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The Effects of Heparin Releasing Hydrogels on Vascular Smooth Muscle Cell Phenotype

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Abstract

Poly(ethylene glycol) diacrylate (PEGDA) hydrogel scaffolds were engineered to promote contractile smooth muscle cell (SMC) phenotype via controlled release of heparin. The scaffold design was evaluated by quantifying the effects of free heparin on SMC phenotype, engineering hydrogels to provide controlled release of heparin, and synthesizing cell-adhesive, heparin releasing hydrogels to promote contractile SMC phenotype. Heparin inhibited SMC proliferation and up-regulated expression of contractile SMC phenotype markers, including smooth muscle alpha actin, calponin, and SM-22 alpha, in a dose-dependent fashion (6 ug/ml-3.2 mg/ml). Heparin release from PEGDA hydrogels was controlled by altering PEGDA molecular weight (MW 1000-6000) and concentration at polymerization (10-30% w/w), yielding release profiles ranging from hours to weeks in duration. Heparin released from PEGDA gels, formulated for optimized heparin loading and release kinetics (30% w/w PEGDA, MW 3000), stimulated SMCs to up-regulate contractile marker mRNA. A cell-instructive scaffold construct was prepared by polymerizing a thin hydrogel film, with pendent RGD peptides for cell attachment, over the optimized hydrogel depots. SMCs seeded on these constructs had elevated levels of contractile marker mRNA after 3 d of culture compared with SMCs on control constructs. These results indicate that RGD-modified, heparin releasing PEGDA gels can act as cell-instructive scaffolds that promote contractile SMC phenotype.

1. Introduction

Anastomotic intimal hyperplasia (IH) is a significant cause of long-term failure in synthetic vascular grafts. In this pathology, normal smooth muscle cells (SMCs), which exhibit a quiescent, contractile phenotype, de-differentiate into a phenotype characterized by proliferation and excessive synthesis of extracellular matrix (ECM) [1]. These “synthetic” SMCs are implicated in the stenosis of the vascular reconstruction and ultimately contribute to failure. Many stimuli induce this pathology, including mechanical mismatch, flow disturbances, and injury [1,2]. Vascular tissue engineering has the potential to mitigate the response to these stimuli by employing cells and tissues to provide the complex set of responses necessary to maintain long-term patency [3]. Scaffold materials designed to regulate SMC phenotype are an approach to realize this potential.

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Regulation of SMC phenotype is complex and related to soluble signaling factors, mechanical stimuli, and cell substrate [4]. A variety of signaling factors have been implicated that affect the balance between contractile and synthetic phenotype including platelet derived growth factor, basic fibroblast growth factor (bFGF), heparin, and transforming growth factor β1, among many others [5-11]. It also is well known that mechanical stress can affect the phenotype of SMCs [4,12,13]. Furthermore, primary SMCs seeded on fibronectin (FN) de-differentiate more quickly in culture than cells seeded on laminin (LN) [10,14-17]. In general, these studies have focused on factors that mediate de-differentiation of SMCs in culture. Strategies to re-differentiate cultured SMCs toward a contractile phenotype have been investigated less extensively.

In addition to its well-known anti-coagulation function, heparin has been known for several decades to reduce SMC proliferation *in vitro* and *in vivo* [7,8,18,19]. Heparin's antiproliferative effect is believed to be unrelated to its anticoagulant activity [20]. The most important structural determinant of anti-proliferative activity appears to be the overall level of sulfation, although changes in size, structure, and specific O- or N- linked sulfation patterns all contribute to activity [21]. One mechanism of heparin's antiproliferative activity may be related to disruption of exogenous or autocrine bFGF signaling [9,22]. However, heparin may also directly stimulate extracellular receptors [23] and can be internalized [24] allowing it to modulate cytoplasmic signaling pathways [25]. Heparin also can induce expression of smooth muscle α-actin (SMαA) [26-28] and other markers of contractile smooth muscle phenotype [26], in addition to inhibiting SMC proliferation. These results suggest that heparin may be a useful approach to drive SMC differentiation toward a contractile phenotype.

Photopolymerized poly(ethylene glycol) diacrylate (PEGDA)-based hydrogels have been employed widely as tissue engineering scaffolds for a variety of tissues including bone, cartilage, and blood vessels [29-33]. Synthetic peptides can be copolymerized with the network to provide sites for specific cell adhesion to the hydrogel [30]. The network structure of these hydrogels can be controlled quantitatively and independently of peptide incorporation [34] allowing gels to be tailored to serve as depots for the controlled delivery of bioactive agents, such as heparin.

PEGDA and other PEG-based hydrogels systems have been used as scaffolds to study SMC biology by several groups [31,35-39]. This work has established the ability of PEG scaffolds to support basic cell functions, such as attachment, migration, and growth [36,37]. Recently, SMC phenotype in and on these hydrogel constructs has been characterized in greater detail and several mechanisms to modulate phenotype have been explored [35,39]. Our group has recently shown that SMCs cultured on RGD-bearing PEGDA hydrogels can rapidly and robustly shift toward a contractile phenotype [38]. However, these studies utilized exogenous stimuli to drive SMC differentiation. In this report, our goal was to explore the potential of heparin releasing, RGD-bearing PEGDA hydrogels scaffolds to drive SMCs toward a contractile phenotype without extensive exogenous stimulation.

2. Materials and Methods

2.1. Materials

All reagents were obtained from Sigma-Aldrich (St. Louis, MO). Heparin from porcine intestinal mucosa (H9399, Sigma) was further characterized by gel permeation chromatography and multiangle light scattering to determine molecular weight distribution (M_w 16.9±0.5 kD, PDI 1.17±0.08). All other reagents were used as received unless otherwise stated.

2.2. Preparation of PEGDA

PEG (MW 1000, 3000, or 6000) was dried by azeotropic distillation with toluene and stored *in vacuo* for 1 d at 60-80 °C. The dry PEG was dissolved in anhydrous dichloromethane (DCM, 0.2 g/ml), purged with argon, and placed on ice. Triethylamine (dried under molecular sieves, 1.1 molar excess per OH), then acryloyl chloride (1.1 molar excess per OH) were added dropwise. The reaction was stirred overnight at 4 °C. Excess DCM was evaporated to concentrate the acrylated PEG product, which then was filtered to remove triethylamine hydrochloride salt, precipitated in diethyl ether, collected by filtration, reprecipitated, and dried *in vacuo*. The resulting products are denoted PEGDA1k, PEGDA3k, and PEGDA6k, respectively. Substitution was approximately 70%, as determined by 1H NMR [38].

2.3. Preparation of GRGDSP-PEG-Acrylate Derivatives

GRGDSP-PEG-acrylate was prepared as described previously [38]. Briefly, GRGDSP peptide was synthesized using a solid phase peptide synthesizer (Applied Biosystems, Model 433A, Foster City, CA) using standard Fmoc chemistry on an amide (Knorr) resin. Peptides were cleaved and deprotected using trifluoroacetic acid and purified using reverse phase high performance liquid chromatography (HPLC). Peptide synthesis was confirmed by matrix assisted laser desorption/ionization mass spectroscopy (MALDI-MS).

