

Enzymatically Degradable Hydrogel Nanocomposite for Bone Regeneration

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ABSTRACT

A novel approach to bone replacement involves the use of *in situ* crosslinkable biomaterials as scaffolds for cell transplantation and to promote matrix production and tissue growth. Hydrogel/apatite nanocomposites are the ideal biomaterial to mimic the physio-chemical and biologic properties of the bone matrix and to fabricate scaffolds for bone regeneration. The objective of this work was to fabricate hydrogel/apatite nanocomposite scaffolds for bone regeneration. HA nanoparticles were grafted in two steps with hydrophilic unsaturated poly(ethylene glycol) oligomers to improve their suspension stability and interfacial bonding to the hydrogel phase. Poly(lactide-ethylene oxide-fumarate) (PLEOF) unsaturated terpolymer was synthesized by condensation polymerization of low molecular weight PLA and poly(ethylene glycol) (PEG). Hydrogel/apatite porous scaffolds were prepared using PLEOF as the degradable macromer, an MMP degradable peptide crosslinker, and a neutral redox initiation system. Scaffolds were seeded with bone marrow stromal (BMS) cells to study cell attachment and migration. Macropores and micropores were formed inside the scaffold by leaching of the porogen and by the partial phase separation of hydrophilic and hydrophobic domains of PLEOF hydrogel. Cell visualization by confocal fluorescence microscopy demonstrated that BMS cells migrate and attach to the surface of the nanocomposite. Our results demonstrate that the hydrogel/apatite nanocomposite is an attractive alternative as a biomaterial for hard tissue regeneration.

Keywords: hydrogel, nanocomposite, biodegradable, stem cell, bone

1 INTRODUCTION

There are approximately 6.2 million fractures in the United States annually that require bone graft procedures to ensure rapid skeletal repair and achieve union [1]. These include bone loss after skeletal trauma, resection of tumors, spinal arthrodesis, and osteoporotic fractures voids [2]. While the natural process of fracture healing is biologically optimal, acceleration of the repair process has a positive impact on society.

Bone matrix is a composite material consisting of an aqueous and a mineral phase. The inorganic component, made up of apatite crystals, contributes approximately 65%

of the wet weight of the bone and the aqueous phase contributes about 20% [3]. The aqueous gel phase gives bone its form and contributes to its ability to resist bending, while the mineral component primarily resists compression. The aqueous gel component plays a central role in regulation of collagen fibril mineralization, modulation and control of cell division, cell migration, differentiation and maturation, maintenance of matrix integrity, growth factor modulation, and the extent of mineral-collagen interactions. For example matrix metalloproteinases (MMP), secreted by the cellular components, are released to the aqueous gel phase to degrade the collagen network in bone remodeling [4]. Thus, the aqueous gel phase of the bone matrix plays a vital role to support interactions between the mineral component and cellular elements.

Recently developed biodegradable matrices are based on hydrophobic and hydrolytically degradable polymers [5]. These matrices can provide structural support to the regenerating region and their surface can be treated to support adhesion, migration, and proliferation of progenitor bone marrow stromal cells or differentiated osteoblasts. Although the surface of these materials can be functionalized, their bulk is bio-inert and does not bear functional groups for solubilization and interaction with proteins and cells. Hydrogels are three-dimensional crosslinked polymeric structures which are able to swell in aqueous physiological solution and retain water in their structure without dissolving [6]. Hydrogels exhibit excellent biocompatibility and interact less strongly with immobilized biomolecules than hydrophobic materials. Furthermore, biologically active peptides such as MMP can be immobilized within the hydrogel structure.

The objective of this work was to fabricate hydrogel/apatite nanocomposite scaffolds for bone regeneration. The hydrogel phase is made up of the poly(lactide-ethylene oxide-fumarate) (PLEOF) terpolymer. The hydrogel and apatite phases are crosslinked using an MMP degradable peptide crosslinker to modulate the matrix degradation with the migration of BMS cells.

