



The roles of matrix polymer crystallinity and hydroxyapatite nanoparticles in modulating material properties of photo-crosslinked composites and bone marrow stromal cell responses

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ABSTRACT

Two poly(ϵ -caprolactone fumarate)s (PCLFs) with distinct physical properties have been employed to prepare nanocomposites with hydroxyapatite (HA) nanoparticles via photo-crosslinking. The two PCLFs are PCLF530 and PCLF2000, named after their precursor PCL diol with molecular weight of 530 and 2000 g mol⁻¹, respectively. Crosslinked PCLF530 is amorphous while crosslinked PCLF2000 is semi-crystalline with a melting temperature (T_m) of $\sim 40^\circ\text{C}$ and a crystallinity of 40%. Consequently, the rheological and mechanical properties of crosslinked PCLF2000 are significantly greater than those of crosslinked PCLF530. Structural characterizations and physical properties of both series of crosslinked PCLF/HA nanocomposites with HA compositions of 0%, 5%, 10%, 20%, and 30% have been investigated. By adding HA nanoparticles, crosslinked PCLF530/HA nanocomposites demonstrate enhanced rheological and mechanical properties while the enhancement in compressive modulus is less prominent in crosslinked PCLF2000/HA nanocomposites. *In vitro* cell attachment and proliferation have been performed using rat bone marrow stromal cells (BMSCs) and correlated with the material properties. Cell attachment and proliferation on crosslinked PCLF530/HA nanocomposite disks have been enhanced strongly with increasing the HA composition. However, surface morphology and surface chemistry such as composition, hydrophilicity, and the capability of adsorbing protein cannot be used to interpret the cell responses on different samples. Instead, the role of surface stiffness in regulating cell responses can be supported by the correlation between the change in compressive modulus and BMSC proliferation on these two series of crosslinked PCLFs and PCLF/HA nanocomposites.

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1. Introduction

Biodegradable and crosslinkable poly(ϵ -caprolactone fumarate) (PCLF) has been synthesized in our laboratory via polycondensation of poly(ϵ -caprolactone) (PCL) diol and fumaryl chloride in the presence of potassium carbonate [1,2]. By varying the molecular weight of PCL diol, PCLFs with controllable physical properties can be obtained to meet the design requirements of both hard and soft tissue engineering scaffolds [1–4]. Previously, we have reported the structural characterizations and physical properties of both uncrosslinked and crosslinked PCLFs [1–4]. Two-dimensional (2D) disks and three-dimensional (3D) nerve conduits have also been

fabricated from PCLFs via photo-crosslinking [3–5]. *In vitro* cell studies such as cytocompatibility, cell attachment and proliferation, and *in vivo* implantation using a rat sciatic nerve injury model have been conducted and the biological evaluations of crosslinked PCLFs have been correlated with their physical properties [3–5]. Crosslinked PCLFs have demonstrated excellent cell viability with various cell types such as human fetal osteoblast cells, rat bone marrow stromal cells (BMSCs), and rat Schwann cell precursor line (SPL201) cells [2,4,5]. Although their flexibility and toughness make them suitable for fabricating nerve conduits to guide peripheral nerve regeneration [4], the mechanical properties of crosslinked PCLFs are yet to be strengthened for being used as bone-tissue-engineering materials, especially when they are amorphous at body temperature.

Hydroxyapatite (HA), the major inorganic component of bone mineral, has been used for various biomedical applications such as implant coating because of its advantages such as excellent bioactivity, promotion of cellular function, and osteoconductivity [6].

