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Porous collagen-apatite nanocomposite foams as bone regeneration scaffolds

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ABSTRACT

We have created a porous bioresorbable nanocomposite bone scaffold that chemically, structurally and mechanically matched natural bone so that it could be recognized and remodeled by natural bone. Containing collagen fibers and synthetic apatite nanocrystals, our scaffold has high strength for supporting the surrounding tissue. The foam-like scaffold has a similar microstructure as trabecular bone, with nanometer-sized and micron-sized pores. The apatitic phase of the scaffold exhibited similar chemical composition, crystalline phase and grain size as the trabecular bone apatite. The nanocomposite scaffold demonstrated excellent bioactivity for promoting cell attachment and proliferation. It was osteoconductive and successfully healed a non-union fracture in rat femur as well as a critical-sized defect in pig tibia.

1. Introduction

Various artificial materials, such as metals, polymers and ceramics, have been developed as bone substitutes to overcome the problems associated with natural bone grafts in reconstructive surgery. Bioactive synthetic bone implants may aid in regaining shape and function of the defective bone by serving as a scaffold for bone growth, thus contributing to the healing process [1–3]. However, many bone implant materials are not sufficiently osteoconductive and osteoinductive to allow bone regeneration from surrounding bone cells and bone marrow stem cells. We have created a porous bioresorbable nanocomposite bone scaffold that chemically, structurally and mechanically mimics natural bone. Containing collagen fibers extracted from rat skin and synthetic apatite nanocrystals, the foam-like scaffold has a similar microstructure as trabecular bone, with nanopores as well as macropores. The apatitic phase of the scaffold exhibited similar chemical composition, crystalline phase and crystallite size as trabecular bone's apatite. The nanocomposite scaffold demonstrated excellent bioactivity for promoting cell attachment and proliferation. It was osteoconductive and successfully healed a non-union fracture in the femur of Wistar rats. It was also able to heal a critical-sized defect in the tibia of Yorkshire–Landrace pigs. Evidence of mineralization and bone matrix formation even in scaffolds that were ectopically implanted suggested that these novel materials were osteoinductive. This paper describes the synthesis and processing of the nanocomposite foam, and the resulting scaffold's performance *in vitro* and *in vivo*.

On a dry weight basis, 30–35% of bone is composed of organic material, ~95% of which is Type 1 collagen (T1C) fibers. The remaining organic substances are chondroitin sulfate, keratin sulfate, and phospholipids. 65–70% of bone is composed of inorganic substances, most of which is hydroxyapatite (HAP) [4]. Natural bone apatite crystals are 20–40 nm along the c-axis, and one molecule of T1C is ~300 nm in length [5]. The c-axes of the apatite are regularly aligned along the T1C fibers [6]. In general, osteogenesis starts by the production and release of T1C fibrils from the osteoblasts [7]. The fibrous T1C matrices are combined to form bundles, followed by calcification, releasing Ca^{2+} and $HPO4^{2-}$ ions and/or apatite nanocrystals.

Collagen has a low immunogenicity, is bioabsorbable, and is a natural structural protein that cells can attach to and interact with. Collagen sponges and foams have been used as hemostatic agents [8], scaffolds for tissue repair [9], and supports for cell growth [10]. Several patents [11–15] have also described biopolymer foams or freeze-dried sponges for tissue repair and reconstruction that contain collagen and calcium phosphate, hydroxyapatite or demineralized bone. Recently developed three-dimensional scaffolds have been designed to mimic one or more of the bone-forming characteristics of autografts, such as chemical composition [16,17] or microstructure [18,19] in order to facilitate vascularization in the material, and provide a suitable environment for bone formation [16–19]. To date, however, no collagen sponge, biopolymer foam or implant scaffold has been developed with the same or similar composition and structure as that of natural bone. We have created a method for extracting T1C from animal skin using weak acids [20]. By not employing any harsh solvents or

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enzymes in this process, we were able to preserve the original structure of T1C.

