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Affinity-based growth factor delivery using biodegradable, photocrosslinked heparin-alginate hydrogels

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ABSTRACT

Photocrosslinkable biomaterials are promising for tissue engineering applications due to their capacity to be injected and form hydrogels *in situ* in a minimally invasive manner. Our group recently reported on the development of photocrosslinked alginate hydrogels with controlled biodegradation rates, mechanical properties, and cell adhesive properties. In this study, we present an affinity-based growth factor delivery system by incorporating heparin into photocrosslinkable alginate hydrogels (HP-ALG), which allows for controlled, prolonged release of therapeutic proteins. Heparin modification had minimal effect on the biodegradation profiles, swelling ratios, and elastic moduli of the hydrogels in media. The release profiles of growth factors from this affinity-based platform were sustained for 3 weeks with no initial burst release, and the released growth factors retained their biological activity. Implantation of bone morphogenetic protein-2 (BMP-2)-loaded photocrosslinked alginate hydrogels induced moderate bone formation around the implant periphery. Importantly, BMP-2-loaded photocrosslinked HP-ALG hydrogels induced significantly more osteogenesis than BMP-2-loaded photocrosslinked unmodified alginate hydrogels, with 1.9-fold greater peripheral bone formation and 1.3-fold greater calcium content in the BMP-2-loaded photocrosslinked HP-ALG hydrogels compared to the BMP-2-loaded photocrosslinked unmodified alginate hydrogels after 8 weeks implantation. This sustained and controllable growth factor delivery system, with independently controllable physical and cell adhesive properties, may provide a powerful modality for a variety of therapeutic applications.

1. Introduction

Hydrogels are widely applied in many biomedical applications, such as drug delivery [1–3], cell transplantation [4–6], and tissue engineering [7–10]. Hydrogels are frequently used in an injectable format, which allows them to be administered to a site in a minimally invasive manner. The crosslinking method used to form the hydrogels is important as it should not damage or denature any encapsulated bioactive factors or be toxic to incorporated or surrounding host cells; a wide variety of crosslinking approaches have been explored [11]. Photocrosslinking has emerged as a popular method for crosslinking hydrogels, offering the advantages of the capacity to crosslink the material *in situ* upon application of UV light and to produce precise structures in two and three dimension using photopatterning [12]. Recently, biodegradable and photocrosslinked hydrogels have been developed [13–15]; hydrogels such as the photocrosslinked alginate and hyaluronic acid can have tunable biodegradation rates and tunable mechanical properties [13,14]. Our group has developed photocrosslinked alginate hydrogels in which the degradation rates and mechanical properties can be controlled by varying the degree of methacrylation of the alginate backbone [13], and the cell adhesive properties of the material can be independently modulated by covalently coupling cell adhesion ligands, such as those containing the Arg-Gly-Asp (RGD) amino acid sequence, to the polymer [16]. However, in spite of the promising capacity to regulate these physical and biochemical biomaterial properties, these hydrogels typically share a similar problem with many other hydrogel systems regarding delivery of small bioactive factors [17,18]: the release of growth factors from the hydrogels is completed within a few days due to rapid diffusion out of the water-swollen network [19–21] and is thus not sustained over a long period of time. For many tissue regeneration applications, the sustained presentation of growth factors may enhance the growth of new tissue, as the cells in the area may require extended exposure to a specific soluble factor in their microenvironment to elicit certain cellular behaviors or morphogenetic events [22]. The native extracellular matrix in which cells reside in the body stores bioactive growth factors and protects them from degradation [23]. The use of hydrogels, which are able to retain growth factors and then locally deliver them to a specific site over a prolonged time period, may mimic

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this native environment and be beneficial for tissue regeneration. The long-term release of growth factors would allow transplanted cells and cells in tissues adjacent to the hydrogel injection site to be exposed to bioactive growth factors for an extended time.

Several reports have tried to address this issue by introducing growth factor binding ligands to polymer delivery systems [24–26]. Heparin, a highly sulfated glycosaminoglycan, has been used extensively as it is able to bind to many growth factors through affinity interactions [27]. Heparin has been conjugated to natural hydrogels (i.e. fibrin [18], collagen [28], and alginate [29]) and synthetic hydrogels (i.e. poly(ethylene glycol) [30–32] and Pluronic F127 [33]) to elicit the sustained release of heparin-binding growth factors.

Alginate, a naturally derived biocompatible polysaccharide composed of repeating units of α-L guluronic acid and β-D mannuronic acid, has been used in a variety of tissue engineering applications, including for bone [34–36], cartilage [35,37], skin [38,39] and nerve regeneration [40,41]. As a result of its biocompatibility, hydrophilic nature, and ability to form a hydrogel under mild conditions, alginate has great potential as a material for regenerative medicine applications. Several different approaches have been taken to modify alginate systems with heparin. Chitosan-alginate polyelectrolyte scaffolds functionalized with heparin were found to delay the release of fibroblast growth factor-2 (FGF-2), although the majority of the growth factor was released after only 2 days [10]. Heparin has been mixed into alginate prior to making ionically-crosslinked microspheres, and its addition was found to delay the release of a neurotrophin, although again the majority was released within the first couple of days [42]. Alginate and heparin have also been covalently crosslinked by ethylenediamine to form a hydrogel, and the burst release of FGF-2 from these hydrogels was found to be less than that from covalently crosslinked alginate hydrogels without heparin; however these hydrogels would likely not exhibit biodegradability over time due to the stable amide bond between ethylenediamine and alginate or heparin [29]. Alginate modified with sulfate groups using carbodiimide chemistry exhibits growth factor affinity binding capabilities similar to heparin due to the electrostatic interactions of growth factors with the sulfate groups [43], and when mixed into pre-formed freeze-dried calcium-crosslinked alginate scaffolds was shown to delay the release of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β) substantially [44].

