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Preservation of FGF-2 bioactivity using heparin-based nanoparticles, and their delivery from electrospun chitosan fibers

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ABSTRACT

Here we present a novel matrix-mimetic nanoassembly based on polysaccharides. Chitosan electrospun fiber networks are decorated with heparin-containing polyelectrolyte complex nanoparticles (PCNs) that present basic fibroblast growth factor (FGF-2), both stably adsorbed to the surfaces and released into solution. These FGF-2/PCN complexes can be released from the fibers with zero-order kinetics over a period of 30 days. Further modification of fibers with a single bilayer of polyelectrolyte multilayer (PEM) composed of *N,N,N*-trimethyl chitosan and heparin completely prevent release, and the FGF-2/PCN complexes are retained on the fibers for the duration of the release experiment (30 days). We also compare the mitogenic activity of these FGF-2/PCN complexes delivered in two different states: adsorbed to a surface and dissolved in solution. FGF-2/PCN complexes exhibit mitogenic activity with respect to ovine bone marrow-derived mesenchymal stem cells, even after being preconditioned by incubating for 27 days at 37 °C in solution. However, when the FGF-2/PCN complexes are adsorbed to chitosan and coated with PEMs, the mitogenic activity of the FGF-2 steadily decreases with increasing preconditioning time. This work demonstrates a new system for stabilizing and controlling the delivery of heparin-binding growth factors, using polysaccharide-based matrix-mimetic nanomaterials. This work also contributes to our understanding of the preferred mode of growth factor delivery from porous scaffolds.
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1. Introduction

The use of growth factors to guide the differentiation of stem cells is a particularly promising strategy for engineering slow-healing tissues such as bone and cartilage, to treat a variety of injury and disease states [1]. Growth factors from the fibroblast growth factor (FGF) family and transforming growth factor-β (TGF-β) superfamily, which includes the bone morphogenetic proteins (BMPs), affect wound healing, tissue synthesis and mesenchymal stem cell (MSC) differentiation. For example, FGF-2 is involved in osteogenesis [2,3], chondrogenesis [4] and angiogenesis [5]. However, many therapeutic strategies based on growth factor delivery are impeded by the relative instability of growth factors on time scales associated with these biological processes. Members of the FGF family and TGF-β superfamily have plasma half-lives on the order of minutes (1.5 min for FGF-2, and between 11 and 160 min for TGF-β1, for example) [6]. FGF-2 and BMP-2 have been demonstrated to lose their activity within 24 h and become completely degraded within 3 days when adsorbed to and released from mineral-based and ceramic scaffolds for bone tissue engineering [7,8]. Materials for skeletal tissue engineering that use growth factors should be developed that can present the growth factor in a structural and biochemical context similar to native tissue. This could mean presenting the growth factor bound to a surface or slowly releasing the growth factor into nearby tissue [9].

In mammalian tissues, polysaccharides are found in nanostructured proteoglycans, like aggrecan, which impart both biomechanical and biochemical function to the extracellular matrix (ECM) [10]. One of the most important of these biochemical functions is to serve as a reservoir for the binding and stabilization of growth factors. Heparin is a glycosaminoglycan that protects FGF-2 from proteolytic and chemical inactivation [11]. This stabilization likely results from the binding of FGF-2 to specific sulfation patterns in

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heparin that also potentiate the interaction between the growth factor and the growth factor receptor [12]. The heparin-binding sequence of FGF-2 is homologous to a similar sequence in TGF-β1 [2].

Sulfated or sulfonated materials that might mimic this biochemical function of the ECM have been proposed for growth factor delivery. These include sulfonated silk fibroin [13], heparin-conjugated fibrin gels [14] and glycosaminoglycan-containing polyelectrolyte multilayers (PEMs) [15,16]. These materials have been designed either to present growth factors on a surface or to deliver growth factors by releasing them into solution. It is unclear whether one or the other of these particular strategies is the preferred mode of delivery. The present work has two goals. The first goal is to demonstrate an ECM-mimetic polysaccharide-based nanoassembly that can present both surface-bound and soluble FGF-2 that is stabilized by complexation with heparin-containing nanoparticles. The second goal is to compare the ability of these nanoparticles to preserve the mitogenic activity of FGF-2 in the surface-adsorbed and solution states.

In addition to the binding and stabilization of growth factors, polysaccharides possess many other properties that make them attractive biomaterials. These properties include biodegradability, antimicrobial activity and the ability to support mammalian cell growth. Furthermore, polysaccharides can be readily processed by a number of techniques to produce materials that mimic both biophysical and biochemical features of the ECM. Previously we have studied glycosaminoglycan-containing PEMs, polyelectrolyte complex nanoparticles (PCNs) and electrospun fibers [16–21]. We have demonstrated that these polysaccharide-based nanostructures can bind FGF-2 and that FGF-2 mitogenic activity can be enhanced by incorporating it into heparin-containing PEMs [16].

