Biomaterials 30 (2009) 5456–5464

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Multiple factor interactions in biomimetic mineralization of electrospun scaffolds

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ARTICLE INFO

Article history:
Received 3 June 2009
Accepted 21 June 2009
Available online 11 July 2009

Keywords:
Electrospinning
Nanocrystalline hydroxyapatite
Simulated body fluid
Bone engineering
Composite scaffolds
Biomimetic mineralization

ABSTRACT

One of the major limitations in scaffold-based bone tissue engineering has been the inability to increase the loading of biologically active inorganic mineral. The present study introduces a novel two step strategy to increase overall mineral content of electrospun scaffolds and employs multiple factor interaction as a statistic to identify the combination of factors that yields maximal scaffold mineralization. Different amounts of nHA (0, 10, 25 and 50% by wt. of polymer) were electrospun in combination with polydioxanone (PDO) or poly(glycolide: lactide) to generate composite scaffolds. Successful incorporation of nHA within, on and in between nanofibers was confirmed by transmission and scanning electron microscopy. These scaffolds were immersed in different types (conventional, revised, ionic and modified) of simulated body fluid (SBF), prepared at 1× and 4× concentrations and the incubation was carried out either in static or dynamic setting at biomimetic conditions. At 2 weeks, the total amount of mineral within the scaffold was quantified using a modified Alizarin Red-based assay. Each of the five independent factors was analyzed independently and tested for interaction using random effects ANOVA. Statistics revealed significant higher order interactions among factors and the combination of PDO containing 50% nHA incubated in 1× revised SBF resulted in maximum mineralization.

1. Introduction

Bone is a composite material composed of organic collagenous matrix and inorganic nanocrystalline hydroxyapatite (nHA). The interaction between these phases begins at the nanoscale when platelet-like HA crystals nucleate the gap regions of collagen fibrils [1,2] and elongate along the long axis of the fiber to form a mineralized collagen fibril. Such an intimate association of dissimilar material phases is preserved through hierarchical structure and gives bone its unique mechanical properties: high strength, high fracture toughness and low stiffness [3,4]. The ability of bones to undergo cell mediated resorption and remodeling is critical in load-bearing situations and maintaining calcium homeostasis.

Structural bone substitutes made from ceramics, metals and polymers have been popular in load-bearing bone and joint prostheses but problems of wear debris, corrosion, stress-shielding and inadequate integration are still relevant. Efforts to achieve a strong interface between a biomaterial and the host bone resulted in identifying a class of ceramics called bio-glass. These compounds can form a chemical bond to the bone by virtue of forming a calcium-apatite film on the surface [5,6]. Since this discovery, coating of bone implants with hydroxyapatite (or some form of ceramic) has been successfully exploited to induce osseointegration at the interface [7–9]. However, since ceramic coating (by plasma-spraying, sputtering or electrophoresis) requires the biomaterial to be heat resistant or electrically conductive, success of this approach is restricted to metals having simple shapes [10].

In contrast to conventional methods of incorporating ceramic that involves high temperature treatment, Abe et al. demonstrated new mineral deposition at low temperature by immersion of a biomaterial in an aqueous solution of ions [10]. This method was termed biomimetic because it involved incubation in simulated body fluid (whose ionic concentrations are roughly equal to that of plasma) and mineralization occurred at physiological temperature and pressure. Since such mineralization can occur on any biomaterial, independent of shape, structure or composition and in

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doi:10.1016/j.biomaterials.2009.06.043

a temperature range consistent with the glass transition temperature of biocompatible synthetic polymers, we adopted this strategy to increase the mineral content in synthetic polymeric scaffolds. In addition, since low temperature precipitate of calcium phosphate is biologically active *in vivo*, composite bone scaffolds containing this form of apatite would be more amenable for bone tissue engineering.

Scaffold-based bone tissue engineering is a rapidly emerging field that focuses on developing biologically-based substitutes that integrate with and are eventually replaced by host bone [11]. It involves implantation of extracellular matrix (ECM) analogs that can simultaneously support cell function and provide structural support. Incorporation of nHA within the polymer matrix seems to be an attractive strategy in bone tissue engineering because the dispersal of stiff and brittle HA crystals within the tough organic polymer matrix would effectively inhibit crack propagation [12–14]. From a biological perspective, presence of hydroxyapatite enhances protein adsorption, improves osteoblast function [15,16] and confers osteoconductivity [17] to scaffolds.

In the present study, we sought to increase the loading of HA mineral within electrospun scaffolds in two steps: (1) by directly incorporating nanocrystalline hydroxyapatite (nHA) during electrospinning and (2) by subjecting these composite scaffolds to incubation with simulated body fluid (SBF). We hypothesized that in addition to directly contributing to overall mineral content, seeded nHA during electrospinning will act as nucleation sites during SBF treatment for further crystal growth. We tested this novel mineralization strategy on two synthetic polymers (polydioxanone and poly(lactide:glycolide)) containing 0, 10, 25 and 50% (by wt. of polymer) nHA. These scaffolds were then incubated in two concentrations (1× and 4×) of four types of simulated body fluids (conventional, revised, modified and ionic) under either static or dynamic conditions. Finally, we took a statistical approach to analyze the interactions effects of each of these variables to identify the best combination of factors that would yield maximum *in vitro* scaffold mineralization.

