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COMMUNICATION

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

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An in situ cross-linkable hyaluronan hydrogel functionalized with bisphosphonate (BP) groups allows tunable release of bone morphogenetic protein-2 (BMP-2) determined by the amount of BP groups. High affinity of matrix-anchored BP groups towards BMP-2 permits guided differentiation of entrapped progenitor cells in 3-D cultures.

Growth factors are important signalling proteins that stimulate cell growth, differentiation, survival, inflammation, and tissue repair.[1,2] Due to their short half-life and gradual diffusion into extracellular spaces, growth factors usually act swiftly and locally. Moreover, binding ability of a growth factor to domains on extracellular matrices (ECM), degradation of ECM, and overall concentration of the growth factor have pronounced effects on the actual response of a target cell.

Scaffold-assisted repair or replacement of damaged tissues often relies on the ability of a biomaterial to control the delivery of growth factors.[3] However, retention of their bioactivity from the time of encapsulation in the biomaterial until the time of interaction with cells is a major challenge.[4] *In vivo*, growth factor activity is controlled spatiotemporally by non-covalent interactions with sulphated proteoglycans or their glycosaminoglycan (GAG) subunits. Biomimetic approaches to growth factor delivery contemplate biomaterials as a growth factor depot in which stable and local presentation of the growth factor is realized by covalent linking or through specific physical interactions.[5]

A synthetic approach to current ECM mimics involves retro-synthetic deconstruction of ECM macromolecules into functional subunits (epitopes) and their re-assembly using orthogonal combination of site-specific chemical reactions and/or affinity interactions. For example, site-specific immobilization of growth factors to fibrin[6-8] or poly(ethylene glycol)[9] involving a transglutaminase FXIIIa-mediated enzymatic reaction permitted unprecedented control over growth factor release determined exclusively by the proteolytic activity of invading cells. Alternatively, specific affinity interactions, which are engineered between the proteins and different matrices,[10] tuned the rate of release while maintaining bioactivity. Synthetically, these systems include (*i*) chemical incorporation of a pair of complementary binding ligands to both a polymeric matrix and a therapeutic protein,[11,12] (*ii*) modification of the therapeutic protein exclusively,[13] and (*iii*) binding of parent growth factor to the matrix with the immobilized affinity ligand.[14,15] However, all the delivery systems mentioned above, require time and labour-intensive modifications of either the growth factors (via genetic engineering, that might also compromise their activity) or matrices or both with expensive polypeptides. Heparin[16] and heparin-mimetic[17] systems represent the mostly exploited ligands that are naturally available, relatively easy to incorporate and bind native growth factors.[18] However, some growth factors are non-binding or have variable binding to heparin, while others require specific sequences that are difficult to produce easily.[19,20] Clearly, structurally simple affinity ligands that can provide enhanced retention of growth factors and can be easily incorporated *in situ* into a hydrogel-based delivery system that would allow preservation of their activity are highly desirable.

Previously, we reported the effect of bisphosphonate (BP) ligands attached to hyaluronic acid (HA) hydrogels to retain bone morphogenetic protein-2 (BMP-2) in the hydrazone cross-linked HABP matrix.[21] Hyaluronic acid (HA) was used as hydrogel-forming material due to its favourable characteristics as a biomaterial.[22] Earlier studies demonstrated the suitability of HA to delivery of bone morphogenetic protein-2 (BMP-2) to trigger ectopic bone formation,[23] induce bone augmentation,[24] and for functional closure of bone defects.[25] Bisphosphonates (BPs) are well known anti-osteoporotic drugs that were also applied in bone regeneration by non-covalent incorporation into different scaffolds.[26] In the present work we hypothesized that binding properties of BPs can be successfully utilized in the fabrication of 3D cell cultures mimicking native features of ECM such as sequestration and storage of growth factors followed by their spatiotemporal release. Apart of using different (disulfide) cross-linking chemistry, which was more

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suitable for 3D cells encapsulation, we varied the amount of BP groups in the matrix and demonstrated that in this way, one can tune binding of BMP-2 to the matrix which subsequently results in changing the rate of the growth factor release from the matrix. In this work, we also demonstrated for the first time that simultaneous *in situ* incorporation of BMP-2 and C2C12 myogenic progenitor cells in HABP matrix can provide a stable osteogenic microenvironment for the cells (Figure 1). Such *in situ* cross-linkable hydrogel system thus promotes more efficient guiding of cells in 3D.

