Biomaterials 29 (2008) 2839–2848

Contents lists available at ScienceDirect

Biomaterials

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

Physical properties and cellular responses to crosslinkable poly(propylene fumarate)/hydroxyapatite nanocomposites

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ARTICLE INFO

Article history:
Received 12 December 2007
Accepted 20 March 2008
Available online 9 April 2008

Keywords:
Poly(propylene fumarate) (PPF)
Hydroxyapatite (HA)
Nanocomposite
Protein adsorption
Osteoblast response

ABSTRACT

A series of crosslinkable nanocomposites has been developed using hydroxyapatite (HA) nanoparticles and poly(propylene fumarate) (PPF). PPF/HA nanocomposites with four different weight fractions of HA nanoparticles have been characterized in terms of thermal and mechanical properties. To assess surface chemistry of crosslinked PPF/HA nanocomposites, their hydrophilicity and capability of adsorbing proteins have been determined using static contact angle measurement and MicroBCA protein assay kit after incubation with 10% fetal bovine serum (FBS), respectively. *In vitro* cell studies have been performed using MC3T3-E1 mouse pre-osteoblast cells to investigate the ability of PPF/HA nanocomposites to support cell attachment, spreading, and proliferation after 1, 4, and 7 days. By adding HA nanoparticles to PPF, the mechanical properties of crosslinked PPF/HA nanocomposites have not been increased due to the initially high modulus of crosslinked PPF. However, hydrophilicity and serum protein adsorption on the surface of nanocomposites have been significantly increased, resulting in enhanced cell attachment, spreading, and proliferation after 4 days of cell seeding. These results indicate that crosslinkable PPF/HA nanocomposites are useful for hard tissue replacement because of excellent mechanical strength and osteoconductivity.

1. Introduction

Calcium phosphate ceramic materials have been used for skeletal tissue engineering applications because of their excellent biocompatibility and osteoconductive properties [1,2]. Among these materials, hydroxyapatite [HA, $Ca_{10}(PO_4)_6(OH)_2$] is the major inorganic compound and has similar chemical and crystal structure to that of bone mineral. Thus HA has been studied extensively as an excellent osteoconductive material. However, it is very brittle and cannot be applied to the load-bearing site directly [3–5].

To overcome these limitations, HA has been incorporated with natural biomacromolecules such as collagen [6–8] and gelatin [9,10], or synthetic polymers such as poly(α-hydroxyl acids) [11–15], poly(ε-caprolactone) (PCL) [16,17], polyamide [18], and polymethylmethacrylate (PMMA) [19] to prepare composites using a variety of methods including surface coating, grafting, direct mixing, and biomimetic precipitation [10,11,20–23]. Particularly, polymer/HA nanocomposites have improved mechanical properties and enhanced cell attachment, spreading, and proliferation on their surfaces by adding nano-sized HA to modify the polymer's characteristics and/or strengthen the polymer matrix [24,25]. Oftentimes, these nanocomposites still cannot achieve the targeted compressive strength and modulus for load-bearing applications because the matrix polymers themselves do not have sufficient mechanical properties to be improved upon.

One of the promising candidate materials with suitable mechanical properties for load-bearing applications is poly(propylene fumarate) (PPF). It is an unsaturated linear polyester that can be crosslinked through carbon–carbon double bonds along its backbone [26,27] and degraded through simple hydrolysis of the ester bonds into non-toxic products of propylene glycol, poly(acrylic acid-*co*-fumaric acid), and fumaric acid [28]. By controlling the molecular weight of PPF and the crosslinking density, the maximum compressive strength and modulus of crosslinked PPF can be over 10 and 100 MPa, respectively [29–32]. Although many previous studies have been performed to enhance both mechanical strength and osteoconductive properties by adding calcium phosphate such as β-tricalcium phosphate (β-TCP) to PPF [33,34], or preparing PPF/HA composites by either simple mixing [35] or from *in situ* precipitation [36], the correlation between the physical properties and cell responses on PPF/HA nanocomposites has not yet been reported, compared with other poly(α-hydroxyl acids)-based composites.

