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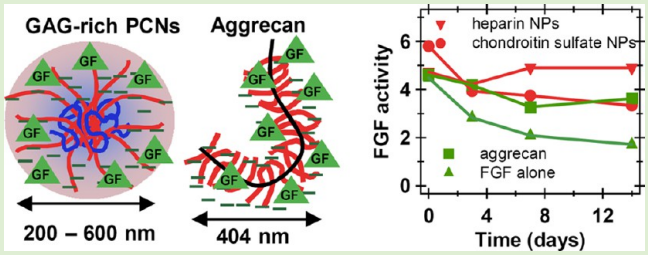
Aggrecan-Mimetic, Glycosaminoglycan-Containing Nanoparticles for Growth Factor Stabilization and Delivery

Laura W. Place,[†] Maria Sekyi,[‡] and Matt J. Kipper*[,†,‡]

[†]School of Biomedical Engineering and [‡]Department of Chemical and Biological Engineering, Colorado State University, 1370 Campus Delivery, Fort Collins, Colorado, United States

Supporting Information

ABSTRACT: The direct delivery of growth factors to sites of tissue healing is complicated by their relative instability. In many tissues, the glycosaminoglycan (GAG) side chains of proteoglycans like aggrecan stabilize growth factors in the pericellular and extracellular space, creating a local reservoir that can be accessed during a wound healing response. GAGs also regulate growth factor-receptor interactions at the cell surface. Here we report the development of nanoparticles for growth factor delivery that mimic the size, GAG composition, and growth factor binding and stabilization of aggrecan. The aggrecan-mimetic nanoparticles are easy to assemble, and their structure and composition can be readily tuned to alter their physical and biological properties. We use basic fibroblast growth factor (FGF-2) as a model heparin-binding growth factor, demonstrating that aggrecan-mimetic nanoparticles can preserve its activity for more than three weeks. We evaluate FGF-2 activity by measuring both the proliferation and metabolic activity of bone marrow stromal cells to demonstrate that chondroitin sulfate-based aggrecan mimics are as effective as aggrecan, and heparin-based aggrecan mimics are superior to aggrecan as delivery vehicles for FGF-2.



INTRODUCTION

Growth factors hold much promise as therapeutics in regenerative medicine. They control cell proliferation, differentiation, and migration and are involved in a number of metabolic pathways.[1−3] Growth factors have been used clinically in wound healing, bone disease, and in clinical trials for treating coronary artery disease.[4] They have also been proposed for tissue engineering of nerves, bladders, blood vessels, and osteochondral defects.[5−11] However, these proteins are unstable in plasma and require high doses to affect clinical outcomes.[12,13] Realizing the potential of growth factors in regenerative medicine will require strategies for maintaining growth factor stability over time scales associated with tissue healing and for delivery of growth factors to the site of disease or injury.

Polymeric nanoparticles can be designed to protect growth factors from enzymatic degradation and to release growth factors at a desired rate. Furthermore, their size allows them to cross the epithelium.[14] For example, Gu et al. formulated nanoparticles from glycidyl methacrylated dextran and gelatin for fibroblast growth factor (FGF) delivery to mouse mesenchymal stem cells.[15] They showed increased cellular activity for as long as 10 days. In another study, Ho et al. synthesized a sulfated chitosan and then complexed it with unmodified chitosan to form polyelectrolyte complex nanoparticles (PCNs). These PCNs were used to deliver FGF to fibroblasts. They tested cell viability for five days, demonstrating that viability was improved using PCNs to deliver FGF but that PCNs did not perform as well as the glycosaminoglycan heparin.[16] Huang et al. demonstrated preservation of the mitogenic activity of vascular endothelial growth factor (VEGF) for up to five days by encapsulation in PCNs of dextran sulfate with three polycations: chitosan, poly(L-lysine), and poly(ethyleneimine)).[17] Tan et al. immobilized chitosan-heparin nanoparticles onto a decellularized vein scaffold and used them to localize vascular endothelial growth factor (VEGF) to improve vascularization of the scaffold. They showed controlled release over 30 days and improved cell infiltration, proliferation, ECM production, and vascularization.[18] Our group has also demonstrated controlled release of FGF complexed to chitosan-heparin PCNs from nanofiber scaffolds, which maintained FGF activity for over 30 days.[19] Rajam et al. was able to show a steady release of growth factor from a nanoparticle impregnated collagen−chitosan scaffold for 50 days.[20]

In normal tissues, growth factors are stabilized and presented to cells in a complex milieu of various other stimuli. Thus, in addition to stabilizing growth factors, a growth factor delivery vehicle might be designed to present growth factors in a biomimetic context that interacts favorably with these other signals. In the extracellular and pericellular space of many tissues, the glycosaminoglycan (GAG) side chains of

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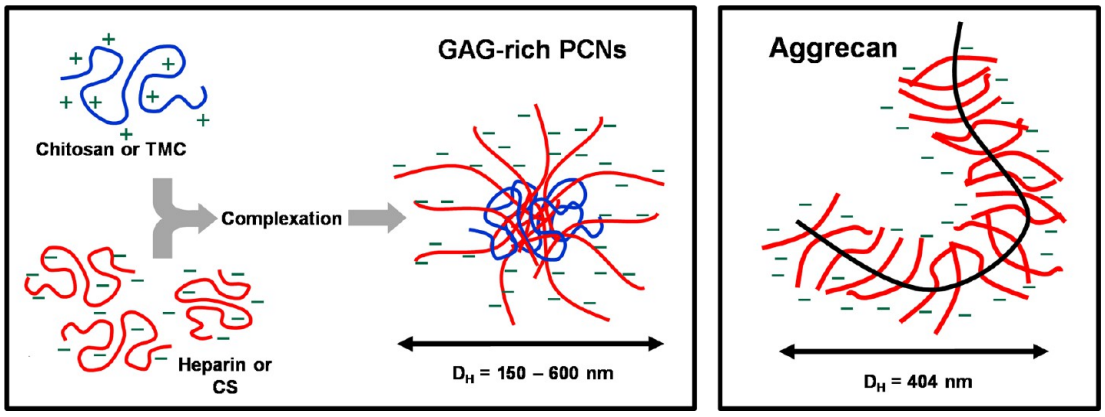

Figure 1. GAG-rich PCNs are formed by the complexation of polycationic chitosan or TMC (blue) with the polyanionic GAGs heparin or CS (red) in excess. This results in negatively charged particles presenting the GAGs that have similar size in solution and composition to the proteoglycan aggrecan. Aggrecan is a highly glycosylated protein (black) with up to 100 CS and keratan sulfate chains (red). The hydrodynamic diameter of aggrecan in solution is taken from Papagiannopoulos et al.[31]

proteoglycans, such as aggrecan, versican, and perlecan serve as a reservoir of stabilized growth factors, and regulate their signaling by also binding to their cell surface receptors.[21−28] GAGs might therefore make excellent materials for growth factor delivery. These GAGs are polyanionic polysaccharides, such as heparin and chondroitin sulfate. The so-called heparin-binding growth factors that bind to GAGs include members of the FGF family and the transforming growth factor-β (TGF-β) superfamily (including some bone morphogenetic proteins). Heparin has been shown to preserve the stability of or to enhance the delivery of a variety of growth factors including FGFs, TGF-β, VEGF, nerve growth factor (NGF), and platelet-derived growth factor (PDGF).[29] This is the motivation for using sulfated glycosaminoglycans like heparin and their analogs as polymers for growth factor delivery.

