Supporting Information

Biosignal-Responsive Polymer Nanorods that Specifically Recognize Hydrogen Polysulfide (H_2S_n) from Reactive Sulfur Species

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1. Materials and Methods.

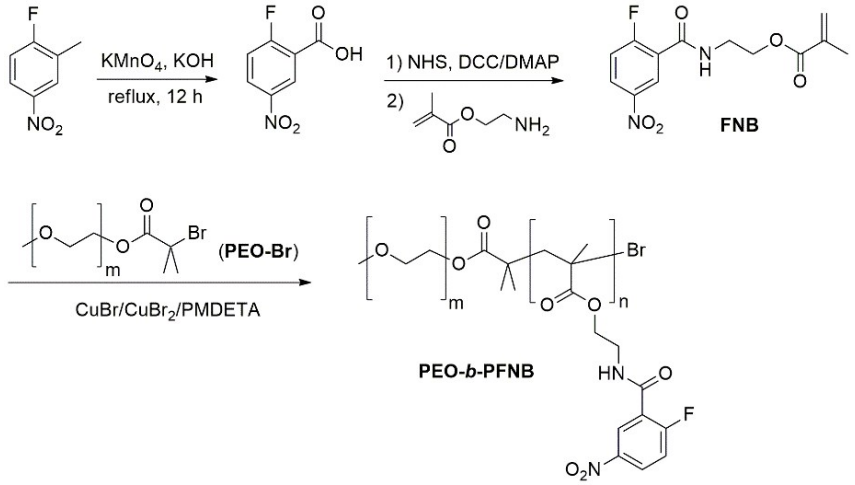
Materials. 1-Fluoro-2-methyl-4-nitrobenzene (Sigma-Aldrich, 99%), 4-dimethylamino pyridine (DMAP, Acros, 98%), *N,N*-dicyclohexyl carbodiimide (DCC, Acros, 98%), 2-aminoethyl methacrylate (AEM, Sigma-Aldrich, 97%), *N*-hydroxyl succinimide (NHS, Acros, 98%), copper (I) bromide (CuBr, Sigma-Aldrich, 99.5%), copper (II) bromide ($CuBr_2$, Sigma-Aldrich, 99.5%), and *N,N',N'',N''*-pentamethyl diethylene triamine (PMDETA, Sigma-Aldrich, 99%) were used as received. Poly(ethylene oxide)-based macro-initiator (PEO-Br, $M_{n,GPC}$ = 2.3 kg/mol, M_w/M_n = 1.03) was prepared according to the literature elsewhere.[1] All the solvents were used as received.

Methods. ^{1}H NMR and ^{19}F NMR spectra of the designed monomers and polymers were recorded by AVANCE III HD-400 (400 MHz) spectrometer with $CDCl_3$ and d6-DMSO as the solvent. The molecular weight and polydispersion ($Đ$) of all polymer samples were measured on a Waters 515 HPLC system (GPC) with ultraviolet (UV) and refractive index (RI) detectors. HPLC-grade tetrahydrofuran (THF) was used as eluent at a flow rate of 1.0 mL/min at 30 ^{o}C and PEOs as standard reference for calibration. Transmission electron microscope (TEM) images were measured on a FEI Tecnai G2-F20 S-TWIN instrument at a voltage of 120 kV. The specimen were prepared by drop-casting polymer assembly solution (10 μL) onto carbon-coated copper grid and freeze-drying before observation. Atom force microscope (AFM) images were performed

from Bruker Dimension-ICON SPM system equipped with a J scanner. The specimen were obtained by droplet adding polymer assembly solution (20 μL) onto mica slice and vacuum drying for observation. The laser light scattering (LLS/DLS/SLS) were performed at a scattering angel of 90^{o} on a Brookhaven (BI-200SM) equipped with a highly sensitive avalanche photodiode detector (Brookhaven, BI-APD), a digital correlator (TurboCorr) that calculates the photo intensity autocorrelation function $g^2(t)$, and a helium-neon laser goniometer (λ = 632 nm). The PEO-*b*-PFNB polymer solutions at various conditions were filtered twice before measuring. UV-Vis absorption bands of polymer solutions at different conditions were measured using an Agilent Cary 6000i UV-Vis-NIR spectrometer. All the polymer samples for the responsive tests were fixed at 2.5×10^{-3} g/L and then measured at given time intervals upon stimuli. The drug release experiments monitored the diagnostic absorbance (λ = 328 nm) of Nicardipine (NP) by UV-Vis spectrometer. Small-angle X-ray scattering (SAXS) analysis were performed by Bruker N8 Horizon. The scattering vector, q, was extracted from SAXS profile and the diameter (d) of polymeric nanorods can be calculated according to the scattering equation, $q = 2\pi/d$.[2] The scattering wavelength (λ = 0.15405 nm). The molecular weight of monomers and other products were conducted on an electrospray ionization mass spectrometry (ESI-MS, Bruker-TOF11). The critical micelle concentration (CMC) of polymers were determined by Hitachi F-7000 spectrofluorometer (FS).

2. Synthesis, Characterization and Preparation.

2.1 Synthesis.



Scheme S1. Synthetic Route of H_2S_n-Sensitive Monomer (FNB) and Block Copolymer (PEO-*b*-PFNB).

2.1.1 Synthesis of 2-fluoro-5-nitrobenzoic acid.

A typical oxidation synthesis was based on literature elsewhere.[3] Potassium hydroxide (5.62 g, 100 mmol) and 1-fluoro-2-methyl-5-nitrobenzene (13.92 g, 90 mmol) were dissolved in deionized water (120 mL) and added potassium permanganate (15.80 g, 100 mmol). The mixture was refluxed for 12 hours with stirring. After the precipitate was filtered, the resulting aqueous phase was acidized by HCl (0.1 M) to pH of 3. The solution was extracted with ethyl acetate (50 mL×3) and the combined organic phase was treated with saturated sodium chloride and dried with Na_2SO_4. Finally, the product, 2-fluoro-5-nitrobenzoic acid, was obtained by concentrated the filtrate under distillation (5.62g, yield: 34%).

^{1}H NMR (d_6-DMSO, δ, ppm): 12.14 (s, 1H, -COO**H**), 8.89 (d, 1H, J = 8.4 Hz, -C**H**-CHCOOH), 8.62 (dd, 1H, J = 6.3 Hz, -C**H**CHNO$_2$), 7.73 (dd, 1H, J = 6.3 Hz, -C**H**CHF).

^{19}F NMR (d_6-DMSO, δ, ppm): -89.2.

ESI-MS: $[C_7H_4FNO_4Na]^+$, calcd. 208.01; found 208.23.

2.1.2 Synthesis of H_2S_n-sensitive functional monomer (2-fluoro-5-nitrobenzamido)ethyl methacrylate (FNB).

The above 2-fluoro-5-nitrobenzoic acid (3.73 g, 20 mmol) in 150 mL of anhydrous THF

solution was added *N*-hydroxyl succinimide (NHS, 2.59 g, 22.5 mmol), 4-dimethyl-amino pyridine (DMAP, 0.28 g, 2.3 mmol) and *N,N*-dicyclohexyl carbodiimide (DCC, 4.64 g, 22.5 mmol). The mixture was reacted with stirring at 0 °C for 3 hours and then reacted at room temperature overnight. After filtrating the DCU byproduct, the organic phase was concentrated to ~50 mL without further purification, and then droplet added 2-aminoethyl methacrylate (AEM, 2.58 g, 20 mmol) for 24 hours of reaction. After removing the precipitate, the resulted organic phase was extracted by saturated sodium bicarbonate (20 mL×3) to remove the unreactive carboxylic acid. The filtrate was dried with Na_2SO_4 and evaporated under reduced pressure and the residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH, 30/1, v/v) to yield a yellow solid product (4.38 g, yield: 74%).

