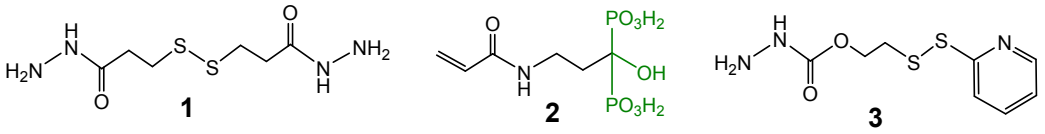
Supporting Information

Control of Growth Factor Binding and Release in Bisphosphonate Functionalized Hydrogels Guides Rapid Differentiation of Precursor Cells *In Vitro*

Sujit Kootala,[†] Yu Zhang,[†] Sara Ghalib,[†] Vladimir Tolmachev,[‡] Jöns Hilborn,[†] Dmitri A. Ossipov*,[†]

General. Linkers used in preparation of modified hyaluronan (HA) derivatives were synthesized according to stated literature procedures. Their structures are given in Scheme S1. Particularly, 3,3'-dithiobis(propionic hydrazide) **1**[1] and 2-(2-pyridinyldithio)ethyl hydrazinecarboxylate **3**[2] were used to attach thiol- and 2-dithiopyridyl-terminated side chains to HA backbone respectively. 3-(Acrylamido-1-hydroxypropane-1,1-diyl) bis(phosphonic acid) **2**[3] was used to attach bisphosphonate ligands to the thiol groups of HA-SH derivative.



Scheme S1. Structures of modifying linkers used in the study.

Synthesis of 3-(acrylamido-1-hydroxypropane-1,1-diyl) bis(phosphonic acid) (BP-acrylamide). Pamidronate hydrochloride (543 mg, 2 mmol) was dissolved in 20 mL of 2 wt. % NaOH. The solution was cooled to 0°C and acryloyl chloride (0.64 mL, 8 mmol) was added into 4 portions (162 µL for each portion) over 45 min while the reaction mixture was kept at 0°C by an external ice-bath cooling. After third addition of acryloyl chloride, the pH was adjusted with 2 wt. % NaOH from 2 to 10. After the addition, the mixture was stirred for 1.5 h with the temperature raising to room temperature. The resulting mixture was extracted with ethyl acetate (2×). The aqueous phase was evaporated and the residue was triturated with methanol. Insoluble in methanol crystals were collected by filtration and dried under vacuo to give 468 mg (81% yield) of BP-acrylamide as a white solid. ^{1}H-NMR (D_2O): 6.21 and 6.12 (2H, dd and d, CH_2=, J = 16.1 Hz, J = 9.9 Hz), 5.69 (1H, d, –CH=, J = 10.3 Hz), 3.53 (2H, t, –NHC$\underline{H_2}$–, J = 7.7 Hz), 2.20 – 2.09 (2H, m, –C$\underline{H_2}$C(OH)(PO_3H_2)$_2$). ^{31}P-NMR: 18.6.

Synthesis of 2-dithiopyridyl HA (HA-SSPy). HA (MW 150000 Da) (400 mg, 1 mmol of disaccharide repeating units) was dissolved in de-ionized water at the concentration of 8 mg/mL. 2-(2-Pyridinyldithio)ethyl hydrazinecarboxylate **3**[2] (49 mg, 0.2 mmol) was dissolved in 4 mL of methanol and the solution was added to the solution of HA. *N*-hydroxybenzotriazole (153 mg, 1 mmol) in 5 mL of 1:1 (v/v) mixture of acetonitrile-water was also added to the HA solution under stirring. The pH of the resultant solution was adjusted to 4.8, after which EDC (48 mg, 0.25 mmol) was finally added to the mixture. The reaction mixture was stirred overnight at room temperature. After the amide coupling, the

mixture was acidified to 3.5 with 1M HCl and transferred to a dialysis tube (M_w cutoff = 3500). After exhaustive dialysis against dilute HCl (pH 3.5) containing 0.1 M NaCl, followed by dialysis against dilute HCl, pH 3.5, the solution was lyophilized to give 385 mg of HA-SSPy. The incorporation of pyridyl group was verified by ^{1}H-NMR. Specifically, the newly appeared peaks at 7.45, 7.92 – 8.06, and 8.45 ppm corresponding to four aromatic protons of the pyridyl group were integrated. This indicated that 6 % of HA disaccharide units were modified.

