Supplementary information for:

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

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This document comprises 10 pages with experimental methods, 1 schematic, 7 figures, and calculations.

EXPERIMENTAL SECTION

Materials

N-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. *N*-(2-hydroxypropyl) methacrylamide (HPMA), *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMA), and *N*-(3-aminopropyl)methacrylamide (APMA) monomers were purchased from Polysciences, and 4-carboxyphenylboronic acid and 4-carboxy-3-fluorophenylboronic acid were purchased from Combi-Blocks. The chain-transfer agent 4-cyano-4-(ethylsulfanylthiocarbonyl) sulfanylvpentanoic acid (ECT) was a generous gift from Anthony Convertine. VA-044 was purchased from Wako Chemicals. Bis-T-23 was purchased from Aberjona Laboratories and dissolved to 30 mM stocks in DMSO. NHS-fluorescein was purchased from Thermo Fisher Scientific.

Characterization

^{1}H NMR spectra were recorded on a Bruker AV 300 (Bruker Corporation, Billerica, MA) nuclear magnetic resonance (NMR) instrument in deuteroxide (D_2O). The molecular weight and molecular weight dispersity ($Đ$) of the polymers were determined by size exclusion chromatography. To prepare materials for analysis, the purified polymer was dissolved at 5 mg/mL in running buffer (0.15 M sodium acetate buffered to pH 4.4 with acetic acid) for analysis by SEC. Samples were then applied to an OHpak SB-804 HQ column (Shodex) in line with a miniDAWN TREOS light scattering detector (Wyatt) and a OptiLab rEX refractive index detector (Wyatt). Absolute molecular weight averages (M_w and M_n) was calculated using ASTRA software (Wyatt). To test if aggregates were formed in the process of drug loading, the mixed solutions were analyzed by dynamic light scattering using a ZetaPLUS (Brookhaven Instruments Corporation, Holtsvile, NY) by preparing samples as described in polymer-drug conjugate drug delivery studies. UV absorption was tested by NanoDrop (Thermo Fisher Scientific).

Copolymer synthesis

p(HPMA$_{70}$-*co*-DMAPMA$_{20}$-*co*-APMA$_{10}$) was synthesized by reversible addition-fragmentation chain transfer polymerization (RAFT). In a typical reaction, HPMA (381 mg, 2.66 mmol), DMAPMA (130 mg, 0.76 mmol), and APMA (68 mg, 0.38 mmol) were dissolved in 5.4 mL acetate buffer (1 M, pH 5.1) with ECT (10 mg, 0.038 mmol) and VA-044 (1.23 mg, 0.0038 mmol). After purging with argon for 10 min, polymerization was initiated by placing the reaction in a 44 °C oil bath. After 24 h, polymerization was stopped and the polymer was purified by dialysis against dH_2O followed by lyophilization. Polymer was characterized by ^{1}H NMR and GPC. Yield: 78%.

To functionalize copolymers with phenylboronic acid, 4-carboxyphenylboronic acid (PBA) (55 mg, 0.33 mmol), EDC (95 mg, 0.495 mmol), and NHS (57 mg, 0.495 mmol) were dissolved in a solution of DMSO (4 mL) and dH_2O (0.1 mL), then stirred at room temperature for 30 min. p(HPMA-*co*-DMAPMA-*co*-APMA) (100 mg) dissolved in 1 mL DMSO was added to the above

solution, and the reaction was allowed to proceed for 24 h. The final product, p(HPMA$_{70}$-*co*-DMAPMA$_{20}$-*co*-(APMA-*g*-PBA)$_{10}$), was obtained by sequential dialysis against methanol and dH_2O followed by lyophilization. Polymers were characterized by ^{1}H NMR. Yield: 90%. A phenylboronic acid derivative, 4-carboxy-3-fluorophenylboronic acid (FPBA), was also conjugated onto p(HPMA$_{70}$-*co*-DMAPMA$_{20}$-*co*-APMA$_{10}$) to produce p(HPMA$_{70}$-*co*-DMAPMA$_{20}$-*co*-(APMA-*g*-FPB)$_{10}$) with the same procedure.

