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In Situ Cross-Linkable High Molecular Weight Hyaluronan−Bisphosphonate Conjugate for Localized Delivery and Cell-Specific Targeting: A Hydrogel Linked Prodrug Approach

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In recent years considerable effort has been given to improve site specific biodistribution and time-controlled delivery of drugs to minimize undesired side effects and to improve the efficacy of the treatment.[1] Most of the polymeric drug delivery systems are designed for intravenous administration on the basis of EPR (enhanced permeability retention) effect[2] and include nanoparticles,[3] dendrimers,[4] liposomes,[5] and polymeric micelles.[6] An injectable hydrogel based delivery system has mainly been employed to deliver large protein based drugs such as growth factors[7] and very recently was used for small molecule drugs such as doxorubicin[8] and cisplatin.[9] The incorporation of small molecule drugs limits the applicability due to their passive diffusion out of highly porous hydrogels. Polymer−drug conjugates, however, having a specific cell targeting ligand could be incorporated into an implantable drug delivery device and may provide precise dose control, high release efficiency, low toxicity, and cell specificity. This provides a platform to overcome disadvantages associated with conventional drug delivery systems by closely mimicking the natural cell targeting mechanism. The localized delivery of such drugs at the surgical resection site could open up new avenues to control tumor recurrence after removal of the tumor.

Our efforts have been focused on developing a hyaluronan (HA) based injectable hydrogel system that acts as a depot for sensitive drugs. Recently, we have demonstrated the delivery of BMP-2 (bone morphogenetic protein-2) using hydrogel to trigger ectopic bone formation at the site of injection.[10] The hydrogel was completely degraded within 4 weeks after injection by the action of HA specific enzyme hyaluronidase (Hase) without any inflammatory response. HA, a nonsulfated glycosaminoglycan polymer, found as a main component of the extracellular matrix (ECM) and in mammalian bone marrow and loose connective tissues, regulates diverse cellular responses including proliferation, differentiation, motility, adhesion, and gene expression.[11] Many of these functions have been attributed to its interaction with specific cell surface receptors, mainly CD44 but also other receptors, like RHAMM, LYVE-1, layilin, HARE, stabilin, and Toll-like receptor 4 (TLR4).[12] High molecular weight HA (HMW-HA)[13] and the low molecular weight drug bisphosphonate (BP)[14] were shown to inhibit osteoclast differentiation thus preventing osteoclastic bone resorption activity. Antiosteolytic activity of BPs provided also the rationale for their use in bone metastases as adjuvants in combination with established antineoplastic drugs. Most cancers such as breast, prostate, colon cancer, etc. metastasize to bone which results in increased osteolytic activity causing release of growth factors that in turn stimulates proliferation of cancer cells (known as the "vicious cycle").[15]

We hypothesized that the toxic drugs, such as BP conjugated to HA, could mask toxicity until specific targeting to CD44 positive cells, e.g., cancer. In addition, its incorporation into an injectable gel provides sustained release of therapeutic doses through the gel degradation. Even though, the individual therapeutic benefits of HA and BP are known, we present the first report of the tailored synthesis of HA−BP conjugate **1** having cross-linkable functionality (Figure 1). The hydrazide group was employed to react with aldehyde functionalized HA **2** which resulted in hydrogel formation in less than 30 s.

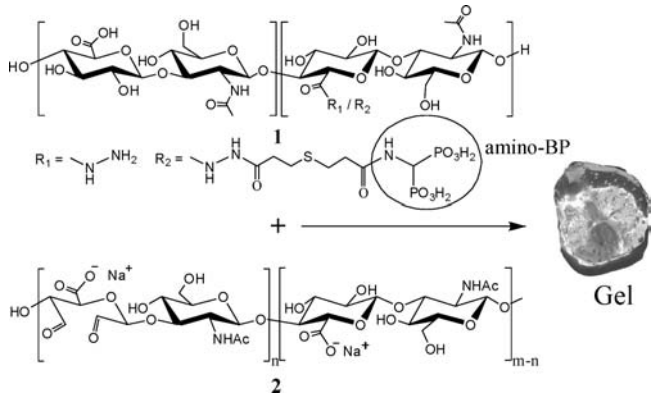
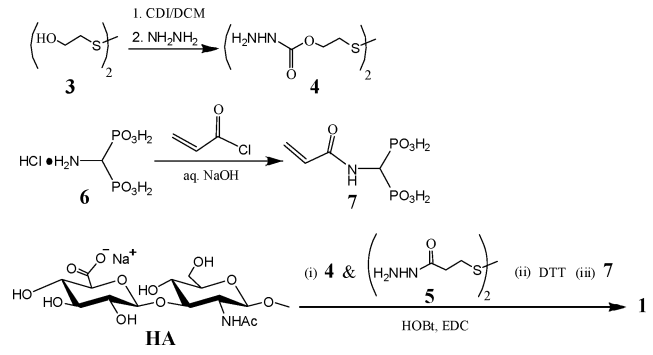


