Coating Electrospun Collagen and Gelatin Fibers with Perlecan Domain I for Increased Growth Factor Binding

Cheryl L. Casper,[†] Weidong Yang,[‡] Mary C. Farach-Carson,*[,†,‡] and John F. Rabolt[†]

Department of Materials Science and Engineering and Department of Biological Sciences, University of Delaware, Newark, Delaware 19716

Received October 18, 2006; Revised Manuscript Received January 17, 2007

Electrospun natural polymer membranes were fabricated from collagen or gelatin coated with a bioactive recombinant fragment of perlecan, a natural heparan sulfate proteoglycan. The electrospinning process allowed the facile processing of a three-dimensional, porous fibril (2−6 μm in diameter) matrix suitable for tissue engineering. Laser scanning confocal microscopy revealed that osteoblast-like MG63 cells infiltrated the depth of the electrospun membrane evenly without visible apoptosis. Tissue engineering scaffolds ideally mimic the extracellular matrix; therefore, the electrospun membrane must contain both structural and functional matrix features. Fibers were coated, after processing, with perlecan domain I (PlnDI) to improve binding of basic fibroblast growth factor (FGF-2), which binds to native heparan sulfate chains on PlnDI. PlnDI-coated electrospun collagen fibers were ten times more effective than heparin−BSA collagen fibers at binding FGF-2. Because FGF-2 modulates cell growth, differentiation, migration and survival, the ability to effectively bind FGF-2 to an electrospun matrix is a key improvement in creating a successful tissue engineering scaffold.

Introduction

Electrospinning is a processing technique in which a high voltage is applied to a polymeric solution to produce small nanometer to micron diameter scale fibers that are ideal for a variety of applications such as filtration,[1,2] biological/chemical resistant clothing,[1,2] and electronic and fuel cell applications.[3−5] Current research in the field focuses on use of electrospun fibers for biomedical applications, including wound dressings,[6] drug delivery vehicles,[7−9] and tissue engineering scaffolds.[7−23] The 3-D, porous membrane provides a microenvironment within the scaffold that contains pores large enough ($\geq$10 μm) for living cells to move throughout the membrane. The porosity allows for nutrient uptake and cellular waste product removal.[24] The nanometer diameter electrospun fibers mimic the size scale of fibrous proteins found in the extracellular matrix (ECM).[17] For these reasons, a variety of natural and synthetic polymers such as: poly(glycolic acid), poly(ethylene-*co*-vinylacetate), poly(ethylene-*co*-vinyl alcohol), collagen, and fibrinogen have been electrospun for tissue engineering and other related purposes.[6,7,11,12,25]

In this work, electrospinning was used to fabricate natural polymers derived from collagen and gelatin. Currently, the most popular methods of bone substitution are external allografts implanted in patients and autografts grafted from one area of the body to another. More recently, the use of synthetic materials has provided another option for patients and are increasingly popular because of lower cost, reduced infection, higher success rate, and fewer surgeries. Scaffolds for bone regeneration have been composed of various materials such as glass beads, ceramics, collagens, tricalcium phosphate, hydroxyapatite, and a variety of synthetic polymers.[26]

A scaffold must mimic the ECM in both structure and function. The structural requirements include small fiber diameters (<250 nm), high-matrix porosity (pores > 10 μm), large void volume, large surface-to-volume ratio, mechanical strength, and a three-dimensional structure to allow for cell infiltration.[24,27] It must be biocompatible and serve as a reservoir for growth factors which must be released on time scales supporting tissue regeneration.[24,28] The electrospinning process is a relatively simple and quick method to produce matrices with the above characteristics, making electrospinning an ideal technique to fabricate tissue engineering constructs.

Although collagen is native to ECM, coupling of additional proteins to the fiber surface can enhance the recruitment and differentiation of engrafted cells. Perlecan (Pln) is a heparan sulfate proteoglycan found as a major component of basement membranes, in the territorial matrix of cartilage, and in other mesenchymal tissues.[29] Pln is large (more than 4000 amino acids) and consists of five distinct domains. Domain I (PlnDI) is unique and contains three glycosaminoglycan (GAG) attachment sites. The GAG chains found in PlnDI are typically heparan sulfate (HS) chains, but can be substituted with chondroitin sulfate (30). Domains II−V contain motifs that are similar to those found in neural cell adhesion molecules, epidermal growth factor, and laminin.[30] The presence of these five domains, together, play a direct role in the function of Pln in thrombosis,[31] ECM formation,[32] cell proliferation, cellular uptake,[33] tumor invasion,[34,35] angiogenesis,[35,36] and chondrogenesis.[37] Therefore, the presence of Pln in an electrospun matrix would aid in tissue regeneration.

HS proteoglycans have found clinical use in the area of inflammation, cancer, and wound repair partially because of their ability to bind growth factors.[38] The HS chains provide a reservoir to stabilize and store growth factors while preventing them from thermal denaturation and proteolytic degradation.[38] Therefore, PlnDI, acting as a mini-proteoglycan, can bind heparin-binding growth factors (HBGFs), including basic fibroblast growth factor (bFGF or FGF-2), vascular endothelial

* Corresponding author. Address: Room 326 Wolf Hall, Department of Biological Sciences, University of Delaware, Newark, DE 19716. Phone: (302) 831-2277. Fax (302) 831-2281. Email: farachca@udel.edu.
[†] Department of Materials Science and Engineering.
[‡] Department of Biological Sciences.

10.1021/bm061003s CCC: $37.00
Published on Web 02/28/2007

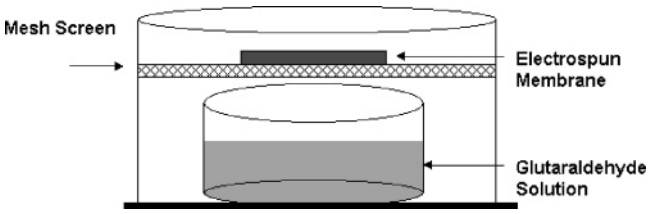

Figure 1. Schematic of the crosslinking apparatus used in these experiments.

growth factor (VEGF), heparin-binding epidermal growth factor (HBEGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β).[39] PlnDI thus endows a tissue engineering construct with the property of sequestering and releasing HBGFs in a physiologically relevant context.[40,41]

FGF-2 modulates growth, differentiation, migration, and survival in a variety of cell types.[42,43,44] FGF-2 is expressed by osteoblastic cells and is present during early stages of fracture repair.[28] Kato et al.[45] showed that local FGF-2 injection stimulated healing of a bone defect in rabbit tibia with a single injection found to increase bone volume by 95% and bone-mineral content by 36%. Pln regulates the activity of FGF-2 both in vivo and in vitro.[36] Binding of FGF-2 to heparan sulfate significantly increases its biological activity.[30,42,35] Therefore, we tested the idea that having PlnDI associated with an electrospun matrix should increase binding, activation, and delivery of FGF-2.