In addition to their biochemical functions, proteoglycans also perform important biophysical functions that are dependent upon their nanoscale structure.[30] Aggrecan is the most highly glycosylated of the proteoglycans, with up to 100 GAGs (chondroitin sulfate and keratan sulfate) attached to a core protein. In solution it adopts a dense, bottle-brush structure bearing a high concentration of negatively charged sulfate groups, with a hydrodynamic diameter of about 400 nm.[31−35] The high negative charge density and resulting high osmotic pressure of aggrecan give cartilage its compressive strength and lubricity.[34]

The aim of this work is to develop nanoparticles for growth factor delivery that mimic the composition, biochemical function, and size of aggrecan for growth factor stabilization and delivery. We use FGF-2 as a model growth factor because it is relatively unstable, and its binding to GAGs is known to influence both its stability and its presentation to growth factor receptors.[36] The aggrecan-mimetic nanoparticles are formed by the complexation of anionic and cationic polysaccharides to form polyelectrolyte complex nanoparticles (PCNs), which has been extensively studied by several groups.[37−42] The cationic polysaccharides chitosan (Chi) and *N,N,N*-trimethyl chitosan (TMC) are complexed with the anionic GAGs heparin (Hep) and chondroitin sulfate (CS) in solution with the polyanion in excess, so that the resulting PCNs have a colloidally stable, negatively charged structure that mimics the size and chemistry of aggrecan (Figure 1). The structures of the polyelectrolytes are shown in Figure 2.

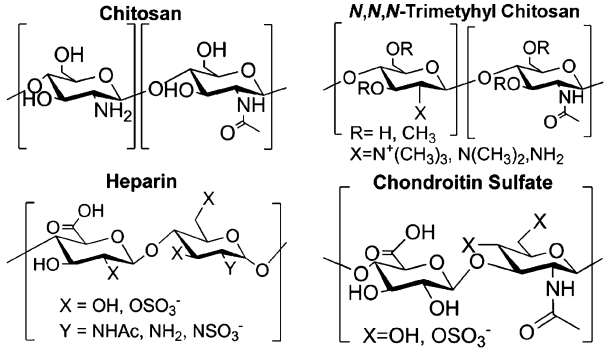

Figure 2. Chemical structures of the polysaccharides used in this work.

Our group has previously studied growth factor binding, stabilization, and delivery using Hep-Chi PCNs and Hep-Chi and Hep-TMC polyelectrolyte multilayers.[1,19,43] In the current work, PCNs from the four polyanion−polycation pairs, Hep-Chi, Hep-TMC, CS-Chi, and CS-TMC, are characterized, including the particle yield, size, zeta potential, and ability to bind FGF-2. The ability of the PCNs to maintain FGF-2 activity over 21 days is also demonstrated here, using assays that measure both the overall metabolic activity and the mitogenic activity of ovine marrow stromal cells (MSCs) to FGF-2. The ability to stabilize and deliver FGF-2 is compared to natural aggrecan and to FGF-2 delivered in solution with no polymer protection.

MATERIALS AND METHODS

Materials. Chitosan was purchased from Novamatrix (Protosan UP B 90/20, 5% acetylated determined by ^{1}H NMR, M_w = 80 kDa; PDI = 1.52; Sandvika, Norway). Heparin sodium was purchased from Celsus Laboratories (from porcine intestinal mucosa, 12.5% sulfur, M_w = 14.4 kDa; PDI = 1.14, Cincinnati, OH). Chondroitin sulfate sodium salt (CS; from shark cartilage, 6% sulfur, 6 sulfate/4 sulfate = 1.24, M_w = 84.3 kDa; PDI = 1.94), rhodamine B isothiocyanate, and aggrecan from bovine articular cartilage were purchased from Sigma-Aldrich (St. Louis, MO). *N,N,N*-Trimethyl chitosan (TMC) was synthesized following a procedure described by De Britto and Assis and characterized as we have done previously.[43,44] Sodium acetate, sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA). Glacial acetic acid and glutaraldehyde 8% in water were purchased from Acros Organics

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(Geel, Belgium). Recombinant human FGF basic (FGF-2) 146 aa (carrier free) and Quantikine ELISA Human FGF basic were purchased from R&D Systems (Minneapolis, MN). 4′,6-Diamidino-2-phenylindole·2HCl (DAPI) was purchased from Thermo-Scientific (Rockford, IL). LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells was purchased from Invitrogen (Eugene, OR). CellTiter 96 Non-Radioactive Cell Proliferation Assay (modified MTT assay) was purchased from Promega (Madison, WI). Fetal bovine serum (FBS), 0.25% trypsin with EDTA, low-glucose Dulbecco's modified Eagle's medium (D-MEM), minimum essential medium alpha (α-MEM; supplemented with L-glutamine, ribonucleosides, and deoxyribonucleosides), and Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} and Mg^{2+} were purchased from HyClone (Logan, UT). Antibiotic-antimycotic (anti/anti), 1 M HEPES buffer solution, and Dulbecco's phosphate buffered saline with Ca^{2+} and Mg^{2+} were purchased from Gibco (Grand Island, NY). A Millipore synthesis water purification unit (Millipore, Billerica, MA) was used to obtain ultrapure, 18.2 MΩ·cm water (DI water), used for making all aqueous solutions. Fluorimetric and UV−vis spectrophotometric techniques were performed using a fluorescence microplate reader (FLUOstar Omega, BMG Labtech, Durham, NC). Fluorescence microscopy was performed using an Olympus IX70 epifluorescence microscope (Center Valley, PA) with appropriate filters.

Formation and Characterization of Polyelectrolyte Complex Nanoparticles (PCNs). All PCNs were formed by the "one-shot" addition of a solution of the polycation to a solution of the polyanion, under vigorous stirring, with the polyanion in excess.[41,42,45,46] Four different polycation−polyanion pairs were used to create PCNs with different compositions: heparin with chitosan (Hep-Chi), heparin with TMC (Hep-TMC), chondroitin sulfate with chitosan (CS-Chi), and chondroitin sulfate with TMC (CS-TMC). For Hep-Chi PCNs, heparin (0.95 mg mL^{-1}) and chitosan (0.9 mg mL^{-1}) were each dissolved in 0.1 M acetate buffer at pH 5 and then filtered using 0.22 μm polyvinylidene fluoride syringe filters (Fisher Scientific, PA). The chitosan solution was added in one shot to the stirring heparin solution in a 1:6 volume ratio (6 mL chitosan solution to 36 mL heparin solution), vigorously stirred for 3 h, then left to settle overnight to remove any aggregates. The solution containing Hep-Chi PCNs and uncomplexed polymer was decanted from the settled aggregates and centrifuged (9000 rcf for 15 min). The supernatant was decanted to remove any uncomplexed polymer and the pelleted PCNs were resuspended in 2.5 mL of DI water. At this concentration PCNs do aggregate over time and are not used if they are older than 1 month. Before being diluted for analysis or use in an experiment, PCNs were briefly vortexed, which was sufficient to break up aggregates and resuspend them. A similar procedure was used for Hep-TMC, CS-Chi, and CS-TMC PCNs with the following modifications: For CS-Chi and CS-TMC PCNs, the chondroitin sulfate solution was 1.8 mg mL^{-1} because it contains approximately half as many sulfate groups as heparin. The Hep-TMC and CS-Chi PCNs were centrifuged at 2000 rcf for 30 min (instead of 9000 rcf for 15 min) because, at higher speeds, the resulting pellet was not readily resuspended. The Hep-TMC, CS-Chi, and CS-TMC PCNs were resuspended in PBS because they did not readily resuspend in DI water. Different centrifugation conditions might yield different particle size distributions. The conditions used here for each type of PCN were chosen based on our ability to pellet the particles and also subsequently resuspend them.

