Supporting Information

MMP9-sensitive polymers mediate environmentally-responsive bivalirudin release and thrombin inhibition

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1. Experimental Section

Materials

N-(2-hydroxypropyl) methacrylamide (HPMA) and *N*-(3-aminopropyl) methacrylamide hydrochloride (APMA) were purchased from Polysciences (Warrington, PA). The initiator VA-044 was purchased from Wako Chemicals (Richmond, VA). Bivalirudin-MMP9 substrate (BM9) peptide (Ac-(D)FPRPGGGGNGDFEEIPEEYLGGGPRQITAGGC-CONH2) was synthesized by Elim Biopharmaceuticals (Hayward, CA) at >95% purity, bivalirudin peptide (Ac-(D)FPRPGGGGNGDFEEIPEEYL-COOH) was bought from Bachem (Torrance, CA) at > 98% purity, succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) crosslinker from Pierce (Rockford, IL), hyaluronan (1.8 MDa) from Lifecore Biomedical (Chaska, MN), and methylcellulose (4000 cP) from Sigma-Aldrich (St. Louis, MO). All other materials were reagent grade or better and were purchased from Sigma-Aldrich unless otherwise stated.

MMP9-mediated cleavage of BM9

The susceptibility of bivalirudin-MMP9 substrate peptide BM9 to MMP-9-mediated enzymatic cleavage was first evaluated *in vitro*. BM9 peptide solution (25 µL, 3.52 mg/mL) in Reaction Buffer A (500 mM TrisHCl, 5 mM $CaCl_2$, 200mM NaCl, pH 7.7) was mixed with 5 µL of 0.1 mg/mL pre-activated MMP9 stock solution (EMD Millipore, Billerica, MA) and incubated at 37 ºC. At various time points, a 3 µL aliquot of the reaction solution was removed and mixed with 3 µL of 5 mM 1,10-phenanthroline to stop the enzymatic digest. Samples were analyzed using RP-HPLC on a Jupiter Proteo 90A analytical column (Phenomenex, Torrance, CA) following the fluorescence of tryptophan (ex/em 270/330 nm), and peptide fragmentation was determined by MALDI-TOF MS.

HPMA-*co*-APMA copolymer synthesis

Three polymers were synthesized: DP200, DP300, and DP400. Each polymer was synthesized with target degree of polymerization (DP) of 200, 300, and 400, respectively, with 10% mole feed of APMA. Monomers were dissolved in acetate buffer (1 M, pH 5.1) such that the final monomer concentration of the solution was 0.7 M. The RAFT chain transfer agent (CTA) used was ethyl cyanovaleric trithiocarbonate (ECT, molecular weight 263.4 g/mol) and the initiator (I) used was VA-044. The molar ratios of total monomer:CTA:I at the start of polymerization were 200:1:0.1,

300:1:0.1, and 400:1:0.1, respectively. The reaction solutions were transferred to round bottom flasks, capped with a rubber septum, purged with argon for 10 min, and the submerged in a 44 °C oil bath to initiate polymerization. The polymerization was allowed to proceed for 14 hrs. Polymers were dialyzed against DI H_2O and lyophilized.

HPMA -*co*-APMA end terminus capping

Polymers were end-capped to remove the trithiocarbonate terminal group. HPMA-*co*-APMA copolymers (DP200, DP300, DP400) were dissolved to a final concentration of 1 mM in DMF and solutions transferred to round bottom flasks. 40 eq of 2,2′-azobis(2-methylpropionitrile) (AIBN) was added to each reaction flask; flasks were capped and purged for 15 min under argon. Samples were incubated 70 ºC for 4 hrs. After 4 hrs, polymers were recovered by precipitation 3x in diethyl ether. Elimination of the terminal trithiocarbonate group was confirmed by loss of absorbance at 310 nm.

BM9 peptide grafting on HPMA-*co*-APMA copolymers

Peptides were grafted onto the HPMA-*co*-APMA copolymers via thiol-maleimide chemistry. First, HPMA-*co*-APMA copolymers were dissolved to a concentration of 1 mM in DMF. 2 eq of SMCC (relative to total primary amines) was added and reaction proceeded for 4 hrs at room temperature. Polymers were purified by precipitation in diethyl ether 3x.

BM9 peptide was dissolved in PBS, pH 6.5 and added as a 2 eq excess to maleimide-functionalized polymers. Thiol-maleimide reaction proceeded overnight at room temperature. Polymers were purified by dialysis for 4 days against 25 mM phosphate buffer, pH 6.5 and then against distilled H_2O for 3 days. Polymers were lyophilized dry.

