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A cyotosol-selective nitric oxide bomb as a new paradigm of an anticancer drug

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Experimental section

Materials

tert-Buthanol (*t*-BuOH; 99.0%), methanol (MeOH, 99.5%), diethyl ether (Ether, 94.5%), dichloromehtane (99.5%), acetic acid (99.5%), sodium acetate (anhydrous, 98.5%), sodium bicarbonate (99%), and sodium sulfate (anhydrous, 98.5%) were obtained from Samchun Chemicals (Pyeongtaek, Korea). Nitric oxide (NO) gas was purchased from HANA gas (Gimhae, Korea). Nitrogen (N_2) and argon (Ar) gas were purchased from Linde Korea (Pohang, Korea). 3-Aminomethyl-1,2-propanediol (>98%) and *N*-phenylacrylamide (>98%) were obtained from TCI (Tokyo, Japan). Phenylboronic acid (98+%) was purchased from Alfa aesar. Poly(ethylene glycol) methyl ether methacrylate (OEGMA, average M_n = 500), 3-(acrylamide)phenylboronic acid (98%), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (88-112%), 1-fluro-2,4-dinitrobenzene (99.0%), toluene (anhydrous, 99.8%), dimethylformamide (Anhydrous, 99.8 %), dimethyl sulfoxide (DMSO, ≥ 99.9% for molecular biology), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffered solution (1 M, pH 7.0-7.6, Bioreagent), 2,4-dinitrophenol (≥ 98.0%), L-glutathione reduced (GSH, ≥ 98.0%), glutathione S-transferase (from equine liver), albumin from human serum (HSA), Hoechst 33258, thiazolyl blue tetrazolium bromide (MTT, Bioreagent, ≥ 97.5%), and Annexin V-FITC apoptosis detection kit and glycerol (≥ 99% for molecular biology) were acquired from Sigma Aldrich. Hydrazine monohydrate (80 %) was acquired from JUNSEI (Tokyo, Japan). Sodium nitrite ($NaNO_2$, >98.5%) was obtained from KANTO Chemical Co., Inc. (Tokyo, Japan). 10 % neutral buffered formalin (NBF) solution was obtained from Biosesang (Seongnam, Korea). Dulbecco's modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) medium, Dulbecco's Phosphate Buffered Saline (DPBS) solution, 100 x Penicillin-streptomycin

(P/S) solution (10 KU/mL penicillin and 10 mg/mL streptomycin) and Fetal Bovine Serum (FBS) was obtained from Capricorn Scientific (Ebsdorfergrund, Germany). 10 X Phosphate buffered saline (PBS) solution was purchased from GIBCO. Trypsin (0.25 %) and RPMI 1640 media with 25 mM HEPES were obtained from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA). DAF-2 diacetate (DAF-2 DA) was acquired from Abcam (Cambridge, UK). The deuterium NMR solvents such as D_2O (D, 99.9 %), methylene chloride-D_2 (CD_2Cl_2, D 99.8%), Chloroform-d ($CDCl_3$, D 99.8%), and Methanol-d_4 (MeOD, D 99.8%) were purchased from Cambridge Isotope Laboratories, Inc (Cambridge, UK).

Instruments

^{1}H-NMR spectra were obtained by using Bruker Advance 500 or 850 MHz FT-NMR with deuterium NMR solvent. The chemical shifts were recorded in parts per million (ppm) and referenced to the NMR solvent peak as following previously reported paper.[1] The patterns of the splitting peaks were represented as s (singlet), d (doublet), t (triplet) and m (multiplet). The coupling constant (J) of each proton peak was analyzed in Hertz (Hz). ^{11}B-NMR spectra were obtained by using Bruker Advance 500 MHz FT-NMR with $CDCl_3$ solvent. Gel permeation chromatography (GPC) measurements were equipped with two different solvent system separately. The GPC profile of OEGMA and POEGMA were conducted by Water Alliance e2695 GPC system equipped with three linked styragel columns (HR1, HR2, and HR4) and refractive index (RI) detector (2414 refractometer). In this system, THF was used as the eluent at a flow rate of 1 mL/min. The molecular weights were calibrated using polystyrene standards (1 kDa, 3 kDa, 10 kDa, 30 kDa, 100 kDa, 300 kDa). The GPC profile of POEGMA and POEGMA-*b*-PPBA was conducted by SHIMADZU GPC system equipped with two consecutive polyhydroxymethacrylate columns and RI detector (RID-10A). In this

system, MeOH was used as the eluent at a flow rate of 0.5 mL/min. The molecular weight distribution was calculated using linear polyethylene glycol standards. (300 Da, 1 kDa, 4 kDa, 8 kDa, 12 kDa) UV-Visible spectra were obtained by using UV 2550 spectrophotometer (Schimadzu). The hydrodynamic size of each sample was obtained by dynamic light scattering (DLS) (Zetasizer Nano S90 system, Malvern Instruments, Worcestershire, U.K.). Transmittance electron microscope (TEM) (JEM-1011, JEOL, Tokyo, Japan) was used to confirm the morphology and size of POEGMA-*b*-PPBA and P-NO-PMs on copper TEM grid. The polymeric constructs were visualized after negative staining with uranyl acetate.

Synthesis of *N*-diazeniumdiolates-incorporated 3-aminomethyl-1-2-propanediol (1-[*N*-methyl-(2,3-dihydroxypropyl)amino]-diazenium-1,2-diolates; Am-diol-NO)

Am-diol-NO was synthesized with modified synthetic protocol.[2] 1.243 g of 3-aminomethyl-1,2-propanediol (11.8 mmol) was taken into 70 mL vial filled with 25 mL MeOH/Ether. (1:4, v/v). After homogenous dispersion, 2.3 mL of 30 wt% NaOMe solution (12.3 mmol of NaOMe) was added into the solution. The solution was moved into the high-pressure NO reactor, followed by argon purging several times. The reactor was then immediately filled with 90 psi of NO gas. After 2 days, NO gas was removed from the reactor and the reactor was flushed with 20-40 psi argon gas. Am-diol-NO was precipitated inside the vial. After collecting the compounds by removing solvent, the Am-diol-NO was washed with cold diethyl ether. The white sticky Am-diol-NO was dried under high vacuum at 0 ˚C. Am-diol-NO was stored under the - 20 ˚C refrigerator with sealing. Yield: 2.1859 g (11.68 mmol, 98.9%) ^{1}H-NMR (D_2O, 500 MHz, δ; ppm) 3.62 (d, J = 11.7 Hz, 1H), 3.56 (s, 1H), 3.49 (dd, J = 11.6, 5.8 Hz, 1H) 3.32 (s, 1H), 3.05 (dd, J = 13.3, 3.5 Hz, 1H), 2.88 (dd, J = 13.1, 8.0 Hz, 1H), 2.74 (s, 3H)

Synthesis of Protected NO donor (P-NO)

P-NO (*O*^{2}-(2,4-dinitrophenyl)-1-[*N*-methyl-(2,3-dihydroxypropyl)amino]-diazenium-1,2-diolates) was synthesized according to a nucleophilic aromatic substitution with modification.[3] 22 mL of *t*-BuOH was added into the 100 mL round flask under 30 ˚C oil bath. Saturated $NaHCO_3$ solution was prepared at room temperature and kept in the ice bath for 20 min. 1.1 mL (8.78 mmol) of 1-fluoro-2,4-dinitrobenzene was added to round flask containing the *t*-BuOH. When 1-fluoro-2,4-dinitrobenzene was dispersed, Am-diol-NO (1.5 eq) was dissolved in 22 mL of the prepared saturated $NaHCO_3$ solution. The AM-diol-NO solution was immediately added into the 1-fluoro-2,4-dinitrobenzene solution. This mixture was stirred overnight at 30 ˚C. The product was collected with extraction with methylene chloride (MC) several times. After extraction, Na_2SO_4 was used for removing traces of water from the solution. The product was purified with flushed silica column chromatography. The yellow solid product was stored at – 20 ˚C with sealing. Yield: 1.53 g (4.17 mmol; 47.6 %) ^{1}H-NMR ($CDCl_3$, 500 MHz, δ; ppm) 8.89 (dd, J = 2.0, 1H) 8.46 (dd, J = 9.2, 2.1 Hz, 1H), 7.68 (d, J = 9.2 Hz, 1H), 4.15-4.00 (m, 1H), 3.78 (dd, J = 12.9, 3.9 Hz, 2 H), 3.73 (dd, J = 14.7, 7.2 Hz, 1H), 3.63 (dd, J = 11.1, 5.1 Hz, 1H), 3.42 (s, 3H). M/S [M+Na+] = 354.1

