Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting Information

Poly(Piloty's Acid): A Slow Releasing Macromolecular HNO Donor

Sarah N. Swilley,[a] Evan M. Zajkowski[a], and John B. Matson*[a]

[a]Department of Chemistry, Virginia Tech Center for Drug Discovery, Macromolecules Innovation Institute, 1040 Drillfield Dr., Blacksburg, VA 24061, USA.

Materials and Methods

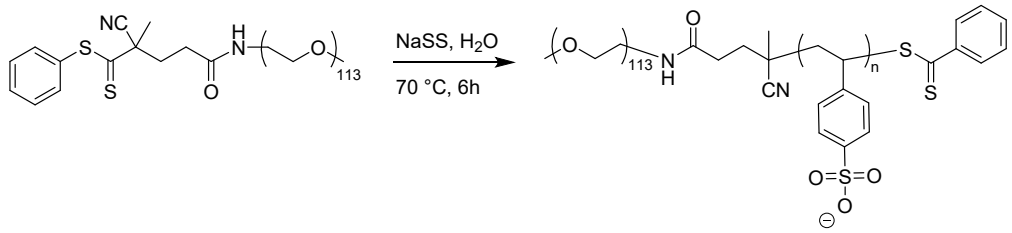
All reagents and solvents were obtained from commercial vendors and used as received unless otherwise stated. NMR spectra were measured on Agilent 400 MHz or Bruker 500 MHz spectrometers. ^{1}H and ^{13}C NMR chemical shifts are reported in ppm relative to internal solvent resonances. Yields refer to compounds as isolated after requisite purification unless otherwise stated. Dialysis was performed using Vivaspin 15R Centrifugal Filtration Units, Sartorius (Mwco 5 kg/mol). Thin-layer chromatography (TLC) was performed on glass-backed silica plates and visualized by UV. Fluorescence experiments were conducted on a BioTek Cytation3 imaging reader (specific parameters defined below).

Chemicals: 5 kg/mol poly(ethylene glycol monomethyl ether) (PEG-MME) (Aldrich), mesyl chloride (MsCl) (BeanTown), triethylamine (NEt_3) (MilliPore), 25% ammonia solution (VWR), bromobenzene (Aldrich), iodine (I_2) (Wards), carbon disulfide (CS_2) (MilliPore), magnesium (Mg) (Macron), 4,4′-azobis(4-cyanovaleric acid) (ACVA) (BeanTown), *N*-hydroxysuccinimide (NHS) (Chemimpex), dicyclohexyl carbodiimide (DCC) (Oakwood), sulfonated styrene (NaSS) (Sigma), thionyl chloride ($SOCl_2$) (TCI), hydroxylamine hydrochloride (NH_2OH-HCl) (BeanTown), magnesium oxide (MgO) (Acros), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Chemimpex), dimethylamino pyridine (DMAP) (Matrix), sodium sulfate (Na_2SO_4) (Wards), 7-hydroxycoumarin (7-HC) (Oxchem), diphenyl phosphino benzoic acid (Alfa Aesar), hydrochloric acid (HCl) (Ward's), sodium hydroxide (NaOH) (Macron), azobisisobutyronitrile (AIBN) (Matrix Scientific), 2-(diphenylphosphino)benzoic acid Alfa Aesar, and Piloty's acid (Cayman Chemical).

Synthetic Procedures

The PEG_{110} macroCTA was prepared as previously reported by Armes.[1] The HNO Probe (P-CM) was synthesized as previously reported by Yu.[2]

PEG_{110}-*b*-PSS_n Polymerization



In a typical experiment, 2 g macroCTA (0.0278 mmol, 1 equiv), 213 mg ACVA (0.0556 mmol, 0.2 equiv), and 3.7 g sulfonated styrene (5.6 mmol, 20 equiv) were dissolved in H_2O (10 mL) in a Schenk flask. The solution was subjected to freeze-pump-thaw cycles until bubbling ceased completely (typically ~5 cycles). The flask was then placed in an oil bath at 70 °C for ~4 h, or until the polymerization reached 50% conversion as monitored by ^{1}H NMR spectroscopy to give **PEG_{110}-*b*-PSS_6**. The reaction mixture was allowed to cool and then directly transferred to a

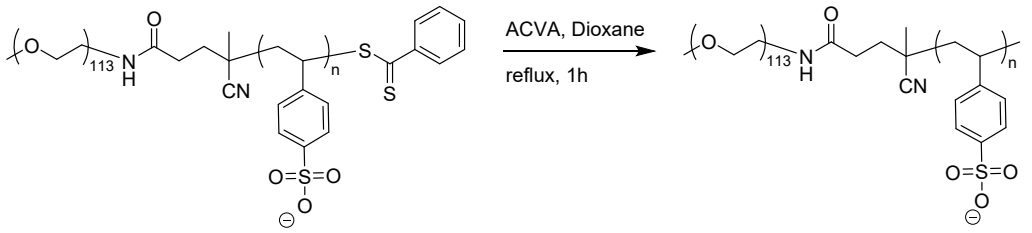
dialysis tube and dialyzed for 24 h against water. After dialysis the polymer was dried via lyophilization.

PEG_{110}-*b*-PSS_{15} was prepared in a similar manner using 2 g macroCTA (1 eq), 2.12 mg ACVA (0.2 eq), 3.76 g NaSS (50 eq), and 30 mL H_2O. Once the polymerization reached ~50% conversion, the polymer was purified as stated above.

PEG_{110}-*b*-PSS_{36} was prepared in a similar manner using 2 g of macroCTA (1 eq), 21.3 mg of ACVA (0.2 eq), 3.76 g of NaSS (70 eq), and 40 mL of H_2O. Once the polymerization reached ~54% conversion, the polymer was purified as stated above.

PEG_{110}-*b*-PSS_{57} was prepared in a similar manner using 2 g of macroCTA (1 eq), 15.9 mg of ACVA (0.2 eq), 9.4 g of NaSS (120 eq), and 50 mL of H_2O. Once the polymerization reached ~85% conversion, the polymer was purified as stated above.