Size exclusion chromatography

Molecular weight analysis was carried out by size exclusion chromatography. HPMA-*co*-APMA copolymers were dissolved at 2 mg/mL in running buffer (150 mM acetate buffer, pH 4.4) for analysis by size exclusion chromatography-multiangle laser light scattering (SEC-MALLS). Analysis was carried out on an OHpak SB-804 HQ column (Shodex, New York, NY) in line with a miniDAWN TREOS multiangle laser light scattering detector (Wyatt, Santa Barbara, CA) and an OptiLab rEX refractive index detector (Wyatt). HPMA-BM9 copolymers were dissolved at 2

mg/mL in running buffer (100 mM phosphate buffer, 300 mM NaCl, 0.05% NaN_3 pH 7.0) with analysis carried out on a TSK-Gel G3000SWXL column (Tosoh Bioscience, King of Prussia, PA). Absolute molecular weight averages (M_n, M_w) and polydispersity index (PDI) were calculated using ASTRA software (Wyatt).

Amino acid analysis

The incorporated amount of peptide and HPMA in the final copolymers was determined through modified amino acid analysis following the method of Bidlingmeyer and coworkers.[1] Briefly, HPMA-BM9 copolymers were hydrolyzed by reflux for 24 hrs at 110 ºC in 6N HCl. Hydrolyzed copolymers were derivatized with o-phthalaldehyde/β-mercaptopropionic acid and run on a ZORBAX Eclipse Plus C18 (Agilent Technologies, Santa Clara, CA) HPLC column with pre-column derivatization to label glycine and 1-amino-2-propanol (results from hydrolyzed HPMA). Calibration curves were generated using serial dilutions of glycine and reagent grade 1-amino-2-propanol.

MMP9-mediated polymer degradation

MMP9 mediated enzymatic release of bivalirudin peptide was determined. 25 µL of 5 mg/mL polymer solution in Reaction Buffer A (500 mM TrisHCl, 5 mM $CaCl_2$, 200mM NaCl, pH 7.7) was mixed with 5 µL of 0.1 mg/mL pre-activated MMP9 stock solution and incubated at 37 ºC. At various time points, a 5 µL aliquot of the reaction solution was removed and mixed with 5 µL of 5 mM 1,10-phenanthroline to stop the enzymatic digest. Samples were analyzed via SEC-MALLS following shifts in molecular weight distribution using the RI detector.

***In vitro* thrombin inhibition**

The thrombin inhibitory effects of BM9 peptide and HPMA-BM9 copolymers were assayed using a thrombin colorimetric activity assay. To 95.75 µL of 104.4 µM BM9 (or polymer equivalent) in thrombin reaction buffer (100 mM Tris, 150 mM NaCl, 0.1% PEG-8000, pH 7.5), 1 µL of thrombin (10 µg/mL) was added and the mixtures were incubated for 10 min at room temperature. After 10 min, 3.25 µL of thrombin substrate S-2238 (1 mg/mL) was added to each reaction and absorbance at 405 nm was read every minute for 30 minutes using a Tecan Safire^2 plate reader (Männerdorf, Switzerland).

To evaluate the effects of MMP9 treatment on HPMA-BM9 polymer activity, 9.5 μL of the HPMA-BM9 polymers was mixed with 2 μL of 0.1 mg/mL pre-activated MMP9 for 30 min at 37 °C; samples treated with HAMC (1.5% methylcellulose, 0.5% hyaluronic acid in HBSS HAMC) had an additional 0.5 μL of HAMC added. Following 30 min incubation, samples were diluted with 83.8 μL of thrombin reaction buffer and 1 μL of thrombin (10 μg/mL) was added. Samples were incubated for 10 min at room temperature and then 3.25 μL of S-2238 was added and the absorbance read at 405 nm every minute for 30 minutes.

Hydrogel release kinetics

Release kinetics of the HPMA-BM9 copolymers and the native bivalirudin peptide from HAMC hydrogels was evaluated. Polymers were dissolved at 10 mg/mL and bivalirudin peptide at an equivalent molar peptide concentration of 4.68 mg/mL in a cold 1.5% methylcellulose (w/v), 0.5% hyaluronic acid (w/v) HBSS solution and mixed thoroughly. In triplicate, 30 μL of each HPMA-BM9 hydrogel solution was aliquoted into microcentrifuge tubes and incubated at 37 ºC overnight to ensure complete gelation. 300 μL of pre-warmed HBSS was added to each tube and tubes were incubated at 37 ºC. At various time points, 30 μL of HBSS was removed and 30 μL of fresh pre-warmed HBSS was added.