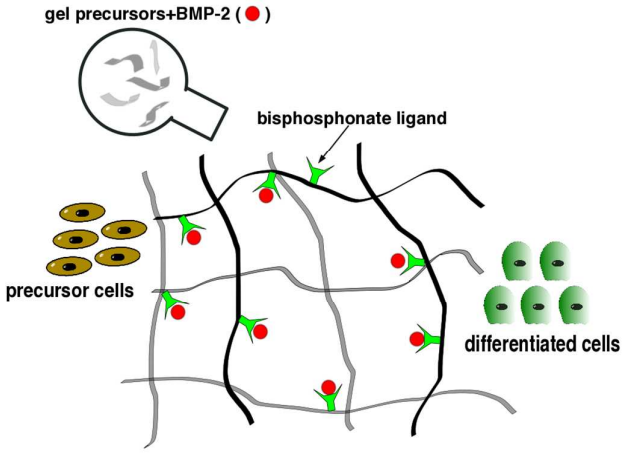
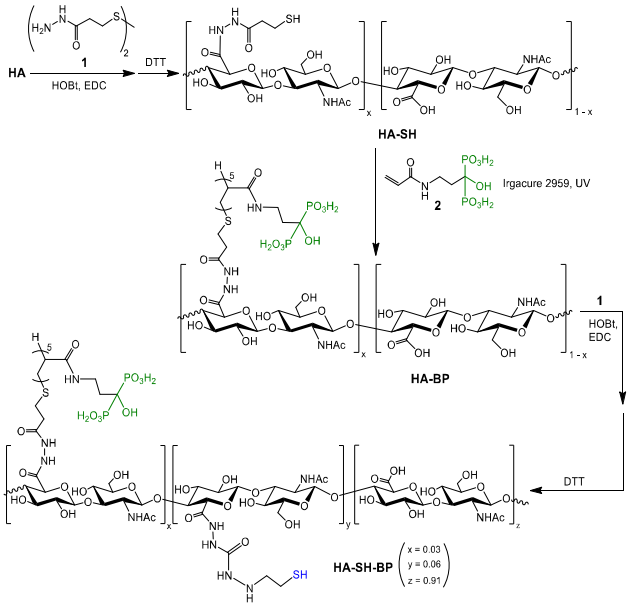


Figure 1. In situ encapsulation of myotube forming cells and BMP-2 in hyaluronic acid hydrogel to induce 3D differentiation of the cells through the controlled retention and release of the growth factor by the matrix-linked bisphosphonate (BP) ligands.

Entrapment of cells and BMP-2 in BP-functionalized hydrogels, should be implemented *in situ*, i.e. cross-linking should be chemo-selective to ensure cytocompatibility. We have chosen a thiol-disulfide exchange reaction to cross-link HA chains because it proved to be the most cytocompatible for several different cell types *in vitro*.[27] To ensure quick setting time for the hydrogel, we prepared two HA derivatives, HA-SH-BP and HA-SSPy. The HA backbone in these derivatives was functionalized with thiol (-SH) and 2-dithiopyridyl (-SSPy) groups respectively to form a mixing-triggered disulphide hydrogel (Figure 1). Preparation of HA-SSPy has been reported by us previously[28] and the details of its synthesis can be found in Supporting Information. Synthesis of HA derivative dually modified with thiol groups and BP ligands was realized in three steps (Scheme 1). First, 3% of HA disaccharide units were thiolated with a linker **1** according to literature procedure to afford HA-SH.[29] BP groups were subsequently attached to sulfhydryl groups of HA-SH by photochemically induced thiol-ene reaction.[28] On average, five BP groups (n = 5) were linked per sulfhydryl group according to NMR and elemental analysis. Finally, the obtained HA-BP derivative was again thiolated during the course of water-soluble carbodiimide-mediated coupling of **1** followed by one-pot treatment with reducing agent. This afforded bisphosphonated hyaluronan with 6% *in situ* cross-linkable thiol groups.