^{1}H NMR ($CDCl_3$, δ, ppm): 8.92 (d, 1H, J = 8.2 Hz, -C**H**-CHCONH), 8.77 (dd, 1H, J = 5.8 Hz, -C**H**CHNO$_2$), 8.10 (s, 1H, -CON**H**-), 7.75 (dd, 1H, J = 6.0 Hz, -C**H**CHF), 6.48 (d, 2H, C**H$_2$**=C), 4.27 (t, 2H, -NHCH$_2$C**H$_2$**-), 2.30 (t, 2H, -NHC**H$_2$**CH$_2$-), 1.87 (s, 3H, -CH$_3$).

^{19}F NMR ($CDCl_3$, δ, ppm): -83.2.

ESI-MS: $[C_{13}H_{13}FN_2O_5Na]^+$, calcd. 319.11; found 319.75.

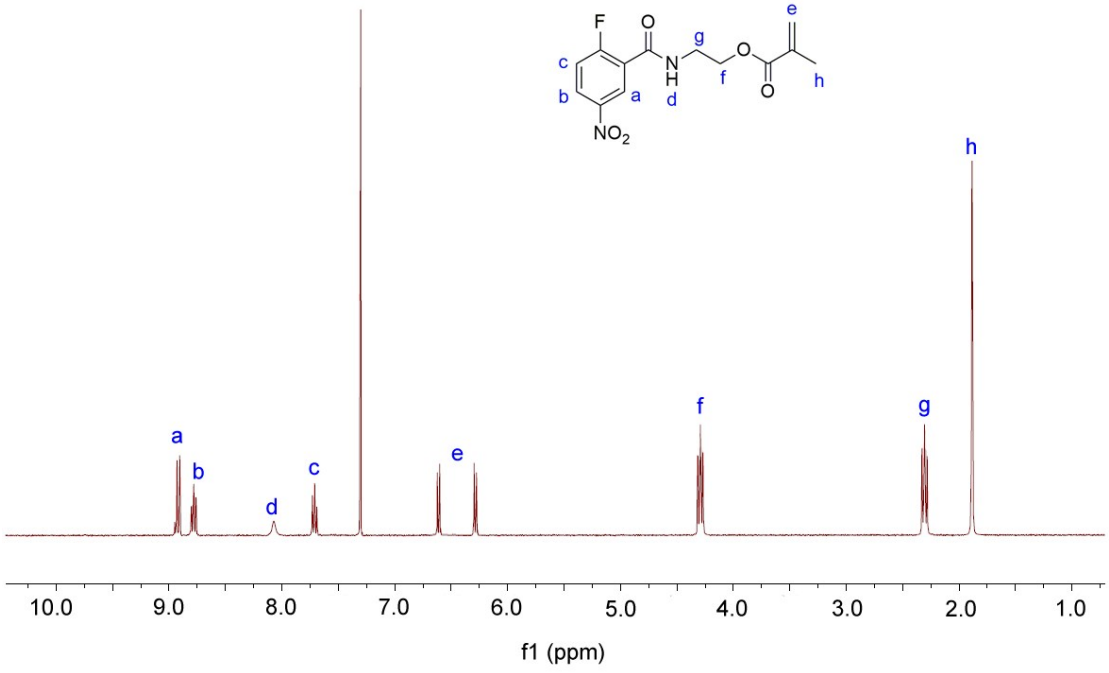


Fig. S1 ^{1}H NMR spectra of functional monomer FNB in $CDCl_3$.

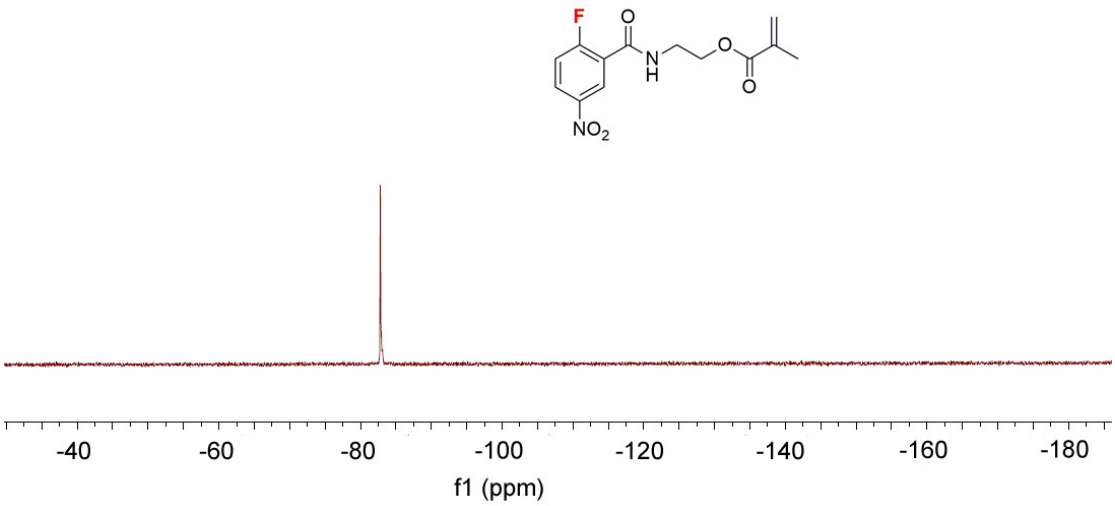


Fig. S2 ^{19}F NMR spectra of functional monomer FNB in $CDCl_3$.

2.1.3 Synthesis of H_2S_n-sensitive block copolymer, poly(ethylene oxide)-block-poly((2-fluoro-5-nitrobenzamido)ethyl methacrylate) (PEO-b-PFNB).

The polymerization adopted the atom-transfer radical polymerization (ATRP) protocol according to literature.[4] The PEO-Br macro-initiator (M_w = 2.3 kg/mol, 0.232 g, 0.10 mmol) in toluene solution was added CuBr (14 mg, 0.1 mmol), $CuBr_2$ (4 mg, 0.02 mmol) and the above FNB monomer (3.55 g, 12 mmol). Followed by three standard freeze-vacuum-thaw cycles, the reactive tube was injected into PMDETA ligand (17 μL, 0.1 mmol) and heated at 75 °C with stirring for 10 hours. The molar fraction of [initiator]: [CuBr]:[$CuBr_2$]:[PMDETA]:[FNB monomer] = 1:1:0.2:1:120. After reaction, the tube was immersed to liquid nitrogen to cease the polymerization. Then removing toluene in vacuum the residue was dissolved in THF (10 mL) and removed the copper catalyst by neutral alumina gel column chromatography. The resulted solution was precipitated in cold diethylene ether (50 mL) twice to obtain target PEO-*b*-PFNB copolymer (2.28 g, conversion: 76%).