On the other hand, synthetic HAP has been used in many orthopedic [7,21] and dental [22] implant materials as it is bioactive and displays osteoconductive and osteoinductive properties [23]. However, conventional synthetic HAP exhibits poor mechanical properties due to its lack of phase purity and homogeneity. Its crystallite size also tends to be much larger than that of natural bone apatite. We have developed a method to synthesize HAP using nanostructure processing techniques that allowed us to control the stoichiometry, crystalline phase, crystallite size, and particle morphology of the final apatite product [24]. This also enabled us to increase the chemical and thermal stability of the apatites. Our nanostructured HAP monoliths also demonstrated superior compressive (900 MPa) and bending (200 MPa) strengths, and fracture toughness (1.3 MPa $m^{1/2}$) [25,26]. The high volume fraction of grain boundaries associated with nanocrystalline HAP also led to better cell adhesion, proliferation and mineralization compared to conventional coarse-grained HAP [25,26]. We have also successfully derived carbonated apatite (CAP) by nanostructure processing [24]. In this study, we have employed a mixture of CAP and HAP nanocrystals as the inorganic component in the scaffold processing, so as to best match the natural bone apatite.

To maximize the surface area for cell attachment and proliferation, we have processed the scaffold into a highly porous three-dimensional structure using freeze drying. The freeze drying process [27] created porosity in the scaffold to enable cell attachment and motility, as well as to allow blood vessels to infiltrate the scaffold and for fluid transport throughout the scaffold. We have also chemically crosslinked [28] the scaffolds with a solution containing 14 mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 5.5 mM of N-hydroxysuccinimide (NHS) to increase the compressive modulus of collagen, and improve its resistance against degradation [28].

2. Materials and methods

2.1. Extraction and characterization of T1C

T1C was extracted from freshly obtained, shaven skins of animals (e.g. rats and rabbits) that were sacrificed after *in vivo* studies. The sample pieces (1–3 mm) were soaked up to 2 weeks in 95% ethanol for disinfection and dehydration. They were then stirred for 24 h in 0.1% acetic acid solution. The resulting solution was discarded, and the remaining skin was stirred for 4–14 days in fresh 0.1% acetic acid solution. The product was then centrifuged for 1–3 h at 4000 rpm to separate the transparent, jelly-like suspension from the remaining solid. The supernatant was then skimmed to remove the fats, and transferred into vessels for freeze drying to obtain dry, white collagen foams. Remaining impurities were separated from the foam surface, and removed from the purified collagen. Photoacoustic Fourier-transform infrared (PA-FTIR) spectra of the extracted T1C and commercial Type 1 bovine tendon collagen (Sigma) were recorded on a Digilab FTS 7000 FTIR spectrometer equipped with a MTEC-300 photoacoustic detector.

2.2. Synthesis and characterization of apatites

HAP nanocrystals were synthesized from a solution of 0.167 M of $Ca(NO_3)_2 \cdot 4H_2O$ (Sigma) and 0.1 M of $(NH_4)_2HPO_4$ (Sigma). The solution was mixed with Tween 80 surfactant at a weight ratio of 150:2, and aged at room temperature for 100 h. The precipitate was centrifuged, oven dried at 120 °C, ground into powder, and calcined at 550 °C for 5 h. CAP nanocrystals were synthesized from a solution of 0.167 M of $Ca(NO_3)_2 \cdot 4H_2O$, 0.1 M of $(NH_4)_2HPO_4$ and 0.1 M of $(NH_4)HCO_3$ (Sigma). The solution was mixed with Tween 80 surfactant at a weight ratio of 150:2, and aged at room temperature for 100 h. The precipitate was centrifuged, oven dried at 120 °C, and ground into powder. PA-FTIR spectra of the HAP and CAP nanocrystals were recorded on a Digilab FTS 7000 FTIR spectrometer equipped with a MTEC-300 photoacoustic detector.

2.3. Preparation and characterization of collagen-apatite nanocomposite scaffolds

The nanocomposite scaffold was prepared by mixing T1C (4 g) with 0.05 M of H_3PO_4 (100 ml) at 14,000 rpm for 3 h in an ice water bath. Powders of nanocrystalline apatite (CAP, HAP or CAP-HAP mixture) were added to this slurry, and mixed for 3 h. The mixture was then centrifuged for 30 min at 0–1000 rpm to remove excess liquids. The remaining slurry was rehomogenized by vortexing, placed in a −20 °C freezer, and cooled at −0.36 to −0.66 J/s. The frozen samples were then placed in the freeze dryer (Virtis), and allowed to sublime for 48 h to obtain the collagen-apatite foam-like nanocomposite. The nanocomposite samples were further crosslinked by EDC/NHS. This treatment [29] involved sample immersion in a carbodiimide solution containing 14 mM of EDC and 5.5 mM of NHS (Sigma) at room temperature for 2 h. The sample was then removed from the solution, incubated in 0.1 M of phosphate buffered saline (PBS) for 2 h, and washed at least five times in sterile deionized H_2O to eliminate any excess carbodiimide.

