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Materials

N-Hydroxysuccinimidal ester of tetramethyl rhodamine (NHS-TMR) was purchased from the Invitrogen Company. Cyanine 5-NHS ester was purchased from the Lumiprobe Corporation. Cysteamine 4-methoxytrityl resin was bought from EMD Millipore Corporation. *N*,*N*,*N*,*N*,*N*-Pentamethyldiethylenetriamine (PMDETA) was purchased from Sigma-Aldrich. PEG macroinitiator, MeO-PEG_{114}-Br, was prepared from 2-bromo-2-methyl propanoyl bromide and MeO-PEG_{114}-OH according to the procedure in literature.[1] Monomers such as 2-(diethylamino)ethyl methacrylate (DEA-MA) and 2-aminoethyl methacrylate (AMA-MA) were purchased from Polyscience Company. AMA-MA was recrystallized twice with isopropanol and ethyl acetate (3:7) before use. Methacrylate monomers including 2-(dipropylamino) ethyl methacrylate (DPA-MA), 2-(dibutylamino) ethyl methacrylate (DBA-MA), 2-(dipentylamino) ethyl methacrylate (D5A-MA) and 2-(hexamethyleneimino) ethyl methacrylate (C7A-MA) were synthesized following previous publications.[2] JetPEI was bought from Polyplus-transfection. PEG_{5000}-PLA_{5000} was purchased from Advanced Polymer Materials Inc. Amicon ultra-15 centrifugal filter tubes (M_W = 10 K or 3,500 Da) were obtained from Millipore. Other solvents and reagents were used as received from Sigma-Aldrich or Fisher Scientific Inc.

Synthesis *S*-(2-Thiopyridyl)cysteine hydrochloride

A solution of cysteine hydrochloride (3.16 g, 20.0 mmol) in methanol (40 mL) was added dropwise to a magnetically stirred mixture of 2,2'-dithiodipyridine (8.80 g, 40.0 mmol) and acetic acid (1.5 mL) in methanol (40 mL).[3] After stirring at room temperature for 24 h, the solvent was removed from the mixture by rotary evaporation. The product was dissolved in methanol and isolated by precipitating with diethyl ether. This washing/precipitation procedure was repeated 4 times to lead to a white product. ^{1}H NMR (400 MHz, DMSO-d_6, ppm): 8.72 (br s, 3H), 8.50 (ddd, *J* = 4.8, 1.8, 0.9 Hz, 1H), 7.81 (td, *J* = 7.7, 1.8 Hz, 1H), 7.71 (dt, *J* = 8.2, 1.0 Hz, 1H), 7.29 (ddd, *J* = 7.5, 4.9, 1.1 Hz, 1H), 4.17 (dd, *J* = 7.1, 5.2 Hz, 1H), 3.43-3.27 (m, 2H). $[M-Cl]^+$: 231.0 (calculated 231.3).

Synthesis of TMR-SH

TMR-SH was synthesized following a previous report with minor modification.[4] Firstly, cysteamine 4-methoxytrityl resin (18 mg) was pre-swelled in dimethylformamide (DMF) for 2 h at room temperature. NHS-TMR (10.6 mg, 0.02 mmol) was dissolved in dry DMF (0.3 mL) and then added to the resin suspension with *N*,*N*-diisopropylethylamine (DIEA, 15 μL, 0.08 mmol). The reaction was proceeded in the dark for 24 h. The resin was washed with DMF for 3 times followed by dichloromethane (DCM). After vacuum drying, the functionalized resin was treated with a mixture of trifluoroacetic acid (TFA, 0.2 mL) and DCM (0.4 mL). The solvent was evaporated at room temperature under vacuum. The resulting TMR-SH product was collected and its molecular weight was confirmed by HPLC-MS. $[M+H]^+$: 490.0 (calculated 490.2).

Synthesis of small molecule of qRAS

Compound *S*-(2-Thiopyridyl)cysteine hydrochloride (0.64 mg, 2.4 μM) and Cy5-NHS (1.54 mg, 2.5 μM) were dissolved in 0.2 mL of Ar saturated dry DMSO. Triethylamine (0.76 mg, 7.6 μM) was added. The resulting mixture was added into 100 mM phosphate-buffered saline (PBS, pH 7.6), then TMR-SH (1.23 mg, 2.5 μM) in Ar saturated DMSO was added in the mixture. The reaction was allowed at r.t. in the dark under Ar for 4 h. The product was isolated using HPLC, and collected fractions were concentrated under reduced pressure to remove acetonitrile and lyophilized. $[M\text{-}Cl]^+$: 1073.6 (calculated 1073.9).

This above compound was modified through the available carboxylic acids in order for further conjugation.[5] The obtained compound (1.66 mg, 1.5 μM) was reacted with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (0.96 mg, 5 μM) and *N*-hydroxysuccinimide (0.58 mg, 5 μM) in DMSO for 6 h. The amine-reactive qRAS product was separated by chromatography using carboxymethyl cellulose, the solvent was removed and stored at -80 ℃. $[M\text{-}Cl]^+$: 1170.2 (calculated 1170.0). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.03 (d, J = 6.0 Hz, 1 H), 8.67 (s, 1 H), 8.29 -8.27(m, 3 H), 7.60 (d, J = 7.4 Hz, 2 H), 7.37 (dd, J = 9.4, 7.2 Hz, 4 H), 7.22 (q, J = 8.1 Hz, 2 H), 7.13-6.86 (m, 6 H), 6.53 (t, J = 12.3 Hz, 1 H), 6.25 (d, J = 13.8 Hz, 2 H), 4.59-4.41 (m, 1H), 4.04 (t, J = 7.4 Hz, 2 H), 3.62 (d, J = 6.4 Hz, 3 H), 3.24-3.13 (m, 20 H), 2.89 (s, 4 H), 2.52 (s, 2 H), 1.66 (s, 18 H), 1.54 (dt, J = 20.1, 7.4 Hz, 3 H), 1.39 (t, J = 7.8 Hz, 3 H), 1.22 (s, 1 H).

