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Heparin-decorated, hyaluronic acid-based hydrogel particles for the controlled release of bone morphogenetic protein 2

Xian Xu^a, Amit K. Jha^a, Randall L. Duncan^{b,c,d}, and Xinqiao Jia^{a,b,d,*}

^aDepartment of Materials Science and Engineering, Delaware Biotechnology Institute, University of Delaware, Newark DE 19716, USA

^bDepartment of Biological Sciences, University of Delaware, Newark, DE 19716, USA

^cDepartment of Mechanical Engineering, University of Delaware, Newark, DE 19716, USA

^dCenter for Translational Cancer Research, University of Delaware, Newark DE 19716, USA

Abstract

We are interested in developing hydrophilic particulate systems that are capable of sequestering growth factors, regulating their release and potentiating their biological functions. Towards this end, heparin (HP)-decorated, hyaluronic acid (HA)-based, hydrogel particles (HGP) were synthesized using an inverse emulsion polymerization technique employing divinyl sulfone as the crosslinker. By varying the feed composition of the aqueous phase, the amount of heparin integrated in the particles can be systematically tuned. The resulting microscopic particles are spherical in shape and contain nanosized pores suitable for growth factor encapsulation. The covalently immobilized heparin retained its ability to bind bone morphogenetic protein 2 (BMP-2) specifically, and its release kinetics can be adjusted by tuning the particle composition. Compared to the pure HA particles, the hybrid HA/HP HGPs show a higher BMP-2 loading capacity. While BMP-2 was released from HA HGPs with a significant initial burst, a near zero-order release kinetics was observed from HA/HP hybrid particles with an optimized heparin content of 0.55 µg per milligram HGPs. The ability of HA/HP hybrid particles to present BMP-2 in a controlled manner, combined with the innate bioactivity of HA, induced robust and consistent chondrogenic differentiation of murine mesenchymal stem cells, as evidenced by the up-regulation of mRNA levels of chondrogenic markers and the production of cartilage-specific extracellular matrix components. The simplicity of the particle synthesis, combined with the defined biological activities of the constituent building blocks, renders the HP-decorated, HA-based hydrogel particle system an attractive candidate for the sustained release of BMP-2, possibly for cartilage repair and regeneration.

Keywords

heparin; hyaluronic acid; hydrogel particles; controlled release; bone morphogenetic protein-2

1. Introduction

Cartilage is a highly specialized musculoskeletal tissue that provides frictionless movement and facilitates the distribution of mechanical loads across the joints [1]. Traumatic rupture or age-related degenerative diseases, such as osteoarthritis (OA), are the major cartilage-related

*Corresponding author: Xinqiao Jia, 201 DuPont Hall, Department of Materials Science and Engineering, University of Delaware, Newark, DE, 19716. Phone: 302-831-6553; Fax: 302-831-4545; xjia@udel.edu..

health problems around the world [2]. Despite recent advances in surgical or nonsurgical therapies, cartilage related disorders still remain largely intractable. This challenge is mainly due to the non-vascularized, non-innervated and acellular environment of the cartilaginous tissue, which significantly limits its self-regeneration capacity [2-4].

Recent studies have demonstrated the efficacy of growth factor therapy as an alternative and promising treatment modality for cartilage lesions [5]. Among various growth factors that show chondrogenic effects, bone morphogenetic protein-2 (BMP-2) has been extensively studied and widely used in the clinic [6, 7]. Expressed in the condensing mesenchyme of the developing limb, BMP-2 has been shown to enhance the recruitment of mesenchymal progenitors to cartilage condensation, regulate their chondrogenic development and stimulate the synthesis of cartilage matrix components by articular chondrocytes both *in vivo* and *in vitro* [8]. BMP-2's high diffusivity in the cartilage tissue, combined with its susceptibility to enzymatic degradation [9], necessitates the employment of drug delivery systems for its immobilization or sequestration. The ultimate goal of the growth factor delivery systems is to modulate the release of BMP-2 in a spatial and temporal manner so that its chondrogenic potential is maximized [10-12].

The simplest strategy to slow down the diffusion and degradation of BMP-2 is to encapsulate the protein physically in a polymeric matrix [13, 14]. However, the inherently high initial burst and the short duration of the release significantly limit the utility of these materials in clinic settings. When BMP-2 is covalently conjugated to polymeric scaffolds [15], prolonged retention and sustained release of the growth factor were achieved. Chemical modification of growth factors may potentially compromise biological activities of growth factors. Alternatively, growth factors have been physically immobilized in hydrogel matrices functionalized with custom-designed aptamers with tunable affinity for the growth factor of interest [16, 17]. Similarly, a basement membrane proteoglycan, perlecan domain I (PlnDI), capable of binding various growth factors specifically [18], has been utilized as to provide the control release mechanism. In our previous study [19], HA-based hydrogel particles (HGP) covalently conjugated with PlnDI were employed as the growth factor depot. Although biologically active BMP-2 was released in a controlled manner over a prolonged period of time, the sophisticated multi-step biosynthetic procedure for the production of PlnDI, as well as the subsequent bioconjugation process necessary for PlnDI immobilization, prohibit the large scale production of the delivery device. This limitation is further compounded by the lack of control over the amount of PlnDI coupled to HA HGPs. Consequently, the BMP-2 release kinetics cannot be adjusted over a wider time scale.

