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Research paper

Effects of hydroxyapatite reinforcement on the architecture and mechanical properties of freeze-dried collagen scaffolds

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ARTICLE INFO

Article history:
Published online 25 September 2011

Keywords:
Collagen
Composite
Hydroxyapatite
Tissue engineering scaffold
Freeze-dried
Synthetic bone graft substitute

ABSTRACT

Freeze-dried collagen scaffolds reinforced with hydroxyapatite (HA) are of clinical interest for synthetic bone graft substitutes and tissue engineering scaffolds, but a systematic evaluation of the effects of the HA reinforcement weight fraction and morphology on the mechanical properties is lacking. Therefore, freeze-dried collagen scaffolds were reinforced with either HA whiskers or an equiaxed HA powder at 1:1, 1:2, or 1:4 collagen:HA by weight (which corresponded to approximately 28, 44, and 61 vol% HA, respectively) to investigate the effects of the HA reinforcement weight fraction and morphology on the architecture and compressive mechanical properties. All scaffolds exhibited a highly elongated linear pore structure containing 90%–96% porosity, which decreased with increased HA content, and a pore width of ~50 μm. HA reinforcement resulted in up to a ten-fold increase in compressive modulus at high reinforcement levels (~200 kPa at 1:4 collagen:HA by weight) compared to scaffolds with no reinforcement or low reinforcement levels (~20 kPa at 1:1 collagen:HA by weight). This effect could not be explained by the concomitant decrease in the scaffold porosity (from 95% to 90%) with HA reinforcement, which could only account for up to a two-fold increase in compressive modulus. At moderate reinforcement levels (1:2 collagen:HA by weight), HA whisker reinforced scaffolds exhibited a nearly four-fold greater modulus compared to the equiaxed HA powder, while there were no differences with the HA reinforcement morphology at high and low reinforcement levels. Therefore, the elongated morphology of HA whiskers enabled a reinforcing effect at a lower level of reinforcement compared to a conventional, equiaxed HA powder.

1. Introduction

Over 500,000 bone graft procedures are performed each year in the United States making bone the most transplanted tissue. Autografts are the "gold standard", but are limited in size, shape, and availability (Arrington et al., 1996; Greenwald et al., 2001). Moreover, autograft harvesting can result in donor site morbidity, especially as patient age increases (Arrington et al., 1996; Seiler and Johnson, 2000). Allograft tissue alleviates size and shape constraints, but is limited by donor tissue availability and an increased risk of immunogenic response, disease transmission, and infection

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doi:10.1016/j.jmbbm.2011.09.010

(Greenwald et al., 2001; Lord et al., 1988). Therefore, the limitations associated with autograft and allograft tissue have motivated extensive research and commercial product development for synthetic bone graft substitutes.

Synthetic bone graft substitutes available for clinical use are under continuous change, but currently include: calcium sulfate (e.g., Osteoset®, Wright Medical), porous hydroxyapatite (Pro-Osteon®, Biomet; FRIOS Algipore®, Dentsply; Bio-Oss®, Osteohealth), porous silicon substituted hydroxyapatite (Actifuse®, Baxter Healthcare), porous beta-tricalcium phosphate (ChronOS, Synthes; Vitoss®, Orthovita), calcium phosphate cements (Norian SRS®, Synthes; BoneSource®, Stryker; α-BSM®, ETEX), collagen sponges loaded with bone morphogenetic protein (InFuse®, Medtronic Somafor Danek; OP-1®, Olympus Biotech), and hydroxyapatite reinforced porous collagen (CopiOs®, Zimmer; Healos®, DePuy), among others (De Long et al., 2007; Friedlaender et al., 2006) and not including demineralized bone matrix. Each graft substitute offers advantageous properties, but also suffers from its own limitations. Calcium phosphates provide bioactivity, but are brittle, particularly in highly porous scaffolds which may also exhibit low strength. Collagen materials alone lack the stiffness and strength required to bear load prior to or during healing. An ideal or widely applicable synthetic bone graft substitute does not yet exist.

