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Perlecan domain I-conjugated, hyaluronic acid-based hydrogel particles for enhanced chondrogenic differentiation via BMP-2 release

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Abstract

We have developed a biomimetic growth factor delivery system that effectively stimulates the chondrogenic differentiation of the cultured mesenchymal stem cells via the controlled presentation of bone morphogenetic protein 2 (BMP-2). Hyaluronic acid (HA)-based, microscopic hydrogel particles (HGP) with inherent nanopores and defined functional groups were synthesized by an inverse emulsion polymerization technique. Recombinantly produced, heparan sulfate (HS)-bearing perlecan domain I (PlnDI) was covalently immobilized to HA HGP (HGP-P₁) via a flexible poly (ethylene glycol) (PEG) linker through the lysine amines in the core protein of PlnDI employing reductive amination. Compared to HGP without PlnDI, HGP-P₁ exhibited significantly ($p < 0.05$) higher BMP-2 binding capacity and distinctly different BMP-2 release kinetics. Heparitinase treatment increased the amount of BMP-2 released from HGP-P₁, confirming the HS-dependent BMP-2 binding. While BMP-2 was released from HGP with a distinct burst release followed by a minimal cumulative release, its release from HGP-P₁ exhibited a minimal burst release followed by linear release kinetics over 15 days. The bioactivity of the hydrogel particles was evaluated using micromass culture of multipotent mesenchymal stem cells (MSCs), and the chondrogenic differentiation was assessed by the production of glycosaminoglycan, aggrecan and collagen type II. Our results revealed that BMP-2 loaded HGP-P₁ stimulates more robust cartilage specific ECM production as compared to BMP-2 loaded HGP, due to the ability of HGP-P₁ to potentiate BMP-2 and modulate its release with a near zero-order release kinetics. The PlnDI conjugated, HA HGP provide an improved BMP-2 delivery system for stimulating chondrogenic differentiation in vitro, with potential therapeutic application for cartilage repair and regeneration.

Keywords

perlecan domain I; hyaluronic acid; hydrogel particles; controlled release; bone morphogenetic protein-2; chondrogenic differentiation; mesenchymal stem cells

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1. Introduction

Cartilage is a highly specialized, avascular connective tissue that provides low friction, shock absorbance, load bearing and load distribution for effective joint movement. Degradation and degeneration of articular cartilage, resulting from excess mechanical stresses, normal aging as well as genetic factors, give rise to osteoarthritis (OA) that is characterized by pain, stiffness and loss of mobility. Due to the limited regenerative capacity of chondrocyte, the damaged cartilage cannot fully repair itself [1,2]. Growth factor therapy has gained importance in recent years as an alternative strategy for the regeneration of functional cartilage [3]. Among various growth factors investigated, bone morphogenetic protein-2 (BMP-2) is the cytokine that most effectively enhances the recruitment of mesenchymal stem cells (MSCs) to cartilage condensations, modulates expansion of condensation size, and initiates BMP-dependent signaling cascades in mesenchymal progenitor cells for the induction of chondrogenesis [4]. The importance of BMP-2 activity for normal joint formation, articular cartilage development and maintenance, the chondrogenic activity of BMP-2 when applied to MSC cultures, and the encouraging *in vivo* outcomes have been well-documented [2].

In order to exploit the full potential of BMP-2 in modulating MSC differentiation without triggering inflammation, a biomimetic delivery system needs to be developed so as to stabilize the growth factor, preserve their biological activities and prolong the *in vivo* residence time [5]. In the native extracellular matrix (ECM), growth factors are stored as an intact, latent complex through their specific binding to ECM molecules including heparan sulfate proteoglycans (HSPGs). HSPGs alone or through their specific binding with heparin binding growth factors (HBGFs) effectively modulates cellular growth, development, angiogenesis, and tissue regeneration [6]. Perlecan (Pln) is an important HSPG expressed in many ECM and basement membranes. It is remarkably enriched in the cartilage of developing and adult animals [7]. It has a protein core of approximately 400 kDa and consists of five distinct domains. Perlecan domain I (PlnDI) is unique to Pln and contains three consensus serine-glycine-aspartate (SGD) motifs that act as glycosaminoglycan (GAG) attachment sites. Through the attached GAG chains, PlnDI can function as a ligand reservoir for storage and release of HBGFs, such as BMP-2, protecting these proteins from inactivation by proteolytic digestion and potentiating their biological functions [8,9].

However, PlnDI alone cannot be used as an effective delivery vehicle for BMP-2 due to its high diffusivity and susceptibility to degradation when injected into cartilage. We therefore aim to design a biocompatible, bioactive and nanoporous particulate system that permits PlnDI immobilization and improve its applicability to treat OA. In this investigation, we strategically improved the utility of PlnDI via its covalent conjugation to hyaluronic acid (HA)-based hydrogel particles (HGP) [10–12] for the non-covalent sequestration of BMP-2. Our motivation for using HA HGP as the carrier system stems from HA's natural abundance in cartilage ECM, inherent biocompatibility and diverse biological functions including wound healing, morphogenesis and embryonic development [13–15]. In cartilage, HA interacts with aggrecan to form large aggregates that not only help organize the cartilage ECM but also provide compression resistance to the tissue [16]. In addition, HA HGP exhibit large surface area, faster responses, defined pore size and enhanced stability that are attractive for use as growth factor release depots [10,11,17]. Sustained release of biologically-active BMP-2 was accomplished using PlnDI-conjugated HA HGP. Chondrogenic differentiation of MSCs was demonstrated using micromass cultures of C3H10T1/2 cells in the presence of BMP-2-loaded, PlnDI-conjugated HA HGP.

2. Materials and Methods

2.1. Materials

Hyaluronic acid (HA, sodium salt) was a gift from Genzyme Corporation (Cambridge, MA, USA). Poly(ethylene glycol) dibutylaldehyde (PEGdiALD, $M_w=3400$) was purchased from Nektar™ Therapeutics (San Carlos, CA). Sodium cyanoborohydride (NaBH_3CN), span 80, mineral oil, and fluorescein isothiocyanate (FITC)-dextran of different molecular weight was obtained from Aldrich (Milwaukee, WI). Acetone, hexane and isopropyl alcohol were obtained from Fisher Scientific (Pittsburgh, PA). Bovine testicular hyaluronidase, heparitinases I, II, and III, bovine serum albumin (BSA), Tween-20, D-(+)-glucose were obtained from Sigma (St. Louis, MO). Recombinant human BMP-2, mouse monoclonal anti-human BMP-2 antibody (IgG2B) and BMP-2 Quantikine ELISA kit were obtained from R&D Systems (Minneapolis, MN). Rabbit anti-mouse collagen II polyclonal antibody (1:80) was purchased from Biodesign International (Saco, ME). Rabbit anti-mouse aggrecan polyclonal antibody (10 $\mu\text{g/mL}$) was obtained from Chemicon International Inc. (Temecula, CA). Rhodamine Red™-X-conjugated donkey anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Cascade blue hydrazide (CB, sodium salt) and Syto-13 (1:1000 in DPBS) were purchased from Molecular Probes (Carlsbad, CA). All reagents were used as received unless otherwise noted.

