Biomimetic organization of collagen matrices to template bone-like microstructures

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Abstract

The mineralized extracellular matrix (ECM) of bone is essential in vertebrates to provide structure, locomotion, and protect vital organs, while also acting as a calcium and phosphate reservoir to maintain homeostasis. Bone's structure comprises mainly structural collagen fibrils, hydroxyapatite nanocrystals and water, and it is the organization of the densely-packed collagen matrix that directs the organization of the mineral crystallites. Biogenic mineralization occurs when osteoblasts release “mineral bearing globules” which fuse into the preformed collagen matrix, and upon crystallization of this amorphous precursor, the fibrils become embedded with [001] oriented nanocrystals of hydroxyapatite. Our prior work has shown that this nanostructured organization of bone can be reproduced in vitro using the polymer-induced liquid-precursor (PILP) process. In this report, our focus is on using biomimetic processing to recreate both the nano- and micro-structure of lamellar bone. We first applied molecular crowding techniques to acidic, type-I collagen solutions to form dense, liquid crystalline collagen (LCC) scaffolds with cholesteric order. We subsequently mineralized these LCCs via the PILP process to achieve a high degree of intrafibrillar mineral, with compositions and organization similar to that of native bone and with a “lamellar” microstructure generated by the twisting LCC template. In depth characterization of the nano- and micro-structure was performed, including optical and electron microscopy, X-ray and electron diffraction, and thermogravimetric analyses. The results of this work lead us closer to our goal of developing hierarchically structured, collagen-hydroxyapatite composites which can serve as fully synthetic, bioresorbable, load-bearing bone substitutes that are remodeled by the native BRU.

Introduction

The extracellular matrix (ECM) of bone is a dynamic environment which serves multiple functions, ranging from mechanical roles such as protection of vital organs and providing a means for locomotion and stability, to metabolic functions acting as an ionic reservoir to help maintain homeostasis [1]. Despite decades of research, bone related diseases (e.g. osteoporosis, osteogenesis imperfecta, etc.) and injuries remain a significant clinical challenge and exacerbate the need for a synthetic, bioresorbable substitute [2–4]. Unfortunately, bone morbidities account for half of all chronic conditions worldwide in people over the age of 50, and this is expected to increase with global growth of an aging population [5–7]. Many researchers are investigating composite-based biomaterials for potential use as bone grafts due to the demanding requirements of scaffold design for bone regenerative engineering. However, the lack of current viable options for the treatment of load-bearing, critical-sized bone defects illustrates the need for novel, synthetic materials where mechanical integrity of the scaffold must be maintained during bioresorption and replacement with host tissue [5]. The use of hierarchical, collagen-hydroxyapatite composites as biocompatible scaffolds for tissue regeneration strategies seems a promising approach due to their osteoconductivity, cell compatibility, bioresorptivity, and synergistic mechanical properties [8–14]. Toward the long-term goal of preparing a

bioresorbable, load-bearing bone substitute, a crucial first step is the development of a bulk scaffold material with a dense, bone-like organization and composition. Our biomimetic approach to achieve this goal is to emulate the natural processes of bone formation to create a material with the nano- and micro-structure of lamellar bone, which we anticipate will provide mechanical properties and resorption potential of native bone.

The composition and organization of bone extracellular matrix

Bone is composed primarily of type I collagen protein (~20 wt.%), highly substituted hydroxyapatite mineral (~65 wt.%), water (~10 wt.%), and various non-collagenous proteins (NCPs, ~5 wt.%); the latter of which are believed to play important roles in the biomineralization process [15,16] (see [17] for a detailed discussion). Collagen fibrils are composed of a periodic arrangement of assembled tropocollagen, which results in a characteristic 67 nm banding periodicity, comprising the gap and overlap zones [18]. Load-bearing tissues commonly have fibrils organized in parallel arrays, which then are arranged into array patterns at the next level of hierarchy [19]. Lamellar bone has a mesoscale arrangement where adjacent lamellae of dense, parallel fibril arrays have offset orientations between neighboring layers. The thickness and rotational offset of lamellae can vary with anatomical location and from species to species [19], but is generally on the order of a few microns. This microstructural arrangement is often referred to as a 'rotated-plywood' motif, and has been suggested to arise from the cholesteric liquid crystalline order that can result from concentrated solutions of collagen [20,21]. Soon after osteoid deposition, the process of biomineralization begins as the collagen is infiltrated with "mineral bearing globules" that are secreted by osteoblasts into the extracellular milieu [22,23].

The mineralized collagen fibril is considered to be a basic building block of mineralized tissue. The morphology of hydroxyapatite in bone has often been described as thin, irregular, platelet-shaped crystals with average dimensions of 50 × 25 × 5 nm [19]. The crystallographic *c*-axis of the mineral is aligned with the fibril axis producing an interpenetrating, organic–inorganic nanocomposite with remarkable mechanical properties [24,25]. Throughout an organism's lifetime, bone remodeling units (BRU), also referred to as basic multicellular units (BMU), work to resorb old bone and replace it with new (unmineralized) collagenous osteoid [26–28] to maintain mechanical integrity and repair damaged bone [29]. The interpenetrating, composite organization of bone has important implications for its bioresorptive potential, as well as the overall mechanical properties of bone (and bone substitutes) across multiple length scales.

Biomimetic processing and structures for bone tissue engineering

Most of the current knowledge regarding collagen self-assembly is a result of in vitro studies in which fibrils are reconstituted from purified type-I collagen isolated from bovine tendon and skin [30]. Densification of dilute collagen solutions has enabled researchers to generate a variety of collagen scaffolds with control over parameters such as final concentration, homogeneity, and shape [31–34]. Giraud-Guille and colleagues conducted experiments which formed liquid crystalline, or cholesteric, collagen phases by densifying acidic collagen solutions beyond a critical concentration (~90 mg/ml) and stabilizing the organization through fibrillogenesis induced via neutralization by ammonia vapor diffusion [31,34–39]. Here we use a similar molecular crowding/confining mechanism to generate dense, liquid crystalline collagen (LCC) scaffolds. However, by permitting the system to equilibrate with a neutral poly(ethylene glycol) (PEG) solution placed outside a dialysis cassette, which also acts as an osmicant to concentrate the solution, fibrillogenesis can be induced while avoiding cytotoxicity associated with ammonia [32,33]. Additionally, by selecting a PEG concentration which balances the osmotic pressure across the dialysis membrane, densification proceeds in a manner that eliminates heterogeneities associated with the dual interface method (i.e. combined evaporation/dialysis). In both the Giraud-Guille and Ruberti systems, the processing is not fully biomimetic because the densification reaction in the biological realm consists of enzymatic cleavage to produce tropocollagen, which is neither easy nor affordable in the beaker; but the outcome is a biomimetic structure of densely-packed collagen scaffolds with similar cholesteric order.

With respect to the biomineralization reaction, previous work from our group has shown that the polymer-induced liquid precursor (PILP) process is capable of reproducing the fundamental nanostructure of bone. Recently, in vivo observations have shown evidence of amorphous calcium phosphate (ACP) phases involved in the mineralization of zebrafish caudal fin arrays [22,23]. The ACP phase appears to be transported to the pre-formed collagen matrix by secretion of cellular vesicles that contain "mineral-bearing globules", which fuse to and infiltrate the collagen matrix. The PILP process is a biomimetic in vitro mineralization model in which acidic polypeptides, used as synthetic analogs of the NCP's present in bone, stabilize a liquid-like amorphous mineral precursor that is also able to penetrate into dense collagen