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Engineering Bone Formation with Peptidomimetic Hybrid Biomaterials

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Abstract— **Bone exhibits hierarchical levels of organization from macroscopic to microscopic, and nano- length scales. Furthermore, multiple bioactive peptides, as part of the collagenous and non-collagenous water soluble glycoproteins and proteoglycans in the bone ECM, interact with progenitor BMS cells to initiate the cascade of chemotaxis, differentiation, and mineralization. In this work, a nanofiber hydrogel/apatite composite matrix is developed to mimic the laminated structure of the osteons in bone and to determine the effect of RGD and BMP peptides, grafted to the composite, on osteogenic differentiation and mineralization of BMS cells. For four-layer laminates, the Young's modulus of the laminated composites was four times that of the nanofibers alone. BMS cells seeded on RGD+BMP peptide modified composites showed synergistic 4.9- and 11.8-fold increase in calcium content from day 7 to 14 and 21. These findings are potentially useful in developing engineered scaffolds for bone regeneration.**

I. Introduction

Bone is a composite matrix consisting of a mineral and a collagenous phase [1]. The mineral phase provides mechanical strength in compression and an osteoconductive substrate for bone formation [2,3], while the collagenous phase plays a central role in differentiation and maturation of progenitor bone marrow stromal (BMS) cells and the extent of mineral-collagen interactions [4]. Bone exhibits hierarchical levels of organization from macroscopic to microscopic, and nano- length scales [5]. On the microscale, layers of fibrils are glued together by ECM proteins to form laminated structures, called osteons, that makes bone elastic and allows diffusion of nutrients and oxygen to all cells embedded in the bone matrix [6]. The apatite crystals in the bone matrix provide mechanical support in compression, the laminated structure of the osteons confers elasticity, while the hydrophilic ECM proteins allow diffusion of nutrients and oxygen to cells in the bone matrix.

Furthermore, multiple bioactive peptides, as part of the collagenous and non-collagenous water soluble glycoproteins and proteoglycans in the ECM, interact with progenitor BMS cells to initiate the cascade of chemotaxis, differentiation, and mineralization. Among these peptides, RGD sequences associated with ECM matrix proteins (collagen I, fibronectin, sialoprotein, and osteopontin) interact with BMS cells through integrin cell surface receptors to facilitate cell spreading and focal-point adhesion to the ECM [8]. The peptide KIPKA SSVPT ELSAI STLYL (hereafter referred to as the BMP peptide), corresponding to residues 73-92 of the knuckle epitope of *recombinant human* bone morphogenetic protein (rhBMP-2), is implicated in osteogenic differentiation of BMS cells [9].

Biodegradable hydrogel/calcium phosphate (CaP) composites are ideal biomaterials as scaffolds for regeneration of skeletal tissues. The CaP phase provides mechanical strength in compression while the hydrogel phase provides a high water content environment for cell-matrix interactions. We have recently synthesized a novel macromer, poly(lactide-co-ethylene oxide fumarate) (PLEOF), that can be crosslinked in aqueous environment with redox or ultraviolet initiators to produce biodegradable inert hydrogels The crosslink density can be adjusted by the initiator concentration and density of fumarate groups on PLEOF chains [10]. The objective of this work was to develop a composite matrix to mimic the laminated structure of the osteons in bone and to determine the effect of RGD and BMP peptides, grafted to the inert PLEOF composite, on osteogenic differentiation and mineralization of BMS cells.

II. Experimental Procedure

A. PLEOF Synthesis and PLA Fiber Processing

Low molecular weight polylactide (LMW PLA) was synthesized by ring opening polymerization of lactide (LA) [11]. PLEOF was synthesized by condensation polymerization of ULMW PLA and polyethylene glycol (PEG) with fumaryl chloride (FuCl) [10]. The product was purified by precipitation in cold ether and dried in vacuum. The synthesized PLEOF was characterized by 1H-NMR and GPC. The L-PLA fiber mesh was prepared by electrospinning from HFIP solvent (9 wt% polymer concentration) as described [12]. The use of HFIP as the solvent resulted in the production of bead-free fibers at the lowest L-PLA concentration of 9 wt%. The optimum conditions of 1.0 mL/h injection rate, 25 kV electric potential, and 7.0 cm needle-to-collector distance were used in the electrospinning process [12].

