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Boronate-crosslinked polysaccharide conjugates for pH-responsive and targeted drug delivery†

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A pH-responsive and targeted polysaccharide conjugate was fabricated from the boronate linkage of *N*-(2-aminoethyl)-gluconamide-grafted hyaluronic acid with anticancer drug bortezomib, which could exhibit targeted drug release behaviors at acidic pH and possess lower cytotoxicity and a higher inhibition effect toward cancer cells.

Although there is a growing consensus that chemotherapy is a leading therapeutic approach in clinical cancer treatment, it still faces substantial drawbacks such as poor water solubility, lack of specificity, and some unpleasant side effects, which greatly impede the therapeutic efficiency.[1–4] To solve these problems, the construction of environmental stimulus-responsive targeted drug delivery systems has drawn tremendous attention in the biomedical field.[5–9] In this regard, the incorporation of cell-specific targeting ligands with stimulus-responsive building blocks may endow several advantages in pharmacotherapy. Firstly, by introducing targeting ligands, an anticancer drug could distinguish tumor cells from normal ones, thus enhancing the accumulation of drug in tumor tissue and decreasing side effects.[10–16] Besides, an anticancer drug could be integrated into water-soluble carriers, which could increase the solubility and biocompatibility.[17–21] Furthermore, many endogenous stimuli, such as pH, redox potential, and enzymes could be exploited as triggers for drug release, especially in cancer treatment.[22–29]

It is well known that boronic acid could bind with *cis*-diols reversibly to form boronate esters, which could be used as a stimulus-responsive site for drug delivery because of its fast responsiveness to pH change and competing diols.[30–33] For instance, Messersmith and co-workers developed pH-responsive catechol polymers for targeted bortezomib delivery to cancer cells and investigated their anticancer abilities *in vitro*, demonstrating that the pH-sensitive catechol-boronate binding mechanism provides a versatile strategy for controlled drug release.[34] Lam and co-workers reported a class of dual-responsive boronate-crosslinked micelles for targeted paclitaxel delivery, and the resulting dendrimer-type micelles displayed improved stability and quick drug release in response to pH and diols.[35]

In this work, *N*-(2-aminoethyl)-gluconamide (Glu) was chosen as the diol site to be grafted on hyaluronic acid (HA), a type of biocompatible polymer with specific recognition ability toward cancer cells. Then, the anticancer drug bortezomib (BTZ) was added to the HAGlu chain to form boronate esters with diols. Finally, the boronate-crosslinked targeted polysaccharide nano-conjugate was conveniently constructed for pH-dependent drug delivery (Scheme 1). This delivery carrier possesses three desirable advantages: (1) this system was achieved by the facile synthesis of HAGlu, followed by complexation with BTZ through reversible ester bonds; (2) the boronic acid moiety in BTZ could form covalent boronate esters with *cis*-diols under neutral pH; when the boronate esters encountered low pH, BTZ could be dissociated readily from the conjugate, which makes the conjugate responsive to the local tumor environment; (3) the water solubility, biocompatibility and targeting ability could be concurrently enhanced in the obtained supramolecular assembly. In our case, the water-soluble and biocompatible polysaccharide, HA polymer, was utilized as the targeting agent and building block for the construction of a pH-dependent targeted drug delivery system, which is considered as an effective strategy for cancer treatment.[36–39]

HAGlu was synthesized by the condensation reaction of the HA chain with Glu moieties, and the molecular weight of HA used in this work was 550 kDa. As shown in Fig. S1 (ESI†), ^{1}H NMR spectra of HAGlu showed the characteristic methylene proton signals of the ethylenediamine group around the chemical shift of 3.16–3.21 ppm, and the proton signals at 2.0 ppm and 4.5 ppm were assigned to HA, which indicated the

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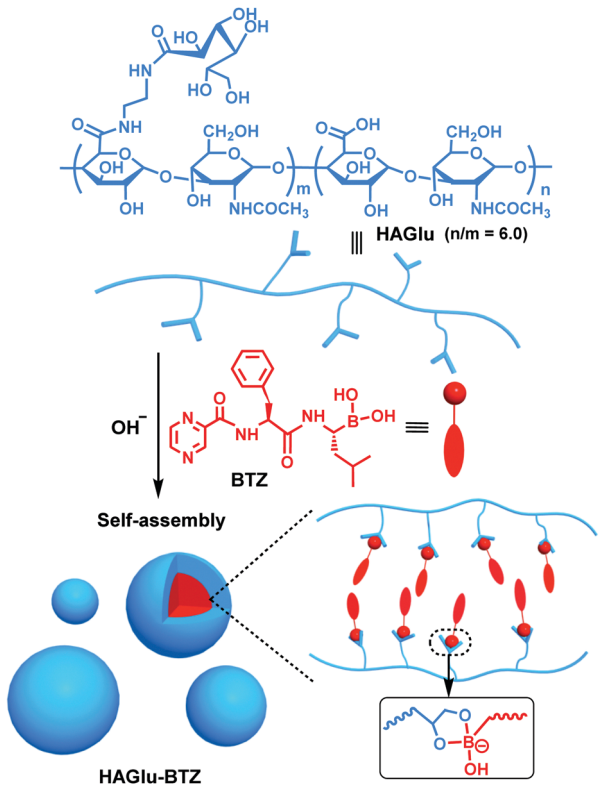

Scheme 1 Construction of HAGlu–BTZ polysaccharide conjugates.