GRGDSP (added dropwise, 0-15% molar excess) was reacted with acrylate-PEG-N-hydroxysuccinimide (ACRL-PEG-NHS, 40 mg/ml, MW ~3400, Laysan Bio, Huntsville, AL) in aqueous sodium bicarbonate (pH 8.4) under argon for 2 h. Salts and unreacted peptide were removed by dialysis against water for 1 day (1:400 volume ratio, 3 exchanges minimum, 1000 MWCO). The purified product was lyophilized and stored at -20 °C. Conjugation of the peptide was confirmed by MALDI-MS.

2.4. Cell Culture

Human coronary artery SMCs (HCASMCs, Lonza, Walkersville, MD) were routinely cultured in SmGM-2 (Lonza) growth medium which contains 5% fetal bovine serum (FBS) and proprietary amounts of basic fibroblast growth factor, epidermal growth factor, and insulin. Media used for hydrogel experiments were supplemented with gentamycin and amphotericin (1X, Lonza). All cell culture was performed at 37 °C, 5% CO_2. HCASMCs were used between passage 6 and 9. Unless otherwise noted, all materials used for cell culture were received sterile or were steam sterilized prior to use.

2.5. Effects of Heparin on HCASMC Proliferation

HCASMCs were cultured to confluence, growth arrested in serum-free medium (SFM: insulin-transferrin-selenium supplement [ITS-X, 1X, Invitrogen, Carlsbad, CA], taurine [5 mM], bovine serum albumin [BSA, 1 mg/ml] in Dulbecco's Modified Eagle Medium [DMEM, Invitrogen]) for 3 d, and seeded on fibronectin (FN, 1 μg/cm^2) coated 24-well culture plates (5,000 cells/cm^2) overnight. Medium was then changed to DMEM containing FBS (10% v/v) supplemented with heparin (0-3200 μg/ml). SFM supplemented with 0.5% FBS was used as a no-growth control and SmGM-2 medium was used as proliferation control. After 6 d, relative cell populations were determined using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (MTS Assay, Promega, Madison, WI). Inhibition was calculated as relative decrease in growth relative to 10% FBS (0% inhibition) and 0.5% FBS (100% inhibition). The half maximal inhibitory concentration (IC_{50}) was determined by logistic fit of the inhibition data using Origin 8 (OriginLab, Northampton, MA).

2.6. Effects of Heparin on HCASMC Differentiation

HCASMCs were cultured to confluence in 6-well plates. Medium was changed to low serum medium (LSM: 2% v/v FBS in DMEM) supplemented with heparin (0-3200 μg/ml). SmGM-2 was used as a control for the synthetic phenotype. Medium was changed every 2 d. After 6 d, protein was collected in radioimmunoprecipitation assay (RIPA) lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) on ice. The soluble protein content was determined using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnologies, Rockford, IL). Expression of contractile marker proteins was determined by western blotting.

2.7. Western Blotting

Samples were suspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (final composition: 62.5 mM Tris [pH 6.8], 2% w/v SDS, 10% v/v glycerol, 5% v/v β-mercaptoethanol, 25 μg/ml bromophenol blue). Protein samples (5 μg per lane) were separated on a Tris-HCl Ready Gel (12% resolving, 4% stacking, Bio-rad) and transferred to a nitrocellulose membrane. Membranes were blocked in 4% (w/v) dried milk and probed for SMαA (200 ng/ml IgG_{2a}, clone 1A4, Santa Cruz), calponin (400 ng/ml IgG_1, clone CALP, Santa Cruz), and SM-22α (800 ng/ml, rabbit polyclonal, H-75, Santa Cruz). The blot was visualized using horseradish peroxidase conjugated secondary antibodies (goat-anti-mouse IgG_{2a}: 1:20,000, goat-anti-mouse IgG_1: 1:2000, goat-anti-rabbit: 1:2000, Santa Cruz) and the SuperSignal West Pico Chemiluminescent detection system (Pierce) using CL-XPosure film (Pierce). Membranes were then stripped in stripping buffer (62.5 mM Tris, 2% SDS, 100 mM β-mercaptoethanol) for 20 min at 50 °C and reprobed for GAPDH (400 ng/ml, clone 0411, Santa Cruz) using the same procedure. Films were scanned using a Hewlett-Packard ScanJet IIcx and densitometry was performed on the digitized images using ImageJ (NIH).

2.8. Heparin Release from PEGDA Hydrogels

Hydrogel precursor solutions were prepared containing PEGDA (10-30% w/w, MW 1-6 kD), Irgacure 2959 (0.1% w/v, 1-[4-[2-Hydroxyethoxy]-phenyl]-2-hydroxy-2-methyl-1-propane-1-one, Ciba Specialty Chemicals, Tarrytown, NY), and heparin (2 mg/ml) dissolved in phosphate buffered saline (PBS, pH 7.4). Hydrogel disks were polymerized in a stainless steel mold (D = 10 mm, H = 1.2 mm) for 10 min under ultraviolet irradiation (365 nm, 0.4-0.5 mW/cm^2). The resulting hydrogels were transferred to a 24-well plate containing loading buffer (2 ml/well, 2 mg/ml heparin, 0.1% w/v sodium azide in PBS) to swell and load overnight (12-16 h). A set of control hydrogels (20% w/w PEGDA, MW 1-6 kD) were formed and swollen in the same manner but without heparin. At the start of the release experiments, the loading buffer was removed, the gels were rinsed with PBS, and fresh release buffer (1 ml/well, 0.1% w/v sodium azide in PBS) was added to each hydrogel. The plates were tightly sealed with parafilm and incubated at 37 °C and 80 rpm. At predetermined intervals, the release buffer was completely removed and replaced.

The heparin concentration in the recovered samples was determined using dimethylmethylene blue (DMMB) [40] on the same day the samples were collected. Release buffer recovered from heparin-free control hydrogels was used as a blank to form a standard curve. Heparin release was obtained by multiplying the sample concentration by the volume of release buffer recovered, which was measured gravimetrically. The overall heparin release was calculated by summation of released heparin over the 34-d experiment.

2.9. Characterization of Hydrogel Networks

PEGDA hydrogels were formed as described in the release study, swollen in release buffer for 2 d and the swollen mass determined. Buffer salts were leached in excess distilled water, the

gels lyophilized, and the polymer network mass determined. The mass swelling ratio, q, was calculated as the ratio of the swollen hydrogel and polymer network masses. Since the hydrogels studied here were formed in solution, the method of Bray and Merrill was adapted to calculate v_e, the effective cross-linked chains per gram of polymer, using the following equation [41] as previously described [34]:

$$v_e = \frac{\ln\left(1 - v_{p,s}\right) + v_{p,s} + \chi v_{p,s}^2}{-\bar{V}_s \rho_p v_{p,r}\left[\left(\frac{v_{p,s}}{v_{p,r}}\right)^{\left(\frac{1}{3}\right)} - \frac{2}{F}\left(\frac{v_{p,s}}{v_{p,r}}\right)\right]} \quad (1)$$

where:

$$v_{p,s} = \frac{1}{\frac{\rho_p}{\rho_s}(q-1)+1} \quad (2)$$

$$v_{p,r} = \frac{1}{\frac{\rho_p}{\rho_s}\left(\frac{1}{f}-1\right)+1} \quad (3)$$

and χ is the Flory-Huggins interaction parameter for PEG and water (0.426) [42], $\bar{V}_s$ is the molar volume of the solvent, water (18.0 cm^3 mol^{-1}), ρ_p is the bulk density of PEG (1.18 g ml^{-1})[43], F is the junction functionality (arbitrarily set to 4, see Discussion), ρ_s is the density of PBS (1.01 g ml^{-1}), and f is the mass fraction of polymer in solution at the time of cross-linking. Because dangling ends and other defects cannot be calculated explicitly in PEGDA systems (as is the case for networks formed from cross-linking of long chains), we have proposed using the number of effective chains, estimated from swelling data, per PEGDA molecule at the time of polymerization, denoted v_e^*, to characterize these systems [34].

