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Hydroxyapatite reinforced collagen scaffolds with improved architecture and mechanical properties ☆

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ABSTRACT

Hydroxyapatite (HA) reinforced collagen scaffolds have shown promise for synthetic bone graft substitutes and tissue engineering scaffolds. Freeze-dried HA–collagen scaffolds are readily fabricated and have exhibited osteogenicity *in vivo*, but are limited by an inherent scaffold architecture that results in a relatively small pore size and weak mechanical properties. In order to overcome these limitations, HA–collagen scaffolds were prepared by compression molding HA reinforcements and paraffin microspheres within a suspension of concentrated collagen fibrils (~180 mg/mL), cross-linking the collagen matrix, and leaching the paraffin porogen. HA–collagen scaffolds exhibited an architecture with high porosity (85–90%), interconnected pores ~300–400 μm in size, and struts ~3–100 μm in thickness containing 0–80 vol% HA whisker or powder reinforcements. HA reinforcement enabled a compressive modulus of up to ~1 MPa, which was an order of magnitude greater than unreinforced collagen scaffolds. The compressive modulus was also at least one order of magnitude greater than comparable freeze-dried HA–collagen scaffolds and two orders of magnitude greater than absorbable collagen sponges used clinically. Moreover, scaffolds reinforced with up to 60 vol% HA exhibited fully recoverable elastic deformation upon loading to 50% compressive strain for at least 100,000 cycles. Thus, the scaffold mechanical properties were well-suited for surgical handling, fixation, and bearing osteogenic loads during bone regeneration. The scaffold architecture, permeability, and composition were shown to be conducive to the infiltration and differentiation of adipose-derive stromal cells *in vitro*. Acellular scaffolds were demonstrated to induce angiogenesis and osteogenesis after subcutaneous ectopic implantation by recruiting endogenous cell populations, suggesting that the scaffolds were osteoinductive.

1. Introduction

Bone grafts are commonly used to repair bone defects, such as those caused by traumatic injuries or the resection of osteosarcoma, and to promote implant fixation, such as in spinal fusion and dental implants [1–3]. Autograft is the current gold standard used in the majority of procedures but is limited by supply, graft size, and donor-site morbidity [1–5]. Allograft can be used to obviate the limitations of autograft, but is limited by the need for rigorous tissue screening, processing, and preservation methods to minimize the risk of immunogenicity or transmitting pathogens, and these procedures can compromise other aspects of graft function [1–3]. Therefore, a large number of synthetic bone graft substitutes have been investigated as alternatives and many are used clinically, including porous calcium phosphate ceramics and cements, collagenous sponges and putties, and various composites thereof [1–3,6].

The use of an absorbable collagen sponge to deliver osteoinductive recombinant human bone morphogenetic protein-2 (rhBMP-2) has comprised the most commercially successful synthetic bone graft substitute (InFuse®, Medtronic Somafor Danek) due to demonstrated potency in regenerating bone [6–8], although clinical efficacy has been clouded by controversy surrounding BMP dosing, complications, and off-label use [8,9]. Type I collagen is advantageous as a scaffold biomaterial due to partially mimicking the extracellular matrix (ECM) of bone for cell attachment, undergoing enzymatic degradation for cellular resorption, enabling cross-linking for tailored properties, and preparing highly porous scaffolds via freeze-drying [7,10–13]. However, collagen scaffolds

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are limited by weak mechanical properties [12,13], such that clinical use is restricted to spatially confined and mechanically shielded sites, e.g., within an interbody spinal fusion cage.

Hydroxyapatite (HA) reinforced collagen scaffolds have been investigated as a means to improve mechanical properties and provide bioactivity, while further mimicking the ECM of bone [14–25]. HA–collagen scaffolds have been shown to support the attachment and proliferation of osteoblasts and osteoblast-like cells [15,21–24]. HA–collagen scaffolds were also recently shown to exhibit greater or comparable new bone formation *in vivo* when implanted alone compared to when implanted as a cell carrier or BMP delivery vehicle in ectopic and orthotopic models [26–28] for reasons that are not yet well understood. Thus, HA–collagen scaffolds have shown promise as a synthetic bone graft substitute and tissue engineering scaffold.

HA–collagen scaffolds are most commonly prepared by freeze drying a suspension of collagen fibrils and HA particles [14–19,22–28], or precipitating HA within a freeze-dried collagen scaffold [16,20,21]. Highly porous (>90%) freeze-dried scaffolds are readily fabricated, but exhibit a relatively small pore size, typically <100 μm. A pore size >300 μm is generally thought to be most advantageous for cellular infiltration, vascularization, and bone in growth [29]. Freeze-drying also results in a scaffold architecture with inherently thin struts, typically ~1–3 μm in thickness, which readily buckle and therefore limit compressive stiffness and strength even with HA reinforcement. Compressive moduli for freeze-dried HA–collagen scaffolds under hydrated conditions have been reported up to ~200–300 kPa in highly oriented scaffolds [18] or using HA whisker reinforcements [25], but are more commonly ~1–20 kPa [20–23,25,26]. Thus, the mechanical properties of HA–collagen scaffolds have remained less than ideal for surgical handling, fixation (e.g., with a pin), and bearing osteogenic loads during healing.