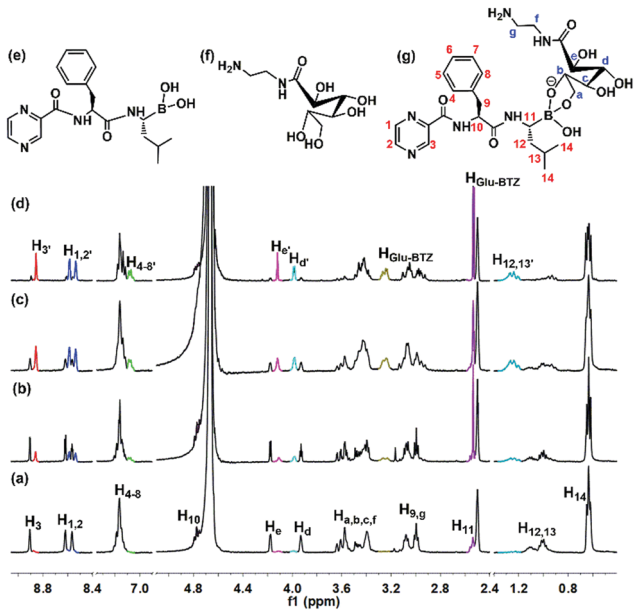

Fig. 1 ^{1}H NMR spectra of Glu and BTZ pH-dependent interactions in deuterated PBS at pH (a) 5.7, (b) 6.5, (c) 7.2, and (d) 8.5. The structure of (e) BTZ, (f) Glu, and (g) Glu–BTZ conjugate.

successful grafting of Glu onto the HA chain. From the integral values of the peak area of the methylene proton in the ethylenediamine group and *N*-acetyl protons of HA at 2.0 ppm, we could calculate that one Glu was modified by every six polysaccharide units of HA, that is *ca.* 16.8% of the carboxyl groups of HA reacted with Glu, which was suitable for the specific recognition of HA toward CD44 and RHAMM receptor-overexpressing cancer cells.[40]

The pH-responsive binding reversibility of Glu with BTZ was analyzed by ^{1}H NMR spectroscopy. As shown in Fig. 1a, there was almost no Glu–BTZ interaction observed at pH 5.7, and its spectrum was similar to the superposition of their individual ones, which demonstrated that most of the BTZ existed as free form. In contrast, the spectral changes and peak splitting were observed upon the increase of pH from 6.5 to 8.5 (Fig. 1b–d). Moreover, the proton signals $H_{Glu-BTZ}$ assigned to the Glu–BTZ complex were gradually increased. Thus, we could deduce that the Glu–BTZ conjugates could be formed under near neutral and alkaline conditions. These results also indicated that the binding of Glu with BTZ was pH-dependent. Since the tumor's intracellular environment was acidic (pH 5.0–6.5) compared with normal tissue or blood (pH 7.4), the pH-dependent Glu–BTZ complexation would be beneficial to the drug release from the polysaccharide conjugates and then improve the anticancer ability.[41]

Taking advantage of the satisfactory boron esterification interactions between the *cis*-diols in HAGlu and the boronic acid group in BTZ, the polysaccharide conjugate (HAGlu–BTZ) was successfully constructed by simply mixing the two components in aqueous solution. Then the resulting solution was dialyzed against an excess amount of deionized water for 2 h to remove any unbound BTZ and freeze-dried. The drug loading efficiency and encapsulation efficiency of BTZ on HAGlu were calculated as 3.6% and 24.7%, respectively, *via* the ultraviolet standard curve of HAGlu–BTZ with absorption at 270 nm (Fig. S2c, ESI†). The morphological and structural information of the targeted polysaccharide delivery system comes from the high-resolution transmission electron microscopy (HR-TEM), scanning electron microscopy (SEM), dynamic light scattering (DLS), and zeta potential experiments. The TEM image in Fig. 2a showed that the polysaccharide conjugates existed as spherical nanoparticles with an average diameter of *ca.* 120 nm, and the SEM image (Fig. 2b) gave similar morphological information. Moreover, as shown in Fig. 2c, the DLS result indicated that the conjugates had a narrow distribution with a hydrodynamic diameter of *ca.* 143 nm. Besides, the zeta potential (Fig. 2d) of the polysaccharide conjugates was measured to be *ca.* −14.78 mV, indicating that the surfaces of the binary nanoconjugates were negatively charged owing to the ionization of carboxyl groups on the HA skeleton, and this negatively charged surface would extend the circulation time and eventually facilitate the stability and biocompatibility of the conjugates under the cellular environment, as described below.[42]