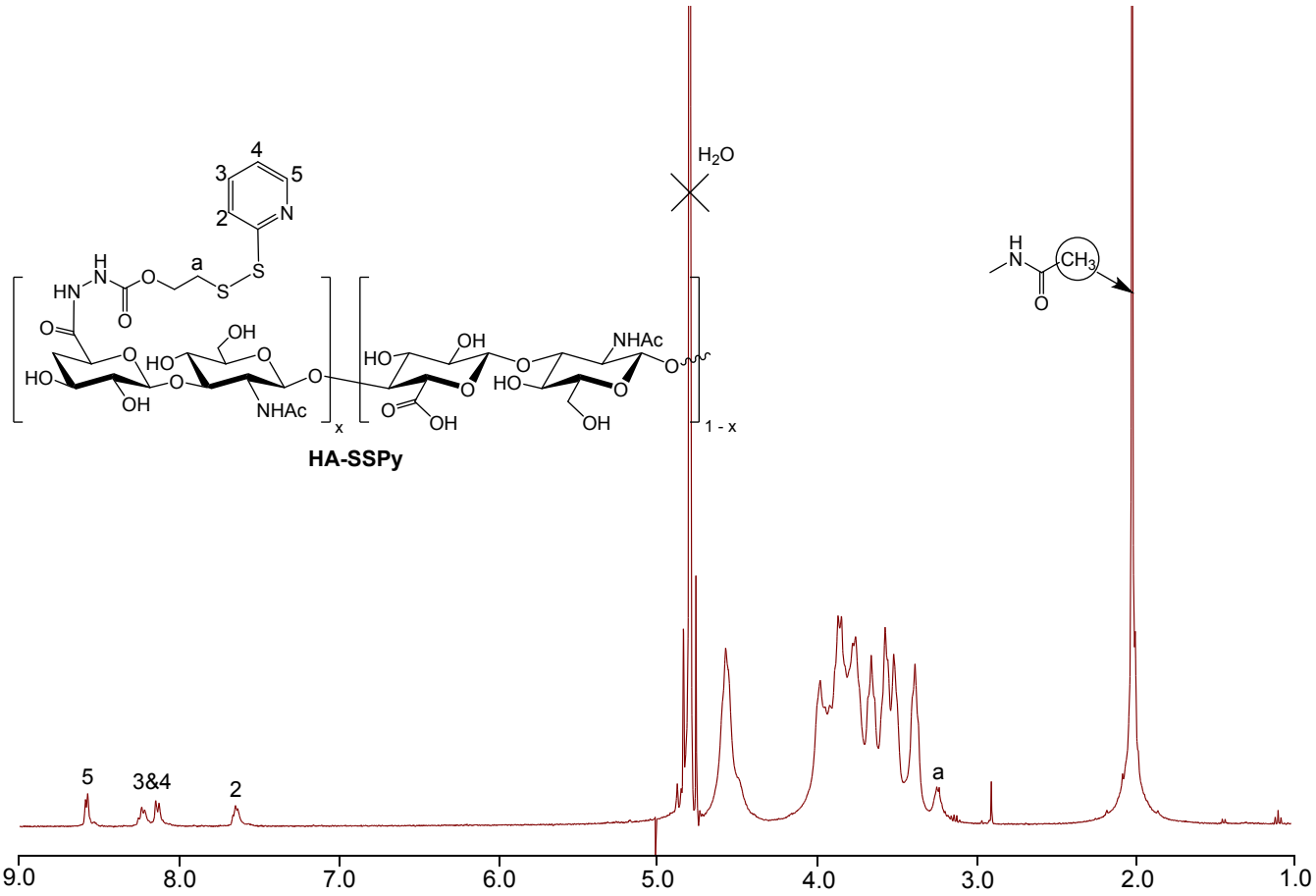


Figure S1. ^{1}H-NMR spectrum of HA-SSPy derivative.

Synthesis of thiol-modified HA (HA-SH). HA (MW 150000 Da) was dissolved in de-ionized water at concentration 8 mg/mL. Dihydrazide linker **1** was added to the HA solution at the reagent/HA disaccharide molar ratios 0.15 : 1. *N*-hydroxybenzotriazole (HOBt) was separately dissolved in a 1:1 (v/v) mixture of acetonitrile-water at concentration 0.2 M and added to the solution of HA. Molar ratio of HOBt to HA disaccharide was 1 : 1. The pH of the resultant solution was adjusted to 4.7 after which the coupling reaction was initiated by addition of solid EDC (0.15 molar equivalents per HA disaccharide units) to the reaction mixture. The mixture was stirred overnight and then basified to 8.5 with 1M NaOH. DTT was added to the solution. 10-fold molar excess of DTT relative to the estimated amount of disulfide linkages in the HA derivative was used to ensure the cleavage of disulfide bond by the reagent. The mixture was stirred overnight, after which the solution was transferred to a dialysis tube (M_w cutoff = 3500). After exhaustive dialysis against dilute HCl (pH 3.5) containing 0.1 M NaCl, followed by dialysis against dilute HCl, pH 3.5 two times. After lyophilization of the dialyzed solution, it was obtained 369 mg of the thiol-modified HA (HA-SH) (92 % yield for the last step). The degree of incorporation of thiol (6.2 %) groups in HA-SH was verified by comparison of integration of the –

CH_2CH_2SH side chain peaks at 2.58 and 2.73 ppm with the acetamido moiety of the *N*-acetyl-D-glucosamine residue of HA.