Acid-catalyzed hydrolysis of polymers followed by amine concentration determination via Fluoraldehyde assay (Pierce) was used to quantitate release. 15 μL of each sample was dissolved in 200 μL of 6N HCl, and refluxed at 110 ºC for 24 h. Hydrolyzed samples were dried using a SpeedVac (ThermoFisher Scientific, Waltham, MA) and resuspended in 100 μL of 10 mM borate, 10 mM phosphate, 0.05% sodium azide pH 8.2. To assay, 10 μL of sample was mixed with 100 μL of Fluoraldehyde reagent in 96 well round-bottom black plates. Samples were incubated in the dark for 4 min at room temperature and then fluorescence read using a plate reader at ex/em 360/456 nm.

Spinal cord contusion injury model

All animal work was completed using approved procedures and following the strict guidelines from the University of Washington IACUC. Spinal cord contusion injuries were performed on adult female Long-Evans rats as previously described.[2] Briefly, animals were anesthetized with ketamine/xylazine and a laminectomy was performed at the C4 spinous process of the lamina

ipsilateral to the animal's dominant paw. The animals were then placed in a spinal frame and a contusion injury was conducted using a fourth generation Ohio State Injury Device. An electromagnetically controlled probe (0.7 mm end diameter, Ling Dynamics, Inc) was lowered to the surface of the cord just lateral to midline. The probe was oscillated on the surface of the spinal cord to achieve a common starting force of 3000 dynes for all animals. The spinal cord was displaced 0.8 mm for 14 ms to induce the injury. One hr post-injury, 1 μL (10 mg/mL) of either DP400 HPMA-BM9 copolymer or DP400 APMA-HPMA copolymer physically encapsulated in a 1.5% methylcellulose, 0.5% hyaluronic acid hydrogel (in HBSS) was administered to the site of injury. The surgical site was closed by suturing muscle in layers and closing skin with wound clips.

Quantification of proliferating cells

Bromodeoxyuridine (BrdU) incorporation is a commonly used as a marker for mitotically active cells. Following hydrogel administration, a single injection of BrdU (50 mg/kg, Sigma) was administered intrapertoneally. 24 hrs after injection, animals were anesthetized and perfused with saline and then 4% paraformaldehyde in 0.1M phosphate buffer. Spinal cords were removed, post-fixed overnight and transferred to 30% sucrose for cryopreservation. 1 mm coronal sections surrounding the lesion were cut, embedded in OCT medium, and flash frozen. 20 μm sections were cut and stored at -80C.

For the stereological quantitation of BrdU-labeled cells, cryostat sections were stained for diaminobenzadine (DAB) immunohistochemistry as previous reported.[3] Briefly, sections were pretreated with 50% formamide in 2x saline-sodium citrate (SSC), 2x SSC, 2N HCl, 0.1 M borate buffer, and then rinsed six times with TBS. Nonspecific labeling was blocked with TBS + 0.1% Triton X-100 and 3% normal donkey serum. A monoclonal mouse antibody against BrdU (1:400, Boehringer Mannheim, Indianapolis, IN) was incubated with the tissue for 2 d at 4 ºC. After primary antibody incubation, sections were quenched with 0.6% H_2O_2 in TBS and tissue rinsed in TBS before incubation with a polyclonal donkey α mouse IgG secondary antibody (Accurate Chemicals, Westbury, NY; 1:200 in TBS). Sections were then rinsed with TBS and incubated with avidin-biotin complex (ABC-Elite; Vector Laboratories, Burlingame, CA). BrdU labeling was visualized using DAB (0.25 mg DAB, 0.009% H_2O_2, and 0.04% NiCl in TBS). DAB incubation was terminated by a tap water rinse, and slides were then dehydrated through alcohols and

coversliped with permount. BrdU nuclei were excluded from the study if they exhibited punctate staining in part of the nucleus or the DNA appeared to be condensed.

Quantification of astrocytes

30 days post-injury, rats were sacrificed, spinal cords harvested, and sections processed as discussed above. To stain for astrocytes, sections were treated with rabbit αS-100β (1:10,000; Swant, Bellinzona, Switzerland) for 2 days at 4 ºC in TBS + 0.1% Triton X-100 + 5% donkey serum. Sections were rinsed twice with TBS and once with TBS + 0.1% Triton X-100 for 15 min. Sections were treated with secondary antibody donkey α rabbit conjugated to Cy5 (1:500; Jackson ImmunoResearch) for 1-2 hrs with 0.1% Tween-20. Sections were washed three times for 15 min with TBS. Slides were immediately coversliped and imaged. Cells populations within the spinal cord and lesion epicenter were calculated via fractionator Stereology (an unbiased sampling method) by Stereo Investigator (Microbrightfield, Inc.). A grid size of 400x400 μm and counting frame of 75x75 μm was used to assure unbiased sampling of a randomized grid in a 1 in 6 series of tissue sections to generate averaged populations for each animal.

