FUNCTIONALIZATION OF POLY(ETHYLENE GLYCOL) HYDROGELS BY HEPARIN PROMOTES OSTEOGENIC DIFFERENTIATION OF HMSCS

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Abstract: Heparin was modified with methacrylate groups, copolymerized with dimethacrylated poly(ethylene glycol), and analyzed as a localized delivery vehicle for bFGF and synthetic extracellular matrix for the differentiation of hMSCs. By deriving cues from molecules normally present in the extracellular matrix (ECM), synthetic cell scaffolds can be designed that actively sequester important bioactive signals. Heparin binds reversibly with many proteins, therefore, poly(ethylene glycol) based biomaterials, normally resistant to cell adhesion, functionalized with heparin in order to sequester important proteins, can actively and selectively stimulate desired cell functions. Results demonstrate that methacrylate-modified heparin retained its ability to bind heparin-binding proteins both in solution and when copolymerized with dimethacrylated PEG in a hydrogel. In addition, the heparin functionalized gels can deliver biologically active bFGF for up to 5 weeks. Finally, the gels were examined as a potential osteogenic scaffold for hMSC culture and were found to promote adhesion and osteogenic differentiation.

Introduction

Biomaterials selected for their bulk properties (e.g., modulus, biodegradability, water content) may not possess qualities conducive to cell adhesion, proliferation, and tissue evolution. Consequently, adsorption or immobilization of proteins is an attractive method to improve biomaterial-cell interactions. Fibronectin has been coupled to the surfaces of poly(vinyl alcohol) gels and shown to regulate endothelial cell ingrowth and attachment [1]; however, modification of biomaterials with full proteins can be very complex. Protein coupling to biomaterials requires mild reaction conditions, as the proteins are subject to both denaturation and degradation. As an alternative to inclusion of full proteins, significant interest has emerged in the design of cell scaffolds that actively sequester proteins and other important bioactive signals. Many of these cues are derived from molecules normally present in the extracellular matrix (ECM). The extracellular matrix (ECM) is complex, composed of collagens, laminin, fibronectin, and glycosaminoglycans. In native tissues, ECM molecules have been shown to influence strongly proliferation, differentiation, migration, and particularly important to tissue engineering, regeneration of damaged tissues [2].

The ECM is also known to function as a reservoir of endogenous growth factors [3], serving to localize growth factor activity, prevent growth factor degradation, and in some cases, enhance binding to cell surface receptors [4]. Growth factors contribute to tissue regeneration at various stages of cell proliferation and differentiation [3]. Polymeric growth factor delivery systems based on heparin can store growth factors in a manner similar to the native ECM. Based on this *in vivo* storage mechanism, controlled release of growth factors has been described from heparin-carrying polystyrene-bound collagen substrata [5], acid gelatin hydrogels [6], alginate gels containing heparin [7], photocrosslinkable chitosan hydrogels [8], and a fibrin-based system incorporating a heparin-binding peptide [9]. Also, heparin has been studied previously as a means to control the orientation of adsorbed fibronectin [10]. In addition, evidence exists that heparin can also promote cell adhesion [11]. Interestingly, heparin is capable of interacting with numerous proteins associated with human mesenchymal stem cell (hMSC) adhesion (e.g., fibronectin, vitronectin), proliferation (e.g., basic fibroblastic growth factor (bFGF)), and osteogenic differentiation (e.g., bone morphogenetic proteins, pleiotrophin) [12].

Exploiting the knowledge of cell interactions with natural ECM molecules can be beneficial in the development of biomaterials that actively and selectively stimulate desired cellular functions important for tissue growth and healing. In this regard, poly(ethylene glycol) (PEG) based biomaterials provide an especially useful platform from which to initiate fundamental studies. PEG-based materials are resistant to non-specific protein adsorption, and thus, PEG-based scaffolds can be used to introduce systematically selected cell signaling epitopes

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and study how this influences cell attachment and subsequent cellular functions.

Toward the end of designing a PEG-based gel that specifically sequesters growth factors that influence cell function, macromolecular heparin monomers were utilized in a novel growth factor sequestering biomaterial. Many methods have been explored to deliver growth factors, including microparticles [13] and chemical tethering [14]. However, these methods have problems such as growth factor damage or degradation, maintaining biological activity upon release, and the reliance on chemical degradation for the release of the molecule. Hence, taking advantage of the natural binding mechanism of heparin for the design of a bioactive matrix is very promising. In the present study, macromolecular heparin monomers were synthesized and copolymerized with dimethacrylated PEG monomers to yield hydrogels of varying composition. These gels were analyzed as a possible delivery vehicle for bFGF and synthetic extracellular matrix for osteogenic differentiation of hMSCs.