The position of Cy5 and TMR was switched and synthesized according to the same protocol. $[M\text{-}Cl]^+$: 1170.1 (calculated 1170.0). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.25 (d, J = 8.0 Hz, 1 H), 8.73 (s, 1 H), 8.37-8.27(m, 3 H), 7.60 (d, J = 7.5 Hz, 2 H), 7.37 (dd, J = 9.5, 7.2 Hz, 4 H), 7.22 (q, J = 7.2 Hz, 2 H), 7.12-6.92 (m, 6 H), 6.51 (t, J = 12.3 Hz, 1 H), 6.24 (d, J = 13.8 Hz, 2 H), 4.79 (t, J = 11.0 Hz, 1 H), 4.05 (t, J = 7.2 Hz, 2 H), 3.56 (s, 3 H), 3.28-3.11 (m, 20 H), 2.77 (s, 4 H), 2.05 (s, J = 7.1 Hz, 2 H), 1.66 (s, 18 H), 1.57-1.45 (m, 3 H), 1.32 (p, J = 7.5, 7.0 Hz, 3 H), 1.22 (s, 1 H).

Redox activation of qRAS

To examine the fluorescence emission intensity of qRAS in response to a reducing stimulus, qRAS was firstly dissolved in methanol. Then 5 mM tris(2-carboxyethy)phosphine (TCEP) was added to trigger the cleavage of disulfide bond. The fluorescence spectra were recorded on a Hitachi Fluorometer with an excitation wavelength of 550 nm. In the meantime, the emission spectra of Cy5 was also measured as excited at 640 nm. The normalized ratio of the emission intensities of the TMR (F_{575}) and Cy5 (F_{665}) in response to redox cleavage was plotted over time.

Conjugation and characterization of qRAS to ovalbumin (OVA)

To a solution of OVA (4.5 mg, 0.1 μM) in PBS (100 mM, pH 7.6) was added a solution of qRAS (0.36 mg, 0.3 μM) in 100 μL DMSO. The reaction mixture was vortexed, and then allowed to react at r.t. in the dark for 12 h. The mixture was passed through a PD-10 gel filtration column to

separate the product from unreacted qRAS and NHS byproduct. The obtained protein (OVA^{qRAS}) was lyophilized and stored at -80 ℃.

To assess its redox response, OVA^{qRAS} was added into a solution of PBS (pH 7.4) containing 5 mM GSH. The emission spectra of TMR was measured on a Hitachi Fluorometer with an excitation wavelength of 550 nm. The fluorescent images of OVA^{qRAS} solution before and after the addition of GSH (5mM) were obtained using the Maestro imaging system (CRI, Inc., Woburn, MA) with corresponding band pass excitation filter and long-pass emission filter according to the instrument manual.

Syntheses of PEG-*b*-PR block copolymers

PEG-*b*-PR copolymers were synthesized by atom transfer radical polymerization (ATRP) following similar procedures previously reported.[6] PEG-*b*-PDBA ($UPS_{5.3}$) is used as an example to illustrate the procedure. First, DBA-MA (1.93 g, 8 mmol), PMDETA (23 μL, 0.1 mmol), and MeO-PEG_{114}-Br (0.5 g, 0.1 mmol) were charged into a polymerization tube. Then a mixture of 2-propanol (2 mL) and DMF (2 mL) was added to dissolve the monomer and initiator. After three cycles of freeze-pump-thaw to remove the oxygen, CuBr (15 mg, 0.1 mmol) was added into the polymerization tube under nitrogen atmosphere, and the tube was sealed *in vacuo*. The polymerization was carried out at 40 ℃ for 12 h. After polymerization, the reaction mixture was diluted with 15 mL tetrahydrofuran (THF), and passed through a neutral Al_2O_3 column to remove the catalyst. The THF solvent was removed by rotovap. The residue was dialyzed against distilled water and lyophilized. After synthesis, the polymers were characterized by gel permeation chromatography (GPC).

Syntheses of PEG-*b*-(PR-*r*-qRAS) block copolymers

AMA-MA was used for the conjugation of qRAS. Synthesis of PEG-*b*-(PR-*r*-AMA) copolymers followed the procedure described above. Three primary amino groups were introduced into each polymer chain by controlling the feeding ratio of AMA monomer to the initiator. After synthesis, PEG-*b*-(PR-*r*-AMA) and NHS-qRAS were dissolved in 1 mL DMF. After overnight reaction, the copolymers were purified by preparative gel permeation chromatography (PLgel Prep 10 m 10E3 Å 300×250 columns by Varian, THF as eluent at 5 mL/min) to remove the free dye molecules. The produced copolymers were lyophilized and kept at -80 ℃ for storage.

Preparation of polymeric nanoparticles

Nanoparticles were prepared following a previously published procedure.[2] In the example of $UPS_{5.3}$, 10 mg of the copolymer was dissolved in 1 mL THF and then added into 4 mL distilled water dropwise under sonication. The mixture was filtered 3 times to remove THF using the ultrafiltration system (M_W = 10 KD). Then distilled water was added to adjust the polymer concentration to 10 mg/mL as a stock solution. The nanoparticles were characterized by dynamic light scattering for hydrodynamic diameter (Dh).