Synthesis of poly(oligo(ethylene glycol)methyl ether methacrylate) (POEGMA)

POEGMA was synthesized by following RAFT polymerization.[4] Before polymerization, 2,2-azobisisobutyronitrile (AIBN) was re-crystallized with methanol. The re-crystallized AIBN was stored at -20 ℃ before use. In addition, purchased OEGMA was passed through a basic alumina column to remove the inhibitor. The purified OEGMA was stored in the refrigerator at 4 ˚C before use. After purification of these materials, OEGMA500 (1.790 g, 3.58 mmol), 4-cyanopentanoic acid dithiobenzoate (100 mg, 0.358 mmol) and AIBN (5.9 mg, 0.0358 mmol) were dissolved

in 5 mL toluene. The solution was transferred to the schlenk flask with stirring bar. The solution was then deoxygenated by a stream of nitrogen for 30 min with stirring. After deoxygenation, schlenk flask was sealed and put into the 70 ˚C heated oil bath. The solution was then stirred during 18 h. The reaction was monitored by ^{1}H-NMR and GPC and conversion ratio was calculated by ^{1}H-NMR. The reaction flask was cooled to room temperature and the toluene was removed by rotary evaporation. The crude compound was precipitated with cold diethyl ether to afford the product. The molecular weight was determined by GPC by using THF as an eluent (M_n = 4557, theoretical molecular weight: 4430) Yield: 1010.9 mg.

Synthesis of poly(oligo(ethylene glycol)methyl ether methacrylate)-block-poly ((3-acrylamido)phenylboronic acid) (POEGMA-*b*-PPBA)

POEGMA-*b*-PPBA was synthesized by using POEGMA as macro-chain transfer agent. 168 mg of 3-(acrylamido)phenylboronic acid, 150 mg of POEGMA, and 1 mg of recrystallized AIBN were dissolved in 3 mL DMF. This solution was transferred into the schlenk flask with stirring bar and the solution was deoxygenated by a stream of nitrogen with stirring for 30 min. The schlenk flask was sealed and put into 70 ˚C pre-warmed oil bath for reaction. During reaction, a small amount of solution was taken to confirm the progress of the polymerization. Conversion ratio was calculated by ^{1}H-NMR. After 36 h, the schlenk flask was removed from oil bath to terminate the reaction. After cooling it to room temperature, the DMF was evaporated and the crude product was dissolved in small amount of MeOH. The product was purified by precipitation in cold ether. Furthermore, remaining monomer was removed by further dialysis in against MeOH (1 kDa cut-off). After purification, MeOH was evaporated and the product was placed under the vacuum to remove residual solvent. The product was stored it in -20 ˚C condition with sealing. Yield: 249 mg. (Light pink product)

Measurement of the NO release profile in real time

The NO release profile was measured by Sievers 280i Nitric Oxide Analyzer (Boulder, CO). The analysis is based on the chemiluminescence between NO and ozone.[5] NO-donors (Am-diol-NO and P-NO) or P-NO-PMs were added to DPBS (40 mL) w/ or w/o 20 μM or 2 mM GSH, and 10 mM of acetate buffer (pH 5.0) w/ or w/o 2 mM GSH. NO release was evaluated by representing various parameter such as $[NO]_t$ (the number of moles of NO release per mg of the particle), t_d (duration time of NO), $[NO]_m$ (maximum instantaneous concentration of NO released), t_m (time required to reach $[NO]_m$), $t_{1/2}$ (half-life of NO release).

The NO release from P-NO in the presence of GSH or HSA

The effects of GSH concentrations and HSA on NO release from P-NO was investigated by Griess assay. Based on the reaction between nitrite (the product from auto-oxidation of NO) and Griess reagent to form azo dye,[5] $NaNO_2$ was used to plot the calibration curve for the quantitative analysis of NO. P-NO was dissolved in DMSO to make the 500 mM stock solution. The stock solution was diluted it in PBS buffered solution to make the 5 mM P-NO solution. GSH containing solution or HSA containing solution were added to the 5 mM P-NO solutions to make the final samples as following; 50 μM P-NO solution, 50 μM P-NO solution with 2 mM GSH, 50 μM P-NO solution with 50 μM GSH, and 50 μM P-NO with 50 g/L HAS. After 3 h incubation, 50 μL of each sample was transferred in 96 well plate. 50 μL of sulfanilamide solution was then added into the sample-containing well and incubated at room temperature for 5 min with the protection from light. After 5 min, 50 μL of naphthylethylenediamine was added to each solution. The absorbance at 540 nm was measured to quantify the amount of NO release.

Study of the molecular recognition property between PBA and P-NO

The molecular recognition between PBA and diol group of P-NO was monitored by the following procedure. 5 mg of PBA was dissolved in 400 μL MeOH and equivalent P-NO (13.6 mg) was dissolved in 300 μL MeOH. Both solutions were cooled to 0 ˚C and PBA containing solution was mixed with P-NO donor solution with stirring. The solution was allowed to warm to room temperature with stirring for 4 h. The light yellow compound was precipitated during the reaction. After removing MeOH, The precipitated compound was dried under vacuum condition. The structure of the compound was confirmed by 1H-NMR and ^{11}B-NMR. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ; ppm) 8.87 (d, J = 2.7 Hz, 1H), 8.42 (dd, J = 9.3, 2.6 Hz, 1H), 7.78 (d, J = 7.7 Hz, 2H), 7.64 (d, J = 9.3 Hz, 1H), 7.49 (t, J = 7.4 Hz, 1H), 7.37 (t, J = 7.6 Hz, 2H), 5.00-4.84 (m, 1H), 4.52 (t, J = 8.8 Hz, 1H), 4.17 (dd, J = 9.4, 6.8 Hz, 1H), 4.02 (dd, J = 14.9, 3.1 Hz, 1H), 3.74 (dd, J = 15.0, 7.0 Hz, 1H), 3.48 (s, 3H). ^{11}B-NMR ($CDCl_3$, 500 MHz, δ; ppm) 31.91.

Preparation and characterization of P-NO complexed micelles (P-NO-PMs)

The P-NO complexed micelles (P-NO-PMs) were prepared by the co-solvent evaporation method.[6] 1.7 mg of P-NO and 1.9 mg of POEGMA-*b*-PPBA was dissolved in 1 mL MeOH with stirring at 0 ˚C and the temperature was spontaneously warmed up to room temperature by removing ice bath. After 2 h for stirring, 1 mL of deionized (DI) water was added into the solvent and the solution was stirred for 30 min. After evaporating MeOH, DI water was added into the solution to adjust concentration.

Colloidal stability of P-NO-PMs in various physiological conditions

Stability of P-NO-PMs at physiological condition was evaluated by measuring hydrodynamic size of P-NO-PMs in DLS. P-NO-PMs stock solution was mixed with 10 X PBS solution, 0.5 M acetate buffer solution (pH 5.0), concentrated glucose solution (45 g/L), or bovine serum solution to make samples; P-NO-PMs in 1 X PBS solution

(10 mM phosphate buffered solution), 1 X PBS with 1 g/L glucose, 1 X PBS with 4.5 g/L glucose, and 1 X PBS with 10 % serum solution. Each sample was incubated in 37 ˚C water bath and aliquots of the sample were used to measure hydrodynamic volume at pre-determined time intervals. (1, 3, 6, 9, 12 and 24 h)

Cell culture

MCF-7 cell (human breast carcinoma) was incubated in Roswell Park Memorial Institute (RPMI) medium. 4T1 (murine mammary carcinoma) and A549 (human lung carcinoma) cells were incubated in DMEM medium. All of the mediums contain 9 % FBS, 1 % P/S solution. Cells were cultured with the medium and incubated in 5 % CO_2 humidified incubator at 37 °C.