In order to investigate the intracellular distribution of the polymer, p(HPMA-*co*-DMAPMA-*co*-APMA) was labeled with fluorescein followed by functionalization with PBA. p(HPMA-*co*-DMAPMA-*co*-APMA) (100 mg) was first dissolved in DMSO (4 mL) and dH_2O (0.1 mL), and the solution was purged with argon for 10 min followed by the addition of 38 μL NHS-fluorescein solution in DMF (1 mg/mL). After 2 h, PBA pre-activated with EDC and NHS was added, and the reaction was conducted for an additional 24 h. Afterwards, the mixture was dialyzed against dH_2O for 2 days and purified using a PD-10 column (GE). The labeled polymer, p(HPMA$_{70}$-*co*-DMAPMA$_{20}$-*co*-((APMA-*g*-fluorescein)$_1$-co-(APMA-*g*-PBA)$_9$)), was obtained after lyophilization. Yield: 75%.

Drug loading

Bis-T-23 was dissolved in DMSO (100 mg/mL), and a series of polymer was dissolved in 1 mL H_2O at various polymer:drug mass ratios (0:1-20:1). Drug (1 μL) was added to the polymer solution and then vortexed, and loading was tested after 2 h. The UV spectra of all samples was measured using λ = 410 nm and compared. Drug was considered completely loaded when no apparent increase in UV spectra was observed with increasing polymer:drug ratio.

Drug release

To investigate the copolymer-drug conjugate release behavior under different pH conditions, 1 mL of polymer-drug conjugate solution (100 μg Bis-T-23 at polymer:drug mass ratio = 14) was dissolved in 1 mL buffer, transferred inside dialysis tubing (MWCO 7 kDa), and placed within a 250-mL beaker containing 50 mL buffer. The following citric acid/sodium phosphate pH buffers were prepared: pH 4.6, 5.6, 6.8, and 7.4. Samples were incubated at 37 °C with rotation at 125 RPM. At each time point, 5 mL dialysate sample was collected and replaced with fresh buffer. Samples were then lyophilized, dissolved in 1 mL 10% DMSO, and quantified for drug content by absorbance spectroscopy using λ = 357 nm. In further experiments, PBA copolymer-drug conjugate release was tested in glucose (1.0 or 1.5 mg/mL) or mannitol (1.5 mg/mL) as described above.

Cell culture

Immortalized murine podocytes were cultured as previously described.[1] Briefly, cells were maintained in growth-permissive conditions (33 °C, mIFN-γ, bovine collagen I-coated plates) and then passaged into 24- or 96-well plates at 37 °C overnight prior to experiments. Jurkat cells were cultured according to supplier instruction (ATCC).

Cytotoxicity

Immortalized podocytes (1×10^4 per well in a 96-well plate) were washed twice with PBS and then incubated with various concentrations of polymer (0-200 μg/mL) dissolved in PBS for 20 min at 37 °C. After, cells were washed with PBS, and fresh PBS was added. Cells were allowed to incubate for another 10 min at at 37 °C. Viability was determined by CellTiter-Glo 2.0 kit (Promega).

Polymer uptake

Immortalized podocytes (2×10^5) were cultured on bovine collagen I (Corning)-coated 12-mm glass coverslips overnight, and then incubated with 50 nM LysoTracker Deep Red (Thermo Fisher Scientific) for 30 min according to manufacturer instructions. Fluorescently labeled polymers dissolved in PBS (200 μg/mL) were added, and cells were incubated for 20 min in the presence of 50 nM LysoTracker. After, the treatments were replaced with fresh PBS to emulate treatment conditions. For flow cytometry analysis, cells were washed with PBS and then lifted with trypsin; LysoTracker was not used for flow cytometry experiments. For microscopy analysis, cells were washed with PBS, fixed with 4% PFA, washed, and stained with DAPI according to manufacturer instructions. Images were collected with a confocal microscope (Leica).

Bis-T-23 drug efficacy by image analysis

Immortalized podocytes cultured on glass coverslips were treated with DMSO (0.2% v/v) or 60 μM Bis-T-23 for 4 h. After, cells were processed as above and stained with rhodamine-phalloidin (Cytoskeleton, Inc.) and DAPI for F-actin and nuclei, respectively, according to manufacturer instructions. Images were taken using an EVOS fluorescence microscope and F-actin was quantified by ImageJ analysis. First, 100 individual cells across all treatments were analyzed for size, and the middle 50% of cell sizes was used to determine the size range inclusion criteria for F-actin analysis. For each individual cell, F-actin was thresholded using the "Moments" method, and this area was recorded as the F-actin area and divided by the total cell area. Both image capture and analysis were performed in a blind manner.