End Group Removal



In a typical procedure, 800 mg PEG-*b*-PSS_6 (0.1 mmol, 1 equiv) and 0.56 mg of ACVA (2 mmol, 20 equiv) were dissolved in dioxane (5 mL) and refluxed at 80 °C for 1.5 h. After cooling to rt, the polymer was isolated by precipitation of the reaction mixture into a large volume of cold Et_2O. End groups were removed for each of the three other **PEG-*b*-PSS_n** block copolymers using the same procedure.

PEG_{110}-*b*-PSS_6: NMR (D_2O): δ 7.94-7.14 (s, 21 H), 7.14-6.0 (s, 19 H), 3.63-3.65 (s, 450 H) 3.24-3.21 (s, 3 H), 2.40-0.55 (s, 37 H).

PEG_{110}-*b*-PSS_{15}: NMR (D_2O): δ 7.41-7.13 (s, 34 H), 6.85-5.92 (s, 39 H), 3.63-3.65 (s, 450 H) 3.24-3.21 (s, 3 H), 2.23-3.85 (s, 53 H).

PEG_{110}-*b*-PSS_{36}: NMR (D_2O): δ 7.64-7.11 (s, 57 H), 6.85-6.02 (s, 57 H), 3.63-3.65 (s, 450 H) 3.24-3.21 (s, 3 H), 2.10-1.00 (s, 78 H).

PEG_{110}-*b*-PSS_{57}: NMR (D_2O): δ 7.67-7.02 (s, 133 H), 6.87-6.07 (s, 164 H), 3.63-3.65 (s, 450 H) 3.24-3.21 (s, 3 H), 1.90-0.90 (s, 183 H).

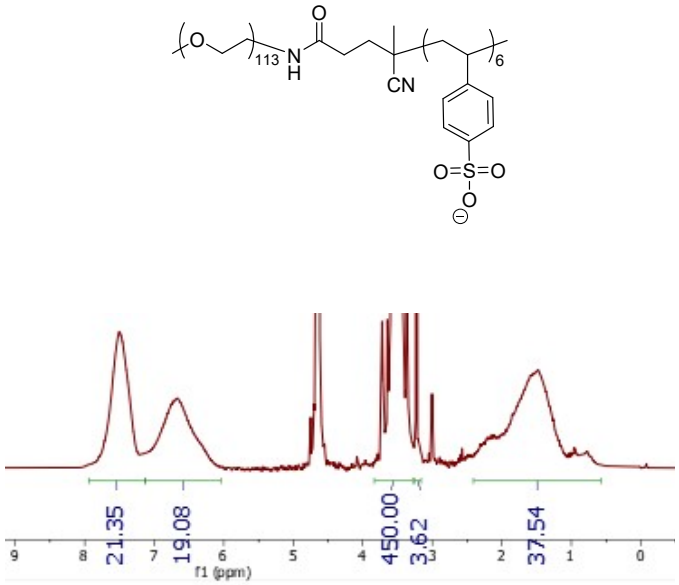


Figure S1. ^{1}H NMR spectrum (D_2O) of **PEG_{110}-*b*-PSS_6** after dialysis and endgroup removal.

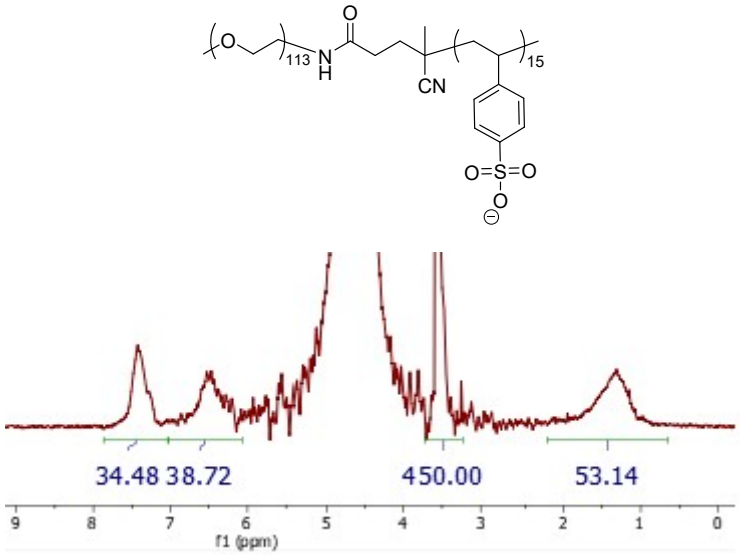


Figure S2. ^{1}H NMR spectrum (D_2O) of **PEG_{110}-*b*-PSS_{15}** after dialysis and endgroup removal.

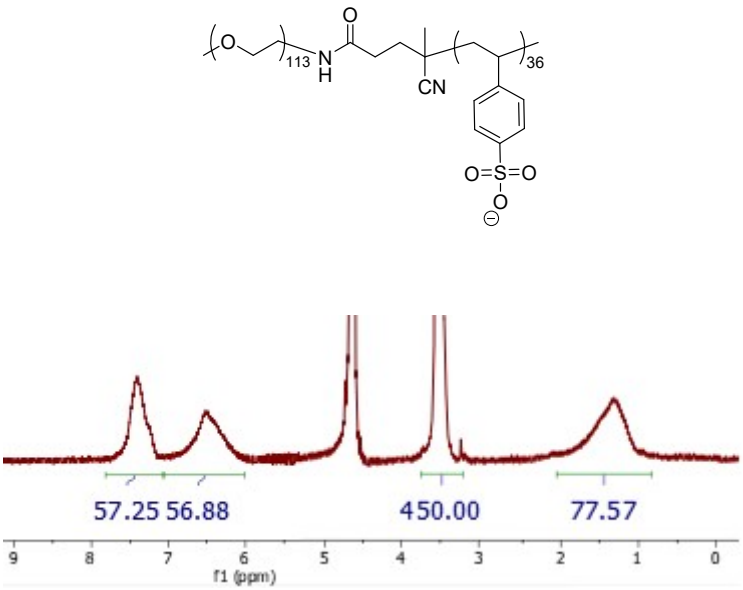


Figure S3. ^{1}H NMR spectrum (D_2O) of **PEG_{110}-*b*-PSS_{36}** after dialysis and endgroup removal.