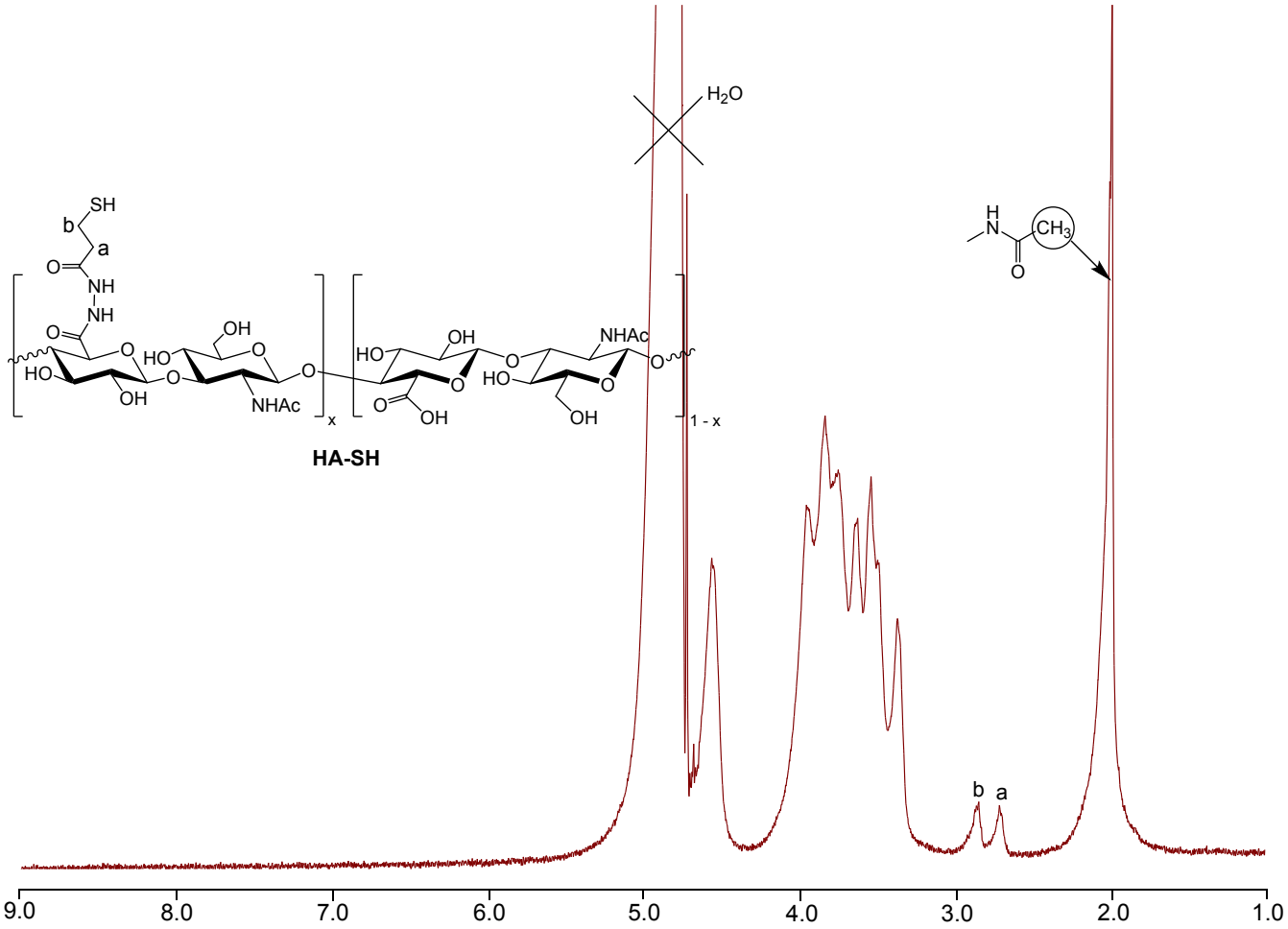


Figure S2. ^{1}H-NMR spectrum of HA-SH derivative.

Synthesis of bisphosphonate-modified HA (HA-BP). HA-BP derivatives were synthesised from thiolated HA (HA-SH) containing 3 molar % of thiol groups per HA disaccharide unit via thiol-ene photopolymerisation as reported previously.[4] In brief, BP-acrylamide **2** (24 mg, 0.2 mmol) was added to 100 mg of HA-SH in 12 mL degassed distilled water in order to obtain BP-to-thiol molar ratios of 4:1. Subsequently, 2.9 mg of Irgacure® 2959 was added and the mixture was stirred for 10 min under ultraviolet light (36 W UV timer lamp, CNC international BV, Netherlands). Thereafter, the mixture was dialysed against 0.1 M NaCl at pH 3.5 (MW cutoff of 3.5 kDa) and subsequently dialysed (48 h) against distilled water at pH 3.5 twice. The solution was neutralised to pH 7.4 and lyophilised. Yield – 108 mg. The resulting polymers were analysed by ^{1}H NMR and ^{31}P NMR and elemental analysis (colorimetric spectrophotometric method by OEA Labs). Specifically, ^{1}H NMR peaks corresponding to the native HA protons (such as acetamide protons at 1.9 ppm; 2′, 3′, 4′, 5′ and 6′-protons of the HA disaccharide unit between 3.2–4.0 ppm as well as anomeric 1′-protons at 4.4 ppm) were compared with peaks corresponding to the methylene protons 2 and 3 of the grafted side chains. The peak at 2.2 ppm corresponds to two methylene protons –CH_2C(OH)(PO_3H_2)$_2$ that are adjacent to a bridging carbon of the BP group. Elemental analysis revealed that sulphur and phosphorous constitute 0.2% and 2.2% by mass respectively. Assuming that bisphosphonate monomers can polymerize by addition of a thiyl radical, the

elemental analysis data corresponds to five BP-acrylamide molecules oligomerized from one thiol group.