Limb-use asymmetry test

The limb-use asymmetry test (LUAT) was used to assess forelimb preference during vertical exploration in a clear Plexiglas cylinder. Animals were scored by blinded-observers on independent and simultaneous use of their left and right forelimbs when rearing to make wall contacts.[4] Each session lasted until an animal made 20 wall contacts. To determine paw preference, an asymmetry score was calculated for each test session, asymmetry = [(affected forelimb) + .5(both forelimbs)] / 20, with a score below 0.5 indicating preference for the unaffected forelimb. No training is required for this test, and all animals prior to injury showed no paw preference (average 0.4875 ± 0.08 SD).

IBB forelimb usage test

Forelimb function was tested using the Irvin, Beatties and Bresnahan (IBB) forelimb scale to assess recovery of different forelimb function such as joint position, object support, digit movement and grasping technique.[5] To do this animals are video taped while eating a spherical and doughnut shaped piece of cereal that are of similar size. The testing environment is a clear Plexiglas cylinder

that is 19.5cm in diameter and 30cm in height and a mirror is placed around the cylinder, which allows for filming of an animal from any side. The week prior to injury each animal was filmed while eating each piece of cereal to determine an uninjured IBB score. Animals were then filmed only once a week for four weeks following their SCI. IBB videos are scored by evaluation of digit usage (in slow-motion) by an observer that was blinded to the animal's experimental group. Forelimb function was scored according to a 9-point scale to assess forelimb position and movement, volar support, wrist movement, and digiti usage for cereal adjustment after SCI.

References

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Table S1. Molecular weights of peptide fragments

Sequence	MW (Da)
Ac-(D)F-PRPGGGGNGDFEEIPEEYLGGG*PRQITAG*GC-CONH2	3275
Ac-(D)F-PRPGGGGNGDFEEIPEEYLGGGPRQ-COOH	2774
Ac-(D)F-PRPGGGGNGDFEEIPEEYLGGGP-COOH	2490