Particle yield was determined on a polycation (chitosan or TMC) basis by fluorescence. Chitosan and TMC were tagged with rhodamine.[19] Chitosan or TMC (10 mg mL^{-1}) was dissolved in 10 mL of 0.1 M acetate buffer, pH 5, then an equal volume of methanol was added. Rhodamine B isothiocyanate was dissolved in methanol (2 mg mL^{-1}) and 3.25 mL of this was added to the 20 mL of chitosan or TMC solution. These were left to stir overnight and then dialyzed against DI water for 24 h using 20 kDa MWCO dialysis cassettes (Slide-A-Lyzer, Thermo Scientific, PA). The resulting solutions were then lyophilized. The entire procedure and storage were done in the dark to protect against photobleaching. Each of the tagged polycations was mixed with untagged polycation in a 1:10 ratio. These were dissolved in 0.1 M acetate buffer, pH 5, filtered, and then used to make PCNs as described above. The fluorescence intensity of the PCNs (λ_{ex} = 544 nm and λ_{em} = 590 nm) was compared to a standard curve created from the 1:10 mixture of tagged and untagged chitosan or TMC to calculate particle yield on a polycation basis. Fluorescently labeled polycations were only used for determining the PCN yields. All other experiments used only nonlabeled polycations.

Hydrodynamic radius of the PCNs was measured by dynamic light scattering (DLS) using a 90Plus/BI-MAS (Brookhaven Instruments, Holtsville, NY). All PCN samples were diluted to 2−5 mg mL^{-1} in PBS to give samples of 1−2 mL. Measurements were taken at 25 °C at a fixed angle of 90°, 1 min per measurement. The values reported are the mean effective diameter and the mean polydispersity for each sample. Zeta potential was measured using the same instrument and the same samples used for DLS via electrophoretic light scattering (ELS). Each sample was measured five times at 25 °C and the values reported are the mean zeta potential for each sample ± the standard error of the mean.

FGF-2 PCN Loading. An ELISA (Quantikine ELISA Human FGF basic, R&D Systems) was used to determine the loading efficiency of FGF-2 to the PCNs and to aggrecan. FGF-2 standards had concentrations from 11 pg mL^{-1} to 700 pg mL^{-1}. FGF-2 loading was performed in duplicate for all conditions. FGF-2 (100 ng mL^{-1}) in DPBS was mixed with each PCN type and with aggrecan (1 mg mL^{-1}) for 30 min. These were then centrifuged (9000 rcf for 15 min). The supernatants were removed and diluted 1:166 for the FGF-2 concentration to be within the range of the ELISA. The FGF-2 concentration in the supernatants was subtracted from the original amount in the solution used for loading to determine the amount of FGF-2 bound to the PCNs and to aggrecan.

Cell Harvest and Culture. The cell harvesting and expansion procedure is described in detail in our previous work.[1] Bone marrow aspirates from the iliac crest of three different female sheep were centrifuged. The supernatant containing the nucleated cells was mixed with growth media (low-glucose D-MEM with 10% FBS, 1% anti/anti, and 2.5% HEPES) and seeded into culture flasks. After 24 h, the media was changed to remove all nonadherent cells. The marrow stromal cell (MSC) colonies were allowed to grow for at least seven days, and then the cells were trypsinized, counted, and reseeded in culture flasks using maintenance media for culture expansion (α-MEM with 10% FBS, 1% anti/anti, and 2.5% HEPES). MSCs were cryo-preserved prior to seeding into experimental conditions. Cells were not used beyond the fifth passage.

PCN Cytocompatibility. The compatibility of the PCNs toward MSCs was evaluated by dosing cell cultures from one donor animal with different PCN concentrations. MSCs were seeded in a 48-well plate at 10000 cells cm^{-2} and allowed to attach for 3 h with 0.5 mL of untreated α-MEM containing 10% FBS. Each of the treatments, Hep-Chi, Hep-TMC, CS-Chi, and CS-TMC, were diluted with low-serum media (α-MEM containing 2.5% FBS) to five different concentrations (0.5, 5, 10, 20, and 40 μg mL^{-1}). After the 3 h attachment, the media was aspirated and replaced with 0.5 mL of low-serum media containing PCNs. Two untreated wells in low-serum media were also cultured. In this low-serum condition, we have previously found that ovine MSCs survive but do not proliferate well unless exogenous FGF-2 is added to the cultures.[1] Cells were then incubated at 37 °C for 48 h. After 48 h, one untreated well was exposed to 70% ethanol for 30 min as a control for the dead stain. The media was aspirated, each well was rinsed with DPBS with Ca^{2+} and Mg^{2+}, and Live/Dead stain was applied according to the vendor's instructions. The Live/Dead kit contained calcein AM (4 mM in DMSO) and ethidium homodimer-1 (2 mM in DMSO). These stock solutions were diluted to 2 μM calcein AM and 0.8 μM ethidium homodimer-1 in DPBS without Ca^{2+} and Mg^{2+}. Dye solution (200 μL) was added to each well and the plates were incubated in the dark at room temperature for 45 min before being imaged by fluorescence microscopy. For each well, three nonoverlapping fields of view were imaged, totaling six images per well, at 4× magnification, approximately covering 25% of the surface area in each well. The corresponding images taken with each filter were merged and qualitatively evaluated using Adobe Photoshop.

Preconditioning FGF-2, PCNs, and Aggrecan. To evaluate the ability of PCNs to stabilize FGF-2, FGF-2-loaded PCNs, FGF-2 bound to aggrecan, and uncomplexed FGF-2 were first preconditioned by incubating in media containing 10% FBS for 0, 3, 7, 14, or 21 days to allow destabilization of the FGF-2. After preconditioning the activity of FGF-2 was assayed by measuring the mitosis and metabolic activity of ovine MSCs. These assays are described in the next section.