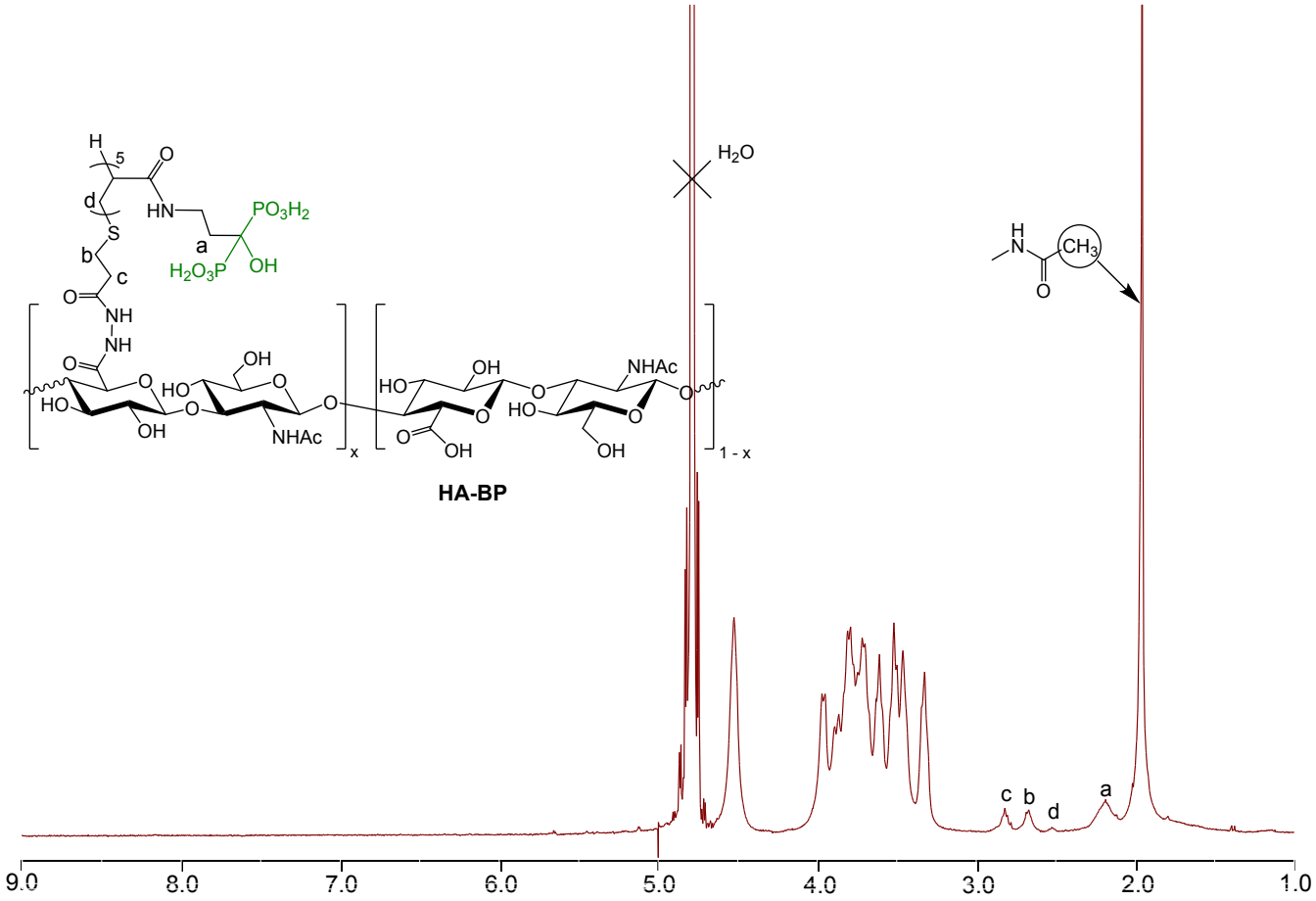


Figure S3. ^{1}H-NMR spectrum of HA-BP derivative.

Synthesis of bisphosphonate and thiol dually modified HA (HA-SH-BP). HA-BP was further derivatized with thiol groups. 105 mg of HA-BP was dissolved in 10.5 mL of de-ionized water. Linker **1** (9.4 mg, 0.039 mmol) was added to the HA solution. *N*-hydroxybenzotriazole (HOBt) (35.4 mg, 0.263 mmol) was separately dissolved in 1.4 mL of a 1:1 (v/v) mixture of acetonitrile-water at and added to the solution of HA. The pH of the resultant solution was adjusted to 4.5 after which the coupling reaction was initiated by addition of solid EDC (9.1 mg, 0.047 mmol) to the reaction mixture. The mixture was stirred overnight. The reaction solution was basified to 8.5 with 1M NaOH and DTT (60.7 mg, 0.3937 mmol) was added to the solution to provide cleavage of disulfide bond of the coupled linker. The mixture was stirred overnight, after which the solution was acidified to 3.5 with 1M HCl and transferred to a dialysis tube (M_w cutoff = 3500). After exhaustive dialysis against dilute HCl (pH 3.5) containing 0.1 M NaCl, followed by dialysis against dilute HCl, pH 3.5 two times, the solution was lyophilized to give HA-SH-BP with 81% yield. Elemental analysis revealed that sulphur and phosphorous constitute 0.7% and 2.4% by mass respectively. The incorporation of free thiol groups was verified by Ellman's assay. It indicated that 6% of the HA disaccharide units were modified with thiol-terminated side chains.

