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Effect of Selective Heparin Desulfation on Preservation of Bone Morphogenetic Protein-2 Bioactivity after Thermal Stress

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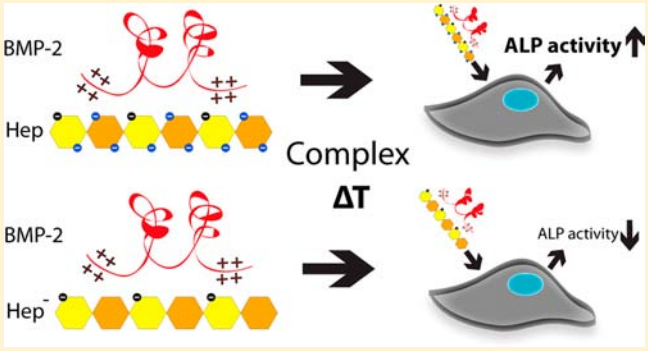
Supporting Information

ABSTRACT: Bone morphogenetic protein-2 (BMP-2) plays an important role in bone and cartilage formation and is of interest in regenerative medicine. Heparin can interact electrostatically with BMP-2 and thus has been explored for controlled release and potential stabilization of this growth factor *in vivo*. However, in its natively sulfated state, heparin has potent anticoagulant properties that may limit its use. Desulfation reduces anticoagulant properties, but may impact heparin's ability to interact and protect BMP-2 from denaturation. The goal of this study was to characterize three selectively desulfated heparin species (N-desulfated (Hep^{-N}), 6-O,N-desulfated ($Hep^{-N,-6O}$), and completely desulfated heparin (Hep^{-})) and determine if the sulfation level of heparin affected the level of BMP-2 bioactivity after heat treatment at 65 °C. BMP-2 bioactivity was evaluated using the established C2C12 cell assay. The resulting alkaline phosphatase activity data demonstrated that native heparin maintained a significant amount of BMP-2 bioactivity and the effect appeared to be heparin concentration dependent. Although all three had the same molecular charge as determined by zeta potential measurements, desulfated heparin derivatives Hep^{-N} and $Hep^{-N,-6O}$ were not as effective as native heparin in maintaining BMP-2 bioactivity (only ~35% of original activity remained in both cases). These findings can be used to better select desulfated heparin species that exhibit low anticoagulant activity while extending the half-life of BMP-2 in solution and in delivery systems.



INTRODUCTION

Bone morphogenetic protein-2 (BMP-2) participates in bone and cartilage formation and is attractive for tissue regeneration applications. It is currently approved for spinal fusion, open tibial fractures, and sinus augmentation, and can be delivered via an absorbable collagen sponge (e.g., INFUSE Bone Graft, Medtronic Sofamor Danek USA, Inc., Memphis, TN, USA).[1,2] However, BMP-2 has been shown to have a short half-life, which may limit its potency *in vivo*.[3] To counter this, delivery vehicles are often loaded with supraphysiological doses of BMP-2 (e.g., mg quantities in INFUSE Bone Graft), which increases overall costs and has been shown to cause adverse effects.[1,4,5] Thus, alternate delivery strategies are needed to prevent loss of BMP-2 bioactivity and improve clinical efficacy.

Heparin, a glycosaminoglycan (GAG), is highly negatively charged, due to sulfate groups at specific locations along its backbone.[6] Heparin itself is usually found natively in mast cells of many animal tissues,[6] but its molecular cousin, heparan sulfate, is present on cell surfaces and plays a large role in signaling of particular growth factors.[7] Heparin and heparin sulfate can bind growth factors, including BMP-2, either electrostatically or through carbohydrate-specific sequences.[8] Therefore, heparin-based biomaterials have been explored previously as controlled delivery vehicles for BMP-2.[9,10] Moreover, heparin binding to another class of positively charged growth factors, fibroblast growth factor (FGF), has been studied extensively and was shown to protect FGF from acidic, enzymatic and high temperature conditions,[11−13] suggesting that GAG-binding may also be beneficial to preserve bioactivity of BMP-2.

Heparin is employed clinically as an anticoagulant,[14] which may limit its use *in vivo* for regenerative medicine applications unless the anticoagulant aspect is ablated. Although it has been demonstrated that selective desulfation of heparin can significantly reduce the anticoagulant activity of heparin,[15−19] heparin desulfation may disrupt any protective interactions between heparin and bound growth factors. Therefore, better understanding of the interactions between desulfated heparin derivatives and BMP-2 can help in developing safe and effective delivery systems.