MCF-7/ADR cells were incubated in RPMI with 25 mM HEPES (RPMI containing 25 mM HEPES, Hyclone) and 4 mg/L insulin. In order to maintain drug resistance of MCF-7/ADR, doxorubicin (final concentration of doxorubicin = 400 ng/mL) was treated to the medium once a week.

Intracellular NO release by confocal fluorescence microscopy Intracellular NO production was detected by using DAF-2 DA as NO-sensitive fluorescence probe.[7] MCF-7 cells were seeded as 3 X 10^4 cells per each well (12 well plate) and incubated at 37 ˚C 5% CO_2 incubator overnight. The medium was then changed to fresh medium containing 10 μM Hoechst 33258 and 10 μM of DAF-2-DA. After 1 h incubation, cells were washed twice with DPBS and each sample (P-NO, P-NO-PMs or POEGMA-*b*-PPBA; final PBA concentration is 50 μM.) with medium was treated to the cells. After 2 h incubation with samples, the medium was changed to 10 % NBF solution to fix the cells. After mounting with Vectashield antifade, cells was observed by using an Olympus FV-1000 confocal laser scanning microscope (CLSM). CLSM image was analyzed by the Olympus Fluoview ver. 3.1 viewer software.

Cell viability test

Cell viability tests after treatment proceeded under various concentration. Cells (A549, 4T1, MCF-7, and MCF-7/ADR) were seeded on 96 well culture plates at a density of 6 X 10^3 cells/well and incubated overnight. After incubation, each sample (Am-diol, Am-diol-NO, 2,4-dinitrophenol, POEGMA-*b*-PPBA, P-NO or P-NO-PMs at the final concentration of 0-100 μM) was treated with fresh medium. The dose of POEGMA-*b*-PPBA treatment was determined by the concentration of PBA. Cell viability was evaluated by MTT assay. After 48 h incubation with samples, cells were washed twice with DPBS, in which fresh 180 μL medium was added. 20 μL of 5 mg/mL MTT solution was treated at each well and the well plates were incubated at 37 °C for 4 h. After removing supernatants carefully, purple crystals on the well plates were completely dissolved by 200 μL of DMSO. 100 μL of the solution was transferred into the new 96 well plate and the absorbance of each well at 570 nm was measured by a microplate spectrofluorometer (VICTOR 3V multilabel counter). The absorbance from the non-treated cells was considered as 100 % of cell viability and the relative percentages of the absorbance were used to represent the cell viability of sample-treated group.

Live/Dead assay

NO-mediated anti-cancer mechanism study was conducted by Annexin-V FTIC staining assay. MCF-7 cells were seeded onto 12-well plates at a density of 3 X 10^4 cells per well. After overnight incubation, the culture medium was changed with fresh media containing each sample (50 μM of PBA containing POEGMA-*b*-PPBA, 50 μM of P-NO and 50 μM of P-NO-PMs; 2 wells per 1 group) and incubated at 37 °C CO_2 incubator for 48 h. Small amount of trypsin was used for detachment of the cells from the well. After centrifuge (2000 rpm, 1.5 min), trypsin medium was removed. The collected cells are dispersed in DPBS and then centrifuged again. After washing twice with DPBS,

cells were dispersed in 900 μL of DPBS. After each solution was transferred into 1.75 mL e-tube, 100 μL of 10 X binding buffer, 5 μL of 50 μg/mL Annexin V FITC conjugate solution, 3 μL of 100 μg/mL propidium iodide solution were added to each cell-containing tube under dark condition. After incubation at room temperature for 10 min, the cells were analyzed by flow cytometry. The flow cytometry data was analyzed with a FACS Calibur (Becton Dickinson) and BD Cell Quest software (Becton Dickinson).

***In vivo* antitumor effects**

All animal experiments were approved by the POSTECH Biotech Center Ethics Committee. MCF-7 cells were inoculated subcutaneously into the flank of each female *balb/c-nu/nu* mice (3 X 10^7 cell/mouse). Tumor volume were calculated following the equation of the ellipsoid formula: [tumor volume = $ab^2/2$], where "a" is the longest length and "b" is the shortest length of tumor. Each length was measured using a caliper. When the average tumor volume was around 100 mm^3, the mice were randomly divided into 4 groups (5 mice per group). The mice were then injected with 200 μL samples via intravenously (*i.v.*) twice (Day 1 and Day 4); 1) P-NO (6 mM solution; 20 mg/kg mice), 2) POEGMA-*b*-PPBA (22 mg/kg mice) 3) P-NO-PMs (including 6 mM P-NO, 42 mg/kg mice). 5 mice without any treatment were considered as a control group. Each tumor volume and body weight were recorded during experimental progress. (n = 5, represented as mean ± SEM). All statistical results were presented using one-way ANOVA. Mouse were sacrificed at Day 23.

Synthesis of thiolated POEGMA-*b*-PPBA

In order to conjugate NIR dye to POEGMA-*b*-PPBA, thiolated POEGMA-*b*-PPBA was synthesized by the aminolysis of dithioester group.[8,9] 10 mg of POEGMA-*b*-PPBA was dissolved in EtOH and the solution was deoxidized by nitrogen purging. 20 μL of 1/125 diluted monohydrated hydrazine solution (2.6 X 10-3 mmol) was added into the solution

with stirring for 1.5 h. After the reaction, the solution was under dialysis in MeOH (1 KDa cut off). The purified solution was then under evaporation to remove the MeOH. Non-colored product was isolated after the evaporation. (5.6 mg; Non-colored product)

Synthesis of NIR-dye conjugated POEGMA-*b*-PPBA

NIR dye conjugated POEGMA-*b*-PPBA was synthesized by the reaction between thiol and maleimide.[10] 5.6 mg of thiolated POEGMA-*b*-PPBA and 0.2 mg of TCEP·HCl were dissolved in MeOH for reduction of unwanted disulfide bond. After 30 min, 0.5 mg of Maleimide-functionalized NIR dye (Flamma 749-Maleimide) and 10 μL of 1/10 diluted TEA were added into the solution with stirring overnight. The product was purified with dialysis (MWCO 1 kDa). The product was obtained by evaporation of MeOH. (4.2 mg; green-colored product)

Hemolysis test

Hemolysis test was followed the previously reported method with slight modification.[11] Fresh mouse blood from *balb/c-nu/nu* mouse was 10X diluted with PBS buffer. The blood solution was centrifuged at 2000 rpm for 15 min to isolate red blood cells (RBC). Supernatant was removed from the sample and the RBC was re-dispersed in PBS buffer. 100 μL of RBC solution was added into 1.75 mL e-tube including 25 μL PBS buffer. 125 μL of each sample (6 mM P-NO, POEGMA-*b*-PPBA containing 6 mM PBA, or P-NO-PMs containing 6 mM P-NO, 6 mM DOX and 6 mM PTX) was added to RBC containing solution, respectively. (n = 3 for each group) After treatment, each solution was incubated at 37 °C for 4 h. After centrifuge (2000 rpm, 15 min), the absorbance of supernatants was measured at 541 nm in order to calculate the hemoglobin release. Saline and 1 X lysis buffer were used as negative control (0 % hemolysis) and positive control (100 % hemolysis), respectively.

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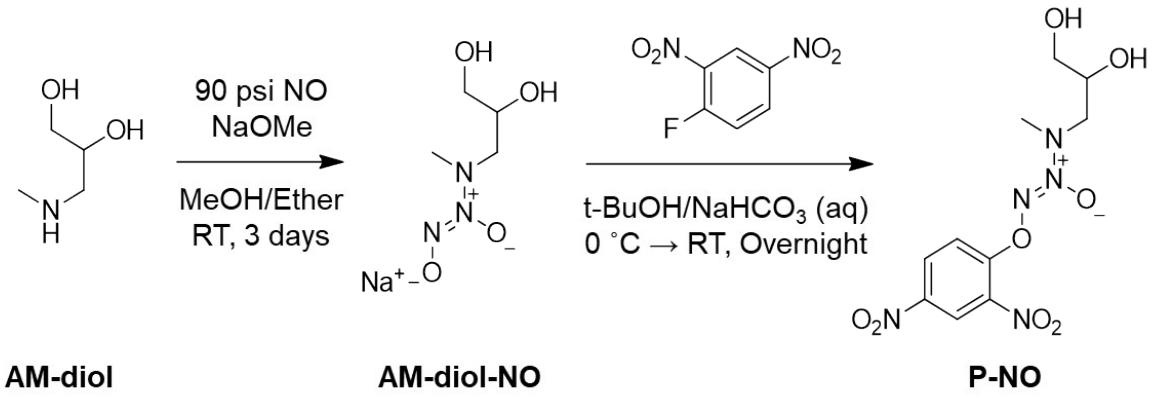

Fig. S1 Synthetic scheme for O^2-(2,4-dinitrophenyl)-1-[*N*-methyl-(2,3-dihydroxypropyl)amino]-diazenium-1,2-diolates (P-NO).