^{1}H NMR ($CDCl_3$, δ, ppm): 9.86 (s, NH), 8.62~8.98 (m, in benzene), 7.45~7.75 (d, in benzene), 4.19 (s, -C**H$_2$**CH$_2$NH-), 3.58 (s, -C**H$_2$**C**H$_2$**O- in PEO), 2.28 (s, -CH$_2$C**H$_2$**NH-), 1.12~1.78 (m, C**H$_2$**=C(C**H$_3$**) in main chain).

^{19}F NMR ($CDCl_3$, δ, ppm): 81.4.

$M_{w,GPC}$ = 29.2 kg/mol, $M_{n,GPC}$ = 24.1 kg/mol, $Đ$ = 1.21.

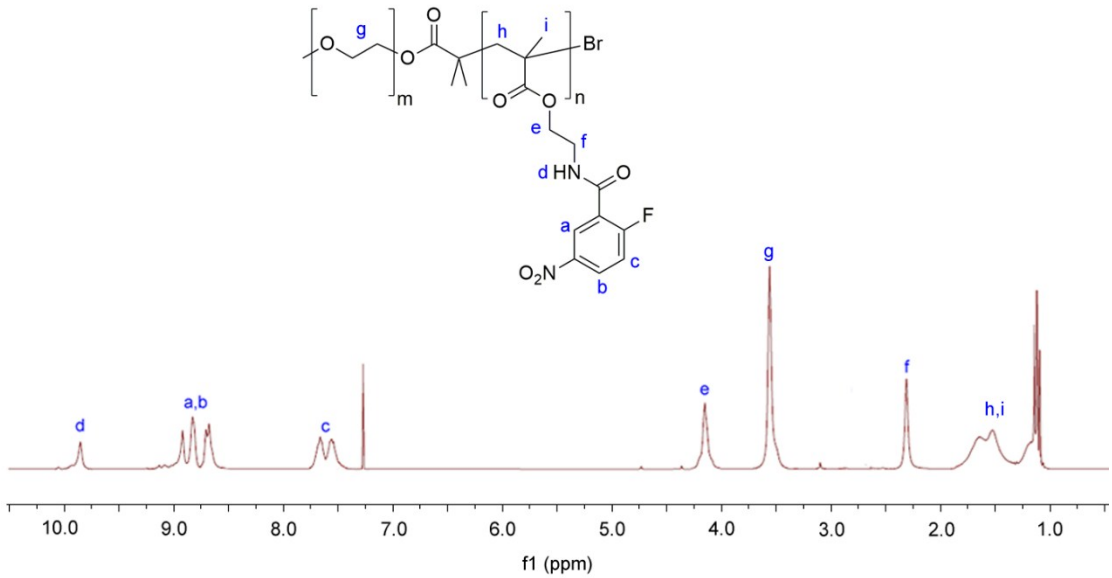


Fig. S3 ^{1}H NMR spectra of block copolymer PEO-*b*-PFNB in $CDCl_3$.

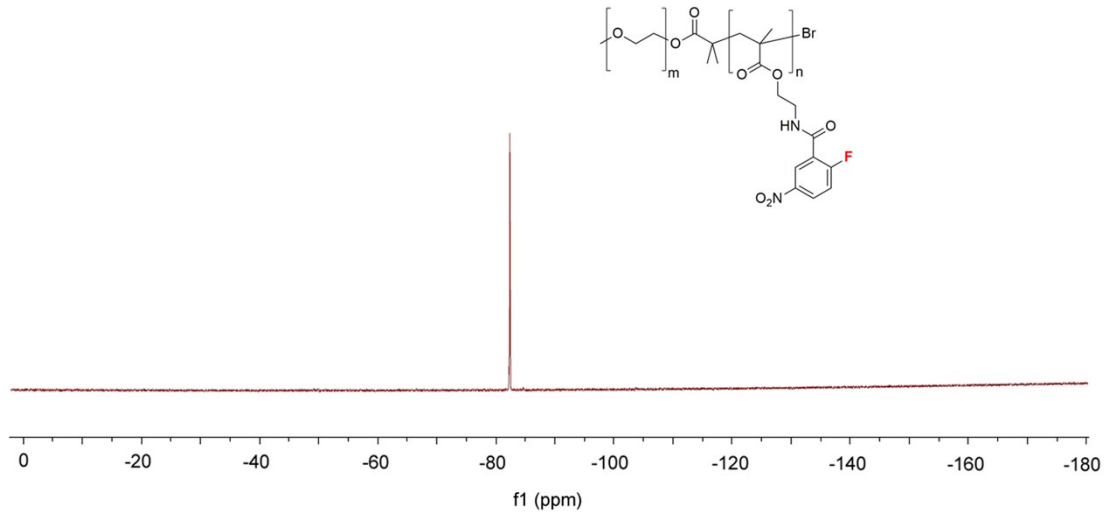


Fig. S4 ^{19}F NMR spectra of block copolymer PEO-*b*-PFNB in $CDCl_3$.

2.2 Preparation of Reactive Sulfur, Oxygen, Nitrogen Species.

Sulfate (SO_4^{2-}), Sulfite (SO_3^{2-}), Thiosulfate ($S_2O_3^{2-}$). SO_4^{2-}, SO_3^{2-} and $S_2O_3^{2-}$ were used as their relative sodium salts and prepared a stock solution of 1.0 mM.

Cysteine (Cys), Homocysteine (Hcy), Glutathione (GSH). Cys, Hcy and GSH were all used as received and prepared a stock solution of 1.0 mM.

Hydrogen Sulfide (H_2S). Na_2S was used as a H_2S donor.[5] It was prepared in a stock

solution at 5 mM, and then dilute to required concentrations.

Hydrogen Polysulfide (H_2S_n, n = 2). We used Na_2S_2 as the H_2S_2 donor and prepared a stock solution of 1.0 mM for dilution to a series of required concentrations for further experiments.

Hydrogen Peroxide (H_2O_2). H_2O_2 (30 wt%) was diluted to 1.0 mM and added to the polymer assemblies for selective responsiveness analysis.

Superoxide Anion ($O_2^{\bullet-}$). $O_2^{\bullet-}$ was formed by xanthine and xanthine oxidase. Xanthine oxidase was added firstly. After xanthine oxidase was dissolved, xanthine (1.0 mM) was injected to the polymer solution with stirring at 25 °C.

Hyperchlorite (OCl^-). 5% NaOCl solution was purchased from Sigma. It was diluted with PBS to gain a 1.0 mM stock solution. The pH was adjusted to 7.4 for adding to the polymer assemblies.