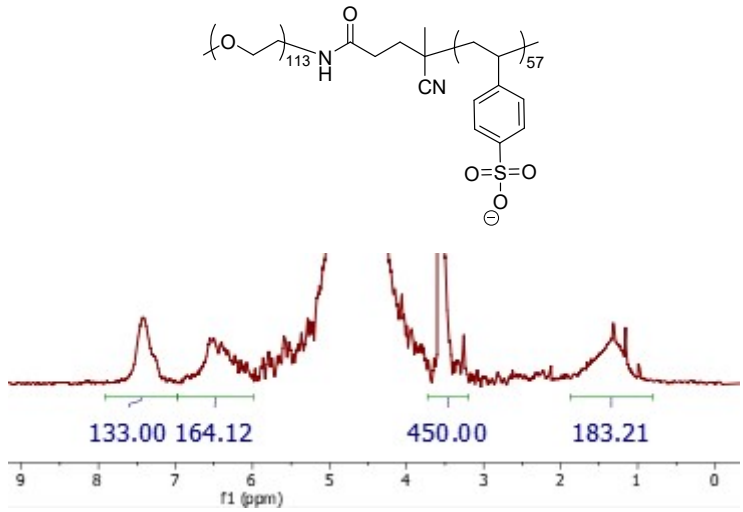
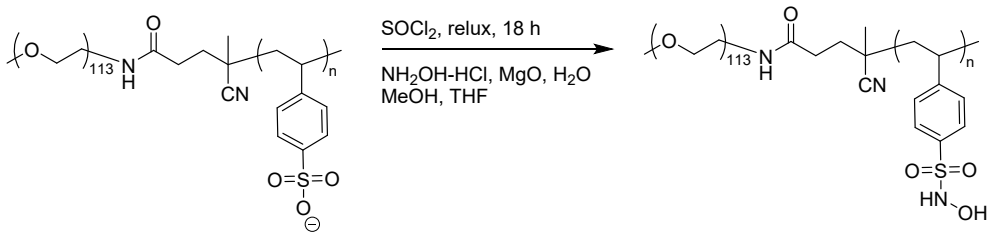


Figure S4. ^{1}H NMR spectrum (D_2O) of **PEG_{110}-*b*-PSS_{57}** after dialysis and endgroup removal.

Conversion of PEG_{110}-*b*-PSS to PEG_{110}-*b*-PPA



To convert the **PEG_{110}-*b*-PSS** block copolymers into **PEG_{110}-*b*-PPA** block copolymers we modified a literature procedure for the synthesis of small molecule PA.[3] In a typical procedure, **PEG_{110}-*b*-PSS_6** (10 mg, 0.133 mmol of sulfonate groups) was dissolved in $SOCl_2$ (15 mL) and refluxed at 70 °C overnight. Excess $SOCl_2$ was removed under vacuum by utilizing a secondary trap connected to a Schlenk line and quenched after isolation by slowly adding water until bubbling ceased. The solids left over after $SOCl_2$ removal were then dissolved in dry DCM and dried via rotary evaporation; this dissolution and rotary evaporation process was repeated 10 times to ensure the complete removal of $SOCl_2$.

Next, NH_2OH-HCl (18 mg, 0.266 mmol, 2 equiv) was dissolved in CH_3OH (0.3 mL) and H_2O (0.2 mL) along with MgO (11 mg, 0.266 mmol, 2 equiv). The dried polymer was dissolved in THF (0.5 mL) and added dropwise to the NH_2OH-HCl solution. Next, a second portion of MgO (5.5 mg, 0.133 mmol, 1 eq) was added all at once, upon which the solution turned cloudy. The reaction mixture was stirred for 16 h, at which point excess organic solvent was removed by rotary evaporation. The solids were then dissolved in H_2O and filtered to remove any remaining MgO before transferring the filtrate into dialysis tubing and dialyzing for 24 h against water. After dialysis, the polymer was dried via lyophilization. All other **PEG_{110}-*b*-PPA_n** block copolymers were synthesized in a similar manner.

Elemental Analysis:

Elemental analysis was performed by Midwest Microlabs. We chose to utilize ratios between C/S, C/N, and S/N due to the hygroscopic nature of these polymers.

Table S1: Elemental analysis of **PEG_{110}-*b*-PSS_n** and **PEG_{110}-*b*-PPA_n** block copolymers.

Sample	%C	%H	%N	%S
PEG_{110}-*b*-PSS_6	53.70	0.17	1.53	4.11
PEG_{110}-*b*-PSS_{15}	38.64	4.93	0.30	4.65
PEG_{110}-*b*-PSS_{36}	36.67	4.08	0.09	6.78
PEG_{110}-*b*-PSS_{57}	41.96	4.61	0.23	9.69
PEG_{110}-*b*-PPA_6	49.78	7.55	2.07	5.12
PEG_{110}-*b*-PPA_{15}	38.27	5.93	2.51	6.25
PEG_{110}-*b*-PPA_{36}	38.10	5.17	1.66	8.50
PEG_{110}-*b*-PPA_{57}	29.18	4.73	2.32	7.19

Data reported from Midwest Microlabs; limit of detection for C/H/N is 0.15% and S is 0.4%; measurements are accurate to 0.3%.

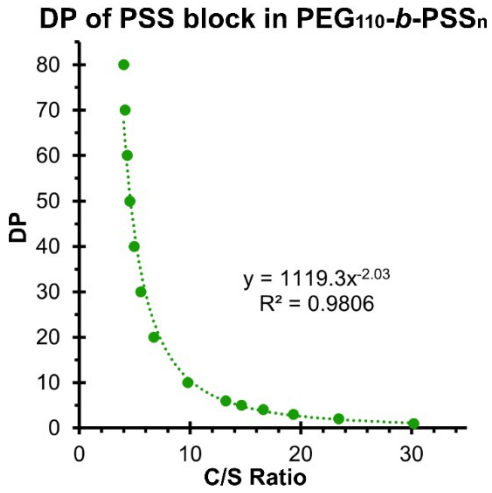


Figure S5: C/S ratios (by mass) based on molecular formulas determined by varying DPs of PSS in the block copolymer PEG_{110}-*b*-PSS_n; the equation of the line from this graph was utilized to determine the DPs reported in Table S2.