$$v_e^* = v_e \cdot MW_{PEGDA} \quad (4)$$

where MW_{PEGDA} is the number averaged molecular weight of the PEGDA. Ignoring defects, the molecular weight between cross-links, M_c, can be estimated as:

$$M_c \approx \frac{v_e^*}{MW_{PEGDA}} = v_e^{-1} \quad (5)$$

From M_c, the mesh size, ε, of the hydrogel can be estimated using the method of Canal and Peppas [44]:

$$\varepsilon = l \cdot \left(2 \cdot C_n \cdot \frac{M_c}{M_r}\right)^{\frac{1}{2}} \cdot v_{p,s}^{\frac{1}{3}} \quad (6)$$

where l is the weighted average bond length in PEG (1.5 Å) [45], M_r is the PEG repeat unit (44 g mol^{-1}) and C_n is the characteristic ratio for PEG (3.8) [42].

2.10. Effects of Heparin Release on SMC Phenotype

PEGDA3k (30% w/w, 0.2 μm filtered) hydrogel discs (D=19 mm, H=1.6 mm) containing heparin (2 mg/ml) were formed between glass plates separated by a silicone sheet with punched circles and prepared as in release studies except the gels were swollen and loaded in a cell compatible loading buffer (DMEM, 2% FBS v/v, 1X gentamycin/amphotericin, 2 mg/ml heparin). Control gels without heparin were prepared in parallel. Hydrogel discs were rinsed in PBS and transferred to transwell culture inserts (3 μm pore, Corning, Lowell, MA) over confluent, synthetic cultured HCASMCs in a 6-well plate. Fresh LSM was added to both chambers. Cultures in LSM supplemented with heparin (400 μg/ml, denoted LSM+H) and SmGM-2 were also maintained as controls for the contractile and synthetic phenotype, respectively. Media were removed and replaced every 2 d. RNA was collected 0, 2, 4, and 6 d after first exposure to the heparin releasing gels. The expression of contractile marker mRNA was determined by real-time RT-PCR. Samples of the media also were collected just before RNA isolation and heparin concentrations were determined using DMMB with LSM as a blank.

2.11. Modulation of SMC Phenotype on Heparin Releasing PEGDA Scaffolds

PEGDA3k (30% w/w, 0.2 μm filtered) hydrogel discs (D=25 mm, H=1.6 mm) containing heparin (1 mg/ml) were formed between glass plates. After initial polymerization, PEGDA supplemented with GRGDSP-PEG-acrylate (PEGDA3k: 30 % w/w, GRGDSP-PEG-acrylate: 5 mM, 0.2 μm filtered, 40 μl) was spread evenly on the surface of the hydrogel, covered with a glass plate and polymerized for 10 min to form a thin cell-adhesive hydrogel film linked to the bulk hydrogel. Control gels containing the negative control glycosaminoglycan (GAG), chondroitin sulfate (1 mg/ml) were prepared in parallel. Composite constructs were transferred to a 6-well plate, gently secured with a piece of silicone tubing (ID = 2.5 cm), and swollen in DMEM containing heparin or chondroitin sulfate (matching loaded GAG, 1 mg/ml, 3 exchanges) overnight at 37 °C. Just before seeding, the hydrogels were quickly rinsed with PBS and HCASMCs were seeded on the constructs in LSM (60,000 cell/cm^2). After 4 h, non-adherent cells were rinsed from the construct by replacing the medium with fresh LSM. RNA samples were collected from the constructs after 3 d.

2.12. Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA purification, reverse transcription, and PCR were performed as previously reported [38]. Briefly, RNA was purified using RNeasy spin columns (Qiagen, Valencia, CA). Total RNA yield was determined spectroscopically and RNA was reverse transcribed using the Superscript First Strand Synthesis System (Invitrogen). The resulting cDNA template was diluted in ultra pure water (final template: 11-25 ng/PCR well assuming 1:1 RNA to cDNA conversion) and was used for real-time PCR analysis using the iQ SYBR Green Supermix (Bio-rad, Hercules, CA) and an iCycler fluorescence detection system (Bio-rad). Gene specific primers for SMαA, calponin, SM-22α, smooth muscle myosin heavy chain (SM-MHC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were previously reported [38]. Confirmatory western blots were not performed. Previous work has demonstrated that mRNA and protein levels for these markers follow similar expression patterns [38]. Primers for myocardin expression were ATGAAGATGCCGTAAAGCAG and CTTCGGGAAGATCTGGGTAT. Relative transcript abundance was normalized to GAPDH expression assuming 100% PCR efficiency.

2.13. Statistics

Statistical analysis was done using Microsoft Excel and Minitab 15. Data are represented as mean ± standard deviation. Single comparisons were made using an un-paired student's t-test. Analysis of variance (ANOVA) followed by Tukey's *post hoc* test was used for data sets with

multiple comparisons. Statistical analysis of real time PCR data was performed in the logarithmic domain. A value of $\alpha < 0.05$ was considered significant.

3. Results

3.1. Effects of Heparin on HCASMC Proliferation

Soluble heparin (0-3200 μg/ml) inhibited proliferation of serum (10% FBS) stimulated SMCs in a dose dependent fashion (Fig. 1A). Heparin concentrations greater than 6.25 μg/ml resulted in significantly decreased cell population ($p < 0.05$) after 6 d of culture. Proliferation decreased with increasing heparin concentration to levels that were indistinguishable ($p \geq 0.09$ for [heparin] ≥ 1600 μg/ml) from the no proliferation control (0.5% FBS). The IC_{50} for heparin inhibition of proliferation was 260±60 μg/ml (Fig. 1B).

3.2. Effects of Heparin on Contractile Phenotype Marker Expression

Soluble heparin (0-3200 μg/ml) increased expression of the contractile phenotype marker proteins SMαA, calponin, and SM-22α (Fig. 2). After 6 d of culture, 6.25 μg/ml heparin was sufficient to induce increases in the level of SMαA, calponin, and SM-22α protein with a clear dose-dependent up-regulation apparent in each of the independent experiments studied (N=4). The magnitude of the response, determined by semi-quantitative densitometry showed variability between experiments (Fig. 2B). Marker protein expression also was greater for SMCs cultured in LSM (all heparin concentrations) than SMCs in SmGM-2.