Hydroxy Radical ($HO^{\bullet}$). $HO^{\bullet}$ was generated by Fenton reaction between Fe^{2+} and H_2O_2. Fresh $FeCl_2$ and H_2O_2 stock solution were prepared for dilution to 1.0 mM.[6]

Nitric Oxide (NO). NO donor adopted 1-hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyltriazene stock solution (1.0 mM) that prepared in 0.1 M NaOH to generate NO. The final mixtures were confirmed to have no notably pH change from 7.4.[7]

Peroxynitrite ($ONOO^-$). $ONOO^-$ was formed according to the literature and modified.[8] Briefly, to a solution of sodium hydroxide (30 mL, 1.5 M) was added sodium nitrite (30 mL, 0.6 M) and the mixture of hydrogen peroxide (30 mL, 0.7 M) and hydrochloric acid (0.6 M). It is noted that sodium nitrite and acidified hydrogen peroxide was added dropwise via a Y-type pipe with the same pump rate. Then activated manganese dioxide (4.0 g) was added to the resulted solution for removing the excess hydrogen peroxide. After 15 min later the mixture was filtrated under reduced pressure and bright yellow filtrate was split into small aliquots and stored at lower than -18 °C. The operation of all of the above was carried out at 4 °C. The concentration of the prepared peroxynitrite was determined by testing the absorption of the solution at 302 nm. The extinction coefficient of $ONOO^-$ solution in 0.1 M NaOH is 1670 M^{-1} cm^{-1} at 302 nm. $C_{ONOO^-} = Abs_{302nm}/1.67$ (mM).

Nitrate (NO_3^-). NO_3^- was used as its relative sodium salts and prepared a stock solution

of 1.0 mM.

3. Supporting Characterization.

3.1 ESI-MS Showing the H_2S_2 Specific-Sensitivity of mFNB Model Monomer

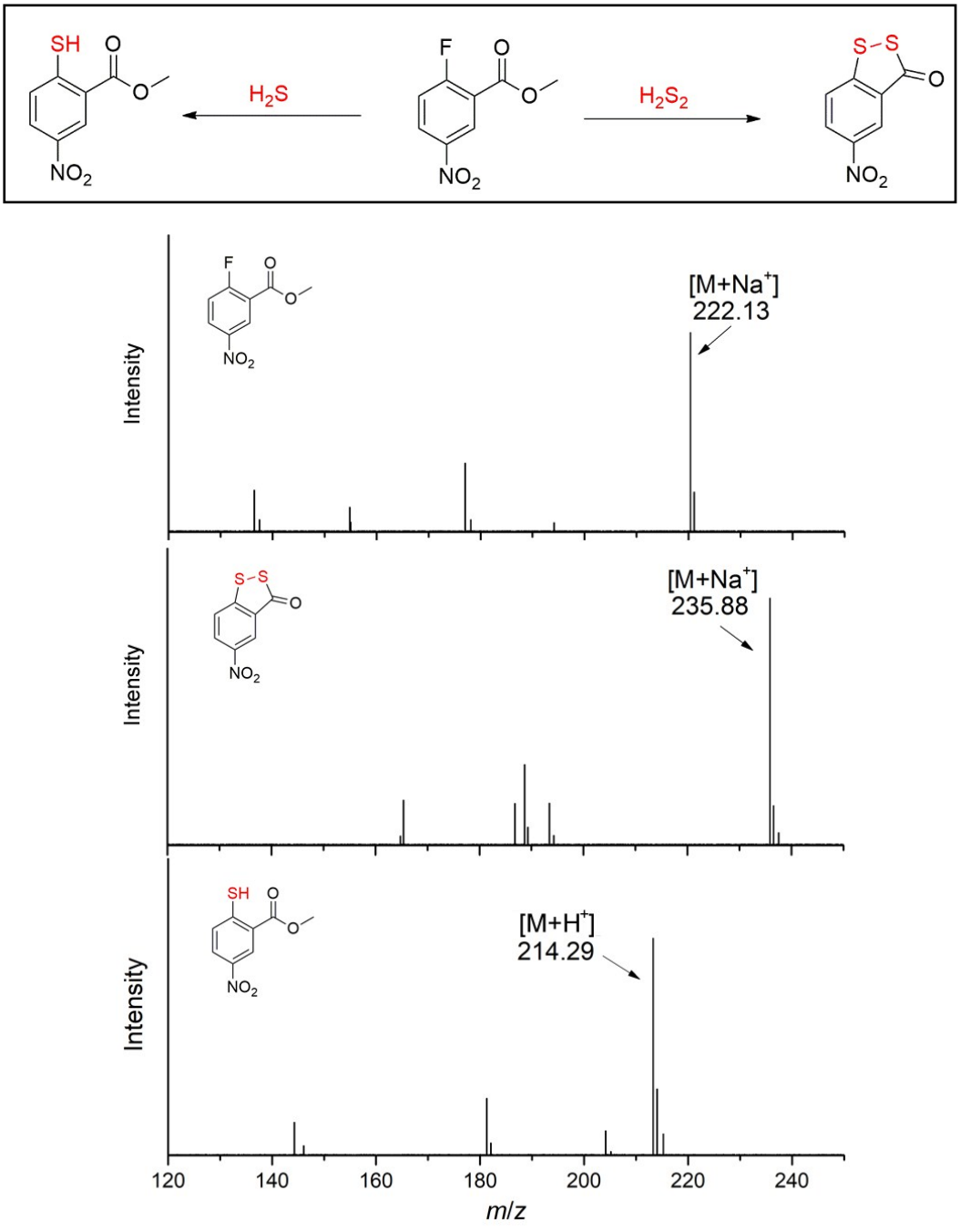


Fig. S5 ESI-MS of the model monomer, methyl 2-fluoro-5-nitrobenzoate (mFNB) under different conditions: without stimuli (top, $[M+Na^+]$=222.13); with H_2S_2 irritant (middle, $[M+Na^+]$=235.88, yield: 94%); with H_2S irritant ($[M+H^+]$=214.29, yield: 91%).

