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Molecular weight and concentration of heparin in hyaluronic acid-based matrices modulates growth factor retention kinetics and stem cell fate

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ABSTRACT

Growth factors are critical for regulating and inducing various stem cell functions. To study the effects of growth factor delivery kinetics and presentation on stem cell fate, we developed a series of heparin-containing hyaluronic acid (HyA)-based hydrogels with various degrees of growth factor affinity and retention. To characterize this system, we investigated the effect of heparin molecular weight, fractionation, and relative concentration on the loading efficiency and retention kinetics of TGFβ1 as a model growth factor. At equal concentrations, high MW heparin both loaded and retained the greatest amount of TGFβ1, and had the slowest release kinetics, primarily due to the higher affinity with TGFβ1 compared to low MW or unfractionated heparin. Subsequently, we tested the effect of TGFβ1, presented from various heparin-containing matrices, to differentiate a versatile population of Sca-1$^+$/CD45$^-$ cardiac progenitor cells (CPCs) into endothelial cells and form vascular-like networks in vitro. High MW heparin HyA hydrogels stimulated more robust differentiation of CPCs into endothelial cells, which formed vascular-like networks within the hydrogel. This observation was attributed to the ability of high MW heparin HyA hydrogels to sequester endogenously synthesized angiogenic factors within the matrix. These results demonstrate the importance of molecular weight, fractionation, and concentration of heparin on presentation of heparin-binding growth factors and their effect on stem cell differentiation and lineage specification.

1. Introduction

Growth factors are signaling proteins involved in harnessing and activating numerous cell functions such as mitotic cell division, differentiation, and synthesis of extracellular matrix (ECM) proteins. Growth factors transmit signals in a spatially controlled biological niche through specific binding to their corresponding transmembrane cell surface receptors, which results in subsequent receptor phosphorylation of intracellular residues that amplify the signal and regulate cell function. Although growth factor-based therapies have emerged as a novel strategy for enhancing stem cell behavior and inducing functional regeneration of biological tissues, their short half-life due to proteolytic degradation/denaturation has limited their success [1–3]. To overcome this limitation, large doses of soluble growth factors are commonly used, which has the potential to induce tumors [4], form aberrant blood vessels [5–7], and induce unwanted ectopic tissue formation [8,9]. Efforts to prolong retention of growth factors at the targeted tissue site have employed various biomaterial-based approaches including strategies for physical entrapment or covalent conjugation of growth factors within a polymer scaffold [10–15]. These systems are still limited by their inability to maintain bioactivity of growth factors due to their short half-life, limited physical and chemical stability, and uncontrolled release kinetics [16].

To overcome these limitations, several synthetic scaffolds have been developed to exploit the natural affinity between heparin and heparin binding domains on native growth factors [2,12,17–20]. Heparinized scaffolds present the growth factor biomimetically from the matrix, which protects the growth factors from proteolytic degradation, enables their prolonged bioactivity, and releases them in response to cellular influences [2]. In this regard, Hubbell and co-workers have reported on heparin-containing fibrin-based matrices made by covalent conjugation of a heparin-binding peptide in the matrix to control the release of several growth factors including basic fibroblast growth factor (bFGF) and β-nerve growth factor (β-NGF) [2,19,21–23]. Prestwich and co-workers developed covalently linked heparin-containing hyaluronic acid-based hydrogels and demonstrated efficient in vivo neovascularization due to improved loading efficiency and slow release of several heparin-binding growth factors including FGF-2, VEGF, KGF, PDGF, and

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TGFβ1 [20,24–26]. More recently, heparin-containing PEG based hydrogels have been reported to act as an efficient reservoir and a tunable delivery system for various growth factors including FGF-2, VEGF, SDF-1α, and EGF, which resulted in better angiogenesis in the chicken chorioallantoic membrane (CAM), in vitro tumor, and in vivo murine kidney models [17,18,27,28]. Collectively, these studies indicate that the presence of heparin in a synthetic matrix significantly enhances the encapsulation and retention of growth factors within the matrix, facilitates maintenance of their bioactivity for prolonged periods, and modulates biological response both in vitro and in vivo.

Although the aforementioned studies clearly demonstrated the value of heparin for growth factor delivery from bioinspired matrices, the attributes of heparin such as the extent of sulfation, pattern of sulfation, molecular weight, and polydispersity of heparin have been less emphasized and not explored in depth [29–32]. For example, heparin derivatives with different sulfation patterns differ significantly in their specific binding to $VEGF_{165}$, which affects the subsequent bioactivity of $VEGF_{165}$ [24], and desulfated heparin-derivatives lose their stabilizing features and stimulatory capacity [33,34]. To partially address these issues, we investigated the effect of molecular weight and relative concentration of heparin within HyA-based hydrogels on growth factor loading efficiency, retention kinetics, and the subsequent effects of the retained growth factor on stem cell behavior.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HyA, sodium salt, 500 kDa) was purchased from Lifecore Biomedical (Chaska, MN). Adipic dihydrazide (ADH), 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC), sodium hydroxide (NaOH), hydrochloric acid (HCl), tris(2-carboxyethyl)phosphine (TCEP) and 1-hydroxybenzotriazole (HOBt) were purchased from Aldrich (Milwaukee, WI). Dimethyl sulfoxide (DMSO), N-Acryloxysuccinimide (NAS), acetone, ethanol, low molecular weight heparin (LMWH), were obtained from Fisher Scientific (Waltham, MA). Paraformaldehyde (16% in H_2O) was obtained from Electron Microscopy Sciences (Hartfield, PA). Calcein was purchased from BD Biosciences (Pasadena, CA). High molecular weight heparin (HMWH), and unfractionated molecular weight heparin (UMWH) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, Texas). The MMP-degradable crosslinker peptide (CQPQGLAKC) and the 15 amino-acid bsp-RGD(15) adhesion peptide (CGGNGEPRGDTYRAY) were synthesized by United BioSystem Inc. (Herndon, VA). Dialysis membranes (10000 MWCO, SpectraPor Biotech CE) were purchased from Spectrum Laboratories (Rancho Dominguez, CA). All chemicals were used as received. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). 1× Dulbecco's phosphate buffered saline (DPBS) was purchased from Invitrogen.