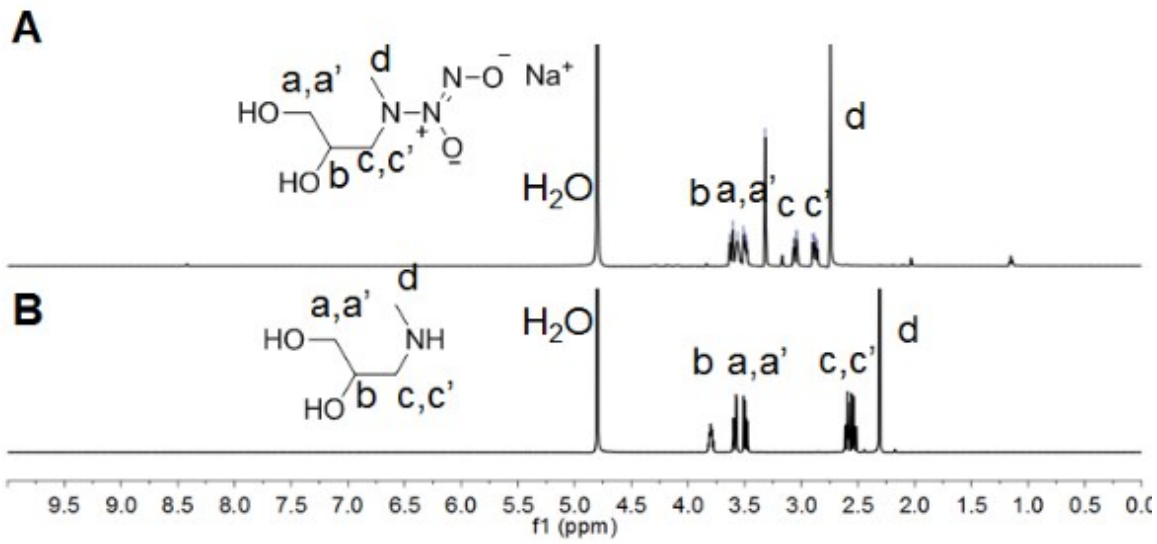

Fig. S2 ^{1}H-NMR spectrum of (A) 1-[*N*-methyl-(2,3-dihydroxypropyl)amino]-diazenium-1,2-diolates (Am-diol-NO) and (B) 3-methylamino-1,2-propanediol (Am-diol) in D_2O.

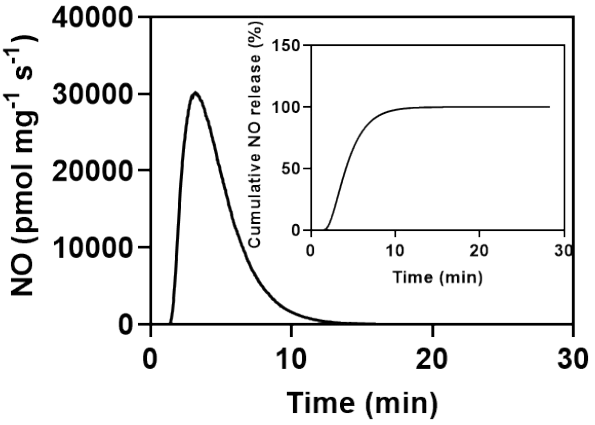

Fig. S3 NO releasing profile of Am-diol-NO.

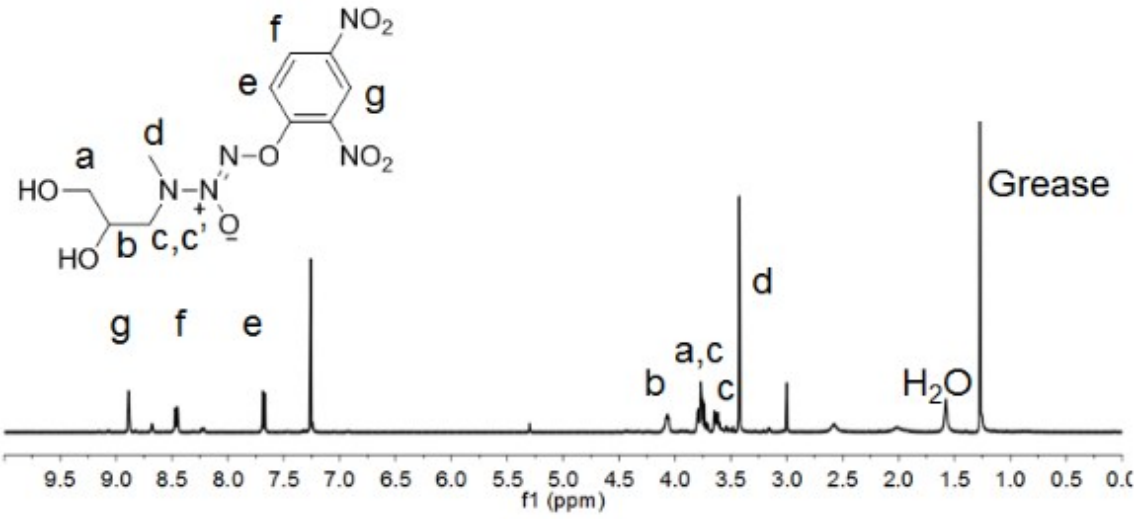

Fig. S4 ^{1}H-NMR spectrum of *O*^{2}-(2,4-dinitrophenyl)-1-[*N*-methyl-(2,3-dihydroxypropyl)amino]-diazenium-1,2-diolates (P-NO) in $CDCl_3$.

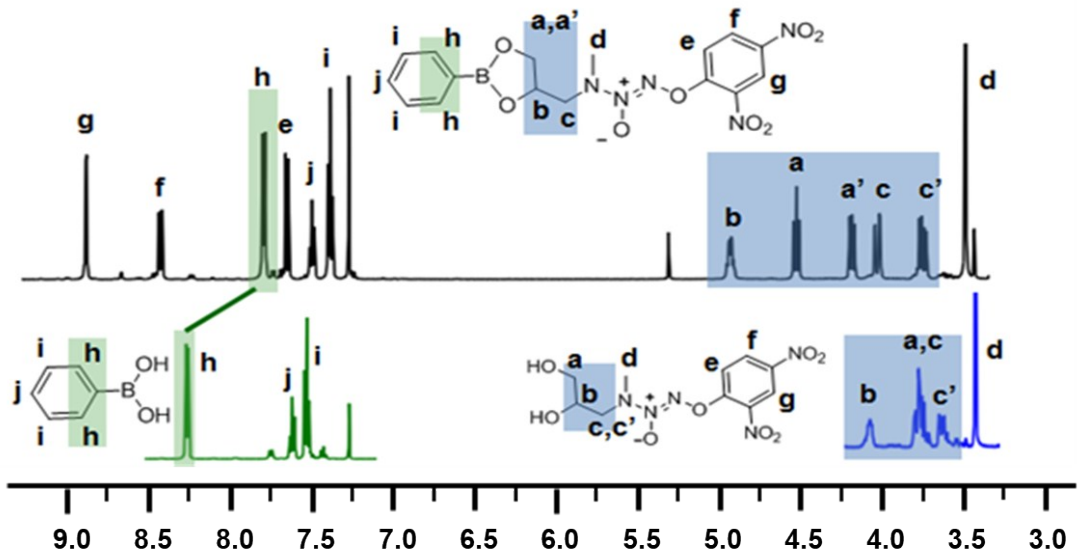

Fig. S5 ^{1}H-NMR spectrum of PBA:P-NO mixture.