Cells

A549 lung cancer cells were cultured in DMEM medium (Invitrogen, CA) supplemented with 5% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin at 37 ℃ in 5% CO_2 atmosphere.

High-throughput quantification protocol

A549 cancer cells were seeded into 96-well black plate at a density of 10,000 cells per well and incubated for 24 h. The UPS polymer (100 μg/mL) and qRAS-labeled protein (OVA^{qRAS}, 25 μg/mL) were co-incubated with cells for 40 min at 37 ℃. Polyethylenimine was used as positive control (15 μg/mL, normalized based on the amount of nitrogen group) and PEG-PLA (100 μg/mL) as the negative control. The cells were washed twice with PBS (pH7.4) and cultured in complete culture medium. The fluorescence of TMR and Cy5 were measured on a Tecan fluorescent plate reader at different times. At 24 h, 5 mM DTT was added to cleave the intracellular disulphide bond and completely release the TMR signal. The excitation and emission settings: TMR: Ex: 545 nm with bandwidth 9 nm, Em: 595 nm with bandwidth 20 nm; Cy5: Ex: 640 nm with bandwidth 9 nm, Em: 690 nm with bandwidth 20 nm.

The activation percentage (%) of OVA^{qRAS} was calculated using the equation described below:

$$\text{Activation (\%)} = (F_{TMR} - F_0)/(F_{TMR,DTT} - F_0) * 100$$

where F_{TMR} is the fluorescence intensity of TMR at different times, $F_{TMR,\,DTT}$ is the fluorescence intensity of TMR after the addition of DTT at 24 h, and F_0 is the background of TMR fluorescence.

For the *N*-ethylmaleimide (NEM) blocking experiments, the same procedures were used except that the cells were pretreated with 50 μM NEM for 30 min prior to the treatment with polymer and OVA^{qRAS}.

Confocal microscopy analysis

A549 lung cancer cells were plated into glass bottom dishes (MatTek, MA) in 1 mL phenol red-free DMEM medium and were allowed to grow to 60-70% confluence. Cells were co-incubated with qRAS-labeled protein (OVA^{qRAS} or IgG^{qRAS}, 25 μg/mL) and $UPS_{4.4}$ or PC7A for 40 min at a polymer concentration of 100 μg/mL. The cells were then stained with LysoSensor Green DND-189 (1 μM) for 10 min at 37 ℃. The medium was exchanged to complete DMEM medium and confocal images were acquired at different time points using the ZEISS LSM700 laser-scanning confocal microscope with a 60× objective lens.

Cytotoxicity analysis of polymers

A549 cells were seeded into 96-well plate at 10,000 cells per well and incubated for 24 h. Then the cells were exposed to a series of polymers at 100 μg/mL for 40 min and washed twice with PBS (pH7.4), and the fresh medium was added into plates. The cells were incubated for 48 h

before determination of cell viability. The cell viability was measured using MTT assay.[7] Briefly, the cells were incubated with 0.5 mg/mL MTT solution for 4 h, after which the medium was removed. Then 200 μL of DMSO was added into cell plates for OD determination at 570 nm using a microplate reader (SpectraMax M5, Molecular Devices, CA).

In vitro efficacy of cytotoxic protein delivery

Ribonuclease A was used as a cytotoxic protein to evaluate the cytosolic efficacy of JetPEI and PEG-PLA. Firstly, the lysine residues of proteins were reacted with cis-aconitic anhydride to convert lysines into negatively charged carboxylate groups (RNase A-Aco). This modification is reversible in the acidic intracellular environment (e.g, endosomes and lysosomes), leading to the restoration of the biological activity of the modified proteins.[8] To evaluate the efficacy, A549 cells were seeded into 96-well plates at a density of 10,000 cells per well. The cells were exposed to JetPEI (15 μg/mL) and PEG-PLA (100 μg/mL) with RNase A-Aco (5 μg/mL) for 40 min and washed twice with PBS (pH7.4), and the fresh cell culture medium was added into plates. The cells were incubated for 48 h before determination of cell viability using MTT assay.

Statistical analysis

Statistical analysis was performed using Prism 5.0 (GraphPad). Data are expressed as means ± S.D. Data were analyzed by Student's t test, and considered statistically significant if $p< 0.05$. (*, $p < 0.05$; **, $p < 0.01$ unless otherwise indicated)

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Table S1. Chemical compositions and physical properties of UPS nanoparticles.

Polymers	Composition[a]	pKa[b]	Dh (nm)[c]	PDI[c]
$UPS_{7.4}$	$P(DEA_{80})$	7.43	9.9±1.1	0.25±0.03
$UPS_{7.1}$	$P(DEA_{58}\text{-}DPA_{42})$	7.05	28.4±2.0	0.24±0.01
$UPS_{6.8}$	$P(DEA_{39}\text{-}DPA_{61})$	6.77	35.1±4.8	0.24±0.01
$UPS_{6.5}$	$P(DEA_{21}\text{-}DPA_{79})$	6.45	45.1±1.3	0.15±0.01
$UPS_{6.2}$	$P(DPA_{80})$	6.19	57.0±2.7	0.18±0.02
$UPS_{5.9}$	$P(DPA_{60}\text{-}DBA_{20})$	5.89	68.1±4.7	0.19±0.03
$UPS_{5.6}$	$P(DPA_{30}\text{-}DBA_{50})$	5.58	62.9±6.5	0.15±0.01
$UPS_{5.3}$	$P(DBA_{80})$	5.31	71.8±3.3	0.15±0.06
$UPS_{5.0}$	$P(DBA_{56}\text{-}D5A_{24})$	4.93	60.6±5.4	0.19±0.02
$UPS_{4.7}$	$P(DBA_{28}\text{-}D5A_{52})$	4.65	65.2±1.0	0.15±0.01
$UPS_{4.4}$	$P(D5A_{80})$	4.36	73.8±3.1	0.18±0.03
PC7A	$P(C7A_{80})$	6.97	32.4±2.0	0.30±0.02

