HA-silk mixture was kneaded by hand into a homogenous paste and molded into silicone molds ($ø = 10$ mm, $h = 10$ mm). Molded green bodies were incubated in a 60 °C oven for 24 h to induce silk stabilization to render the silk insoluble in aqueous environments by inducing beta sheet formation. After 24 h, the HA-silk green bodies were sintered in a Lindberg Blue-M Tube furnace at 1300 °C or 1400 °C for 3 h at maximum temperature with linear heating and cooling rates of 8 °C per minute. Sintered scaffolds were stored at ambient conditions until testing.

2.4.3. Silk freeze-drying (SFD) method

Silk solution (15% wt/v) was mixed with HA powder in HA/silk mass ratios of 99/1, 90/10, and 80/20. Additional DI water (approximately 1 mL per gram of HA-silk material) was added to the mixture to obtain a moldable HA-silk paste. The HA-silk mixture was kneaded by hand into a homogenous paste and molded into silicone molds ($ø = 10$ mm, $h = 10$ mm). The molded green bodies were frozen at −20 °C for 24 h and then lyophilized at a pressure of 0.020 Torr for 24–48 h to induce silk stabilization. After lyophilization, the HA-silk green bodies were sintered in a Lindberg Blue-M Tube furnace at 1300 °C or 1400 °C for 3 h at maximum temperature with linear heating and cooling rates of 8 °C per minute. Sintered scaffolds were stored at ambient conditions until testing.

2.4.4. Silk macroporogens (SMPs)

SMPs could be added to any of the three silk-based fabrication methods (SS, SP, SFD). A 60% HA/40% silk ratio using the Silk Solvent Method was chosen in which 50% of the silk material by mass was replaced with SMPs. The SMPs were first mixed with the 15% wt/v concentrated 20-min boiled aqueous silk solution. HA powder was then mixed into the silk-SMP mixture and additional DI water (approximately 1 mL per gram of HA-silk material) was added to the mixture to obtain a moldable HA-silk paste. The paste was kneaded by hand to achieve a homogenous paste and packed into silicone molds ($ø = 10$ mm, $h = 10$ mm). The green bodies were incubated in a 60 °C oven for 24 h to induce silk stabilization and then sintered in a Lindberg Blue-M Tube furnace at 1300 °C or 1400 °C for 3 h at maximum temperature with linear heating and cooling rates of 8 °C per minute. Sintered scaffolds were stored at ambient conditions until testing.

2.5. Characterization of ceramic scaffolds

2.5.1. X-ray diffraction (XRD)

Phase composition of the sintered scaffolds ($n = 2$) was analyzed using a Scintag PAD-X powder x-ray diffractometer (Scintag, Inc., Cupertino, CA). X-rays were generated using a sealed glass Cu-K_α x-ray source (wavelength 1.54 Å; Advanced Technical Products & Services, Inc., Douglassville, PA) operated at an accelerating voltage of 45 kV and a beam current of 40 mA, using 1 mm divergent and 2 mm