Protein labeling

Bovine serum albumin (BSA, Thermo Fisher Scientific) and human holo-transferrin (R&D Biosystems) were dissolved in 0.1 M sodium bicarbonate buffer, pH 8.56 (20 mg/mL) and then labeled with NHS-fluorescein at an 8-fold molar excess for 1 h at room temperature. Afterwards, proteins were purified by Zeba column (Thermo Fisher Scientific) twice into water.

Polymer-drug conjugate drug delivery

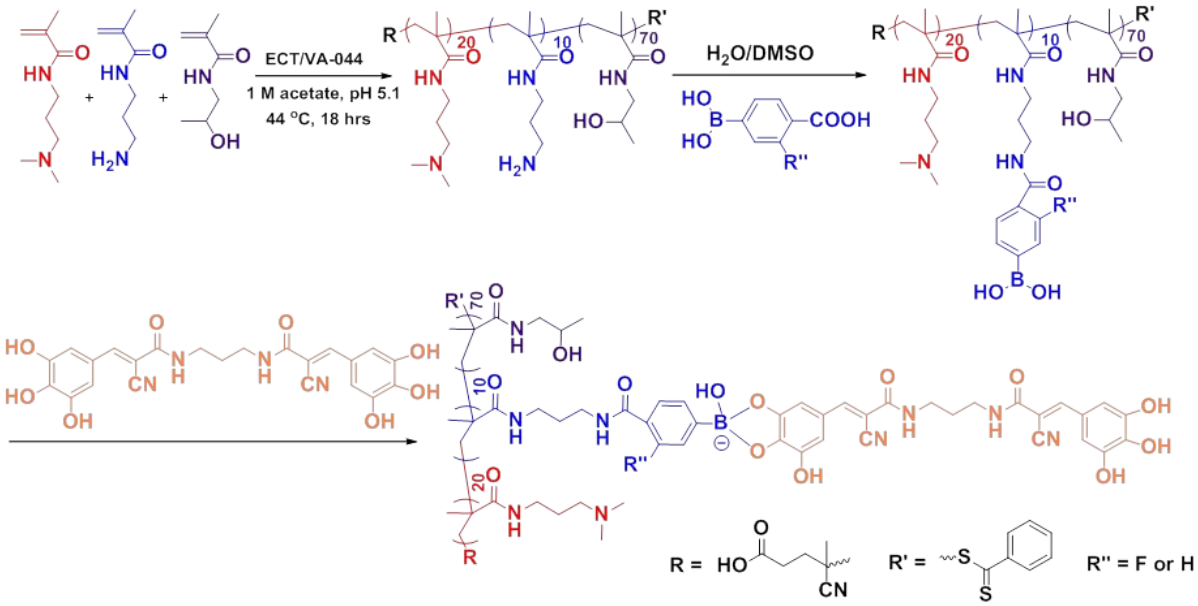
Polymer-drug conjugates were prepared as follows: in a total volume of 100 μL in molecular-grade H_2O, PBA copolymer (at a 14-fold mass excess to drug, typically 67.3 μg/mL final concentration) was added, and then Bis-T-23 (typically 10 μM or 4.8 μg/mL final concentration) was added. The solution was then vortexed and allowed to incubate at room temperature for at least 2 h. After, PBS was added to achieve the final drug concentration (typically 10 μM). DMSO and free drug treatments were prepared immediately before treatment.

For drug delivery studies, podocytes (5-6×10^4 cells per well in a 24-well plate) were washed twice with PBS, incubated with treatments in PBS for 20 min at 37 °C, and then incubated with 50-100 μg/mL fluorescein-BSA in PBS for 10 min at 37 °C. After, cells were washed twice with ice-cold PBS, lifted with trypsin, resuspended in PBS+0.5% BSA, and then analyzed for FITC fluorescence using an Attune NxT flow cytometer (Thermo Fisher Scientific). Two independent experiments were performed.