[a] Only the composition of the PR segment is shown. The subscripts indicate the number of repeating unit for each monomer; [b] The apparent pKa values for UPS nanoparticles were determined by pH titration of polymer solutions using 4 M NaOH in the presence of 150 mM NaCl. The maximum buffer pH corresponds to the apparent pKa of each copolymer; [c] The hydrodynamic diameter (Dh) and polydispersity index (PDI) were measured using dynamic light scattering analysis, mean ± S.D (n=3).

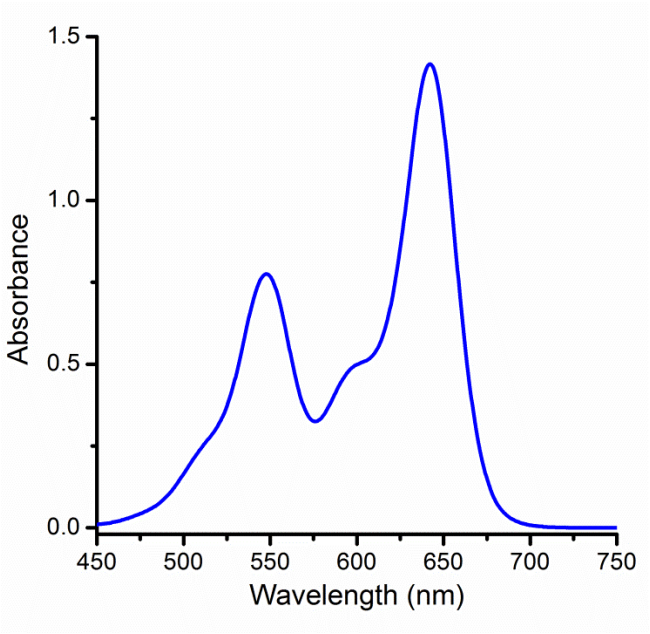

Figure S1. The UV-Vis absorbance spectrum of qRAS. qRAS was dissolved in methanol, the absorbance was recorded on UV-Vis spectrometer from 450 to 750nm. The peaks at 550 nm and 645 nm represent the absorbance of donor dye (TMR) and acceptor dye (Cy5), respectively.

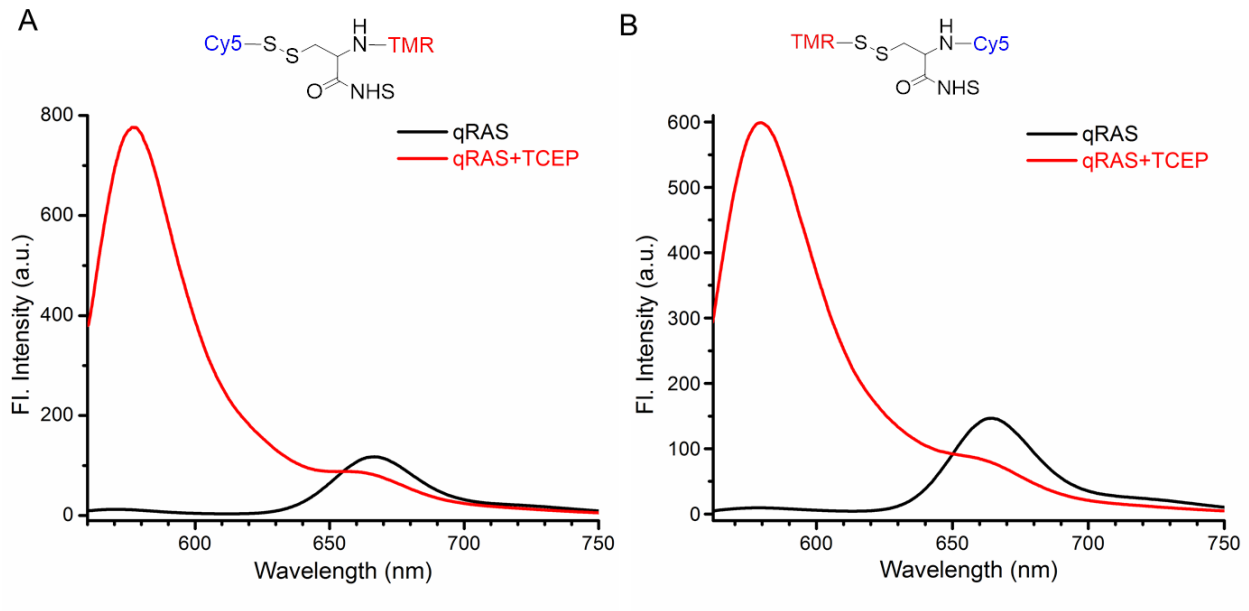

Figure S2. Fluorescence emission spectra of two qRAS probes with switched donor/acceptor positions before and after the addition of TCEP to cleave the disulfide bond in methanol. The samples were excited at 550 nm.