2.2. Synthesis of PlnDI-conjugated HA HGP (HGP-P₁)

2.2.1. Preparation of HA HGPs—Prior to the preparation of HA HGPs, HA derivatives containing hydrazide (HAADH) and aldehyde (HAALD) functional groups were synthesized and characterized following established procedures [17,18]. Inverse emulsion polymerization was employed for the synthesis of HA HGPs [17]. Specifically, inverse emulsion was prepared by homogenizing HAADH (1 mL, 1 wt % in deionized water, DI H_2O) in mineral oil (25 mL) containing 0.1 mL of Span 80 for 5 min using a Silverson L4R homogenizer (Silverson Machines Ltd., Cheshire, England). HAALD (1 wt % in DI H_2O) was subsequently added to the emulsion dropwise, and the resulting emulsion was homogenized for an additional 5 min. The aqueous phase was allowed to evaporate overnight at 40 °C with constant stirring. HGPs were isolated by centrifugation at 3,000 rpm for 5 min. The particles were thoroughly washed by hexane, isopropanol and acetone before drying under vacuum overnight.

2.2.2. Glycine passivation—Prior to PlnDI conjugation, HA HGPs (50 mg) were allowed to react with glycine (18 mg) in DI H_2O (5 mL) for 2 h at 37 °C under constant stirring in order to block residual aldehyde groups [19] in HGPs. The glycine-treated particles were washed with water, isopropanol, acetone (three times each) and vacuum dried. All subsequent modifications were carried out using glycine-treated HGPs.

2.2.3. Modification of HGPs with PEGdiALD. (HGP-PEG)—PEGdiALD (157 mg) was dissolved in 5 mL of phosphate buffered saline (PBS). Under constant stirring at 37 °C, 200 μL of HGP suspension (10 mg/mL in PBS) was added every 15 min for a total of 150 min. The final mixture was stirred for another hour at 37 °C before the particles were collected by centrifugation. The particle pellets were immediately resuspended in PBS (5 mL) containing 5.8 mg of NaBH_3CN . The reaction was allowed to proceed under constant stirring at ambient temperature for 4 h. The PEG-modified HGPs (HGP-PEG) were thoroughly washed with DI water, isopropanol and acetone before being dried under vacuum.

2.2.4. PlnDI conjugation—Mouse PlnDI was recombinantly produced from stably transfected human kidney cells following the previously reported protocol [20,21]. The presence of surface disposed lysine residues available for conjugation was assessed using the protein homology/analogy recognition engine, Phyre [22]. Only the sequence of the mature

PlnDI including the SEA domain was submitted for analysis. Vacuum-dried HGP-PEGs (20 mg) were dispersed in a sodium acetate buffer (pH 4.0, 5 mL), to which a total of 5.2 mg of PlnDI was added. The reaction mixture was stirred at 37 °C for 4 h, after which 5.8 mg of NaBH₃CN was added and the reaction was allowed to proceed for an additional 4 h at 37 °C. After exhaustive washing and vacuum drying, the PlnDI-conjugated HGPs again were treated with glycine following the procedure described above. The final product, HGP-P₁, was purified by thorough washing with water, isopropanol and acetone and before being dried under vacuum.

2.3. Particle Characterization

2.3.1. Particle pore size analysis [23,24]—Commercial FITC-dextran was purified using a Shimadzu (Columbia, MD) high-performance liquid chromatography (HPLC) system operating in size exclusion chromatography (SEC) mode using a RID-10A refractive index detector and two TSK gel columns (Tosoh Bioscience, Montgomeryville, PA) calibrated by HA molecular weight standards (Lifecore Biomedical LLC, Chaska, MN). Only fractions corresponding to the designated molecular weight (4 kDa: FITC-dex-4; 40 kDa: FITC-dex-40; 70 kDa: FITC-dex-70; 250 kDa: FITC-dex-250; 500 kDa: FITC-dex-500; 2000 kDa: FITC-dex-2000) were collected and used as the molecular probes. FITC-dex was dissolved in DI water at a concentration of 1 mg/mL. Five hundred microliters of particles dispersion (1mg/mL in DI water) were mixed with 200 µl of FITC-dex solution, to which fresh DI water was added so that the final volume was 1 mL. After equilibration for 2 days at 37 °C, the supernatant was collected and FITC-dex concentration was determined (C₁ mg/mL) using a FluoroMax-3 spectrofluorometer (Jobin Yvon, NJ) at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The collected particles were re-suspended in fresh DI water (1 mL) and incubated at 37 °C for 2 days under constant stirring. Upon reaching the second equilibrium, the supernatant was collected and FITC-dex concentration was analyzed similarly (C₂ mg/mL). The relative retention of FITC-dex with different molecular weights was determined by the

equation: $100 \times \frac{0.2 - C_1 - C_2}{0.2 - C_1}$. All experiments were carried out in triplicate.

2.3.2. Particle functional group analysis—Cascade blue hydrazide (CB) staining was utilized to verify the presence of aldehyde groups in HGPs following previously reported protocols [10,11]. Briefly, hydrogel particles were allowed to react with CB in DI water at 37 °C in the dark for 2 h. After centrifugation (1,200 rpm for 5 min), particles were re-dispersed in DI H₂O and imaged with a Zeiss 510 NLO Laser Scanning Microscope (LSM) with a Zeiss 40X C-Apochromat (numerical aperture 1.2) water immersion objective lens.

2.3.3. Particle size and size distribution—The average size and size distribution of HGPs was obtained using a Coulter Counter Multisizer 3 (Beckman Coulter, Fullerton, CA) with a 2–70 µm orifice. Prior to the measurement, HGPs were diluted with large excess of isotonic solution to a final concentration of approximately 10 µg/mL.

2.3.4. Alcian blue staining for PlnDI—Alcian blue staining was utilized to confirm the presence of the GAG chains in PlnDI-conjugated hydrogel particles (HGP-P₁). Five hundred microliters of Alcian blue (0.5 wt% in 3% (v/v) glacial acid, pH=1.0) was added to 0.3 mg of HGP-P₁ and the mixture was left on the benchtop at room temperature overnight. Unconjugated HGPs were included as the control. Both types of particles were washed five times with 3% (v/v) glacial acid prior to imaging with a Nikon SMZ 1500 microscope (Optical Apparatus Co, Ardmore, PA).