Table S2: DP of **PEG_{110}-*b*-PSS_n** polymers determined based on observed (experimental) C/S ratios

Sample	Observed C/S ratio[a]	DP[b]
PEG_{110}-*b*-PSS_6	13.1	6.1
PEG_{110}-*b*-PSS_{15}	8.3	15.2
PEG_{110}-*b*-PSS_{36}	5.4	36.4
PEG_{110}-*b*-PSS_{57}	4.3	57.1

[a]Observed C/S ratio was determined from the experimental data received from Midwest Microlabs in Table 1. [b]Estimated DP of each PSS block based on C/S ratios. Each observed C/S ratio in the table above was input into the equation of the line in the graph in Fig. S8 in order to solve for DP.

FTIR Analysis

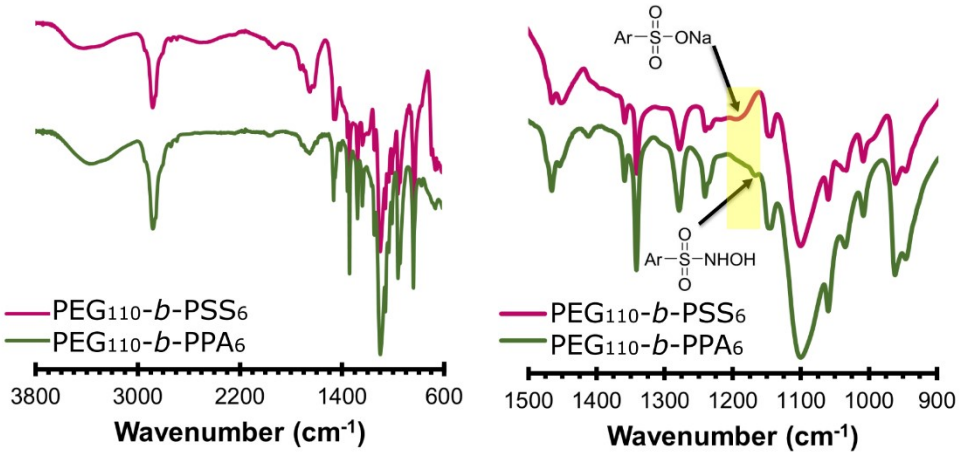


Figure S6. IR spectra of **PEG_{110}-*b*-PSS_6** (pink) and **PEG_{110}-*b*-PPA_6** (green); left: full spectrum of before and after conversion to PPA. Right: Zoomed-in spectrum highlighting the conversion of the sulfate (1180 cm^{-1}) to sulfonamide (1160 cm^{-1}) peaks.

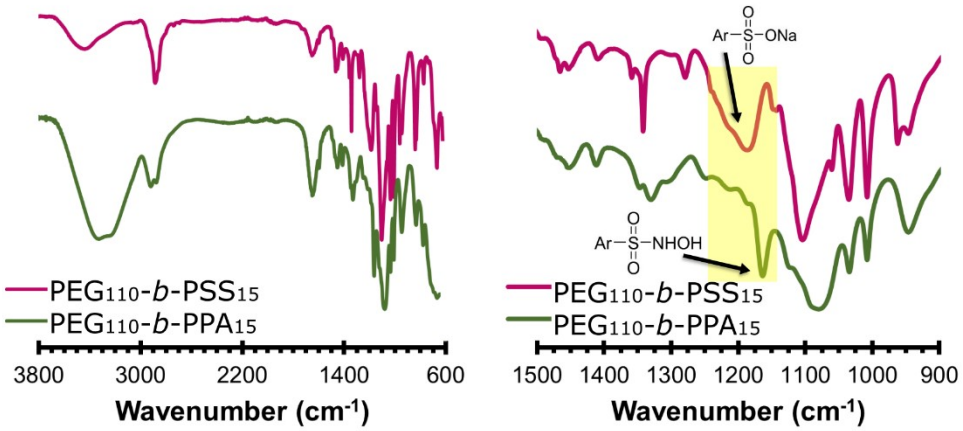


Figure S7. IR spectra of **PEG_{110}-*b*-PSS_{15}** (pink) and **PEG_{110}-*b*-PPA_{15}** (green); left: full spectrum of before and after conversion to PPA. Right: Zoomed-in spectrum highlighting the conversion of the sulfate (1180 cm^{-1}) to sulfonamide (1160 cm^{-1}) peaks.

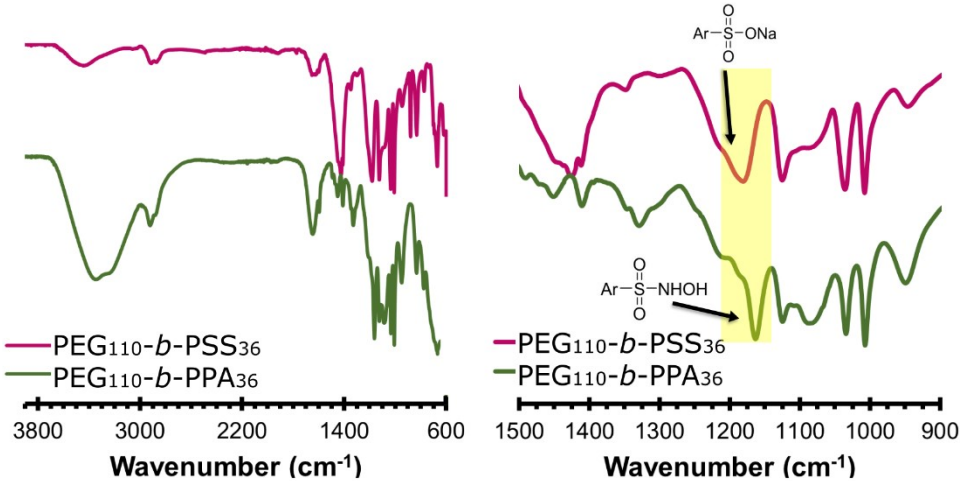


Figure S8. IR spectra of **PEG_{110}-*b*-PSS_{36} (pink)** and **PEG_{110}-*b*-PPA_{36}** (green); left: full spectrum of before and after conversion to PPA. Right: Zoomed-in spectrum highlighting the conversion of the the sulfate (1180 cm^{-1}) to sulfonamide (1160 cm^{-1}) peaks.