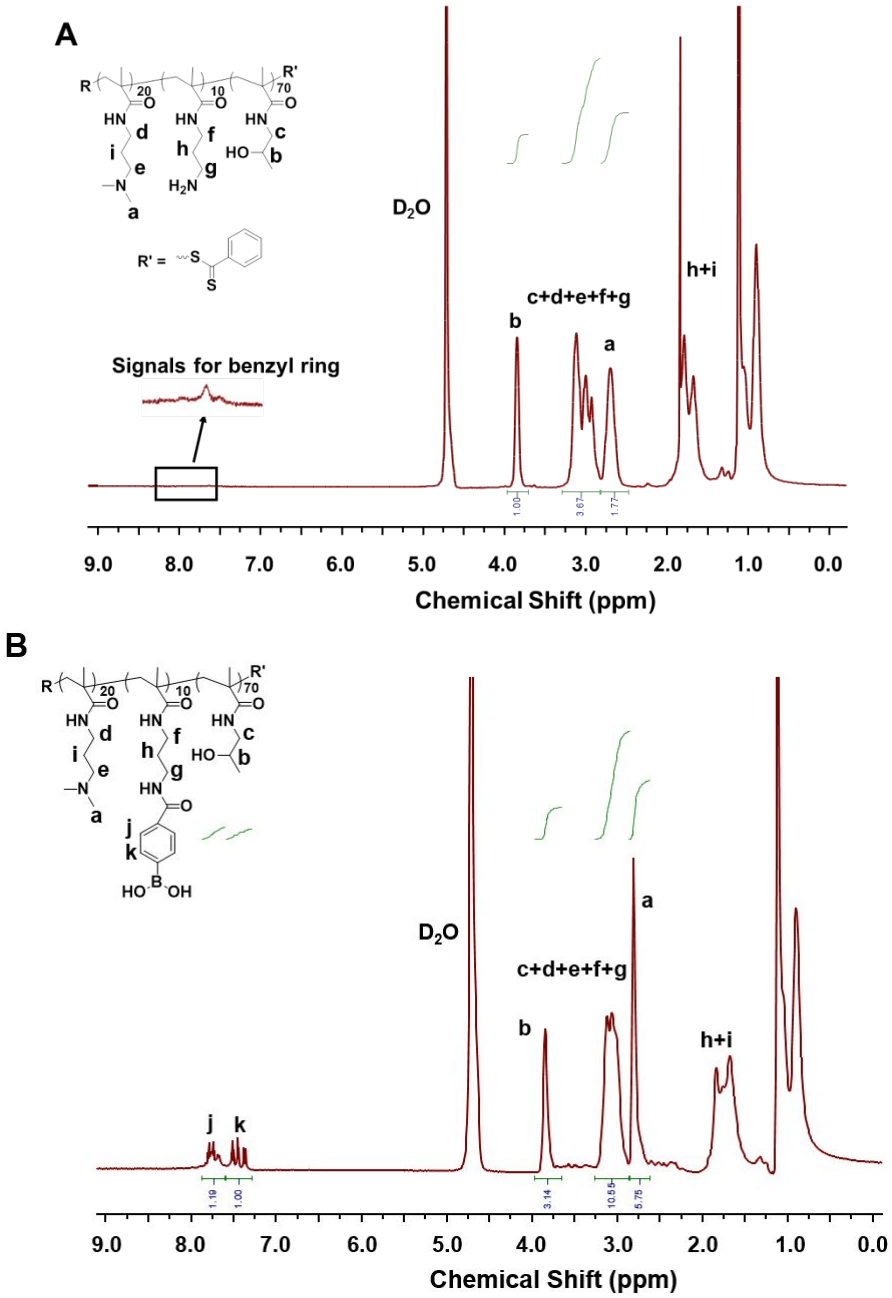
For Jurkat studies, cells (1×10^5 cells per well in a round-bottom 96-well plate) were washed twice with PBS, incubated with treatments as above, and then incubated with 10 μg/mL fluorescein-transferrin as above. After, cells were washed, resuspended in PBS+0.5% BSA, and analyzed by flow cytometry as above. Three independent experiments were performed.

Data analysis

Data analysis was performed using GraphPad Prism software. Statistical analysis was performed using a one-way ANOVA with post-hoc Dunnett's multiple comparisons test to untreated cells, or a Student's *t*-test for single comparisons.