After purification, the structure of HA-SH-BP was confirmed by NMR analysis. The ^{1}H-NMR spectrum of HA-SH-BP showed signals in the range between 2.9 and 2.5 ppm (Figure 2) corresponding to the –COC***H*****$_2$**–C***H*****$_2$**S[C***H*****$_2$**–CHCO]$_n$– sequence of the attached chains of n repeating units (n ≈ 5) each carrying BP moiety. Characteristic methylene protons adjacent to the bridging carbon of bisphosphonate residue (–C***H*****$_2$**C(OH)(PO$_3$H$_2$)$_2$) were observed at 2.2 ppm. Comparison of integration of these methylene protons with acetamido moiety of the *N*-acetyl-D-glucosamine allowed us to approximately determine the degree of BP modification, which was close to 15% (3% of thiol groups to which 5 BP moieties were linked). Attachment of BP groups was also confirmed by ^{31}P-NMR, which showed a characteristic peak at 18.9 ppm (Figure S4).

For evaluation, a control thiolated hyaluronan lacking BP groups (HA-SH) was also synthesized and the amount of thiol groups in both derivatives was kept the same (6%). Mixing of new HA-SH-BP derivative or its HA-SH analogue with 2-dithiopyridyl derivatized HA (HA-SSPy) afforded hydrogels with disulfide cross-links in less than a minute. Equal volumes of 2%



Scheme 1. Synthesis of chemically "clickable" bisphosphonate-derivatized HA (HA-SH-BP).

(w/v) solutions of each component in serum-free cell culture medium (pH 7) were used to ensure equimolar ratio between the cross-linking functional groups (provided that the same degree of modification around 6% was achieved in all the derivatives). The hydrogels were set overnight and their mechanical properties were evaluated by rheology. We observed higher elastic moduli for HABP gels (G′ = 1256 ± 72 Pa) than for HA gels (G′ = 990 ± 82 Pa). This can be explained by the presence of Ca^{2+} ions in cell culture medium. As a result, additionally to disulfide chemical cross-linking of HA chains, BP•Ca^{2+} coordination bonds can be formed in HABP gels which thus counterbalance electrostatic repulsion of the BP groups.[30]

Release of BMP-2 was investigated using the ^{125}I labelled protein.[31] Due to the instability of BMP-2 it is equally

important to correlate release of the protein from hydrogel and biological activity of the released protein on cells.[31] We therefore conduct a preliminary screening of two different commercial sources of rhBMP-2, i.e. Wyeth and Peprotech, to ascertain efficiency of labelling and to probe the activity of the

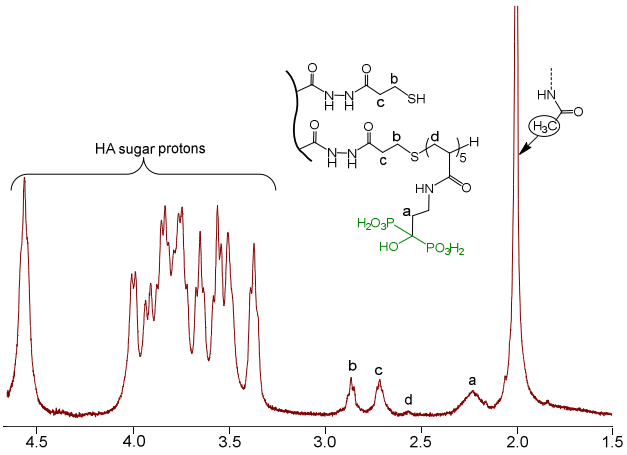


Figure 2. ^{1}H NMR spectrum of HA-SH-BP.