Materials and Methods

Material Synthesis: Poly(ethylene glycol) dimethacrylate (PEGDM) was synthesized by dissolving PEG (MW~4600) in methylene chloride. Triethylamine (TEA) at 20% molar excess was added dropwise, and the solution was mixed under argon for 5 min. Methacryloyl chloride at 20% molar excess was mixed with methylene chloride and added dropwise to the PEG/TEA solution, and the final mixture was stirred overnight. The product was precipitated in ice-cold diethyl ether, filtered, and dried in a dessicator. After drying, the PEGDM was redissolved in diH_2O and dialyzed (Spectrum, 1000MW cutoff) over 24 h with two distilled water exchanges. ^{1}H NMR analysis (in chloroform-d, Cambridge Isotopes) on the PEGDM revealed an average of 88% methacrylation.

Heparin (sodium salt from porcine intestinal mucosa, MW ~ 16 kDa) was methacrylated following a previous method [15]. Briefly, a 2% (w/v) solution of heparin in diH_2O was prepared and reacted with 5-fold excess of methacrylic anhydride. The pH of the reaction mixture was adjusted to 8.5 using 5N NaOH, and the reaction was allowed to proceed overnight at 4 ºC. The product, methacrylated heparin, was precipitated once in 95% ethanol, dried, and dialyzed (Spectrum, 1000MW cutoff) for 48 hours against diH_2O. ^{1}H NMR analysis (in D_2O, Cambridge Isotopes) revealed an average of 6 and 22% methacrylation, respectively, for two separate reactions. The percentage of methacrylation refers to the number of methacrylate groups per heparin disaccharide unit. With exception to the native PAGE (see section 2.3), the 22% methacrylated heparin was utilized for all experiments herein. Structures of PEGDM, heparin, and methacrylated heparin are shown in Figure 1.

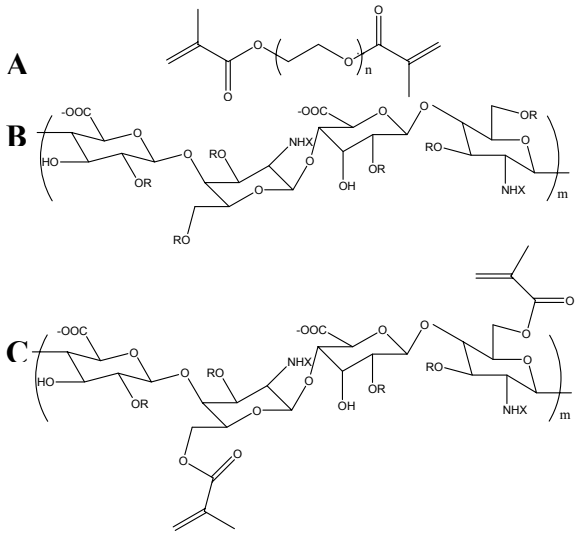

Figure 1: Chemical structures of the dimethacrylated macromer PEGDM (A), n = 105, heparin (B), m = 15, R = -H or -OSO_3H, X = -OCH_3 or -OSO_3H, and methacrylated heparin (C), m = 15, R = -H or -OSO_3H, X = -OCH_3 or -OSO_3H.

Interactions of functionalized heparin monomer with heparin-binding proteins: Methacrylated heparin with different degrees of methacrylate modification and nonmethacrylated heparin (as a control) were dissolved in PBS and combined with 25 μg/ml basic fibroblastic growth factor (bFGF). These solutions were diluted 1:2 with native PAGE sample buffer (0.0625 M Tris-HCl pH 6.8, 30% (v/v) glycerol, and 0.1% (w/v) bromophenol blue in diH_2O) and electrophoresed on a 4-15% Ready-Gel precast polyacrylamide Tris-HCl gels (Bio-Rad, Hercules, CA) using a vertical electrophoresis system (Mini-Protean II, Bio-Rad) at 150 V in native PAGE running buffer (3 g Tris base and 14.4 g glycine per 1 L diH_2O). bFGF bands were detected with a BioSafe Coomassie Stain (Bio-Rad), and band intensity was quantified using Kodak 1D software. bFGF bands indicate free or unbound bFGF, while the bFGF complexed with heparin appears as a higher molecular weight band. Results are reported as the difference in bFGF band intensity of the bFGF + methacrylated heparin sample from pure bFGF divided by the difference in band intensity of the bFGF + heparin sample from pure bFGF.