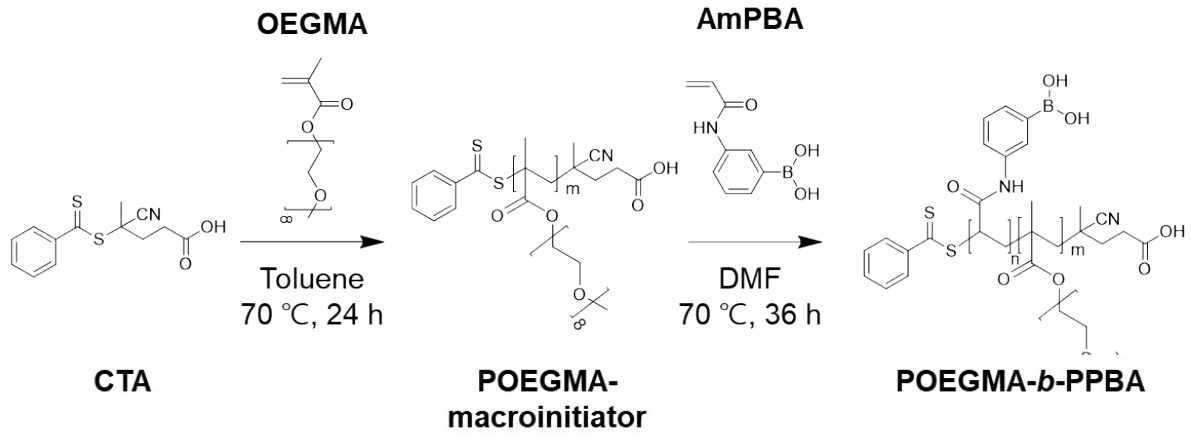

Fig. S6 Synthetic scheme for POEGMA-*b*-PPBA.

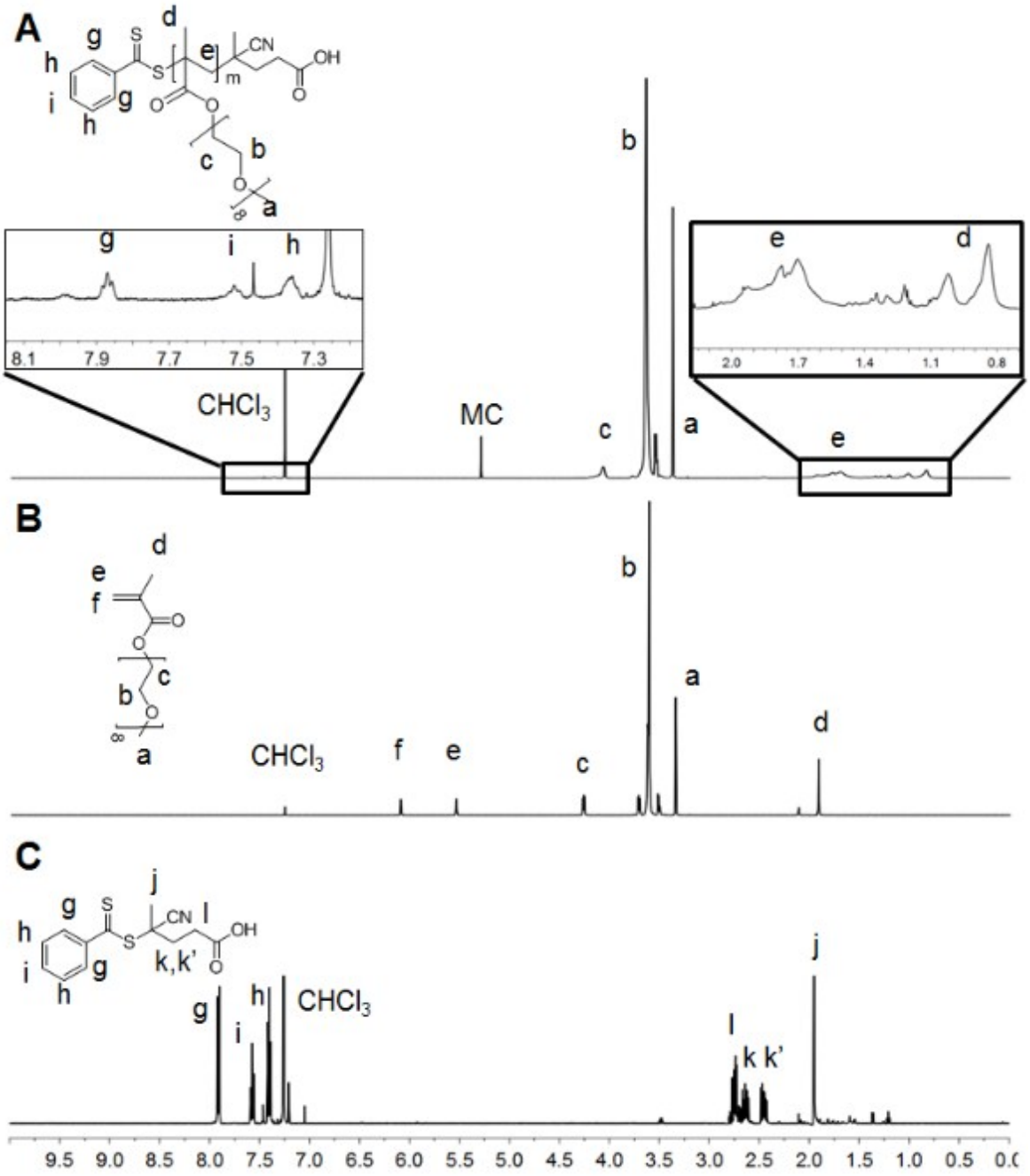

Fig. S7 ^{1}H spectrum of (A) POEGMA, (B) OEGMA and (C) Chain transfer agent (CTA) (4-cyano-4-(phenylcarbonothioylthio)pentanoic acid) in $CDCl_3$

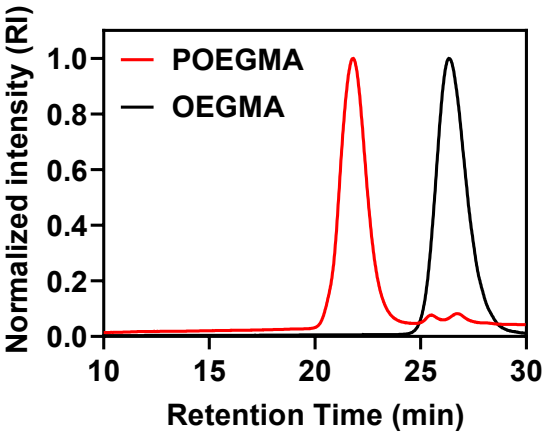

Fig. S8 GPC profiles of POEGMA and OEGMA. THF was used as eluent.