resultant protein-adduct in cells. We were able to label both the BMPs with similar efficiencies. However, we noticed a loss of biological activity of labeled Wyeth™ BMP-2 over time, even though the *in vitro* release profiles were more distinctive for HABP and HA hydrogels (Figure S4) and no loss in the cells proliferation rate was observed (data not shown). BMP-2 from Peprotech™ when labeled, however, did not show significant loss in activity and therefore, we chose to use this protein in our further studies.

Previously, we observed sequestration of Wyeth™ BMP-2 in HABP hydrogels cross-linked through hydrazone chemistry.[21] In this work, almost the same effect was observed for the release of Wyeth™ BMP-2 from the disulphide cross-linked HABP hydrogel (Figure S5). The Peprotech™ BMP-2 was not however sequestered in HABP hydrogel (green curve in Figure 3) as it was observed with Wyeth™ BMP-2. Nevertheless, a distinctive binding affinity of BP groups toward Peprotech™ BMP-2 could still be detected upon varying the amount of these groups in HA matrix. Our injectable hydrogel

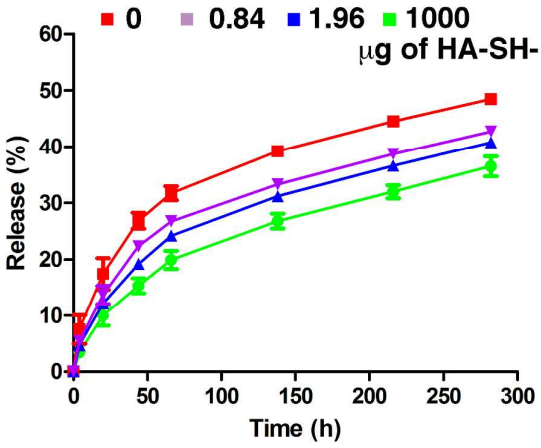


Figure 3. Tunable *In vitro* release profile of ^{125}I-labelled BMP-2 (Peprotech™) (0.1 μg) from 2% (w/v) disulfide cross-linked HA hydrogels of 100 μL by volume and different content of HA-SH-BP. 1000 μg, 1.96 μg, 0.84 μg, and 0 μg of HA-SH-BP loading correspond to green, blue, pink, and red release curves respectively.

system allowed such variation through simple mixing of pre-determined volumes of HA-SH-BP and HA-SH solutions. It allowed loading of equal quantities (0.1 mg/mL) of BMP-2 into hydrogel samples with variable amounts of HA-SH-BP component in situ by addition of the growth factor into HA-SSPy solution followed by addition of the thiolated counterpart. It was noteworthy that the change in retention of Peprotech™ BMP-2 could be achieved in the range of [BP]/[BMP-2] molar ratios between 0 to 10 and corresponding to the amounts of HA-SH-BP varying from 0 to 2 μg (red, pink, and blue curves in Figure 3). These amounts are 1000 times less than the total HA content in the hydrogel. It demonstrates that BP groups have indeed very high affinity to the protein which can be effectively used for tuning the release of the protein from HA hydrogel. The smaller differences between the release curves as well as relative retention of BMP-2 in the control HA hydrogel can be explained by the presence of different forms of iodinated BMP-2 including aggregates or radioactive impurities. This could impair the radioactivity read-out from the release study.