2 METHODS

2.1 Synthesis of LMW PLA

Low molecular weight poly(L-lactide) (LMW PLA) was synthesized by ring opening polymerization of the lactide monomer (LA) in a dry glass ampoule with diethylene glycol (DEG) as the bifunctional initiator and tin octoate

(TOC) as the polymerization catalyst. The molar ratio of DEG to TOC was 25:1. The molar ratio of LA to DEG was varied from 10 to 30 to produce LMW PLA with number average molecular weights (M_n) in the range of 1000 to 4000 Da. The ampoules were sealed under nitrogen atmosphere at 140°C and the reaction was continued for 12 h at the same temperature. The resulting polymer mixture was dissolved in methylene chloride (MC) and precipitated in methanol. Next, the methanol was removed by rotovaporation, the polymer was redissolved in MC and precipitated in hexane. The precipitate was dried in vacuum for at least 12 h and stored in a dry atmosphere. The synthesized LMWPLA was characterized by ^1H NMR and gel permeation chromatography (GPC).

2.2 Synthesis of PLEOF Terpolymer

PLEOF was synthesized by condensation polymerization of LMW PLA and PEG with fumaryl chloride (FuCl) and triethylamine (TEA) as the catalyst as described [7]. The molar ratio of FuCl:PEG and TEA:PEG was 0.9:1.0 and 1.8:1.0, respectively. PLEOF terpolymer was synthesized using PEG with M_n ranging from 1 to 5 kDa and PLA with M_n ranging from 1 to 7 kDa. The weight ratio of PEG to PLA was varied from 100/0 to 80/20 to produce a hydrophilic water-soluble terpolymer. In a typical reaction, dried PEG and LMW PLA were dissolved in MC under dry nitrogen atmosphere in a reaction flask. Next, FuCl and TEA, each dissolved in MC, were added dropwise to the reaction with stirring. The reaction was continued for 6 h under ambient conditions. After completion of the reaction, solvent was removed by rotovaporation and residue was dissolved in ethyl acetate to precipitate the by-product triethylamine hydrochloride and the salt was removed by filtration. Ethyl acetate was removed by distillation. The macromer was redissolved in MC and precipitated in ethyl ether. It was dried in vacuum for at least 12 h and stored at -20°C. The structure of PLEOF macromer was characterized by ^1H -NMR and FTIR.

2.3 Synthesis of Crosslinker

MMP-13 degradable lysine-alanine-isoleucine-glycine-glutamine-histidine-lysine peptide sequence with unsaturated reactive end groups were synthesized manually in the solid phase using a novel method on the Rink Amide NovaGel™ resin with Fmoc-, Mtt-, and Trt- protected amino acid derivatives [8]. Resin was swelled and washed in dimethyl formamide (DMF). The Fmoc-protected amino acid derivative, 1-hydroxybenzotriazole (HOBt), and 1,3-diisopropylcarbodiimide (DIC) in dry DMF were mixed and added to the resin. 0.2 ml of 0.05 M 4-Dimethylaminophenol (DMAP) was added to this mixture, the resin was treated with 20% piperidine in DMF and washed with DMF; other amino acids were coupled successively using the same procedure. After coupling the

last amino acid of the peptide chain, the resin was washed with DMF and DCM and the Mtt protecting group was selectively deprotected by treating the peptidyl resin with trifluoroacetic acid/ dichloromethane (TFA/DCM) (1:99 v/v) and filtered. The resin was washed with MC and DMF and the Fmoc amino acids were deprotected by treatment with 20% piperidine in DMF.

Bifunctional peptide with unsaturated acrylate end groups was synthesized in the solid phase by coupling acrylic acid to the amine groups of glutamine and lysine residues at the two ends of the peptide sequence. Briefly, acrylic acid, HoBt, and 1,3-diisopropylcarbodiimide (DIC) were mixed in DMF, added to the peptidyl resin, and mixed. Next, the resin was washed with DMF and DCM and treated with a mixture of 95% TFA, 2.5% triisopropylsilane (TIPS), and 2.5% water for 2 h to cleave the peptide from the resin and deprotect the side chains. The peptide crosslinker was precipitated in cold ether, suspension was centrifuged, and the supernatant was decanted, and freeze-dried. The product was purified by preparative HPLC on a Prep RP18 column using a gradient 5% acetonitrile (MeCN) and 95% 0.1% aqueous TFA solvent mixture. A photodiode array detector was used for detection at a wavelength of 214 nm. Mass spectrometric (MS) studies were performed with a Fannigan 4500 Electro Spray Ionization (ESI) spectrometer. The peaks in the ESI-MS spectrum at 870 and 848 mass numbers corresponded to sodium cation and hydrogen cation of the peptide crosslinker, respectively.