Next, the time-dependent and pH-triggered release behaviors of BTZ in polysaccharide conjugates were investigated at pH 5.7, 6.5 and 7.2, respectively. According to the photometric standard curve of HAGlu–BTZ, 14.3% of BTZ was released from the HAGlu–BTZ polysaccharide conjugates at pH 7.2 over a 24 h period, whereas the corresponding values increased to 35.1% and 57.9% at pH 6.5 and 5.7, respectively (Fig. S2 and S3, ESI†). That is, the release rate at pH 5.7 (the endosomal pH of cancer

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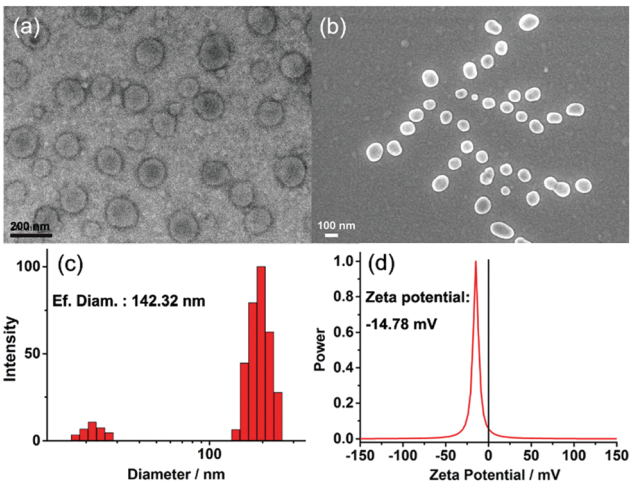

Fig. 2 (a) TEM image, (b) SEM image, (c) DLS and (d) zeta potential results of the polysaccharide conjugate.

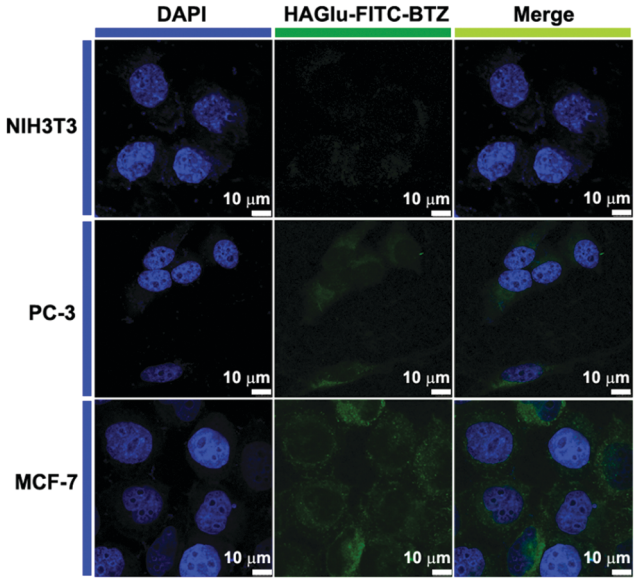

Fig. 3 Confocal fluorescence images of NIH3T3, PC-3 and MCF-7 cells incubated with FITC@HAGlu–BTZ for 6 h.

cells) was 4 times higher than the one at pH 7.2 (physiological pH). Combining the pH-responsive binding reversibility of Glu with BTZ, this result further confirmed the pH-dependent BTZ dissociation from HAGlu–BTZ polysaccharide conjugates. This pH-dependent release would not only improve the therapeutic efficacy of the anticancer drug but also reduce the toxicity to normal tissues, suggesting that the polysaccharide conjugates could potentially be a controlled delivery system in cancer therapy.

Subsequently, the HA-mediated cellular uptake of HAGlu–BTZ polysaccharide conjugates was evaluated by fluorescent confocal image experiments. Fluorescein isothiocyanate (FITC) was grafted on the HAGlu chain through an amide condensation reaction to form FITC@HAGlu (Fig. S4, ESI†), thus giving fluorescently labelled polysaccharide conjugates with BTZ. Then, PC-3 human prostatic cancer cells and MCF-7 human breast cancer cells that over-express HA receptors on their surface were used as the HA receptor positive group,[36,43] and a type of mouse embryonic fibroblast NIH3T3 cell line was used as the HA receptor negative group.[36,44] As shown in Fig. 3, both PC-3 and MCF-7 cells exhibited intense green fluorescence of FITC after incubation with FITC@HAGlu–BTZ for 6 h, but in contrast, only weak green fluorescence was observed in NIH3T3 cells due to the lack of HA receptor on the cell surface. These phenomena jointly indicated that the internalization of the polysaccharide conjugate could be sufficiently adjusted by the HA-receptor mediated cellular endocytosis.