The ability of the PEG:heparin copolymer hydrogels to non-covalently interact with heparin-binding proteins, fibronectin (FN) and bFGF, was determined. A native protein solution (either FN or bFGF) in PBS was spiked with ^{125}I-labeled protein as a percentage of total protein. The solution was added to the hydrogels and proteins were adsorbed for 48 hours while shaking. After the incubation period, gels were washed with copious amounts of PBS, and the protein adsorbed to the hydrogels was quantified using scintillation counting.

Mesenchymal stem cell culture: Human mesenchymal stem cells (hMSCs) were purchased from Cambrex and cultured in low-glucose Dulbecco's modified eagle

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medium (Gibco) supplemented with 10% FBS (Invitrogen), 1% penicillin/streptomycin (Gibco), 0.25% gentamicin (Gibco), and 0.25% fungizone (Gibco). hMSCs after passage 3 were used in this study.

bFGF sequestering and localized delivery by heparin functionalized gels: Hydrogels were fabricated as above and allowed to swell overnight in PBS. bFGF (750 ng/gel) in PBS with 0.1% sodium azide was added to the gels and allowed to adsorb over 48 hours while shaking. The bFGF solution was then removed and fresh PBS was added. The desorption of bFGF was monitored over 5 weeks. Samples, taken at intervals, were stored until assayed with an ELISA (Quantikine, R & D Systems) using standard manufacturer's instructions.

Adhesion of hMSCs on heparin functionalized gels: hMSCs were trypsinized from culture plates, counted, centrifuged, resuspended, and seeded onto sterile hydrogel disks at a density of 2 x 10^4 cells/cm^2. After 4 hours, the disks were rinsed with PBS and cells were fixed in 4% paraformaldehyde in PBS for 10 min. Phase contrast images (Nikon Eclipse TE300) were taken of the fixed hMSCs.

hMSC gene expression on heparin functionalized gels: hMSC gene expression was analyzed using reverse transcription polymerase chain reaction. Cells were trypsinized from culture plates, counted, centrifuged, resuspended, and seeded onto sterile hydrogel disks at a density of 5 x 10^3 cells/cm^2. After 2 days, 1 week, and 2 weeks, gels with attached cells were removed from culture and rinsed three times with PBS. Total RNA was isolated using a guanidinium thiocyanate/phenol reagent (TRI reagent, Sigma) and standard manufacturer's protocols. After allowing the RNA pellet to dry, it was resuspended in nuclease-free water, and any residual genomic DNA in the samples was digested (DNase I, Invitrogen). RNA was then quantified using the RiboGreen assay (Molecular Probes) based on the manufacturer's instructions.

Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad). A 15 ng total RNA sample was used for the single strand cDNA synthesis. The reverse transcription reaction was incubated at 25 ºC for 5 min, 42 ºC for 30 min, and terminated at 85 ºC for 5 min. PCR was conducted using the iCycler Real-Time PCR machine (Bio-Rad), and primers and probes were designed using the Beacon Designer primer design program. Primers (Invitrogen) and probes (Integrated DNA Technologies) for alkaline phosphatase (ALP), osteopontin (OPN), collagen type I (Col I), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used in a multiplex format. The following PCR parameters were utilized: 95 ºC for 90 s followed by 45 cycles of 95 ºC for 30 s and 55 ºC for 60 s. Threshold cycle (C_T) analysis was used to quantify PCR products, normalized to GAPDH and relative to expression of hMSCs seeded on the homopolymer PEG gels.

Results

Macromolecular heparin monomers were synthesized and copolymerized with dimethacrylated poly(ethylene glycol) monomers to form copolymer hydrogels and investigated as a possible delivery vehicle for bFGF and as a synthetic ECM material to promote osteogenic differentiation of hMSCs. Heparin interacts reversibly with hundreds of proteins. This quality can be exploited for tissue engineering applications by creating heparin functionalized gels that combine a synthetic component to modulate bulk properties with a native ECM component to sequester proteins to which cells will interact and alter their function.

