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Boronic acid copolymers for direct loading and acid-triggered release of Bis-T-23 in cultured podocytes

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Abstract

We report an acid-reversible linker for triggered release of Bis-T-23, an experimental small molecule drug for kidney disease treatment that restores podocyte morphology during disease. Bis-T-23 contains catechols, which form an acid-reversible, covalent boronate ester bond with boronic acids. We synthesized phenylboronic acid-containing polymers using reversible addition-fragmentation chain transfer polymerization that were able to directly load and solubilize Bis-T-23. Because of the reversibility of the boronic ester bond, drug was released in its native form in a pH-dependent manner. The polymers rapidly trafficked into acidic compartments and did not exhibit cytotoxicity, and polymer-drug conjugates successfully delivered Bis-T-23 into cultured podocytes.

Graphical Abstract

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SUPPORTING INFORMATION

Experimental methods, additional figures, and calculations may be found in supporting information.

CONFLICTS OF INTEREST

The authors declare no competing financial interest.

Keywords

Bis-T-23; boronic acid; drug delivery; polymers; triggered release

More than 850 million people worldwide are afflicted with kidney disease.¹ Because of limited therapies to halt disease advancement, many patients progress to chronic kidney failure and ultimately require dialysis or kidney transplantation. However, kidney disease patients continue to face high morbidity and poor five-year survival rates.² Human and experimental studies have shown that loss and dysfunction of podocytes, highly specialized and terminally differentiated epithelial cells within the kidney glomerulus essential to filtration, directly underlies scarring of kidney filtration units that leads to deteriorating kidney function and proteinuria.³ However, current therapies do not specifically modulate podocyte dysfunction, and burden the patient with significant side effects that further complicate the disease.^{4–6}

A common response to injury in podocytes is actin cytoskeleton derangement, which precedes morphological changes that lead to disruption of the filtration barrier.⁷ Therefore, drugs that modulate the actin cytoskeleton could have widespread clinical impact in treating chronic kidney disease. Schiffer *et al.* recently reported on the therapeutic efficacy of a small molecule, Bis-T-23, which promotes actin polymerization via dynamin oligomerization.⁷ In various rodent models of podocyte injury, Bis-T-23 restored overall podocyte morphology and reduced proteinuria, a clinical signature of podocyte injury. However, low drug solubility and the ubiquitous expression of dynamin preclude systemic administration of Bis-T-23 in the clinic due to off-target toxicity.^{8, 9} Therefore, targeted Bis-T-23 delivery to glomerular podocytes could significantly expedite clinical translation of this experimental drug.

A critical component of targeted drug delivery is spatiotemporal control of drug release. Ideally, drugs are conjugated or loaded onto carriers in a manner that minimizes drug release during circulation to mitigate drug loss and reduce side effects, and releases drug after target cell binding and uptake. Drug linkers have been engineered to be sensitive to a variety of local cues (pH, enzymes, or reduction/oxidation) for programmed drug release within the target tissue or cell.^{10, 11} For example, the antibody-drug conjugate Adcetris contains cathepsin-sensitive linkers for drug release after cellular internalization and trafficking into lysosomes,¹² enabling targeted delivery of the highly potent drug monomethyl auristatin E. These advances in linker chemistry have subsequently improved the safety and efficacy of

chemotherapeutics, and similar technologies could improve the standard of treatment for kidney diseases.

Here, we report an acid-labile, boronate ester linkage for programmed release of Bis-T-23. We exploited the fact that Bis-T-23 contains catechol groups, which bind tightly to boronic acids to form a reversible covalent boronate ester bond.¹³ Importantly, no drug modification is required for conjugation to boronic acid-functionalized carriers, and drugs are released in original form. Boronic acids bind tightly to diols, and reactions with catechols such as Bis-T-23 are especially favored because of the aromatic hydroxyl groups (association constant $K_a \sim 830 \text{ M}^{-1}$ and 4.6 M^{-1} for catechols and D-glucose, respectively).¹³ The conjugated Bis-T-23 can be released by bond-breaking triggered by acidic pH conditions.^{14, 15} Therefore, the drug remains in a pro-drug form during circulation, but is released in active form after cellular binding and internalization into acidic, intracellular vesicles (Figure 1). In this work, we developed boronic acid-containing copolymers for loading and pH-sensitive release of the catechol-containing drug Bis-T-23. We characterize these materials for drug loading, release kinetics, cellular trafficking, and cytotoxicity, and demonstrate polymer-drug conjugate delivery in cultured podocytes. Moreover, we report a new method of evaluating Bis-T-23 delivery to podocytes.