2.4 Synthesis of Grafted HA

Hydroxyapatite (HA) nanoparticles were grafted with hydrophilic unsaturated PEG oligomers to improve interfacial bonding between the hydrogel and apatite phases by inter-crosslinking between the unsaturated fumarate (in the hydrogel) and methacrylate (on the surface of the HA nanoparticles) groups. The grafting reaction was carried out in two steps. In the first step, poly(ethylene glycol) methacrylate (PEGMA) was condensed with 3-isocyanatopropyltrimethoxysilane (iCPTMS) to form a PEGMA-PTMS urethane with unsaturated methacrylate and trimethoxysilane end-groups. In the second step, the trimethoxysilane end of the urethane was reacted with reactive phosphate and carbonate groups on the HA surface using ammonium hydroxide and methanol as the catalysts to produce HA with grafted PEGMA oligomers (gHA). In a typical reaction, DMF and iCPTMS were added dropwise with stirring PEGMA in a reactor with a refluxing condenser. The condensation reaction was allowed to proceed for 1 h under reflux conditions and with excess PEGMA. The reaction was allowed to cool to ambient conditions, HA was added, the mixture was sonicated, 1 ml of ammonium hydroxide and 1 ml of methanol were added to the reaction as catalysts, and grafting reaction was allowed to continue for 12 h under reflux conditions. The grafted HA was washed with MC, centrifuged, re-

suspended, and dried under vacuum. gHA was characterized with FTIR, TGA, and TEM.

2.5 Fabrication of Nano-Biocomposite

Hydrogel/apatite porous scaffolds were prepared using PLEOF as the degradable macromer, MMP degradable crosslinker, a neutral redox initiation system, and sodium chloride crystals as the porogen. The redox system consisted of ammonium persulfate (APS) and tetramethylethylenediamine (TMEDA), respectively [7]. To prepare a scaffold with 60% HA and 60% porosity, 63mg PLEOF was added to 165 μ l of a 0.24 M solution of BISAM. To this mixture, 550 mg of gHA was added and mixed to a paste. As the porogen, 1.6 g of NaCl crystals was added and mixed. Next, 21 μ l of APS and 21 μ l of TMEDA were added and the polymerizing mixture was transferred to a mold and allowed to crosslink at 37°C. After crosslinking, scaffolds were removed, the porogen was leached out, and scaffolds were dried in vacuum. The pore morphology was studied with an environmental scanning electron microscope (ESEM).

2.6 Cell Attachment and Migration

Cell behavior on the hydrogel/apatite nanocomposite surfaces was investigated with bone marrow stromal cells. Wistar rats were housed in an AAALAC-approved facility. All experiments were approved by the University of South Carolina Institutional Animal Use and Care Committee and were performed following the recommendations of the Guide for the Care and Use of Laboratory Animals (NIH). Disk-shaped samples were sterilized by transferring to excess 70% ethanol. A sterile sample was added to fibrinogen-coated wells at a density of 1×10^4 cells/cm². The plate was incubated for 48 h for cell attachment. The disks were fixed in 4% paraformaldehyde and permeabilized by soaking in PBS with 0.1% triton x100 and 0.1M glycine. Cell nucleus was stained with 4',6-Diamidino-2-phenylindole (DAPI, violet, 1:5000 dilution) or SYTOX Green (1:5000 dilution) and counterstained with Texas Red-X Phalloidin (1:1000 dilution).