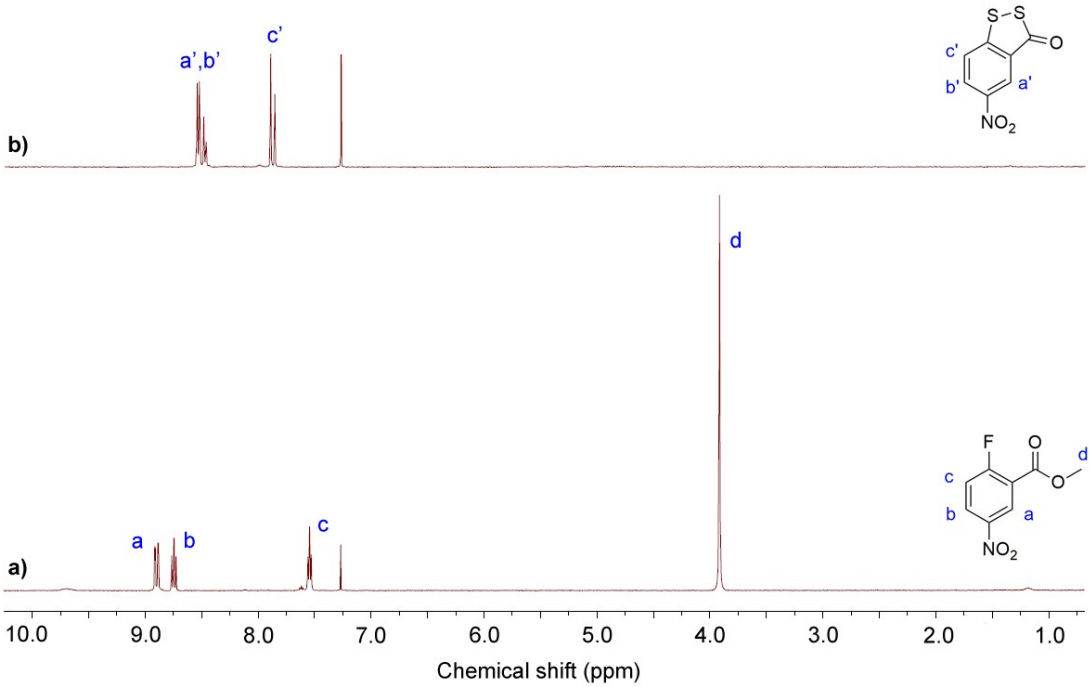


Fig. S6 ^{1}H NMR spectra of the model monomer, methyl 2-fluoro-5-nitrobenzoate (mFNB) before (a) and after (b) H_2S_2 treatment. The chemical shifts of proton signals on aromatic region ($H_a \rightarrow H_{a'}$: 8.85→8.52 ppm, $H_b \rightarrow H_{b'}$: 8.74→8.46 ppm, and $H_c \rightarrow H_{c'}$: 7.54→7.76 ppm), as well as the disappearance of methyl proton signal (H_d), together prove the cascade substitution-cyclization reaction, which can covert mFNB into cyclic benzodithiolone and site-specific cleave the benzoate bond.

3.2 Critical Micelle Concentration (CMC) of PEO-*b*-PFNB Block Copolymer.

The CMC experiment was briefly carried out as follows:[9] A 2 mL of solution of 1.0 g/L PEO-*b*-PFNB block copolymer in THF was added into 3 mL of deionized water under sonication. Then the solution followed by dialysis against deionized water to obtain an aggregate solution at a concentration of 0.2 g/L for further experiments. The CMC was measured by pyrene fluorescent probe method. A 10 μL of 1.0×10^{-4} g/L pyrene acetone solution was mixed with the copolymer to get a series of mixtures with various polymer concentrations, and the solutions were sonicated for 1 hour before fluorescent tests. The results showed that the CMC of PEO-*b*-PFNB is determined to be 1.3×10^{-3} g/L (pyrene probe producing a decrease in the I_1/I_3 ratio with increase of polymer concentration).

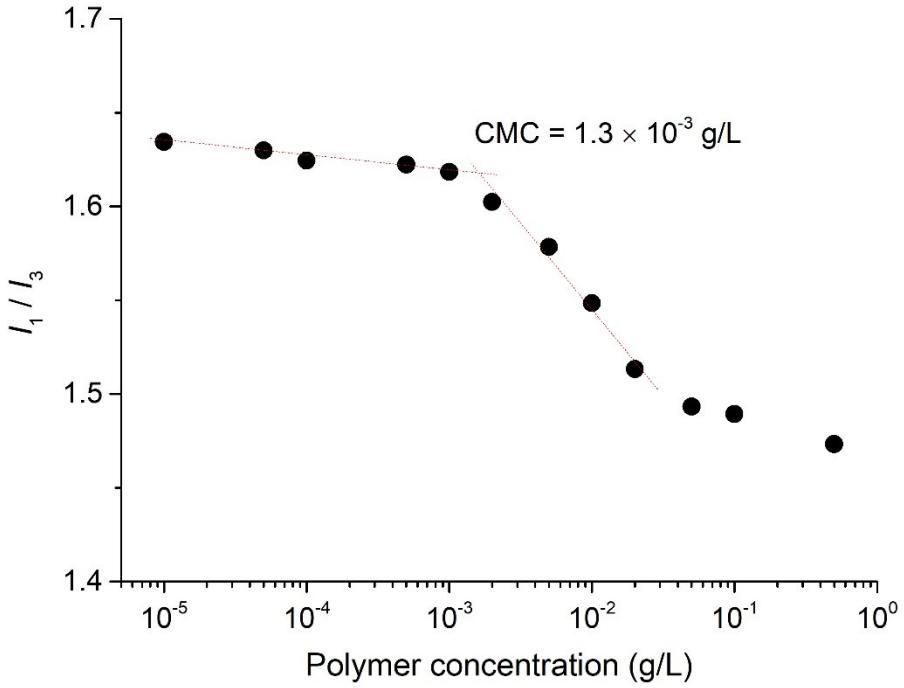


Fig. S7 The CMC of PEO-b-PFNB block copolymer in aqueous solution is 1.3×10^{-3} g/L measured by a pyrene fluorescent probe method.

3.3 Shape Factor of the PEO-*b*-PFNB Polymer Assemblies.

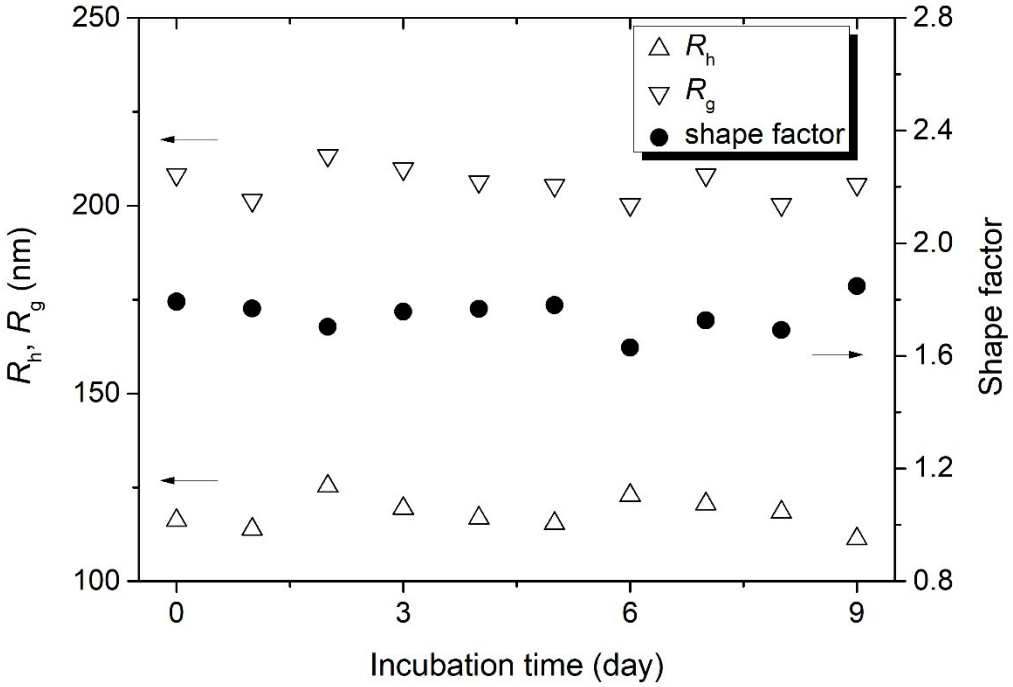


Fig. S8 The apparent gyration radius (R_g) measured by static light scattering (SLS), the hydrodynamic radius (R_h) measured by dynamic light scattering (DLS), and the shape factor ($\rho = R_g/R_h$) changes of PEO-*b*-PFNB nanorods plotted against incubation time. The polymer concentration is 2.5×10^{-3} g/L and the average ρ is calculated to be 1.75, corresponding to the theoretical value of columnar aggregate, $\rho_T = 1.732$.[10]

3.4 Stability of Polymer Assemblies in Mimetic Cytoplasmic Environment.

Table S1. DLS studies of PEO-*b*-PFNB polymer assemblies after treated with viscous glycerol solution and high salty PBS buffer solution.

