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Induction and quantification of collagen fiber alignment in a three-dimensional hydroxyapatite–collagen composite scaffold

Richard F. Banglmaier [a,*], Edward A. Sander [b], Pamela J. VandeVord [a,1]

[a] Biomedical Engineering Department, Wayne State University, Detroit, MI 48202, USA
[b] Department of Biomedical Engineering, University of Iowa, Iowa City, IA 52242, USA

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ABSTRACT

Hydroxyapatite–collagen composite scaffolds are designed to serve as a regenerative load bearing replacement that mimics bone. However, the material properties of these scaffolds are at least an order of magnitude less than that of bone and subject to fail under physiological loading conditions. These scaffolds compositionally resemble bone but they do not possess important structural attributes such as an ordered arrangement of collagen fibers, which is a correlate to the mechanical properties in bone. Furthermore, it is unclear how much ordering of structure is satisfactory to mimic bone. Therefore, quantitative methods are needed to characterize collagen fiber alignment in these scaffolds for better correlation between the scaffold structure and the mechanical properties. A combination of extrusion and compaction was used to induce collagen fiber alignment in composite scaffolds. Collagen fiber alignment, due to extrusion and compaction, was quantified from polarized light microscopy images with a Fourier transform image processing algorithm. The Fourier transform method was capable of resolving the degree of collagen alignment from polarized light images. Anisotropy indices of the image planes ranged from 0.08 to 0.45. Increases in the degree of fiber alignment induced solely by extrusion (0.08–0.25) or compaction (0.25–0.44) were not as great as those by the combination of extrusion and compaction (0.35–0.45). Additional measures of randomness and fiber direction corroborate these anisotropy findings. This increased degree of collagen fiber alignment was induced in a preferred direction that is consistent with the extrusion direction and parallel with the compacted plane.

1. Introduction

Hydroxyapatite–collagen (HAp/C) composites are biocompatible materials that possess the mineral and organic constituents of bone. The mineral phase formation, via precipitation, is well characterized in the absence [1–11] and presence [12–17] of collagen. The mechanical properties of mineralized collagen composites, however, are often one or more orders of magnitude less than bone [18–23] even though these scaffolds are compositionally similar to bone. One morphological characteristic of bone that is absent in these HAp/C composites is an ordered arrangement of the collagen fibers. Since collagen alignment and mechanical properties are correlated in bone [24–30], an ordered arrangement of collagen within the HAp/C composite should improve scaffold mechanical performance. Thus, structural organization, in addition to composition, is an important component for producing a functional load-bearing scaffold for bone repair.

It should be possible to produce collagen fiber alignment in HAp/C composites by adopting methods used to produce alignment in fiber reinforced polymer and cementitious composites. In these methods, extrusion [31,32] and planar fiber flow due to compression [33,34] are used to induce fiber alignment. Both modes produce fiber alignment that improves the composite's mechanical properties in the direction of fiber alignment [31,33]. Thus, an important structural property to assess in a HAp/C composite is the extent of collagen fiber alignment.

A number of methods have been devised to measure collagen fiber alignment, including methods that utilize polarized light microscopy (PLM) [35–38] and digital image processing algorithms [39–42]. Collagen is birefringent. Its optical axis is aligned with the longitudinal axis of the molecule. Under polarized light, collagen appears bright or extinct depending on its alignment with respect to the polarizers. When viewed with plane polarized light, the transmitted light traveling through collagen appears brightest

* Corresponding author at: 2700 Ellwood Ave., Berkley, MI 48072, USA. Tel.: +1 248 302 0789.
E-mail address: rbanglmaier@gmail.com (R.F. Banglmaier).
[1] Present address: Department of Biomedical Engineering & Mechanics, 447 Kelly Hall, Virginia Tech, Blacksburg, VA 24061, USA.

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when its optical axis is aligned 45° to the polarizing direction. The transmitted light diminishes in intensity as its axis is rotated to align either parallel or perpendicular to the polarizing direction at 0° or 90°. In circularly polarized light, collagen is extinct when aligned perpendicular to the plane of polarization. Based on this behavior, the amount of collagen fiber alignment in a sample can be estimated by taking the ratio of bright and dark field light intensities transmitted through the sample section [35,36,38].

Other quantitative measurements can be made with digital image processing algorithms such as mean intercept length (MIL) [43–45], line fraction deviation (LFD) [46,47], and Fourier transform methods (FTM) [39–42]. MIL and LFD are rooted in stereological techniques and are most commonly employed to quantify the orientation of trabecular struts in cancellous bone [43–47]. FTM are a relatively new technique with widespread application in biomedical research, including quantification of cell, extracellular matrix, and scaffold alignment [39–42]. In a comparison of these three techniques on simulated fiber networks of known orientation and alignment, Sander and Barocas [42] concluded that the FTM were the most accurate technique for recovering the fiber orientation distribution and predicting the anisotropy (or degree of fiber alignment) and the principle direction of fiber orientation. Together, PLM and FTM are complimentary techniques for measuring collagen fiber alignment, where the former enhances the observed collagen structure in an image, and the latter extracts quantitative structural information from an image.

The goal of this study was to assess the suitability of extrusion and compaction fabrication methods for producing HAp/C composite scaffolds with enhanced collagen fiber alignment. PLM and FTM were combined to assess that fiber alignment in scaffold histological sections. This quantification technique is the first such application to fabricated hydroxyapatite–collagen scaffolds. Assessment of the fiber alignment is necessary for the structural properties to be correlated with the previously reported mechanical properties [48].