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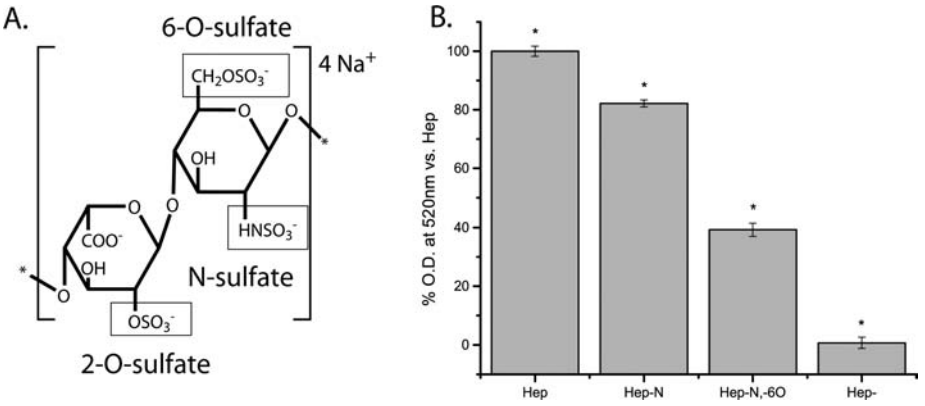

Figure 1. Common sulfation positions on heparin disaccharide and effect of desulfation protocols on native heparin. (A) Boxed regions represent positions that are sensitive to the desulfation protocols. (B) Overall sulfation levels of modified heparins compared to native heparin as measured by absorbance of DMMB. * indicates that normalized absorbance of sample was significantly different than all other derivatives, $p < 0.05$, $n = 3$, mean ± s.d.

While a previous study has examined the effect of codelivery of desulfated heparin species and BMP-2 in standard cell culture conditions,[20] the protective effects of desulfated heparin derivatives on BMP-2 when exposed to denaturing environments have not yet been investigated. Unlike FGF-2, which requires heparin for signaling, BMP-2 has a heparin-binding domain that is separated from the active site of the protein.[21] In addition, in contrast to other growth factors of the TGF-β superfamily, BMP-2's heparin-binding region is not covalently attached to the protein core by disulfide bonds, making this region relatively flexible,[22] which may influence how it interacts with various native and desulfated GAGs.[22,23]

Therefore, the objective of this study was to evaluate how the level and position of sulfation of heparin affects the stability of nonglycosylated BMP-2 when exposed to thermal stress as a model of denaturing conditions. We first synthesized three desulfated derivatives, all known to have reduced anticoagulant properties:[15,24] N-desulfated (Hep^{-N}), 6-O,N-desulfated ($Hep^{-N,-6O}$), and completely desulfated heparin (Hep^{-}). The hypothesis of this study was that soluble complexes formed between heparin and BMP-2 could protect BMP-2 against thermal denaturation, and that this protection would decrease with decreasing levels of sulfation. Using an established cell-based assay, the bioactivity of nonglycosylated BMP-2 was evaluated following exposure to high temperature over 15 min in order to probe the effect of presence of the various heparin derivatives.

RESULTS

Heparin Desulfation and Characterization. The DMMB assay was used to assess overall sulfation levels of heparin derivatives desulfated by the various protocols. In comparison to the native heparin molecule, Hep^{-N}, $Hep^{-N,-6O}$, and Hep^{-} were approximately 82.2%, 39.2%, and 0.7% as sulfated, indicating a stepwise decrease in sulfation due to the desulfation protocols (Figure 1A and B). The molecular weight for each heparin derivative was significantly different than that of the other samples (Table 1). $Hep^{-N,-6O}$ and Hep^{-} had significantly lower number-average molecular weights than Hep and Hep^{-N}.

The charge profile of each heparin derivative was measured at ~pH 7.4 and indicated that all heparin species that still contained sulfate groups had an equal negative net charge (−40 to −50 mV) whereas Hep^{-} exhibited a significantly lower negative charge (−30 to −40 mV) due to the lack of sulfate groups (Table 1). Determination of the isoelectric point of each derivative was difficult to achieve under the given experimental conditions, but could be extrapolated from zeta potential titration curves. The isoelectric points for all derivatives was between pH 0 and pH 2.5 (data not shown), indicating that under physiological conditions all heparin species carry a permanent negative charge.

Table 1. Relative Number Average Molecular Weight (M_n) and Polydispersity (PI) of Heparin Species Compared to PEG Standards

	M_n	PI	ζ at pH 7.4 ± 0.3 [mV]
Hep	24 000 ± 1200[a]	1.4 ± 0.0[b]	−48 ± 1
Hep^{-N}	27 700 ± 200[a]	1.4 ± 0.0[b]	−45 ± 3
$Hep^{-N,-6O}$	20 300 ± 20[a]	1.7 ± 0.0[a]	−45 ± 3
Hep^{-}	16 900 ± 30[a]	1.5 ± 0.0[a]	−33 ± 2[c]

[a]Value is significantly different from values of all other samples. [b]Value is different from values for $Hep^{-N,-6O}$ and Hep^{-}. $p < 0.05$, $n = 3$, mean ± s.d. Zeta potential titration at pH 7.4 was measured for each heparin species. [c]Significant difference from all other groups ($n = 3$, $p < 0.05$, mean ± s.d.).

