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Heparin microparticle effects on presentation and bioactivity of bone morphogenetic protein-2

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ABSTRACT

Biomaterials capable of providing localized and sustained presentation of bioactive proteins are critical for effective therapeutic growth factor delivery. However, current biomaterial delivery vehicles commonly suffer from limitations that can result in low retention of growth factors at the site of interest or adversely affect growth factor bioactivity. Heparin, a highly sulfated glycosaminoglycan, is an attractive growth factor delivery vehicle due to its ability to reversibly bind positively charged proteins, provide sustained delivery, and maintain protein bioactivity. This study describes the fabrication and characterization of heparin methacrylamide (HMAm) microparticles for recombinant growth factor delivery. HMAm microparticles were shown to efficiently bind several heparin-binding growth factors (e.g. bone morphogenetic protein-2 (BMP-2), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (FGF-2)), including a wide range of BMP-2 concentrations that exceeds the maximum binding capacity of other common growth factor delivery vehicles, such as gelatin. BMP-2 bioactivity was assessed on the basis of alkaline phosphatase (ALP) activity induced in skeletal myoblasts (C2C12). Microparticles loaded with BMP-2 stimulated comparable C2C12 ALP activity to soluble BMP-2 treatment, indicating that BMP-2-loaded microparticles retain bioactivity and potently elicit a functional cell response. In summary, our results suggest that heparin microparticles stably retain large amounts of bioactive BMP-2 for prolonged periods of time, and that presentation of BMP-2 via heparin microparticles can elicit cell responses comparable to soluble BMP-2 treatment. Consequently, heparin microparticles present an effective method of delivering and spatially retaining growth factors that could be used in a variety of systems to enable directed induction of cell fates and tissue regeneration.

1. Introduction

Recombinant growth factor delivery has been effective for a number of tissue engineering applications. In particular, bone morphogenetic proteins (BMPs), which are potent osteoinductive growth factors, have been used extensively to treat bone defects in both research and clinical settings [1–3]. However, current treatment strategies require supraphysiological levels of recombinant proteins, such as BMPs, in order to stimulate endogenous mechanisms of repair. This inefficient use of growth factor is largely due to the inability of biomaterial delivery vehicles to provide adequate sustained and localized presentation of growth factors necessary to stimulate repair over long periods of time. Current biomaterial delivery vehicles have major limitations, such as the rapid release of molecular cargo upon deployment, causing low retention of soluble factors at the site of interest [4–6], or alternatively, reliance upon growth factor tethering strategies that can significantly reduce growth factor bioactivity [7,8]. Thus, materials with the ability to strongly, but reversibly, interact with their molecular payload are necessary, and may significantly decrease the amount of growth factor required for therapies, while improving physiological response.

Recently, glycosaminoglycan-containing biomaterials have become an attractive delivery method for recombinant growth factors, due to their ability to strongly bind a variety of growth

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factors in a reversible manner. Glycosaminoglycans (GAGs) are linear polysaccharide chains that bind positively charged growth factors primarily through their negatively charged sulfate groups and exist both as free chains and covalently-linked components of glycosylated proteins known as proteoglycans [9,10]. GAGs such as heparin, heparan sulfate, and chondroitin sulfate are ubiquitous components of natural extracellular matrices (ECM) that are involved in sequestering and immobilizing growth factors within the cellular microenvironment [11–13]. Thus, GAG-based materials present the opportunity to harness the natural growth factor binding capacity of the ECM and deliver growth factors in a biomimetic manner with spatiotemporal control. Heparin, in particular, is highly negatively charged and has a strong affinity for a class of positively charged growth factors known as "heparin-binding growth factors," for which specific growth factor binding sequences on heparin chains have been identified [14–16]. The non-covalent, reversible interactions between heparin and heparin-binding growth factors ensure that binding occurs with minimal impact on growth factor structure. Heparin-binding growth factors such as transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), and bone morphogenetic proteins (BMPs), are especially influential in many developmental and regeneration processes, and it is thought that heparin itself may play an influential role in the preservation and presentation of molecules through electrostatic interactions [17,18].