In this study, we present an affinity-based growth factor delivery system using photocrosslinked heparin-alginate (HP-ALG) hydrogels to allow for the controlled, prolonged release of therapeutic proteins. Solutions of methacrylated alginate and methacrylated heparin are mixed together, and during the photopolymerization of the alginate hydrogels, the heparin is covalently bound to the alginate via free radical polymerization, and thus is covalently incorporated throughout the hydrogels. The alginate crosslinks and heparin linkages contain ester groups that are hydrolytically labile, thus permitting the degradation of the hydrogels by hydrolysis. Furthermore, growth factors incorporated into these hydrogels can bind to the heparin via affinity binding, and these bioactive factors can then be released through disassociation from the heparin and subsequent diffusion out of the hydrogels and through hydrolytic degradation of the heparin linkages and alginate crosslinks. Here, we determine if heparin modification has an effect on the biodegradation profiles, swelling ratios, and elastic moduli of the hydrogels and report on the release profiles and bioactivity of growth factors from these materials. We also examine the capacity of BMP-2-loaded HP-ALG hydrogels to enhance bone formation at an ectopic site in mice compared to BMP-2-laden alginate hydrogels without covalently coupled heparin. To our knowledge, this is the first report on an injectable alginate hydrogel system that offers the advantages of tunable physical properties [13], cell adhesive properties [16], and bioactive factor release properties; thus it may prove useful for a variety of therapeutic applications.

2. Materials and methods

2.1. Synthesis of methacrylated heparin

The methacrylated heparin was prepared by reacting heparin (Mw 17,000, Sigma, St. Louis, MO) with 2-aminoethyl methacrylate (AEMA, Sigma). To synthesize the methacrylated heparin with theoretical methacrylation of two carboxylic acid groups, heparin (1 g) was dissolved in a buffer solution (1% w/v, pH 6.5) of 50 mM 2-morpholinoethanesulfonic acid (MES, Sigma) containing 0.5 M NaCl. N-hydroxysuccinimide (NHS, 13.8 mg; Sigma) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 45.1 mg; Sigma) (molar ratio of NHS:EDC = 1:2) were added to the solution to activate the carboxylic acid groups of the heparin. After 5 min, AEMA (21.7 mg) (molar ratio of NHS:EDC:AEMA = 1:2:1) was added to the product and the reaction was maintained at room temperature for 24 h. The mixture was precipitated with the addition of excess acetone, dried under reduced pressure, and rehydrated to a 1% w/v solution in ultrapure deionized water (diH_2O) for further purification. The methacrylated heparin was purified by dialysis (MWCO 3500; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against diH_2O for 3 days, filtered (0.22 μm filter), and lyophilized. To verify the methacrylation of heparin, unmodified and methacrylated heparin were dissolved in deuterium oxide (D_2O, Sigma) and placed in separate NMR tubes. The ^{1}H-NMR spectra of the samples were recorded on a Varian Unity-300 (300 MHz) NMR spectrometer (Varian Inc., Palo Alto, CA, USA) using 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid (Sigma) as an internal standard.

2.2. Photocrosslinking for physical characterization of hydrogels

Low molecular weight sodium alginate (37,000 g/mol) was prepared by irradiating Protanal LF 20/40 (196,000 g/mol, FMC Biopolymer, Philadelphia, PA, USA) at a gamma dose of 5 Mrad. Methacrylated alginate (ALG) [13] and RGD-modified methacrylated alginate (RGD-ALG) at a theoretical methacrylation of 45% (25% actual) were prepared as previously reported [16]. To fabricate photocrosslinked HP-ALG hydrogels, methacrylated alginate (0.182 g) and methacrylated heparin (0.018 g) were dissolved in 10 ml of diH_2O or Dulbecco's Modified Eagle Medium (DMEM, Sigma) with 0.05% w/v photoinitiator (Irgacure-2959, Sigma). These solutions were placed between two glass plates separated by 0.75 mm spacers and photocrosslinked with 365 nm UV light (Model ENF-260C, Spectroline, Westbury, NY) at ~1 mW/cm^2 for 10 min to form the hydrogels. To fabricate ALG hydrogels without heparin as a comparative group, methacrylated alginate (0.2 g) was dissolved in 10 ml of diH_2O or DMEM with 0.05% w/v photoinitiator. These solutions were photocrosslinked as described above. Photocrosslinked hydrogel disks were created using a 6 mm diameter biopsy punch and placed in diH_2O or DMEM for mechanical testing, swelling, and degradation studies. To verify the completeness of methacrylated heparin and methacrylated alginate photocrosslinking, methacrylated alginate (0.0182 g) and methacrylated heparin (0.0018 g) were dissolved in D_2O (Sigma) with 0.05% w/v photoinitiator, placed in an NMR tube, and photocrosslinked as described above. The ^{1}H-NMR spectra of the HP-ALG before and after crosslinking were then determined as described above.

2.3. Mechanical testing

The elastic moduli of the photocrosslinked HP-ALG or ALG hydrogels formed with diH_2O or DMEM were determined by performing constant strain rate compression tests using a Rheometrics Solid Analyzer (RSAII, Rheometrics Inc., Piscataway, NJ, USA) equipped with a 10 N load cell. The photocrosslinked HP-ALG or ALG hydrogel disks were prepared as described above and maintained in DMEM or diH_2O at 37 °C. After 24 h incubation in DMEM or diH_2O,