*Bivalirudin sequence is bolded; MMP9 linker sequence is italicized

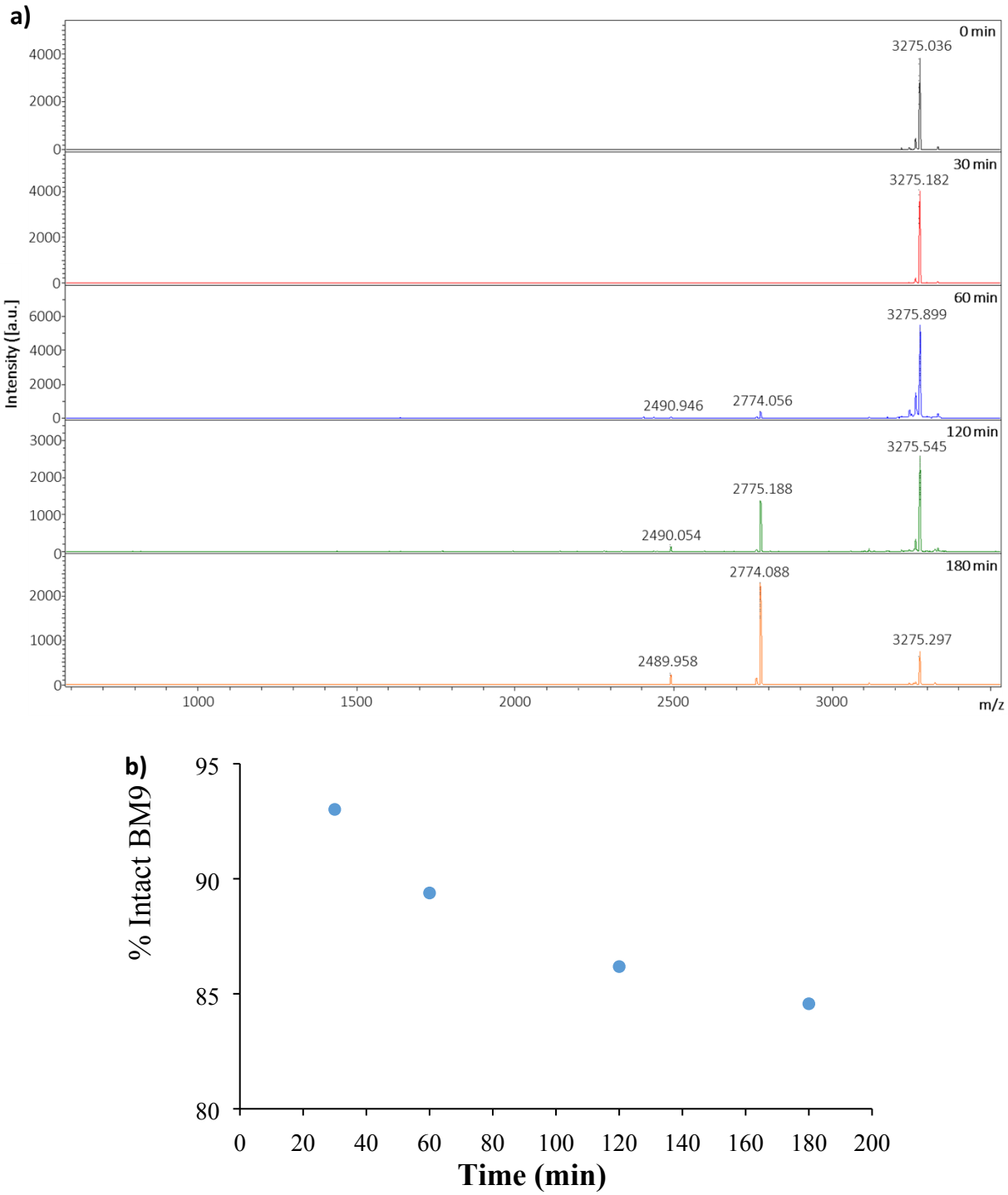

Figure S1. (a) MALDI-TO MS time-point study of MMP9-mediated peptide digest of BM9. (b) Kinetics of MMP9 digest of BM9 peptide by tyrosine fluorescence via RP-HPLC.

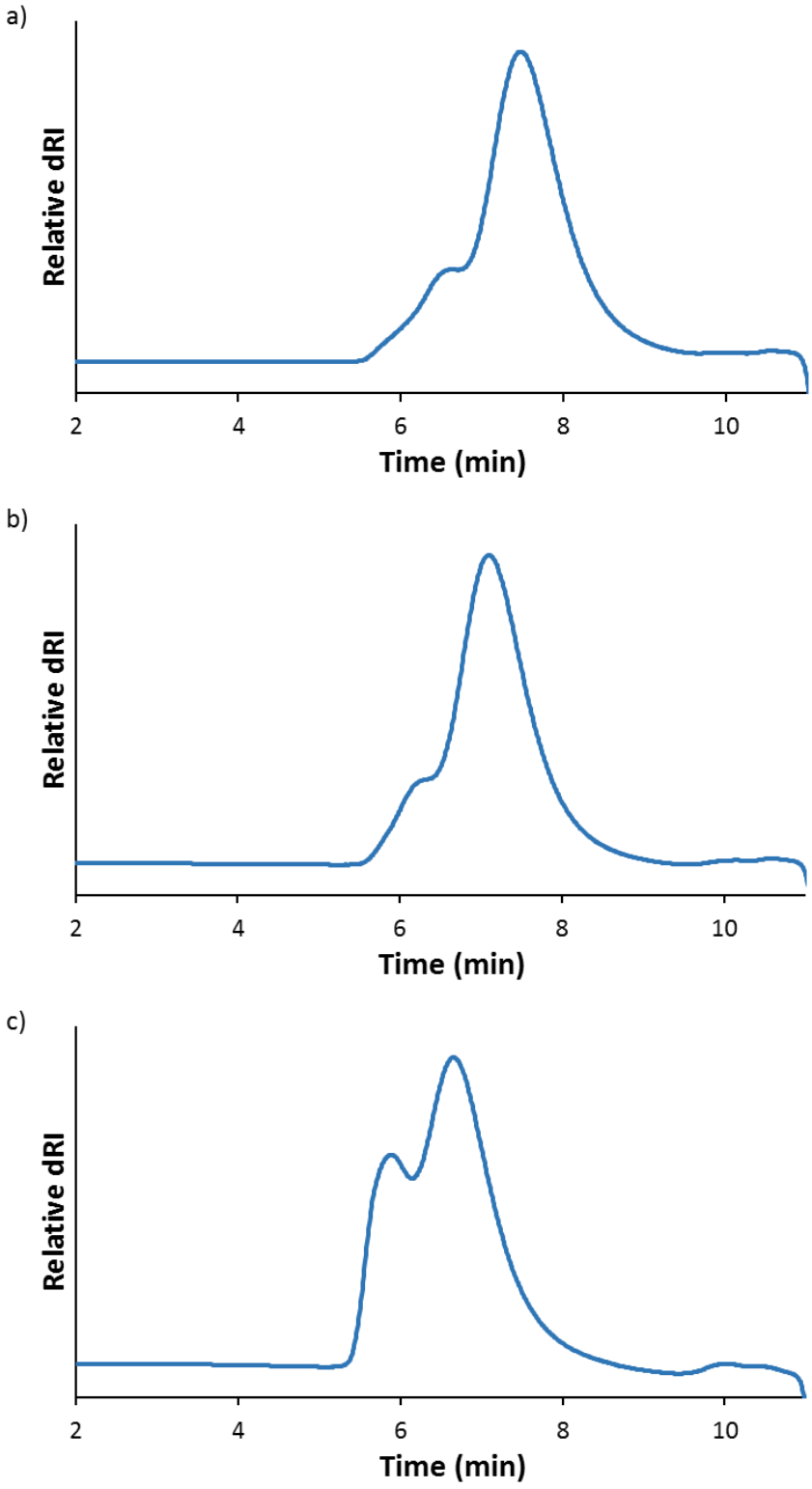

Figure S2. Gel-permeation chromatography traces of (a) DP200, (b) DP300, and (c) DP400 copolymers.