A logical approach for the design of a synthetic bone graft substitute is to mimic the structure and composition of natural bone tissue, potentially resulting in properties similar to graft tissue without the associated limitations and risks. The extracellular matrix (ECM) of bone tissue is a composite composed of type I collagen fibers reinforced with calcium-deficient hydroxyapatite (HA) crystals which are elongated and plate-like (Rho et al., 1998). Therefore, dense (Du et al., 2000; Itoh et al., 2002; Kikuchi et al., 2001; TenHuisen et al., 1995) and porous (Al-Munajjed et al., 2009; Gelinsky et al., 2008; Liu et al., 2003; Tampieri et al., 2003; Wahl et al., 2007; Wang et al., 1995; Yunoki et al., 2007) collagen–HA composites have been investigated for synthetic bone graft substitutes and bone tissue engineering scaffolds. Collagen-HA scaffolds are typically prepared by freeze drying to achieve high levels of interconnected porosity (~80%–99%) required for cell infiltration, nutrient transport, vascularization, and tissue regeneration (Al-Munajjed et al., 2009; Cunniffe et al., 2010; Harley et al., 2010; Jones et al., 2010; Lyons et al., 2010; Wahl et al., 2007; Yunoki et al., 2007). Moreover, the porosity, pore size, and pore morphology of freeze-dried collagen scaffolds can be tailored by controlling the freezing rate, freezing temperature, and collagen concentration (Harley et al., 2007, 2010; O'Brien et al., 2004, 2005; Schoof et al., 2001; Tierney et al., 2009; Wahl et al., 2007; Yunoki et al., 2010).

The mechanical properties of freeze-dried collagen–HA scaffolds are typically investigated in unconfined uniaxial compression but comparisons between studies are complicated by differences in the HA content, HA morphology, porosity, pore architecture, and fabrication methods (Al-Munajjed and O'Brien, 2009; Cunniffe et al., 2010; Gelinsky et al., 2008; Gleeson et al., 2010; Harley et al., 2010; Yunoki et al., 2007). The inherently thin struts within a freeze-dried collagen scaffold result in a low apparent compressive modulus typically reported within the range of 1–150 kPa. The maximum reported compressive modulus for a freeze-dried collagen–HA scaffold was ~280 kPa in an anisotropic scaffold containing highly aligned struts (Yunoki et al., 2007). A systematic evaluation of the effects of the HA reinforcement weight fraction and morphology on the mechanical properties of freeze-dried collagen scaffolds is lacking.

Therefore, the objective of this study was to investigate the effects of the HA reinforcement weight fraction and morphology on the architecture and compressive mechanical properties of freeze-dried collagen scaffolds. Scaffolds were reinforced with either HA whiskers or an equiaxed HA powder at 1:1, 1:2 and 1:4 collagen:HA by weight. The scaffold porosity and pore size were also measured to account for possible concomitant effects on the mechanical properties.

2. Materials and methods

2.1. *Collagen purification*

Soluble type I collagen was extracted from bovine dermis using acid extraction and salt fractionation (Trelstad, 1982; Huang-Lee and Nimni, 1993). Bovine dermal tissue was trimmed of hair and excess fat. Strips of tissue, weighing 500 g and measuring ~10 × 50 mm, were defatted in 1 L of 50/50 chloroform/methanol by volume for 8 h and washed for 2 h each in 100% methanol, 50% methanol in water by volume, and finally physiological phosphate buffered saline (PBS). Defatted dermal tissue strips were then placed in 1.5 L of 0.5 M acetic acid and blended for 5 min in a commercial blender (Model 909, Hamilton Beach/Procter-Silex, Inc., Washington, NC). Porcine pepsin (P7000, Sigma-Aldrich, Milwaukee, WI) was added at a concentration of 2 mg/mL to increase the fraction of extractable soluble collagen. Pepsin preferentially attacks the globular ends of the collagen proteins, breaking crosslinks that bind collagen molecules to each other. Pepsin can increase the extractable yield by 400% and the resulting collagen displays lower immunogenicity compared to collagen not treated with pepsin (Trelstad, 1982). The acid solution was adjusted to a pH of 2.5 with HCl for optimum pepsin activity and incubated at 4 °C for 24 h.

After acid extraction, the solution was centrifuged at 5000 g for 1 h to remove all non-soluble collagen fragments. The supernatant contained 5–10 mg/mL of soluble type I collagen. Other minor contaminants likely included residual elastin, type III collagen, and keratin. Type I collagen was separated by adding NaCl to a concentration of 2.5 M to induce precipitation. The precipitated collagen was collected by centrifugation at 7500 g for 1 h, re-dissolved in 0.1 M acetic acid, centrifuged again at 7500 g for 1 h, and the remaining precipitate was discarded. The resulting supernatant was nearly pure type I collagen as demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylimide gel and comparing the sample profile to a commercial protein standard (Fig. 1). The final collagen solution was freeze-dried and stored at −20 °C until further use.