Interactions of the modified heparin monomer with heparin-binding proteins: Heparin possesses specific binding interactions with many proteins, of which bFGF is one, and this property was retained after modification with methacrylate groups and after copolymerizing methacrylated heparin with dimethacrylated PEG. When bFGF is combined with heparin or methacrylated heparin, decreased staining intensity for bFGF on native PAGE gels indicates bFGF binding to heparin or methacrylated heparin. The results in Table 1 are reported as a percent of control binding.

Table 1: Percent of control (native heparin) binding based on native PAGE analysis of heparin demonstrated that bFGF binding to heparin decreased with increasing extent of methacrylation (% methacrylation show in parentheses). * $p < 0.05$, n = 3 samples per condition.

Heparin + bFGF	Methacrylated heparin (6%) + bFGF	Methacrylated heparin (22%) + bFGF
1 ± 0.07	0.92 ± 0.05	0.77 ± 0.08*

The degree of bFGF association with methacrylated heparin was dependent on the extent of methacrylation of heparin, with a higher degree of modification corresponding to a decreased bFGF binding.

Copolymerized methacrylated heparin and dimethacrylated PEG gels were also shown to specifically retain noncovalently bound proteins, bFGF and FN, as evidenced by the radioactivity of gels swelled with ^{125}I-protein solutions (Table 2).

Table 2: Methacrylated heparin copolymerized with PEGDM into hydrogels retained its ability to specifically bind to heparin-binding proteins fibronectin (FN) and basic fibroblastic growth factor (bFGF) in a heparin dose-dependent manner. * $p < 0.05$ of sample versus control (10% PEGDM), n = 10 samples per condition.

	10% PEGDM	10% PEGDM + 0.01 mM methacrylated heparin	10% PEGDM + 0.025 mM methacrylated heparin
bFGF (μg/gel)	0.0001 ± 0.00001	0.003 ± 0.0003*	0.007 ± 0.0002*
FN (μg/gel)	0.068 ± 0.007	1.52 ± 0.047*	3.69 ± 0.3*

To demonstrate that the proteins were binding specifically to heparin contained in the gels, negative control gels (PEG only) were also assayed and showed no significant

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protein adsorption. Results indicate that proteins, including bFGF and FN, are still able to associate and bind with methacrylated heparin that has been copolymerized with dimethacrylated PEG to form a hydrogel.

bFGF sequestering and localized delivery by heparin functionalized gels: The release of bFGF from heparin/PEG copolymerized gels into PBS was measured over time as shown in Figure 2.

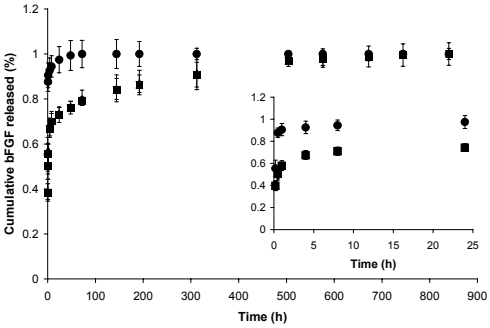

Figure 2: Release of bFGF adsorbed in 10% PEGDM gels (●), 10% PEGDM + 0.01 mM methacrylated heparin gels (▲), and 10% PEGDM + 0.025 mM methacrylated heparin gels (■) at 37 ºC.

The release of bFGF from unmodified PEG gels was very rapid, releasing all bFGF after 8 hours. The release from 0.01 mM and 0.025 mM methacrylated heparin gels was initially rapid, but then continued releasing slowly over a 5-week time period, following zero order release kinetics.

Adhesion of hMSCs on heparin functionalized gels: Heparin, due to its ability to bind proteins, including extracellular matrix proteins such as fibronectin and collagen, was methacrylated and copolymerized with dimethacrylated PEG. Due to its hydrophilic and uncharged nature, PEG-based hydrogels are inherently non cell-adhesive. hMSCs, adhesion-dependent cells, were seeded on PEG:heparin copolymer hydrogels and are shown in Figure 3.

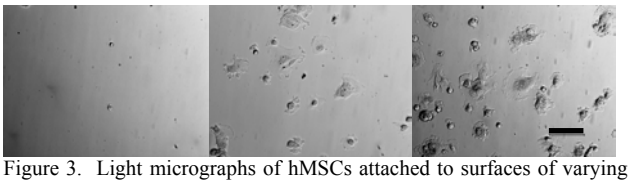

Figure 3. Light micrographs of hMSCs attached to surfaces of varying composition, bar = 100 μm.