Furthermore, cytotoxicity experiments were carried out by MTT assay to evaluate the anticancer activities of HAGlu–BTZ *in vitro*. As shown in Fig. 4a, the HAGlu–BTZ polysaccharide conjugates exhibited a satisfactory malignant cell inhibition effect toward PC-3 cells. After 24 h incubation, the relative cellular viability of PC-3 cells for the HAGlu–BTZ polysaccharide conjugate was 31.1%, which was lower than the corresponding value for the commercial anticancer drug BTZ (45.8%). In contrast, the relative cellular viability for normal cells with HAGlu–BTZ was 68.7%, which was higher than that with free BTZ (52.4%). However, when the receptors on the PC-3 cell surface were blocked by an excess amount of HA, the anticancer

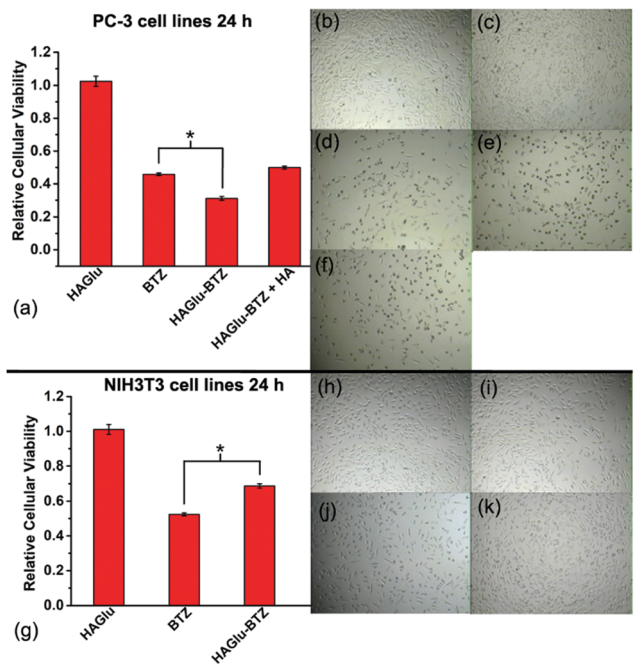

Fig. 4 Relative cellular viability and cellular photographs of (a–f) PC-3 and (g–k) NIH3T3 cell lines after 24 h of treatment with blank (b and h), HAGlu (c and i), BTZ (d and j), HAGlu–BTZ (e and k), and HAGlu–BTZ with an excess of HA (f). The statistically significant differences are indicated with asterisks ($P < 0.05$).

activity of the HAGlu–BTZ conjugates decreased distinctly, which was nearly the same as the value obtained using free BTZ. These phenomena further confirmed the cellular uptake of polysaccharide conjugates *via* HA receptor-mediated endocytosis, followed by the pH-triggered release of BTZ from the conjugate by low pH in cancer cells. Moreover, it is noteworthy that HAGlu was nontoxic to all the examined cell lines due to its

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satisfactory biocompatibility. Besides, the morphological changes of PC-3 cells (Fig. 4c–e) and NIH3T3 cells (Fig. 4i–k) treated with HAGlu, BTZ and HAGlu–BTZ revealed that the HAGlu–BTZ conjugate presented more effective damage to cancer cells but with less toxicity toward normal cells than free BTZ. These results jointly indicated that the polysaccharide conjugates exhibited a specific targeting ability with low cytotoxicity, which could facilitate the selective and rapid accumulation of BTZ in cancer cells and hold great promise to be a safe and efficient tool for anticancer therapy.

In conclusion, a pH-responsive and targeted polysaccharide conjugate was constructed by the boronate cross-linkage of *N*-(2-aminoethyl)-gluconamide-grafted hyaluronic acid (HAGlu) with anticancer drug bortezomib (BTZ), thereby displaying a satisfactory stimulus-responsive drug release at acidic pH values along with good stability and biocompatibility under physiological conditions. Moreover, as investigated using cytotoxicity experiments, it can be seen that the polysaccharide conjugate exhibited a higher anticancer activity and lower cytotoxicity than the free drug. Taking the simple decoration of polysaccharide and its specific targeting ability into account, we can envision that the obtained nanoconjugate can be further functionalized with other drug molecules and/or imaging agents, thus providing us with a novel toolbox for the design of more efficient drug carriers.

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Conflicts of interest

The authors declare no competing financial interest.

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