FGF-2 was bound to each PCN type and to aggrecan using the same procedure as in the loading study above, without the final centrifugation step. Aliquots (125−250 μL in PBS) of FGF-2-loaded PCNs, FGF-2-loaded aggrecan, or FGF-2 alone were stored frozen. The aliquots reserved for 21 days of preconditioning were removed first and added to α-MEM with 10% FBS. These were placed in the incubator at 37 °C for 21 days. After 7 days, the 14 day aliquots were removed from the freezer and the same procedure was done. These remained in the incubator for 14 days. This was repeated for the 7 and 3 day aliquots on the appropriate days. On the day on which MSCs were seeded, the zero day aliquots were thawed and prepared.

MSC Response to FGF-2, PCNs, and Preconditioned FGF-2-Loaded PCNs and Aggrecan. To evaluate the ability of PCNs and aggrecan to stabilize growth factors, MSCs were treated with the preconditioned FGF-2 in low-serum media. MSC mitogenic activity was evaluated after 4 days of culture by staining, imaging, and counting cell nuclei, and their metabolic activity was measured using the CellTiter 96 assay after 48 h of culture.

Mitogenic Activity Assay. The FGF-2 activity after 0, 3, 7, and 14 days of preconditioning in media containing 10% serum at 37 °C was evaluated in an MSC proliferation assay. All treatments were conducted in duplicate wells on cells from each of the three donor animals seeded at 7000 cells cm^{-2}. These were allowed to attach for 3 h in a 48-well plate with 0.5 mL of untreated, α-MEM containing 10% FBS. After the 3 h attachment period, the seeding media was aspirated and replaced with 0.5 mL of low-serum media containing the treatment (preconditioned FGF-2-loaded PCNs, FGF-2 bound to aggrecan, PCNs without FGF-2, aggrecan without FGF-2, or FGF-2 in solution). PCN and aggrecan treatments were diluted to 10 μg mL^{-1} (PCN or aggrecan), which corresponds to 1 ng mL^{-1} total FGF-2 (including both bound and unbound FGF-2). Unbound FGF-2 was also diluted to 1 ng mL^{-1}. These were cultured for four days with one media change after two days of culture, where the media change also contained the treatment. The treatment-containing media used for the media change was also stored in the jacketed incubator at 37 °C in α-MEM 2.5% FBS (5% CO_2) for the first two days of culture.

After the 4 day culture period, the cells were stained with calcein AM, fixed with 2% glutaraldehyde, and counter-stained with DAPI. The media was aspirated and each well was rinsed with DPBS with Ca^{2+} and Mg^{2+}. A 2 μM solution of calcein AM in DPBS with Ca^{2+} and Mg^{2+} was added to each well and incubated at 37 °C in the dark for 30 min. This dye was aspirated, wells were rinsed, and 2% glutaraldehyde in DPBS with Ca^{2+} and Mg^{2+} was added to each well. This was incubated at 4 °C in the dark for 40 min. The glutaraldehyde solution was aspirated, each well was rinsed with DPBS with Ca^{2+} and Mg^{2+}, DAPI (1 μg mL^{-1}) in DPBS with Ca^{2+} and Mg^{2+} was added to each well, and this was incubated at room temperature for 15 min. The dye solution was aspirated and a final rinse was done with DPBS with Ca^{2+} and Mg^{2+}. The well plates were stored at 4 °C in the dark until imaging was performed. Three nonoverlapping images were taken per well (25% of the surface area). Images were processed using the ImageJ 1.41o software (National Institutes of Health, U.S.A.). The blue channel contained only the DAPI stained nuclei and was counted using the particle analyzer algorithm in the ImageJ software to obtain cell numbers per area. The FGF-2 activity is reported as the average cells per area for each treatment, normalized by the average cells per area in the untreated control samples.

Metabolic Activity Assay. To further understand how the FGF-2 was affecting the cultures during the mitosis assay, the FGF-2 activity after 0, 7, 14, and 21 days of preconditioning in media containing 10% serum at 37 °C was evaluated using a modified MTT assay for cell metabolic activity. Ovine MSCs isolated from one donor animal were used for each treatment. All treatments were conducted in triplicate wells on cells seeded at 10000 cells cm^{-2}. These were allowed to attach for 3 h in a 48-well plate with 0.5 mL of untreated α-MEM containing 10% FBS. After the 3 h attachment period, the seeding media was aspirated and replaced with 0.5 mL of low-serum media containing the treatment (preconditioned FGF-2-loaded PCNs, FGF-2 bound to aggrecan, PCNs without FGF-2, aggrecan without FGF-2, or FGF-2 in solution). Treatments were all diluted to the same concentrations used for the mitosis assay described above. Each treatment was evaluated in triplicate.

After a 48 h culture period, the CellTiter 96 (modified MTT) assay was performed according to the manufacturer's instructions. The FGF-2 activity is reported as the average of nine readings (triplicate readings from each of three replicates) for each condition, normalized by the negative control (no FGF-2).

Statistics. Data analysis was performed using Minitab (Minitab, Inc., State College, PA), version 16. For the FGF-2 stability/activity assay, comparisons between groups were performed via analysis of variance (ANOVA) models with Tukey's multiple comparison tests. Comparisons of each treatment group to the negative control were done using ANOVA with Dunnett's tests. Differences with $p < 0.05$ were considered statistically significant. Mitogenic activity data are expressed as the mean ± standard error of the mean ($n = 6$). Cell metabolic activity data were obtained from triplicate samples taken from each well; each treatment was also performed in triplicate ($n = 9$).

RESULTS AND DISCUSSION

PCN Formation and Characterization. Four different compositions of PCNs were made by electrostatic complexation of a polyanion (either Hep or CS) and a polycation (either Chi or TMC) with the polyanion in excess. The PCN yield was determined on a polycation basis from fluorescence measurements of PCNs formed with rhodamine-labeled polycations. The FGF-2 loading was determined by difference using an ELISA for FGF-2, and the size and zeta potentials of PCNs before and after FGF-2 loading were determined by DLS and ELS, respectively. These results are shown in Table 1.

The Hep-Chi PCNs exhibited the highest yield (63%) while the other three types were all in the 35−40% range. The Hep-TMC PCNs were the most difficult to resuspend and material was lost in the purification process. All PCNs had hydrodynamic diameters <1 μm and each type had a monomodal size distribution with polydispersity index less than 0.2.[37] The Hep-

Table 1. PCN Characterization[a]

composition	yield (%)	FGF-2 loading (ng/mg)	mean D_h (nm)	PDI	ζ-potential (mV)
Hep-Chi	63 ± 7		260	0.152	−48 ± 0.3
Hep-Chi-FGF-2		51 ± 14	260	0.153	−3.87 ± 0.05
Hep-TMC	39 ± 3		170	0.005	−10 ± 2
Hep-TMC-FGF-2		56 ± 11	[b]	[b]	[b]
CS-Chi	34 ± 5		260	0.05	−40 ± 3
CS-Chi-FGF-2		47 ± 3	230	0.038	−4.1 ± 0.1
CS-TMC	39 ± 6		540	0.161	−34 ± 0.8
CS-TMC-FGF-2		48 ± 12	380	0.161	−4.55 ± 0.05
aggrecan		50 ± 5	400[c]		