Figure 1. Structure of aminomethylene bisphosphonate/hydrazide-dually functionalized HA **1** that forms a hydrazone cross-linked hydrogel with periodate-oxidized HA **2**.

To prepare an HA derivative bearing both cross-linkable groups and a bioactive ligand, we have chosen two orthogonally reactive and chemoselective groups, namely hydrazide and thiol, that can be individually accessible. We preferred to use identical functionalization conditions which would allow simultaneous installation of both types of groups in one pot and under mild conditions. For this purpose, we have developed a self-immolative bifunctional linker that can generate a free hydrazide group on an HA carboxylate. The linker was synthesized by activating 2,2′-dithiodiethanol **3** with 2 equiv of carbonyldiimidazole (CDI) followed by condensation with hydrazine to give **4** (Scheme 1). Structurally,

Scheme 1



the linker **4** is a biscarbazate and differs from previously reported thiol generating linker **5** that is bishydrazide.[16] Contrary to the linker **5**, cleavage of the central disulfide bond in the newly developed linker **4** should result in the formation of unstable 2-thioethyl carbazate residues that can undergo spontaneous elimination of ethylene episulfide and carbon dioxide and eventually liberate free hydrazide group. This type of elimination has been reported previously.[17] We therefore employed this chemistry to generate hydrazide and thiol groups on HA carboxylates by subjecting the linker **4** and **5** in a 1:1 molar ratio by standard EDC-mediated coupling to HA at pH 4.8, followed by mild disulfide cleavage with DTT at pH 8.5. Thus, dual functionalization of HA having hydrazide and thiol groups was successfully accomplished in one step.

Second, we investigated whether the newly formed thiol group could be further functionalized *in situ* by providing a suitable Michael acceptor bearing a bioactive ligand. For this purpose we have chosen acrylamidomethylenebisphosphonate **7**. The Michael acceptor **7** was synthesized by acrylating aminomethylene bisphosphonate **6**[18] using acryloyl chloride in aqueous NaOH. The Michael addition reaction was indeed successful after coupling of **4** and **5** to HA under standard EDC conditions followed by a DTT unmasking step and subsequent treatment with an excess of **7** in one pot (Scheme 1). After overnight stirring, the reaction mixture was acidified to pH 3.5 followed by exhaustive dialysis against dilute HCl (pH 3.5) affording the HA−BP conjugate **1**.

After purification, the structural integrity of HA−BP **1** was confirmed by NMR analysis. The ^{1}H NMR spectrum of **1** showed signals at δ 2.67 and 2.81 ppm corresponding to the $-COCH_2-CH_2SCH_2-CH_2CO-$ sequence of the side chains terminated with the BP moiety (Figure 2). Comparison of the integration of methylene protons with the acetamido moiety of the *N*-acetyl-D-glucosamine allowed us to determine the degree of BP modification, which was found to be 5%. We were unable to see the very characteristic tertiary proton of the BP residue that is coupled to two phosphorus and one nitrogen atom due to its overlapping with the signals of H1′ saccharide protons of HA. However, the presence of the coupled BP moiety was confirmed by detecting a phosphorus signal at 14.2 ppm by ^{31}P NMR (Figure 2). The amount of hydrazide groups (9%) in **1** was assessed by a trinitrobenzene sulfonic acid (TNBS) assay as well as by reaction with 2-furaldehyde followed by ^{1}H NMR.