2.2. Determination of molecular weight and molecular weight distribution of heparins

A size exclusion chromatography-multi-angle light scattering (SEC-MALS) instrument equipped with DAWN-HELEOS II 18-angle light scattering detector and an Optilab T-rEX refractive index detector (Wyatt Technology, Santa Barbara, CA) was used to measure the molecular weight and polydispersity index (PDI) of the different heparins. A Shodex polymer-based packed column (OHpak SB-803 HQ) with a molecular weight range of up to 100,000 Da was used for the separation of heparin polymeric chains. DPBS + 0.02% sodium azide was used as the mobile phase, the flow rate was 0.3 mL/min and the sample concentration was in the range 1–5 mg/mL.

Normalization of the multi-angle detectors, peak alignment, and band broadening correction between the UV, MALS, and RI detectors was performed using BSA (2 mg/mL) as a standard in DPBS mobile phase at a flowrate of 0.3 mL/min using Astra software algorithms. Analysis in the Astra software (Wyatt Technology, Santa Barbara, CA) was restricted to moderate angle detectors to eliminate high noise from the extreme angle detectors. The differential refractive indices for each heparin sample were determine using a 100% mass recovery method at a wavelength of 690 nm with the Optilab T-rEX refractive index detector. The UV extinction coefficients were determined by analysis of the UV peak during inline analysis with the diode array detector at a wavelength of 280 nm. First-degree Zimm plots were used in the Astra software to determine the molecular weight and polydispersity index (PDI) of the heparin samples. Measured molecular weight and molecular weight distribution of the heparins are shown in the Table 1.

2.3. Synthesis of AcHyA hydrogel

Recently, we reported the synthesis of HyA-based hydrogel used in this study. Briefly, HyA derivatives carrying hydrazide groups (HyAADH) were synthesized using previously described methods [35–37], and acryloxysuccinimide (700 mg) was subsequently reacted to the HAADH solution (300 mg, 100 mL DI water) to generate acrylate groups on the HyA (AcHyA) [35,38]. The presence of the acrylate group on AcHA was confirmed by ^{1}H NMR [39]. Then, AcHyA-RGD derivative was synthesized by reacting CGGNGEPRGDTYRAY (bsp-RGD(15)) (10 mg) with AcHyA solution (25 mg, 10 mL DI water) at room temperature. Separately, thiolated-heparin was synthesized by reacting heparin (50 mg, 10 mL DI water) with the excess of cysteamine in the presence of EDC and HOBt at pH 6.8. AcHyA (4 mg), AcHyA-RGD (6 mg), and heparin-SH (0.03 wt.%) were dissolved in 0.3 mL of triethanolamine-buffer (TEOA; 0.3 M, pH 8), and incubated for 15 min at 37 °C. HyA hydrogels were generated by in situ crosslinking of the HyA precursors with bis-cysteine containing MMP-13-cleavable peptide sequence CQPQGLAKC (3 mg, 50 μL TEOA buffer) [40–42].

2.4. Incorporation of TGFβ1 and measurement of retention kinetics

Hydrogel macromers of AcHyA, AcHyA-RGD, and heparin-SH were dissolved at various ratios in 0.3 mL of triethanolamine-buffer (TEOA; 0.3 M, pH 8) for 15 min at 37 °C. Then, TGFβ1 (Cell Signaling Technology, Inc., Beverly, MA) was mixed in the solution of HyA derivatives and incubated for another 15 min at 4 °C. Subsequently, MMP-13 crosslinker (50 μL TEOA buffer) was added to form TGFβ1 loaded hydrogel, then TGFβ1 was allowed to release into 400 μL of cell culture media. At predetermined time points over the course of 3 weeks, the supernatant was withdrawn and fresh media was replenished. The mass of TGFβ1 in each supernatant was determined with sandwich ELISA kits (RayBiotech, Inc., Norcross GA). Retention of TGFβ1 was calculated by subtraction of released TGFβ1 from the calculated initial loading amount of TGFβ1.

2.5. Fluorescence recovery after photobleaching (FRAP) diffusivity measurement

FRAP measurements were performed on HyA hydrogels containing fluorescein isothiocyanate (FITC) labeled TGFβ1. For FRAP measurements, two sets of hydrogels were formed as described above using heparin of different molecular weights (HMWH, LMWH, UMWH), and a second set of hydrogels were formed by varying the wt% of HMWH (0.01, 0.02, 0.03) in HyA hydrogels containing 40 nM TGFβ1. Total

Table 1
Molecular weight and molecular weight distribution of heparins were measured using SEC-MALS.

Sample	∂n/∂c (mL/g)	ε_{abs} (mL/mg cm)	MW_n (kDa)	MW_w (kDa)	PDI
LMWH	0.125	0.010	4.0	4.1	1.02
HMWH	0.125	0.005	10.6	12.0	1.14
UMWH	0.136	0.012	9.3	12.8	1.38