Proton NMR showed specific chemical shifts upon the desulfation protocols (Figure 2 and Supporting Information). Hydrogen atoms on the heparin carbohydrate ring clearly confirmed N-desulfation (b,s), and N,6O-desulfation (f,f′). There was some evidence of 2-O desulfation upon the N-desulfation protocol, although shifts of IduA protons are less pronounced upon desulfation. Therefore, 2-O desulfation was difficult to monitor with ^{1}H NMR. Completely desulfated heparin spectra did not show any signals for sulfated groups at the N or 6-O positions. Epimerization from IduA to GlcA was observed in the spectra of Hep^{-N} ($\delta = 4.85$ ppm, k in Figure 2, C5 GlcA). The signal further increased in height with progress of desulfation to Hep^{-}.

Cell-Based BMP-2 Bioactivity Assay. For these results, the ALP activity of each sample was normalized to the activity level induced by untreated BMP-2 in order to minimize differences across plates. Negative controls of low serum (1% FBS) basal medium and medium supplemented with heparin species did not induce significant ALP activity in C2C12 cells over 3 days (Figure 3A). When BMP-2 was pretreated with heat, a significant drop in bioactivity was observed with as short as a 2 min heating period compared to untreated ("0 min")

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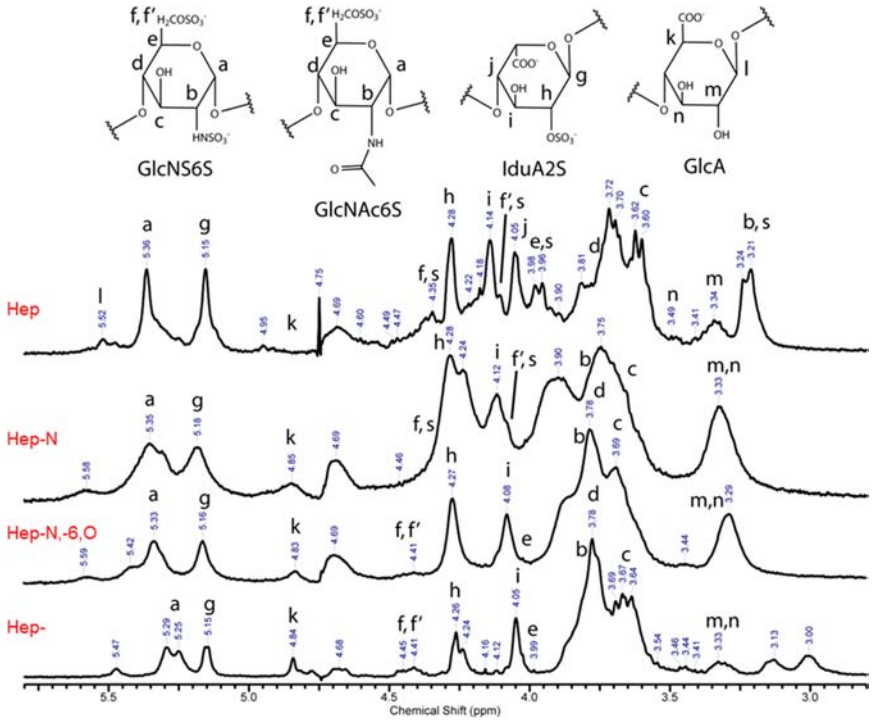

Figure 2. ^{1}H NMR spectra for heparin and its desulfated heparin species. At the top of the figure the monosaccharide units that are commonly found in native heparin are depicted. A small letter 's' indicates the sulfated position. Other letters correspond to the position of the hydrogen depicted on the chemical structure. The signal of at δ = 3.21−3.24 ppm (b,s) corresponds to ring proton 2 of GlcNS6S that completely vanished after N-desulfation while the signals δ = 4.35 ppm and δ = 4.06 ppm for the 6-O sulfate group (f,s and f′,s) were maintained. After N,6-O-desulfation, the signals of the desulfated 6-O groups shifted to δ = 4.4−4.5.