Experimental Procedures

Materials. Collagen (type I acid soluble from calf skin, Sigma Aldrich, St. Louis, MO) was dissolved in a 1:1 ratio of acetic acid (Fisher Scientific, Atlanta, GA) to 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma Aldrich) at an 18% (w/v) concentration. Gelatin (courtesy of Eastman Kodak Corporation, Rochester, NY) was dissolved in HFIP at a 15% (w/v) concentration. An antibiotic/antimycotic (Sigma Aldrich) was used to prevent microbial growth.

Electrospinning. The electrospinning apparatus consisted of a 5 mL syringe (Popper & Sons, Inc., New Hyde Park, NY) connected to a syringe pump (Orian Sage, Fisher, Fair Lawn, NJ). The syringe pump allowed for a steady flow of polymer solution to the tip of the needle. A high-voltage power supply (Glassman Series EH, High Bridge, NJ) was used to apply a voltage to the needle. The collector consisted of a 3" $\times$ 3" metal sheet covered in non-stick aluminum foil, which was placed 22 cm from the tip of the needle to collect the dried fibers. For fabrication of collagen fibers, a needle (0.51 mm inner diameter, Hamilton, Reno, NV) 9 mL/hr flow rate, +14 kV voltage, and 22 cm working distance were used. The gelatin fibers were electrospun using a needle (0.51 mm inner diameter), 1 mL/ min flow rate, +15 kV voltage, and 22 cm working distance.

Crosslinking. The collagen and gelatin fibers readily dissolved in aqueous media, necessitating that they be crosslinked before any cellular work could begin. Initially, glucose (Sigma Aldrich) was investigated as a crosslinking agent by directly adding the glucose to collagen and gelatin solutions prior to electrospinning. The aldehyde group of the glucose reacts with the amino group of the collagen to allow for the crosslinking of adjacent collagen chains.[46]

Glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA), a commonly used crosslinking agent, also was studied as an alterative to glucose. Glutaraldehyde cross-links collagen and gelatin fibers via the reaction between the carboxyl groups on the glutaraldehyde and the amide groups of the collagen or gelatin to form cross-links between polymer chains. Glutaraldehyde was employed at various concentrations ranging from 0.5 to 6% (w/w) (19 h) to determine the optimal concentration needed for crosslinking. Figure 1 shows the setup for the glutaraldehyde vapor-phase crosslinking experiments. A scanning electron microscope (Hitachi S-4700, Pleasanton, CA) was used to determine if crosslinking affected fiber structure or size. The electrospun fibers were placed on a carbon adhesive tab to secure the fibers onto a mount during imaging.

Table 1. Samples Tested Using Kaiser Test

sample	concentration of glutaraldehyde used for crosslinking (% w/w)
polystyrene fibers (negative control)	N/A
collagen Fibers	1
collagen Fibers	4
gelatin Fibers	1
gelatin Fibers	4

Measures of Crosslinking. The Kaiser Test Kit (Sigma Aldrich (St. Louis, MO) was used to determine if any free amines remained after the glutaraldehyde crosslinking reaction.[47] The reaction of ninhydrin with free primary amines produces a blue colored solution because of the presence of Ruhemanns Blue complex. The presence of free amines allows for further chemical coupling to be completed on the fibers. Table 1 lists the samples tested for the presence of free amines.

Cell seeding and Attachment. The osteoblastic cell line, MG63 (American Type Culture Collection [ATCC], Manassas, VA), was cultured in DMEM medium supplemented with 10% (v/v) FBS, 100U/mL of penicillin and 100ug/mL of streptomycin, and incubated at 37 °C in a humidified atmosphere of 5% CO_2. Culture medium was changed every 2−3 days. The MG63 cells then were detached with 1% (w/v) trypsin/EDTA at 80−90% confluence, pelleted by centrifugation, and resuspended. Before cell seeding, the electrospun membranes were sterilized by 70% (v/v) ethanol for 30 min and irradiated under UV light for 2 h. MG63 cells were seeded on electrospun membranes as described previously.[40,41] After washing extensively with phosphate buffer saline (PBS), electrospun membranes were placed into 24-well tissue culture dishes containing an MG63 cell suspension (2×10^5/mL) in DMEM supplemented with 10% (v/v) FBS. Using a dynamic seeding method, the 24-well plates were placed on an orbital shaker (Lab-Line Instruments, Inc. Melrose Park, IL) and rotary agitated (100rpm) in an incubator at 37 °C for 4 h. After gently washing with DMEM to remove non-adherent cells, the cell-seeded electrospun membranes were transferred to new 24-well plates and incubated in DMEM containing 1% (v/v) FBS in a humidified atmosphere of 95% air and 5% CO_2. Culture medium was changed every 3 days. For confocal microscopic analysis of the cells attached on electrospun membranes, cell-seeded electrospun membranes after 3 days of culture were washed with DMEM three times and transferred to Nalge-Nunc "Permanox" chambered slides (4 well, Nalge-Nunc, Naperville, IL), and then were stained with SYTO 13 (Molecular Probes) green fluorescent nucleic acid stain (1:1000) in DMEM for 30 min. Data were acquired on a Zeiss inverted 100M Axioskop equipped with a Zeiss 510 LSM confocal microscope (Zeiss, Oberkochen, Germany).

Cellular Viability and Proliferation Assay. Cell viability and proliferation was measured using an MTS assay (CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay, Promega, Pittsburgh, PA). The AQ_{ueous} One Solution Reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-t etrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). The principle mechanism of this assay is that metabolically active cells react with a tetrazolium salt in MTS agent to produce a soluble formazan dye that can be absorbed at 490 nm wavelength. The cultures of cell-seeded electrospun membranes were harvested at 4, 12, and 24 h, and on days 3, 5, 7, and 10. At each time point, three samples were used to measure a number of attached cells on electrospun membranes. Each sample was rinsed three times with PBS, followed by incubation with MTS reagent in serum-free culture medium for 3 h. Aliquots then were pipetted into three wells

of a 96-well culture plate and the absorbance at 490 nm of the content in each well was measured using a spectrophotometric plate reader.

Heparin−BSA Synthesis and Biotinylation. Heparin−BSA (bovine serum albumin) was synthesized by mixing 34 mg of BSA (67 000 g/mol, Sigma Aldrich), 912 mg heparin (12 500 g/mol, Sigma Aldrich), and 25 mg of sodium cyanoborohydride (Sigma Aldrich) in 5 mL of potassium phosphate (0.2 M solution, pH 8.0). The solution was incubated at 37 °C for 48 h. Aliquots (5 mL) then were removed and placed into dialysis (SpectraPor Membrane, molecular weight cut off MWCO = 3500 g/mol, Spectrum Laboratories Inc., Rancho Dominquez, CA) against 1000 mL of distilled water for 24 h. The solution then was concentrated using a filter device (Amicon Ultra-15, MWCO= 5000 g/mol, Millipore, Burlington, MA) and centrifuged for 1 h. In order to biotinylate the heparin−BSA complex, 459 μL of 10 mM sulfo-NHS-LC−biotin solution (EZ-Link Sulfo-NHS-LC-Biotinylation Kit, Pierce Biotechnology Inc., Rockford, IL) was added to 454 μL of the heparin−BSA solution. The biotin and heparin−BSA molecule cross-link via an amide bond. This mixture was placed on ice for 2 h, separated by gel filtration chromatography, and the protein fractions were identified by monitoring at 280 nm. The high molecular weight fractions were pooled and filtered (Amicon Ultra-15, MWCO= 30,000 g/ mol, Millipore). The Coomassie Assay (Coomassie plus-200 protein assay, Pierce Biotechnology Inc. Biotechnology Inc.) was employed to determine protein concentration. BSA (2.0 mg/mL) was used to construct a calibration curve to determine the concentration of the heparin−BSA solutions based on their absorbance at 595 nm. The final concentration of the heparin−BSA−biotin protein was determined to be 10.7 mg/mL.