2.4. BMP-2 binding and Release

2.4.1. BMP-2 binding—HGP-P₁ (0.2 mg) was incubated with 3% (w/v) bovine serum albumin (BSA) in PBS at room temperature for 2 h. To this mixture was added 100 ng of BMP-2 dissolved in 3% BSA (in PBS), and the suspension was left overnight at 4 °C. After three consecutive washes with PBS, the particles were incubated with HRP-conjugated mouse monoclonal anti-human BMP-2 at room temperature for 2 h. Finally, tetramethylbenzidine (TMB, from BMP-2 Quantikine ELISA Kit) was added to detect particle binding BMP-2 specifically, according to the manufacturer's instructions. Unconjugated HGPs were included as the control.

2.4.2. Heparitinase treatment—After rinsing with PBS, hydrogel particles were incubated in a BMP-2 solution (1 µg/mL in PBS) at a particle concentration of 5 mg/mL at 4 °C overnight for BMP-2 loading. The BMP-loaded particles (1 mg) were subsequently rinsed with PBS and subjected to heparitinase digestion (2.5 U/mL) in PBS containing 1 mM Ca²⁺ and Mg²⁺ for 2 h at 37 °C. Particles incubated under the same conditions in the absence of heparitinase were used as the controls. Upon completion of heparitinase digestion, the supernatant was collected and the amount of BMP-2 was measured with BMP-2 ELISA as described above.

2.4.3. Quantification of BMP-2 release from HGPs—One milligram of hydrogel particles (HGP and HGP-P₁) was incubated with 200 ng of BMP-2 in 200 µL of binding buffer [0.1% (v/v) BSA, 1% (v/v) Penicillin-Streptomycin (PS), 0.1mM phenylmethylsulfonyl fluoride (PMSF) in PBS] for 2 h at room temperature. The supernatant was subsequently removed and the amount of BMP-2 in the medium was measured. Subsequently, 400 µL of release buffer (CMRL-1066, Gibco Life Sciences, Rockville, MD) containing 100 U/mL PS was added and the particles were incubated at 37 °C. At predetermined time points, the supernatant was collected and the release media was replenished with an equal amount of fresh buffer. The amount of BMP-2 in the release medium was measured using a BMP-2 ELISA kit. The solution absorbance was measured at 450 nm using a plate-reader (Universal Microplate Analyzer, PerkinElmer, Waltham, MA).

2.5. In vitro chondrogenesis

2.5.1. High-density micromass culture—To measure the chondrogenic differentiation potential of BMP-2 loaded HGPs (HGP-B₂ and HGP-P₁-B₂), micromass of murine mesenchymal stem cells (MSCs, C3H10T1/2 cell line, ATCC, Manassas, VA) was cultured following previously reported procedures [25]. Particles (3 mg) suspended in 200 µL of media were subsequently added directly to the micromass. Blank particles (HGP and HGP-P₁) and pure media were included as the controls. Media was changed every three days and the chondrogenic differentiation of C3H10T1/2 cells was assessed by histological and immunohistochemical analyses.

2.5.2. GAG production by Alcian blue staining—After 9 days of incubation, micromass cultures were washed with PBS, fixed with 10% (v/v) buffered formalin acetate containing 0.5% (w/v) cetylpyridinium chloride for 10 min at room temperature, briefly rinsed with 3% (v/v) glacial acetic acid (pH 1.0) and incubated in 1 mL of 0.5% (w/v) Alcian blue in 3% (v/v) glacial acetic acid (pH 1.0) overnight at room temperature [25]. The stained micromass culture was photographed with a digital camera attached to a Nikon microscope (Coolpix 990, Nikon, Kanagawa, Japan).

2.5.3. Immunofluorescent detection of ECM components—Upon completion of micromass culture, cells were rinsed once with ice-cold PBS. The specimens were subsequently fixed (see below), washed with ice-cold PBS, and incubated with 0.02% (w/v) type IV-S testicular hyaluronidase in PBS for 30 min at room temperature. The micromass cultures then

were rinsed with PBS three times and blocked with 3% BSA (in PBS) for 1 h at room temperature. The micromass cultures then were incubated with the primary antibody in 3% BSA for 2 h at room temperature. After three washes for 5 min each at room temperature, the micromass cultures were incubated with the secondary antibody (Rhodamine RedTM-X-conjugated donkey anti-rabbit IgG) in 3% BSA for 1 h at room temperature. Syto 13 was added to stain the nucleic acid in the cells (5 min at room temperature). The micromass was washed again with PBS 5 times. The stained micromass was lifted up from the tissue culture plates using a surgical scalpel (# 12) and mounted on glass slides for confocal imaging. For type II collagen detection, samples were fixed for 30 min on ice in ethanol/acetic acid (95/5, v/v) and the primary antibody was rabbit anti-mouse collagen II polyclonal antibody. For aggrecan staining, cells were fixed with 4% (v/v) paraformaldehyde in PBS on ice and the primary antibody was rabbit anti-mouse aggrecan polyclonal antibody. Images were acquired using a Zeiss Axiovert confocal microscope (Axiovert LSM 510).

2.6. Statistical analysis

All quantitative measurements were performed on 2–3 replicates. All values are expressed as means $\pm$ standard deviations (SD). Statistical significance was determined using a two-tailed student t-test. A p value of less than 0.05 was considered to be statistically different.

3. Results

3.1. Synthesis and Characterization of HA HGPs

HA HGPs were synthesized by *in situ* crosslinking of HAALD and HAADH within the inverse emulsion droplets. The resulting HGPs were spherical in shape and had an average particle size of 10 μ m [17]. HA HGPs are chemically and enzymatically more stable than their macroscopic counterparts (bulk gels made with the same chemical composition). No degradation was observed after the particles were soaked in buffers at pH of 4 or in the presence of hyaluronidase (100 U/mL at pH 7.4) for one month, whereas the bulk gel completely disintegrated overnight under the same conditions.

The average particle pore size was determined by a solute retention experiment [23]. FITC-labeled dextran with molecular weights varying from 4 kDa to 2000 kDa, corresponding to an estimated Stokes radius ranging from 1.5 nm to 27.4 nm [24] was used as the molecular probe. By combining two equilibration steps (uptake and release), one can obtain information not only on the average particle mesh size but also the relative distribution. The average FITC-dex retention as a function of the molecular weight follows a bell-shape distribution (Figure 1), and the lowest retention was observed for FITC-dex-4 ($13.4 \pm 6.8\%$) and FITC-dex-2000 ($25.5 \pm 10.8\%$). The highest retention was observed for probes of intermediate sizes. The smallest probe, FITC-dex-4, diffuses readily in and out of the particles, resulting in an overall low retention. On the other hand, the largest probe was excluded from the majority of the pores in the particles, and only approximately 25% of pores had comparable sizes to FITC-dex-2000, thus being retained. When the average mesh size of the hydrogel particles became comparable to the size of the probe molecules, a high percentage (84–86%) of the probe molecules that were taken up by the particle remained trapped in the particles during the second equilibrium process. Because the Stokes radius for FITC-dex-40 and FITC-dex-250 is 4.5 nm and 10.5 nm, respectively [24], we estimated that the average mesh size of the hydrogel particles is in the range of 5–10 nm.