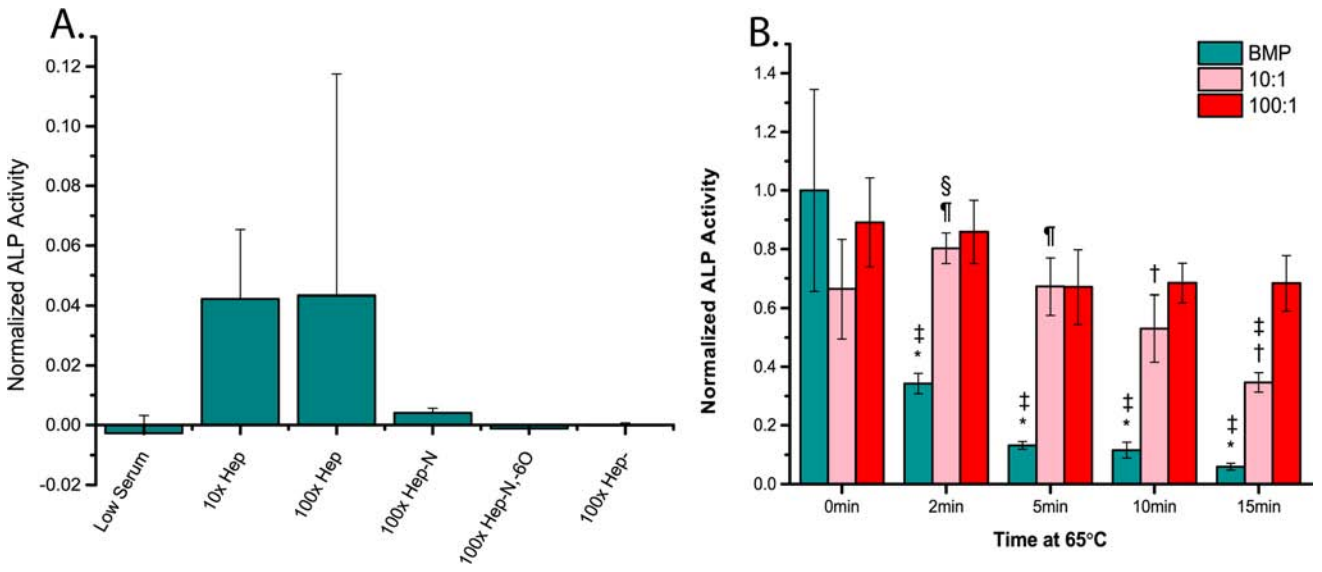

Figure 3. Normalized BMP-2 bioactivity levels after pretreating BMP-2 with heat for various times prior to administration to C2C12 cells. (A) ALP activity for BMP-free control cultures showing no significant differences. (B) BMP-2 saw a significant drop in bioactivity after 2 min of heating, while 10:1 heparin:BMP did not exhibit a significant drop until 10 min of heating. There were no significant differences in bioactivity of 100:1 heparin:BMP groups over treatment time. All BMP activity values were normalized to an untreated BMP-2 control. * indicates significantly different than other samples within same treatment time, † lower than 100:1 within same treatment time, ‡ different from 0 min for same sample, § different from 10 min for same sample, ¶ different from 15 min for same sample. $p < 0.05$, $n = 4$, mean ± s.d.

BMP-2 (Figure 3B). When BMP-2 was complexed with 10 molar excess heparin, no drop in bioactivity was detected until the complex was pretreated with 10 min of heat. Unlike the BMP-2-alone and 10:1 complex, the 100:1 complex did not show a significant decrease in bioactivity over heat treatment times. In addition, the 100:1 complex exhibited significantly higher ALP activity than BMP-2-alone over all heat treatment times. With 15 min of heat treatment, the 100:1 complex retained approximately 70% of the ALP activity induced by untreated BMP-2 (Figure 3B).

Initial differences in ALP activity were observed when BMP-2 was codelivered with chemically desulfated heparin species.

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Codelivery with Hep^{-N} or Hep$^{-N,-6O}$ showed significantly lower ALP activity than BMP-2-alone (Figure 4). In addition,

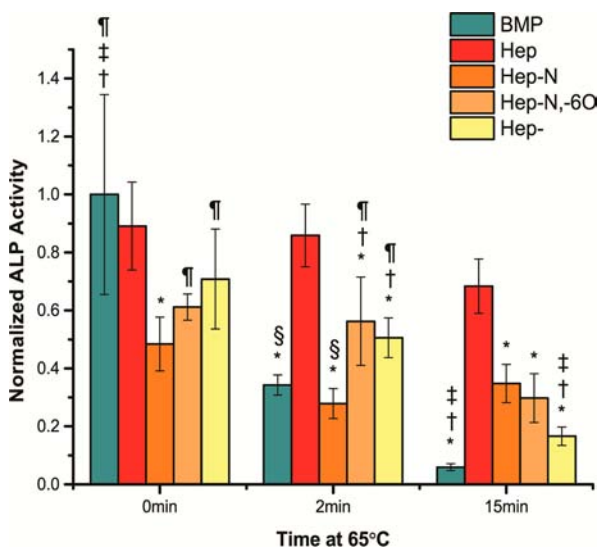

Figure 4. Normalized BMP-2 bioactivity over heat treatment time and codelivery with modified heparins. With 15 min of heat treatment, BMP-2 codelivered with Hep, Hep-N, or Hep-N,-6O showed higher bioactivity than BMP-2 alone. All BMP activity values were normalized to an untreated BMP-2 control. * indicates significantly different than Hep within the same treatment time, † different from Hep-N within the same treatment time, ‡ different from Hep-N,-6O within the same treatment time, § different from 0 min of same sample type, ¶ different from 15 min of same sample type. $p < 0.05$, $n = 4$, mean ± s.d.