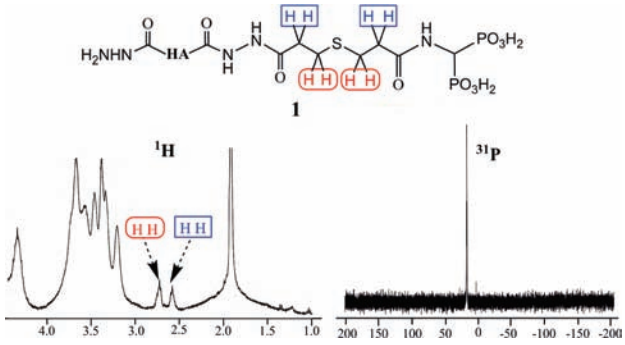


Figure 2. ^{1}H and ^{31}P NMR spectra of **1**.

Mixing of new HA hydrazide derivatives with di- or polyaldehyde compounds should afford hydrogel formation via a hydrazone cross-linking reaction. To test the hydrogel forming ability of the dually modified HA hydrazide **1**, we have chosen a well-known periodate-oxidized HA **2** (degree of aldehyde modification 10%).[19] Previously, we invented a series of poly(vinyl alcohol)-based cross-linkers that upon mixing with **2** allowed quick transitioning from liquids to gels.[20] Here, to prepare a hydrogel covalently incorporating bisphosphonates, the two HA derivatives **1** and **2** were dissolved in PBS buffer (pH 7.4) at concentrations providing equimolar amounts of aldehyde and hydrazide functionalities. Equal volumes of these solutions were injected using a double-barreled syringe to obtain the hydrogels with the concentration of solid contents at 2%. The hydrogel was formed in just 30 s which shows the potential of administering these gels in the intra-articular space of the osteoarthritic animal model. The localized delivery would also reduce BP toxicity associated with systemic delivery. The gel exhibited viscoelastic properties. A cylindrical hydrogel sample of 16-mm diameter and 1 mm thickness was obtained and analyzed after setting for 24 h. The mechanical properties were measured at a frequency of 1 Hz giving storage modulus $G' = 108$ Pa and loss modulus $G'' = 11$ Pa, respectively. To our knowledge, this is the first example of an injectable preparation of the HA hydrogel with bisphosphonates covalently linked to the matrix.[21]

The cytotoxic evaluation of the HA−BP prodrug was another aspect that we wanted to explore since BPs not only inhibit osteoclast differentiation but also can directly exert a similar apoptotic effect on cancer cells.[22] For this purpose we have chosen CD44 positive HCT-116 cells and less CD44 positive HEK-293T cells. Both these cell lines have been previously studied for HA specific uptake because of their difference in the levels of cell surface HA receptors.[23,24] Recently, Harada et al. have shown that fluorescently labeled HA could be internalized by engineered HEK-293 cells that stably express human CD44 but not by normal HEK-293 cells as it lacks this receptor.[25] To visualize the selective uptake of HMW-HA we have fluorescently tagged HMW-HA (FITC labeled HA **11**, see Supporting Information (SI)) and evaluated its uptake in HCT-116 cell lines. It was indeed surprising that there was no uptake of this fluorescently tagged HA, even after 4 days of incubation. It had been reported earlier that HA internalization by cell surface receptors is very much size-dependent.[26] Specifically, increasing the size of HA beyond a threshold limit sterically hinders its internalization, *in vivo*. In this scenario, internalization is facilitated with enzymatic degradation of the ECM. To prove this assumption *in vitro*, we simulated *in vivo* enzymatic degradation of cell-bound HA by the addition of Hase into the culture medium. Indeed, incubation of HCT-116 cells and FITC-labeled HA (**11** in SI) in the presence of 20 U/mL Hase allowed its internalization, as it was evident from inverted fluorescent microscopy images after 3 days of incubation (Figure 3).