An especially attractive alternative is to immobilize BMP-2 to a synthetic matrix *via* its specific, non-covalent interaction with heparin (HP) that has been previously installed in the matrix (Scheme 1A). Heparin exhibits growth factor binding capacity similar to PlnDI [20]. Unlike PlnDI, HP can be obtained in large quantities with high purity from commercial sources. The ability of HP to associate with various heparin binding growth factors (HBGFs) effectively slows down their diffusion, thus diminishing the initial rapid burst. The hydrated microenvironment provided by the delivery systems can also protect the HBGFs from enzymatic degradation, thereby, potentiating their biological functions [21-23]. In the present investigation, HA HGPs decorated with HP were utilized for the sequestration and the release of BMP-2. The HA/HP HGPs were prepared via an inverse emulsion crosslinking technique. The release profile of encapsulated BMP-2 was systematically tuned by varying the amount of HP incorporated. Sustained release of BMP-2 with a near zero-order release kinetics up to 13 days was accomplished using HGPs with a heparin content of 0.55 µg/mg. BMP-2-loaded, HP-decorated HA HGPs effectively induced the chondrogenic differentiation of C3H10T1/2 multipotent stem cells cultured in high density micromass.

2. Materials and method

2.1. Materials

Hyaluronic acid (HA, sodium salt, 500 kDa) was a gift from Genzyme Corporation (Cambridge, MA, USA). Heparin (HP, sodium salt, 18 kDa, 177 USP unit/mg) from porcine intestinal mucosa, dioctyl sulfosuccinate sodium salt (Aerosol OT, AOT, 98%), 2,2,4-trimethylpentane (isooctane, anhydrous), divinyl sulfone (DVS), 1-heptanol, Toluidine blue O, bovine serum albumin (BSA), fluorescein isothiocyanate-dextran (FITC-DX) with various molecular weights, Alcian blue 8GX and bovine testicular hyaluronidase were purchased from Sigma Aldrich (Milwaukee, WI). Recombinant human BMP-2 (rhBMP-2) and BMP-2 Quantikine ELISA kit were obtained from R&D Systems (Minneapolis, MN). Rabbit anti-mouse collagen II polyclonal antibody was purchased from Abcam Inc. (Cambridge, MA). Rabbit anti-mouse aggrecan polyclonal antibody was obtained from Millipore Corporation (Temecula, CA). Rhodamine Red™-X-conjugated, donkey anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Syto 13 was obtained from Molecular Probes (Carlsbad, CA). All reagents were used as received unless otherwise noted.

2.2. Synthesis of HA/HP HGP

HA/HP HGPs with varying HP content were synthesized using an AOT/isooctane/H₂O reverse micelle system as previously described with minor modifications [24, 25]. Briefly, the aqueous phase was prepared by dissolving HA and HP separately in a NaOH (0.2 M) solution at various pre-determined concentrations (Table 1). The organic phase was composed of 0.2 M AOT and 0.04 M 1-heptanol in isooctane. The aqueous solution with a total volume of 270 μ L containing different amounts of HA and HP (Table 1) was injected into a scintillating vial containing 7.5 mL of AOT/1-heptanol/isooctane. The mixture was immediately vortex-mixed (3,200 rpm) until a clear suspension was obtained. DVS (0.4 μ L) was subsequently added to the microemulsion, and the mixture was vortexed (3,200 rpm) again to disperse DVS. The reaction was allowed to proceed for 1 h at ambient temperature under vigorous stirring (2,000-2,200 rpm). Particles were collected by precipitation in acetone, followed by centrifugation at 12,000 rpm for 10 min. The collected particles were thoroughly washed with water, ethanol and acetone before being dried at 37°C overnight. Four different particle formulations were prepared and the products were designated as HA-HP_x, with x being the amount of HP in feed (micrograms of HP per milligram HGPs, x=0, 1, 10, 100).

2.3. Particle characterization

2.3.1. Quantification of heparin content—The amount of HP covalently incorporated in the hydrogel particles was quantitatively analyzed using Toluidine blue assay [26]. Briefly, HA-HP_x (0.5 mg) were suspended in a Toluidine blue solution (0.005%, w/v) containing 0.2 wt% NaCl and 0.02 N HCl. The suspension was vigorously vortex-mixed for 30 s, followed by centrifugation at 12,000 rpm for 10 min. The absorbance of the supernatant (100 μ L) was determined at 590 nm by a plate reader (Universal Microplate Analyzer, PerkinElmer, Waltham, MA). Absorption background from the control samples consisting of HA alone was subtracted from the test samples. The amount of heparin incorporated in the final particles was derived from a series of standards at a heparin concentration of 2.5-40 μ g/mL.