B. Fabrication of laminated Composites

The polymerizing mixture was prepared by mixing the PLEOF macromer with BISAM as the cross-linking agent

and a redox initiation system in aqueous solution. Equimolar concentrations of initiator and accelerator were used to keep the pH at 7.4. For samples with 10 wt% hydroxyapatite (HA), 32 mg HA nanoparticles with a diameter of 50 nm was added and the solution was sonicated to break down the aggregates. For samples with 1 wt% Ac-GRGD, 3.2 mg of Ac-GRGD was first dissolved in 100 µl water and then added to the polymer solution. Then PLA fiber layers were dipped in the PLEOF polymerizing mixture, stacked together, the assembly was compressed at a pressure of 4.7 kPa, and allowed to crosslink at 50 ºC. The final thickness of the stack was 0.085±0.015 mm and the weight ratio of the nanofiber in the crosslinked composite was 10±4 wt%.

C. RGD and BMP Peptides Synthesis Grafting

GRGD peptide was synthesized manually on Rink Amide NovaGel resin in the solid phase using a previously described procedure for the synthesis of QPQGLAK peptide [13]. The peptide was functionalized directly on the peptidyl resin by coupling acrylic acid to the N-terminal amine group under conditions used for the amino acid coupling reaction. The BMP peptide was synthesized on Rink Amide NovaGel resin using a procedure similar to GRGD and GRGDK peptides with modification [13]. The pseudoproline dipeptides (oxazolidine) Fmoc-Ser(tBu)-Thr(ΨMe,Mepro)-OH, Fmoc-Leu-Ser(ΨMe,Mepro)-OH, and Fmoc-Ala-Ser(ΨMe,Mepro)-OH were used for the synthesis of BMP peptide to improve the coupling efficiency and product yield. To increase solubility of the azide functionalized BMP peptide in aqueous solution and reduce steric hindrance in the grafting reaction, a mini-PEG spacer was inserted between the BMP peptide and functional azide group. The PEGylated BMP peptide was functionalized directly on the resin by coupling the end amine group of mini-PEG spacer with 4-carboxybenzenesulfonazide to produce the Az-mPEG-BMP peptide. The peptide was purified by preparative HPLC and characterized by a MALDI-TOF/TOF spectrometer. RGD was conjugated PLEOF by the reaction of acrylamide group of Ac-GRGD with fumarate group of PLEOF. Click chemistry was used to covalently attach Az-mPEG-BMP peptide to PLEOF hydrogel by the reaction between the reactive azide group of the peptide and propargyl group of the hydrogel.

D. Osteogenic Differentiation of Stromal Cells

BMS cells were isolated from the bone marrow of young adult male Wistar rats. BMS cells were seeded on composite samples at a density of $2x10^5$ cells/mL and incubated in primary media. After 24 h, the media was replaced with standard osteogenic media and cultured for up to 21 days. At each time point (7, 14, 21 days), BMS cell seeded samples were lysed and aliquots were used for measurement of DNA content, ALPase activity and calcium content.