Dot Blot Assay. A dot blot assay with a Bio-Dot Microfiltration apparatus was used to determine if the heparin−BSA−biotin complex bound HBGFs. A nitrocellulose membrane was pre-wetted with PBS and placed in the dot blot apparatus. To each well, 100 μL of PBS was added and vacuum-dried. Growth factor (recombinant human bFGF/rhFGF-2, R&D Systems Inc., Minneapolis, MN) was added to nine wells at a concentration of 200 ng/100 μL per well. BSA (3% w/ v in PBS−T) was added to the other nine wells. BSA served as a negative control for this assay. The solutions were gravity filtered through the wells for 2 h at room temperature, then pump filtered to remove any remaining solution. A 5% (w/v) skim milk in PBS−T solution was made and this solution (200 μL) was added to each well and filtered for 2 h at room temperature. A vacuum was used to remove any excess milk. The wells then were washed three times with blocking buffer (200 μL). To the first six wells was added the heparin−BSA−biotin complex (3 μg protein in 100 μL per well). The next six wells contained 100 μL of PlnDI−biotin (0.1 μg protein per well) to serve as a positive control. The negative control consisted of 100 μL of BSA−biotin (3 μg protein) per well. These solutions were incubated for 2 h at room temperature. Any remaining solution was vacuum filtered from the membrane. The membrane was removed from the dot blot apparatus and blocked overnight using 30 mL of BSA (3% w/v in PBS-T) using slight agitation at 4 °C. The next day, 1 μL of NA-HRP (1 mg/mL) was added to 50 mL of blocking buffer. Six mL of this solution then were diluted to 24 mL with blocking buffer and was placed on the membrane for gentle agitation for 40 min. The membrane then was rinsed with 35 mL of PBS-T for a total of four washes. Using the SuperSignal West Kit (Pierce Biotechnology Inc.), 3 mL of each SuperSignal West solution was mixed and added to the membrane for a 1 min incubation period. The membrane was dried and placed in plastic wrap. The membrane then was exposed on film for 10 s and developed.

Attachment of Heparin−BSA−Biotin and PlnDI−Biotin to Electrospun Fibers. PlnDI and heparin−BSA−biotin proteins were attached to the electrospun collagen and gelatin fibers using a coupling reaction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce Biotechnology Inc.) and *N*-hydroxysulfosuccinimide (NHS, Pierce Biotechnology Inc). EDC is a water-soluble carbodiimide that is used to activate carboxyl groups for reaction with primary amines. An unstable, amine-reactive intermediate (*O*-acylisourea) is formed by the reaction of EDC with a carboxyl group. This intermediate can react with another amine or can be hydrolyzed. To stabilize this intermediate, NHS is added. NHS converts the amine-reactive *O*-acylisourea into an amine-reactive NHS ester. This stabilization helps to increase the overall efficiency of the coupling reaction and allows for "two-step" crosslinking to occur where only one carboxyl group on a protein is coupled, leaving the other free for further coupling chemistry.[48] A graphic depiction of the essentials of this reaction is available in the Pierce Biotechnology catalog.

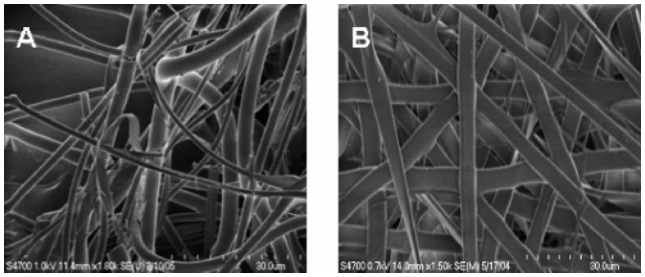

Figure 2. FESEM micrographs of electrospun (A) collagen and (B) gelatin fibers. Collagen fibers were approximately 3−6 μm in diameter and had a circular or tube-like appearance (panel A), whereas gelatin produced uniform fibers that appeared flat and were 2−6 μm in diameter.

Before coupling proteins to the electrospun membranes, the fibers were dried in a 37 °C oven for 2 days and then rinsed three times with PBS. Ten crosslinked collagen and ten crosslinked gelatin membranes were placed in separate tubes. To the collagen tube was added MES buffer (20 mL) (50 mM, 2-(N-morpholino)ethanesulfonic acid, Acros Organics, Fairlawn, NJ), and 10 mL of the MES buffer was added to the gelatin samples; the membranes then were incubated at room temperature for 30 min. The membranes were transferred to separate glass tubes containing 30 mM EDC, 6 mM NHS (in MES buffer), and 100 μL (unless otherwise noted) of protein (either PlnDI or heparin−BSA). The samples were incubated in this solution for 2 h at room temperature. The fibers then were washed for a total of six times with PBS over a 24 h period.

To determine if the EDC/NHS coupling of biotinylated species to the fibers was successful, horseradish peroxidase-conjugated NeutrAvidin (NA-HRP, Pierce, 0.1 μg/mL) was used. All experiments were completed at room temperature in 24-well polystyrene assay plates (Corning Inc., Corning, NY), blocked with 3% (w/v) BSA (Sigma Aldrich) in PBS-T (Invitrogen, Carlsbad, CA). The assay plates were blocked for 24 h, and the electrospun fiber samples were blocked for 1 h. Samples were placed in the wells and incubated with 500 μL of NA-HRP (in SuperBlock buffer) at room temperature for 30 min. The fibers then were washed three times with PBS−T and incubated in 500 μL per well, of TMB substrate solution (Pierce Biotechnology Inc.) for 10−12 min. Aliquots (50 μL) of the colored solution were removed and mixed with 50 μL of sulfuric acid (1 M) to stop the reaction and allow for optical density measurements. The absorbance, measured at 450 nm, corresponded to the amount of heparin−BSA−biotin or Pln−biotin bound to the electrospun fibers. Because this is a colorimetric assay, the presence of heparin−BSA−biotin or Pln−biotin complex is indicated by the appearance of a dark blue color on the fibers. The controls for this experiment consisted of collagen and gelatin electrospun fibers that were exposed to the EDC/NHS reagents but not to the heparin−BSA−biotin or Pln−biotin molecules.

