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The impact of critical point drying with liquid carbon dioxide on collagen–hydroxyapatite composite scaffolds

Eleftherios Sachlos [a,*], Denys A. Wahl [a], James T. Triffitt [b], Jan T. Czernuszka [a]

[a] Department of Materials, University of Oxford, Parks Road, Oxford OX1 3PH, UK
[b] Botnar Research Centre, Institute of Musculoskeletal Sciences, University of Oxford, Oxford OX3 7LD, UK

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Abstract

Collagen–hydroxyapatite composites for bone tissue engineering are usually made by freezing an aqueous dispersion of these components and then freeze-drying. This method creates a foamed matrix which may not be optimum for growing cell colonies larger than a few hundred micrometres due to the limited diffusion of nutrients and oxygen, and the limited removal of waste metabolites. Incorporating a network of microchannels in the interior of the scaffold which may permit the flow of nutrient-rich media has been proposed as a method to overcome these diffusion constraints. A novel three-dimensional printing and critical point drying technique previously used to make collagen scaffolds has been modified to create collagen–hydroxyapatite scaffolds. This study investigates the properties of collagen and collagen–hydroxyapatite scaffolds and whether subjecting collagen and hydroxyapatite to critical point drying with liquid carbon dioxide results in any changes to the individual components. Specifically, the hydroxyapatite component was characterized before and after processing using wavelength-dispersive X-ray spectroscopy, X-ray diffraction and infrared spectroscopy. Critical point drying did not induce elemental, crystallographic or molecular changes in the hydroxyapatite. The quaternary structure of collagen was characterized using transmission electron microscopy and the quarter-staggered array characteristic of native collagen remained after processing. Microstructural characterization of the composites using scanning electron microscopy showed the hydroxyapatite particles were mechanically interlocked in the collagen matrix. The in vitro biological response of MG63 osteogenic cells to the composite scaffolds were characterized using the Alamar Blue™, PicoGreen™, alkaline phosphate and Live/Dead™ assays, and revealed that the critical point dried scaffolds were non-cytotoxic.

Keywords: Bone tissue engineering; Critical point drying; Supercritical carbon dioxide; Rapid prototyping; Collagen

1. Introduction

Bone is a composite material made of an organic matrix, which is predominantly (90%) collagen type I and non-collagenous proteins, reinforced with hydroxyapatite mineral. For bone tissue engineering, hydroxyapatite (HA) has been incorporated with collagen to create composite scaffolds that chemically resemble the natural extracellular matrix components of bone [1–6]. HA is osteoconductive [7] and acts as a reservoir of calcium and phosphate ions whilst collagen can provide the natural binding sites for cell attachment [8–10]. Collagen–HA scaffolds have traditionally been fabricated into foam structures using the freeze-drying method [4,5]. This method involves freezing an aqueous dispersion of collagen and HA, which leads to the aggregation of these components in the interstitial spaces of the ice crystal network, generating a porous structure. The ice is then removed by sublimation, leaving a foamed collagen matrix embedded with HA particles. However, cell penetration into foam scaffolds has been reported to be only within a few hundred micrometres from the scaffold's periphery

* Corresponding author. Present address: School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138, USA. Tel.: +1 617 496 3034.
E-mail address: sachlos@seas.harvard.edu (E. Sachlos).

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and the lack of diffusion of metabolites and oxygen in and waste products out has been proposed as a limitation in scaffold design [11–14].

Creating internal microchannels which permit the flow of nutrient-rich medium throughout the scaffold interior is one strategy proposed for maintaining cell survival deep within the scaffold. Particularly suited to this approach is the use of rapid prototyping for scaffold fabrication, and several methodologies have been developed for making scaffolds from bioceramics [15,16] and synthetic [17–23] or natural polymers [24–26].