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Because HA nanoparticles are brittle and cannot be applied to the load-bearing sites directly, they are often used as physical fillers to enhance the mechanical properties and osteoconductivity of numerous polymers such as PCL, poly(L-lactide) (PLLA), poly(lactide-co-glycolide) (PLGA), and poly(methyl methacrylate) (PMMA) [6–18]. Besides being used as pre-made HA nanoparticles with polymer matrices, they can be also generated *in situ* during polymer precipitation or synthesis to improve the interface between fillers and matrix [6,19]. Compared with the above-mentioned matrix polymers with saturated backbones, crosslinkable or injectable polymeric biomaterials exemplified by PCLFs have many advantages because they can be injected and hardened *in situ* to fill tissue defects or fabricated into pre-formed scaffolds [20–22]. There only exist a few crosslinked nanocomposites based on HA nanoparticles and crosslinkable polymer matrices such as polypropylene fumarate (PPF) and polyanhydride [16,23–27]. In the crosslinked PPF/HA nanocomposites reported by us, the addition of HA nanoparticles did not improve the compressive modulus significantly possibly because crosslinked PPF was intrinsically very rigid [23]. Nevertheless, dramatically enhanced cell attachment and proliferation have been observed on the crosslinked PPF/HA nanocomposite disks cut from cylinders because the HA nanoparticles exposed on the disk surface greatly increased hydrophilicity and the capability of adsorbing protein from cell culture medium [23].

In this study, we present novel photo-crosslinked biodegradable and bioactive nanocomposites for orthopedic applications by incorporating HA nanoparticles with PCLF matrix. Two PCLFs, PCLF530 and 2000, synthesized from the PCL diol precursors with the nominal molecular weights of 530 and 2000 g mol⁻¹ have been used. Because of its amorphous characteristics, crosslinked PCLF530 has much lower mechanical properties compared with crosslinked PCLF2000 that has a higher crystallinity and a melting temperature (T_m) higher than the body temperature [3,4]. Thus the enhancement of HA nanoparticles in these two matrices is expected to differ significantly. Another focus of this study is on regulating cell responses using different PCLFs and their nanocomposites. Recently the mechanical properties of biomaterials have been revealed to be crucial in influencing cell responses [28–30]. For instance, we reported that SPL201 cell proliferation could be enhanced dramatically on the bare crosslinked PCLF2000 surface with higher crystallinity and mechanical properties compared to crosslinked PCLF530 [4]. Using rat BMSCs, we will present *in vitro* cell attachment and proliferation on the crosslinked PCLFs and PCLF/HA nanocomposites. Through this study, we not only supply a series of crosslinkable polymer/HA nanocomposites for bone-tissue engineering applications, but also use them as model polymeric systems to suggest the roles of matrix polymer crystallinity and HA nanoparticles in regulating material properties and cell responses without greatly modifying the materials' surface chemistry.

2. Materials and methods

2.1. Materials

All chemicals used in this study were purchased from Sigma–Aldrich Co. (Milwaukee, WI) unless noted otherwise. PCLF530 and PCLF2000 were synthesized in our laboratory as described in our previous report [1], having a weight-average molecular weight (M_w) of 6050 and 12900 g mol⁻¹, and a number-average molecular weight (M_n) of 3520 and 7300 g mol⁻¹, respectively. HA nanoparticles (~100 nm × ~20 nm, length × width) were purchased from Berkeley Advanced Biomaterials (Berkeley, CA).

2.2. Photo-crosslinking of PCLF/HA nanocomposites

Photo-crosslinking was initiated with ultraviolet (UV) light ($\lambda = 315–380$ nm) using a photoinitiator phenyl bis(2,4,6-trimethyl benzoyl) phosphine oxide (BAPO, IRGACURE 819™, Ciba Specialty Chemicals, Tarrytown, NY). Similar to the

preparation of crosslinked PCLFs [3–5], 150 μ L of BAPO/CH₂Cl₂ (300 mg/1.5 mL) solution was mixed with a slurry formed by 500 μ L CH₂Cl₂ and 1.5 g of PCLF/HA with varied HA weight composition (ϕ_{HA}) of 0%, 5%, 10%, 20%, and 30%. Homogeneous PCLF/HA/BAPO/CH₂Cl₂ mixture was transferred into a mold formed by two glass plates (2.1 mm, thickness) and a Teflon spacer (0.37 mm, thickness). The filled mold was placed under UV light with a distance of ~7 cm from the lamp head for 30 min to allow crosslinking. Crosslinked PCLF/HA sheets were removed from the mold after cooled down to ambient temperature. Strips and disks with different dimensions cut from the sheets were extracted in acetone for two days and dried in vacuum for various experiments.