III. RESULTS AND DISCUSSION

Figure 1 shows the Young's moduli of the electropsun fibers, hydrogel/apatite nanocomposite, and the laminated fiber-reinforced composites in the wet and dry states. The modulus of the fiber mesh under dry and wet conditions was 140±3 MPa, but the modulus of hydrogel under wet condition was two orders of magnitude lower than dry condition (0.50±0.07 versus 139±23 MPa). Addition of HA to the hydrogel did not improve modulus under wet condition (0.50±0.07 MPa without HA versus 0.35±0.06 with HA). The residual stresses at the filler-polymer interface as a result of matrix swelling can decrease modulus in the wet state. For four-layer laminate, the Young's modulus significantly increased to 569 ± 127 MPa which is roughly four times of the modulus of a single nanofiber. This indicates that the number of the layer of nanofibers is one of the most important parameters to determine the final mechanical properties of the fiber-reinforced polymer composites. Addition of 10% HA to the hydrogel reduced modulus of the laminate under dry and wet conditions, which can be explained by the low interfacial interaction

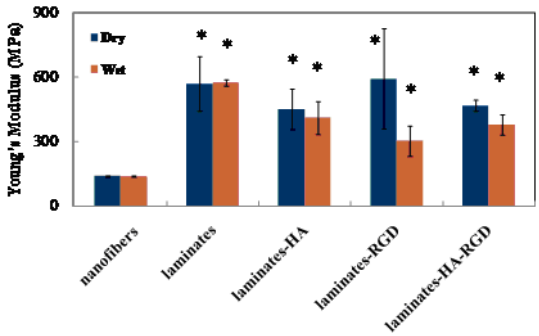

Figure 1. Comparison of Young's moduli for L-PLA nanofibers, laminates, laminates-HA, laminates-RGD, and laminates-HA-RGD under both dry and wet states at 37°C. One star indicates statistically significant difference between the test group and control group (hydrogel). Error bars correspond to means ± standard deviation for n = 3.

between the apatite and hydrogel phases. We and others have demonstrated that energetic interactions at the interface of the hydrogel and filler particles significantly affect viscoelastic properties of the composite [7,14]. For example, addition of an acrylamide-terminated glutamic acid sequence to apatite nanocrystals improved shear modulus of the composite by an order of magnitude [7].

To determine synergistic effects of RGD and BMP peptides on the extent of osteogenic differentiation of BMS cells, experimental groups included composites without peptides (control group), with Ac-GRGD peptide (RGD group), with Ac-mPEG-BMP peptide (BMP group), with Ac-GRGD and Ac-mPEG-BMP peptides (RGD+BMP group), and with Ac-GRGD and mutant Ac-mPEG-BMP peptides (RGD+muBMP group). The DNA content of the composites with incubation time is shown in Figure 2. For all time points, the DNA content of the control group was lower than the other peptide-modified groups. The RGD,

RGD/BMP, and RGD/muBMP groups supported cell growth and exhibited only minor differences between each other. For example, at day 7, the DNA content on RGD, BMP, RGD+BMP, and RGD+muBMP groups showed a statistically significant increase of 2.0, 1.4, 1.9 and 1.7-fold in DNA content compared with control group. After 21

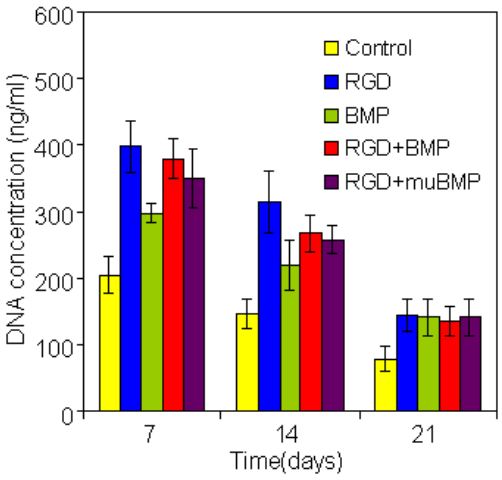

Figure 2. DNA content of BMS cells as a function of incubation time seeded on PLEOF hydrogels and cultured in osteogenic media. Error bars correspond to means ± 1 SD for n = 3.

days, all peptide modified groups had relatively similar DNA contents. The DNA contents demonstrate that conjugation of RGD peptide to the hydrogel improves adhesion of BMS cells to the substrate, especially in the first week after cell seeding.