For migration studies, cells were placed on the top surface of a sterile porous hydrogel/apatite scaffold at a density of 2×10^6 cells per scaffold. The scaffold/cell construct was placed in fibrinogen-coated well plates and incubated in primary media (DMEM, 10% FBS, 5% neonatal calf serum, and antibiotics) at 37°C, 5% CO₂, and 95% humidity for 7 days with media change every two days. A commercial kit containing the fluorescent dyes calcein AM and ethidium homodimer-1 (EthD) was used to visualize migrating cells with confocal microscopy. A Zeiss LSM 510 META confocal microscope was utilized to take images in the z-plane, perpendicular to the plane of the microscope.

3 RESULTS AND DISCUSSION

A singlet chemical shift with peak position at 1.6 ppm, two triplets with peaks positions at 3.6 and 4.2 ppm, and a quartet with peak location at 5.1 ppm were observed in the ¹H NMR spectrum of the LMW PLA. The degree of polymerization depended on the reaction time, temperature, catalyst concentration, and the ratio of the monomer to initiator. The PLA chain length and distribution was measured by GPC. The ratio of the peaks in the NMR spectrum due to the chemical shifts at 3.6 ppm and 5.1 ppm was related to the degree of polymerization. The PLA with M_n and PI of 3.3 kDa and 1.6 based on GPC, respectively, resulted in the NMR monomer to initiator peak ratio of 42, corresponding to M_n of 3.1 kDa.

Four singlet chemical shifts with peak positions at 1.6, 3.5, 6.8, and 6.9 ppm, two triplets with peaks positions at 3.6 and 4.2 ppm, and a quartet with peak position at 5.1 ppm were observed in the ¹H NMR spectrum of the PLEOF terpolymer. The presence of peaks at 6.90 ppm in the NMR spectrum attributable to the hydrogens of the fumarate group, and the presence of a band due to the ester carbonyl stretching vibration centered at 1725 cm⁻¹ in the FTIR spectrum, confirmed the incorporation of fumarate monomers into the PLEOF macromer. The PLEOF macromer with PLA and PEG molecular weights of 1.2 kDa (PI of 1.4) and 4.3 kDa (PI of 1.1) had a M_n and PI of 7.4 kDa and 2.3, respectively, as determined by GPC.

The FTIR spectrum of the grafted HA (gHA) subtracted from the untreated HA is shown in Figure 1(left). The shoulder at 2950 cm⁻¹ was due to the symmetric and asymmetric C-H stretching vibrations of the -CH₃ groups of the methacrylate and methoxy silane. These absorption bands were absent in the spectrum of the untreated HA. The absorption with peak location at 1670 cm⁻¹ was assigned to the carbonyl absorption band of the urethane group (amide I) and the broad absorption with peak location at 1580 cm⁻¹ was collectively attributed to the N-H bending and C-N vibration (amide II) of the urethane groups. These peaks were absent in the spectrum of the untreated HA. The absorptions with peak locations at 1420 and 1460 cm⁻¹ was attributed to vibrations of the P-O-Si complex on the surface of HA by the reaction of methoxysilane with phosphate groups. The absorptions in the FTIR spectrum of gHA with untreated HA as the reference confirmed the grafting of PEGMA-PTMS urethane on the surface of HA.

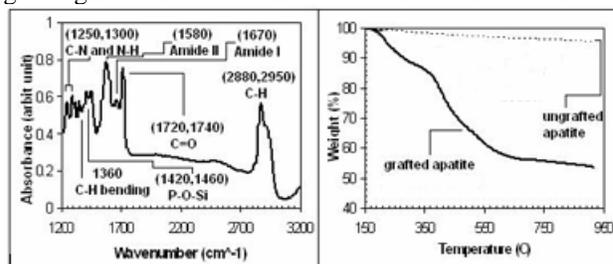


Figure 1: FTIR (left) and TGA (right) spectra of gHA.

Figure 1 (right) shows the weight loss of HA and gHA as a function of temperature. The weight loss of HA and gHA after reaching 900°C was 5.0% and 46.0%, respectively. After subtracting the weight loss of 5.0% due to the bound water, the percent grafting of gHA was 41.0%. Figure 2 shows the electron micrograph of the scaffold before (left) and after (right) the salt leaching step, respectively, at 26x magnification.