There is a dose-dependent improvement of attachment of hMSCs to hydrogels incorporating heparin over hydrogels with no modification.

hMSC gene expression on heparin functionalized gels: Gene expression profiles for the cultured hMSCs, normalized to GAPDH and relative to expression of cells cultured on PEG alone, are quantified in Figures 4, 5, and 6 for Col I, ALP, and OPN, respectively. For Col I expression, an initial (day 2) decrease (2-fold compared to 10% PEGDM for both concentrations of heparin), followed by an increase over unmodified PEG gels at days 7 (1.05-fold and 1.4-fold for 0.01 mM and 0.025 mM heparin) and 14 (2.4-fold and 2.4-fold for 0.01 mM and 0.025 mM heparin).

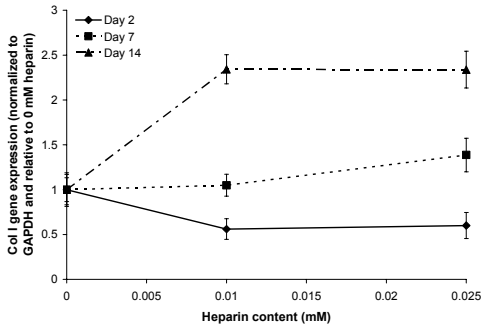

Figure 4: Collagen type I (Col I) gene expression normalized to levels of GAPDH of hMSCs cultured on 10% PEGDM (control), 10% PEGDM + 0.01 mM methacrylated heparin, and 10% PEGDM + 0.025 mM methacrylated heparin hydrogels at 2 days, 1 week , and 2 weeks (error bars designate standard deviation). n = 4 samples per condition.

Similar trends are found for ALP expression (Figure 5), with an initial decrease at day 2 for heparin-containing gels, followed by an increase at days 7 (1.1-fold and 1.4-fold for 0.01 mM and 0.025 mM heparin) and 14 (1.7-fold and 1.9-fold for 0.01 mM and 0.025 mM heparin) over unmodified PEG gels. The expression of Col I and ALP was greatest at day 14, increasing over time with the study.

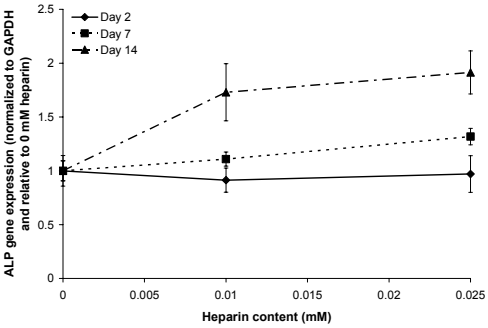

Figure 5: Alkaline phosphatase (ALP) gene expression normalized to levels of GAPDH of hMSCs cultured on 10% PEGDM (control), 10% PEGDM + 0.01 mM methacrylated heparin, and 10% PEGDM + 0.025 mM methacrylated heparin hydrogels at 2 days, 1 week , and 2 weeks (error bars designate standard deviation). n = 4 samples per condition.

The expression of OPN by hMSCs on heparin-containing gels (Figure 6) again showed an initial decrease at day 2 (3-fold reduction compared to 10% PEGDM for both concentrations of heparin) followed by an increase at days 7 (6.7-fold and 8.7-fold for 0.01 mM and 0.025 mM heparin) and 14 (3.1-fold and 4.5-fold for 0.01 mM and 0.025 mM heparin) versus unmodified PEG gels. The trend, however, was slightly different where the greatest expression was at day 7 and slightly lower at day 14.

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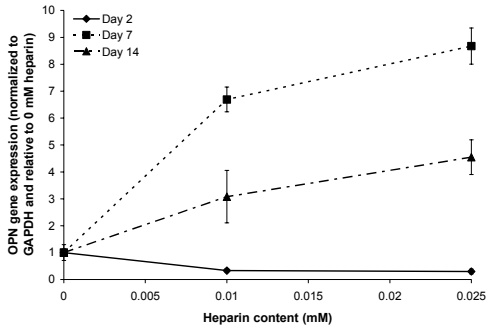

Figure 6: Osteopontin (OPN) gene expression normalized to levels of GAPDH of hMSCs cultured on 10% PEGDM (control), 10% PEGDM + 0.01 mM methacrylated heparin, and 10% PEGDM + 0.025 mM methacrylated heparin hydrogels at 2 days, 1 week , and 2 weeks (error bars designate standard deviation). n = 4 samples per condition.