Elemental Analysis:

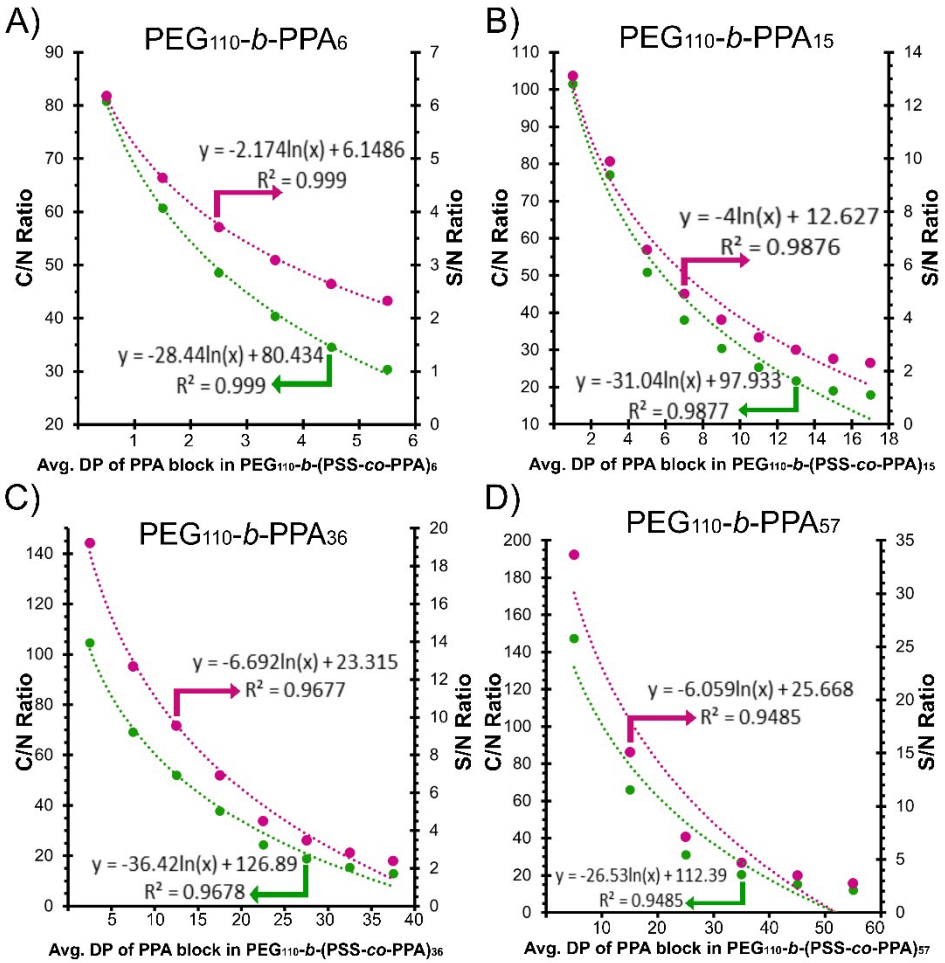


Figure S9: C/N (left axis) and S/N (right axis) ratios (by mass) based on molecular formulas determined by varying DPs of PPA (n=6, 15, 36, or 57 in panels A-D, respectively). The equations of the lines from these graphs were utilized to determine the DP of the PPA block (i.e., how many PSS units were converted into PPA units) as explained in Table S3.

Table S3: Percent functionalization (funct.) of **PEG_{110}-*b*-PPA_n** polymers determined via C/N and S/N ratios.

Sample	Found C/N ratio[a]	% Funct. based on C/N ratio[b]	Found S/N ratio[a]	% Funct. based on S/N[b]	% Funct. (avg)[c]
PEG_{110}-*b*-PPA_6	24.0	119%	2.47	89%	104%
PEG_{110}-*b*-PPA_{15}	15.2	94%	2.49	83%	89%
PEG_{110}-*b*-PPA_{36}	23.0	48%	5.12	42%	53%
PEG_{110}-*b*-PPA_{57}	12.6	75%	3.10	73%	74%

[a]Both the C/N and S/N ratios were calculated from the data provided from Midwest Microlabs. [b]Percent functionalization was determined by inputting either C/N or S/N ratio into the equations of the lines for the corresponding block copolymers in Fig. S9. From this, the DP of the PPA block was determined, and by subtracting this value from the calculated DP in Table S2, we were able to determine how many repeat units were unreacted PSS. Finally, by comparing the ratio of PPA/PSS we determined the degree of functionalization. [c]The average percent functionalization was determined by averaging both of the found percent functionalization values together.

HNO release with P-CM probe:

Fluorescence measurements were carried out on a BioTek Cytation3 imaging reader with the following parameters: fixed excitation 370 nm, emission start/stop 400 nm-700 nm, optics top, gain 50, lamp energy high, read speed normal, delay 100 msec, read height 7 mm.

First a stock solution of P-CM was prepared with 4.5 mg dissolved in 2.5 mL of DMF to reach a final concentration of 4 mM P-CM. Next, to 4.388 mL of buffer (type and strength noted below) was added 112.5 μL of the P-CM solution to reach a final concentration of 100 μM of P-CM; 225 μL of this solution was then added to a 1 mL centrifuge tube. Each of the PPA polymers were dissolved individually in MilliQ water to obtain a final concentration of 2 mM Piloty's acid functional groups. Next, 25 μL of each polymer solution was added to the P-CM solution to reach a final concentration of 200 μM Piloty's acid, and these solutions were vortexed. Finally, 200 μL of each of the final solutions was pipetted onto a 96-well plate. For polymers without probe, each solution was prepared similarly, but with pure buffer instead of the buffer+P-CM stock solution. All **PEG_{110}-*b*-PPA_n** polymers were analyzed at rt in pH 7.4 PBS, pH 8, 9, and 10 boric acid, and pH 11 piperidine buffers at 20 mM. All **PEG_{110}-*b*-PSS_n** polymers were analyzed at rt in pH 10, 100 mM boric acid buffer (the sulfonate groups were not completely buffered at lower buffer concentrations).