Growth Factor Binding. Growth factor binding assays were employed to determine if the electrospun fibers containing either heparin−BSA or PlnDI bound FGF-2. These assays also were completed in order to determine if there are any functional differences between heparin−BSA and PlnDI in binding growth factors. All growth factor experiments were completed at room temperature in 24-well polystyrene assay plates, blocked with 3% (w/v) BSA in PBS. All electrospun samples were washed with PBS at room temperature.

Samples were placed in the wells and incubated with 500 μL of a solution of FGF-2 (10 ng/mL in PBS) for 2 h. After seven washes with PBS-T, FGF-2 antibody (500 μL) (R&D Systems, 2 μg/ mL in PBS) was added to each well and incubated for 2 h. The samples were washed seven more times with PBS-T then incubated for 30 min in 500 μL solution prepared from 1 μL NA-HRP, 8 mL PBS, and 2 mL SuperBlock Blocking Buffer. The fibers then were washed seven times with PBS-T and incubated in 500 μL of TMB substrate solution for 10−12 min. The absorbance, measured at 450 nm, from the ELISA assay corresponded to the amount of growth factor bound to the electrospun fibers. The experiments were performed in triplicate, with single measurements of three separately prepared samples. Variation is reported as standard deviation from the mean.

Results

Preparation and Crosslinking of Electrospun Collagen and Gelatin Membranes. Electrospinning conditions for the fabrication of collagen fibers were first optimized. Collagen can be electrospun from a variety of solvents including formic acid, acetic acid, glycolic acid, and HFIP.[27,49] We found a 1:1 ratio of HFIP:acetic acid to be the best choice on the basis of the relatively short time needed for electrospinning and the characteristics of the electrospun fibers produced. The electrospun collagen fibers (Figure 2A) were approximately 3−6 μm in diameter and had a circular or tube-like appearance. Gelatin, or denatured collagen, also was used to optimize processing parameters as a cheaper alternative to collagen type I. Gelatin electrospun from 15% (w/v) solution in HFIP, produced fibers that appeared flat and were 2−6 μm in diameter (Figure 2B).

A protocol[49] was optimized which used 70% (v/v) ethanol/water solution and a UV lamp to sterilize all glassware, materials, and equipment.[50] The electrospinning box and all accompanying materials were thoroughly washed with the ethanol solution and then subjected to the UV lamp, located inside the electrospinning box, for 10 min before each use. The fibers then were incubated in an antibiotic/antimycotic solution overnight. To test for fiber sterilization, fibers were placed in media and incubated at 37 °C for 24 h. At that time, the media was visually inspected to ensure no clouding of the solution had occurred, indicating there had been no contamination. Next, the media was tested using polymerase chain reaction (PCR)[51] to further confirm the absence of mycoplasma DNA. We thus confirmed that the sterilization process was effective in preventing microbial contamination over the course of these experiments.

Because of the solubility of collagen and gelatin fibers, crosslinking was used to stabilize the fibers for cellular assays. Initially glucose was investigated as a possible crosslinking agent because of its nontoxic nature. Glucose (6 mM) was mixed with the collagen and gelatin solutions before electrospinning. The electrospun glucose/collagen and glucose/gelatin mats were UV irradiated for 2 h. The fibrous mats then were placed in a 70% (v/v) ethanol/water solution for 48 h. Unfortunately, incorporation of glucose into electrospun collagen or gelatin mats failed to stabilize the fibrous structures when placed in ethanol/water rinse. Thus, glutaraldehyde, another crosslinking agent, was investigated.

Although glutaraldehyde is commonly used in the crosslinking of biomaterials, it can be cytotoxic.[52,53] Therefore, limiting the amount of glutaraldehyde that is exposed to the fibers was essential for this reason and for preservation of the free amines present in the polymer backbones, which are needed for coupling chemistry to attach proteins to the fiber surface. For these reasons, the minimum amount of glutaraldehyde required to sufficiently cross-link the fibers was investigated in the range from 0.5 to 6% (w/w). Figure 3 demonstrates that as little as 0.5% glutaraldehyde was sufficient to cross-link electrospun gelatin or collagen fibers. These fibers maintained their fiber structure after immersion in an ethanol/water solution. Thus, all subsequently manufactured collagen and gelatin fibers were crosslinked using a 0.5% glutaraldehyde solution.

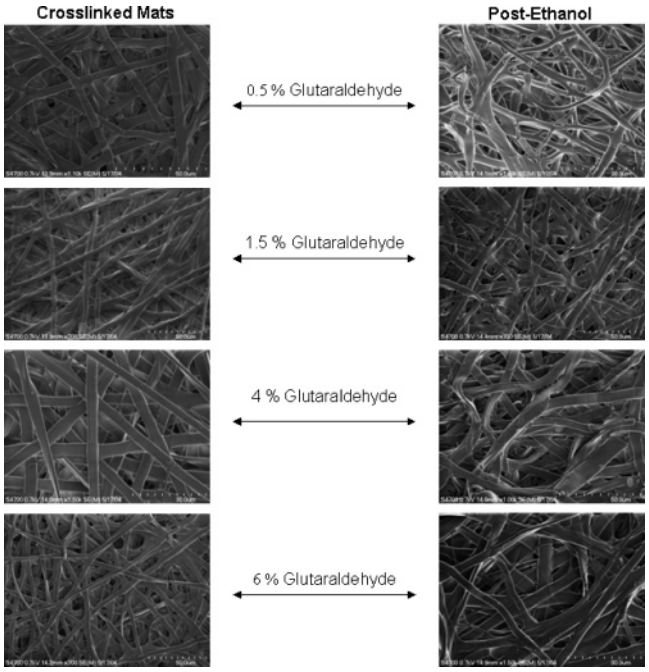

Figure 3. Scanning electron micrographs of crosslinked electrospun gelatin fibers before (left-hand column) and after (right-hand column) immersion in ethanol/water solution. 0.5% glutaraldehyde was found to be sufficient to stabilize fiber structure.

An electrospun gelatin mat initially was studied to determine if cells (MG63, human osteosarcoma cell line) would attach and infiltrate the crosslinked, fiber membrane without added protein. We were concerned about the small and inconsistent spacing between fibers that could hinder cell infiltration through the matrix. Laser Scanning Confocal Microscopy (LSCM) images showed that cells could attach and infiltrate the electrospun matrix over a short time period (Figure 4, panels A−C). Figure 4B shows a 3-D LSCM image illustrating the ability of the cells to move through the spaces between the fibers. Figure 4C shows cell infiltration throughout the depth of the electrospun matrix. A MTS assay was completed to ensure the viability of the cells. Figure 5 shows that over a 10 day period, cells remain alive and proliferate through approximately two doublings on the gelatin fiber scaffold, indicating that the glutaraldehyde at this concentration (0.5%) was not harmful to the cells. These results showed that the crosslinked electrospun membrane provides a suitable three-dimensional scaffold for the attachment and infiltration of osteoblastic cells. Additionally, there was no evidence of apoptosis or cell death on the electrospun fiber scaffold.