2.3.2. Particle size and morphology—Scanning electron microscope (JSM 7400F SEM, operating voltage: 3 kV, current: 8 μ A) was used to reveal the particle size and morphology. Particles dispersed in acetone were deposited on aluminum stubs, and acetone was allowed to evaporate at ambient temperature. Dry particles were coated with Pt/Pd

before imaging. The average size and size distribution were analyzed by dynamic light scattering (DLS) using a Malvern Zetasizer NanoZS apparatus (Malvern Instruments, UK). HGP's were dispersed in deionized water at a concentration of ~0.1 mg/mL for the analysis. Size analyses were carried out at 25°C using dynamic light scattering combined with Malvern's DTS software (v.6.01).

2.3.3. Particle pore size analysis—Prior to the analysis, commercial FITC-DX was purified using a Shimadzu (Columbia, MD) high-performance liquid chromatography (HPLC) as previously described [24]. Only fractions corresponding to the designated molecular weight (4 kDa, FITC-DX-4; 70 kDa, FITC-DX-70; 250 kDa, FITC-DX-250; 500 kDa, FITC-DX-500; 2000 kDa, FITC-DX-2000) were collected and used as the molecular probes. FITC-DX was dissolved in DI water at a concentration of 0.8 mg/mL. Five hundred microliters of FITC-DX solution was then mixed with 500 µL of HGP suspension (1 mg/mL in DI water). The mixture was incubated at room temperature under constant shaking for 2 days. The supernatant was collected and hydrogel particles were washed with DI water. The concentration of FITC-DX (C_1 mg/mL) in the combined supernatant and the washing solution (total volume V_1) was determined by the plate reader at an excitation wavelength of 490 nm and an emission wavelength of 530 nm. The amount (M_1 , mg) of FITC-DX that was excluded from the particles was determined from C_1 and V_1 . Next, the collected particles were re-suspended in fresh DI water (1 mL) and the molecular probe was allowed to release from the particles for 2 days under constant shaking. Upon completion of the experiment, the supernatant was collected and the amount of FITC-DX released (M_2 , mg) was measured similarly. The retention of FITC-DX by HGP was determined via the equation

$$\frac{0.4-M_1-M_2}{0.4-M_1} \times 100.$$

2.4. BMP-2 loading and release

In vitro BMP-2 release was performed according to the reported procedure [19]. Briefly, one milligram of hydrogel particles was incubated with 200 ng of BMP-2 in 200 µL binding buffer [0.1% (v/v) BSA, 1% (v/v) Penicillin-Streptomycin (PS) in PBS] for 2 h at room temperature. The supernatant was collected by centrifugation and the amount of free BMP-2 was measured using a BMP-2 Quantikine ELISA kit. 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 a predetermined time (day 1, 3, 5, 7, 9, 11 and 13), the supernatant was collected and the release medium was replenished with an equal amount of the fresh one. The amount of BMP-2 released in the medium was measured by ELISA. The cumulative release was calculated as the total amount of BMP-2 released to the medium at a particular time relative to initial loading content. The amount of BMP-2 remaining associated with the particles was calculated by subtracting the amount released from the initial loading at a predetermined time.

2.5 *In vitro* chondrogenesis

2.5.1. High density micromass cultures—Multipotent mouse embryonic fibroblast stem cell line, C3H10T1/2, was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in DMEM/F12 containing 10% (v/v) FBS, 100 U/mL PS at 37°C in a humid atmosphere of air and CO₂ (95:5, v/v). To evaluate the chondrogenic potential of BMP-2-loaded HGPs, high-density micromass culture of C3H10T1/2 cells was conducted according to a previously reported method [27-29]. Briefly, the trypsinized cells were re-suspended in the medium at a concentration of 10⁷ cells/mL. Ten microliters of the cell suspension were spot-seeded at the center of the well in a standard 24-well cell culture plate. Cells were allowed to adhere for ~2 h at 37°C in the incubator before the medium [0.5 mL, CMRL-1066 containing 15% (v/v) FBS, ascorbic acid (50 µg/mL), 1%

(v/v) PS] containing 2 mg of BMP-2-loaded HGPs or BMP-2 free HGPs were added. Cells cultured in media alone were included as the controls. The medium was carefully changed every two days and the chondrogenic differentiation was assessed by quantitative polymerase chain reaction (qPCR) and (immune)histochemical analysis after 10 days of *in vitro* cultivation.

2.5.2. Gene expression by qPCR analysis—The total RNA was extracted from C3H10T1/2 micromass culture at day 10 using a RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instruction. The RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) at 260 nm. Reverse transcription of mRNA into cDNA was implemented using the QuantiTect Reverse Transcription Kit from QIAGEN following the manufacturer's protocol. The mRNA levels of the respective genes were determined using qPCR employing SYBR Green PCR Maser Mix (Applied Biosystems, Warrington WA1 4SR, UK). The PCR reactions were carried out using an ABI Prism 7300 Sequence Detection System (AB Applied Biosystems, Foster City, CA). A two-step cycling protocol was used with an annealing and elongation temperature of 60 °C and a denaturation temperature of 95°C. Primer sequences for collagen type II (forward: 5'CTCATCCAGGGCTCCAATGA3', reverse: 5'TCCTTCAGGGCAGTGTATGTGA3'), aggrecan (forward: 5'CAGGGTTCCCAGTGTTCAGT3', reverse: 5'CCAGAAGACTCTCCACTGCC3'), and SOX9 (forward: 5'GAGGCCACGGAACAGACTCA3', reverse: 5'CAGCGCCTTGAAGATAGCATT3') were adapted from a previous publication, and β -actin (forward: 5'AAATCGTGCGTGACATCAAAGA3', reverse: 5'GCCATCTCCTGCTCGAAGTC3') was used as the house keeping gene [30]. The relative gene expression was calculated according to equation (1) [31]:

$$\text{Ratio} = \frac{E_{\text{target}}^{\Delta C_{\text{target}}(\text{control-sample})}}{E_{\text{ref}}^{\Delta C_{\text{ref}}(\text{control-sample})}} \quad (1)$$

where E_{target} and E_{ref} are the real-time PCR efficiencies of the target and the reference genes, respectively. ΔC_{target} is the difference in terms of the threshold cycle (Ct) of the target genes between the control and the test samples, and ΔC_{ref} is the difference in the Ct values of the control vs the test samples with respective to β -actin. Cells cultured in the presence of the pure medium were used as the controls.

2.5.3. Alcian blue staining—At the end of the micromass culture, the cell pellets were rinsed with PBS, fixed with 4% (v/v) paraformaldehyde for 10 min at room temperature, washed with PBS and 0.1M HCl and finally incubated with 0.5 mL Alcian blue solution [0.5% (w/v) in 0.1M HCl, pH1.0] overnight at room temperature. The stained micromass cultures were washed again with 0.1M HCl and PBS before being imaged with a Zeiss M²BIO light microscope (Zeiss, Germany). The blue stain taken up by the micromass culture was subsequently solubilized using guanidine hydrochloride (6 M in PBS) overnight, and the respective solution absorbance was analyzed using a plate reader at 590 nm. The normalized absorbance was calculated from the ratio of the absorbance of the samples to that of the medium controls.

2.5.4. Immunohistochemical analysis—Immunohistochemical study was performed according to previous protocol [9]. Briefly, upon completion of micromass culture, the cell pellets were first extensively washed three times by PBS to remove HGPs. Then cells were fixed for 30 min on ice with ethanol/acetic acid (95/5, v/v) for collagen II staining or with 4% (v/v) paraformaldehyde for aggrecan staining. After that, samples were rinsed with PBS

three times and digested with 0.02% (w/v) type IV-S testicular hyaluronidase in PBS for 30 min at room temperature. The micromass cultures then were washed with PBS three times and blocked by 3% BSA (in PBS) overnight at 4°C. Subsequently, specimens were incubated with the primary antibodies against collagen type II (rabbit anti-mouse collagen II polyclonal antibody, 1:200 in 3% BSA) or aggrecan (rabbit anti-aggrecan polyclonal antibody, 1:50 in 3% BSA) respectively, for 1 h at room temperature. After rinsing 5 times with PBS, micromass cultures were incubated with secondary antibody of Rhodamine Red™-X-conjugated donkey anti-rabbit IgG (1:100 in 3% BSA) for another 1 h at room temperature. Finally, after washing 5 times with PBS, Syto 13 (1:1000 in PBS) was added to stain the nucleic acid in the cell (5 min at room temperature). The micromass cultures were again washed with PBS for 3 times, and images were acquired using a Zeiss 5 Live Duo Laser Scanning Microscope (LSM, Zeiss 5 Live Duo Laser Scanning Microscope (LSM, Carl Zeiss Inc. Thornwood, New York).

2.6. Statistical analysis

All quantitative measurements were performed on two or three replicates. All values are expressed as means $\pm$ standard deviations (SD). Statistical significance was evaluated using a two-tailed student's t-test. A p value of less than 0.05 was considered to be statistically different.

3. Results

3.1. Particle synthesis and characterization

Heparin-decorated, hyaluronic acid-based, hydrogel particles were successfully synthesized *via* an inverse emulsion polymerization technique using an AOT/isooctane/H₂O emulsion system employing DVS as a crosslinker (Scheme 1B). A decrease in HP/HA feed ratio led to a reduction of HP content in the corresponding hybrid particles (Table 1). An HP incorporation factor (IF) of 0.55 was determined from the slope of the working curve constructed from various formulations with HP in feed ranging from 0 to 125 $\mu\text{g}/\text{mg}$ (Figure 1). The actual HP content in HA-HP10 and HA-HP1 were estimated to be 5.5 and 0.55 $\mu\text{g}/\text{mg}$ based on the linear relationship and an IF value of 0.55.