Phenylboronic acid-grafted copolymers (PBA copolymers) were synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization (Scheme S1) of the monomers *N*-(2-hydroxypropyl) methacrylamide (HPMA), *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMA), and *N*-(3-aminopropyl)methacrylamide (APMA). HPMA was selected due to its biocompatibility and water solubility;¹⁶ DMAPMA, which contains a tertiary amine and is positively charged at physiologic pH, was selected to facilitate cellular internalization in lieu of a targeting ligand;¹⁷ and APMA was selected for facile functionalization with 4-carboxyphenylboronic acid by EDC/NHS conjugation post-polymerization. Notably, cationic polymers containing similar tertiary amines were observed to be rapidly internalized and trafficked into acidic endosomes and lysosomes.^{18, 19} This internalization route is an important requirement for the polymers studied here to reverse the boronate ester bond and trigger intracellular drug release.

Monomer conversion and copolymer composition was monitored by ¹H NMR during polymerization. Monomer conversion was nearly 100% after 18 h, and polymer composition was determined to be 70:20:10 for HPMA:DMAPMA:APMA (Figure S1). The copolymers exhibited a number average molecular weight (M_n) of 22 kDa and low dispersity ($\text{PDI} = 1.05$) by GPC analysis, indicating good control of polymerization (Figure S2 and Table 1). Functionalization of the copolymers with 4-carboxyphenylboronic acid was confirmed by ¹H NMR via emergence of new peaks at 7.9 and 7.6 ppm, indicating successful and complete reaction of primary amine side chains with PBA (Figure S1). Comparison of the integrals of the protons in PBA at 7.6 and 7.9 ppm with that of the protons in HPMA at 3.8 ppm confirmed that the final PBA copolymer was p(HPMA₇₀-co-DMAPMA₂₀-co-(APMA-*g*-PBA)₁₀).

When conjugated to PBA copolymers, Bis-T-23 gained a new absorbance at $\lambda = 410 \text{ nm}$, which was used to monitor polymer drug loading. In the absence of polymer, Bis-T-23

exhibited low water solubility and formed a yellow precipitate in aqueous solutions (Figure 2A). At increasing polymer:drug mass ratios, precipitation decreased, and drug absorbance increased until at 14:1 ratio, after which the observed drug absorbance plateaued (Figure 2A and 2B). Therefore, a polymer:drug ratio of 14:1 was used for subsequent drug loading and studies. In these conditions, 24.2% of the available PBA are loaded with drug (see Supporting Information for calculations). Dynamic light scattering analysis was also performed to test if aggregation occurred during drug loading, as Bis-T-23 is symmetrical and may crosslink polymers. No differences in size distribution were observed between polymer only and polymer-drug conjugates, suggesting that these loading conditions do not result in cross-linking and aggregation (Figure S3). This may be due to steric hindrance of the benzyl in both the polymer and Bis-T-23.

Polymer-drug conjugates were then tested for pH-dependent drug release in buffers at pH 7.4, 6.8, 5.6, and 4.6, with the latter two values within the late endosomal and lysosomal pH spanning 6.5–4.5.^{20, 21} While polymer-conjugates at pH 4.6 and 5.6 exhibited rapid and nearly complete drug release, conjugates at pH 6.8 and 7.4 exhibited minimal drug release within the same time frame (Figure 2C). After 48 h, PBA copolymers exhibited 14.5%, 19.3%, 80.4%, and 98.1% cumulative Bis-T-23 release in pH 7.4, 6.8, 5.6, and 4.6, respectively. These observations demonstrate that the copolymers exhibit pH-sensitive drug release, and that the PBA copolymers could release Bis-T-23 in pH ranges relevant to acidic endosomes and lysosomes.