Collagen-based scaffolds with an internal microchannel system have been made by interfacing an indirect three-dimensional (3-D) printing rapid prototyping technique and critical point drying method [24]. The cell-binding properties of collagen make it a suitable material for creating scaffolds. Collagen contains the RGD [8] and DGEA [9] sequences, which mediate cell binding via integrin receptors [10], a quality that is absent in bioceramics and synthetic polymers such as polylactic acid, polyglycolic acid and polycaprolactone. Briefly, the process begins by designing a mould for the scaffold using computer-aided design (CAD) software. The mould can possess a branching network of shafts which will define the microchannels in the scaffold. Voxel data of anatomical structures acquired with computerized tomography (CT) or magnetic resonance imaging can also be used at this point to design a mould for creating a patient-tailored scaffold. The mould is then manufactured using a 3-D phase-change inkjet into which an aqueous-based dispersion of collagen with or without HA is cast and frozen. Ice crystals form which aggregate the collagen or collagen–HA in the interstitial space and create a porous structure within the mould. The scaffold is relieved by dissolving the mould and ice crystals in ethanol and then critical point drying. Critical point drying involves exchanging the ethanol with liquid carbon dioxide before heating to above the critical point and venting the supercritical CO_2. It is noteworthy that at ambient temperature, liquid CO_2 exists at pressures of >5 MPa.

This process combines the advantageous biological properties of collagen and HA with the design capabilities of 3-D printing to create microchannel vasculature that may be used to overcome the diffusion limitations of current foam scaffolds. The critical point drying method was employed to make collagen–HA scaffolds. The objectives of this investigation were to (i) characterize the impact of subjecting collagen and HA to the critical point drying process; (ii) evaluate the influence of incorporating HA particles on the mechanical properties of the scaffold; and (iii) assess the in vitro biological responses of osteogenic cells to these scaffolds.

2. Experimental

2.1. Scaffold fabrication

The general scaffold fabrication method has been reported previously [24]. This method was modified for composite scaffold fabrication. Briefly, a 1% (w/v) collagen dispersion was made by adding 1 g of bovine collagen type I from Achilles tendon (Sigma–Aldrich) in 100 ml of distilled water adjusted to pH 3.2 by the dropwise addition of analytical grade acetic acid (Sigma–Aldrich). Taking into consideration the weight of the collagen present in the dispersion, the respective weight of HA particles (Captal® 60-1, Plasma Biotal), of approximately 40 μm diameter measured by scanning electron microscopy (SEM), was manually mixed with the collagen dispersion, cast into poly(tetrafluoroethylene) (PTFE) moulds, frozen at −30 °C and dehydrated in ethanol for 3 h before critical point drying (CPD) with liquid carbon dioxide for 3 h. The collagen–HA ratio was varied by weight from 100:0 to 10:90.

2.2. Scaffold microstructure characterization by SEM

Composite scaffolds were cryofractured by immersing in liquid nitrogen for 30 s and sectioning with a razor blade to reveal the interior before dehydration in ethanol and CPD. Once critical point dried, samples were gold-sputter coated (E5400, Biorad, Polaron Division) and viewed in the secondary electron mode with a field emission gun scanning electron microscope (JSM-840F, JEOL) operated at an accelerating voltage of 2.5 kV.

2.3. Elemental analysis

HA particles were also cold pressed into discs using a 10 mm stainless steel die (Evacuable Pellet Die, Specac Inc.) at a compaction force of 100 kN. Some discs were processed by subjecting to 3 h of dehydration in ethanol and 3 h of CPD. The HA discs were then carbon coated and analysed with an electron probe microanalyser (JXA-8800, JEOL) possessing four wavelength-dispersive X-ray (WDX) spectrometers. The spectrometers were calibrated using a wollastonite standard. The microscope was operated at an accelerating voltage of 10 kV.

2.4. Phase identification

HA particles were secured on glass slides coated with an adhesive and analysed with an X-ray diffractometer (PW1710, Philips Co.) operated at 35 kV and a current of 50 mA using Cu Kα radiation with a wavelength of 0.15406 nm. Data were acquired between 15° and 60° (2θ) with a step size of 0.02° and time per step of 1 s. The HA particles were analysed as received (control) and after being subjected to a 3 h ethanol bath and 3 h CPD protocol.

2.5. Infrared spectroscopy

HA particles, as received from the manufacturer or processed, were diluted with ground KBr powder and then pressed into a disc using a 10 mm diameter stainless steel

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