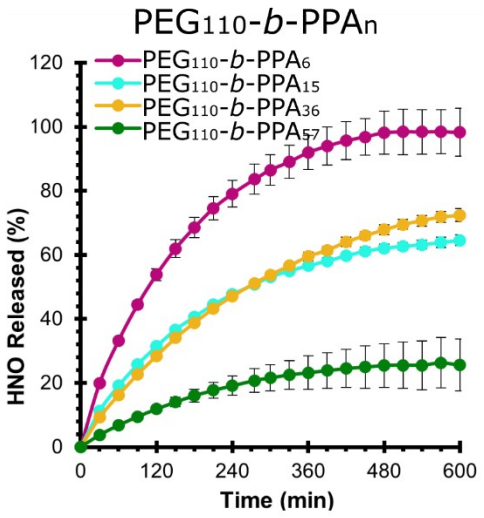


Figure S10. Release of **PEG_{110}-*b*-PPA_n** polymers in 20 mM boric acid buffer (pH 10).

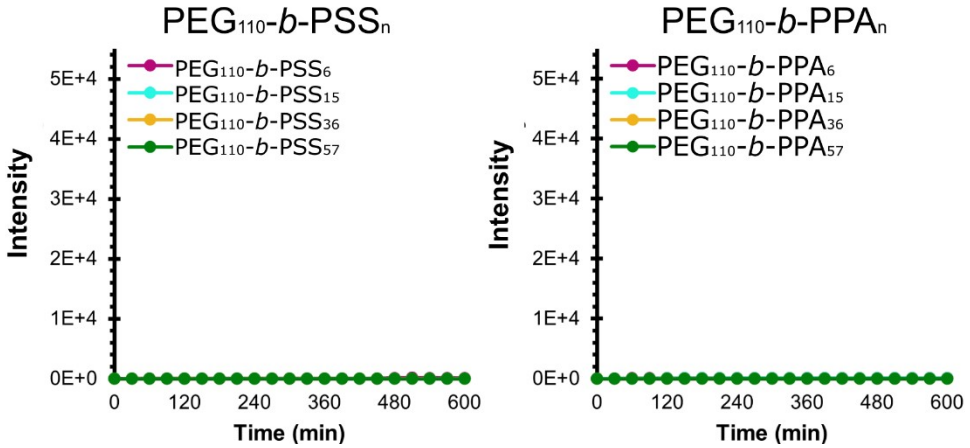


Figure S11. **PEG_{110}-*b*-PSS_n** and **PEG_{110}-*b*-PPA_n** polymers without P-CM probe at pH 10.

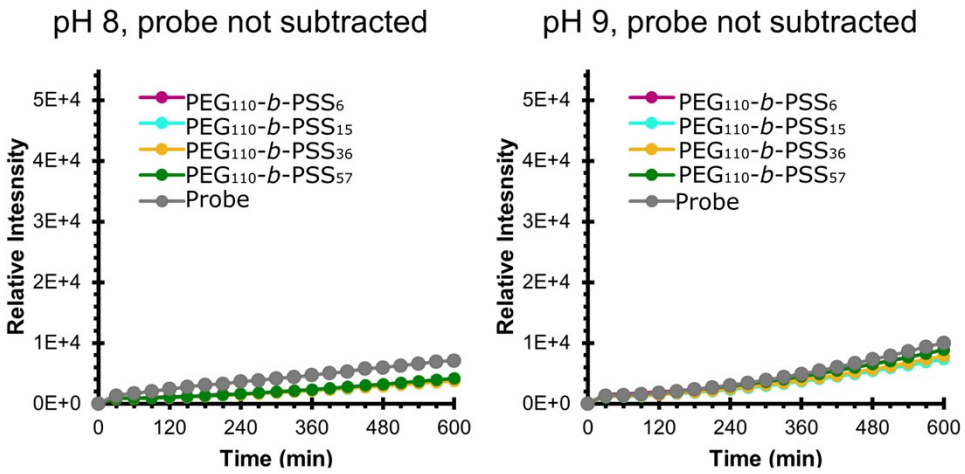


Figure S12. **PEG_{110}-*b*-PSS_n** polymers with probe at pH 8 (left) and 9 (right); background fluorescence from the probe degradation is not subtracted. Some fluorescence was observed due to degradation of the P-CM probe over time.

Table S4. Statistical analysis of the four PEG_{110}-*b*-PSS_n polymers in Fig. S12 (left) at the 600 min time point.

PEG_{110}-*b*-PSS_{57} vs **PEG_{110}-*b*-PSS_6**	***
PEG_{110}-*b*-PSS_{57} vs **PEG_{110}-*b*-PSS_{15}**	*
PEG_{110}-*b*-PSS_{57} vs **PEG_{110}-*b*-PSS_{36}**	ns
PEG_{110}-*b*-PSS_{36} vs **PEG_{110}-*b*-PSS_6**	***
PEG_{110}-*b*-PSS_{36} vs **PEG_{110}-*b*-PSS_{15}**	ns
PEG_{110}-*b*-PSS_{15} vs **PEG_{110}-*b*-PSS_6**	***

Group comparisons are indicated as determined by a one-way analysis of variance (ANOVA) with a Student−Newman−Keuls comparisons post hoc test. *** indicates $p < 0.001$, ** indicates $p<0.01$. * indicates $p<0.05$, ns indicates no significance among indicated treatment groups.

Table S5. Statistical analysis of the four PEG_{110}-*b*-PSS_n polymers in Fig. S12 (right) at the 600 min time point.