Here we demonstrate the use of nanostructured chitosan-based electrospun fibers with high porosity and a large surface area for the binding, stabilization and controlled release of heparin-binding growth factors. Chitosan is an amine-containing polysaccharide. Its pendant amine is protonated in acidic medium, making chitosan a weak polycation. Chitosan has excellent film- and fiber-forming properties, but is difficult to electrospin due to its solution properties. Several reports have demonstrated successful electrospinning of chitosan by blending with other water-soluble polymers [22–24]. Sangsanoh and Supaphol [25] reported the electrospinning of pure chitosan using a trifluoroacetic acid/dichloromethane (TFA/DCM) solvent. This solvent system likely facilitates the electrospinning because of the formation of ammonium–TFA salt with the amine groups in chitosan [26]. In this work, we develop chitosan fiber networks that can present growth factor-containing nanoparticles both on the surface and by releasing them into solution. Chitosan fiber networks are modified with chitosan–heparin PCNs containing of FGF-2 as a model heparin-binding growth factor. Zero-order release of FGF-2 from the fibers is demonstrated for up to 30 days. Release of FGF-2 is controlled by additional complexation of the adsorbed nanoparticles with polysaccharides. When fibers are further modified with a single bilayer of polysaccharide-based PEM, composed of *N*,*N*,*N*-trimethyl chitosan (TMC) and heparin, release of the FGF-2/PCN complexes is prevented for the duration of the analysis (30 days). The mitogenic activity of the FGF-2/PCN complexes is also evaluated, with respect to the proliferation of ovine bone-marrow derived MSCs. Complexation is found to preserve FGF-2 activity for 30 days.

2. Materials and methods

2.1. Materials

Highly purified chitosan (80 kDa, 5% acetylated as determined by ^{1}H NMR, Protasan UP B 90/20) was purchased from NovaMatrix (Sandvika, Norway). Heparin sodium (from porcine intestinal mucosa, 12.5% sulfur) was purchased from Celsus Laboratories (Cincinnati, OH). TMC was synthesized following a procedure described by de Britto and Assis [27]. Recombinant human FGF-2 146 aa was purchased from R&D Systems (Minneapolis, MN). 5(6)-Carboxyfluorescein *N*-hydroxysuccinimide ester was purchased from Sigma–Aldrich (St. Louis, MO). Sodium bicarbonate and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA). TFA, DCM, ammonium hydroxide, dimethyl sulfate, sodium hydroxide and sodium chloride were purchased from Acros Organics (Geel, Belgium). The following were purchased from HyClone (Logan, UT): fetal bovine serum (FBS), 0.25% trypsin with EDTA, low glucose Dulbecco's modified Eagle's medium, minimum essential medium alpha (α-MEM) (supplemented with L-glutamine, ribonucleosides and deoxyribonucleosides), and Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}. The following were purchased from Gibco (Grand Island, NY): antibiotic-antimycotic (anti/anti), 1 M HEPES buffer solution and PBS with Ca^{2+} and Mg^{2+}. Calcein-AM in DMSO (4 mM) was purchased from Invitrogen (Eugene, OR). 4′6-Diamidino-2-phenylindole-2HCl (DAPI) was purchased from Thermo Fisher Scientific (Rockford, IL). Human fibronectin was purchased from BD Biosciences (Bedford, MA). All of the polymers, growth factor and solvents were used without further purification. All aqueous solutions were prepared using ultrapure, 18.2 MΩ cm, water (DI water).

Rhodamine-modified chitosan was prepared by dissolving 100 mg of chitosan in 10 ml of 0.1 M acetic acid, adding 10 ml of methanol to the solution, adding 3.25 ml of 2 mg ml^{-1} rhodamine B isothiocyanate (Sigma, St. Louis, MO) in methanol, and allowing the solution to react overnight. Rhodamine-modified chitosan was purified via dialysis, freeze dried and stored at 4 °C protected from light until use. Fluorescein-labeled FGF-2 (FGF-2^{LB}) was prepared by dissolving of FGF-2 in 0.1 M $NaHCO_3$ (pH 8) at a concentration of 25 μg ml^{-1}. The solution was vigorously stirred in an ice bath to avoid protein denaturation. The dye solution was prepared by the dissolution of 5(6)-carboxyfluorescein *N*-hydroxysuccinimidyl ester in DMSO at a concentration of 10 mg ml^{-1} [28]. Next, 10 μl of the dye solution was slowly added to 1 ml of protein solution and allowed to react for 4 h with vigorous stirring in an ice bath. The unreacted dye was removed by the dialysis of the labeled protein solution in PBS (pH 7.4) for 24 h, using 3 kDa MWCO dialysis cassettes (Slide-A-Lyzer, Thermo Scientific, PA). A fluorescence microplate reader (FLUOstar Omega, BMG Labtech, Durham, NC) was used to confirm labeling of the FGF-2^{LB} and to determine the calibration curve used to quantify labeled FGF-2 in solution (λ_{ex} = 485 nm and λ_{em} = 520 nm).

2.2. Electrospinning chitosan

The electrospinning apparatus consisted of a high-voltage (1-30 kV) direct current power supply (GAMMA High Voltage Instruments, ES30P-10 W/DAM), a syringe pump (Harvard Apparatus, UK) and a rectangular brass collector covered with aluminum foil. The metal needle of the syringe, connected to the power supply, and the collection target were kept at a horizontal distance of 18 cm. Chitosan (or rhodamine-labeled chitosan) was dissolved in TFA:DCM (7:3) overnight at 7 wt.%, at room temperature. The condition was selected after preliminary analysis of conductivity and viscosity as well as evaluations on the stability and homogeneity of the solutions. Electrospinning of the 7 wt.% chitosan was achieved with a flow rate of 0.5 ml h^{-1} and an applied voltage of 18 kV. Although chitosan is insoluble in neutral aqueous solutions, the presence of residual solvent or ammonium–TFA salts render the electrospun fibers soluble in water. To eliminate residual solvent, a procedure for fiber network stabilization has been

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