3.2. Bioconjugation

Our previous investigations showed that the as-synthesized HGPs contain both residual aldehyde and hydrazide groups that can be utilized as the reactive handles for bioconjugation [17]. A predictive structural analysis of PlnDI core protein including the conserved SEA

domain (Figure 2) [22,26] showed that although the core protein folds into a defined secondary structure with a hydrophobic core, the charged lysine residues (Lys 85, 97, 104 and 117 in the mature secreted form of Pln) are well exposed on the surface, thus readily accessible for covalent conjugation. The fifth lysine residue (Lys 164) is not shown in the 3D structure. The as-synthesized HGP's first were treated with glycine to convert the free aldehyde groups on the particles to carboxylic acid prior to the conjugation reaction [19]. The glycine-treated particles were allowed to react with large excess of PEGdiALD, converting the residue hydrazide groups into the aldehyde groups that were extended from the surface of the nanopores by approximately 77 ethylene glycol repeats (Figure 3). A positive CB staining (Figure 4A and 4C) confirmed the presence of aldehyde groups in HGP and HGP-PEG. The negative CB staining (Figure 4B) for glycine-treated HGP's indicated the effectiveness of free glycine to block the reactive aldehyde groups. HGP-PEG subsequently was reacted with PlnDI under acidic conditions at a solution pH slightly below the pI value of PlnDI (4.26). Lysine amines were employed to establish the covalent linkage between the core protein and the PEG-modified HGP's by reductive amination.

The PlnDI-conjugated HA HGP's (HGP-P₁), along with the as-synthesized HGP's, were subjected to particle size analysis using a Coulter Counter Multisizer (Figure 5). Our results showed that under isotonic conditions, the majority of the hydrogel particles appeared to be 5–6 μ m in diameter, smaller than they appeared to be microscopically (Figure 4A and 4C). Upon PEG modification and PlnDI conjugation, the average particle size increased to around 8 μ m, as indicated by the shift in the size distribution profile for HGP-P₁ relative to that for HGP. Such a distinct and consistent shift does not necessarily mean a net particle size increase of ~2 μ m upon PlnDI conjugation. Rather, it implies the altered ability of the electrolyte to pass freely through the hydrogel particles, which can be attributed to the presence of covalently anchored PlnDI heparan sulfate “brushes” within the nanopores of HGP-P₁. Particles also were analyzed by Alcian blue staining to identify their GAG content (Figure 6). HGP-P₁ showed positive Alcian blue staining performed at pH 1.0, whereas HGP remained negative. The robust blue stain seen for HGP-P₁ confirmed the presence of GAG side chains on PlnDI, and hence the presence of PlnDI in HGP's. The immobilized PlnDI retained its ability to bind BMP-2, as shown in Figure 6. In this experiment, BMP-2 loaded particles were incubated with HRP-conjugated anti-human BMP-2. Upon addition of the colorimetric reagents, HGP-P₁-B₂ gave rise to deep blue staining, whereas HGP-B₂ remained colorless under the microscope although the particle suspension exhibited a faint blue hue. The difference in the degree of BMP-2 association between HGP and HGP-P₁ further confirms the presence of covalently immobilized PlnDI in HGP-P₁.

3.3. BMP-2 loading and release

BMP-2 ELISA analysis showed that for every milligram of hydrogel particles, 168 $\pm$ 3 ng BMP-2 was associated with HGP-P₁ in contrast to only 89 $\pm$ 10 ng for HGP, suggesting a higher binding capacity of HGP-P₁. This calculation agrees well with the qualitative BMP-2 binding results shown in Figure 6. In order to assess the effect of enzymatic treatment on BMP-2 release, various hydrogel articles with loaded BMP-2 were subjected to a 2-h heparitinase treatment and the BMP-2 concentration in the supernatant was analyzed using BMP-2 ELISA (Figure 7). Hydrogel particles without the loaded BMP-2 were included as the controls. As expected, the heparitinase treatment did not change the overall amount of BMP-2 released from HGP. However, BMP-2 release from HGP-P₁ was significantly ($p<0.05$) modulated by the enzyme activity, with heparitinase treatment more than doubling the amount of BMP-2 released into the supernatant. In the absence of heparitinase, both HGP and HGP-P₁ released similar amounts of BMP-2 after 2-h incubation. Taken together, these findings indicate that PlnDI was stably immobilized to HA HGP's and its presence greatly promoted HS-dependent BMP-2 binding to HA HGP-P₁.

In vitro BMP-2 release from HGP and HGP-P₁ was evaluated by incubating BMP-loaded hydrogel particles in a physiological buffer for up to 15 days and the results are summarized in Figure 8. After 3 days, 30±7 ng of BMP-2 was retained by one milligram of HGP, corresponding to a cumulative release of 66±3%. After 15 days of incubation, one milligram HGP retained only 4±5 ng BMP-2, with a total of 95±5% of initially bound BMP-2 being released. In contrast, a sustained release with a reduced initial burst was observed for HGP-P₁. After 3 days, 129±4 ng of BMP-2 was retained by a total of one milligram HGP-P₁, indicating a cumulative release of 23.6±0.3%. After 15 days of incubation, one milligram of HGP-P₁ retained 51±4 ng of BMP-2, releasing 69.5±0.5% of BMP-2 initially bound. The experiments were terminated before 100% release was achieved. Examination of the release curves suggests that BMP-loaded HGP exhibited a release profile with two distinct slopes (43% per day vs 2.2% per day), indicating a variable release rate and the presence of an initial burst release. On the other hand, PlnDI-conjugated HGPs maintain a steady state of BMP-2 over the entire course of the experiments with a cumulative release of 3.8%/day over 15 days of incubation. Collectively, these results confirm that PlnDI, when conjugated to HGPs, permits a close to zero-order release kinetics.

3.4. *In vitro* chondrogenesis of micromass cultures

C3H10T1/2 cells were plated at a high density in direct contact with various particles, including HGP, HGP-B₂, HGP-P₁, and HGP-P₁-B₂, to assess their chondrogenic potential. Micromass cultures displayed intense Alcian blue staining when incubated in the presence of HGP-P₁-B₂ and HGP-B₂, indicating GAG accumulation in both cases. HGP-P₁-B₂ treated micromass exhibit more robust blue staining and the cells were confined in a circle with a distinct boundary. On the other hand, HGP-B₂-treated micromass displayed a dark, but diffuse, staining with a ill-defined cell boundary. Interestingly, micromass cultured with carriers alone (HGP-P₁ and HGP) was stained weakly positive by Alcian blue. It is worth mentioning that the HGP treated micromass was bigger in diameter and more diffuse in overall appearance than that treated with HGP-P₁. Cells cultured in control media exhibited the least Alcian blue staining.