Hep^{-N} demonstrated lower ALP activity than Hep codelivery at 0 min. While BMP-2 complexed to Hep showed no significant difference in bioactivity over heating time, Hep^{-N} codelivery exhibited an initial drop in bioactivity after 2 min of heating yet retained approximately 35% of the ALP activity of the untreated BMP-2 positive control after 15 min of heating. Hep$^{-N,-6O}$ codelivery demonstrated a lower ALP activity after 15 min of heating compared to its unheated control, and was significantly lower than the ALP activity induced by Hep codelivery, but also retained ∼35% of the unheated BMP-2 ALP activity. When Hep^{-} was codelivered with BMP-2, it had a similar level of bioactivity as BMP-2 at 0 min (∼71% of untreated BMP-2 bioactivity), but had only approximately 17% of bioactivity after 15 min of heating. In summary, with 15 min of heating, BMP-bioactivity was significantly higher when codelivered with Hep, Hep^{-N}, and Hep$^{-N,-6O}$ (but not Hep^{-}) when compared to BMP-2-alone.

DISCUSSION

Heparin with reduced anticoagulation activity that would be amenable for use in *in vivo* delivery systems was synthesized in our laboratory through selective desulfation. Given that similar desulfation protocols were used as those that have been described in prior studies,[15,26,27] it is expected that the anticoagulant properties of these materials would be greatly reduced compared to native heparin, as previously reported. Therefore, the primary goal of this study was to determine if sulfation level of the modified heparin species correlated to the level of BMP-2 bioactivity after heat treatment.

Both overall sulfation level and molecular weight changes after chemical treatment were evaluated for all desulfated heparin species produced. For the same mass concentration of heparin species, the DMMB assay showed that overall sulfation level decreased as heparin was N-desulfated, 6-O,N-desulfated, and completely desulfated (Figure 1). While remaining similar overall, there was a significant decrease in molecular weight for Hep$^{-N,-6O}$ and Hep^{-} derivatives, indicating that the solvolytic desulfation protocols may affect the glycosidic linkages between monomers (Table 1). Proton NMR clearly showed the loss of N-sulfate groups upon N-, N,6-O, and complete desulfation (Figure 2 and Supporting Information). Signals for 6-O sulfation were found in spectra of N-desulfated heparin, and subsequently vanished with decreasing sulfate content over the different processes. Evidence of iduronic to glucuronic epimerization was noted for all desulfated heparin species (Figure 2). This epimerization, in addition to the loss of sulfate groups, may lead to different heparin chain conformations compared to the native heparin helix,[8] leading to changes in solution interactions with proteins such as BMP-2.

In these studies, a nonglycosylated version of BMP-2 was used to probe the effect of thermal stress. Thermal stress was chosen as a model denaturing environment due to the ease of reproducibility of the denaturing conditions. The benefit of using nonglycosylated BMP-2 in these studies was the suitability in understanding how the protein backbone may be affected by external factors and interactions. With glycosylated BMP-2, glycans attached to the protein backbone may affect protein folding and response to thermal stress.[29,30] Thus, the form of BMP-2 used in this study could more readily provide information on molecules that can stabilize its activity without the confounding effects of glycans.

The results of these experiments show that nonglycosylated BMP-2 was sensitive to heat treatment (bioactivity was reduced to 34 ± 0.03% after a 2 min exposure to heat) and was protected by heparin codelivery (Figure 3). The thermal stress of heating at 65 °C appeared to cause nonreversible unfolding as bioactivity was not recovered after removal from heat. While heparin alone at similar concentrations did not produce a response from the BMP-2-sensitive cell line used, after 15 min exposure to heat, complexes of 10:1 heparin:BMP-2 demonstrated significantly greater BMP-2 bioactivity (∼35%) over free BMP-2 (∼0.06%) and an even higher excess of heparin seemed to have additional protective effects on BMP-2 bioactivity (Figure 3B). Native heparin at 100 molar excess was shown to maintain BMP-2 bioactivity at ∼68% over 15 min of heating. This heparin concentration dependence was similar to a past study with FGF-1, in which FGF-1 experienced more thermal stability when incubated with increasing concentrations of heparin up to approximately 3 molar excess of heparin to FGF-1.[31]