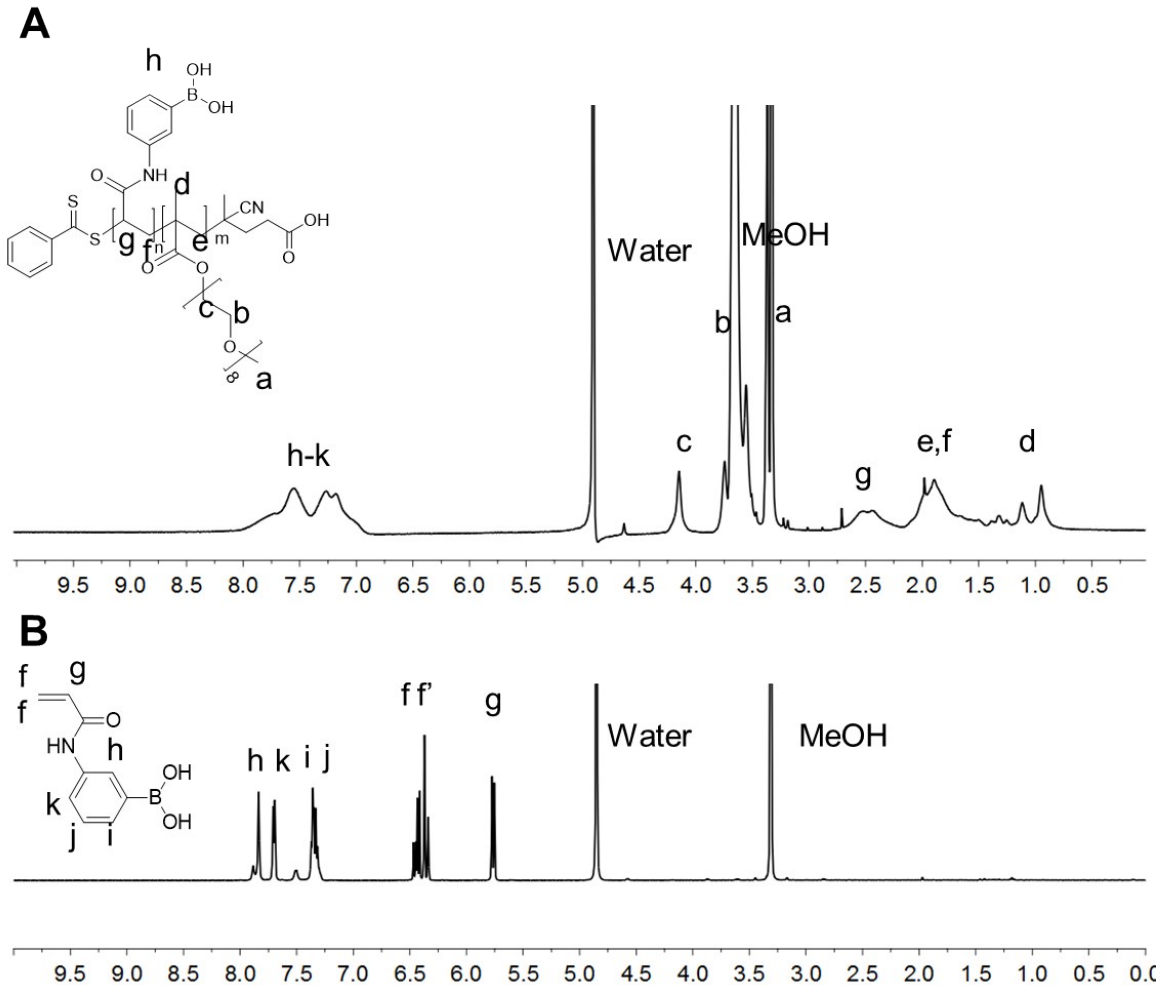

Fig. S9 ^{1}H spectrum of (A) POEGMA-*b*-PPBA and (B) 3-(acrylamido) phenylboronic acid in MeOD.

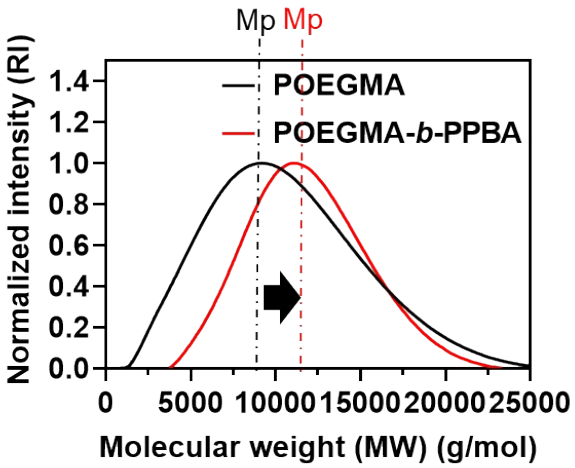

Fig. S10 GPC profiles of POEGMA and POEGMA-*b*-PPBA in MeOH eluent condition. Linear PEG was used as standard materials for calculating relative molecular weight distributions.

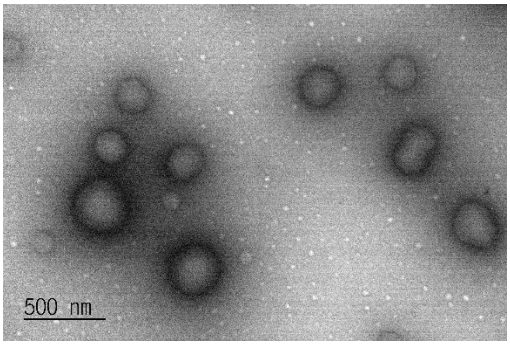

Fig. S11 TEM image of POEGMA-*b*-PPBA.

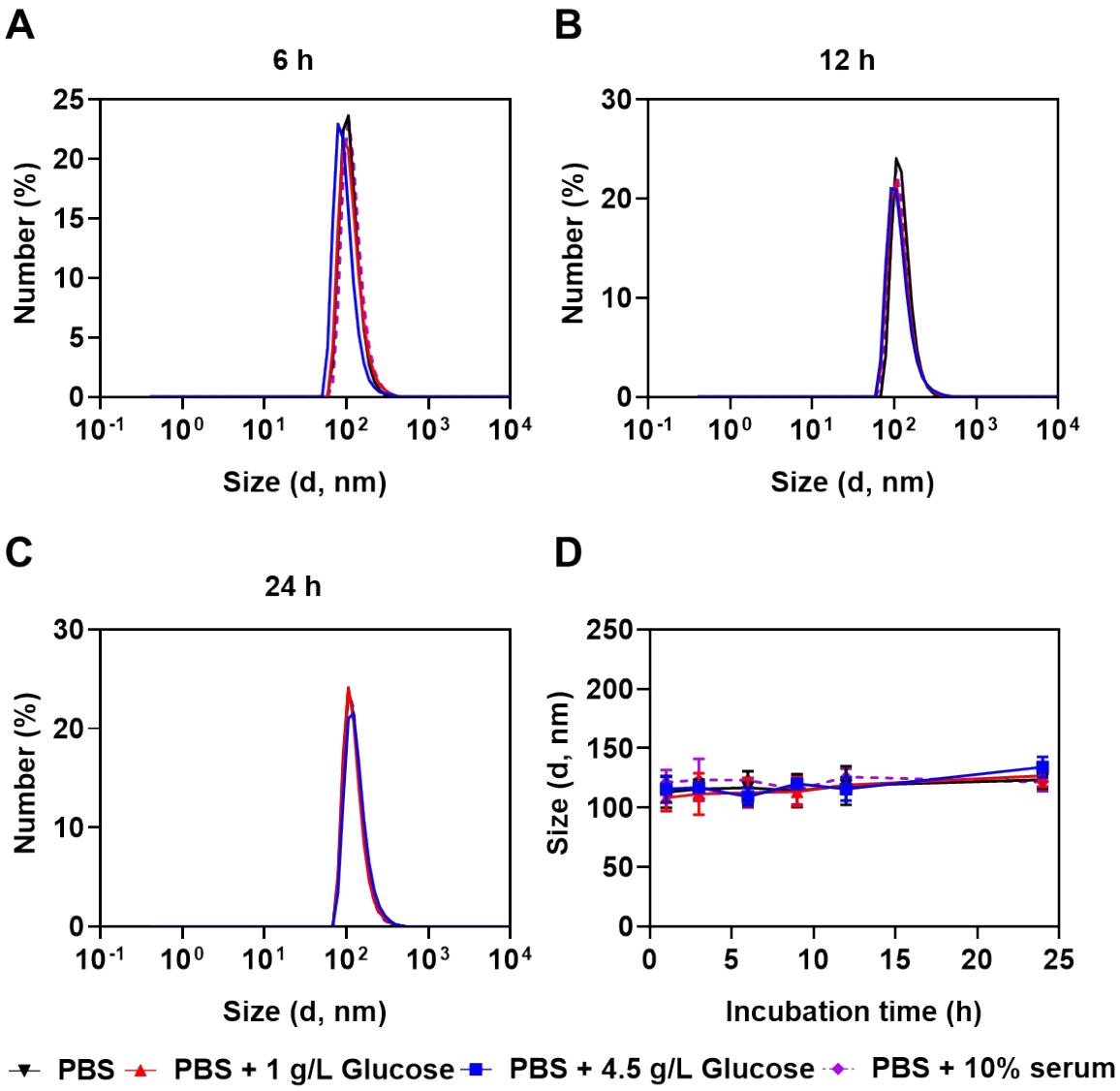

Fig. S12 Size distribution of P-NO-PMs measured at (A) 6 h, (B) 12 h, (C) 24 h after incubation under various physiological conditions. (D) Time-dependent average size in physiological conditions.