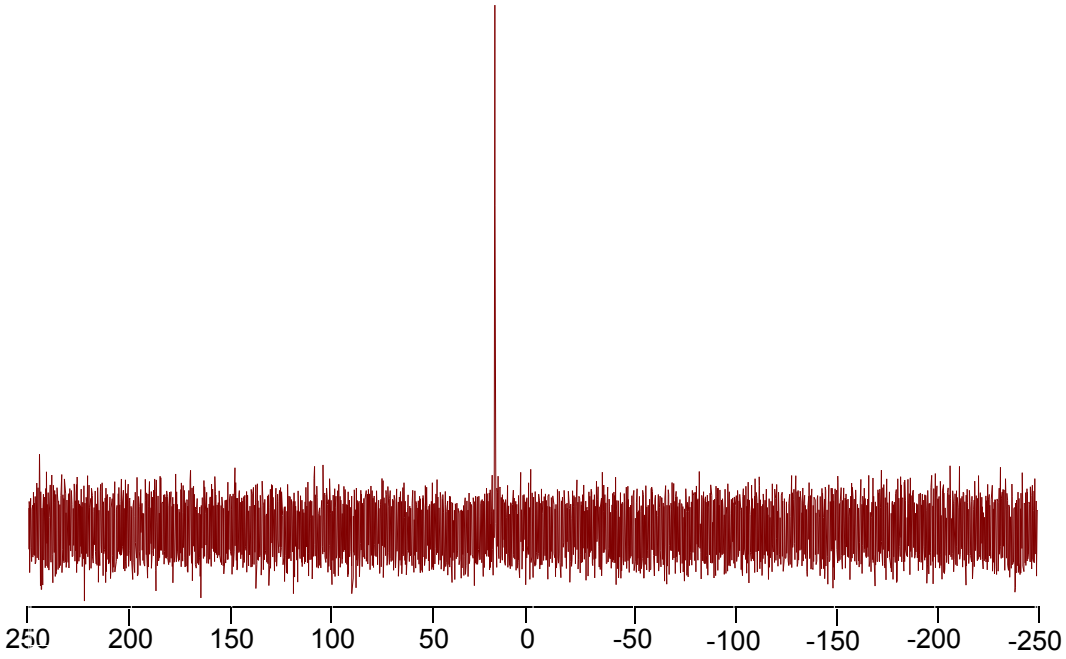


Figure S4. ^{31}P-NMR spectrum of HA-SH-BP derivative.

Preparation of hydrogels. Control hyaluronic acid hydrogels lacking matrix-linked bisphosphonate groups (HA gels) and the hydrogels with matrix-linked bisphosphonate groups (HABP gels) were prepared by disulfide cross-linking reaction. HA derivatized with 2-dithiopiridyl groups (HA-SSPy) containing ~6% of the functional groups was used as a common component for HA and HABP gels. Thiolated HA (HA-SH) and HA derivative dually modified with bisphosphonate and thiol groups (HA-SH-BP) was used to prepare HA and HABP gels respectively. Degree of thiolation was also ~6% for the both HA derivatives. Specifically, thiol-containing HA derivatives and HA-SSPy were dissolved in serum-free cell culture medium at 2% concentration. The obtained solutions were neutralized with 2M NaOH. For fabrication of 2% (w/v) hydrogel of 0.3 mL by volume, 150 μL of the neutralized thiolated HA (either HA-SH or HA-SH-BP) was mixed with 150 μL of the neutralized HA-and the obtained mixture was quickly mixed for 3 seconds and immediately transferred into a syringe mold (a 2 mL syringe with the cut off ending). The hydrogels were set for 24 hours in the molds sealed with Parafilm to prevent the hydrogels drying. After setting, the hydrogels were swollen in PBS for another 24 hours.

Labeling of BMP-2. 10 μg of solid BMP-2 from Peprotech™ (U.K.) was dissolved in 25 μL of PBS by vortexing and sonication for 15 min. The obtained solution was further diluted with 25 μL of PBS resulting in 0.2 μg/μL solution. 18 μL of $Na^{125}I$ (around 37 MBq) and 20 μL of strong oxidizing agent, chloroamine-T were added to BMP-2 solution. After two minutes of labeling reaction, excess of chloroamine-T was reduced with 10 μL of sodium metabisulfite to stop the iodination reaction. The resulting reaction solution (98 μL) was loaded to a NAP-5 size exclusion column. The column was pre-washed with 1% BSA to saturate nonspecific binding sites on the column. Because dead volume of the column was 0.5 mL, 0.4 mL of PBS was additionally added to the column and completely eluted. As expected, no radioactivity was detected in the first 0.5 mL fraction. More PBS (2 mL) was then added to the column to wash out the labeled protein and excess of $Na^{125}I$. The second high molecular weight fraction (1 mL) contained 15.6 MBq of radioactivity. Final low molecular weight fraction (1 mL)

contained 6.6 MBq of radioactivity. After completion of elution, column itself contained 5.2 MBq of radioactivity. This means that some labeled BMP-2 stack in the column. Moreover, the reaction vial after emptying was radioactive (9.4 MBq of radioactivity). This means that the protein also stack in the vial. 30.2 MBq corresponded to all labeled BMP-2, while 36.8 MBq was total amount of radioactivity after the labeling reaction. It was almost equal to the amount of radioactivity in feed (37 MBq). Thus, the efficiency of labeling reaction was 82% and the yield of ^{125}I-labeled BMP-2 was 52%. Because initially 10 μg of BMP-2 was used for the labeling reaction, 10 μg × 0.52 ≈ 5 μg of the labeled protein was recovered from the column. This amount was dissolved in 1000 μL PBS and contained 15.6 MBq of radioactivity.