3.3. Heparin Release from PEGDA Gels

Both PEGDA concentration (10-30% w/w) at the time of polymerization and PEGDA molecular weight (1-6 kD) were used to alter the release kinetics of heparin from PEGDA gels (Fig. 3). Increasing PEGDA concentration from 10-30% w/w resulted in extended kinetics of heparin release (Fig. 3A) and a longer time required for 90% of heparin to be released from the gels ($t_{90\%}$, Table 1) for all PEGDA molecular weights explored. Decreasing PEGDA molecular weight from 6 to 1 kD resulted in extended kinetics of heparin release (Fig. 3B) and a longer $t_{90\%}$ (Table 1), although these trends were only clear for 30% w/w gels. The time scale of release kinetics, $t_{90\%}$, ranged from 0.7±0.3 to 20.4±4.2 d. Heparin loading in the hydrogels (determined by cumulative heparin release after 34 d) also varied with PEGDA concentration and molecular weight (Table 1). Loading was greatest in gels with high PEGDA concentration and molecular weight.

The mass swelling ratio, q, of the gels was determined and used to characterize the hydrogel networks (Table 2). The value of q increased with decreasing PEGDA concentration and increasing molecular weight (Table 2). Hydrogels formed from 10% w/w PEGDA1k or PEGDA3k formed flimsy hydrogels that were noticeably weaker than the other hydrogels studied. Heparin loading was correlated inversely with the mass swelling ratio of the hydrogels. The presence of heparin during polymerization did not affect the swelling ratio of the hydrogels ($p > 0.52$). Analysis of v_e* suggested that the 30% PEGDA hydrogels formed highly entangled networks, whereas 10% PEGDA hydrogels formed weak networks with few entanglements (especially for the PEGDA3k and PEGDA1k) which correlates with the qualitatively weak mechanical properties of these hydrogels (Table 2). The estimated mesh sizes of the hydrogels spanned a broad range from 16-185 Å (Table 2). Based on these estimates, a mesh size of approximately 50 Å was required to effectively impede heparin release from the hydrogels.

3.4. Effect of Heparin Release on HCASMC Phenotype

Heparin released from 30% w/w PEGDA3k gels resulted in increased expression of contractile phenotype marker genes in SMCs over a 6 d culture period (Fig. 4). Over the first 2 d of the

experiment, expression of SMαA, calponin, SM-22α, and smooth muscle myosin heavy chain (SM-MHC) increased in SMCs exposed to heparin releasing hydrogels ($p < 0.05$ for calponin, SM-22α, and SM-MHC) and approached the same level as the SMCs in differentiation control medium, LSM+H ($p > 0.49$ for SMαA, calponin, and SM-22α compared with LSM+H). SMCs cultured with empty gels also increased expression of marker genes, but to a lesser degree than SMCs cultured with heparin releasing gels ($p < 0.025$ for calponin, SM-22α, and SM-MHC). During the remainder of the experiment (2-6 d), SMCs cultured with heparin releasing hydrogels maintained 2.0-, 2.6-, 1.5-, and 8.1-fold greater expression of SMαA, calponin, SM-22α, and SM-MHC, respectively, than SMCs cultured with empty gels ($p < 0.0026$). From 2-6 d, the expression level generally plateaued for SMCs cultured with either hydrogel type, while expression continued to increase slightly in the LSM+H control cultures. SMCs cultured in SmGM-2 as controls for the synthetic phenotype, maintained a relatively constant expression that was significantly less than the other conditions ($p < 0.022$). The heparin concentration in the lower culture chamber, which contained the SMCs, decreased from 48±3 μg/ml after 2 d to 7±1 μg/ml after 6 d (Table 3).

The expression of the transcription factor myocardin was distinctly different from the other contractile marker genes (Fig. 5). Over the 6 d culture, myocardin mRNA remained constant in SMCs cultured with heparin releasing hydrogels. Myocardin mRNA increased slightly in the synthetic SMCs in SmGM-2 and in the SMCs cultured with empty hydrogels ($p < 0.046$ vs. heparin hydrogels, 4-6 d). Myocardin mRNA dramatically decreased over the 6 d culture in SMCs cultured in LSM+H and, by 6 d, was at least 3.9-fold less than in any of the other conditions ($p < 0.0001$).

3.5. Modulation of SMC Phenotype on Heparin Releasing PEGDA Scaffolds

SMCs cultured on GRGDSP-bearing, heparin releasing PEGDA composite scaffolds up-regulated a panel of contractile marker genes (Fig. 6). Expression of SMαA, calponin, SM-22α, and SM-MHC increased by 1.5-, 1.6-, 1.4-, and 1.5-fold, respectively, after 3 d of culture in LSM compared with SMCs cultured on scaffolds loaded with the negative control GAG, chondroitin sulfate ($p = 0.061$, 0.046, 0.028, and 0.151, respectively).

4. Discussion

The goal of this study was to investigate the ability of heparin releasing scaffold systems to actively drive SMC differentiation toward a contractile phenotype. We have previously shown that GRGDSP-bearing PEGDA hydrogels can support rapid and robust expression of markers of contractile phenotype given appropriate external stimuli [38]. By altering the network structure of these hydrogels we sought to expand their utility to also include controlled release of heparin as a mechanism to modulate SMC phenotype.

To assess the potential of heparin to promote contractile SMC phenotype, its effect on SMC proliferation and differentiation was characterized. The effect of soluble heparin on serum stimulated proliferation has been well-established [8,46]. Inhibition of SMC proliferation stimulated by serum (Fig. 1) confirmed the activity of our heparin with the cultured HCASMCs used here. The use of heparin to promote expression of contractile marker proteins has been studied less extensively [26], especially in human vascular SMCs. Therefore, we also explored the ability of heparin to promote the re-expression of the contractile marker proteins SMαA, calponin, and SM-22α in cultured HCASMCs (Fig. 2). The dose-response relationship observed suggested that heparin concentrations near 6-25 μg/ml were sufficient to induce changes in cell phenotype, although higher doses (> 1 mg/ml) could greatly augment this response without any observed cytotoxicity. These results were encouraging since they suggested that a diffusion controlled release system with heparin would be a feasible strategy to modulate SMC phenotype. Using such a system, a high initial heparin concentration can be

achieved, but this concentration decreases rapidly over time. Our results suggest that a high initial heparin concentration could shift SMCs toward the contractile phenotype very effectively. Lower heparin doses obtained at later times in the release profile also would promote the contractile phenotype, though to a lesser degree.

PEGDA6k (20% w/w) hydrogels, used as scaffold materials in our previous work [38] and the work of others [37,39], provided minimal resistance to heparin release (Table 1). Altering PEGDA concentration during polymerization and molecular weight were explored as strategies to engineer the hydrogel scaffold system to provide better control of heparin release. By increasing the PEGDA concentration or decreasing the molecular weight, we obtained hydrogels with decreased estimated mesh size (Table 2) that extended heparin release by several weeks (Fig. 3, Table 1).

The library of formulations employed resulted in a range of mesh sizes from 16-185 Å. Because of the complex network structure of photopolymerized PEGDA hydrogels, it is difficult to estimate the network characteristics accurately. We utilized a model that did not consider all network defects, since it is not possible to explicitly account for these with PEGDA systems, as it is for networks formed from cross-linking long polymer chains. However, we anticipate that our networks contained varying amounts of unreacted acrylate ends and cycles. Furthermore, because the cross-linking nodes of these networks are formed by polyacrylate kinetic chains the junction functionality, F, is generally larger than 4, which was used here to provide consistency with previous studies of PEGDA networks [45]. These issues suggest that the mesh sizes presented here may not estimate the actual network mesh size accurately, but the direction of the error is difficult to predict, since accounting for network defects will tend to decrease and using $F > 4$ will tend to increase mesh size estimates. Despite these limitations, we found a mesh size of approximately 50 Å was required to effectively impede heparin release from the hydrogels, which correlated well with the 55 Å radius of gyration (as a rough estimate of heparin size), determined by small angle x-ray scattering, for ~20 kD heparin reported by others, which is similar is size to the heparin used here [47].