A major focus of this work was to optimize the attachment of proteins to electrospun collagen and gelatin fibers to not only enhance cell attachment and proliferation, but also to modulate cell behavior and further promote tissue regeneration. To enhance the tissue regeneration process on electrospun scaffolds, heparan sulfate bearing PlnDI was chosen for its known ability to bind HBGFs and to aid in cellular processes. Heparin−BSA−biotin complex was synthesized to serve as an artificial proteoglycan to optimize processing protocols and as a com-

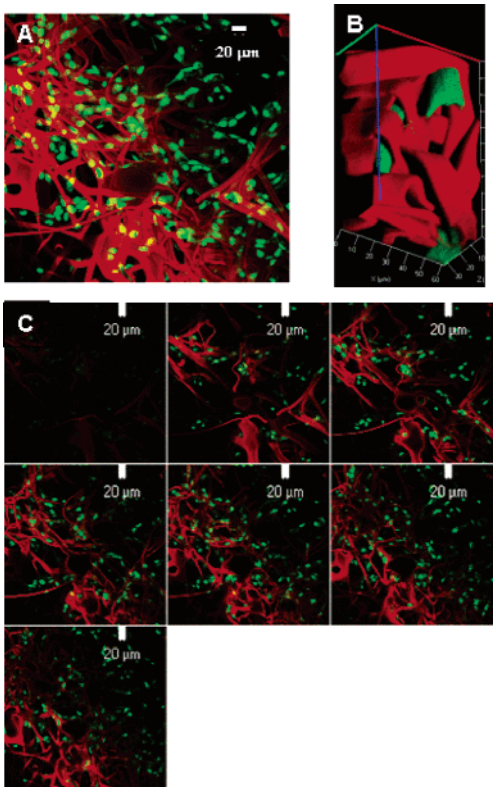

Figure 4. (a) LSCM images of gelatin scaffold seeded with MG63 cells, (b) three-dimensional rendering of LSCM image, and (c) *z*-stack images of cells infiltrating throughout the depth of the membrane.

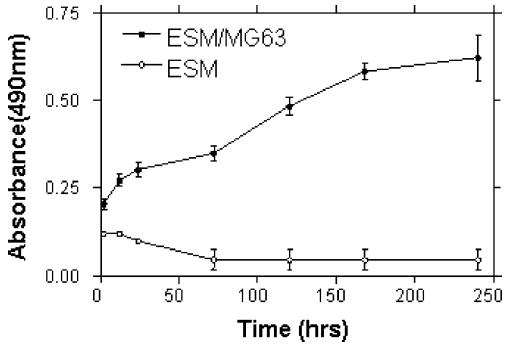

Figure 5. Viability and growth of MG63 osteoblastic cells on electrospun gelatin membrane over a 10 day period. Cells remained viable and underwent two doublings under these conditions. Note that nuclei remain condensed, indicating little if any apoptosis occurred.

parison to the PlnDI. Biotin was added for detection purposes and does not interfere with growth factor binding to heparin or heparan sulfate. The heparin−BSA−biotin complex is structurally similar to that of PlnDI in that it contains a polyanion (heparin), which is similar to the heparan sulfate in PlnDI, and is attached to a protein core (BSA).

The heparin−BSA−biotin complex and PlnDI−biotin were tested for their ability to bind FGF-2 using a dot blot assay on nitrocellulose membrane. BSA−biotin complex served as the negative control. As seen in Figure 6, both the heparin−BSA−biotin complex and the PlnDI−biotin complex bound FGF-2 in a concentration dependent manner, which indicated that the biotin conjugation did not interfere with growth factor binding. Note that the PlnDI concentration used on the membrane had to be decreased to 0.1 μg/well to prevent saturation of the film exposure, whereas the heparin−BSA−biotin complex was tested at a 3.0 μg/well concentration. This reflected the increased ability of PlnDI to bind FGF-2 when compared to heparin−

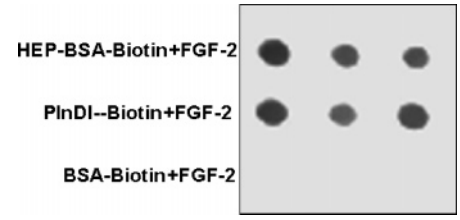

Figure 6. Heparin−BSA−biotin complex and PlnDI−biotin complexes bind FGF-2 when attached to nitrocellulose membrane ("dot blot assay"). The PlnDI concentration used on the membrane was 0.1 μg/well (middle row), whereas the heparin−BSA−biotin complex was tested at a 3.0 μg/well concentration (upper row).

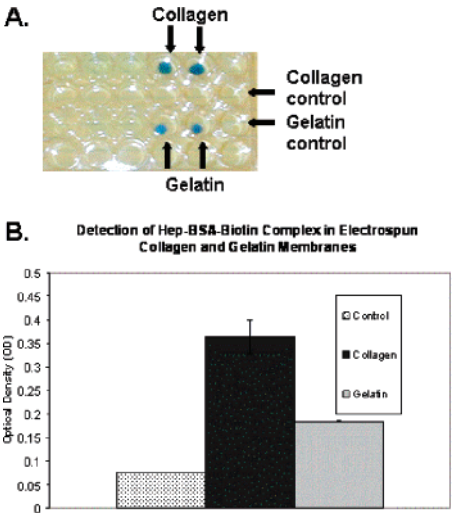

Figure 7. Heparin−BSA−biotin attaches to electrospun fiber mats made of either electrospun collagen or gelatin. The intensity of the blue color in panel A indicates the amount of biotin conjugate remaining associated with the fibril mat in the bottom of the well. This is quantified in panel B as described in Experimental Procedures.

BSA and may reflect an increased number of growth factor binding sites or a tighter binding.

The heparin−BSA−biotin complex next was attached to the surface of the electrospun fibers via EDC/NHS coupling reaction. The use of heparin−BSA−biotin allowed for EDC/NHS reaction optimization using this model system before using PlnDI. The success of the EDC/NHS reaction in coupling the heparin−BSA−biotin to the electrospun fibers was determined by using NA-HRP, which binds to biotin. The blue color indicates the presence of the heparin−BSA−biotin complex. As seen in Figure 7A, both collagen and gelatin were found to have heparin−BSA−biotin attached to the fibers (blue color) and the control samples showed no color change. This data was not, however, quantitative. Optical density measurements next were performed on the collagen, gelatin, and control samples to determine if there was a difference in the relative amount of heparin−BSA−biotin that binds to collagen versus gelatin. The data shown in Figure 7B indicate that collagen binds more heparin−BSA−biotin than does gelatin. This finding suggests that the collagen has a greater number of free amine sites available for EDC/NHS coupling. These results indicate that although collagen binds more heparin−BSA−biotin complex, both collagen and gelatin are suitable candidates for the EDC/NHS coupling reaction of proteins to the surface of these electrospun fibers. The effect of coupling on mat pore size is difficult to assess because of the nature of the electrospinning process and the nonuniformity of the fibril spacing. Spacing between fibers is a function of the jet instability, jet velocity, and humidity, among other factors. Therefore the spacing