In vitro release of BMP-2 from hydrogels. BMP-2 from two sources has been used for studying the growth factor release from HA and HABP hydrogels.

In the first round of experiments, BMP-2 from InductOS® (Wyeth, Madison, NJ) was reconstituted in a formulation buffer (0.07% L-glutamic acid, 2.5% glycine, 0.5% sucrose, 0.029% sodium chloride, and 0,01% polysorbate, Sigma-Aldrich Inc., St Louis, MO, US) according to the manufacturer's protocol at 1.5 mg/mL concentration. HA-SSPy was dissolved in PBS at 2.5% concentration, while HA-SH and HA-SH-BP were dissolved in PBS at 2% concentration. First, 1.2 mL of HA-SSPy solution was mixed with 286.8 μL of BMP-2 stock solution and 13.2 μL of ^{125}I-labeled BMP-2. ^{125}I-labeled BMP-2 had 16.7 μg/mL concentration and 13.2 μL of this solution had 77.9 kBq of radioactivity. HABP gels were prepared by mixing 60 μL aliquot of HA-SH-BP solution with 60 μL aliquot solution of HA-SSPy containing non-labeled and labeled growth factors. [BP]/[BMP-2] ratio in HABP hydrogel was 570/1. For preparation of HA gels, HA-SH was used in place of HA-SH-BP. For preparation of hydrogels with [BP]/[BMP-2] ratios of 11.4/1 and 5.7/1, 58.8 μL and 59.4 μL of HA-SH solution was mixed with 1.2 μL and 0.6 μL of HA-SH-BP solution respectively and the combined thiol-modified HA derivatives were subsequently in situ cross-linked with the HA-SSPy solution containing BMP-2. Hydrogel plugs of 120 μL volume were prepared at the bottom of low protein binding Eppendorf tubes and set for 24 hours. Final HA concentration in each gel was 20 mg/mL (2%), BMP-2 concentration was 143.4 μg/mL, and the amount of radioactivity was 10 kBq/sample. After setting, 0.5 mL of PBS was added to the hydrogels. At determined time points, liquid phase was carefully separated from the gels and collected for measuring of radioactivity. Fresh PBS was added to the gels in palace of withdrawn one. Radioactivity of the hydrogels without release media was also measured. The results from both measurements were consistent to each other and the resulting release profiles were plotted basing on data from both liquid and gel samples. Results of these studies are shown in Figure S4. The obtained curves represent the cumulative radioactivity arising from all forms of ^{125}I-labeled BMP-2.

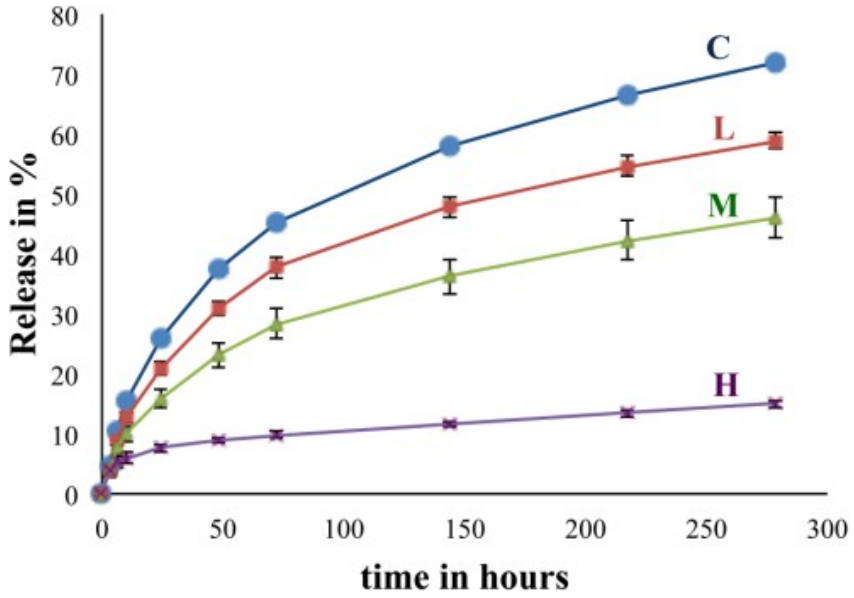


Figure S5. *In vitro* release profile of ^{125}I-labelled BMP-2 (Wyeth™) from 2% (w/v) disulfide cross-linked hyaluronic acid (HA) hydrogels of 120 μL by volume is controlled by different content of bisphosphonate linked HA (HA-SH-BP). Designations H, M, L, and C correspond to 1.2 mg, 0.024 mg, 0.012 mg, and 0 mg of HA-SH-BP loading respectively.