[a]For Hep-Chi, CS-Chi, and CS-TMC PCNs the mean hydrodynamic diameter, PDI, and zeta potential are shown both before and after loading with FGF-2. [b]Instability of Hep-TMC PCNs prevented the measurement of their size and ζ-potential after isolation by dialysis. [c]From Papagiannopoulos et al.[31]

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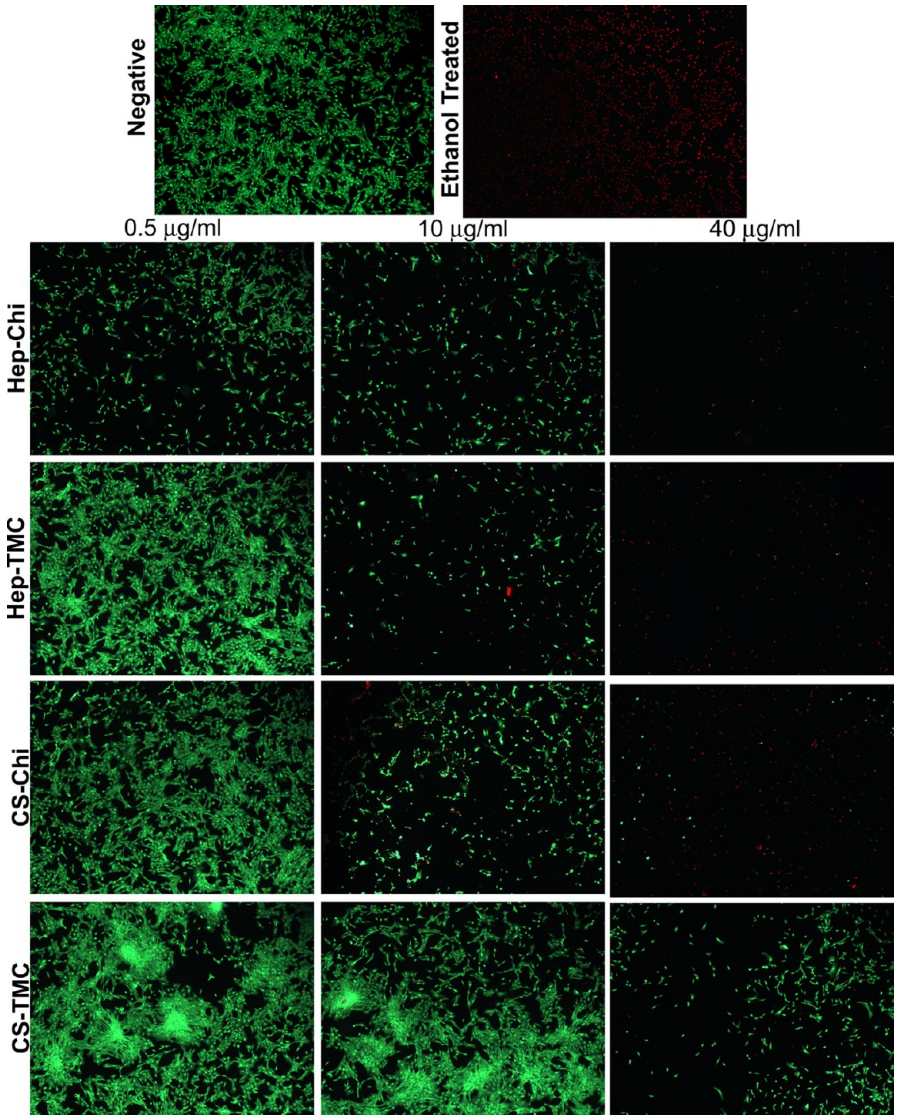

Figure 3. Cytocompatibility using Live/Dead stain. Each row represents a different PCN type dosed with increasing PCN concentrations (0.5−40 μg mL^{-1}) from left to right. Some cytotoxicity of the PCNs is observed for doses greater than 10 μg mL^{-1}.

TMC particles were the smallest (mean D_h = 170 nm), the CS-TMC particles were the largest (mean D_h = 540 nm), and both Hep-Chi and CS-Chi had diameters of 260 nm. These results are similar to those observed by other groups making similar particles.[37,47,48] The hydrodynamic diameter of aggrecan reported by others is in the middle of the observed range of sizes for these PCNs.[31] (We did not independently measure the aggrecan by DLS or zeta potential because of the large sample size and concentration required.) The Hep-Chi particles displayed the most negative zeta potential of −48 mV and the Hep-TMC particles had the least negative zeta potential of −10 mV. The size and zeta potential of PCNs after FGF-2 loading are also reported in Table 1. The magnitude of the zeta potential drops substantially after complexing PCNs with FGF-2 for all PCNs. For the Hep-TMC PCNs, they become colloidally unstable so that their size and zeta potential cannot be measured.

The size and zeta potential of PCNs can be affected by their composition and by the molecular weight and charge density of the constituent polyelectrolytes.[46,49,50] For both the heparin-containing and the CS-containing PCNs, the charge density of the polycation affects the zeta potential. Pairing the polyanion with chitosan results in a more negative zeta potential than the corresponding PCNs formed with TMC as the polycation. The two types of PCNs formed with TMC as the polycation also provide an interesting comparison of PCNs made with a small polyanion (heparin, MW = 14.4 kDa) and a large polyanion (CS, MW = 84.3 kDa), both containing strong anionic sulfate groups. The Hep-TMC particles formed here have the smallest size and the least negative zeta potential of all of the PCNs. This would make them relatively unstable and likely contributes to the observed difficulty in resuspending them after binding FGF-2. The CS-TMC particles are the largest PCNs. The CS used here has a much higher molecular weight than the heparin. A previous study done in our lab showed that ion pairing between these two strong polyions at surfaces leads to hydrophilic complexes that swell with water.[51] This could be due to inefficient ion pairing, requiring that the PCNs retain a large number of small-molecular weight counterions. Dautzenberg and Jaeger also found that particle size increases when polyelectrolyte ion pairing is reduced, due to increased swelling

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with water.[50] This leads to retention of counterions, high osmotic pressure, and swelling of the complexes.

Each of the PCNs and aggrecan all bound approximately 50 ng FGF-2 per mg of PCN or aggrecan. The polyanion GAG FGF-2 binding occurs primarily via electrostatic interactions between the sulfate groups in the GAG and pendent amine groups in the FGF-2.[52] In work by Asada et al., the authors observed that heparin may bind more FGF-2 than CS, likely because heparin is more highly sulfated.[53] In the present work, we used more polyanion when preparing the CS-containing PCNs than when preparing the heparin-containing PCNs to compensate for the reduced degree of sulfation of CS. Hence, the FGF-2 loading of the heparin- and CS-containing PCNs is approximately the same (Table 1).