	R_h (nm) glycerol 8%	R_h (nm) glycerol 18%	R_h (nm) PBS 5 mM	R_h (nm) PBS 15 mM
Initial State	118	114	120	120
One Week	127	138	128	135
$\Delta R_h/R_h$	+7.6%	+14.0%	+6.7%	+12.5%

3.5 GPC Traces Revealing the Side Group Cleavage of PEO-*b*-PFNB.

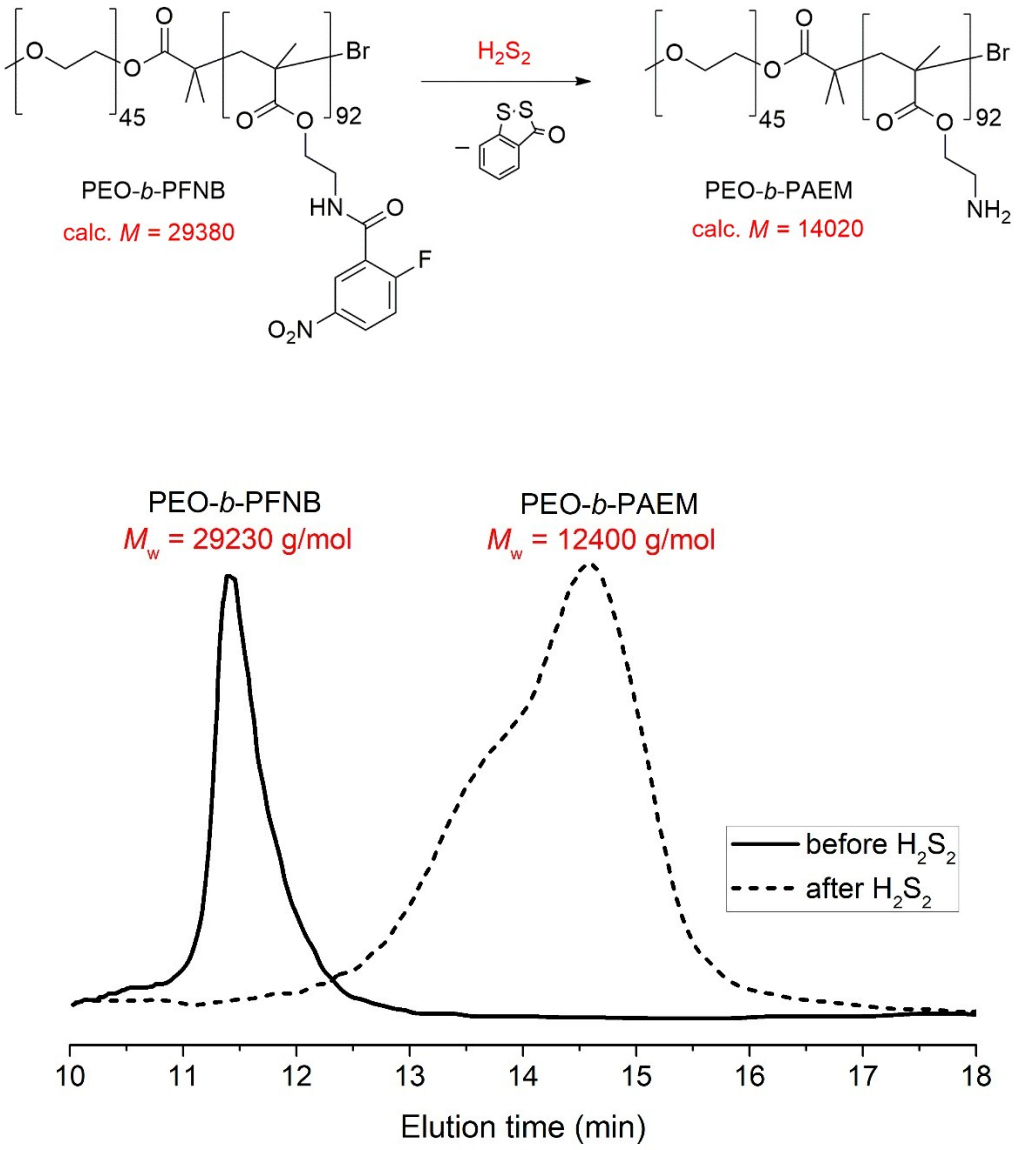
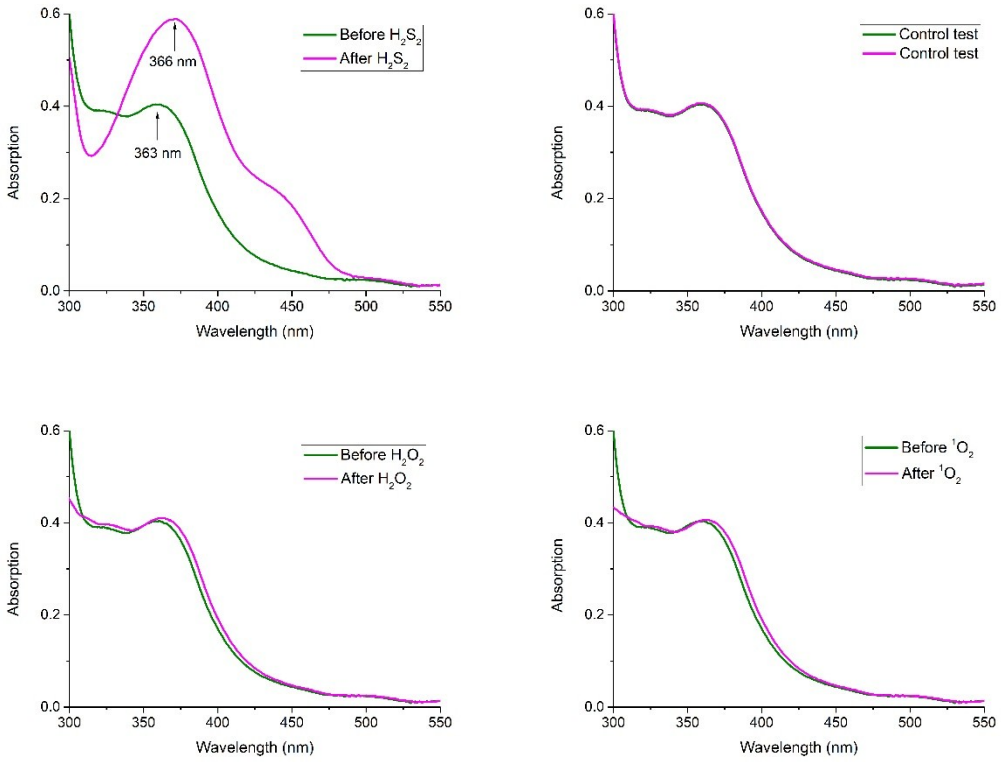
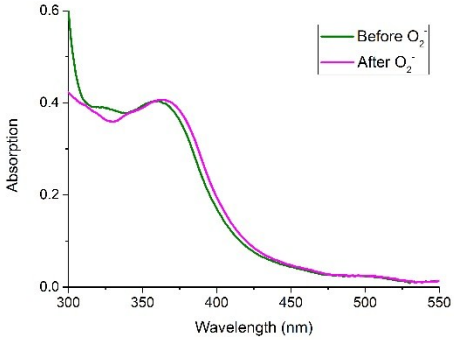