Despite the pK_a of the PBA studied here being greater than 8.0,^{22, 23} Bis-T-23 was retained on the PBA copolymer at pH 7.4, likely due to the high binding affinity between PBA and catechols. As the pK_a of the boronic acid may affect drug loading and release, polymers containing fluoroPBA (FPBA), which exhibits a lower pK_a around 7.2–7.8 due to an electronegative fluorine,^{24, 25} were similarly synthesized and characterized (Figure S1). FPBA copolymers exhibited optimal drug loading at polymer:drug ratio 12:1 with similar trends of pH-dependent drug release as PBA copolymers (Figure S4). As no major differences in drug loading and release were observed between PBA and FPBA copolymers, PBA copolymers were selected for further study due to greater aqueous solubility. Therefore, polymer-drug conjugates may be formed at pH 7 without the need to modulate the pK_a of the phenylboronic acid.

Drug release was also tested in the presence of glucose, as vicinal diols might prematurely displace conjugated Bis-T-23. Physiologically, blood glucose concentrations range from 0.63 to 0.99 mg/mL in normal children and adults.²⁶ Glucose at concentrations up to 1.5 mg/mL did not cause significant drug release from the PBA copolymers (Figure S5). Moreover, mannitol, which contains multiple hydroxyls and exhibits a higher K_a with boronic acid than glucose ($K_a = 120 \text{ M}^{-1}$ and 4.6 M^{-1} , respectively),¹³ did not cause any significant Bis-T-23 release at the same concentration (Figure S5). The stability of the boronate ester is likely due to the 180-fold higher K_a of catechols and boronic acids compared to diols. Therefore, diols at physiological concentrations do not displace Bis-T-23, indicating that boronate ester reversal is more sensitive to pH than diols (Figure S5).

Bis-T-23-loaded polymers were then tested for drug delivery in cultured podocytes. First, the trafficking of PBA copolymers in podocytes was examined by flow cytometry and confocal microscopy. As early as 20–30 min, fluorescently labeled PBA copolymers were mostly internalized (Figure 3A) and colocalized with LysoTracker, a marker of lysosomes (Figure 3B). This is consistent with other studies reporting that polycationic materials rapidly traffic into acidic compartments as early as 5 min, with greater trafficking into more acidic late endosomes and lysosomes observed 10–20 min later.^{27, 28} Bis-T-23 promotes dynamin oligomerization, which causes actin polymerization and crosslinking and focal adhesion maturation.^{7, 29–31} By image analysis, while Bis-T-23 significantly increased F-actin content in cultured podocytes, polymer-drug conjugates did not (Figure S6), likely due to the low dynamic range of this method. Therefore, we sought to develop a more high-throughput method of evaluating Bis-T-23 delivery in cultured podocytes. Given that podocytes endocytose albumin³² and the importance of dynamin in this process,³³ we hypothesized that Bis-T-23 would modulate uptake of albumin. Indeed, in initial studies, Bis-T-23 inhibited albumin uptake up to 27% (Figure S7). Therefore, flow cytometry analysis of fluorescein-albumin uptake was used to quantify polymer-drug delivery. In podocytes, free Bis-T-23 and polymer-Bis-T-23 significantly inhibited albumin uptake (Figure 4A). Notably, cells were incubated with treatments for 20 min in PBS; due to minimal drug release within this time in pH 7.4 (<5%), polymer-Bis-T-23 effects was more likely due to acid-triggered release rather than basal release. Additionally, free Bis-T-23 and polymer-Bis-T-23 significantly inhibited transferrin uptake in a human T cell line (Figure 4B), consistent with previous studies showing Bis-T-23 inhibition of transferrin uptake,³⁴ and did not exhibit significant toxicity towards cultured podocytes (Figure 4C). These findings indicate that polymer-Bis-T-23 conjugates are internalized into acidic compartments, Bis-T-23 is being released intracellularly, and the polymers are not significantly toxic in the conditions tested.

While recent high-throughput screening campaigns have identified new small molecule drug candidates for treating kidney disease,^{35, 36} solubility and off-target side effects remain major obstacles to clinical translation of experimental drugs such as Bis-T-23. Polymeric drug carriers have emerged as an answer to some of these challenges by altering drug properties and pharmacokinetics. An important aspect of these carriers is the drug linker that controls drug release under specific conditions. Phenylboronic acid-containing polymers have been recently reported in applications in drug delivery due to their various stimuli-sensitive properties.^{14, 15, 37} In this work, we developed PBA copolymers for acid-triggered release of the catechol-containing drug Bis-T-23, taking advantage of the fact that catechols form an acid-reversible boronate ester with boronic acids. This chemistry has been utilized in a number of drug delivery applications for stimulus-triggered drug release from polymer systems. Su *et al* reported on polymer-drug conjugates comprising the boronic acid-containing chemotherapeutic, bortezomib, and catechol-containing polymers.¹⁵ The materials exhibited acidic pH-selective drug release and maintained *in vitro* cancer cell killing. Aguirre-Chagala *et al* and Ma *et al* reported on boronic acid-containing polymers for loading the diol-containing chemotherapeutic, capecitabine. These materials self-assembled into micelles and exhibited pH-sensitive drug release.^{38, 39} Li *et al* developed drug-loaded, polymeric micelles through crosslinking of adjacent boronic acid- and catechol-containing polymers. These micelles also exhibited acidic pH- and mannitol-triggered drug release, as