PCN Cytocompatibility. The cytocompatibility of PCNs with respect to MSCs was evaluated to determine how the dose of PCNs affects MSC viability. Five doses of PCNs (0.5, 5.0, 10, 20, and 40 μg mL^{-1}) in low-serum media were compared to the negative control (no PCNs) and to a cytotoxic ethanol treatment (70% ethanol) in a 48 h cytotoxicity assay. Representative images of each PCN treatment (Hep-Chi, Hep-TMC, CS-Chi, and CS-TMC) at 0.5, 10, and 40 μg mL^{-1}, the negative control, and the ethanol-treated cells are shown in Figure 3. The Live/Dead stain indicates live cells in green and dead cells in red. At the 10 μg mL^{-1} dose, the total number of live cells and the degree of cell spreading appears to be reduced for all PCN formulations. At the 40 μg mL^{-1} dose, of all PCN formulations, there are far more dead cells than there are at the lower PCN doses. Since these cells are anchorage-dependent, this inhibition of attachment and spreading may contribute to the apparent cytotoxicity observed at high PCN doses. The CS-TMC PCNs appear to have the least negative effect on cell spreading and viability, while Hep-Chi PCNs caused reduced cell spreading, even at the lowest dose, 0.5 μg mL^{-1}. While some effect of PCN delivery on MSC cultures is observed for all formulations at 10 μg mL^{-1}, we chose to use this dose for the subsequent FGF-2 activity assays to achieve a 1 ng mL^{-1} total dose of FGF-2. The 1 ng mL^{-1} total dose of FGF-2 was identified as the optimally mitogenic dose for ovine MSCs in our previous work.[1]

MSC Response to FGF-2, PCNs, and Preconditioned FGF-2-Loaded PCNs and Aggrecan. *Mitogenic Activity Assay.* To evaluate the ability of PCNs to preserve the activity of FGF-2, cells were treated with PCNs, FGF-2-loaded PCNs, FGF-2 bound to aggrecan, or uncomplexed FGF-2 that had been preconditioned at 37 °C in media containing 10% FBS for 0, 3, 7, or 14 days. In separate experiments, cells were also treated with PCNs and aggrecan with no FGF-2.

We previously reported a biphasic dose response of ovine MSCs to FGF-2 in solution, with an optimally mitogenic dose in the range of 1 to 10 ng mL^{-1} under the same conditions used here (4 day culture in low-serum media).[1] We also showed that the response to FGF-2 was enhanced when the FGF-2 was bound to heparin-containing surface coatings on glass rather than delivered in solution.[1] More recently, we demonstrated significant response to FGF-2 bound to Hep-Chi PCNs at an equivalent FGF-2 dose of approximately 0.2 ng mL^{-1} under the same conditions.[19] These experiments demonstrate that binding FGF-2 to heparin-containing PCNs or surfaces may reduce the dose required to achieve a functional response, compared to FGF-2 delivered in solution. Hence, in the present work, we used an equivalent FGF-2 dose of 1 ng mL^{-1} and 10 μg mL^{-1} PCNs or aggrecan. The FGF-2 loading of each PCN type is displayed in Table 1. In all cases, approximately 50% of the FGF-2 is bound to the PCNs or aggrecan. For the FGF-2 activity experiments, there is no separation step to remove unbound FGF-2. Thus, each condition uses an equal amount of total FGF-2.

Figure 4 shows the cell counts (cells per area after four days of culture) for the PCNs and aggrecan with no FGF-2. The

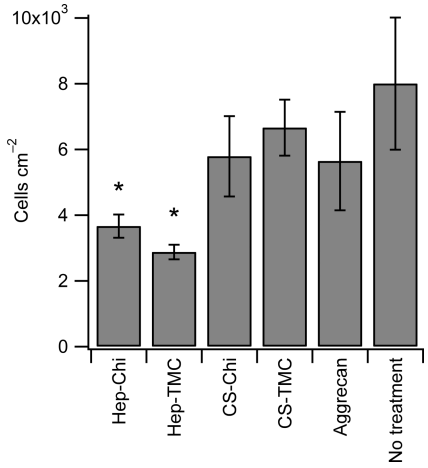

Figure 4. Cells per cm^2 obtained after four days of culture in low-serum media, treated with PCNs and aggrecan, compared to no treatment; *indicates statistical difference from the negative control ($p < 0.05$). Cells were seeded at 7000 cells per cm^2.

negative control (no treatment) shows that MSCs do not proliferate in this low-serum condition over four days of cell culture without exogenous FGF-2. (Cells were initially seeded at 7000 cells per cm^2.) None of the PCN treatments resulted in a positive response over the negative control. The aggrecan and CS-containing PCNs were not statistically different from the negative control. However, the heparin-containing PCNs had cell numbers statistically lower, indicating cytotoxicity or interference with cell attachment. Representative microscopy images of DAPI-stained nuclei from which these cell numbers were obtained are shown in the Supporting Information.

Previous studies have found that both heparin and CS may inhibit cell attachment.[54] Other sulfated polysaccharides have been shown to exhibit antiproliferative or cytotoxic effects on mammalian cells.[55,56] Delivery of Hep-containing PCNs at 10 μg mL^{-1} may reduce the ability of cells to attach and spread, resulting in the reduced cell numbers seen here.

Figure 5 shows representative fluorescence micrographs (DAPI-stained nuclei) after four days of culture in low-serum media for untreated MSCs, MSCs treated with FGF-2 bound to PCNs (Hep-Chi and CS-Chi) or aggrecan, and FGF-2 delivered in solution, for different preconditioning times. The normalized cell numbers for each type of FGF-2-PCN treatment is compared to the FGF-2-aggrecan and FGF-2 only treatments in Figure 6. In the low-serum condition, untreated cells do not proliferate; cells cultured with FGF-2 alone do proliferate, but not to as high of an extent as cells exposed to PCN-bound FGF-2. After 0 days preconditioning, the normalized cell numbers for the FGF-2 alone are statistically lower than the CS-Chi PCNs, and after 7 or 14 days of preconditioning, they are statistically lower than the Hep-containing PCNs but are not statistically different from the negative control after all preconditioning periods.