Chondrogenic differentiation further was evaluated by immunohistological analyses for aggrecan and collagen type II, two well accepted markers for mature hyaline cartilage tissues [27]. In both analyses, the cell nuclear DNA was stained green while the cell-secreted aggrecan and collagen type II were stained red. Non-specific binding of the secondary antibody alone was negative (data not shown). No significant aggrecan production was detected for the vehicle-treated and the untreated micromass culture. Aggrecan and collagen II accumulation was detected in the micromass cultured in the presence of BMP-2-loaded particles, both HGP-P₁ and HGP. However, HGP-P₁-B₂ stimulated the cells to produce larger amount of aggrecan and collagen type II than the HGP-B₂, as evidenced by the more striking red stain for HGP-P₁-B₂-treated micromass cultures in Figure 10 (A and E). Syto 13 staining revealed that cells in all micromass cultures exhibited a spherical-shaped morphology, indicative of the absence of cell differentiation into spread osteoblastic or fibroblastic phenotype.

4. Discussion

The limited regenerative capacity of chondrocyte poses significant challenges for long term, functional repair of the damaged cartilage [28]. Despite the usefulness of BMP-2 in chondrogenic differentiation, its promise has not been fully delivered to clinics, owing to its short *in vivo* half-life and susceptibility to degradation when injected *in vivo* in a soluble form [29,30]. A delivery system that releases BMP-2 in a controlled manner over a long period of time would maximize its capability to trigger chondrogenesis of MSCs and avoid undesirable side effects including inflammation. Traditionally, growth factors have been physically encapsulated into various synthetic and natural hydrogel materials to allow for diffusion-

controlled passive delivery [31,32]. Such strategy often results in poor temporal or spatial control of delivery due to the rapid diffusivity of growth factors through the hydrogel matrix. Undesirable burst release is inevitable. Alternatively, covalent immobilization [33,34] has been utilized to prolong the bioavailability of the growth factors. However, chemical transformation of growth factors inevitably compromises their biological activities. Finally, growth factors have been non-covalently immobilized in hydrogels via their association with HS, heparin or heparan-like polymers that are capable of potentiating the *in vitro* biological activities of HBGFs by stabilizing them against denaturation and by enhancing functional binding with cellular receptors [35–37]. Such interactions also restrict passive diffusion of growth factors and permit their release by complex dissociation and enzymatic degradation.

The utility of HS, heparin or HS-like polymers, however, is limited due to the absence of the core protein. In domain I of perlecan, each core protein contains three attached HS side chains with a native sulfation pattern maximizing binding and permitting release of heparin binding growth factors. The presence of HS-bearing core protein creates a microenvironment that outperforms free chains of heparin or HS [38,39]. The core protein contains a sperm, enterokinase and agrin (SEA) module enhances HS assembly and facilitates its interaction with other constituents of the ECM. Therefore, the core protein is not simply a scaffold for HS attachment; it actively participates in biological events, playing important roles in cell adhesion as well as structural and matrix organization [7]. Collectively, both the core protein and the HS side chains are indispensable for the proper function of PlnDI. Taking advantage of the ability of PlnDI to sequester and release BMP-2, the prevalence of HA in cartilage ECM and the ability of HA to elicit chondrogenic effects on MSC, presumably through CD44 [16,40, 41], we have developed a novel BMP-2 carrier system that combines HA, PlnDI and BMP-2 in a biomimetic fashion. Our long-term goal is to apply such system for the controlled release of BMP-2 in the presence of MSCs for repairing localized cartilage defects in a mouse OA model.

4.1. PlnDI bioconjugation

The recombinantly produced PlnDI contains a core protein with 173 amino acids and three HS side chains that are substantially shorter than HS chains on native Pln. The overall molecular mass of PlnDI is estimated to be 45–50 kDa[21]. Our particle pore size analysis confirmed the presence of nanopores in HA HGP large enough to accommodate PlnDI throughout the particles. The presence of PlnDI-accessible, nanopores within the particles further increases the overall surface area available for PlnDI immobilization and subsequent BMP-2 binding (see below). Unlike microspheres derived from the esterified HA that are non-porous and highly hydrophobic [42], our HA-based HGPs present a porous, hydrated microenvironment that contributes to the maintenance of the biological functions of entrapped growth factors.

Our conjugation strategy relies on the covalent attachment of PlnDI to HGPs via the amino groups in the core protein through a flexible PEG linker employing reductive amination. Compositional and predicted conformational analysis of PlnDI indicates that the core protein contains 5 surface deposited lysine residues, well separated from the HS attachment sites. These residues thus should be readily accessible for bioconjugation. Aldehyde-functionalized, telechelic PEG with a molecular weight of 3.4 kDa was utilized to convert the residual hydrazide groups in HGPs to aldehyde groups. Such reaction essentially regenerated the reactive aldehyde groups, but extended them away from the nanopore surfaces, eliminating the steric hindrance to the anchored PlnDI. In order to avoid the establishment of covalent linkage between the particles and the unsubstituted amines along HS backbone [43] and to facilitate the direct coupling of HGP with the lysine amines in the core protein, the conjugation reaction was carefully carried out at the solution pH (4.0) slightly below the pI value of the core protein (4.26). This process allowed both the HGPs and the HS side chains to remain negatively

charged while the core protein was slightly positively charged. The charge repulsion between HS side chains HGPs reduced the possibility of the unsubstituted HS amines to couple with HGP directly. On the other hand, weak attractive force between the core protein and the hydrogel particles effectively lowers the energy barrier for the direct coupling of HGP with the core protein. The resulting particle complex was treated with glycine to deactivate any unreacted aldehyde group.

Particle sizing using a Coulter Counter Multisizer revealed an average diameter of 5–6 μm for HGPs, larger than what was estimated from the microscopy analysis. While the confocal images were acquired with HGPs fully swollen in DI H_2O , the particle sizing was performed with the particles suspended in an isotonic solution. Obviously, HGPs swell to a larger degree in DI H_2O than in isotonic solutions where the charge repulsion between HA chains was screened out. Secondly, while the confocal image reveals the overall size of the particles, particle sizing correlates directly to the three dimensional volume of the electrolyte solution being displaced by the individual particles. The porous nature of HGPs allows for the electrolyte solution to permeate freely, rendering their apparent size smaller. Upon PlnDI conjugation, a net size increase of approximately 2–3 μm was observed. Such size increase cannot be attributed solely to the physical dimension of PlnDI. It can be explained in terms of the increased restriction of the porous hydrogel particles to the free passage of the electrolyte solution, presumably due to the presence of PlnDI brushes and their large hydration spheres on surfaces of the nanopores.