A maximum of 15% BMP-2 (Wyeth™) was released after 279 hours from the hydrogel made from HA-SH-BP and HA-SSPy as a cross-linker (H-curve in Figure S4). In this formulation, the molar ratio between the matrix-linked BP groups and protein molecules was around 570:1. This ratio can be easily altered by using a mixture of thiolated HA-SH-BP and HA-SH and varying the mass ratio between the two components. When the hydrogel was initially prepared using the equal masses of HA-SH-BP and HA-SH derivatives, the obtained release curve did not change from the one corresponding to the H-hydrogel in Figure S4 (data not shown). Only by lowering the [BP]/[BMP-2] molar ratios to 11.4:1, we observed lower retention (the corresponding M-curve was disposed between the H-curve and a curve for the control HA gel, i.e. C-curve in Figure S4). Decreasing this ratio to 5.7 : 1 allowed us to further decrease retention of BMP-2 (L-curve in Figure S4).

In the second round of experiments, 10 μg of recombinant human BMP-2 (*E.coli* derived) from Peprotech™ was dissolved in 10 μL of sterile water and was then diluted with 90 μL of sterile water to give 0.1 mg/mL stock solution. HA-SSPy was dissolved in PBS at 2.07% concentration, while HA-SH and HA-SH-BP were dissolved in PBS at 2% concentration. HA-BP-SH solutions at 1.96 mg/mL and 0.84 mg/mL concentrations were also prepared by dilution of 2% solution with PBS. First, 725.4 μL of HA-SSPy solution was mixed with 15 μL of BMP-2 stock solution and 9.6 μL of ^{125}I-labeled BMP-2. ^{125}I-labeled BMP-2 had 5 μg/mL concentration and 9.6 μL of this solution had 149.8 kBq of radioactivity. HABP gels were prepared by mixing 50 μL aliquot of 2% HA-SH-BP solution with 50 μL aliquot solution of HA-SSPy containing non-labeled and labeled growth factors. [BP]/[BMP-2] ratio in HABP hydrogel was 7140/1. For preparation of HA gels, HA-SH was used in place of HA-SH-BP. For preparation of hydrogels with [BP]/[BMP-2] ratios of 14/1 and 6/1, 49 μL of HA-SH solution was mixed with 1 μL of 1.96 mg/mL and 0.84 mg/mL HA-SH-BP solution respectively. The combined thiol-modified HA derivatives were subsequently in situ cross-linked with the HA-SSPy solution containing BMP-2. Hydrogel plugs of 100 μL volume were prepared at the bottom of low protein binding Eppendorf tubes and set for 24 hours. Final HA concentration in each gel was 20 mg/mL (2%), BMP-2 concentration was 0.1 μg/mL, and the amount of radioactivity was 10 kBq/sample. After setting,

0.5 mL of PBS was added to the hydrogels. At determined time points, liquid phase was carefully separated from the gels and collected for measuring of radioactivity. Fresh PBS was added to the gels in palace of withdrawn one. The results of these studies are shown in Figure 3.

Cell viability study. Myoblast C2C12 cells were maintained in DMEM, 10% FCS and 100 U/mL P/S. All Cells were maintained at 37°C/5% CO_2 and used between passage 5 & 6. All solid HA derivatives were UV-sterilized in a sterile lab-hood for 30 minutes prior to dissolution in serum-free cell culture medium at 1% v/w concentration. The solutions were neutralized with sterile 3 M NaOH. Around 1.5 million C2C12 cells were spun down into a pellet and then re-dispersed in 2.6 mL of neutralized HA-SSPy solution providing a solution containing 600000 cells/mL. 12 µL of 0.1 mg/mL rhBMP-2 from PeproTech (U.K.) was added to 600 µL of 1% HA-SH. Another 12 µL of 0.1 mg/mL rhBMP-2 from PeproTech (U.K.) was added to 600 µL of 1% HA-SH-BP. HA gels without BMP-2 were prepared by mixing 50 µL of 1% HA-SH with 50 µL of 1% HA-SSPy + cells suspension in 96 well plate affording 30000 cells/hydrogel. HA gels with BMP-2 were prepared by mixing 50 µL of 1% HA-SH + BMP-2 solution with 50 µL of 1% HA-SSPy + cells suspension in 96 well plate also providing 30000 cells/hydrogel. Analogous HABP gels with and without BMP-2 were prepared by using HA-SH-BP instead of HA-SH. Mixing of the component was performed by quick pipetting and the obtained mixtures were set for 30 – 40 minutes in incubator at 37°C/5% CO_2 without the addition of medium at this stage. After 40 minutes of setting in incubator, 500 µL cell culture medium was added on top of the formed hydrogels. Final concentration of BMP-2 in the respective hydrogels was 1 µg/mL. At day 1, 3, 5 and 7, the medium surrounding the hydrogels was removed and 500 µL fresh medium containing DMEM, 1% FBS and 1% alamarBlue® was added. The gels were placed in the incubator at 37°C/5% CO_2 for 4 h. At 4h, 100 µL of the medium was removed and fluorescence was determined by a spectrophotometer (Tecan plate reader) at 590 nm. All samples were normalized to the controls and statistical significance was analyzed with a t-test and p-value > 0.05.