The calcium content of the composites with incubation time is shown in Figure 3. The calcium content of BMS cells

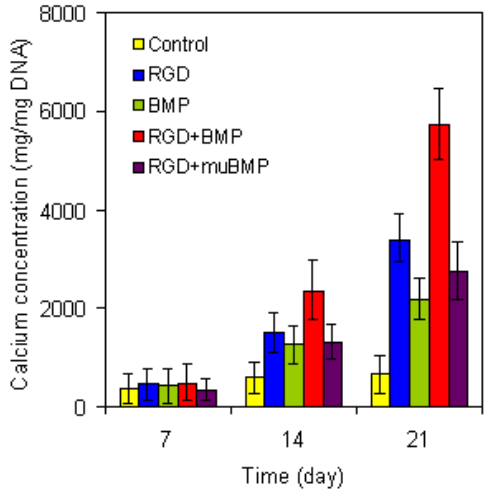

Figure 3. Calcium content of BMS cells as a function of incubation time seeded on PLEOF hydrogels and cultured in osteogenic media. Error bars correspond to means ± 1 SD for n = 3.

seeded on composites without peptide modification did not increase with incubation time in osteogenic media. BMS cells cultured on RGD modified composites showed a 3.2- and 7.3-fold increase in calcium content from day 7 to 14 and 21, respectively. Our results with Ac-GRGD incorporated composites support the fact that RGD peptide functions as a mild promoter of osteogenic differentiation of BMS cells.

BMS cells cultured on BMP peptide grafted composites (without Ac-GRGD) showed a statistically significant 2.9- and 5.1-fold increase in calcium content from day 7 to 14 and 21, respectively. For each time point, calcium content of BMP peptide grafted hydrogels was slightly less than Ac-GRGD conjugated substrates, indicating that RGD peptide modulates osteogenic activity of the BMP peptide by supporting cell spreading and adhesion on the substrate. BMS cells seeded on RGD+BMP peptide modified composites showed 4.9- and 11.8-fold increase in calcium content from day 7 to 14 and 21 (the increase was statistically significant compared to RGD or BMP peptide groups). Furthermore cells seeded on RGD+muBMP hydrogels had calcium content similar to that of RGD only functionalized composites.

The RGD sequence is associated with ECM components like collagen type I, fibronectin, sialoprotein, and osteopontin. It is well established that RGD peptide interacts with BMS cells through integrin cell surface receptors to facilitate spreading and focal-point adhesion [15]. The BMP peptide, corresponding to residues 73-92 of the knuckle epitope of rhBMP-2 protein, has been shown to competitively inhibit the binding of rhBMP-2 protein to type I and type II transmembrane serine/threonine receptor kinase72 and promotes the expression of osteogenic markers like osteocalcin [16]. Our results demonstrate that grafting RGD focal-point adhesion peptide and the BMP peptide to a PLEOF hydrogel synergistically enhances osteogenic differentiation of BMS cells. We speculate that the RGD peptide provides sites for cell attachment to the substrate which enhances the interaction of BMP peptide with type I and type II transmembrane receptors, leading to an increase in ALPase activity and osteogenesis. These findings clearly demonstrate that RGD and BMP peptides, grafted to a composite substrate, act synergistically to increase osteogenic differentiation of BMS cells.

IV. CONCLUSIONS

The objective of this work was to develop a composite matrix to mimic the laminated structure of the osteons in bone and to determine the effect of RGD and BMP peptides, grafted to the composite, on osteogenic differentiation and mineralization of BMS cells. For four-layer laminates, the Young's modulus significantly increased to 569 ± 127 MPa which was approximately four times that of a single layer of electrospun nanofibers. This indicates that the number of the layer of nanofibers is one of the most important parameters to determine the final mechanical properties of the fiber-reinforced polymer composites. Grafting RGD+BMP

peptides to PLEOF composite synergistically increased the extent of mineralization of BMS cells by 4.9 and 11.8-fold after 14 and 21 days, respectively.

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