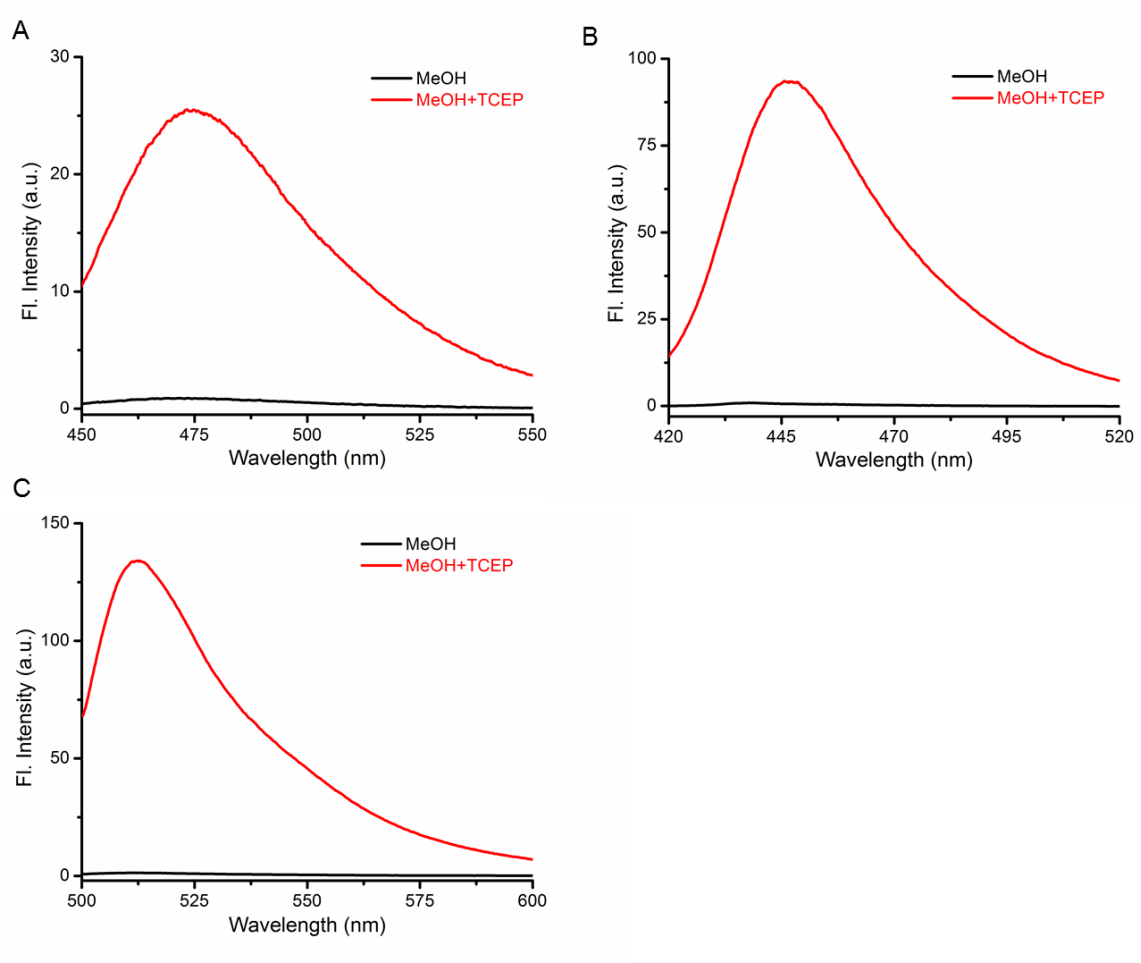

Figure S3. The emission spectra of qRAS probes 7-diethylaminocoumarin/QSY35 (A), 7-hydroxycoumarin/Dabcyl (B) and BODIPY 493/BHQ-1 quencher (C) before and after the addition of reducing reagent TCEP on a Hitachi fluorometer.

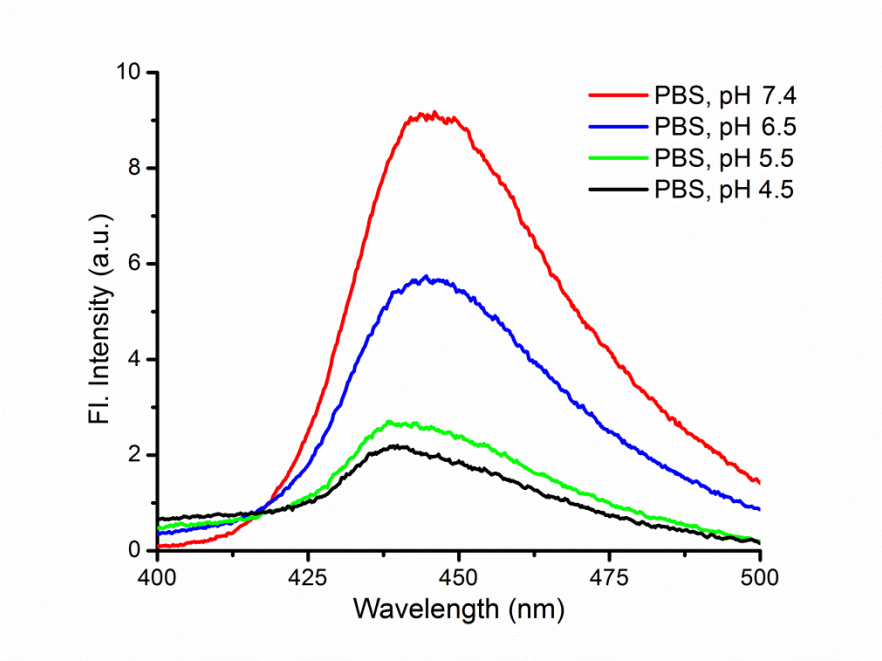

Figure S4. The emission spectra of qRAS probe (7-hydroxycoumarin/Dabcyl) as a function of pH in the PBS buffer. Samples were excited at 390 nm.