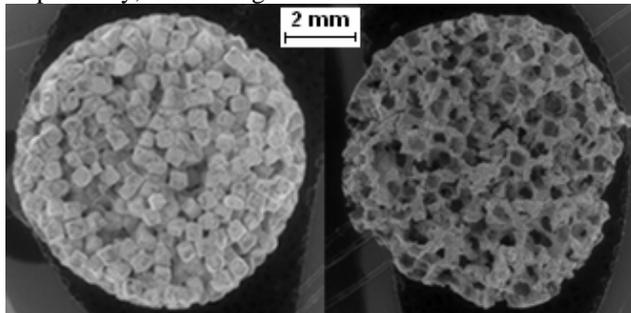


Figure 2: Scaffold before (left) & after (right) salt leaching.

Figure 3 shows the macropores and micropores formed inside the scaffold by the porogen and by the partial phase separation of the PLA and PEG domains of PLEOF at 200x and 3000x magnification, respectively. The average size of the macropores and micropores were 300 μm and 15 μm , respectively. The macropores provide the space for migration of BMS cells while the micropores provide the space for diffusion of nutrients for vascularization if endothelial cells are present.

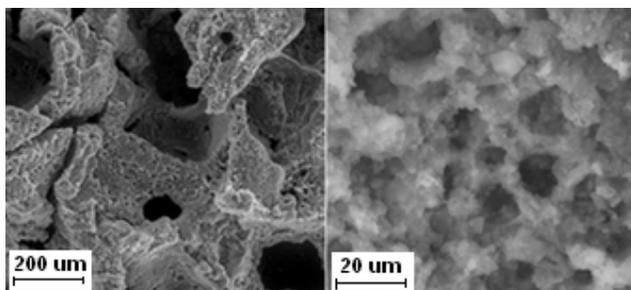


Figure 3: SEM of macropores (left) & micropores (right).

Figure 4 shows fluorescent images of the cells and their morphology on the surface of the samples, respectively.

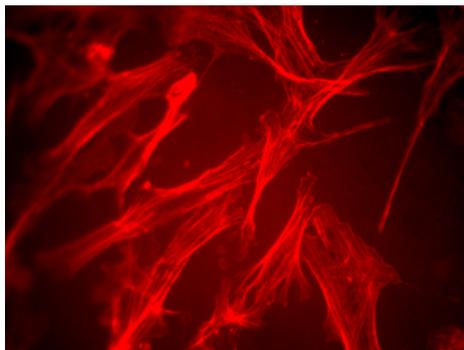


Figure 4: Image of cells and their morphology.

Figure 5 shows the distribution of live (green) and dead (red) cells for a 152 μm thick section, measured from the seeding surface of the scaffold. The image in Figure 5 demonstrates that cells migrated and attached to the pore surfaces of the scaffold.

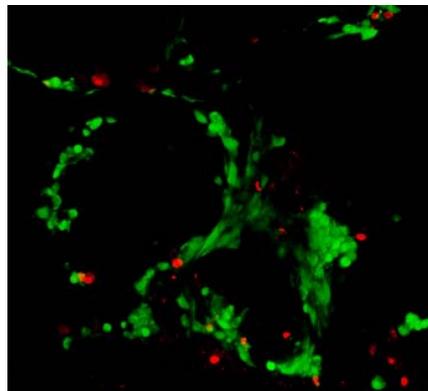


Figure 5: Distribution of live cells in the scaffold.

4 CONCLUSIONS

HA nanoparticles were grafted in two steps with unsaturated oligomers to improve their suspension stability and interfacial bonding to the hydrogel phase. Poly(lactide-ethylene oxide-fumarate) (PLEOF) unsaturated terpolymer was synthesized by condensation polymerization of low molecular weight PLA and poly(ethylene glycol) (PEG). Hydrogel/apatite porous scaffolds were fabricated by porogen leaching. Scaffolds were seeded with bone marrow stromal (BMS) cells to study cell attachment and migration. Cell visualization by confocal fluorescence microscopy demonstrated that cells migrated inside the pore volume of the nanocomposite. Our results demonstrate that the hydrogel/apatite nanocomposite is an attractive alternative as a biomaterial for hard tissue regeneration.

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