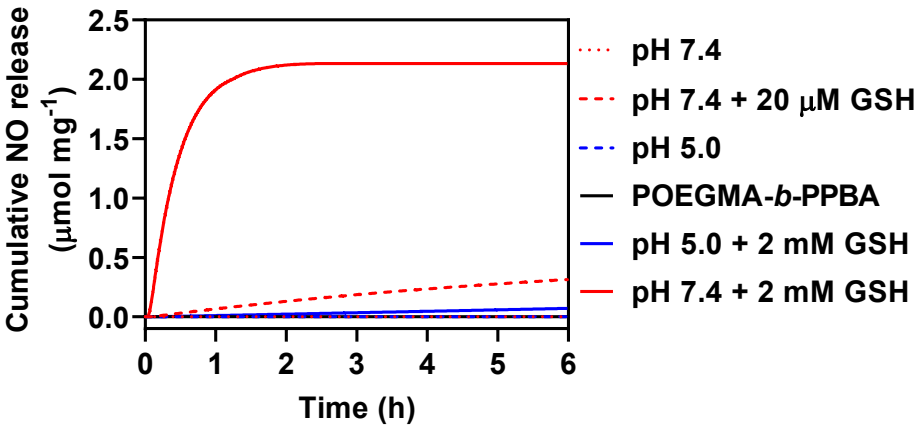

Fig. S13 Cumulative NO release profile of P-NO-PMs under various physiological conditions.

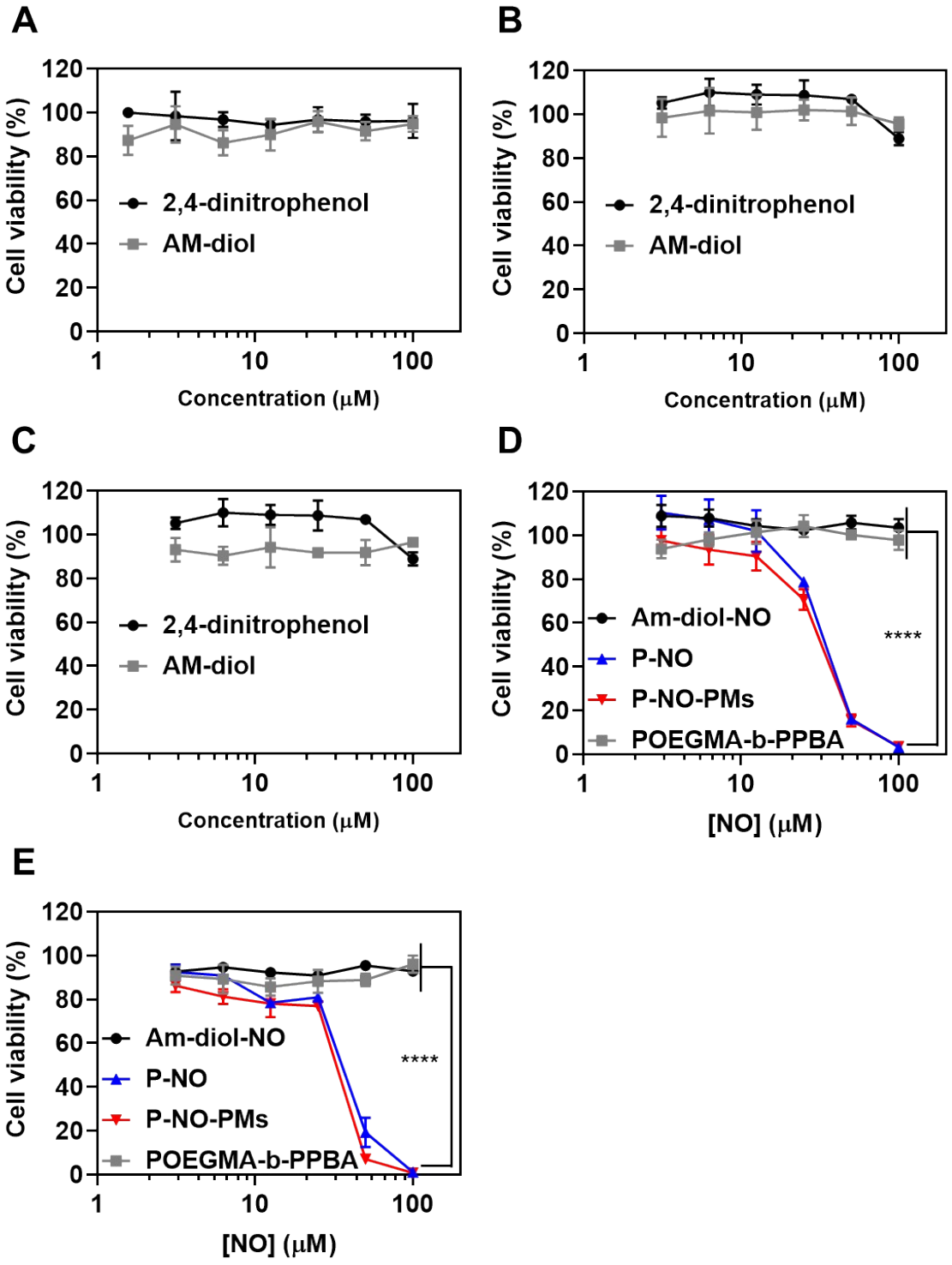

Fig. S14 Cytotoxicity tests. Cell viability profile of Am-diol and 2,4-dinitrophenyl groups assayed in (A) MCF-7, (B) A549, and (C) 4T1 cell lines. Anticancer effects of P-NO-PMs in (D) A549 and (E) 4T1 cell lines.

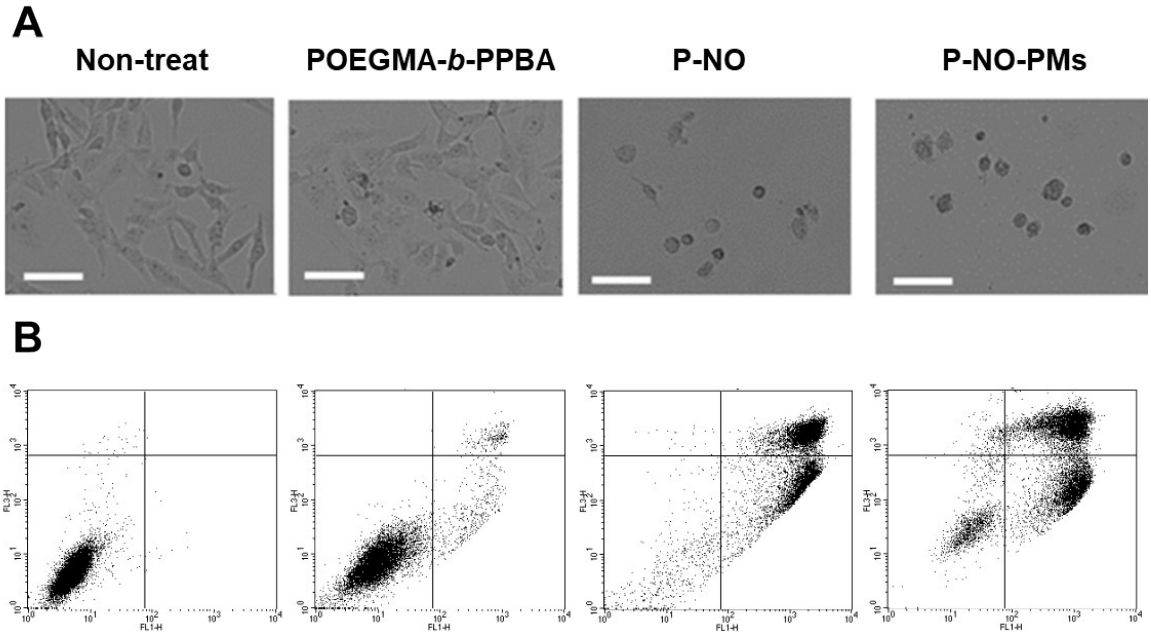

Fig. S15 The mechanism study of NO-induced antitumor therapy. (A) Morphology of the MCF-7 cells after treatment with POEGMA-*b*-PPBA (bare micelles), P-NO (50 uM), and P-NO-PMs (50 uM P-NO). White scale bar is 100 μm. The cells treated with P-NO and P-NO-PMs were mostly detached from the cultured plates. In addition, the cell shrinkage and cytoplasmic condensation were observed, which would be attributed to the cell apoptosis. (B) Annexin-V/PI staining assay to quantify apoptotic pathway.