Examination of the as-synthesized HA-HP1 HGPs by SEM revealed the presence of spherical objects with smooth surfaces (Figure 2A). A visual inspection of the SEM image suggests the presence of two major particle populations with respect to their sizes: one centered around 1 μm and the other in the submicron range. Particle size analysis by DLS (Figure 2B) indicates an overall bimodal distribution, commensurate with the SEM observation. One population of HGPs ranges from 710 nm to 1.7 μm in size and exhibits a median diameter of $\sim 1.1 \mu\text{m}$. Smaller particles varying from 50 nm to 91 nm, with an effective median diameter of 70 nm, are also present. Alteration of the relative HP content in the particles did not cause significant changes in the particle size and morphology (data not shown).

A solute exclusion method was employed to estimate the average mesh size of HA/HP hybrid particles [32]. FITC-DX with molecular weights varying from 4 kDa to 2000 kDa, corresponding to Stokes's radii of 1.5 to 27.4 nm, were used as the molecular probes [32]. A two-step protocol consisting of separate loading and release processes was used to determine the average retention of the probe molecules by HGPs, and thereby their average pore size. As shown in Figure 3, HA-HP1 exhibited the lowest retention capacity for FITC-DX-4; only $\sim 15\%$ of the molecular probes loaded were retained by the particles after 2 days of release. As the size of FITC-DX increases, the average retention increases accordingly, reaching a plateau value of 76% for FITC-DX-500. Further increase in molecular weight did not result

in a significant change in the average retention for FITX-DX-2000. When the size of FITC-DX is smaller than the average mesh size of the particles, unrestricted diffusion of the probe molecules gives rise to a low overall retention. When the average mesh size of the hydrogel particles became comparable to the size of the probe molecules, a high retention was observed. The large molecules forced into the particles during the loading process could not diffuse out within the time frame of the release process. Since the Stoke's radii for FITC-DX-250 and FITC-DX-500 are 10.5 nm and 14.4 nm respectively, we estimate that the average mesh size (diameter) of HA-HP1 to be in the range of 21-28 nm.

3.2. In vitro BMP-2 release

The addition of heparin to HA HGP dramatically altered the loading and the release of BMP-2 (Figure 4A-B). While one milligram of pure HA HGP can only take up 98.3 ± 0.4 ng BMP-2, the same amount of HA-HPx ($x=1, 10$ and 100) can absorb close to twice as much proteins (173.2 ± 1.1 , 182.1 ± 1.6 and 186.6 ± 2.1 ng/mg, respectively, Figure 4B). After 5 days, 34 ± 7 ng of BMP-2 was retained by one milligram of HA HGP corresponding to a cumulative release of $63 \pm 7\%$. After 13 days of release, only 22 ± 9 ng of rhBMP-2 was still retained by HA HGP, with a total $76 \pm 9\%$ of the initial bound BMP-2 being released. BMP-2 was released from HA HGP with a significant initial burst release of 13% per day for 5 days, followed by a minimal cumulative release 1% per day from day 5 to day 13. On the other hand, a high level of heparin incorporation in HGP (HA-HP10 and HA-HP100) dramatically slowed down the release rate of BMP-2, with less than 5% of BMP-2 initially bound released over 13 days. A sustained release of BMP-2 with a close to zero-order release kinetics was detected when the heparin content was optimized to $0.55 \mu\text{g}/\text{mg}$ (HA-HP1). Specifically, 125 ± 4 ng of BMP-2 was retained by a total of one milligram of HA-HP1, reflecting a cumulative release of $28 \pm 3\%$ at day 5. One milligram of HA-HP1 retained 94 ± 5 ng of BMP-2, releasing only $46 \pm 3\%$ of BMP-2 initially bound at day 13 when the experiment was terminated. An estimated 4% of BMP-2 was released per day from HA-HP1 over the entire course of the release study. Our results demonstrate that the BMP-2 release can be readily tuned by varying the heparin content in the particles and the desired, linear release profile was obtained from HA-HP1.

3.3. In vitro chondrogenesis

The BMP-2 releasing, HA/HP hybrid particles were directly added to the micromass culture of murine multi-potential C3H10T1/2 cells and the chondrogenic potential was evaluated by qPCR analyses of representative chondrogenic markers, including collagen type II, aggrecan and Sox 9 (Figure 5A-C). Our results show that the expression levels of all three markers are the highest for HA-HP1-B2 treated samples. The addition of 2 mg HA-HP1-B2 to the micromass culture resulted in a 5.9, 2.0 and 435 fold increase in the expression of collagen II, Sox9 and aggrecan, respectively. The same amount of HA-B2 caused an average of 4.4, 1.6 and 333 fold increase in the mRNA levels of collagen II, Sox9 and aggrecan. Furthermore, cells treated with BMP-2 loaded HGP expressed significantly higher levels of chondrogenic markers than those treated with carrier alone. The mRNA levels of collagen II and Sox 9 were comparable for cells treated with carrier particles (HA or HA-HP1) and the medium alone. A moderate up-regulation (relative to the medium control) of aggrecan was detected for samples treated with HA HGP (fold change: 2.9) and HA-HP1 HGP (fold change 7.3).