2. Materials and methods

Mineralized collagen scaffolds were fabricated by coprecipitating calcium and phosphate ions in a collagen matrix. The starting calcium and phosphate concentrations and pH where chosen based on the stoichiometric calcium to phosphorus molar ratio of HAp (Ca:P = 1.67) and physiologic pH, respectively. Scaffolds were produced by a novel extrusion and compaction method designed to produce varying degrees of collagen alignment. Alignment was measured by a combination of PLM and FTM, as described below.

2.1. Scaffold mineralization

Mineralization precursor solutions were prepared with separate calcium/collagen and phosphate precursor solutions [49]. Collagen was extracted from rat tail tendons [50], dried, and dissolved in 0.01 M HCl at a collagen concentration of 1 mg/mL. Calcium and collagen were combined by adding 72 mL of 0.4 M $CaCl_2$ to 400 mL of the dissolved collagen. Potassium phosphate solution was prepared by adding 1.6 M KH_2PO_4 stock solution to 2.1 M K_2HPO_4 stock solution at a 20:80 volume ratio to achieve a pH of 7.4. Separately, a neutralization buffer was prepared by combining 107 mL of 0.5 M tris(hydroxymethyl)aminomethane (pH 7.4 with HCl) and 73 mL of 2 M NaCl. The potassium phosphate solution (8.6 mL) was added to 108 mL of neutralization buffer and 211 mL of ddH_2O. The mineralization reactions were initiated by combining 328 mL of the phosphate neutralization buffer to 427 mL of the calcium/collagen solution, with a starting pH of 7.4, which decreased to 6.8.

2.2. HAp/C specimen preparation

The calcium/collagen and phosphate precursor solutions were combined in a central reaction vessel and maintained at 37 °C in a water bath. Mineralized collagen was recovered by vacuum filtration after the solutions were allowed to react for 17 h. Wet slurries of HAp/C were placed in the barrel of a custom made screw extruder and extruded into a 5 × 5 × 30 mm mold (Fig. 1). Specimens were extruded to produce one of three *a priori* expected levels of collagen alignment. The three alignment levels were high longitudinal alignment (HLA), low longitudinal alignment (LLA), and random alignment (RA). The alignment levels corresponded to the type of breaker plate, or lack of breaker plate, placed in the path of the extrudate between the extruder barrel and mold. Breaker plates are screens that increase hydrodynamic shear in the extrudate flow. Shear induces fibers to align in the direction of flow [31,32]. The HLA and LLA fabrication methods utilized breaker plates with different sized hole dimensions (HLA holes < LLA holes). The RA method did not use a breaker plate. Five specimens ($n = 5$) were produced for each of the three fabrication methods, for a total number of fifteen specimens. All specimens were compacted under a static pressure of 2.45 MPa for 17 h to produce molded beams that measured 5 mm × 5 mm × 30 mm. After molding and compaction, the beams were removed from the mold and placed in a −80 °C freezer for storage until they were dried by lyophilization.

Sections of the dried specimens were then obtained and prepared for PLM. Three sections were cut from each specimen to obtain PLM sections in each of the three planes: the extrusion plane (E-plane), compaction plane (C-plane), and transverse plane (T-plane) (Fig. 2). To orient the reader: the direction of extrusion coincided with the *x*-direction, i.e., the longitudinal direction of the beam, and the direction of compaction coincided with the *z*-direction. Fiber alignment due to extrusion could be viewed in both the *x*–*y* (E-plane) and *z*–*x* (C-plane) planes. Likewise, fiber alignment due to compaction could be viewed in both the *y*–*z* (T-plane) and *z*–*x* planes (C-plane). As a result, fiber alignment due to extrusion was measured from sections in the E-plane and fiber alignment due to compaction was measured from sections in the T-plane. The combined effect of extrusion and compaction on fiber alignment was measured from sections in the C-plane.

Each of the three specimen sections were embedded in Technovit embedding media (EXAKT Advanced Technologies GmbH, Norderstedt, Germany). Prior to embedding, the sections were fixed in 10% neutral buffered formalin (NBF) for 48 h, washed, demineralized in 10% EDTA for 24 h, washed, dehydrated in successive alcohol concentrations (75%, 95%, 100%, 100%, 100%) for 1 h each, infiltrated in a 50:50 solution of Technovit and alcohol for 8 h, and infiltrated in 100% Technovit for 16 h. Cut sections were then placed in embedding molds, immersed in Technovit media, and cured under a blue light for 24 h. Embedded sections were mounted to slides and then ground and polished to a thickness of 200 μm.

2.3. Polarized light microscopy and FTM image analysis

Specimens were imaged with a Nikon Optiphot-2 microscope (Nikon, Tokyo, Japan) and plane polarized light at a magnification of 100×. A polarizer and an analyzer (Nikon Optiphot-2, Tokyo, Japan) were positioned above and below the sample stage, respectively, with their polarizing directions crossed at 90° to each other (i.e. crossed polars). Specimens were imaged twice, once at an angle 0° and once at an angle 45° relative to the polarizer. A high resolution digital CCD color camera (Evolution MP Color, Media Cybernetics, Bethesda, MD, USA) acquired images in a montage sequence via commercial image acquisition and stage control

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