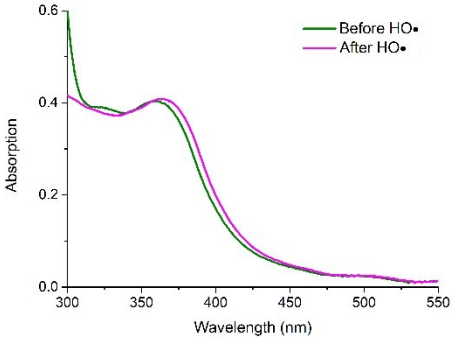
Fig. S9 GPC trace changes of the PEO-*b*-PFNB copolymer before and after H_2S_2 treatment. In the absence of H_2S_2, the molecular weight ($M_{w,GPC}$) of PEO-*b*-PFBN was determined to be 29.2 kg/mol, which is close to the theoretical value of 29.4 kg/mol. In the presence of H_2S_2, the FNB side groups were cleaved and the chain structure turned into PEO-*b*-PAEM. Its actual molecular weight was measured to be 12.4 kg/mol, which approximates the theoretical molecular mass of PEO-*b*-PAEM (14.0 kg/mol). The small deviation is possible to be the further hydrolysis of poly(2-amidoethylene methacrylate) (PAEM) block to poly(methacrylic acid) block (PMAA).

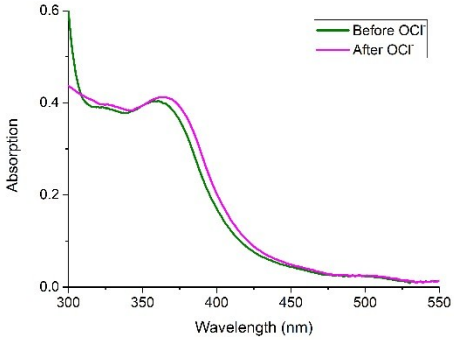
3.6 Ultrahigh Selective Responsiveness of PEO-*b*-PFNB Polymer Nanoparticles to H_2S_2 Biosignal.

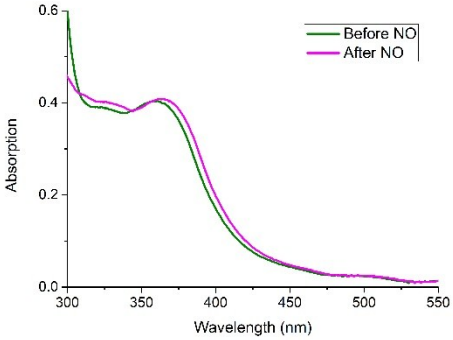
We have demonstrated that the PEO-*b*-PFNB polymer nanorods own H_2S_2-triggered polymer disassembly. Because we anticipated that these smart nanocarriers could be applied in real cellular environment, thus they should possess highly bio-selectivity and bio-specificity to endogenous H_2S_2. However, besides the H_2S_2 biosignal, there are other reactive oxygen, nitrogen and sulfide species (ROS, RNS and RSS) coexisting in our cells. Their strong redox stress is possible to disturb the recognition of our polymer to H_2S_2. To detect whether these bio-analogues produce interference, we used UV-Vis spectroscopy to monitor their reactivity toward the PEO-*b*-PFNB copolymer. First we found that prior to H_2S_2 the initial copolymer showed a typical absorption band at 363 nm, ascribed to the FNB aromatic group. After the copolymer was cleaved by H_2S_2, a new peak with a slight red-shift (366 nm) appeared, consistent with the absorbance band of benzodithiolone final product. Based on this character, if we defined H_2S-induced cleavage as 100% reactivity, the reactive efficiency of other bio-analogues could be quantitatively calculated by the ΔA_{363} based on our previous literature.[11]

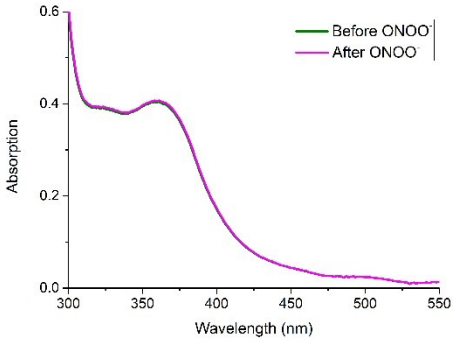


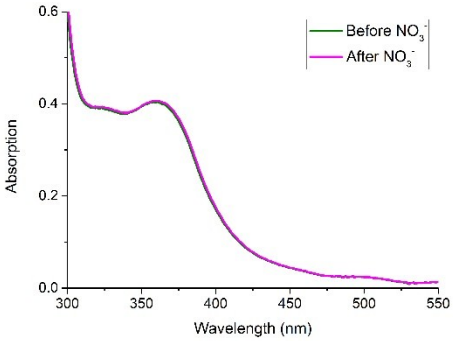

Before $O_2^{\cdot-}$
After $O_2^{\cdot-}$
Absorption
Wavelength (nm)

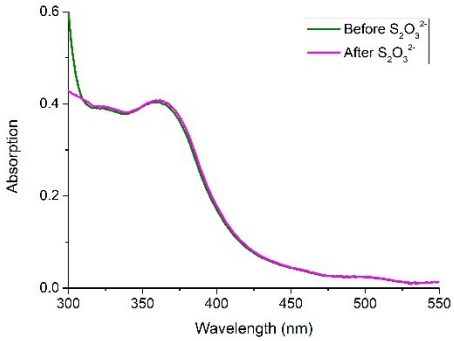

Before HO•
After HO•
Absorption
Wavelength (nm)

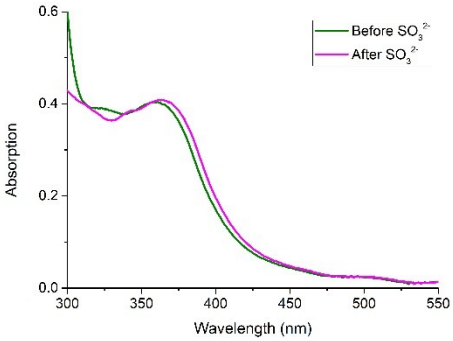

Before OCl$^-$
After OCl$^-$
Absorption
Wavelength (nm)


Before NO
After NO
Absorption
Wavelength (nm)


Before $ONOO^-$
After $ONOO^-$
Absorption
Wavelength (nm)


Before NO_3^-
After NO_3^-
Absorption
Wavelength (nm)


Before $S_2O_3^{2-}$
After $S_2O_3^{2-}$
Absorption
Wavelength (nm)


Before SO_3^{2-}
After SO_3^{2-}
Absorption
Wavelength (nm)