MSCs treated with BMP-2 loaded HGP, both HA HGP and HA-HP1 HGP (HA-B2 and HA-HP1-B2), exhibited a strong Alcian blue staining, indicating the production of glycosaminoglycan (GAG) (Figure 6A-B). Cells cultured in the presence of HA-HP1-B2 exhibit a more robust chondrogenic differentiation, as evidenced by the dark, homogenous blue stain at the concentrated micromass region. A faint and diffuse halo surrounding the

cell pellet is also present. A relatively weak and heterogeneous micromass staining was detected from cells cultivated with HA-B2. Irregular, white gaps inside the micromass are clearly visible. Micromass cultures in the presence of the carrier alone (HA and HA-HP1) exhibited light and diffuse blue hues across the cell monolayer. Cells incubated with the medium alone only had a light background stain. Treatment of the alcian blue-stained micromass with guanidine hydrochloride effectively released the dye into the solution, and the relative intensity of the stain was determined using a plate reader. Consistent with the qualitative assessment from the microscopy images, the normalized absorbance of the alcian blue stained, HA-HP1-B2 treated micromass is significantly higher ($p < 0.05$) than that from HA-B2, both of them being considerably higher than those from the controls (carrier alone and medium alone).

The ability of HA-HP1-B2-treated MSCs to secrete cartilage-specific extracellular matrix (ECM) components was assessed by immunohistochemical staining for collagen type II and aggrecan (Figure 7). In both cases, cells were labeled green by Syto 13 while the ECM components, collagen type II and aggrecan, were stained red. Cells residing in the micromass are clearly spherical-shaped. Non-specific binding of the secondary antibody alone was negative. While robust collagen II and aggrecan staining was detected in cells cultured in the presence of BMP-2 releasing HA-HP1, only faint, diffuse red background can be distinguished from HA-B2 treated cells. When applied to the vehicle-treated (HA-HP1 and HA) cells, collagen II and aggrecan staining were at the level similar to the BMP-2-free medium controls.

4. Discussion

Although the therapeutic potential of BMP-2 is well recognized, a clinically proven, efficient BMP-2 delivery device has not yet been realized. While excess dosage of BMP-2 can cause undesirable side effects to the tissues [33], an inadequate amount of the growth factor will not promote desired therapeutic responses. From a pharmacokinetics perspective, a successful delivery device must exhibit a sustained release profile with the dosage inside the therapeutic window so that the biological functions of the growth factors are maximized [6, 34]. We are interested in developing HA/HP hybrid particulate systems that can retain BMP-2, prolong its *in vivo* residence time and preserve or enhance its biological activities.

HA-based hydrogel particles with varying amounts of heparin were synthesized *via* an inverse emulsion crosslinking method. DVS is capable of reacting with the primary hydroxyls on both HA and HP under basic conditions within the AOT inverse emulsions to afford stably-crosslinked hybrid particles [35]. The presence of highly charged sulfate groups on heparin allows its content in the particles to be quantitatively determined by Toluidine blue assay [26]. Overall, approximately half of HP initially fed to the inverse emulsion system was incorporated in the final particle product. The low incorporation efficiency for HP is not surprising considering the fact that an average HP disaccharide contains 2.7 sulfo groups. Consequently, HP contains significantly fewer numbers of primary hydroxyls per disaccharide repeat than HA [20]. Therefore, HP is less crosslinkable than HA towards DVS under the conditions employed. The high charge density of HP may also contribute to the destabilization of the AOT emulsion system. The un-crosslinked, soluble HP in the reaction mixture was readily washed out during the purification process. It should be mentioned that in addition to its specific growth factor binding capability, heparin also functions as an anticoagulant through its interaction with antithrombin III (AT III) [36]. Our particle formulations were carefully adjusted so as to maximize their therapeutic potential in cartilage repair without triggering the distinguishable blood-thinning effects [37]. Hybrid particles with the lowest HP content (HA-HP1) were the focus of our current investigation.

The HA/HP HGPs have a well-defined spherical shape and can be readily dispersed in aqueous media without aggregation. The bimodal particle size distribution can be attributed to the application of two distinct homogenizing speeds during the particle synthesis [24, 25]. The initial rapid vortex-mixing at 3,200 rpm can effectively break up the emulsion droplets, giving rise to smaller particles with a median diameter of 70 nm. The larger particles with an average diameter of 1.1 μm are formed during the subsequent, milder mixing process (1 h at $\sim 2,000$ rpm). The observed, cumulative BMP-2 release kinetics is a consequence of a combined contribution from two distinct particle populations. In addition to their large surface area, smaller particles also exhibit high diffusivity, potentially decreasing their *in vivo* residence time at the injection site. The particles are inherently porous, with an average mesh diameter of 22 nm. Replacing 0.1% of HA with HP in feed resulted in hybrid particles with larger pore size [24]; the larger pore size further reinforces the notion that HP exhibit a lower reactivity towards DVS, thus lower overall crosslinking density for hybrid gels as opposed to HA HGPs. As a disulfide-linked homodimer with a molecular weight of ~ 26 kDa, BMP-2 can diffuse through the particle readily without encountering any geometry constraints.