X-ray diffraction (XRD) patterns of the collagen-apatite nanocomposites were collected with a PANalytical X-ray diffractometer (Cu Kα) at a scan speed of 4°/min. Scanning electron microscopy (SEM) studies were performed on a JEOL JSM-5310 scanning electron microscope operating at an accelerating voltage of 20 kV. The samples were gold-coated with a SPI-Module sputter coater. Elemental analysis was performed with the Oxford Link ISIS EDX system. Nitrogen adsorption was performed with Micromeritics ASAP 2020 for pore volume and pore size distribution in the range of 1–300 nm. Mercury porosimetry was conducted with Thermofinnigan Pascal 140 and Pascal 240 porosimeters for pore volume and pore size distribution in the range of 1–700 μm. Unconfined compression tests were performed on samples of ~6 mm in diameter and ~4 mm in height in simulated body fluid (SBF: 142 mM of Na^+, 5 mM of K^+, 2.5 mM of Ca^{2+}, 1 mM of Mg^{2+}, 1 mM of SO_4^{2-}, 1 mM of HPO_4^{2-}, 136 mM of Cl^- and 14 mM of HCO_3^-) at 37 °C, using an Instron (Model 5848P8600) mechanical tester. The samples were first stored in PBS, and the dimensions of each hydrated sample were measured using digital Vernier calipers right before mechanical testing. A 2-kN load cell was used to compress the samples at a rate of 2 mm/min up to a maximum of −50% strain. Stress was computed as the compressive load normalized to the initial unstrained disk area. Each data point represented the averaged results of 8 samples. For bone implants, we were interested in the compressive properties of the initial linear region (between 0% and −10% strain) before the pores began to collapse. Compressive stiffness of the porous samples was obtained from a best-fit line through this region of the stress-strain curve. For comparison, ovine trabecular bone samples of the same dimensions were

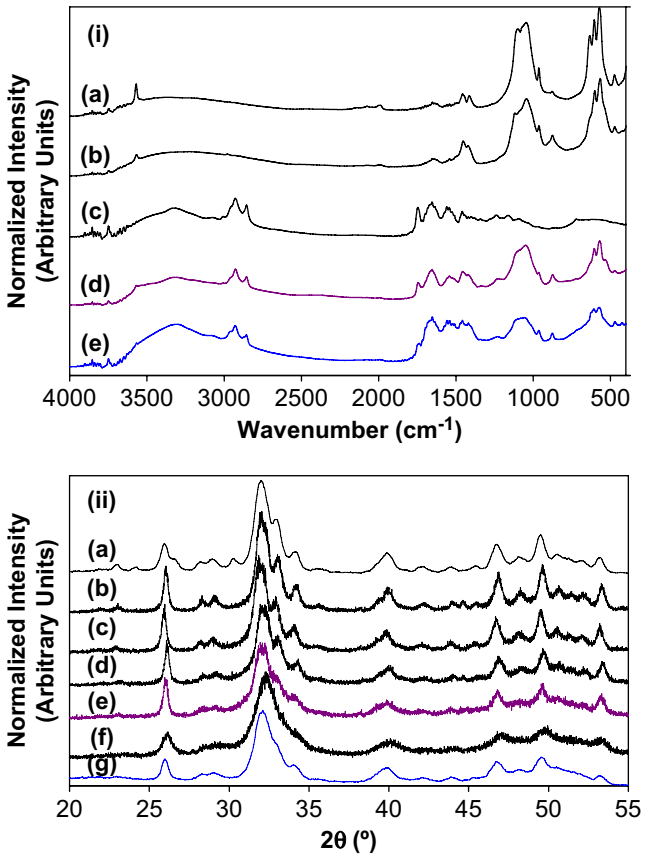

Fig. 1. Chemical and crystalline structure of collagen-apatite nanocomposite scaffold and trabecular bone. (i) PA-FTIR spectra of (a) HAP nanocrystals, (b) CAP nanocrystals, (c) T1C extracted from rat, (d) nanocomposite scaffold of 32.5 wt% of T1C and 67.5 wt% of nanocrystalline apatite (CAP:HAP weight ratio = 4:1), and (e) trabecular bone. (ii) XRD patterns of nanocomposite scaffold with 32.5 wt% of T1C and 67.5 wt% of nanocrystalline apatite, where CAP:HAP weight ratio = (a) 0:5, (b) 1:4, (c) 2:3, (d) 3:2, (e) 4:1 and (f) 5:0, and (g) trabecular bone.