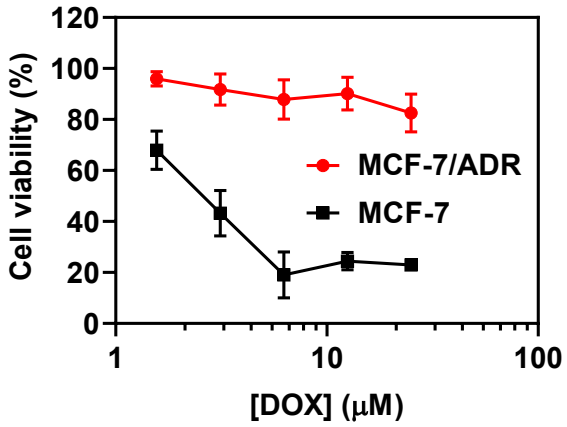

Fig. S16 Anticancer effects in MCF-7 cell and MCF-7/ADR cell lines.

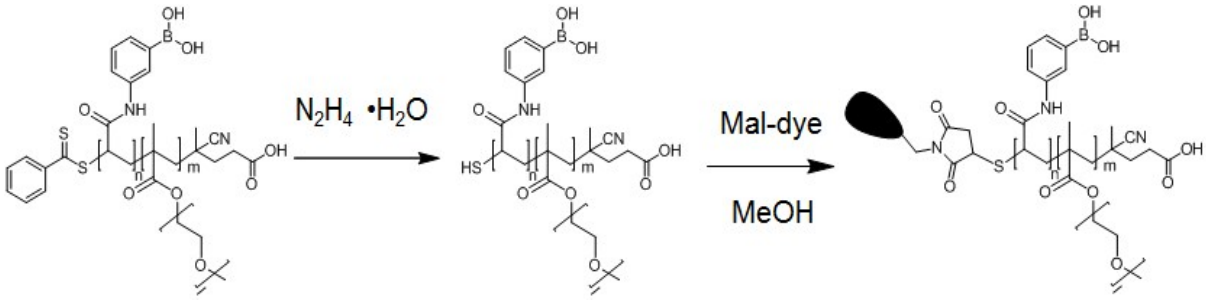

Fig. S17 Synthetic scheme for FCR 749-conjugated POEGMA-*b*-PPBA

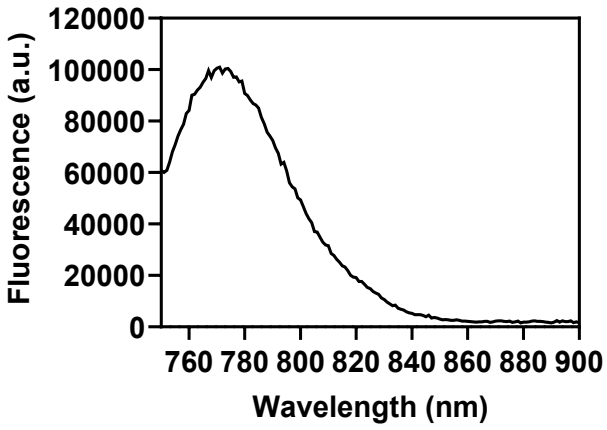

Fig. S18 Fluorescence spectrum of FCR 749-labelled P-NO-PMs.

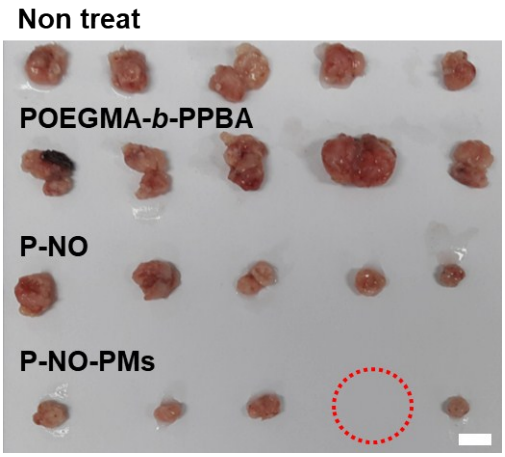

Fig. S19 Optical image of dissected tumor on 23 day. Red dot circle means that tumor was successfully regressed. White scale bar is 1 cm.

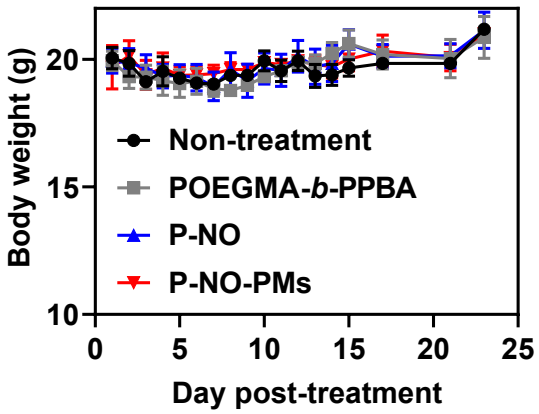

Fig. S20 Body weight changes after *i.v.* administrations.

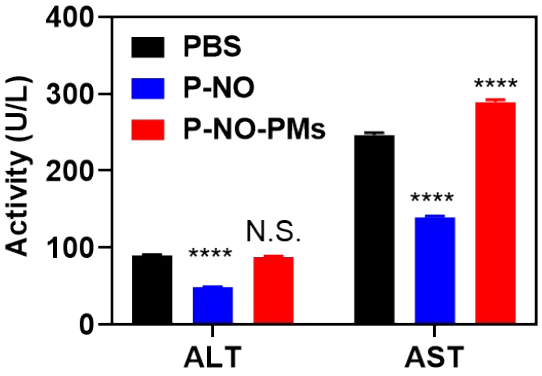

Fig. S21 ALT/AST analysis 5d after administration of P-NO-PMs.

Table S1. NO releasing characteristics of P-NO[a]

Material & conditions	$[NO]_t$[b]	t_d[c]	$[NO]_m$[d]	t_m[e]	$t_{1/2}$[f]
Am-diol-NO pH 7.4	7.28	0.47	30153	3.17	4.15
P-NO pH 7.4 w/o GSH	0	0	0	N.D.	N.D.
P-NO pH 7.4 w/ GSH	4.99	3.88	1890.0	8.18	29.3
P-NO pH 5.0 w/ GSH	0.23	> 4h	20.0	4.8	> 4h

[a] Measured in DPBS at 37 °C by NO Analyzer (NOA 280i chemiluminescence NO analyzer) during 6 h. [b] $[NO]_t$, the number of micromoles of NO release per mg. [c] t_d, duration (h) of NO release above 1 pmol·mg^{-1}·s^{-1}. [d] $[NO]_m$, maximum instantaneous concentration of NO release (pmol·mg^{-1}·s^{-1}). [e] t_m, time required to reach $[NO]_m$ (min). [f] $t_{1/2}$, half-life of NO release (min). N.D., not determined

Table S2. NO releasing characteristics of P-NO-PMs[a]

Conditions	$[NO]_t$[b]	t_d[c]	$[NO]_m$[d]	t_m[e]	$t_{1/2}$[f]
pH 7.4 w/o GSH	0	0	0	N.D.	N.D.
pH 7.4 20 μM GSH	0.3	> 6h	37.8	22.1	> 6h
pH 7.4 2 mM GSH	2.1	2.6	1167.4	7.2	21.3
pH 5.0 w/o GSH	0	0	0	N.D.	N.D.
pH 5.0 2 mM GSH	$7.1X10^{-2}$	> 6h	3.9	357.1	N.D.

[a] Measured in DPBS at 37 °C by NO Analyzer (NOA 280i chemiluminescence NO analyzer) during 6 h. [b] $[NO]_t$, the number of micromoles of NO release per mg. [c] t_d, duration (h) of NO release above 1 $pmol \cdot mg^{-1} \cdot s^{-1}$. [d] $[NO]_m$, maximum instantaneous concentration of NO release ($pmol \cdot mg^{-1} \cdot s^{-1}$). [e] t_m, time required to reach $[NO]_m$ (min). [f] $t_{1/2}$, half-life of NO release (min). N.D., Not determined.