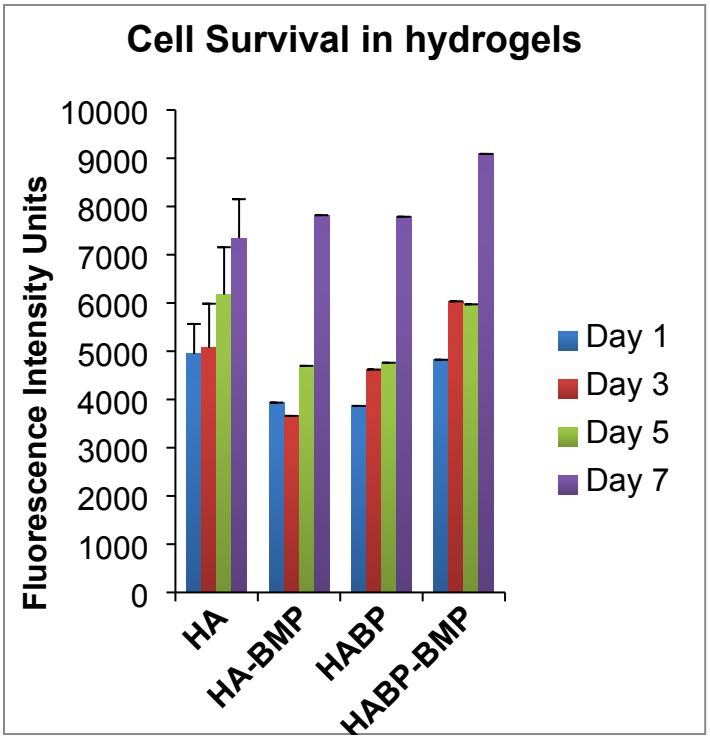


Figure S6. Cell survival of C2C12 cells after culturing in HA and HABP hydrogels either containing or not containing BMP-2.

Cell tracking in hydrogels. Myoblast C2C12 cells were maintained in DMEM, 10% FCS and 100 U/mL P/S. All cells were maintained at 37°C/5% CO_2 and used between passage 5 and 6. Cell culture and hydrogel entrapment was performed as described above. At day 5, representative gels were washed with PBS and stained with Cell Tracker™ Green CMFDA dye (Life Technologies, Sweden) following manufacturers instructions for 30 minutes at 37°C. Subsequently, the gels were immersed in 500 μL PBS and fluorescent images were acquired on the Nikon Eclipse 2000 series microscope using green excitation/emission spectra (492/517 nm maxima).

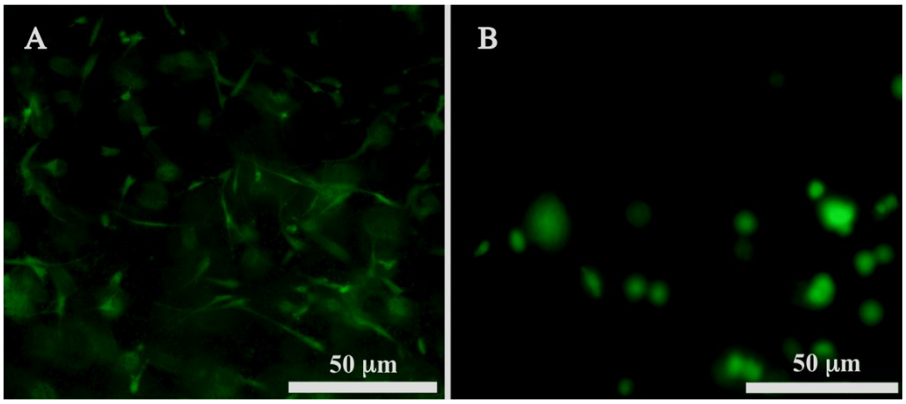


Figure S7. Images of myoblast C2C12 cells entrapped in HABP (A) and HA (B) hydrogels with BMP-2 (1 μg/mL), stained with Cell Tracker™ Green CMFDA dye on day 5.

ALP assay. Approximately 30000 C2C12 cells at passage 5 were encapsulated in the hydrogels of 150 μL volume containing BMP-2 at 1 μg/mL concentration. The encapsulation protocol was similar to that used in cell viability study. Medium was changed after 1 hour and then subsequently each alternate day until day 5. At day 5, cells were washed with PBS and lysed with 200 μL of lysis buffer (20 mM Tris, 1 mM MgCl, 0.1mM ZnCl, 0.1% Triton-X 100) and immediately frozen at -20°C for later analysis. The lysates were freeze/thawed three times at 37°C and a 25 μL aliquot was taken from each well and combined with 50 μL of alkaline phosphatase substrate for 20 minutes. The reaction was stopped by addition of 25 μL of 3M NaOH, and the conversion of substrate p-nitrophenylphosphate (4-NP) into free 4-nitrophenol was determined by spectrophotometer (Tecan plate reader) at 405nm. A 50 μL aliquot was taken from each well and combined with an equal volume of micro-BCA working solution, per the manufacturer's instructions, incubated at 37°C for 1 hour, and the absorbance was read at 562 nm on a Tecan plate reader. ALP and BCA readings were compared to a standard curve of 4-NP and BSA respectively, and the calculated alkaline phosphatase activity was normalized to the protein content for each well. Experiments were replicated twice, with 3 samples per group in each experiment.

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