Scheme S1. Synthetic route of boronic acid-containing copolymers and Bis-T-23 loading.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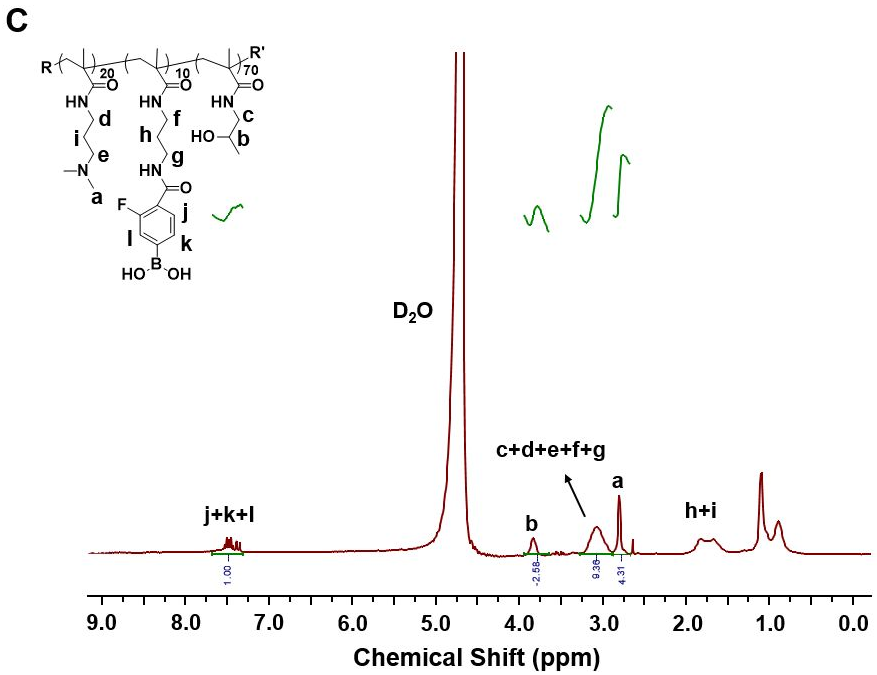

Figure S1. ^{1}H NMR spectra of **A.** p(HPMA$_{70}$-DMAPMA$_{20}$-APMA$_{10}$), **B.** p(HPMA$_{70}$-*co*-DMAPMA$_{20}$-*co*-(APMA-*g*-PBA)$_{10}$), and **C.** p(HPMA$_{70}$-DMAPMA$_{20}$-(APMA-*g*-FPBA$_{10}$)).

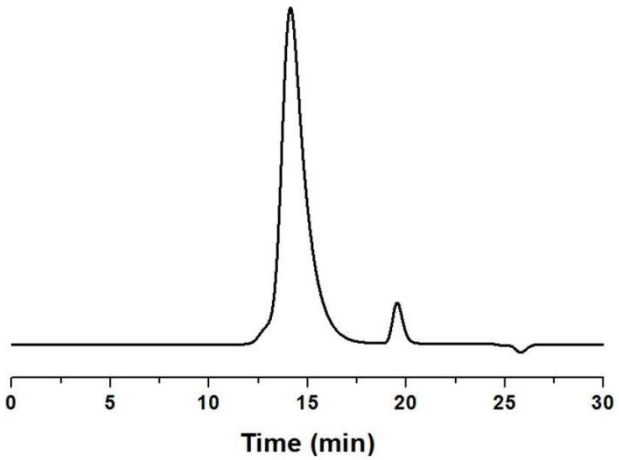

Figure S2. GPC trace of p(HPMA$_{70}$-DMAPMA$_{20}$-APMA$_{10}$).

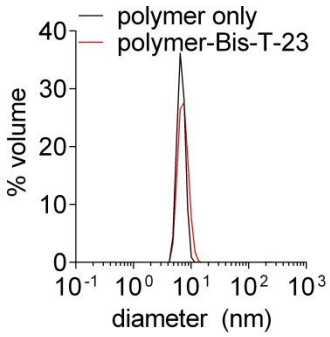

Figure S3. Characterization of polymer and polymer-Bis-T-23 conjugates by dynamic light scattering. A representative spectra of three independent reads is shown.