2. Materials and methods

2.1. Characterization of nanocrystalline hydroxyapatite (nHA)

Commercially available nHA was purchased (Berkeley Advanced Biomaterials Inc, San Leandro, CA) and crystal structure was confirmed to be hydroxyapatite by electron diffraction. The TEM evaluation shows a favorable elongated platelet-like structure that had crystal size in the range of 30–100 nm, similar to that found in bone. The results are presented in Fig. 1.

2.2. Composite scaffold preparation by electrospinning

Two different synthetic polymers – polydioxanone (PDO, Ethicon, Inc. NJ) and 85:15 poly(lactide-glycolide) (PLGA, Lakeshore Biomaterials, AL) were used for the study. The final concentration of the polymers was adjusted to 100 mg per ml of electrospinning solvent, 1,1,1,3,3,3 hexafluoro-2-propanol (HFP) for all scaffold types. Different amounts of nHA (0, 10, 25 and 50% by wt. to polymer) were dispersed in HFP prior to addition of polymer and electrospinning. Since nHA crystals sedimented in HFP, stable dispersion of nHA was achieved by sonicating the solution in pulse mode (on: 50 s, off: 10 s) at 38% maximum amplitude using a tapered tip of a Cole-Palmer Ultrasonic Processor for 10 min. The stability of dispersion of nHA was confirmed after 2 h by visual inspection before adding pre-weighed amounts of PDO or PLGA. Electrospinning conditions were optimized (rate: 7 ml/h, air-gap distance: 20 cm, voltage: 22 kV) to generate continuous non-woven composite nanofibers that were collected onto a rotating rectangular mandrel (7.5 × 2.5 × 0.5 cm). After electrospinning, scaffolds were removed from mandrel, dried in the hood for 30 min and 10 mm discs punched using a dermal biopsy punch. These discs were used for all mineralization experiments.

2.3. Transmission and scanning electron microscopy

Fibers were collected directly onto copper grids during electrospinning and analyzed using Jeol-JEM-1230 transmission electron microscope. For scanning electron microscopy, air-dried electrospun scaffolds were mounted on aluminum stubs, sputter coated with gold and examined at an accelerating voltage of 10 kV using Zeiss EVO 50 XVP scanning electron microscope.

2.4. Biomimetic mineralization with simulated body fluid

Four different types of simulated body fluids- conventional (c-SBF), revised (r-SBF), ionic (i-SBF) and modified (m-SBF) were prepared following a published protocol [18]. The ionic concentrations in these fluids vary with respect to chloride, bicarbonate and calcium. Each type of SBF was prepared in two concentrations (1× and 4×) to study the effect of increased ion concentration on scaffold mineralization. Electrospun scaffolds were incubated in SBF under two conditions: static and dynamic. In static condition, the discs were placed motionless in a tissue culture grade 24-well plate containing 3 ml of corresponding SBF while dynamic incubation involved using a 55 ml slow lateral turning vessel (STLV) bioreactor (Synthecon, Inc. Houston, TX) rotating at 9 rpm. Both static and dynamic experiments were performed in an incubator at 37 °C in 5% CO_2 atmosphere for a total of 14 days with SBF solutions renewed every 5 days.

2.5. Quantification of mineralization using Alizarin Red S (ARS)

The mineralization of scaffolds was quantified using a modification of a published protocol [19]. Alizarin Red S (Sigma, MO) was dissolved in deionized (DI) water to a final concentration of 40 mM and pH was adjusted to 4.1 using 1 M NaOH. The solution was then filtered through a 0.8 μm mesh to remove any particulates

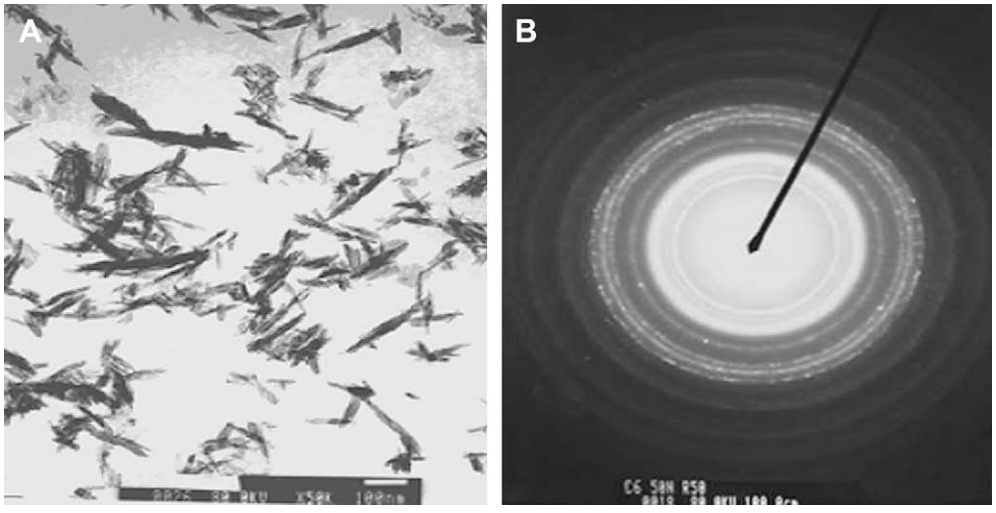

Fig. 1. Initial characterization of commercially available nHA used in the study. (A) Transmission electron micrograph showing platelet-like crystals having sizes ranging from 30 to 100 nm. (B) Electron diffraction pattern confirming the crystal structure to be hydroxyapatite.