PEG_{110}-*b*-PSS_{57} vs **PEG_{110}-*b*-PSS_6**	***
PEG_{110}-*b*-PSS_{57} vs **PEG_{110}-*b*-PSS_{15}**	**
PEG_{110}-*b*-PSS_{57} vs **PEG_{110}-*b*-PSS_{36}**	*
PEG_{110}-*b*-PSS_{36} vs **PEG_{110}-*b*-PSS_6**	***
PEG_{110}-*b*-PSS_{36} vs **PEG_{110}-*b*-PSS_{15}**	ns
PEG_{110}-*b*-PSS_{15} vs **PEG_{110}-*b*-PSS_6**	***

Group comparisons are indicated as determined by a one-way analysis of variance (ANOVA) with a Student−Newman−Keuls comparisons post hoc test. *** indicates $p < 0.001$, ** indicates $p<0.01$. * indicates $p<0.05$, ns indicates no significance among indicated treatment groups.

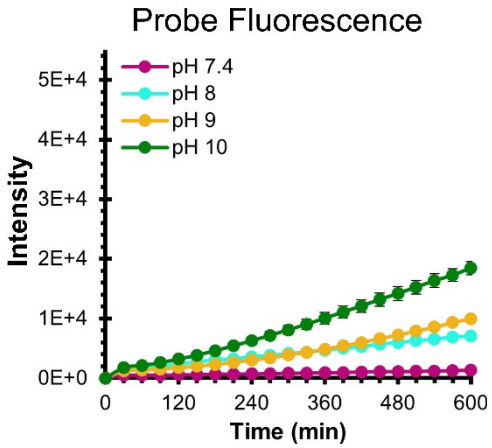


Figure S13. Fluorescence of P-CM probe over 10 h, demonstrating pH-dependent degradation. Statistical analysis between the four pH's at 600 min indicates that all values are significant between each other. Group comparisons are indicated as determined by a one-way analysis of variance (ANOVA) with a Student−Newman−Keuls comparisons post hoc test. *** indicates $p < 0.001$, ** indicates $p<0.01$. * indicates $p<0.05$, ns indicates no significance among indicated treatment groups.

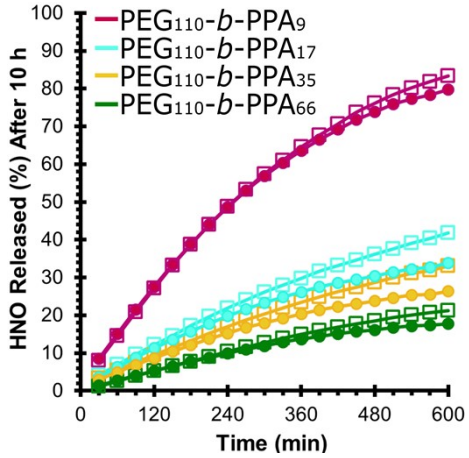


Figure S14. Comparison graph showing subtraction of background fluorescence of the P-CM probe (Fig. S13, which how the data is presented throughout this paper, indicated by closed circles) versus subtraction of the fluorescence signal from both the probe and PEG_{110}-*b*-PSS_n polymers (Fig. S12, indicated by the open squares).

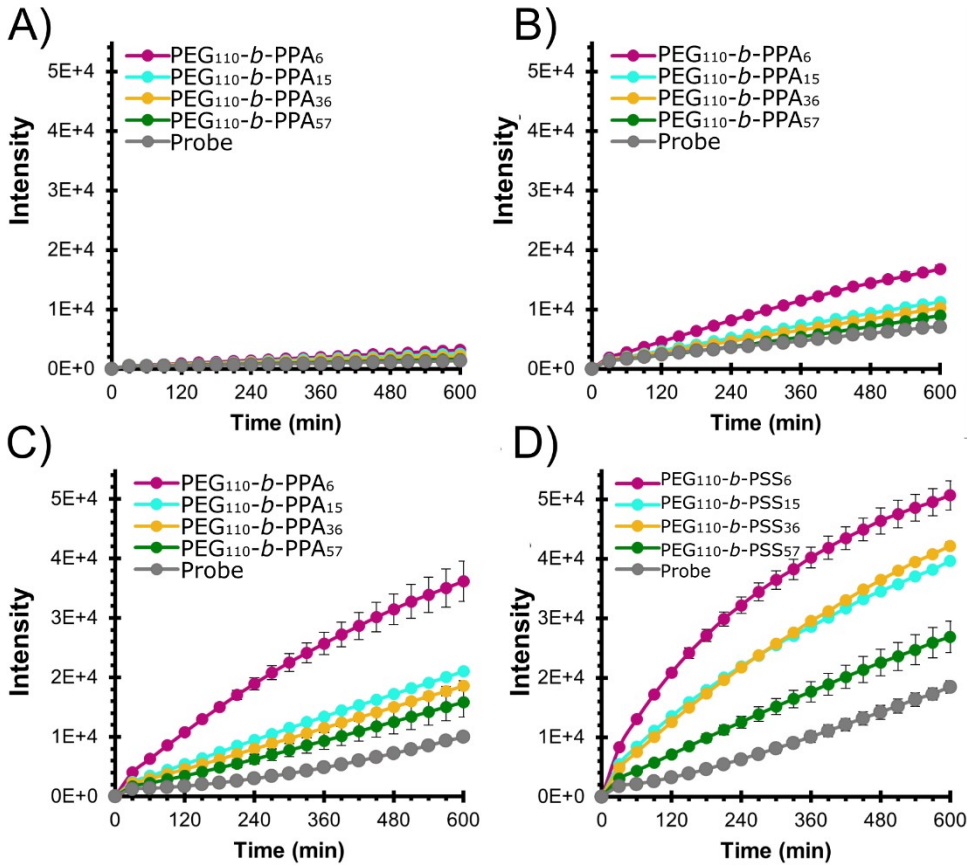


Figure S15. Raw data showing intensity measurements without subtracting background fluorescence from the P-CM probe. A) pH 7.4, B) pH 8, C) pH 9, D) pH 10.