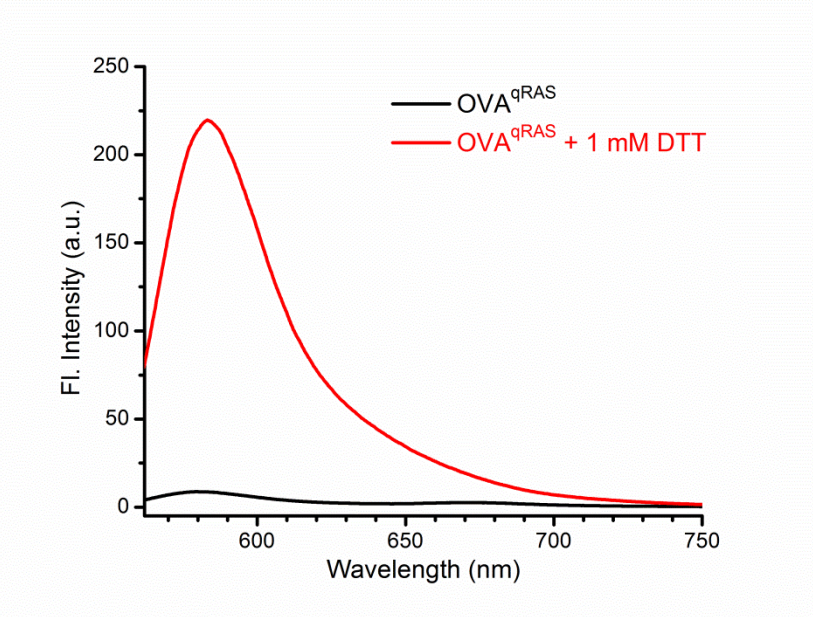

Figure S5. The fluorescence emission spectra of OVA^{qRAS} before and after the addition of 1 mM dithiothreitol (DTT) to trigger the cleavage of the disulfide bond. The excitation wavelength was at 550 nm .

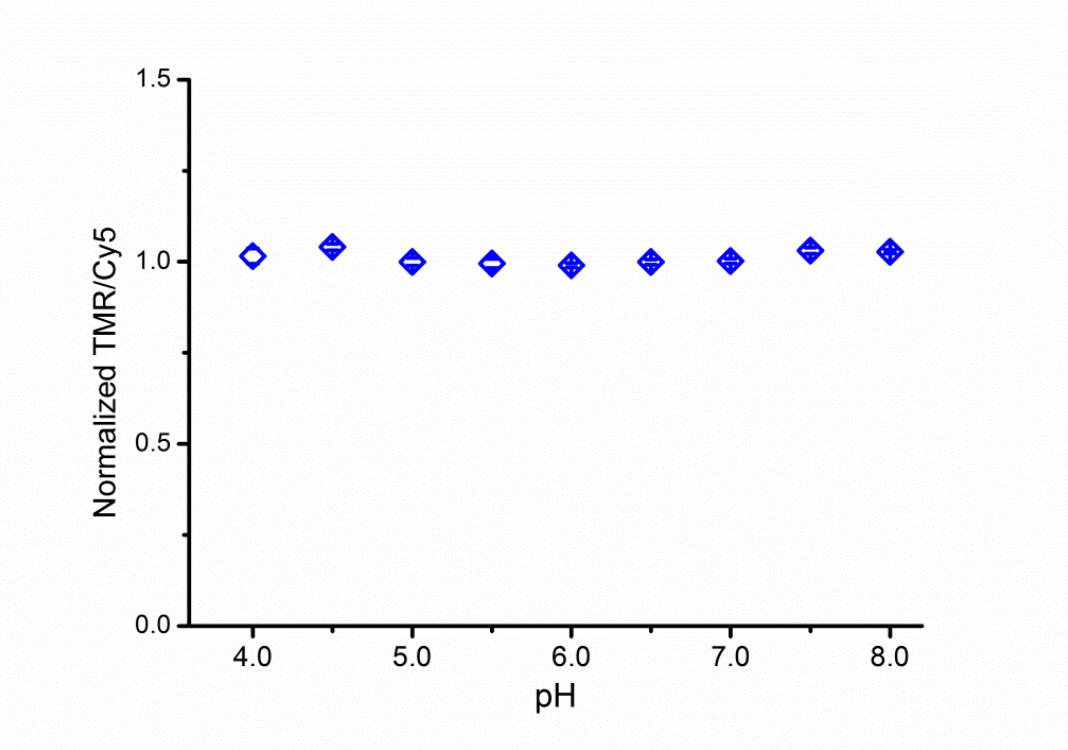

Figure S6. Normalized fluorescence emission ratio of OVA^{qRAS} in PBS at different pH. OVA^{qRAS} (0.5 mg/mL) was incubated in PBS with different pH for 6 h. The fluorescence emission spectra of TMR and Cy5 were recorded using a Hitachi fluorometer with excitation wavelength at 550 nm and 640 nm, respectively.