The use of heparin and heparin-containing biomaterials for BMP-2 delivery, as well as the delivery of several other growth factors, including FGF-2, VEGF, and TGF-β2, has been widely explored in both *in vitro* and *in vivo* test beds [19–24]. Although several studies have investigated heparin-BMP-2 interactions, the effects of heparin-BMP-2 binding on protein bioactivity have been inconsistent and depend largely on the amount of heparin and method of heparin immobilization. Previous studies have demonstrated that co-delivery of soluble heparin with BMP-2 can enhance BMP-2-mediated osteogenesis or, contrastingly, interfere with BMP-2 and BMP receptor binding to inhibit osteogenesis, depending on the cell type and culture conditions [25–31]. Nevertheless, the addition of heparin to biomaterials, including microparticles and bulk gels, has previously resulted in improvement in growth factor retention and BMP-2-induced osteogenesis [32–36]. Heparin-mediated delivery of BMP-2 has also resulted in a wide range of effects *in vivo*, with studies demonstrating variable amounts of mineralization in both ectopic and orthotopic sites [25,37–39], reflecting an inconsistent ability to form functional bone. Furthermore, the majority of these materials consist of relatively small amounts of heparin immobilized within a larger bulk material [23,24,40–43], which may attenuate heparin's ability to effectively bind and present growth factors. As a result, previous reports on heparin-containing biomaterials may significantly underestimate the amount of BMP-2 that can be delivered via heparin-binding. Thus, improving the growth factor binding ability of heparin-containing biomaterials may enable consistent delivery of highly localized BMP-2 concentrations necessary to stimulate more effective bone formation.

Herein, we present a method of fabricating pure heparin microparticles from a modified heparin methacrylamide species that can be thermally cross-linked. Physical and chemical characterization of heparin microparticles was performed, and growth factor binding and release were quantified with different BMP-2 loading concentrations. Additionally, growth factor bioactivity was evaluated by introducing BMP-2 laden heparin microparticles to cultures of C2C12 cells and measuring BMP-2-induced alkaline phosphatase activity, as well as changes in DNA content. Overall, this study marks a crucial first step in developing heparin microparticles as a versatile delivery vehicle and therapeutic platform for growth factor-stimulated tissue engineering, by investigating their capacity to efficiently capture and present BMP-2 to induce a potent functional cell response.

2. Materials and methods

2.1. Heparin methacrylamide modification

Heparin ammonium salt from porcine intestinal mucosa (17–19 kDa; Sigma–Aldrich, St. Louis, MO) was conjugated with N-(3-Aminopropyl)methacrylamide (APMAm; Polysciences, Warrington, PA) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Thermoscientific, Rockford, IL) and N-hydroxysulfosuccinimide (Sulfo-NHS; Thermoscientific, Rockford, IL) as described in previous protocols [44,45] (Fig. 1A). EDC/Sulfo-NHS chemistry causes activation of the carboxyl groups on heparin and subsequent methacrylamide substitution via covalent bonds created with the primary amines on APMAm. Briefly, 325 mg of heparin ammonium salt was mixed with 10-fold molar excess of sulfo-NHS, EDC, and APMAm, in relation to heparin's carboxyl groups, in a reaction volume of 200 mL at room temperature and pH 6.5. The total reaction time was 5 h, with a second set of 10-fold molar excess of EDC and APMAm being added after the first 2.5 h, to replace reactants that had undergone hydrolysis. The reaction volume was then immediately dialyzed against 2 L of water using dialysis tubing with a molecular weight cutoff of 3500 Da (Spectrum Laboratories, Rancho Dominguez, CA) for at least 48 h to remove excess reactants; dialysis water was replaced every 12 h. The remaining volume in the dialysis tubing (~200 mL) was lyophilized (Labconco, Kansas City, MO) for four days.