A 100 molar excess of the desulfated heparin derivatives was chosen for BMP-2 bioactivity assays in order to maximize the potential interaction with the growth factor. These approximate molar ratios of heparin:protein have been used in a previous study investigating heparin with FGF-2 and a delivery vehicle that showed controlled release with 100:1 heparin:growth factor concentrations.[32,33] It has been estimated that 5−6 molecules of BMP-2 can be bound to each heparin chain,[21] so there were heparin molecules that were not saturated with BMP-2 in these studies. It is possible that the amount of unoccupied heparin at 100 molar excess may play an additional role in slowing denaturation of BMP-2. In studies of excipients used to stabilize proteins, increasing concentrations of the sugar trehalose was shown to increase the surface tension of the

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protein solution, which then required greater amounts of energy to denature the protein.[34]

The desulfated species at 100 molar excess delivered less ALP activity in absolute terms compared with 100:1 Hep:BMP-2 when exposed to heat (Figure 4). Perhaps not surprisingly, codelivery with Hep^{-}, which demonstrated less negative charge in zeta potential measurements and therefore would not be able to interact electrostatically as well with BMP-2, resulted in the least bioactivity after 15 min of heat treatment. However, codelivery with Hep^{-N} and $Hep^{-N,-6O}$ resulted in similar BMP-2 bioactivity levels after 15 min of heating, and were higher than the bioactivity of BMP-2 alone, suggesting that there may be protective effects due to residual sulfates on those heparin species, even though $Hep^{-N,-6O}$ retained only ~40% of the sulfate groups compared to native heparin based on the DMMB results (Figure 1). Additionally, zeta potential measurements indicate that the Hep, Hep^{-N}, and $Hep^{-N,-6O}$ derivatives exhibit an equal net negative charge (Table 1) under complexing conditions, so protective effects on BMP-2 bioactivity cannot be explained solely by electrostatic interactions.

Moreover, when normalizing the data after 15 min of heat treatment to the different initial ALP activities of each sample (Figure 4), Hep and Hep^{-N} promoted equal retention of BMP-2 activity, whereas $Hep^{-N,-6O}$ and Hep^{-} protected bioactivity in a less pronounced fashion. As is already established for growth factors of the FGF family,[32,35] heparin-binding proteins may recognize a specific glycan sulfation code. There is evidence that this is the case for BMP-2 as well.[20,36,37] Moreover, the heparin backbone conformation may play a role in interactions between heparin and heparin-binding proteins. Heparin is a relatively flexible molecule, and is able to wrap itself around proteins during binding, driven primarily by the iduronic acid residue that can adopt several conformations.[38,39] It is possible that a change in heparin conformation occurred while undergoing desulfation procedures, as suggested by the increased presence of glucuronic acid, a hexuronic acid with fewer conformations than L-iduronic acid, in ^{1}H NMR results (Figure 2). Any structural difference between Hep^{-}, Hep^{-N}, and $Hep^{-N,-6O}$ may also influence BMP-2 binding and bioactivity. Thus, while further analysis is required to understand the nature of the interactions between the different heparin species and BMP-2, overall results indicate that protective effects are not simply based on number of sulfates or overall charge of the GAG molecules and suggest more complex molecular interactions are at play in BMP-2 stabilization in denaturing conditions.

CONCLUSIONS

These studies build upon what is currently known about BMP-2 interactions with heparin and establish a role of heparin sulfation level in maintenance of BMP-2 bioactivity. Native heparin maintained a significant portion of BMP-2 bioactivity when exposed to thermal stress and, particularly at longer times, the effect appeared to be heparin concentration dependent. Although all three had the same molecular charge as determined by zeta potential measurements, desulfated heparin derivatives Hep^{-N} and $Hep^{-N,-6O}$ were not as effective as native heparin in maintaining BMP-2 bioactivity. These results indicate that overall GAG charge has some effect in maintaining BMP-2 bioactivity, but the backbone structure of the heparin derivatives may also determine the affinity and extent of protective effects they can confer to BMP-2 in solution. Taken together, of the derivatives synthesized, Hep^{-N} has shown the best qualities in terms of retaining high overall sulfation and maintaining BMP-2 bioactivity during heat treatment. Such findings suggest that proper selection of desulfated heparin species, such as Hep^{-N}, that exhibit low anticoagulant activity, improve the half-life of BMP-2 in solution and in delivery systems, and exhibit affinity to BMP-2 will enable the delivery of lower doses of BMP-2, resulting in more effective and cost efficient therapeutics.