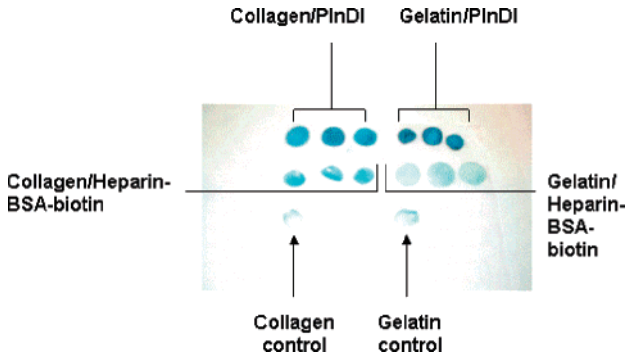

Figure 8. Heparin−BSA and PlnDI bound to collagen or gelatin fiber complexes bind FGF-2. Using the growth factor binding assay described in Experimental Procedures, binding of FGF-2 to various fiber bound complexes was performed in parallel and a digital photograph of the results was taken.

between fibers can vary depending on which section of the sample is examined. Nonetheless, we found that the pore sizes in our spun fibers were, on average, greater than 10 μm, both pre- and post-coupling when examined under the light microscope. Because we saw no observable difference, we did not examine these by SEM.

After the presence of the heparin−BSA−biotin on the surface of the electrospun fibers was confirmed by biotin detection, the ability of the heparin−BSA complex to bind FGF-2 was investigated to ensure the fiber coupling process did not adversely affect heparin's growth factor binding activity. As shown in Figure 8, both the collagen/heparin−BSA and gelatin/heparin−BSA fiber complexes bound FGF-2 (blue color, middle rows). Collagen−heparin BSA bound complexes showed slightly higher amount of binding of FGF-2 (darker blue) than did gelatin−heparin BSA bound complexes in this non-quantitative assay. Both were higher than uncoated collagen or gelatin fiber mat controls (bottom row). Next, PlnDI−biotin and PlnDI (without biotin which was not used in this phase of the study) were attached to electrospun collagen and gelatin fibers using the same procedures employed for the attachment of heparin−BSA−biotin molecules. Interestingly, PlnDI bound to collagen or gelatin fiber mats (upper row) bound more FGF-2 than did heparin−BSA bound to those same fibers. To determine if this was because of a higher degree of binding of PlnDI than heparin−BSA to the fibers, we measured binding of NeutrAvidin to biotinylated complexes on collagen or gelatin fibrils. The amount of heparin−BSA−biotin was varied to determine if very low concentrations of this molecule could be used. A higher concentration solution was made by using 100 μL (or 1.07 mg) of heparin−BSA−biotin and a lower concentration solution was prepared by adding only 20 μL of solution (or 0.0214 mg) to the buffer. As shown in Figure 9, we found a much greater level of NeutrAvidin binding to fibril immobilized PlnDI (0.009 mg) than to heparin−BSA even at the high concentration (1.07 mg), indicating that PlnDI bound very efficiently to electrospun fibrils.

In the final experiment shown in Figure 10, we attached either heparin−BSA or PlnDI to fibril mats of collagen or gelatin and measured binding of FGF-2. As shown, it took 10 times (protein weight basis) as much heparin−BSA to bind approximately the same amount of FGF-2 as bound by PlnDI. This was true regardless of whether the electrospun fibril was composed of gelatin or collagen. Differences were significant with p value <0.01.

Taken together, this study demonstrated that heparin−BSA or PlnDI can be attached to electrospun fibers via EDC/NHS

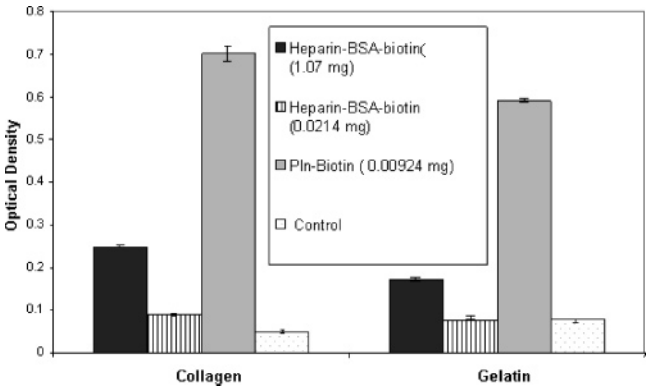

Figure 9. Detection of biotin-containing proteins on electrospun collagen and gelatin fibers coupled via EDC/NHS reaction. NeutrAvidin binding to fibril immobilized biotinylated proteins was performed as described in Experimental Procedures and the results quantified. Results are the mean of triplicates and error bars show standard deviation from the mean.

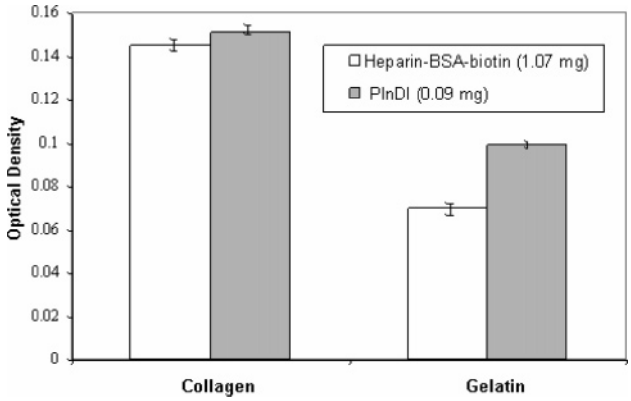

Figure 10. Binding of FGF-2 to heparin−BSA or PlnDI bound to fibril mats of collagen or gelatin. Binding was performed and quantified as described in Experimental Procedures. Results are the mean of triplicates, and error bars show standard deviation from the mean ($p < 0.01$).

coupling and both maintain their ability to bind growth factors such as FGF-2. Comparative binding studies demonstrated that a much higher concentration of heparin−BSA than PlnDI is needed to effectively bind FGF-2.

Discussion

These results showed that PlnDI and heparin−BSA can be chemically attached to electrospun fibers using an EDC/NHS coupling reaction. Although both proteins were effective at binding FGF-2, PlnDI displayed higher binding at lower concentrations, thus suggesting its utility in tissue engineering scaffolds. Other research groups have demonstrated the ability of electrospun membranes to be used in biomedical applications. For example, Xu et al.[20] employed aligned, electrospun poly(L-lactide-*co*-caprolactone) to serve as a scaffold for human coronary artery smooth muscles cells (SMCs) to be used in blood vessel engineering. The researchers showed that SMCs attached to the electrospun fibers and migrated along the axis of the aligned fibers. The SMCs also showed a spindle-like contractile phenotype, which is characteristic of this cell type.[20] Shin et al.[54] cultured cardiomyoctes on electrospun poly(caprolactone) fibers for use as a cardiac graft. Findings showed that the cardiomyoctes began synchronized beating after only 3 days in culture and the cells secreted cardiac-specific and ECM proteins onto the electrospun scaffold.[54] However, these studies

focused on recapitulating the structural aspects of the ECM only and lack the incorporation of biological elements, such as growth factors and other ECM proteins. In order to make a truly ECM-mimetic scaffold, biological elements need to be incorporated so the scaffold mimics the ECM in both structure and function.