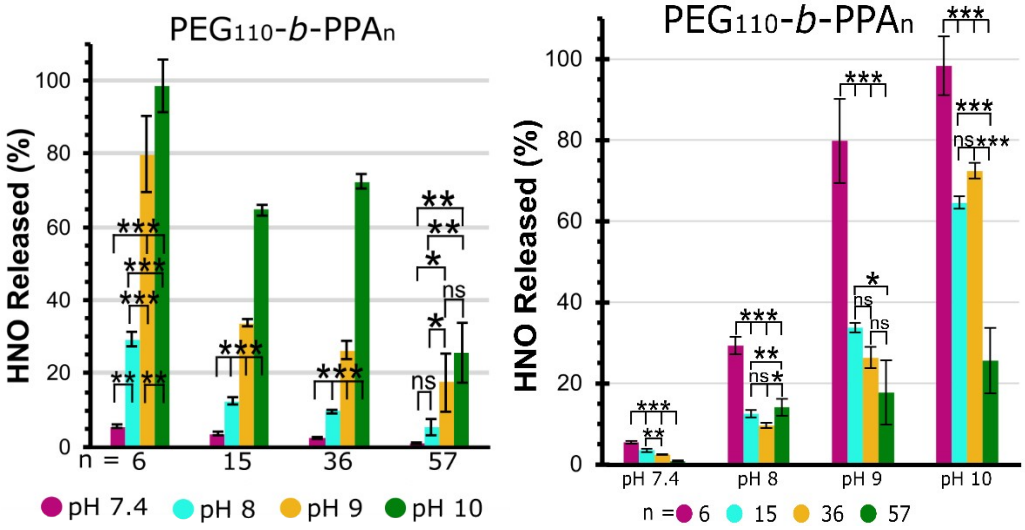


Figure S16. Statistical analysis between HNO release data. The data are presented here in two ways: statistics between the different size polymers at each pH (left panel) as well as statistics between each pH for the different polymers (right panel). Group comparisons are indicated as

determined by a one-way analysis of variance (ANOVA) with a Student−Newman−Keuls comparisons post hoc test. *** indicates $p < 0.001$, ** indicates $p<0.01$. * indicates $p<0.05$, *ns* indicates no significance among indicated treatment groups.

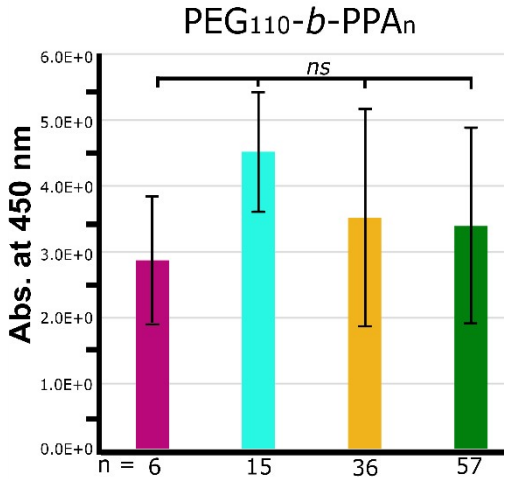


Figure S17. Figure S17. Absorbance at 450 nm of PEG_{110}-*b*-PPA_n polymers in 20 mM piperidine buffer (pH 11) after 12 hours (n = 6). Statistical analysis of these results indicates that there is no significance among any groups, indicating all polymers release similar amounts of HNO over this time period. Group comparisons were determined by a one-way analysis of variance (ANOVA) with a Student−Newman−Keuls comparisons post hoc test.

Dynamic Light Scattering:

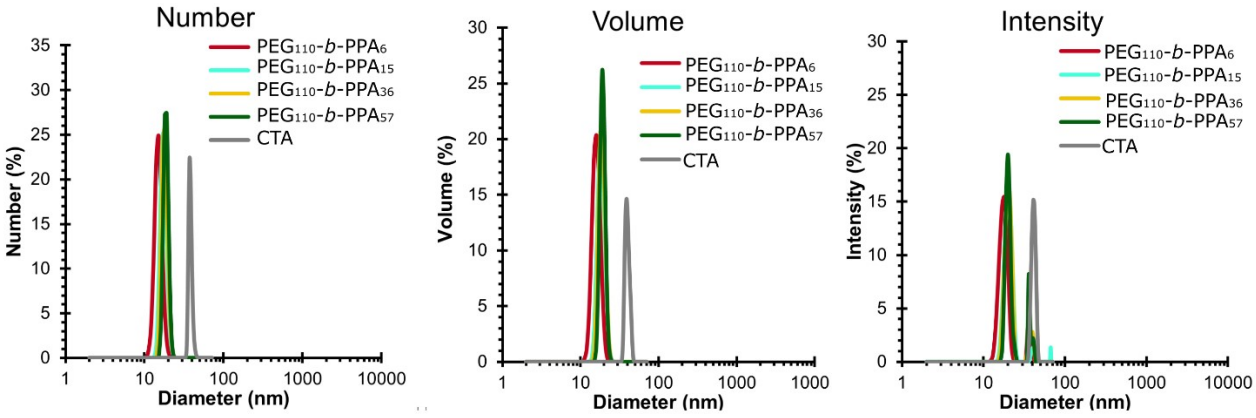


Figure 18: DLS of **PEG_{110}-*b*-PPA_n**; 1 mg/mL polymer in 20 mM pH 9 boric acid buffer.

References

1. Warren, N. J.; Mykhaylyk, O. O.; Mahmood, D.; Ryan, A. J.; Armes, S. P., RAFT Aqueous Dispersion Polymerization Yields Poly(ethylene glycol)-Based Diblock Copolymer Nano-Objects with Predictable Single Phase Morphologies. *J. Am. Chem. Soc.* **2014,** *136* (3), 1023-1033.
2. Mao, G.-J.; Zhang, X.-B.; Shi, X.-L.; Liu, H.-W.; Wu, Y.-X.; Zhou, L.-Y.; Tan, W.; Yu, R.-Q., A highly sensitive and reductant-resistant fluorescent probe for nitroxyl in aqueous solution and serum. *Chem. Commun.* **2014,** *50* (43), 5790-5792.
3. Sanna, D.; Rocchitta, G.; Serra, M.; Abbondio, M.; Serra, P. A.; Migheli, R.; De Luca, L.; Garribba, E.; Porcheddu, A., Synthesis of Nitric Oxide Donors Derived from Piloty's Acid and Study of Their Effects on Dopamine Secretion from PC12 Cells. *Pharmaceuticals (Basel)* **2017,** *10* (3), 74.