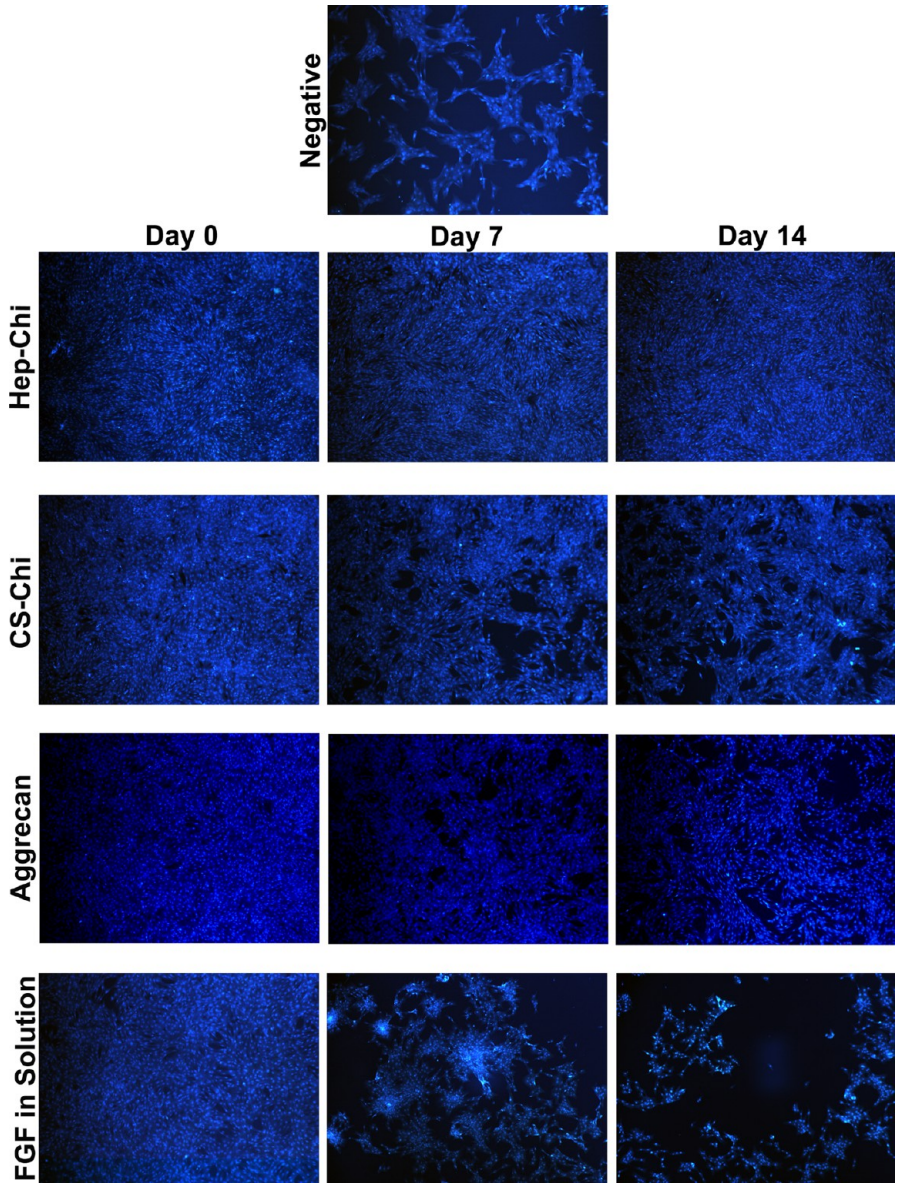

Figure 5. Representative fluorescence micrographs of DAPI-stained MSC nuclei after four days of culture with no treatment, FGF-2-loaded PCNs or aggrecan, and FGF-2 in solution. Preconditioning time does not appear to have an effect on mitogenic activity, however, treatment type does.

Despite the apparent inhibition of cell proliferation observed above for PCNs with no FGF-2 (Figures 4), all of the treatments with FGF-2 result in increased cell numbers compared to cells cultured with no FGF-2 (normalized cell counts are all greater than 1). The mitogenic activity of FGF-2 bound to PCNs is also greater than the mitogenic activity of unbound FGF-2 for all PCNs at all time points. The Hep-Chi and Hep-TMC PCNs maintain a high level of FGF-2 mitogenic activity over the entire 14 day preconditioning, while the CS-Chi and CS-TMC PCNs and aggrecan exhibit some apparent loss of FGF-2 mitogenic activity with increased preconditioning time (Figure 6). Since the unbound FGF-2 in solution has a much lower mitogenic activity than FGF-2 bound to PCNs, we conclude that the FGF-2 bound to the PCNs is primarily responsible for the high level of activity in the PCN conditions. For the heparin-containing PCNs, a substantial amount of FGF-2 must remain bound to the PCNs over the preconditioning time. The CS-containing PCNs and aggrecan may be slowly releasing some FGF-2 into solution, resulting in the observed drop in mitogenic activity with increasing preconditioning time.

Metabolic Activity Assay. To better understand how the cells respond to the preconditioned FGF-2 during the 4 day mitosis experiments, a modified MTT assay was used to evaluate MSC metabolic activity. The modified MTT assay was performed on cells cultured for 48 h, treated with FGF-2, PCNs, FGF-2-loaded PCNs, or aggrecan preconditioned for 0, 7, 14, or 21 days. For treatments with no FGF-2, the MSC metabolic activity was the same across all four PCN types and the same as the negative control (not shown). Hence, although PCNs reduce attachment and spreading (Figures 3 and 4), the MSC metabolic activity is not affected. Figure 7 shows the results of the metabolic activity assay for the FGF-2-containing treatments. The aggrecan and CS-containing PCNs showed no statistical difference across preconditioning time, indicating that FGF-2 activity in relation to metabolic activity for these treatments is stable. However, the Hep-containing PCNs exhibited increased metabolic activity after 21 days of

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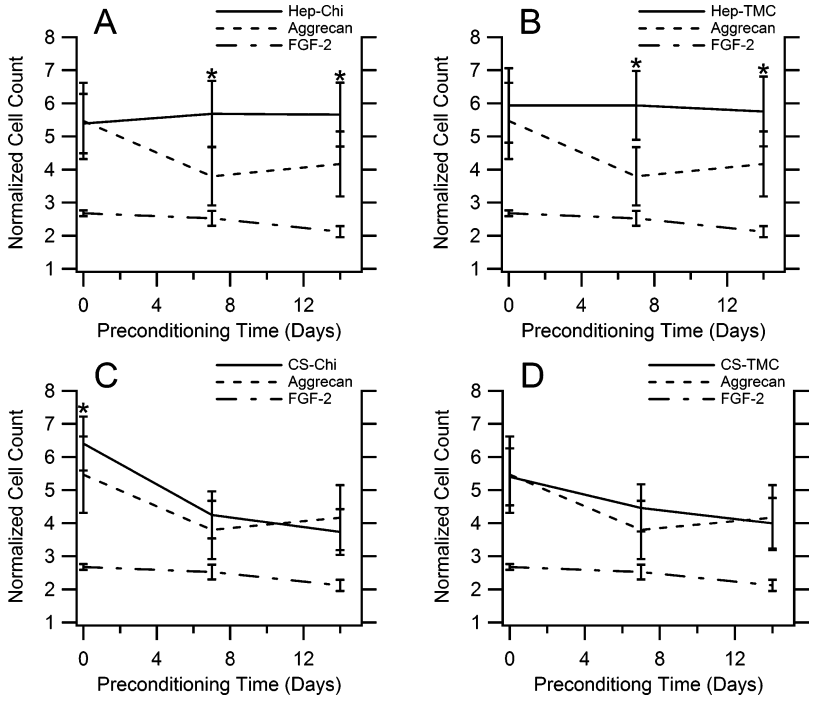

Figure 6. FGF-2 mitogenic activity in 4 day MSC culture for FGF-2-loaded PCNs, FGF-2-loaded aggrecan, and FGF-2 in solution. The numbers represent cell count normalized to cell counts from the negative control. Panels A–D represent Hep-Chi, Hep-TMC, CS-Chi, and CS-TMC, respectively. Each PCN type is compared to aggrecan and FGF-2 in solution; *indicates points that have statistically higher cell counts compared to FGF-2 in solution that was preconditioned for the same amount of time. Hep-containing PCNs remain consistent over preconditioning time, while CS containing PCNs and aggrecan exhibit lower cell numbers for increased preconditioning time.