2.2. Heparin methacrylamide characterization

5 mg of modified or unmodified heparin was dissolved in 750 μL deuterium oxide (Cambridge Isotope Laboratories, Tewksbury, MA) and analyzed using a Bruker Avance III 400 MHz spectrometer (Bruker Biospin Corp, Billerica, MA) for proton nuclear magnetic resonance (^{1}H NMR) spectra analysis. ^{1}H NMR spectra were phase corrected, baseline subtracted, and integrated using ACD/NMR processor 12.0 software. For the determination of methacrylamide modification degree, protons located at the C_1 position of heparin's disaccharide units were integrated between the chemical shifts of $\delta = 5.0$ ppm and $\delta = 5.75$ ppm and assigned a reference value of 1. However, in modified heparin, the protons of the methacrylamide groups ($\delta = 5.41$ ppm and $\delta = 5.66$ ppm) have a similar chemical shift within the aforementioned heparin integration region. Therefore, an alternate region in the heparin spectra ($\delta = 4.0$ ppm and $\delta = 4.33$ ppm) that was not affected by the presence of methacrylamide signals was selected, also determined to have an integral of 1, and compared to the integral of the methacrylamide protons at $\delta = 5.41$ ppm and $\delta = 5.66$ ppm. The number of methacrylamide groups per heparin disaccharide unit was determined by taking the ratio of the integration regions of the methacrylamide protons ($\delta = 5.41$ ppm and $\delta = 5.66$ ppm) and the heparin ($\delta = 4.0$ ppm and $\delta = 4.33$ ppm).

2.3. Heparin microparticle fabrication

Heparin methacrylamide microparticles were fabricated using a water-in-oil emulsion followed by thermal cross-linking of the methacrylamide groups, similar to a previously developed protocol [46]. Briefly, 55.6 mg of heparin methacrylamide was dissolved in 440 μL of phosphate buffered saline (PBS; Corning Mediatech, Manassas, VA) and mixed with equimolar amounts (30 μL of a 0.3 M solution) of the free radical initiators ammonium persulfate (Sigma Aldrich) and tetramethylethylenediamine (Sigma Aldrich). Immediately after mixing, the solution was added dropwise to 60 mL of corn oil and 1 mL of polysorbate 20 (Promega, Madison, WI), and the entire mixture was homogenized on ice at 3000 rpm for 5 min using a Polytron PT3100 homogenizer (Kinematica, Switzerland) to create a water-in-oil emulsion. The emulsion was then submerged in a hot water bath (55 °C) and stirred to activate free radical polymerization and thermal cross-linking of the methacrylamide groups. The reaction proceeded for 30 min under nitrogen purging to prevent depletion of free radicals by the presence of oxygen. The solution was centrifuged at 3000 rpm for at least 10 min and the corn oil phase was removed. The resulting pellet of microparticles was washed once in acetone and centrifuged again (3000 rpm, 10 min) before undergoing several water washes to remove excess oil or loosely cross-linked microparticles that did not settle with centrifugation. Prior to cell culture studies, microparticles were disinfected in a 70% ethanol solution for 30 min followed by three additional washes with sterile water. The microparticles were then lyophilized for two days and stored at 4 °C until use.

2.4. Heparin microparticle characterization

To visualize the microparticles, lyophilized samples were incubated with 0.0016% (w/v) 1,9-dimethylmethylene blue (DMMB; Sigma Aldrich), which specifically stains sulfated GAGs, for 30 min at room temperature, and then washed several times with water before being imaged on a Nikon Eclipse TE2000-U inverted microparticle (Nikon Instruments, Melville, NY). Microparticle size distribution was determined by using CellProfiler image analysis software [47] to evaluate images of microparticle samples. For size analysis, microparticles were stained with 0.1% (w/v)