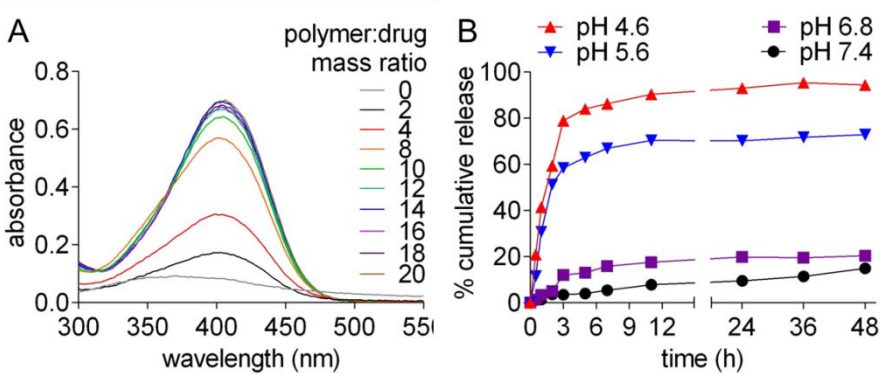

Figure S4. Characterization of FPBA copolymer behavior. **A.** Absorbance quantification of polymer-drug conjugates. **B.** Release profile of Bis-T-23 from polymer-drug conjugates at various pH.

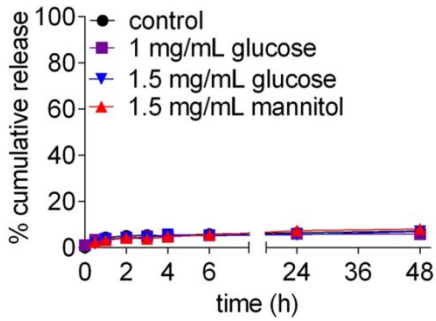

Figure S5. Bis-T-23 release from PBA copolymers in the presence of diols.

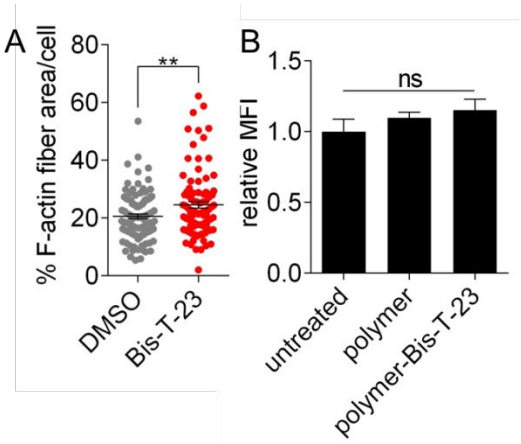

Figure S6. A. F-actin quantification of Bis-T-23-treated podocytes, expressed as percentage of area per cell. At least n = 98 individual cells were analyzed for each treatment. **B.** F-actin intensity quantification of treated podocytes, expressed relative to untreated cells. Bars represent means ± SEM. ***p*-value < 0.01.

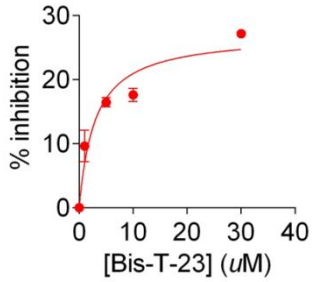

Figure S7. Bis-T-23 inhibition of albumin uptake in cultured podocytes.

CALCULATIONS

Given that a polymer:drug mass ratio of 14 is required to load all drug, the following calculation was used to determine the percent of boronic acids loaded with Bis-T-23:

Bis-T-23 MW = 480.43 g/mol
p(HPMA$_{70}$-*co*-DMAPMA$_{20}$-*co*-(APMA-*g*-PBA)$_{10}$) MW = 16,300 g/mol
per polymer, there are ~10 PBA groups
polymer:drug mass ratio = 14

$$\frac{1\ \mu g\ drug}{14\ \mu g\ polymer} \times \frac{mol\ drug}{480.43\ g\ drug} \times \frac{16300\ g\ polymer}{mol\ polymer} = 2.42 \frac{mol\ drug}{mol\ polymer}$$

each mol of polymer contains 10 PBA groups:

$$2.42 \frac{mol\ drug}{mol\ polymer} \times \frac{1\ mol\ polymer}{10\ mol\ PBA} = 0.242 \frac{mol\ drug}{mol\ polymer} = 24.2\%$$

REFERENCES

(1) Shankland, S. J.; Pippin, J. W.; Reiser, J.; Mundel, P., Podocytes in culture: past, present, and future. *Kidney Int* **2007**, *72* (1), 26-36.