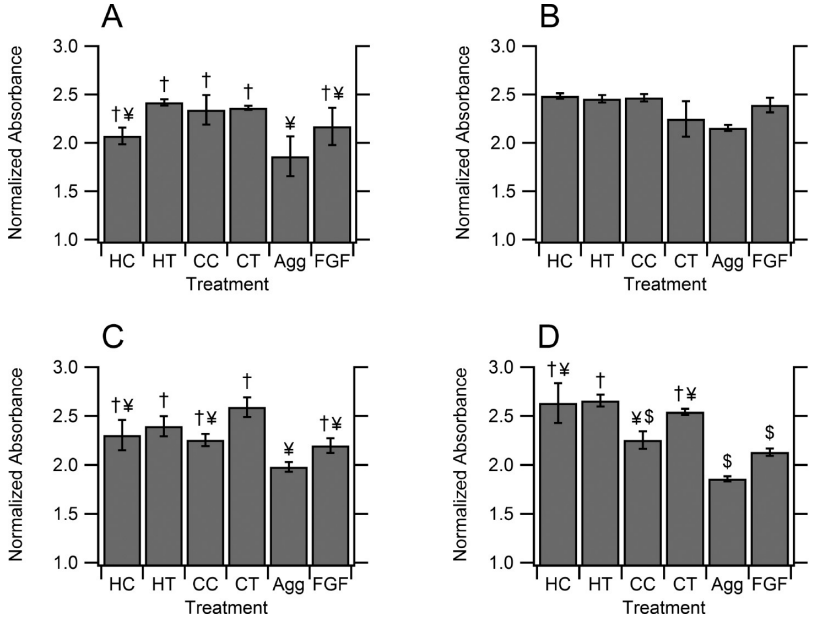

Figure 7. Metabolic activity after 2 days of culture with FGF-2-loaded PCNs, FGF-2-loaded aggrecan, and FGF-2 in solution (treatments were preconditioned in media for (A) 0, (B) 7, (C) 14, or (D) 21 days). Metabolic activity of MSCs exposed to treatment was normalized to the metabolic activity of untreated MSCs. In each panel, bars marked with the same symbol are not statistically different from one another within each figure. There was no statistical difference between any of the treatments after 7 days of preconditioning (B). After 14 days of preconditioning, Hep-TMC and CS-TMC elicit a higher response than aggrecan (C). After 21 days of preconditioning, Hep-TMC, Hep-Chi, and CS-TMC all elicit a higher response than aggrecan and FGF-2 in solution (D). All PCN treatments are statistically higher than the negative control, regardless of preconditioning time.

preconditioning compared to 0 days, and FGF-2 alone showed reduced metabolic activity after 21 days of preconditioning compared to 7 days of preconditioning. When treatments were compared within individual time points, there were statistical differences between groups. At 7 days of preconditioning, all treatments elicit the same MSC response, but as preconditioning time increases, differences emerge indicating that there is some loss of activity over time. After 14 days of preconditioning

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(Figure 7C), CS-TMC and Hep-TMC elicit a higher metabolic response than aggrecan. After 21 days of preconditioning (Figure 7D), three PCN types (Hep-TMC, Hep-Chi, and CS-TMC) are superior to FGF-2 alone and to FGF-2 bound to aggrecan. All PCN treatments stimulate more metabolic activity at all preconditioning times than the negative control, indicating that some FGF-2 activity remains over the 21 days.

Both CS and heparin are highly sulfated polysaccharides (Figure 2) that can bind FGF-2 electrostatically, evidenced by the change in zeta potential upon FGF-2 binding (Table 1). However, the heparin-containing PCNs are more effective at stabilizing FGF-2 than the CS-containing PCNs and the CS-containing aggrecan. The high-specificity binding sequence of heparin and heparan sulfate for FGF-2 requires two *N*-sulfonates and a 2-*O*-sulfated iduronic acid.[21,52] This high specificity binding of FGF-2 to heparin might also impart higher stability to FGF-2 bound to heparin-containing PCNs. The CS used in this work is primarily 4-*O*-sulfated and 6-*O*-sulfated and, therefore, lacks the FGF-2-binding sulfation pattern found in heparin. Aggrecan contains mostly CS and some keratan sulfate, which is also 6-*O*-sulfated.[57,58] The lack of specificity may make CS and aggrecan less effective at stabilizing FGF-2.

CONCLUSIONS

The aggrecan-mimetic PCNs described here have a size and high density of GAGs similar to aggrecan. The manufacturing process is simple, reproducible, and requires no additional cross-linking to form the particles or additional chemical modification to bind the growth factor. At doses of 10 μg mL^{-1} and above they may inhibit cell attachment to tissue-culture polystyrene, and at higher doses, they may be cytotoxic toward MSCs. At a dose of 10 μg mL^{-1}, the Hep-containing PCNs result in reduced cell numbers compared to aggrecan and untreated controls in a 4 day MSC culture assay. Nonetheless, these PCNs bind and stabilize FGF-2 enhancing both the mitogenic activity and the metabolic activity of MSCs in low-serum media.

In these experiments, we did not find a direct correlation between cell number and cell metabolic activity. While the MTT and similar assays can be highly correlated to cell number under some conditions, these cells are approaching confluence in the 4 day cell culture, under the most mitogenic conditions. This likely results in contact inhibition, causing the results of the metabolic activity and mitogenesis assays to diverge.

This work shows that the mitogenic activity of FGF-2 is best maintained by binding to Hep-containing PCNs. Over the same preconditioning time period, delivery of FGF-2 using PCNs results in higher metabolic activity than delivery of FGF-2 using aggrecan. Furthermore, after 21 days of preconditioning, all of the PCN formulations are superior to aggrecan, and the Hep-containing PCNs are superior to FGF-2 in solution at stimulating metabolic activity of MSCs. FGF-2 in solution has no significant mitogenic activity after any of the preconditioning periods (normalized cell numbers were not statistically different from the untreated control). FGF-2 bound to aggrecan and CS-based PCNs lost some mitogenic activity during the 14 day preconditioning. But FGF-2 bound to heparin-containing PCNs exhibited no loss of activity during the 14 day preconditioning. The metabolic activity assay showed that after 14 days of preconditioning, two of the PCN types were superior to aggrecan at maintaining the FGF-2 activity, and after 21 days of preconditioning, the PCNs were superior to both aggrecan and FGF-2 alone.

We have previously demonstrated that similar PCNs can be bound to surfaces and used to deliver heparin-binding growth factors like FGF-2.[19,45] Therefore, these aggrecan-mimetic PCNs might be used for growth factor delivery either in soluble form or bound to surfaces. This could improve the prospects for therapeutic delivery of heparin-binding growth factors and cytokines for tissue engineering and wound healing applications.

ASSOCIATED CONTENT

Supporting Information

Representative fluorescence micrographs of DAPI-stained nuclei from which the data in Figure 4 were obtained. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Fax: 970-491-7369. Tel.: 970-491-0878. E-mail: matthew.kipper@colostate.edu.

Notes

The authors declare no competing financial interest.

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