Discussion

A common objective in regenerative medicine is to design biomaterial niches that actively direct cell functions, especially adhesion, proliferation, and differentiation. An important area of research includes strategies that control the density, clustering, and orientation of cell signaling epitopes, such as proteins and growth factors, to provide avenues for outside-in signaling. Heparin, a protein-binding glycosaminoglycan, possesses numerous attractive qualities with respect to functionalizing scaffolds for regenerative medicine applications. In this study, we explored heparin, modified it with methacrylate groups, copolymerized it with dimethacrylated PEG, and found, in both circumstances, it retained the ability to bind proteins. This is in agreement with work done by Masters and others [16] where methacrylated hyaluronic acid retained the ability to bind fibronectin in solution and when polymerized to form a hydrogel.

The PEG:heparin copolymerized system was explored as a sequestering and delivery system for bFGF. Others have investigated this capacity for heparin containing systems [5-11], and the release of bFGF in our system is very similar to that of a hyaluronate and heparin crosslinked system [6], where bFGF release was initially rapid and then slowly continued for approximately 2 weeks. Similarly, in an alginate and heparin crosslinked matrix [7], there was a bulk release during the first 2 hours and then the release concluded after 7 days.

In our work, the heparin-modified gels promoted hMSC adhesion and spreading. It has been previously reported [17] that a chitosan/heparin complex surface showed enhanced fibroblast adhesion. In comparison to RGD-modified PEG hydrogels at similar concentrations, PEG:heparin hydrogels showed ~10% less adhesion and spreading of hMSCs (data not shown). The increased adhesion and spreading is likely due to matrix proteins adsorbed to the heparin-inclusive surface. Fibronectin, among other adhesion proteins, is a well-known heparin binding protein [12], and that quality was verified in work described here.

PEG:heparin copolymerized hydrogels were also examined as a scaffold to promote osteogenic differentiation of hMSCs. The importance of sulfated glycosaminoglycans for bone formation is inherent to their ability to bind most of the growth factors involved in the regulation of cells of the osteoblast lineage (e.g. FGFs, TFG-β1, BMP2 and 4, IGF-II) [12]. *In vitro* cultures of hMSCs on heparin-containing hydrogels follow the general osteogenic differentiation process with enhancement of various differentiation markers (e.g. Col I, ALP, and OPN) after day 2. However, data at day 2 show a decreased gene expression of Col I and OPN, two important ECM proteins. It is theorized that the hMSCs produced ECM and the heparin functionalized surfaces adsorbed the matrix proteins, initiating feedback inhibition. Because unmodified PEG hydrogels are inherently protein resistant, the absence of matrix caused hMSCs to increase production of these molecules. At later timepoints, the hMSCs are differentiating and, in general, differentiation begins with an increase in cell density, continues with augmented protein levels, a cascade that starts with an increase in ALP and OPN, followed by a heightened production of Col I which continues at a high level until mineralization proceeds [18]. Shibata and others [19] found that soluble heparin stimulates collagen synthesis in mineralized cultures of the osteoblast cell line, MC3T3-E1, and Saos-2 cells, an osteoblast-like cell line, exhibited increased ECM deposition in the presence of soluble heparin [20]. Recently, it has been shown that heparin and bFGF act synergistically in modulating fetal rat calvarial cell synthesis of type I collagen [21] and myoblast differentiation [18]. Gupta and others [22] explored bone marrow stroma, the native hMSC niche, and found that it is the structural specificity of heparin that determines the selective colocalization of cytokines and ECM components that orchestrates their controlled growth and differentiation. Thus, by careful design of hydrogel cell carriers with rationally targeted modification and incorporation of signaling molecules, niches can be synthesized that actively promote cell function such as adhesion and differentiation.

Conclusions

Heparin was modified with methacrylate groups, copolymerized with dimethacrylated poly(ethylene glycol), and analyzed as a delivery vehicle for bFGF and synthetic extracellular matrix for the osteogenic differentiation of hMSCs. The methacrylate-modified heparin retained its ability to bind heparin-binding proteins in solution. In addition, the heparin-modified gels can sequester and deliver biologically active bFGF in

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a controlled dose over a 5-week timeperiod. Finally, the gels were examined as a potential osteogenic scaffold and were found to promote adhesion, proliferation, and osteogenic differentiation of hMSCs.

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