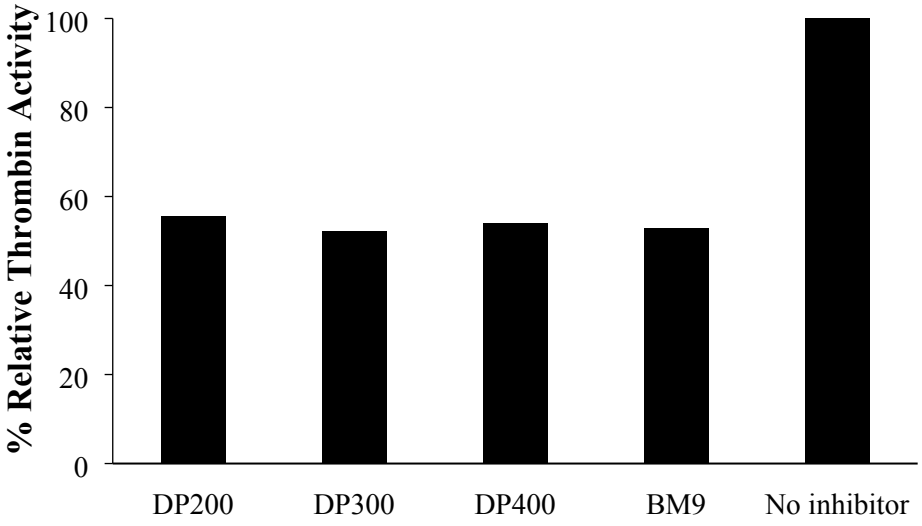

Figure S3. Initial relative thrombin activity measured by colorimetric substrate S-2238.

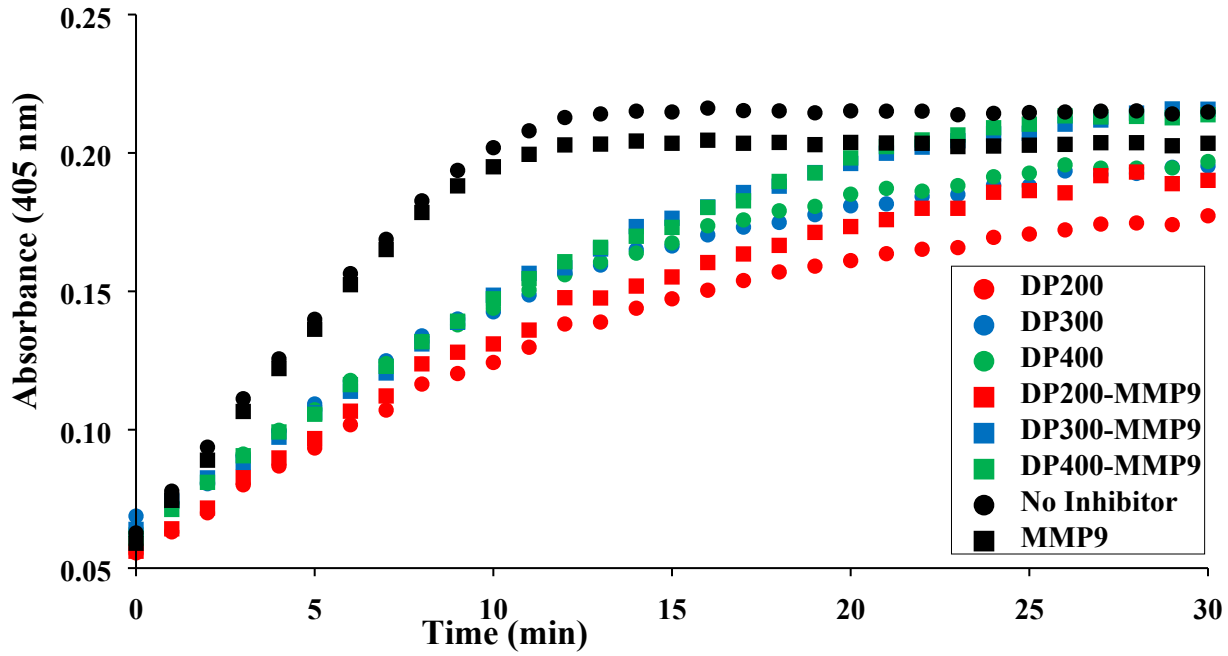

Figure S4. Thrombin activity assay as measured by colorimetric development of S-2238 substrate for polymers that either untreated with MMP9 (○) or treated with MMP9 for 30 min prior to addition of thrombin (□)