Alcian blue staining and BMP-2 binding were employed to further confirm the success of PlnDI conjugation. It is well known that Alcian blue selectively stains sulfated esters (GAGs) rather than the carboxylated (HA) ones at low pH (pH 1.0) [44]. The deep blue color developed by HGP-P₁, as opposed to the negative Alcian blue staining seen for HGP, suggests the presence of GAG chains in HGP-P₁. Particle aggregation observed upon Alcian blue staining can be attributed to the charge neutralization at pH 1.0. The Alcian blue staining for HGP-P₁ does not seem to be co-localized with the individual particles, due potentially to partial particle degradation during at low pH. BMP-2 binding assay serves as the final proof for the success of functional PlnDI conjugation. The dark blue color seen for BMP-2 loaded HGP-P₁, as opposed to the faint blue hues from HGP-B₂ suspension, confirms the presence of PlnDI in HGP-P₁ as well as the specificity of BMP-2 binding. The co-localization of the blue color with the individual particles suggests that both PlnDI and the bound BMP-2 remain associated with the particles. The colorimetric BMP-2 binding analysis agrees well with the quantitative analysis using ELISA. The higher BMP-2 binding capacity observed for HGP-P₁ reinforces the notion that PlnDI is in fact covalently attached to HGPs and that the immobilized PlnDI retains its ability to bind BMP-2 specifically.

4.2. BMP-2 loading and release

Recombinant human BMP-2 contains 103 amino acids and exists as a disulfide-linked homodimer with an apparent molecular weight of 26 kDa and a pI value of 8.21 [45]. Previous investigations confirmed the association of HA with BMP-2 at pH 7.4, presumably due to the ionic bonds between the negatively charged HA and positively charged BMP as well as multiple hydrogen-bonding interactions between HA and BMP-2 [46]. Under the experimental conditions employed in the current study, BMP-2 was driven into the HGPs via ion exchange since its effective size was smaller than the average mesh size of the HGP matrix. With the addition of covalently immobilized PlnDI, the growth factor binding capacity of HGPs was significantly enhanced. Through its three HS side chains and its local high concentration, the immobilized PlnDI amplified the available binding sites through multivalency, favoring the formation of more PlnDI/BMP-2 complexes [47,48]. Moreover, HS is a much stronger polyelectrolyte as compared to HA and is capable of binding more BMP-2 with a higher

association constant. Therefore, the specific interaction between BMP-2 and PlnDI played a key role in increasing the capacity for BMP-2 binding to the HGP-P₁. The observation that heparitinase treatment led to higher amount of BMP-2 being released from HGP-P₁ than from HGP confirmed that HGP-P₁ had a higher BMP-2 binding capacity and that BMP-2 binding activity is HS dependent. heparitinase degradation provides a means for active BMP-2 release that mimics that that would occur during tissue development and wound healing.

PlnDI conjugation not only improved the BMP-2 binding and retention but also modulated its release. Sustained release of growth factors into the ECM improves the cell proliferation, and tissue regeneration and that uncontrolled burst release of growth factors results in pathological conditions and inflammatory responses that hamper the effective tissue repair [20]. Domain I of Pln specifically bind HBGFs, releases them from their local extracellular matrix storage, chaperones them to their cognate receptors, and thus amplifies the growth factor signaling. The *in vitro* BMP-2 release kinetics indicates that the growth factor was released from HGP with a two-phase behavior: rapid initial release at a rate of 43% per day and a slow release of 2.2% per day in the subsequent days. A biphasic release profile usually is associated with simple diffusion in many growth factor release systems. By covalent immobilization of heparin on polymeric micelles, Lee et al.[49] demonstrated the controlled, long-term release of basic fibroblast growth factor (bFGF/FGF2) with a reduced initial burst and minimal loss of bFGF/FGF2 bioactivity. Other groups have shown that heparin immobilization on the surface of porous microspheres[36] or electrospun nanofibers[50] not only provided an opportunity for enhancing the loading amount of bFGF/FGF2 but also improved the sustained release profile with reduced initial burst. In the current study, HS-bearing PlnDI, rather than an isolated heparin, was immobilized in HA HGPs via a stable covalent linkage with an aldehyde end-functionalized PEG that have been previously coupled to HA HGPs. Similar to previously reported literature, the presence of PlnDI effectively dampened the initial burst characteristics of HGP, permitting more linear and sustained BMP-2 release.

The improved control over BMP-2 release from HGP-P₁ as compared to HGP alone can be attributed to the enhanced BMP-2 binding and the restricted BMP-2 diffusion due to the presence of immobilized PlnDI. BMP-2 release from the hydrogel particles is governed by two competing mechanisms: BMP-2/HA association and BMP-2 diffusion through the porous matrix. In the case of HGPs lacking PlnDI, the HA/BMP-2 interaction is relatively weak and non-specific. Thus, the release kinetics is predominantly controlled by BMP-2 diffusion that is not severely restricted due to the small size of the growth factor. Another consequence of the weak association between HA and BMP-2 is the presence of a relatively high percentage of surface bound BMP-2, which was immediately desorbed upon incubation, contributing to the initial burst. In the case of HGP-P₁, the release kinetics likely was dictated by specific binding of BMP-2 to PlnDI. The dissociation rate of BMP-2 from the BMP/PlnDI in the HGP-P₁ hydrogel particles was probably governed mainly by a thermodynamic equilibrium between free BMP-2 in the release medium and the bound BMP-2 in the particles. As discussed above, the presence of extended HS brushes anchored on the surface of the nanopores effectively reduced the apparent particle mesh size as evidenced by the particle size analysis. Consequently, BMP-2 diffusion through PlnDI-decorated nanopores was severely restricted, further decreasing the rate of BMP-2 release. It is noteworthy that only 69.5±0.5% BMP-2 was released at the end of the experiment. The heparitinase digestion experiments indicated that the rest of the bound BMP-2 could be released during prolonged incubation and/or through enzymatic degradation. The long-term, zero-order release of the growth factor demonstrated here would allow tissues at the injection site to be exposed to BMP-2 at a constant concentration for a long period, allowing sufficient time for the generation of functional new cartilage.