Changes in the network structure also altered the swelling of the hydrogels. Since we chose to swell the hydrogels in heparin-containing medium prior to use to facilitate cell culture experiments, the amount of heparin loaded in each formulation also varied as a function of composition (Table 1). To optimize the overall heparin delivery, we employed 30% PEGDA3k hydrogels for our studies with SMCs, which balanced extended release kinetics with high heparin loading.

Indirect heparin release studies were utilized to assess both the bioactivity of released heparin and the effect of release-dependent changes in heparin concentration on the expression of contractile marker mRNA. Heparin released from 30% PEGDA3k gels resulted in a significant up-regulation of markers of contractile phenotype compared with unloaded controls (Fig. 4). Despite a rapid decrease in heparin concentration after 2 d (Table 3), marker gene expression remained elevated in SMCs exposed to heparin releasing hydrogels, suggesting that the SMCs may be latching into a contractile phenotype expression pattern due to the initial heparin exposure that requires little or no heparin to maintain. These results suggest that long term changes in gene expression may be accomplished using a high initial dose followed by a lower maintenance dose, such as is achieved by diffusion controlled release. However, the fact that SMCs treated with constant high concentration heparin (400 μg/ml) continued to induce marker gene expression suggests that the diffusion-based release profile explored here was sub-optimal.

The expression of the transcription factor myocardin followed a pattern distinct from the other contractile markers (Fig. 5) Myocardin interacts with another transcription factor, serum

response factor, in smooth muscle cells to induce expression of many SMC marker genes including SMαA, calponin, SM-22α, and SM-MHC, [48,49] and appears to be regulated, at least in part, at the transcriptional level [50,51]. Therefore, our observation that myocardin mRNA levels were inversely related to contractile marker expression was unexpected. An alternate activation pathway for SMαA expression has been described for myofibroblasts that is myocardin-independent [52]. Since SM-22α expression also has been reported in myofibroblasts [53], it is possible that the SMCs in these studies were trans-differentiating toward this related, but distinct phenotype. However, it is also possible that heparin was driving this or another myocardin-independent pathway in a yet undescribed fashion in cultured human SMCs. The significance of this altered transcriptional control on the functional phenotype of the SMCs is unclear, although cells cultured for 6 d in LSM+H did not exhibit contraction in response to carbachol [38], suggesting that this effect results in incomplete modulation toward a truly contractile phenotype.

To form a hydrogel scaffold system, heparin loaded hydrogels were modified with a thin hydrogel film containing the ubiquitous cell binding peptide GRGDSP to provide cell adhesion. Since this film only contributed to ~4% of the construct volume, had the same PEGDA composition as the bulk hydrogel, and was applied before swelling and loading, it is unlikely that this layer altered the release behavior of heparin from the hydrogels. SMCs on these films attached and spread normally and within 3 d showed increased expression of contractile marker proteins. The results suggest this is an effective scaffold system for modulation of cultured SMCs toward a contractile phenotype. Other studies using mechanical and/or biochemical stimulation have reported increases in SMαA [54] and calponin [39] on the order of ~2 fold, but require longer culture durations and complicated bioreactor set-ups. In contrast, the composite scaffold system employed here can provide similar or greater up-regulation of contractile marker genes in 2-3 d without an extensive apparatus. The approach and scaffold design outlined here may substantially enhance the effects of additional mechanical or biochemical stimulation used by others and may act as an effective stand-alone cell-instructive scaffold system for regeneration of functional contractile smooth muscle tissue. If employed as a component of a vascular graft construct, released heparin also may act on adjacent tissues to minimize IH by inhibiting SMC proliferation and promoting contractile phenotype.

5. Conclusions

Heparin was found to be an effective modulator of SMC phenotype, with the ability to induce expression of markers of contractile SMC phenotype. Manipulation of PEGDA concentration and molecular weight were effective strategies to engineer PEGDA scaffold structures with heparin dose and release kinetics sufficient to induce a significant up-regulation of SMC contractile markers. SMCs seeded on cell-instructive GRGDSP-modified, heparin releasing hydrogels significantly up-regulated contractile marker expression suggesting these scaffold systems show promise as a construct that facilitates the formation of tissue engineered contractile smooth muscle from cultured SMCs.

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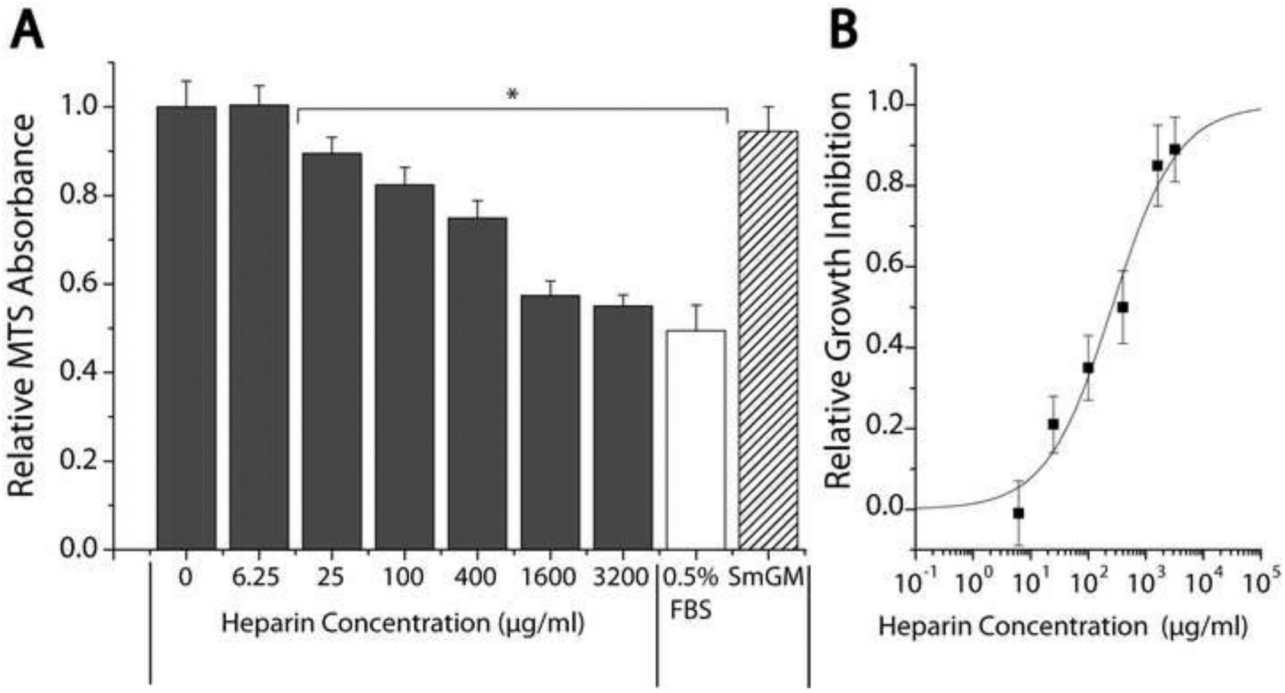

Figure 1.