2.3. Gel fraction and swelling ratio measurements

Two crosslinked PCLF or PCLF/HA disks (5 mm × 0.34 mm, diameter × thickness) were immersed in excess CH₂Cl₂. After two days, the disks were taken out and weighed after blotted quickly to remove the attached solvent on the surfaces. The solvent adsorbed in the disks was subsequently evacuated and the dry disks were weighed. Based on the measured weights of the original (W_0), dry (W_d), and fully swollen (W_s) disks, their swelling ratios and gel fractions were calculated using the equations of $(W_s - W_d)/W_d \times 100\%$ and $W_d/W_0 \times 100\%$, respectively.

2.4. Structural and thermal characterizations

Fourier transform infrared (FTIR) spectra of HA, crosslinked PCLFs and PCLF/HA nanocomposites were obtained on a Nicolet 550 FTIR spectrometer using a zinc selenide ATR crystal. The resolution of the instrument was specified as 4 cm⁻¹ at a wavenumber of 1000 cm⁻¹. Differential scanning calorimetry (DSC) measurements were performed on a Q1000 differential scanning calorimeter (TA Instruments) in a nitrogen atmosphere. To keep the same thermal history, each sample was first heated from room temperature to 100 °C and cooled to -90 °C at a cooling rate of 5 °C/min. A subsequent heating run was performed from -90 to 100 °C at a heating rate of 10 °C/min. Thermogravimetric analysis (TGA) was done in flowing nitrogen at a heating rate of 20 °C/min using a Q500 thermal analyzer (TA Instruments).

In wide-angle X-ray diffraction (WAXD) measurements, HA nanoparticles, crosslinked PCLF and PCLF/HA disks were suspended and run in transmission mode. A Bruker AXS micro-diffractometer with copper radiation, an incident beam monochromator, and a GADDS multi-wire area detector was used for the wide-angle tests. Two frames were collected, one at the Bragg angle $2\theta = 20^\circ$ and one at 50° to cover a range of $5^\circ–65^\circ$ for 2θ . Each frame was collected for 5 min. The data were then integrated and plotted as intensity vs. 2θ peaks were identified using Material Data Incorporated's JADE 7.0 software.

2.5. Microscopic observation

Morphology of HA nanoparticles in the crosslinked PCLF/HA disks was examined by a transmission electron microscope (TEM) (1200-EX II, JEOL Inc., Japan). The sample was placed into plastic capsules filled with resin, hardened into at 60 °C for 24 h, and sectioned with a glass knife as a thick block with a thickness of 0.6 μ m. For TEM imaging, the block was trimmed down to the area of interest and then sectioned with a diamond knife to a thickness of 0.1 μ m. All samples were viewed at 80 kV accelerating voltage. Surface morphologies of the crosslinked PCLF and PCLF/HA disks were examined by a cold-field emission scanning electron microscope (SEM) (S-4700, Hitachi Instruments Inc., Japan). In SEM imaging, the sample was mounted onto an aluminum stub, sputter coated with gold–palladium, and viewed at 3 kV accelerating voltage.

2.6. Rheological and mechanical measurements

Linear viscoelastic properties of crosslinked PCLF and PCLF/HA disks, including storage and loss moduli G' and G'' as well as viscosity η as functions of frequency, were measured using an AR2000 rheometer (TA Instruments) in the frequency (ω) range of 0.5–100 rad/s and at 37 and 60 °C, sequentially. Rheological measurements were performed with a small strain ($\gamma = 0.01$) using an 8 mm diameter parallel plate flow cell and a gap around 0.5 mm, depending on the thickness of polymer disk. Tensile and compressive properties of crosslinked PCLF and PCLF/HA specimens were implemented at room temperature by a dynamic mechanical analyzer (DMA2980, TA instruments). Five specimens were measured and averaged per sample. In tensile measurements, crosslinked PCLF and PCLF/HA strips (~30 mm × ~2 mm × ~0.4 mm, length × width × thickness) were pulled at a rate of 0.5 N min⁻¹ up to a maximum static force of 18 N. In compression measurements, modulus was measured on small crosslinked PCLF and PCLF/HA disks (4.1 mm × 0.36 mm, diameter × thickness) at a compression rate of 2 N min⁻¹ up to a maximum static force of 18 N.

2.7. Contact angle measurement and protein adsorption

A static Knuss drop shape analysis (DSA) system, including a Knuss G10 contact angle measurement instrument and a data analyzing software version of 1.51.026,

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