Recently, Chew et al.[55] successfully mixed human nerve growth factor (NGF) into a copolymer solution of poly(caprolactone) and poly(ethyl ethylene phosphate) (PCLEEP) to produce electrospun fibers containing NGF. Findings suggested that the growth factor was present in aggregate form throughout the fiber matrix at low loading levels ($3.10 \pm 0.53 \times 10^{-4}$ %). The authors believed that the presence of these aggregates, along with the low loading levels, may be attributed to the phase separation that was observed during electrospinning due to the different charge densities of the aqueous growth factor solution and the polymer solution. However, despite the phase separation, NGF was released from the electrospun matrix. The NGF showed a burst release of 20%, then a steady release over a period of 3 months. The authors suggested that it was likely that the electrospinning process may have denatured the NGF, however, they were unable to quantify the NGF bioactivity after release. Our current study shows that electrospun fibers can be successfully used to bind growth factors for biomedical applications. The goal of this work was to couple proteins to the surface of electrospun fibers to enhance growth factor binding for tissue engineering applications. By adding the growth factors to the electrospun membranes, we can circumvent some of the problems that Chew and co-workers[55] encountered such as phase separation and denaturing of growth factors.

These studies have laid the foundation for preparing electrospun collagen and gelatin fibers for tissue engineering applications. Fiber production, crosslinking methods, sterilization protocols, and protein attachment have been studied for optimal conditions to employ these fibers as bioengineered scaffolds. Both electrospun collagen and gelatin consist of 2−6 μm fiber diameters. The electrospun membranes are mechanically stable and remain intact during extensive assays where stress is applied to the membranes (via repeated sample handling, washing and vortexing). Although the membranes are crosslinked to decrease water solubility, there are residual free amines available for coupling chemistry. The use of EDC/NHS proved successful in coupling proteins, including heparin−BSA and PlnDI, to the fibers. PlnDI was shown to bind a greater amount of FGF-2 than heparin−BSA, even though the PlnDI was present at significantly lower concentrations. This suggests that PlnDI is a superior candidate than heparin for use in these tissue engineering constructs because lower concentrations can be employed while still achieving significant growth factor binding.

This research has provided the necessary protocols for preparing electrospun membranes for use as tissue engineering constructs for bone and other tissue regeneration. Ongoing work will determine if the presence of PlnDI versus heparin−BSA enhances cell proliferation or behavior. Future work is planned to investigate the ability of PlnDI bound to electrospun membranes to bind other HBGFs and the effect of this binding on cell behavior. Growth factors such as TGF-β (transforming growth factor), PDGF (platelet-derived growth factor), and BMP (bone morphogenic protein) should be considered because of their roles in bone regeneration.

Acknowledgment. The authors thank Dr. Kirk Czymmek and Debbie Powell for their assistance with LSCM and FESEM. We also thank NSF-NIRT, Delaware INBRE, and NASA for partial support of this work. In addition, C.L.C. thanks the University of Delaware NSF/IGERT program for partial support during this project. W.Y was supported by R01 DE13542.

References and Notes

(1) Schreuder-Gibson, H.; Gibson, P.; Senecal, K.; Sennett, M.; Walker, J.; Yeomans, W.; Ziegler, D.; Tsai, P. P. *J. Adv. Mater.* **2002**, *34*, 44−55.
(2) Gibson, P.; Schreuder-Gibson, H.; Rivin, D. *Colloids Surf. A* **2001**, *187*, 469−481.
(3) Drew, C.; Wang, X. Y.; Samuelson, L. A.; Kumar, J. *J. Macromol. Sci., Pure Appl. Chem.* **2003**, *A40*, 1415−1422.
(4) Lu, J. Y.; Norman, C.; Abboud, K. A.; Ison, A. *Inorg. Chem. Commun.* **2001**, *4*, 459−461.
(5) Dai, H. Q.; Gong, J.; Kim, H.; Lee, D. *Nanotechnology* **2002**, *13*, 674−677.
(6) Kenawy, E. R.; Layman, J. M.; Watkins, J. R.; Bowlin, G. L.; Matthews, J. A.; Simpson, D. G.; Wnek, G. E. *Biomaterials* **2003**, *24*, 907−913.
(7) Kenawy, E. R.; Bowlin, G. L.; Mansfield, K.; Layman, J.; Simpson, D. G.; Sanders, E. H.; Wnek, G. E. *J. Controlled Release* **2002**, *81*, 57−64.
(8) Jiang, H. L.; Fang, D. F.; Hsiao, B. J.; Chu, B. J.; Chen, W. L. *J. Biomater. Sci., Polym. Ed.* **2004**, *15*, 279−296.
(9) Jing, Z.; Xu, X. Y.; Chen, X. S.; Liang, Q. Z.; Bian, X. C.; Yang, L. X.; Jing, X. B. *J. Controlled Release* **2003**, *92*, 227−231.
(10) Bhattarai, S. R.; Bhattarai, N.; Yi, H. K.; Hwang, P. H.; Cha, D. I.; Kim, H. Y. *Biomaterials* **2004**, *25*, 2595−2602.
(11) Boland, E. D.; Bowlin, G. L.; Simpson, D. G.; Wnek, G. E. *Abstr. Papers Am. Chem. Soc.* **2001**, *222*, 31-PMSE.
(12) Boland, E. D.; Wnek, G. E.; Simpson, D. G.; Pawlowski, K. J.; Bowlin, G. L. *J. Macromol. Sci., Pure Appl. Chem.* **2001**, *38*, 1231−1243.
(13) Huang, L.; McMillan, R. A.; Apkarian, R. P.; Pourdeyhimi, B.; Conticello, V. P.; Chaikof, E. L. *Macromolecules* **2000**, *33*, 2989−2997.
(14) Huang, L.; Nagapudi, K.; Apkarian, R. P.; Chaikof, E. L. *J. Biomater. Sci., Polym. Ed.* **2001**, *12*, 979−993.
(15) Jin, H. J.; Chen, J. S.; Karageorgiou, V.; Altman, G. H.; Kaplan, D. L. *Biomaterials* **2004**, *25*, 1039−1047.
(16) Kenawy, E. R.; Abdel-Fattah, Y. R. *Macromol. Biosci.* **2002**, *2*, 261−266.
(17) Li, W. J.; Laurencin, C. T.; Caterson, E. J.; Tuan, R. S.; Ko, F. K. *J. Biomed. Mater. Res.* **2002**, *60*, 613−621.
(18) Luu, Y. K.; Kim, K.; Hsiao, B. S.; Chu, B.; Hadjiargyrou, M. *J. Controlled Release* **2003**, *89*, 341−353.
(19) Min, B. M.; Lee, G.; Kim, S. H.; Nam, Y. S.; Lee, T. S.; Park, W. H. *Biomaterials* **2004**, *25*, 1289−1297.
(20) Xu, C. Y.; Inai, R.; Kotaki, M.; Ramakrishna, S. *Biomaterials* **2004**, *25*, 877−886.
(21) Zong, X. H.; Li, S.; Chen, E.; Garlick, B.; Kim, K. S.; Fang, D. F.; Chiu, J.; Zimmerman, T.; Brathwaite, C.; Hsiao, B. S.; Chu, B. *Ann. Surg.* **2004**, *240*, 910−915.
(22) Casper, C. L.; Yamaguchi, N.; Kiick, K. L.; Rabolt, J. F. *Biomacromolecules* **2005**, *6*, 1998−2007.
(23) Mo, X. M.; Xu, C. Y.; Kotaki, M.; Ramakrishna, S. *Biomaterials* **2004**, *25*, 1883−1890.
(24) Lu, L. C.; Mikos, A. G. *MRS Bull.* **1996**, *21*, 28−32.
(25) Wnek, G. E.; Carr, M. E.; Simpson, D. G.; Bowlin, G. L. *Nano Lett.* **2003**, *3*, 213−216.
(26) Reddi, A. H. *Tissue Eng.* **2000**, *6*, 351−359.
(27) Matthews, J. A.; Wnek, G. E.; Simpson, D. G.; Bowlin, G. L. *Biomacromolecules* **2002**, *3*, 232−238.
(28) Lieberman, J. R.; Daluiski, A.; Einhorn, T. A. *J. Bone Joint Surg., Am. Vol.* **2002**, *84A*, 1032−1044.
(29) Costell, M.; Gustafsson, E.; Aszodi, A.; Morgelin, M.; Bloch, W.; Hunziker, E.; Addicks, K.; Timpl, R.; Fassler, R. *J. Cell Biol.* **1999**, *147*, 1109−1122.
(30) Knox, S.; Merry, C.; Stringer, S.; Melrose, J.; Whitelock, J. *J. Biol. Chem.* **2002**, *277*, 14657−14665.
(31) Nugent, M. A.; Iozzo, R. V. *Int. J. Biochem. Cell Biol.* **2000**, *32*, 115−120.
(32) Iozzo, R. V. *Matrix Biol.* **1994**, *14*, 203−208.
(33) Ebara, T.; Conde, K.; Kako, Y.; Liu, Y. Z.; Xu, Y.; Ramakrishnan, R.; Goldberg, I. J.; Shachter, N. S. *J. Clin. Invest.* **2000**, *105*, 1807−1818.
(34) Tessler, S.; Rockwell, P.; Hicklin, D.; Cohen, T.; Levi, B. Z.; Witte, L.; Lemischka, I. R.; Neufeld, G. *J. Biol. Chem.* **1994**, *269*, 12456−12461.