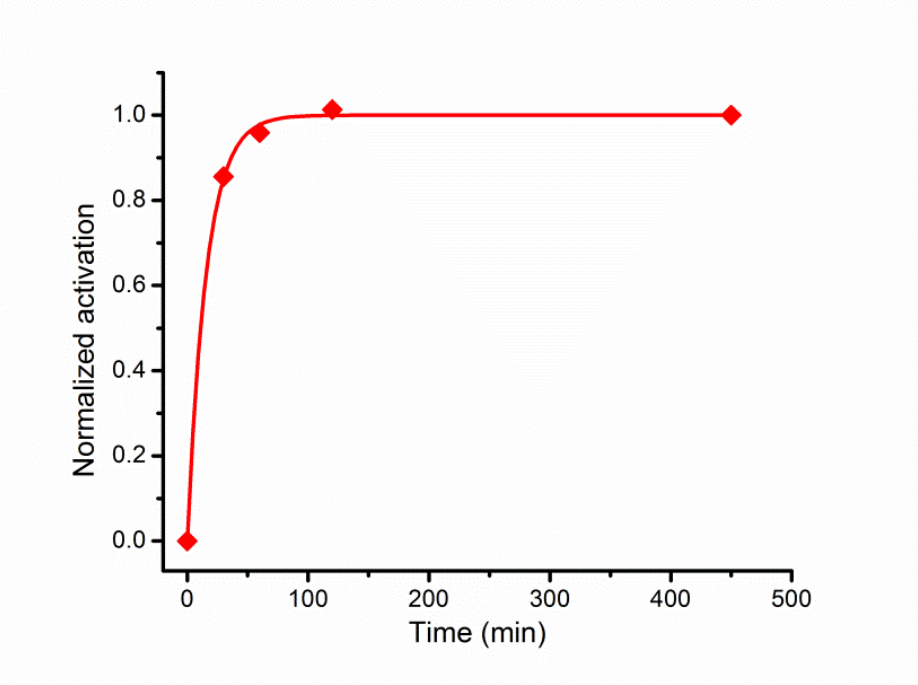

Figure S7. Investigation of redox activation of OVA^{qRAS} by GSH over time on a Tecan plate reader (Infinite 200 PRO). OVA^{qRAS} (0.5 mg/mL) was incubated in PBS solution (pH 7.4) containing 5 mM GSH. Fluorescence emission of TMR was normalized to maximum intensity and plotted versus time.

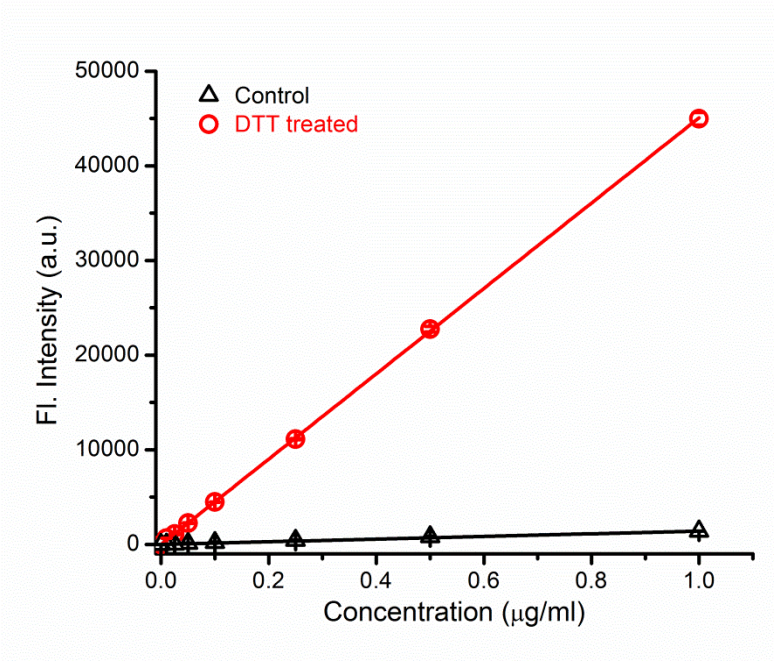

Figure S8. Linear correlation of fluorescence intensity of OVA^{qRAS} as a function of probe concentration on a plate reader. Fluorescence intensity was measured in the TMR channel as excited at 545 nm. OVA^{qRAS} solution before and after the addition of DTT was shown. The linear correlations of the off and on states of OVA^{qRAS} were used to quantify the activation percentage in live cell imaging applications.

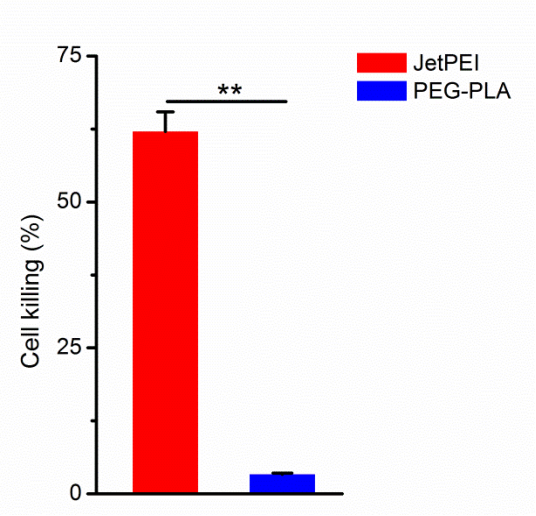

Figure S9. JetPEI enhanced the cytotoxicity of ribonuclease A. A549 cells were exposed to JetPEI and PEG-PLA with ribonuclease A-aco (5 μg/mL) for 40 min at 37 ℃. The cell viability was evaluated by the MTT assay after 48 h incubation. Error bars represent standard deviation of 3 replicate samples.