MATERIALS AND METHODS

Heparin Desulfation. For preparation of Hep^{-N} and $Hep^{-N,-6O}$, heparin sodium salt from porcine intestinal mucosa (Sigma-Aldrich) was first desalted by reconstituting at ~10 mg/mL in distilled, deionized water (ddH_2O) and passing the solution through Dowex 50WX4 resin (mesh size 100−200, Sigma-Aldrich) in a column (460 mm effective length, 10 mm internal diameter, Ace Glass) with a sealed fritted disc (70−100 μm). After washing the column with additional ddH_2O, 2 mL aliquots of pyridine were added to the desalted heparin until the pH of the solution increased to ~6. Excess water and pyridine was removed on a rotary evaporator (Buchi). The solution of heparin pyridinium salt was flash-frozen in liquid nitrogen, and lyophilized to a powder.

For Hep^{-N} production, heparin pyridinium salt was dissolved at 1 mg/mL in a 90% DMSO/10% ddH_2O (v/v) solution[15,25] and mixed at 50 °C for 2 h. The solution was then cooled on ice and precipitated by an equal volume of 95% ethanol (VWR) saturated with sodium acetate (VWR). The precipitate was centrifuged and supernatant decanted. The precipitate was washed once more with ethanol prior to dissolving in distilled water (dH_2O). The solution was dialyzed for 3 days with daily exchanges of dH_2O and then lyophilized.

For $Hep^{-N,-6O}$ synthesis, a solution of 10 mg/mL of heparin pyridine was made in 90% *N*-methylpyrrolidone (NMP, Acros Organics)/10% ddH2O (v/v).[26] The solution was mixed at 90 °C for 48 h. The solution was then cooled on ice and precipitated with 95% ethanol saturated with sodium acetate. The precipitate was stirred for an additional 2 h on ice and then centrifuged to remove excess ethanol and H_2O from heparin. The resulting powder was dissolved in dH_2O and dialyzed for 3 days prior to lyophilization.

Nonselective desulfation was carried out with a protocol adapted from Schubert and Kantor.[27] Heparin sodium salt was stirred at 5.0 mg/mL in methanol (VWR) containing 0.5% v/v acetyl chloride (Acros Organics). The dispersion was centrifuged and acidic methanol was replaced on days 1, 3, and 6 to produce a methyl ester of heparin. The product was then dissolved in 20 mL dH_2O per gram of heparin before precipitation in an excess of 95% ethanol while on ice. The methyl ester of heparin was centrifuged and washed in ethanol twice. In the last wash, the methyl ester was precipitated with ethyl ether (Fisher) and vacuum-dried at <5 mmHg.

The methyl ester of desulfated heparin was demethylated at 25 mg/mL in 0.1 M potassium hydroxide (KOH) for 24 h to produce Hep^{-}. The Hep^{-} product was then neutralized in 4 mL of 100 mg/mL potassium acetate (Fisher) in 10% v/v acetic acid (VWR) per gram of starting product, and precipitated in an excess of ethanol on ice. Hep^{-} was washed in ethanol and ethyl ether, vacuum-dried, and stored at −20 °C until use.

Material Characterization. *Relative Sulfation Degree.* Removal of sulfate groups in all desulfation protocols was confirmed by dimethylmethylene blue (DMMB) assay for

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sulfated GAGs.[28] The absorbance of DMMB at 520 nm (SpectraMax M2e, Molecular Devices, Sunnyvale, CA) was measured for increasing concentrations of heparin derivatives. The absorbance values for 4.5 μg/mL heparin derivatives were normalized to the absorbance value of native heparin at the same concentration to compute relative sulfation levels.

Molecular Weight. The molecular weights of heparin derivatives were approximated by size exclusion chromatography. Samples were dissolved in an aqueous buffer of 150 mM magnesium sulfate (Macron Fine Chemicals) and 10 mM Tris base (Sigma-Aldrich) in ddH_2O. The aqueous buffer was also used as the mobile phase in the size exclusion chromatography instrument equipped with a refractive index detector (Shimadzu). 100 μL samples were injected, and the separation was carried out on a Tosoh TSK Gel G4000PWXL column (Tosoh Bioscience) at a flow rate of 0.5 mL/min ($n = 3$). The column was calibrated with polyethylene glycol (PEG) standards in the range of M_p 14 000−73 500 (Waters).

NMR Analysis. Proton nuclear magnetic resonance (^{1}H NMR) was utilized to determine the shifts observed after each selective desulfation process. Ten mg/mL heparin and its desulfated derivatives were dissolved in deuterated water and ^{1}H NMR was measured on a Bruker Avance III 400 spectrometer at 400 MHz. For structural assignments, ^{1}H-2D NMR experiments were conducted and signals were assigned based on spectra recorded by double quantum filtered correlation spectroscopy (DQF-COSY) and total correlation spectroscopy (TOCSY). Each sample was measured under water suppression. The resulting spectra were analyzed with ACD NMR Processor software version 12.