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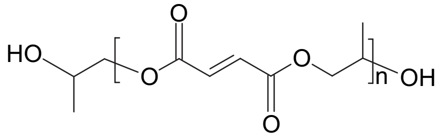
doi:10.1016/j.biomaterials.2008.03.030



Scheme 1. Chemical structure of PPF.

As a prelude to a further study on three-dimensional (3D) PPF/HA nanocomposite scaffolds with different features fabricated using solid freeform technique [37], we report a series of crosslinkable nanocomposites based on PPF and HA nanoparticles and their two-dimensional (2D) disks prepared through thermal crosslinking. In this report, we will reveal the role of HA nanoparticles in modifying the surface properties and controlling *in vitro* cell responses. The structure, morphology, and physical properties of the crosslinked PPF/HA nanocomposites have been characterized using Fourier transform infrared (FTIR) spectroscopy, wide-angle X-ray diffraction (WAXD), thermogravimetric analysis (TGA), transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM), and dynamic mechanical analyzer (DMA). In addition, hydrophilicity and the capability of adsorbing serum proteins on the crosslinked PPF/HA disks have been determined from static contact angle measurement and MicroBCA protein assay kit, respectively. To investigate *in vitro* cellular responses, MC3T3-E1 cells were seeded and cultured on the PPF/HA nanocomposite disks. Cell morphology after 24 h was imaged using SEM. Cell spreading was examined by F-actin staining with rhodamine–phalloidin and cell proliferation was quantified using a colorimetric MTS assay at 1, 4, and 7 days.

2. Materials and methods

2.1. PPF synthesis

As described previously [31,32], diethyl fumarate (DEF) and excess amount of 1,2-propylene glycol were polymerized together with hydroquinone (crosslinking inhibitor) and zinc chloride (catalyst) first at 100 °C for 1 h and then 150 °C for 7 h to obtain the intermediate dimer. Then the intermediate dimer was transesterified to form PPF under vacuum at 130 °C for another 4 h. Gel permeation chromatography (GPC) was used to determine the molecular weight and polydispersity of PPF. GPC was carried out with a Waters 717 Plus Autosampler GPC system (Waters, Milford, MA) connected to a model 515 HPLC pump and model 2410 refractive index detector. Mono-dispersed polystyrene standards (Polysciences, Warrington, PA) with peak molecular weights (M_p) of 474, 6690, and 18,600 g mol^{-1} were used to construct the calibration curve. The M_n and weight average molecular weight (M_w) of the synthesized PPF were 3460 and 7910 g mol^{-1}, respectively. The chemical structure of PPF is shown in Scheme 1.

2.2. PPF/HA nanocomposite preparation

HA nanoparticles were purchased from Berkeley Advanced Biomaterials (Berkeley, CA). The size range of HA nanoparticles is from 20 to 550 nm (average size = 100 nm) and their whiskers have long and short axis of ~100 and ~20 nm, respectively. PPF and HA were crosslinked by a free radical polymerization using 1-vinyl-2-pyrrolidinone (NVP), benzoyl peroxide (BPO) and *N*-dimethyl-*p*-toluidine (DMT) as crosslinker, free radical initiator, and accelerator, respectively. One gram of PPF and 0.4 g of NVP, corresponding to 40% NVP per gram of PPF, were mixed in a glass vial at 37 °C for 2 h. Then, four different amounts of HA were added into PPF solutions to prepare PPF/HA mixtures with HA weight fractions of 0, 10, 20, and 30%. Forty microliters of initiator solution (50 mg of BPO in 250 μL of NVP) and 16 μL of accelerator solution (20 μL of DMT in 980 μL of methylene chloride) were added and mixed thoroughly. Compositions of PPF/HA mixtures are listed in Table 1. The resulting PPF/HA mixtures were poured into cylindrical glass molds with a diameter of 10 mm and a height of 30 mm. The loaded molds were placed in a convection oven at 60 °C for 1 h to facilitate crosslinking. After crosslinking, the molds were cooled down to room temperature. Crosslinked PPF/HA specimens were removed from the molds, and cut into disks with a diameter of 10 mm and a thickness of 1 mm using an Isomet low speed saw (Buehler, Lake Bluff, IL).