Therefore, the objectives of this study were to (1) prepare HA–collagen scaffolds with improved architecture and mechanical properties to overcome the limitations of freeze-dried scaffolds, (2) demonstrate cell infiltration and bioactivity *in vitro*, and (3) demonstrate angiogenesis and osteogenesis *in vivo*. HA–collagen scaffolds were prepared by concentrating a suspension of collagen fibrils, compression molding a mixture of HA, collagen, and paraffin microspheres, cross-linking the collagen matrix, and leaching the paraffin porogen. Effects of the scaffold porosity, HA reinforcement morphology, and HA volume fraction on the scaffold permeability, scaffold mechanical properties, and *in vitro* cellular behavior were investigated. Finally, *in vivo* angiogenesis and osteogenesis were investigated after subcutaneous ectopic implantation of HA–collagen scaffolds in mice.

2. Materials and methods

2.1. Raw materials

Soluble type I bovine collagen was received at a concentration of 3.2 mg/mL in 0.01 M HCl (DM-1, Devro Medical PLC, Glasgow, UK). The collagen solution was adjusted to physiological pH (7.4) and ionic strength (0.05 M) by adding the appropriate amounts of 1 M NaOH (Sigma–Aldrich, St. Louis, MO) and 10× Dulbecco's phosphate buffered saline (PBS, Sigma–Aldrich), respectively. Aliquots from the collagen solution (20 mL) were placed in an incubator overnight at 40 °C and the resulting collagen gels, still at a collagen concentration of ~3.2 mg/mL, were disrupted using a tissue homogenizer (Polytron PT1200, Kinematica, Lucerne, Switzerland) for 30 s. Collagen fibril suspensions were then centrifuged at 6000*g* for 30 min (Sorvall RC-6 Plus, Thermo Scientific, Waltham, MA) to concentrate the collagen fibrils to ~180 mg/mL and combined into a stock suspension which was stored at 8 °C until further use.

Single crystal HA whiskers were precipitated under hydrothermal conditions using the chelate decomposition method and conditions previously reported to result in a mean (± standard deviation) length and aspect ratio of 18 (8.9) μm and 7.9 (3.4), respectively [30,31]. An equiaxed HA powder was obtained commercially (Product #21221, Fluka Chemical Co., Buchs, Switzerland). The as-received powder was ground using a mortar and pestle to minimize agglomerates, and stored at 90 °C to remove residual moisture. The mean (± standard deviation) particle diameter of this powder was previously reported as 1.3 (0.4) μm [31], which was comparable to the width of the HA whiskers.

A paraffin microsphere porogen was produced using methods adapted from the literature [32]. Briefly, 40 g of a low melting point paraffin (Paraplast X-TRA®, Sigma–Aldrich) was heated to 90 °C, added to 400 mL of a solution containing 25 wt% sucrose (American Sugar Refining, West Palm Beach, FL) in deionized (DI) water at 80 °C, and rapidly stirred for 1 h to form a visually homogeneous emulsion. The emulsion was subsequently poured into 2 L of ice water to solidify the paraffin microspheres which were collected, washed five times with DI water to remove residual sucrose, and dried in a vacuum desiccator for 24 h at room temperature. Paraffin microspheres were fractionated to a size range of 300–425 μm using a shaker sieve (Ro-Tap® RX-29, W.S. Tyler, Mentor, OH) and stored in a sealed container at −20 °C. The sieved paraffin microspheres exhibited a mean diameter (± standard deviation) of 370 (56) μm as measured by optical microscopy by sampling over 250 microspheres collected from multiple batches.

2.2. Scaffold fabrication

HA reinforcements were added to the concentrated collagen fibril suspension in amounts calculated to result in 0, 20, 40, 60, and 80 vol% HA within collagen after removal of water, and stirred by hand for ~5 min until uniformly distributed. Paraffin microspheres were then folded into the HA–collagen suspension by hand using a spatula in amounts designed to produce scaffolds with 85% or 90% porosity by volume. The HA–collagen-paraffin suspension was loaded into a 6 mm diameter pellet die and compression molded at 1 MPa for 1 min. As-molded scaffolds were 3–5 mm in height depending on the amount of suspension added to the die and were subsequently dried at 37 °C for 24–48 h. Paraffin microspheres were leached by soaking scaffolds in successive solutions of 2× 100% hexane, 50/50 hexane/ethanol, and 4× 100% ethanol for at least 6 h each. The volume of each leaching solution was at least 20 times greater than the volume of the immersed scaffolds.

After compression molding and porogen leaching, the collagen matrix was crosslinked for 12 h under gentle agitation in a solution containing 20 mM *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, Sigma–Aldrich) and 8 mM *N*-hydroxysuccimide (NHS, Sigma–Aldrich) in 80/20 ethanol/DI water with pH adjusted to 7.4 by 0.1 M HCl. The concentration of EDC was adjusted such that the EDC:NHS:collagen binding site ratio was 5:2:1, which was previously shown to maximize collagen crosslinking [33]. Scaffolds were subsequently washed three times in 100% ethanol to remove unreacted EDC and stored in ethanol at 4 °C until further use.

2.3. Scaffold architecture and microstructure

The percent porosity (P) of as-prepared scaffolds fabricated with 85% or 90% target porosity and reinforced with 0, 20, 40, 60, or 80 vol% HA whiskers or powder (n = 3/group) was calculated as,

$$P = \left(1 - \frac{\rho_{scaffold}}{\rho_{material}}\right) \cdot 100 \quad (1)$$