Inhibition of cell proliferation by heparin (0-3200 μg/ml, in SFM + 10% v/v FBS, solid bars) determined by relative MTS absorbance after 6 d of culture (A). 0.5% FBS was used a no proliferation control (open bar). SmGM-2 was used a positive control for growth (hashed bar). Inhibition by heparin was fit to a logistic regression to calculate the IC_{50} (B, IC_{50} = 260±60 μg/ml). *: $p < 0.05$ compared with 0 μg/ml heparin (10% FBS).

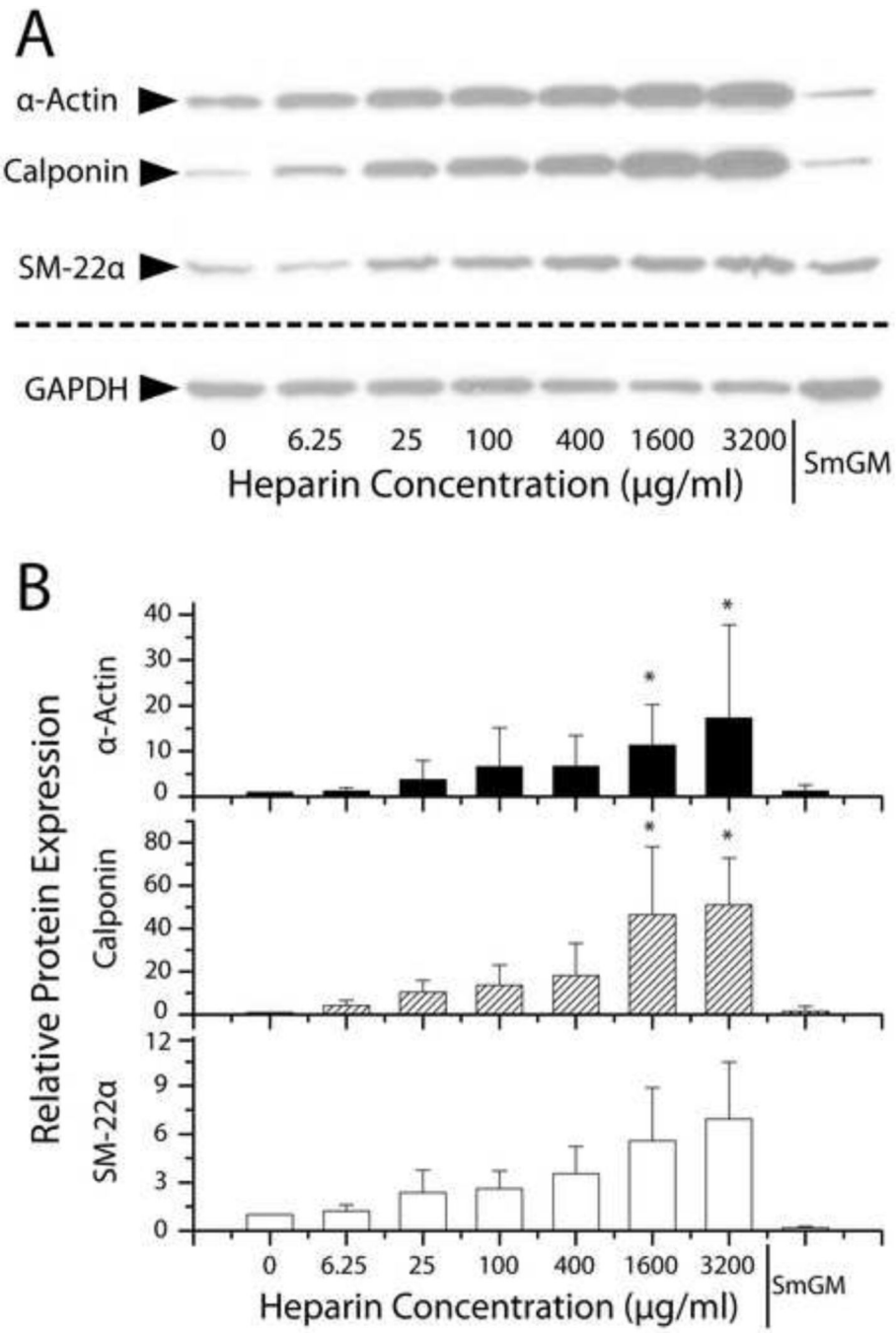

Figure 2.

A representative western blot of SMC contractile marker proteins showing dose-dependent up-regulation with increasing heparin concentration in DMEM + 2% v/v FBS (A). SmGM-2 was used as a control for the synthetic phenotype. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Densitometry also was performed on the blots (B). Results were normalized to GAPDH expression and then to 0 μg/ml heparin before pooling data from independent experiments (N=4). *: $p < 0.05$ compared with 0 μg/ml heparin.