(35) Sharma, B.; Handler, M.; Eichstetter, I.; Whitelock, J. M.; Nugent, M. A.; Iozzo, R. V. *J. Clin. Invest.* **1998**, *102*, 1599−1608.
(36) Aviezer, D.; Hecht, D.; Safran, M.; Eisinger, M.; David, G.; Yayon, A. *Cell* **1994**, *79*, 1005−1013.
(37) French, M. M.; Smith, S. E.; Akanbi, K.; Sanford, T.; Hecht, J.; Farach-Carson, M. C.; Carson, D. D. *J. Cell Biol.* **1999**, *145*, 1103−1115.
(38) Mongiat, M.; Otto, J.; Oldershaw, R.; Ferrer, F.; Sato, J. D.; Iozzo, R. V. *J. Biol. Chem.* **2001**, *276*, 10263−10271.
(39) Ishihara, M.; Sato, M.; Hattori, H.; Saito, Y.; Yura, H.; Ono, K.; Masuoka, K.; Kikuchi, M.; Fujikawa, K.; Kurita, A. *J. Biomed. Mater. Res.* **2001**, *56*, 536−544.
(40) Yang, W. D.; Gomes, R. R.; Alicknavitch, M.; Farach-Carson, M. C.; Carson, D. D. *Tissue Eng.* **2005**, *11*, 76−89.
(41) Yang, W. D.; Gomes, R. R.; Brown, A. J.; Burdett, A. R.; Alicknavitch, M.; Farach-Carson, M. C.; Carson, D. D. *Tissue Eng.*, in press.
(42) Roghani, M.; Mansukhani, A.; Dellera, P.; Bellosta, P.; Basilico, C.; Rifkin, D. B.; Moscatelli, D. *J. Biol. Chem.* **1994**, *269*, 3976−3984.
(43) Sakiyama-Elbert, S. E.; Hubbell, J. A. *J. Controlled Release* **2000**, *65*, 389−402.
(44) Nimni, M. E. *Biomaterials* **1997**, *18*, 1201−1225.
(45) Kato, T.; Kawaguchi, H.; Hanada, K.; Aoyama, L.; Hiyama, Y.; Nakamura, T.; Kuzutani, K.; Tamura, M.; Kurokawa, T.; Nakamura, K. *J. Orthopaedic Res.* **1998**, *16*, 654−659.
(46) Ohan, M. P.; Weadock, K. S.; Dunn, M. G. *J. Biomed. Mater. Res.* **2002**, *60*, 384−391.
(47) Kay, C.; Lorthioir, O. E.; Parr, N. J.; Congreve, M.; McKeown, S. C.; Scicinski, J. J.; Ley, S. V. *Biotechnol. Bioeng.* **2000**, *71*, 110−118.
(48) www.PierceBiotechnologyInc.net.com, 2005.
(49) Stephens, J. S. Ph.D. Dissertation, University of Delaware, Newark, DE, 2003.
(50) Sherwood, J. K.; Riley, S. L.; Palazzolo, R.; Brown, S. C.; Monkhouse, D. C.; Coates, M.; Griffith, L. G.; Landeen, L. K.; Ratcliffe, A. *Biomaterials* **2002**, *23*, 4739−4751.
(51) PCR work was completed by Anissa Brown in the Department of Biology at the University of Delaware, Newark, DE.
(52) Tomihata, K.; Burczak, K.; Shiraki, K.; Ikada, Y. In *Polymers of Biological and Biomedical Significance*; 1994; Vol. 540, pp 275−286.
(53) Han, B.; Jaurequi, J.; Tang, B. W.; Nimni, M. E. *J. Biomed. Mater. Res.* **2003**, *65A*, 118−124.
(54) Shin, M.; Ishii, O.; Sueda, T.; Vacanti, J. P. *Biomaterials* **2004**, *25*, 3717−3723.
(55) Chew, S. Y.; Wen, J.; Yim, E. K. F.; Leong, K. W. *Biomacromolecules* **2005**, *6*, 2017−2024.

BM061003S