Table 1
Compositions of PPF/HA mixtures for nanocomposite fabrication via chemical crosslinking

PPF (g)	NVP (g)	Nano-HA (g) (wt.%)	BPO (μL)	DMT (μL)
1	0.4	0 (0)	40	16
		0.156 (10)		
		0.350 (20)		
		0.600 (30)		

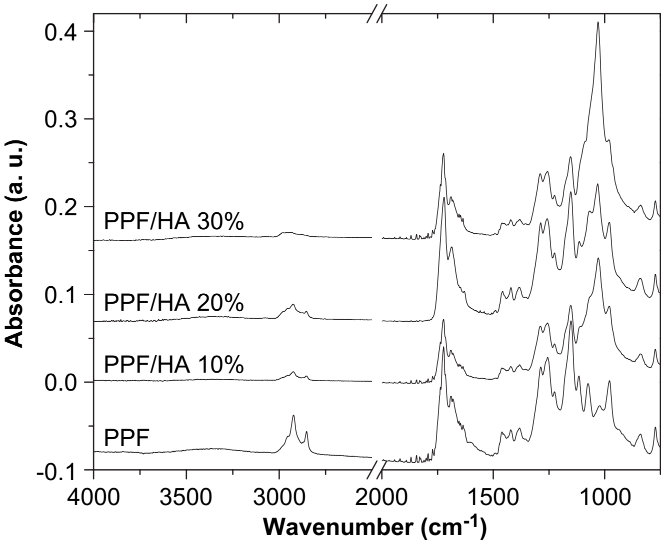


Fig. 1. FTIR spectra of crosslinked PPF and PPF/HA with different HA contents.

2.3. PPF/HA disk characterization

Fourier transform infrared (FTIR) spectra were obtained on a Nicolet 550 spectrometer (Thermo Scientific Inc., Waltham, MA). All samples were analyzed using a zinc selenide ATR crystal. The resolution of the instrument was specified as 4 cm^{-1} at a wavenumber of 1000 cm^{-1}.

Wide-angle X-ray diffraction (WAXD) measurements were performed in the X-ray scattering lab of Characterization Facility at the University of Minnesota. The small pieces from the disks were suspended and run in transmission mode. The microdiffractometer (Bruker AXS Inc., Madison, WI) with copper radiation, an incident beam monochromater, and a GADDS multiwire area detector were used for the wide angle tests. Two frames were collected, one at $2\theta = 20°$ and the other at 50° to cover a range of 5–65° for 2θ. Each frame was collected for 5 min. The data were then integrated and plotted as intensity vs. 2θ.

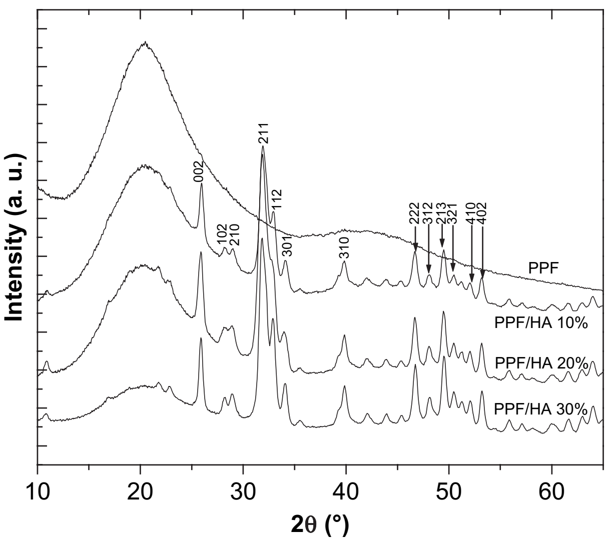


Fig. 2. Wide-angle X-ray diffraction patterns of crosslinked PPF and PPF/HA with different HA contents. HA peaks were indexed according to the JCPDS 9-432 standard.