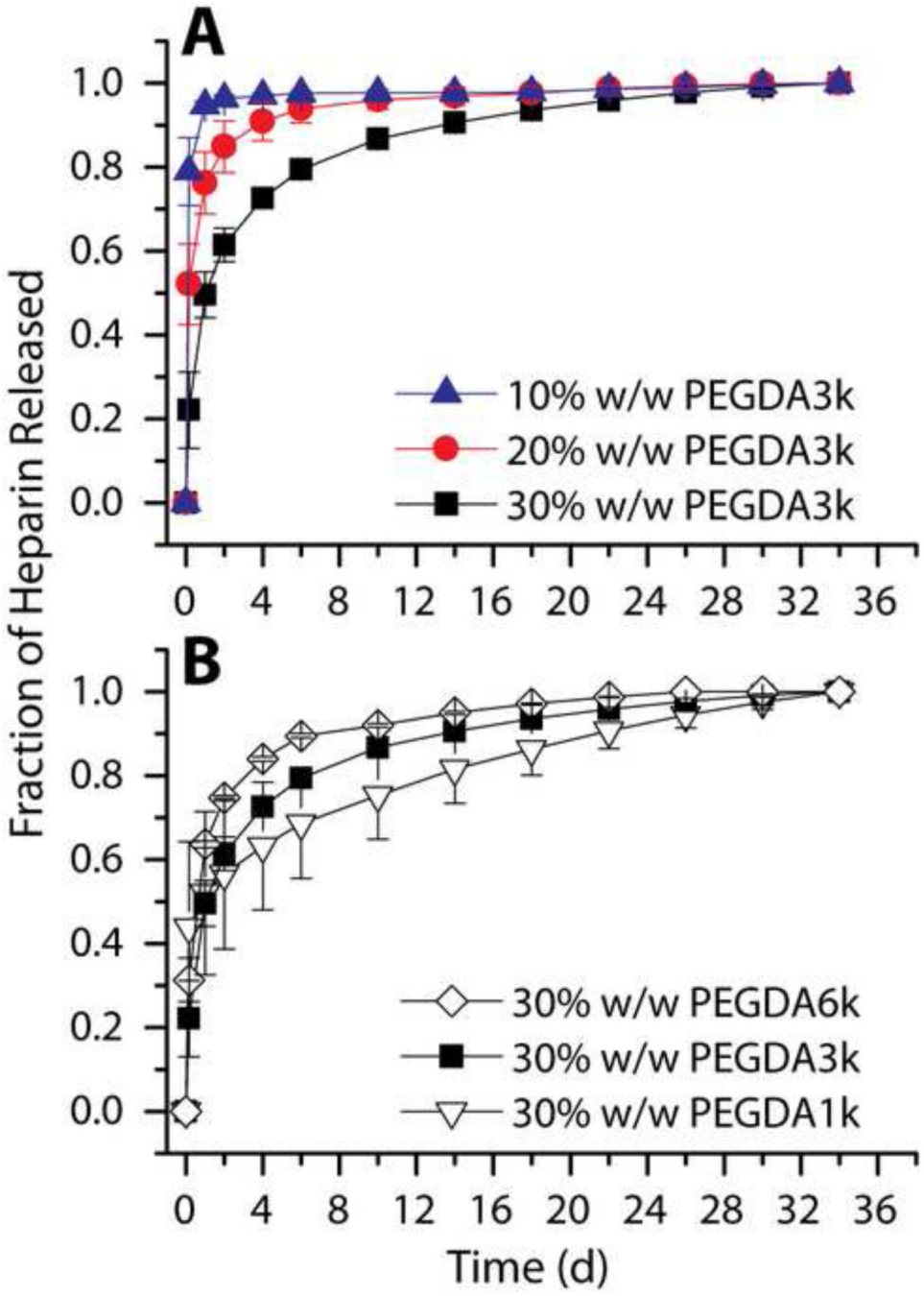

Figure 3.

Heparin release profiles for PEGDA hydrogels of various formulations showing that increasing PEGDA concentration (A: PEGDA3k shown, but PEGDA1k and PEGDA6k followed similar patterns) or decreasing the PEGDA molecular weight (B: 30% w/w PEGDA shown) retards heparin release. The effect of molecular weight was weak for 10 and 20% w/w PEGDA (see Table 1). Cumulative heparin release was determined after 34 d for each sample and used to calculate fractional release (see Table 1 for cumulative release measurements for each formulation).

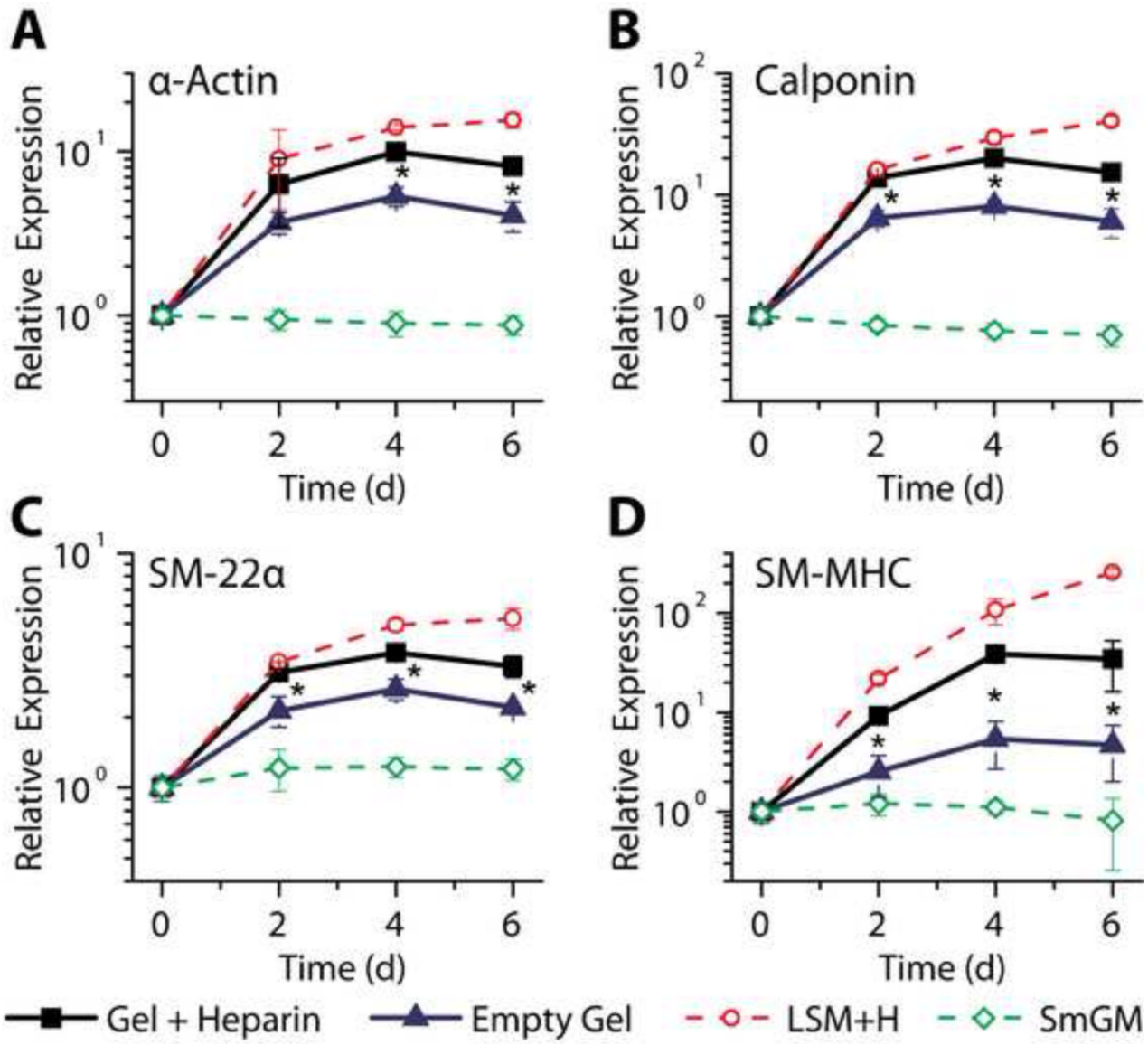

Figure 4.

Effect of heparin released from PEGDA gels on the expression of contractile phenotype marker genes. SMCs cultured in LSM with heparin releasing hydrogels (Gel + Heparin) showed increased mRNA expression of the markers smooth muscle α-actin (A), calponin (B), SM-22α (C), and smooth muscle myosin heavy chain (SM-MHC, D) compared with unloaded control gels (Empty Gel). LSM + 400 μg/ml heparin (LSM+H) was used as a control for differentiation and SmGM-2 was used as control for the synthetic phenotype. Expression of mRNA was determined using real time RT-PCR. Expression levels relative to 0 d are shown. *: $p < 0.05$, heparin releasing hydrogel vs. unloaded hydrogel control.