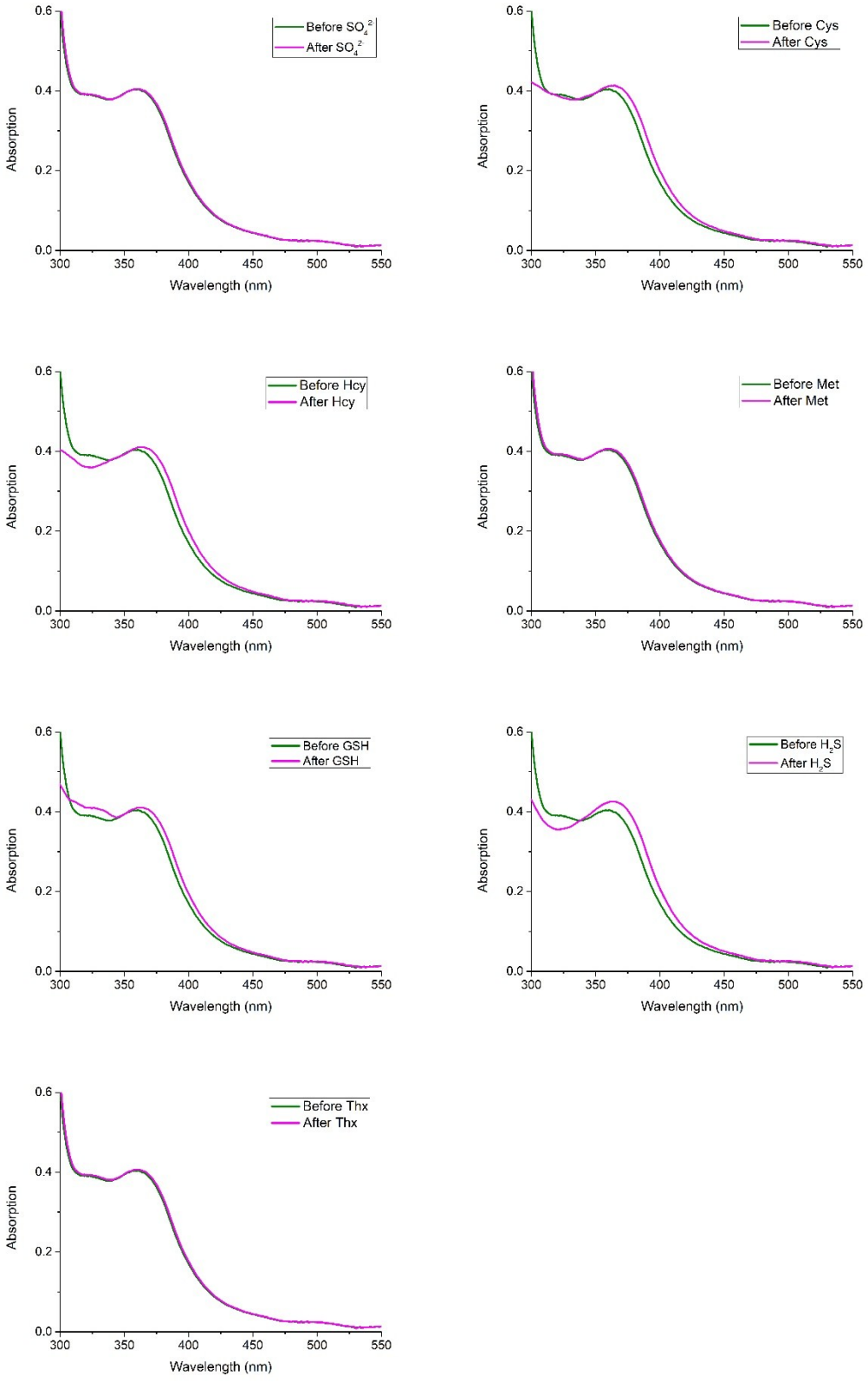


Fig. S10 UV-Vis spectra changes of PEO-b-PFNB polymer nanoparticle solution after exerting a variety of ROS, RNS and RSS stimuli: 1) H_2S_2, 2) control test, 3) H_2O_2, 4) $^1O_2$, 5) $O_2^{\bullet-}$, 6) $HO^{\bullet}$, 7) OCl^-, 8) NO, 9) $ONOO^-$, 10) NO_3^-, 11) $S_2O_3^{2-}$, 12) SO_3^{2-}, 13)

SO_4^{2-}, 14) Cys, 15) Hcy, 16) Met, 17) GSH, 18) H_2S, 19) Thx. The polymer was fixed at the concentration of 2.5×10^{-3} g/L, which contains FNB units of 8 μM. ROS, RNS and RSS levels were fixed at 1.0 mM, 125 equiv. with respect to FNB molar amount, except for H_2S_2 of 8 μM (1.0 equiv.).

3.7 UV-Vis Spectra of Drug Release Process

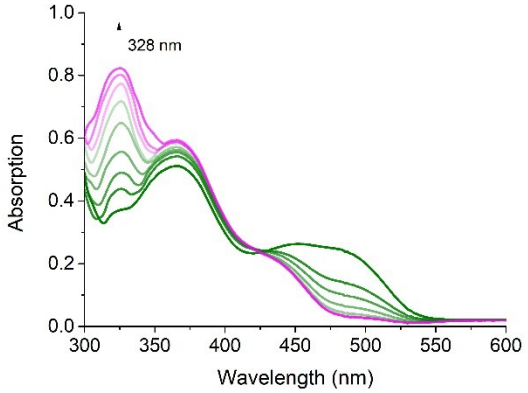


Fig. S11 H_2S_2-triggered controllable cargo release of NP drug from PEO-*b*-PFNB nanorods at 8 μM H_2S_2 stimulation level (1.0 equiv.)

Reference

(1) Du, J. Z.; Chen, Y. M. *Macromolecules* **2004**, *37*, 6322-6328.

(2) Li, T.; Senesi, A. J.; Lee, B. *Chem. Rev.* **2016**, *116*, 11128-11180.

(3) Rosenstrom, U.; Skold, C.; Lindeberg, G.; Botros, M.; Nyberg, F.; Karlen, A.; Hallberg, A. *J. Am. Chem. Soc.* **2006**, *49*, 6133-6137.

(4) Tsarevsky, N. V.; Matyjaszewski, K. *Chem. Rev.* **2007**, *107*, 2270-2299.

(5) Liu, C. R.; Chen, W.; Shi, W.; Peng, B.; Zhao, Y.; Ma, H. M.; Xian, M. *J. Am. Chem. Soc.* **2014**, *136*, 7257-7260.

(6) Lloyd, R. V.; Hanna, P. M.; Mason, R. P. *Free Radical Biol. Med.* **1997**, *22*, 885-888.

(7) Johnson, I. D. *The Molecular Probes Handbook: A Guide to Fluorescent Probes and Labeling Technologies*. 11th ed.; Life Technologies Corporation, 2010.

(8) Reed, J. W.; Ho, H. H.; Jolly, W. L. *J. Am. Chem. Soc.* **1974**, *96*, 1248-1249.

(9) Mohr, A.; Talbiersky, P.; Korth, H.-G.; Sustmann, R.; Boese, R.; Bläser, D.; Rehage, H. *J. Phys. Chem. B* **2007**, *111*, 12985-12992.

(10) Zhang, G.; Wu, C. *Phys. Rev. Lett.* **2001**, *86*, 822-825.

(11) Xu, M. M.; Liu, L. X.; Hu, J.; Zhao, Y.; Yan, Q. *ACS Macro Lett.* **2017**, *6*, 458-462.