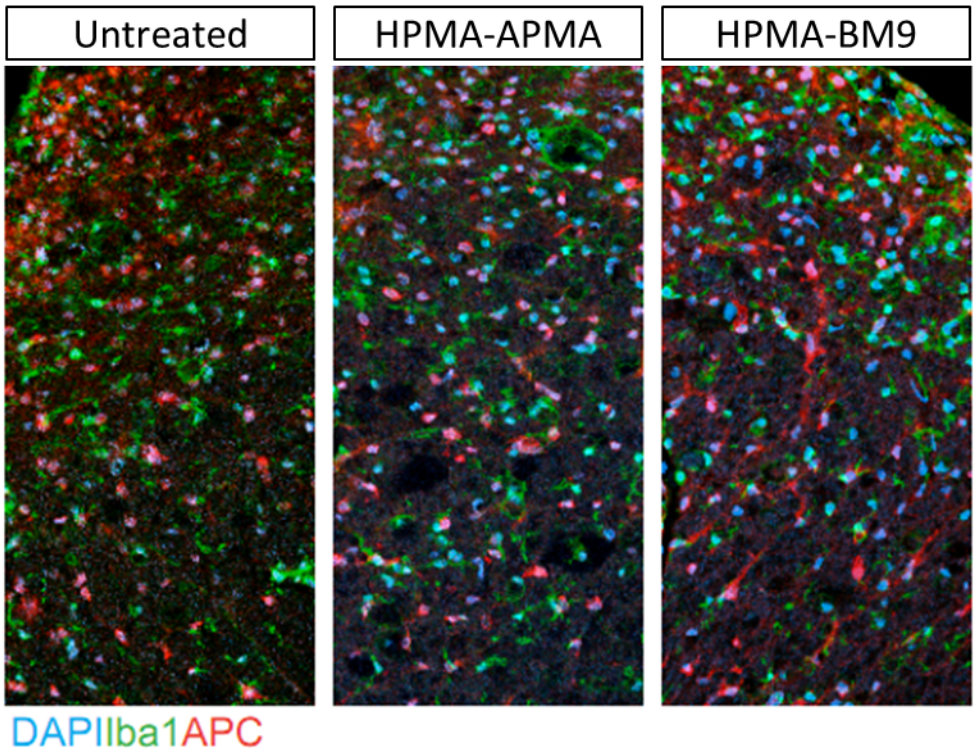

Figure S5. Confocal images of spinal cord sections at the lesion 30 days post-injury stained for microglia (Iba1), immature oligodendrocytes (APC), and nuclei (DAPI).

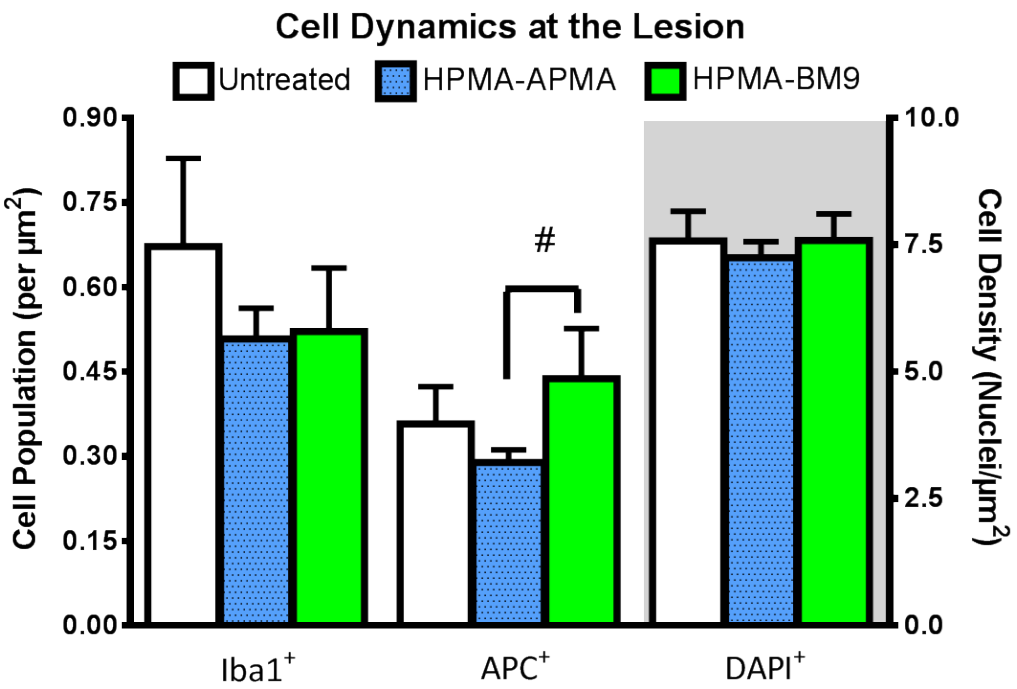

Figure S6. Iba^{+} (microglia), APC^{+} (immature oligodendrocytes), and DAPI^{+} (all) cells per area at the lesion 30 days post-injury. $^{\#}p < 0.08$. Error bars = standard error.