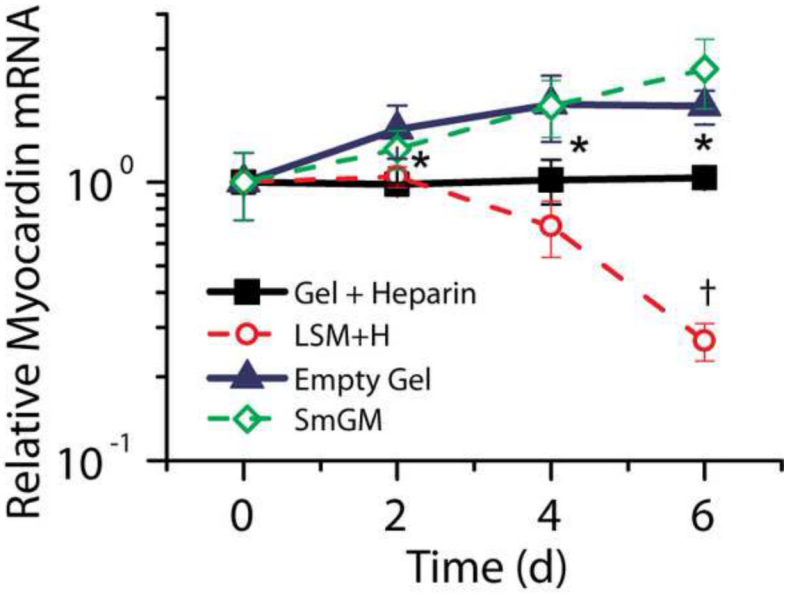

Figure 5.

Effect of heparin released from PEGDA gels on the transcription factor myocardin. LSM + 400 μg/ml heparin (LSM+H) was used as a control for differentiation and SmGM-2 was used as control for the synthetic phenotype. Expression of mRNA was determined using real time RT-PCR. Expression levels relative to 0 d are shown. *: $p < 0.05$, heparin releasing hydrogel vs. unloaded hydrogel control (same day), †: $p < 0.0001$, LSM+H vs. all other conditions (same day)

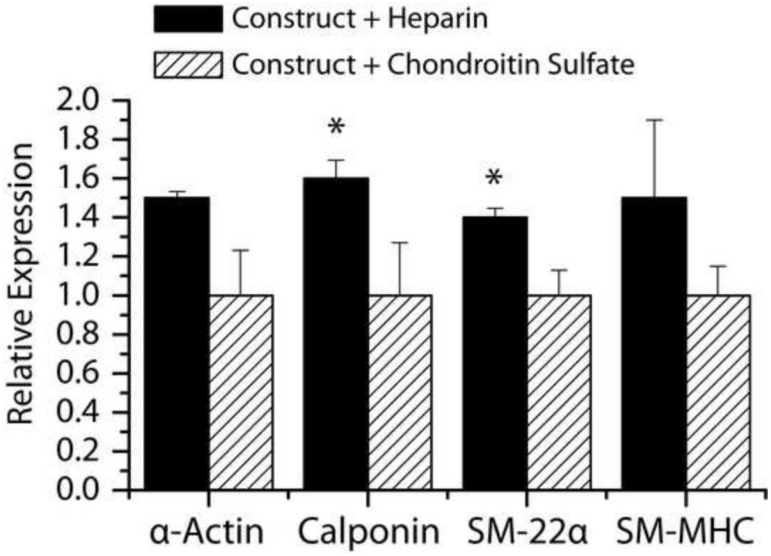

Figure 6.

Expression of markers of contractile SMC phenotype on heparin releasing PEGDA scaffold constructs compared with constructs loaded with the negative control chondroitin sulfate. Expression of mRNA was determined using real time RT-PCR after 3 d of culture. Expression levels relative to chondroitin sulfate controls for each gene are shown. *: $p < 0.05$ compared with chondroitin sulfate loaded constructs.

Table 1

Summary of heparin release from PEGDA hydrogels

Composition†	Cumulative Release (μg)	$t_{90\%}$‡ (d)
30% PEGDA1k	90.3 ± 21.8	20.4 ± 4.2
20% PEGDA1k	86.9 ± 5.4	6.9 ± 1.1
10% PEGDA1k	26.2 ± 7.1	2.5 ± 1.1
30% PEGDA3k	105.9 ± 6.5	13.4 ± 0.3
20% PEGDA3k	68.4 ± 18.1	3.9 ± 2.2
10% PEGDA3k	70.5 ± 8.5	0.7 ± 0.3
30% PEGDA6k	120.8 ± 20.4	6.8 ± 0.8
20% PEGDA6k	68.5 ± 17.0	4.9 ± 3.8
10% PEGDA6k	33.0 ± 7.4	2.9 ± 2.0

† composition provided as % w/w at the time of polymerization

‡ $t_{90\%}$: time at 90% of cumulative release, determined by linear interpolation

Table 2

Network properties of PEGDA hydrogels

Composition[†]	Heparin[**]	$q^{\S}$	$v_e{}^{*\ddagger}$	M_c (g mol^{-1})	Mesh Size (Å)
30% PEGDA1k	+	3.72 ± 0.03	3.8 ± 0.1	260 ± 10	16.0 ± 0.3
20% PEGDA1k	+	5.02 ± 0.06	2.6 ± 0.1	380 ± 10	21.4 ± 0.5
20% PEGDA1k	-	5.14 ± 0.01	2.5 ± 0.0	400 ± 0	22.3 ± 0.1
10% PEGDA1k	+	11.67 ± 0.17	0.7 ± 0.0	1540 ± 60	57.3 ± 1.4
30% PEGDA3k	+	7.13 ± 0.06	2.0 ± 0.0	1510 ± 30	48.0 ± 0.6
20% PEGDA3k	+	9.62 ± 0.18	1.5 ± 0.1	2060 ± 90	62.1 ± 1.7
20% PEGDA3k	-	9.81 ± 0.09	1.4 ± 0.0	2160 ± 50	63.9 ± 0.9
10% PEGDA3k	+	26.11 ± 0.77	0.3 ± 0.0	9430 ± 570	185.6 ± 7.4
30% PEGDA6k	+	8.02 ± 0.08	3.0 ± 0.1	2000 ± 40	57.5 ± 0.8
20% PEGDA6k	+	10.61 ± 0.12	2.3 ± 0.1	2590 ± 70	71.9 ± 1.2
20% PEGDA6k	-	10.66 ± 0.09	2.3 ± 0.0	2620 ± 50	72.4 ± 0.9
10% PEGDA6k	+	19.58 ± 0.29	1.2 ± 0.0	5130 ± 170	124.4 ± 2.6

[†] composition provided as % w/w at the time of polymerization

[**] "+" indicates presence of 2 mg/ml heparin at the time of polymerization

[§] q calculated as ratio of swollen mass to polymer network mass

[‡] v_e* is the ratio of effect network chains to PEGDA molecules at polymerization (F = 4)

Table 3

Heparin concentration in medium during transwell insert study

Day	Culture Condition	Heparin Concentration (μg/ml) †
2	Heparin Releasing Gel	48 ± 3
	Empty Gel	2 ± 2
4	Heparin Releasing Gel	11 ± 1
	Empty Gel	1 ± 0
6	Heparin Releasing Gel	7 ± 1
	Empty Gel	1 ± 0
2-6	LSM+H‡	362 ± 66

† Heparin concentration determined using DMMB with standards made in LSM

‡ LSM+H: DMEM + FBS (2% v/v) + Heparin (400 μg/ml); average for all samples (2, 4, & 6 d) shown