4.3. Biological activities of BMP-2 releasing HA HGPs

The biological activity of BMP-2 releasing hydrogel particles was evaluated for their chondrogenic potential using a micromass culture of C3H10T1/2 mesenchymal progenitor cell. The absence of cell death (data not shown) confirms the biocompatibility of these hydrogel particles [12]. Interestingly, a micromass cultured with carriers alone in the absence of loaded BMP-2 was stained weakly positive by Alcian blue, suggesting enhanced GAG accumulation as compared to cells cultured in control media. Such observation is not surprising considering the relevance of PlnDI and HA in inducing chondrogenesis. PlnDI alone facilitates the attachment and aggregation of C3H10T1/2 fibroblasts and subsequently promotes their chondrogenic differentiation. Although PlnDI alone did not lead to chondrogenic maturation, cells pre-exposed to PlnDI were activated through an early stage of chondrogenic conversion, rendering them more responsive to the subsequent BMP-2 treatment [51]. Thus, PlnDI and BMP-2 synergistically guide the cells through various developmental stages that lead to the formation of mature cartilage tissues [38]. Similarly, exogenous HA elicit a chondrogenic effect on equine MSCs grown in pellet culture, which may be explained by the interaction between HA and HS CD44 receptor [40]. The fact that both HA-based carriers, HGP and HGP-P₁, enhanced GAG production implies that chemically crosslinked HA hydrogel particles and the covalently anchored PlnDI remain biologically active, capable of fulfilling their natural biological functions. Subtle differences, however, exist in Alcian blue staining of a micromass treated with HGP and HGP-P₁. While the former appear larger in diameter and the staining is more diffuse, the latter shows a compact nodule with a distinct boundary with deeper staining as compared to that in the center. Such observation correlates well with HA's role in facilitating cell migration and Pln's ability to enhance the formation of cell condensations [14,15].

Addition of BMP-2 intensified the mesenchymal differentiation as evidenced by greater and more intense Alcian blue staining for HGP-B₂ and HGP-P₁-B₂ treated micromass cultures, respectively. The subtle difference in the staining pattern seen with the carriers only was essentially maintained, with micromass cultures treated with PlnDI-containing HGPs showing cells being restricted to a defined circular area with intensified periphery, whereas the micromass treated with HGPs lacking PlnDI exhibiting larger and more diffuse squares with the center stained more heavily than the edge. The more intense blue staining for a micromass cultured in the presence of HGP-P₁ at the edge relative to the center suggests faster cell proliferation [52] and/or higher GAG production per cell at the periphery. These results prove that the released BMP-2 is biologically active and synergistically enhances and accelerates the chondrogenic differentiation and maturation of MSCs. The diffuse stain observed on HGP-B₂-treated micromass can be attributed to a weaker BMP-2 binding due to the absence of HS chains and/or the stimulatory effect of HA on cell migration. The brighter Alcian blue stain on HGP-P₁-B₂-treated micromass than on HGP-B₂ treated micromass suggests that cells cultured in the former conditions are making more proteoglycan. These observations are in agreement with previous investigations that demonstrate the ability of HS to enhance the interaction of BMP2 with its receptors, dramatically improving its ability to stimulate chondrogenesis and reducing the concentration of BMP-2 needed to stimulate chondrogenesis [53].

To further confirm the biological activities of BMP-releasing HGPs, immunofluorescence staining for early chondrogenic differentiation markers including aggrecan and type II collagen was performed. Consistent with the Alcian blue staining, media alone did not elicit expression of these markers. The production of aggrecan and collagen type II was highest in HGP-P₁-B₂ and moderate in HGP-B₂. Unlike the GAG production, the presence of carriers did not elicit significant aggrecan and collagen type II production, suggesting that GAG production is readily up-regulated by the carriers alone while collagen type II and aggrecan production requires the action of BMP-2. It is also possible that collagen type II and aggrecan have not accumulated to the level that can be detected immunofluorescently.

It is important to recall that HGP-P₁ exhibits higher BMP-2 binding and slower BMP-2 release than HGP. The total amount of BMP-2 released from both types of particles over the course of nine days is in fact similar. Thus, the different chondrogenic patterns seen from HGP and HGP-P₁ are not due to the difference in the total amount of BMP-2 presented. Rather the difference is a direct result of how BMP-2 was presented kinetically to the cultured cells. Our results show that high dosage of BMP-2 during the initial micromass culture followed by relatively slow release, as in the case of HGP-B₂, does not lead to a high degree of chondrogenic differentiation. The rapid initial burst release may further reinforce the cell migration. Additionally, the specific binding between BMP-2 and PlnDI synergistically enhanced the cellular responses. Our results underscore the importance in engineering a biomimetic growth factor delivery system so as to maximize the potential of these regulatory factors. BMP-2 interaction with HS chains of PlnDI is indispensable for the coordinated chondrogenic differentiation. These results, along with the release data, revealed that PlnDI immobilized HA HGPs were capable of reversible, specific binding of BMP-2, which was released in a controlled manner *in vitro*. Using an animal model mimicking early symptoms of OA, we are currently testing the efficacy of the BMP-2 delivery system to promote cartilage repair by inducing the growth and differentiation of chondroprogenitor stem cells in cartilage lining.

5. Conclusion

The current investigation aims to develop a biomimetic growth factor delivery system for spatial and temporal presentation of biologically active BMP-2 that can be used to stimulate chondrogenic differentiation of MSCs and ultimately facilitate cartilage repair and regeneration. Our design is motivated by the growth factor binding ability of PlnDI and the chondrogenic potential of HA, PlnDI and BMP-2. Microscopic, HA-based hydrogel particles with defined functional groups and inherent nanopores were utilized as the delivery vehicle. Recombinantly produced PlnDI was successfully linked to HGP by reductive amination using the aldehyde groups that have been previously extended from the particles through a flexible PEG linker and the lysine amines that are exposed on the surface of the core protein at a solution pH below the pI value of the core protein. Particle size analysis, Alcian blue staining and BMP-2 binding assay collectively confirmed the presence of immobilized PlnDI in HGP-P₁. heparitinase treatment indicates that the BMP-2 binding ability of the anchored PlnDI is HS-dependent. HGP-P₁ not only binds significantly more BMP-2 but also modulates its release in a controlled fashion, in sharp contrast to the unmodified HGP. The initial burst release seen with HGP was significantly reduced and the subsequent BMP-2 release follows a near-zero order release kinetics that is attractive for long term tissue repair. The chondrogenic potentials of BMP-2 loaded HGP-P₁ were assessed using a high density micromass culture of MSCs in a direct contact mode. HGP-P₁-B₂ induced a markedly higher degree of chondrogenesis than HGP-B₂ as evidenced by Alcian blue staining and immunohistochemical staining for aggrecan and collagen type II. Cells within the micromass exhibit rounded cell morphology, confirming their chondrogenic phenotype. The HA-based, PlnDI conjugated HGP is an attractive growth factor delivery system for cartilage tissue repair and regeneration.