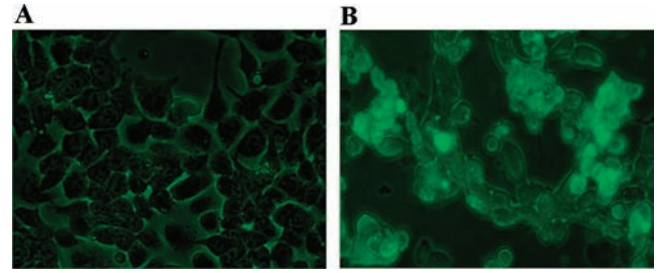


Figure 3. Fluorescence microscopy images of HCT-116 cells incubated for 3 days with 1.6 mM fluorescein-labeled HA derivative **11** either (A) without or (B) with 20 U/mL of hyaluronidase. Panel A shows no internalization of the high molecular weight **11**, while panel B shows binding and uptake of the hyaluronidase-degraded **11** (see SI for the structure of **11**).

Since Hase is known to be cytotoxic[27] we first estimated the tolerant level of this enzyme with the two cell lines. We found that both these cells were completely viable when incubated with native HMW-HA and an increasing amount of Hase up to 20 U/mL

(see SI). The cytostatic evaluation by an MTT assay of the free BP drug **6** on HCT-116 and HEK-293T cells revealed their IC_{50} values (10 μM and 8 μM respectively) to be similar to the third generation bisphosphonate drug zoledronic acid, which exerts a powerful antitumoral effect in a variety of human cancers.[28] As expected from the cell uptake experiments, prodrug **1** showed no cell toxicity at all in the concentration range that is equivalent to the cytotoxic range of the free drug **6** (5 to 80 μM) even after 4 days of incubation. Most interestingly, the toxicity effect on both cell lines were regained in the presence of Hase (18 U/mL) in a BP concentration-dependent manner (Figure 4) albeit to a significantly different extent. The IC_{50} of **1** established for HCT-116 cells was 1.2 mM (corresponding to 60 μM of BP moieties), while for HEK-293T it was much above 1.6 mM (corresponding to 80 μM of BP moieties).[29] By linking bisphosphonate moieties to HA one can envision a loss of cytotoxicity of the resultant high molecular weight polymeric prodrug **1**. The action of hyaluronidase on HA−BP conjugate **1** does not give the free unaltered compound **6**, but its various amide-linked derivatives which might reduce the potency as compared to the free drug. The difference in toxicity between the two cell lines could be correlated to the difference in the CD44 expression in them. The BP dependent toxic effect suggests that the smaller fragments of HA−BP prodrug not only were internalized by endocytosis but also probably would have been released from the endosome to the cytoplasm to give this effect.

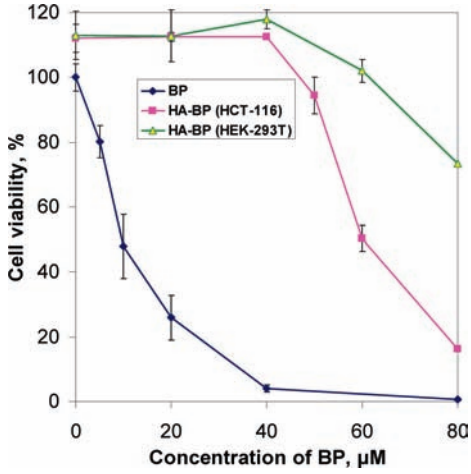


Figure 4. Comparative *in vitro* cytotoxicity of the HA-BP **1** (blue curve) and free BP **6** (pink curve) against HCT-116 cells and the HA-BP **1** against HEK-293T cells (green curve). Cytotoxicity experiments with **1** were performed in the presence of the fixed amount of Hase (18 U/mL).

In conclusion, we present here the novel synthesis of an HMW HA−BP conjugate having free hydrazide functionality that can be used as an antiosteoclastic and antineoplastic drug in the injectable hydrogel formulation. The potency of the prodrug is triggered by an ubiquitous enzyme, Hase, that cleaves the HA−BP to suitable sizes so as to be internalized by CD44 positive cells by the receptor-mediated endocytosis. In the form of hydrogel it would prevent systemic exposure of the drug and allow its controlled release at the site of implantation. The hydrazide group of the HA−BP conjugate, primarily used for cross-linking, could also be used to explore hydrazone linking of other drug molecules such as doxorubicin, etc. that could be integrated into the hydrogel matrix.

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Supporting Information Available: Experimental procedures, Figures S1−S5. This material is available free of charge via the Internet at http://pubs.acs.org.

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