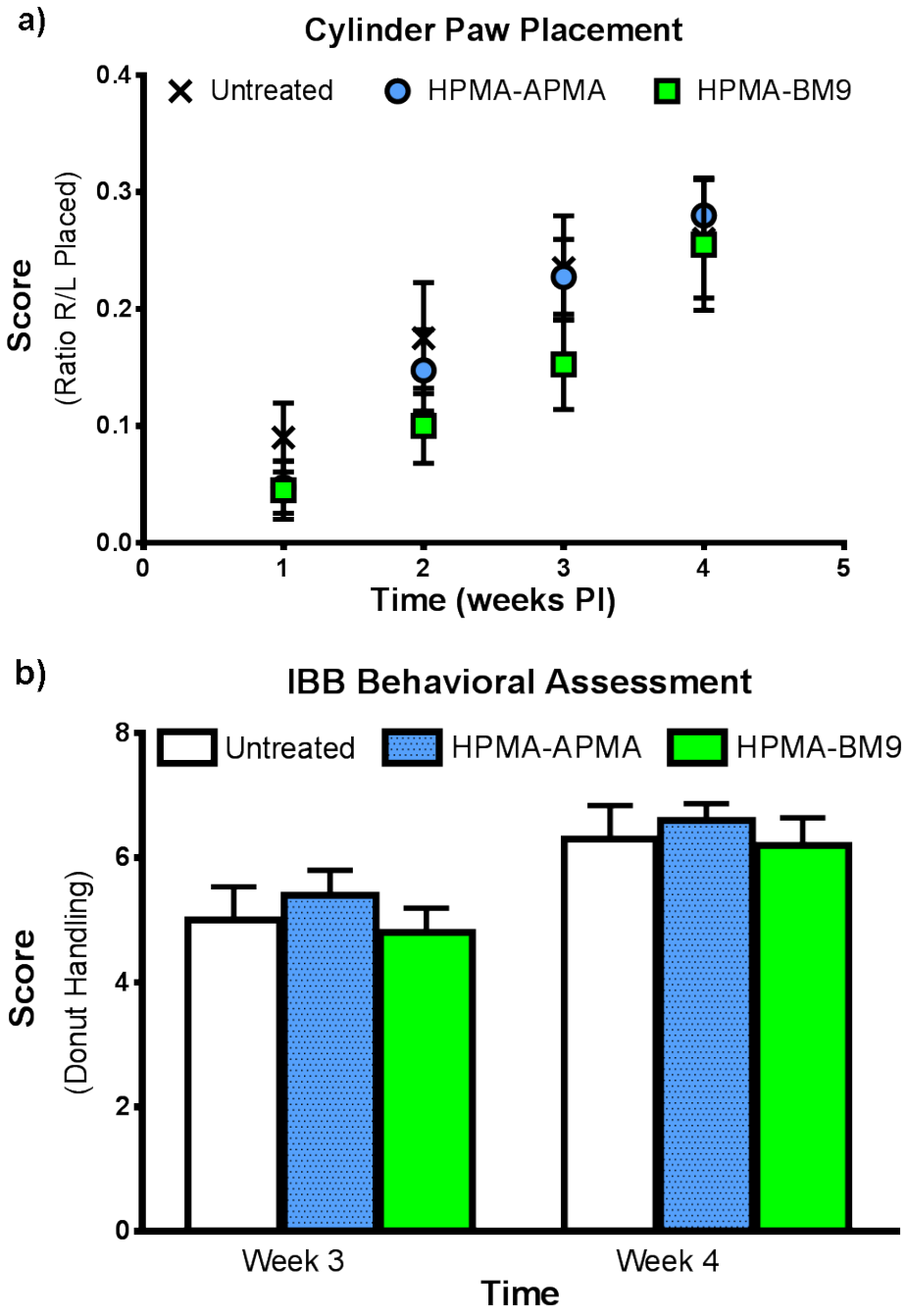

Figure S7. Behavioural recovery of treated rats following injury studied via (a) limb-use asymmetry tests to assess forelimb preference and (b) forelimb function via the Irvin, Beatties and Bresnahan (IBB) forelimb scale.