The *in vitro* release study revealed a well-defined, linear release profile for BMP-2 loaded in HA-HP1 hydrogel particles, contrary to the two-phase release kinetics with significant initial burst seen from HA HGPs. Recombinant human BMP-2 is a member of the BMP subgroup of the TGF- β superfamily with 103 amino acids in length and an isoelectric point of 8.21 [34]. Therefore HA can bind BMP-2 through electrostatic interactions between the negatively charged carboxyls on HA and the positively charged BMP-2 [38]. However, such interaction is rather weak and non-specific. Consequently, homogenous BMP-2 distribution within the HA HGPs and its long-term sustained release cannot be achieved. The initial burst can be attributed to the dissociation of BMP-2 that weakly bound near or on the surface of the particles, and the subsequent trace amount of growth factor diffusing out of the inner compartments of HGP contributes to the second step of the release profile.

Heparin associates with a variety of growth factors with high affinity and specificity *via* the electrostatic interactions between clusters of positively charged basic amino acids on the growth factor and the spatially-defined, negatively charged sulfo or carboxyl groups on heparin chains [20, 39]. Therefore, the release kinetics and the biological functions of HBGFs can be regulated *via* the strategic incorporation of HP in synthetic scaffolds or particulate systems [12, 40]. In our case, incorporation of a small amount of HP in HA-based hydrogel particles dramatically increased their loading capacity, attenuated the initial burst, and afforded a sustained release over a prolonged period. Moreover, the release profiles of the payload can be modulated by tuning the molar ratio of the amount of HP introduced in the particles.

In our system, the overall BMP-2 release rate is mediated by the combined effects of free diffusion induced by the concentration gradient and the association/dissociation of BMP-2 with the heparinized matrix [39]. When the release is diffusion-controlled, a severe initial burst is inevitable, as shown in Figure 4A for the HA HGP control samples. On the other hand, when the HP/protein association becomes the dominating mechanism, the BMP-2 release from the matrix is significantly hindered. In our experiments, the same BMP-2 loading protocol was applied to different particle formulations. The different HP content, therefore, translates to a variation of HP/BMP-2 ratio. In the presence of a large excess of heparin in the particles, the locally dissociated protein molecules are rapidly recaptured by the abundant unoccupied heparin sites in the matrix before they can diffuse out into the surrounding medium [41]. Once the threshold composition is reached, further increase of the HA/HP only induced minute changes in the overall release rate. When the rate of free diffusion and matrix association become comparable, a zero-order sustained release over an

extended period can be obtained. This is the case for HA-HP1 particles having an HP to BMP-2 molar ratio of 4:1. From a therapeutic perspective, extreme release profiles, such as minimal release over a prolonged period of time in the case of HA-HP100 and HA-HP10, or rapid release over a very short period of time, as in the case of HA HGPs, are detrimental to the native tissues and non-conducive to the tissue regeneration [6]. Collectively, the optimized particle formulation with a HP content of 0.55 $\mu\text{g}/\text{mg}$ is potentially useful for cartilage repair because of its ability to present free BMP-2 at a constant dosage over a long period.

Using a high density micromass culture, we evaluated the biological activity of the released BMP-2 and the chondrogenic potential of our hydrogel particle-based growth factor delivery device. A moderate increase in GAG production as compared to the medium controls was detected from C3H10T1/2 cells cultured in the presence of BMP-2-free carriers, both pure HA particles and HP-containing equivalents, suggesting the stimulatory effects of HA. HA is known to play an important role in cartilage homeostasis [42], and is involved in various cellular processes such as cell proliferation and morphogenesis [43]. It is postulated that HA induces chondrogenesis through its binding with the cell surface receptor CD44 [44, 45], abundantly expressed in mesenchymal stem cells [46]. Therefore, the HA-based hydrogel particles are not just passive carriers; they are biological entities whose activities can be further enhanced by BMP-2.

Quantitative PCR analyses and immunohistochemical staining unambiguously confirmed the efficacy of the HA-HP1-B2 system in inducing chondrogenic differentiation of C3H10T1/2 cells. Although cells treated with HA-B2 exhibited an increased expression of genes related to cartilage ECM proteins (collagen type II and aggrecan) as compared to the vehicle controls, such enhancements were not manifested at the protein level. Chondrogenic differentiation of mesenchymal progenitor cells requires the complex interplay of cell-cell contacts, cell-ECM interactions, and most importantly, spatio/temporal presentation of growth factors including BMP-2 [27, 28, 30, 47]. The different degree of chondrogenesis observed from HA-B2 and HA-HP1-B2 can be attributed to their distinctly different release kinetics. Specifically, the amount of BMP-2 released from both types of HGPs within the first 6 days of culture was essentially the same. However, for the subsequent 4 days, only trace amounts of BMP-2 ($\sim 4\text{ng}/\text{mL}$ per day) diffused out of HA HGPs. Conversely, cells cultured in the presence of HA-HP1-B2 were exposed to the free BMP-2 at a constant level in a sustained manner ($28\text{ ng}/\text{mL}$ per day) over the entire course of the culture. The ability of HA-HP1 HGPs to present BMP-2 to the cells in a temporal pattern with the desired dosage suggests its potential application in cartilage repair.