Abbreviations

Pln, perlecan
 PlnDI, perlecan domain I
 HA, hyaluronic acid
 HGPs, hydrogel particles
 BMP-2, bone morphogenetic protein-2
 MSCs, mesenchymal stem cells
 HS, heparan sulfate
 HGP-P₁, PlnDI-conjugated HGPs

PEGdiALD, poly(ethylene glycol) dibutylaldehyde
 GAG, glycosaminoglycan
 HGP-P1-B2, BMP-2-loaded HGP-P1
 HGP-B2, BMP-2-loaded HGP
 OA, osteoarthritis
 ECM, extracellular matrix
 HSPGs, heparan sulfate proteoglycans
 HBGFs, heparin binding growth factors
 SGD motif, serine-glycine-aspartate motif
 NaBH₃CN, sodium cyanoborohydride
 FITC-dex, fluorescein isothiocyanate-labeled dextran
 FITC-dex-n, FITC-dex with a molecular weight of n kDa (n=4, 40, 250, 500 and 2000)
 CB, cascade blue hydrazide
 HAADH, HA derivative carrying hydrazide groups
 HAALD, HA derivative carrying aldehyde groups
 HGP-PEG, PEGdiALD-modified HGP
 BSA, bovine serum albumin
 TMB, tetramethylbenzidine
 PS, penicillin-streptomycin
 PMSF, phenylmethylsulfonyl fluoride
 SEA module, sperm, enterokinase and agrin (SEA) module.

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Figure 1. Characterization of the average particle pore size by FITC-dextran retention

FITC-dextran with molecular weight of 4 kDa (FITC-dex-4), 40 kDa (FITC-dex-40), 250 kDa (FITC-dex-250), 500 kDa (FITC-dex-500) and 2000 kDa (FITC-dex-2000) were used as the molecular probes. The relative retention of FITC-dex was determined by the equation:

$100 \times \frac{0.2 - C_1 - C_2}{0.2 - C_1}$, where C_1 is the dextran concentration in the supernatant after the first equilibrium and C_2 is the dextran concentration in the supernatant after the second equilibrium. All experiments were carried out in triplicate and the results are expressed as the mean $\pm$ SD (*: significant difference between FITC-dex-4 and 250; FITC-dex-250 and 500; and FITC-dex-250 and 2000, $p \leq 0.05$).

Figure 2. Predicted 3D structural model of SEA domain of PlnD I (Tyr 62 - Ile 153) core protein using Phyre (Protein Homology/analogY Recognition Engine

(A): View showing surface lysines in blue; all are visible from this angle except Lys 104. (B) Alternative view showing Lys 104 on the surface. All four lysine residues are located on the surface of the folded protein (shown in white) where they are readily accessible for covalent conjugation. Note that Lys 164 is not included in this model as it is outside the folded SEA domain.

Figure 3. Schematic illustration of PInDI bioconjugation

PInDI was conjugated to HA HGPs via the core protein through a flexible PEG linker. The access aldehyde groups in HGPs were passivated by glycine and the residual hydrazide groups were allowed to react with PEGdiALD (M_w=3,400). The regenerative aldehyde groups were subsequently used to couple with the lysine amines in the core protein. The PInDI-conjugated particles (HGP-P₁) were passivated again with glycine before being used for BMP-2 loading.

Figure 4. Monitoring the presence of aldehyde groups in HGPs by Cascade blue staining
Particles were allowed to react with cascade blue hydrazide in DI H₂O for 2 h in the dark. Particles were collected by centrifugation and re-suspended in fresh DI H₂O for imaging with a Zeiss LSM 510. (A) as-synthesized HGPs; (2) glycine-treated HGPs; (3) PEG-diALD reacted HGPs (HGP-PEG). Scale bar = 10 μm

Figure 5. Size and size distribution as determined by Coulter Counter Multisizer 3

Water-swollen particles were diluted in isotonic solution at a concentration of $\sim 10 \mu\text{g/mL}$ prior to the analysis using a Coulter Counter Multisizer 3. Open circle: HGP; filled circle: HGP-P₁. Three separate runs were carried out on each types of particles.

Figure 6. The presence of PlnDI in HGP-P₁ as assessed by (A) Alcian blue staining and (B) BMP-2 binding using BMP-2 ELISA

In Alcian blue staining, particles were treated with the Alcian blue solution at pH=1.0. In BMP-2 binding assays, particles were treated with HRP-conjugated BMP-2 antibody followed by the addition of the colorimetric reagents from BMP-2 Quantikine ELISA kit. Glycine-treated HGPs (left panels) were included as the controls in both assays. Magnification: 11.25×.

Figure 7. HS-dependent BMP-2 release from HGP-P₁ and HS-independent BMP-2 release from HGP

BMP-loaded hydrogel particles were treated with heparitinase or heparinase I, II, III (2.5 U/mL) for 2 h. BMP-2 concentration in the supernatant was analyzed using BMP-2 ELISA. BMP-2 loaded hydrogel particles were incubated under the same conditions in the absence of heparitinase I, II, III were included as the controls. #:Significant difference between BMP-2 release from heparitinase-treated HGP-P₁-B₂ and HGP-B₂, $p \leq 0.05$. *:Significant difference between BMP-2 release from heparitinase -treated HGP-P₁-B₂ and untreated HGP-P₁-B₂ control, $p \leq 0.05$.

Figure 8. BMP-2 release from HGP-P₁ (filled circle) and HGP (open circle)

BMP-2 loaded hydrogel particles were incubated in the release buffer for up to 15 days. At pre-determined time points, 400 μ L of the supernatant was collected and the release media was replenished with equal amount of fresh buffer. The amount of BMP-2 in the release medium was measured using BMP-2 ELISA. (A) The amount of BMP-2 bound was calculated by subtracting the amount of BMP-2 released from the amount determined to be bound at time 0. (B) BMP-2 release was calculated as the percentage of BMP-2 released at the indicated time relative to the amount bound to particles at time zero. BMP-2 Release from HGP-P₁ was significantly different from its release from HGP at all times, $p \leq 0.05$.

Figure 9. Alcian blue staining of C3H10T1/2 cells in the presence of different hydrogel particles
High density micromass cultures of C3H10T1/2 cells (1×10^7 cells/mL) were plated on tissue culture dishes in direct contact HGP, HGP-B₂, HGP-P₁, HGP-P₁-B₂. Cells cultured in the absence of any particles were included as the control. Alcian blue staining was carried out after 9 days of culture. Magnification: 11.25 $\times$.

Figure 10. Immunohistochemical analysis of chondrogenic markers by C3H10T1/2 cells cultured in the presence of various particles

Micromass cultures were stained for aggrecan (A–E) and collagen II (F–J) after 9 days of culture. A and F: HGP-P₁-B₂; B and G: HGP-P₁; C and H: HGP-P₁; D and H: HGP; E and J: media only. Scale bar = 50 μ m.