Zeta Potential Measurements. Heparin and its desulfated counterparts were desalted with Dowex resin as described above. One mg/mL of each polymeric species was dissolved in water and pH was adjusted with either 0.02 M NaOH (to reach pH 7.4) or 2 M HCl (to reach acidic conditions to estimate isoelectric point) solution. Zeta potential was measured with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a dip cell. Each measurement was performed in triplicate.

C2C12 Cell Culture. Recombinant human BMP-2 expressed in *E. coli* (Peprotech, 26 kDa) was dissolved at 100 μg/mL in sterile ddH_2O and single-use aliquots were frozen until use. Solutions of 1 mg/mL heparin and desulfated heparin derivatives were made in PBS, sterile-filtered, and aliquots were frozen until use. Low adsorption microcentrifuge tubes (Eppendorf) were used for storage of BMP-2 and heparin derivatives.

For the cell-based assay, 2×10^4 mouse myoblast subclone C2C12 (ATCC) cells between passages 4−6 were plated into each well of a 96-well plate in growth medium consisting of 4.5 g/mL glucose Dulbecco's Modified Eagle Medium (DMEM, Cellgro), 10% v/v fetal bovine serum (FBS, Atlanta Biologics), 1% v/v 10 000 IU penicillin/10 000 μg/mL streptomycin (Mediatech), and 1% v/v 200 mM L-glutamine (Cellgro). Cells were allowed to attach for 8 h, at which point, cells were washed with PBS containing 1 g/L $CaCl_2/MgCl_2$ and switched to the bioactivity assay medium. BMP-2 concentrations were tested at 0−600 ng/mL; all bioactivity studies presented here used 500 ng/mL, or 150 ng/well, BMP-2. Heparin was assumed to have a molecular weight of 18 kDa and molar excess of 10 and 100 were used in the studies. Due to the polydisperse nature of the modified heparin species, equivalent mass concentrations of heparin derivatives were used in the studies.

For samples consisting of heparin or heparin derivatives, BMP-2 and heparin were pipetted vigorously before being allowed to incubate at room temperature for 10 min to allow interaction between the moieties. Samples for heat treatment were then placed in a 65 °C heated water bath for 2, 5, 10, or 15 min. Samples were cooled on ice and basal low serum medium consisting of 4.5 g/mL glucose DMEM with 1% v/v FBS was then added to the mixture and transferred to cells. Negative controls consisted of wells free of BMP-2 and included the low serum medium or low serum medium supplemented with the same concentrations of heparin or heparin derivatives used in the experimental wells.

After treatment, the C2C12 cells were cultured in the 96-well plate for an additional 72 h, at which point the wells were aspirated, washed twice with PBS, and incubated with ddH_2O to lyse cells. The plates of cells were frozen, thawed, and mechanically scraped to retrieve cell lysate. Cell lysates were sonicated for 20 min and the freeze−thaw−sonicate cycle was repeated once more, whereafter the supernatant was collected. Cell lysates were spun down and the supernatants were collected into separate microcentrifuge tubes.

Alkaline Phosphatase (ALP) Activity and DNA Content Assays. ALP activity was assayed using the *p*-nitrophenol phosphate substrate and a serial dilution of *p*-nitrophenol standards (0−1000 μM). All samples were assayed in duplicate. Briefly, 50 μL of samples and standards were incubated with 50 μL of 1.5 M 2-amino-2-methyl-1-propanol (Sigma-Aldrich) solution at pH 10.25. Then, 100 μL of a freshly made 1:1 mixture of 20 mM *p*-nitrophenol phosphate disodium salt hexahydrate and 10 mM magnesium chloride was added to each well. The assay plate was sealed and incubated at 37 °C until a colorimetric change was observed, at which point the reaction was terminated with the addition of 100 μL of 1 M NaOH to each well. The plate was read at 405 nm and the assay time was used to normalize the nitrophenol concentration.

In addition, the concentration of double-stranded DNA was assayed by the Quant-iT dsDNA PicoGreen (Invitrogen) assay. Samples and λ DNA standards (0−4 μg/mL) were incubated in PicoGreen reagent and the fluorescence output (ex 485 nm, em 528 nm) was measured on a plate reader. All samples were assayed in duplicate. The ALP activity (nmol of *p*-nitrophenol/mL/min) of each sample was normalized to its dsDNA concentration (μg/mL) and then normalized to the activity level of the positive control of untreated BMP-2 administered to C2C12.

Statistical Analysis. DMMB, M_n, polydispersity (PDI), and BMP-2 bioactivity data are presented as mean ± standard deviation. One- and two-way analyses of variance (ANOVA) were used to identify significant differences or interactions. Tukey's *post hoc* multiple comparison test with a significance value set at $p < 0.05$ indicated significant differences between individual samples. Statistical analysis was performed with Minitab (v 15.1).

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Figures 1−4 contain DQF-COSY and TOCSY spectra obtained from heparin derivatives. This material is available at free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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