In this study, the *in vitro* BMP-2 release was monitored for up to 13 days. The near zero-release kinetics of BMP-2 from HA/HP1 suggests that BMP-2 could be continuously released over a prolonged period of time. However, 100% BMP-2 release is unlikely within a finite time frame because the driving force (i.e. BMP-2 concentration gradient) gradually decreases over time. BMP-2 is known to induce both chondrogenesis and osteogenesis; and osteogenesis follows chondrogenesis after the blood vessel infiltration [48]. Thus, for *in vivo* cartilage repair using the injectable HA/HP HGPs, effective treatment regimen needs to be developed so that BMP-2 is presented at the desired time with an adequate concentration without bone formation induction.

5. Conclusion

Hyaluronic acid-based hydrogel particles containing covalently immobilized heparin were successfully synthesized using the AOT/water/isooctane inverse emulsion system. The resulting particles were spherical-shaped, nano-porous and readily dispersible in aqueous

medium. The heparinized HA HGP provide an affinity-based delivery system for the controlled release of BMP-2. With an optimized HP content of 0.55 µg/mg, the hybrid particles exhibit a near zero-order BMP-2 release kinetics. The controlled release of BMP-2, combined with the inductive role of HA in chondrogenesis, led to an efficient chondrogenic differentiation of mesenchymal stem cells cultured at high density. *In vivo* assessment of the hybrid BMP-2 delivery system is currently underway.

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Scheme 1.

Schematic illustrations of the underlying mechanism for the controlled release of BMP-2 from HP-decorated, HA-based hydrogel particles (A) and possible crosslinked structures established by DVS in the hybrid hydrogel particles (B). The light blue background in (A) represents water.

Figure 1. Quantification of heparin content in the hybrid hydrogel particles using Toluidine blue assay. A working curve was constructed from the initial feed composition and the measured HP content in the products.

Figure 2. Characterization of hydrogel particles by SEM (A) and DLS (B). Only results from HA-HP1 HGP are shown.

Figure 3.

Particle pore size analysis by solute uptake experiments. FITC-DX with molecular weights of 4, 70, 250, 500, 2000 KDa were used as the molecular probes and the analysis was conducted on HA-HP1 HGPs. All experiments were performed in triplicate and the results are expressed as the mean $\pm$ SD. (* indicates significant difference from 500 kDa, $p < 0.05$).

Figure 4. BMP-2 release from hydrogel particles with various compositions. (A) Cumulative release as a function of time; (B) BMP-2 retention as a function of time. The initial loading for HA-

HP100, HA-HP10, HA-HP1 and HA HGPs is 186.6 ± 2.1 , 182.1 ± 1.6 , 173.2 ± 1.1 and 98.3 ± 0.4 ng/mg respectively.

Figure 5. Gene expression of collagen II (A) Sox9 (B) and aggrecan (C) of murine mesenchymal stem cells cultured for 10 days in the presence HGP and BMP-2 loaded HGP. The relative expression of each gene was normalized to the medium control using β -actin as the house keeping gene. Values represent means $\pm$ SD of triplicates of separate RNA isolates. (*indicates a significant difference determined by student t test, $p < 0.05$).

Figure 6.

(A) Optical images of C3H10T1/2 cells stained by Alcian blue after 10 days of micromass culture in the presence of various hydrogel particles. Cells cultured with pure medium were used as the controls. Magnification: x8. (B) Quantification of alcian blue staining using a plate reader. Stained micromass samples were solublized in 6M guanidine hydrochloride and the respective solution absorbance was determined using a plate reader at 590 nm. The normalized absorbance was calculated from the ratio of the absorbance of the samples to that of the medium controls.

Figure 7. Immunohistochemical analyses of cartilage-specific ECM proteins secreted by C3H10T1/2 cells in the presence of various types of particles after 10 days of culture. Collagen II (top) and aggrecan (bottom) were stained red and nucleic acid was stained green by Syto 13.

Table 1

Feed composition for the synthesis of HA/HP HGPs with varying HP contents.

Sample ID	[HA] (mg/mL) ¹	[HP] (mg/mL) ²	V _{HA} (μL) ³	V _{HP} (μL) ⁴	HP (μg/mg) ⁵	HP (μg/mg) ⁶
HA-HP125	4	4	236.2	33.8	125	71.4±0.5
HA-HP100	4	4	243	27	100	52.9±1.6
HA-HP75	4	4	249.8	20.2	75	39.2±1.4
HA-HP50	4	4	256.5	13.5	50	25.5±0.8
HA-HP10	4	4	267.3	2.7	10	5.5 ⁷
HA-HP1	4.04	0.4	267.3	2.7	1	0.55 ⁷
HA	4	0	270	0	0	0

¹ HA concentration in aqueous phase

² HP concentration in aqueous phase

³ Volume of the HA solution added to the microemulsion

⁴ Volume of the HP solution added to the microemulsion

⁵ Relative HP content in feed

⁶ Relative HP content in the product

⁷ Predicted using a HP incorporation factor of 0.55.