well as improved particle stability *in vivo*.¹⁴ Moreover, boronate esters have broad utility in other applications, including isolation of catechol-containing compounds from plasma for quantification,⁴⁰ and saccharide-triggered release of catechol-containing model drugs from boronic acid-functionalized nanoparticles.⁴¹

This work reports PBA copolymers that reversibly load the catechol-containing drug Bis-T-23 without any further drug modification and release active drug after cell internalization. Polymer-drug conjugates were stable at physiological pH and in the presence of sugars, indicating potential stability of the conjugates during circulation *in vivo*. *In vitro*, PBA copolymers distributed into acidic endosomes, and through a flow cytometry assay to evaluate Bis-T-23 delivery, polymer-drug conjugates were able to deliver drug intracellularly. Collectively, these findings suggest that boronate ester chemistry may be an attractive strategy for releasing Bis-T-23 after cellular uptake. The copolymers reported here included the tertiary amine-containing monomer DMAPMA, which mediates cellular internalization for proof-of-concept *in vitro* studies. Future work includes functionalizing uncharged versions of these polymers for targeted podocyte delivery of Bis-T-23 in the kidney. This strategy could also be readily applied to other catechol-containing drugs such as quercetin or EGCG.^{42, 43}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic of polymer-drug conjugates and release mechanism. **A.** Phenylboronic acid (PBA) copolymers load Bis-T-23 through boronate ester formation between boronic acids on copolymer side chains and catechols on Bis-T-23. At acidic pH conditions, the boronate ester bond reverses, and Bis-T-23 is released in its native form. **B.** Copolymers are rapidly endocytosed due to cationic side chains and are trafficked into acidic endosomes and lysosomes, which trigger bond reversal. Bis-T-23 is then released, catalyzing dynamin oligomerization.

Figure 2.

Characterization of PBA copolymer behavior. **A.** Image of polymer-drug conjugates loaded at increasing polymer:drug mass ratios. **B.** Absorbance quantification of polymer-drug conjugates. When loaded onto PBA copolymers, Bis-T-23 exhibits a new absorbance at $\lambda = 410$ nm. **C.** Bis-T-23 release from polymer-drug conjugates at various pH.

Figure 3.

In vitro polymer uptake. **A.** Polymer uptake by podocytes, measured by mean fluorescence intensity (MFI), after 20 min incubation at 37 or 4 °C. **B.** Fluorescent images of podocytes treated with fluorescein-PBA copolymers obtained by confocal microscopy. Green, polymer; red, lysosomes; yellow, merge. Pearson's correlation coefficient = 0.5. Scale bars, 10 μ m.

Figure 4.

In vitro drug delivery and cytotoxicity of polymer-drug conjugates. **A.** Normalized inhibition of fluorescein-BSA uptake in podocytes. **B.** Normalized inhibition of fluorescein-transferrin in Jurkat cells. Treatments were normalized by respective vehicle (DMSO or polymer) controls. **C.** Percent viability of treatments relative to untreated podocytes. Bars represent means $\pm$ SEM. *** p -value < 0.001 ; **** p -value < 0.0001 ; ns, not significant.

Table 1Summary of p(HPMA-*co*-DMPMA-*co*-APMA) copolymers

Polymer	M_n^a (Da)	M_n^b (Da)	
p(HPMA ₇₀ - <i>co</i> -DMPMA ₂₀ - <i>co</i> -APMA ₁₀)	15,200	22,000	1.05
p(HPMA ₇₀ - <i>co</i> -DMPMA ₂₀ - <i>co</i> -(APMA- <i>g</i> -PBA) ₁₀)	16,300	N/A	N/A

^a¹H NMR,^bGPC