Next we evaluated biological activity of BMP-2 measured as cell differentiation induced by culturing myogenic progenitor cells C2C12 inside the hydrogel materials. C2C12 cells were chosen due to their innate ability to express the early stage bone marker, alkaline phosphatase (ALP), upon stimulation with BMP-2.[18] C2C12 cells were shown as a useful cell line to study bone markers in the presence of BMPs.[32] First we confirmed that the cells survival was close to 100 % upon in situ entrapment and further 3D culturing in the disulphide cross-linked HA and HABP hydrogels (Figure S6). The cells proliferated comparably over 7 days in both types of hydrogels irrespective to the presence or absence of the growth factor. Subsequently, approximately 30000 cells were encapsulated in the hydrogels of 150 μL volume containing Peprotech™ BMP-2 at 1 μg/mL concentration. Expression of alkaline phosphatase (ALP), a major early-stage osteogenic differentiation marker, was measured. A 5-fold increase in ALP expression was detected for the cells cultured 3D in HABP hydrogels as compared to HA hydrogels. It is noteworthy that culturing of the cells in HABP hydrogel without BMP-2 did not result in the cells expressing ALP at all (Figure 4). This

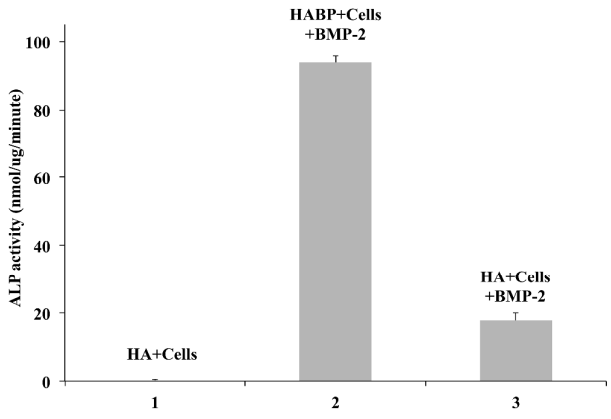


Figure 4. Comparative *in vitro* ALP activity of C2C12 cells after culturing in hydrogels for 5 days.

observation is a direct consequence of the combined effect of matrix-linked BP groups and active BMP-2 on cells. To ascertain the morphology and spreading of the cells after the induction period, the hydrogels were washed and stained with CMFDA green dye. We found that the cells in the HABP gels containing BMP-2 showed spreading at day 5 (Figure S7a) while the cells did not spread in HA gels (Figure S7b). Anti-adhesive properties of HA are very well known. However, attachment of BP groups may provide Ca^{2+} ions mediated interaction of membrane proteins with the BP groups of the matrix. The same effect was observed by us previously for 2D cultures on HABP hydrogels.[21] Taking into account the results obtained from our release study, we can suggest that the cells were exposed to BMP-2 for longer time and at higher concentrations in HABP hydrogel that acted as a pocket-like protective storage and supply for the growth factor in similarity to the native ECM. In addition to this, the products of degradation of HABP hydrogel are expected to have potent anti-osteoclastic activity as was shown by us recently.[33] Contrary, HA hydrogel alone allowed less efficient protection and released BMP-2 too fast to provide local therapeutic doses for the cells during all three days of culturing and induce significant cells differentiation.

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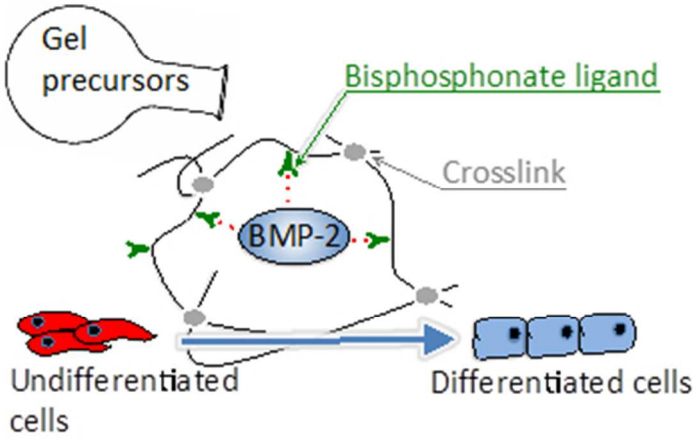

Sequestration and active release of BMP-2 in HA-BP hydrogels to precursor cells aids rapid differentiation to osteoblasts.
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