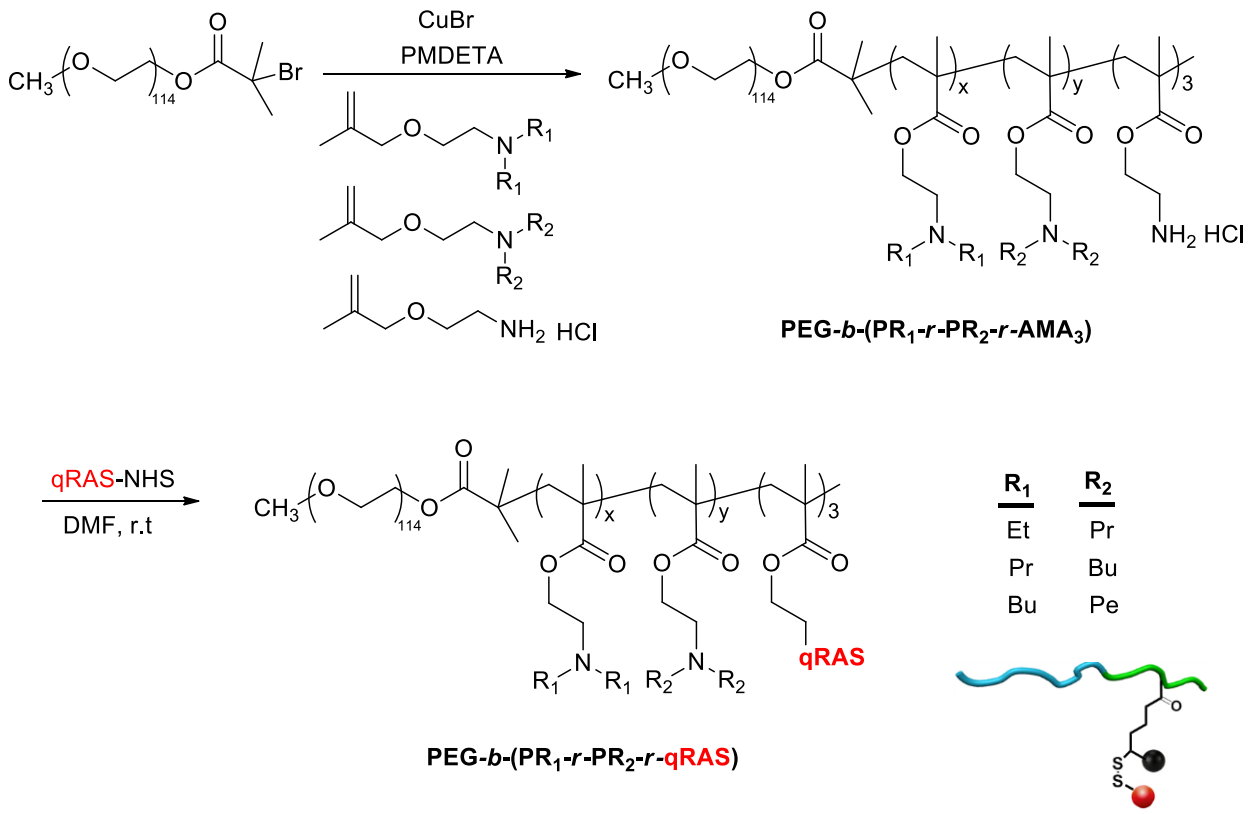

Figure S10. Synthesis of qRAS-labeled UPS copolymers. The PR segment consists of a random block from two monomers with different molar fractions to fine-tune its pH transition (see **Table S1**). (R_1 or R_2 = Et, ethyl; Pr, propyl; Bu, butyl; Pe, pentyl).

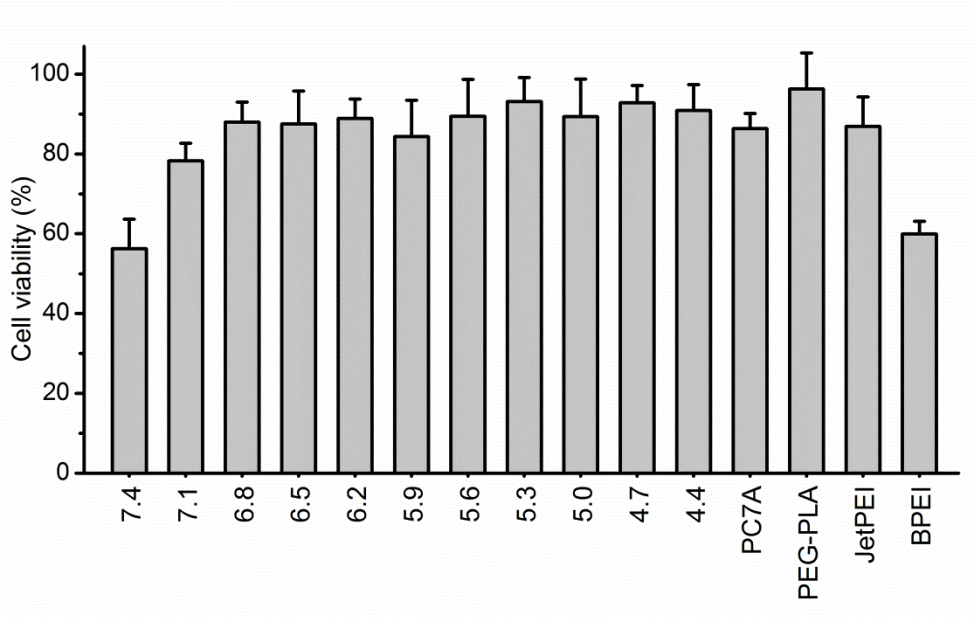

Figure S11. Evaluation of the cytotoxicity of polymers in A549 lung cancer cells. A549 cells were exposed to different polymers for 40 min at 37 ℃. The cell viability was evaluated by the MTT assay after 48 h incubation. Error bars represent standard deviation from 3 replicate samples.

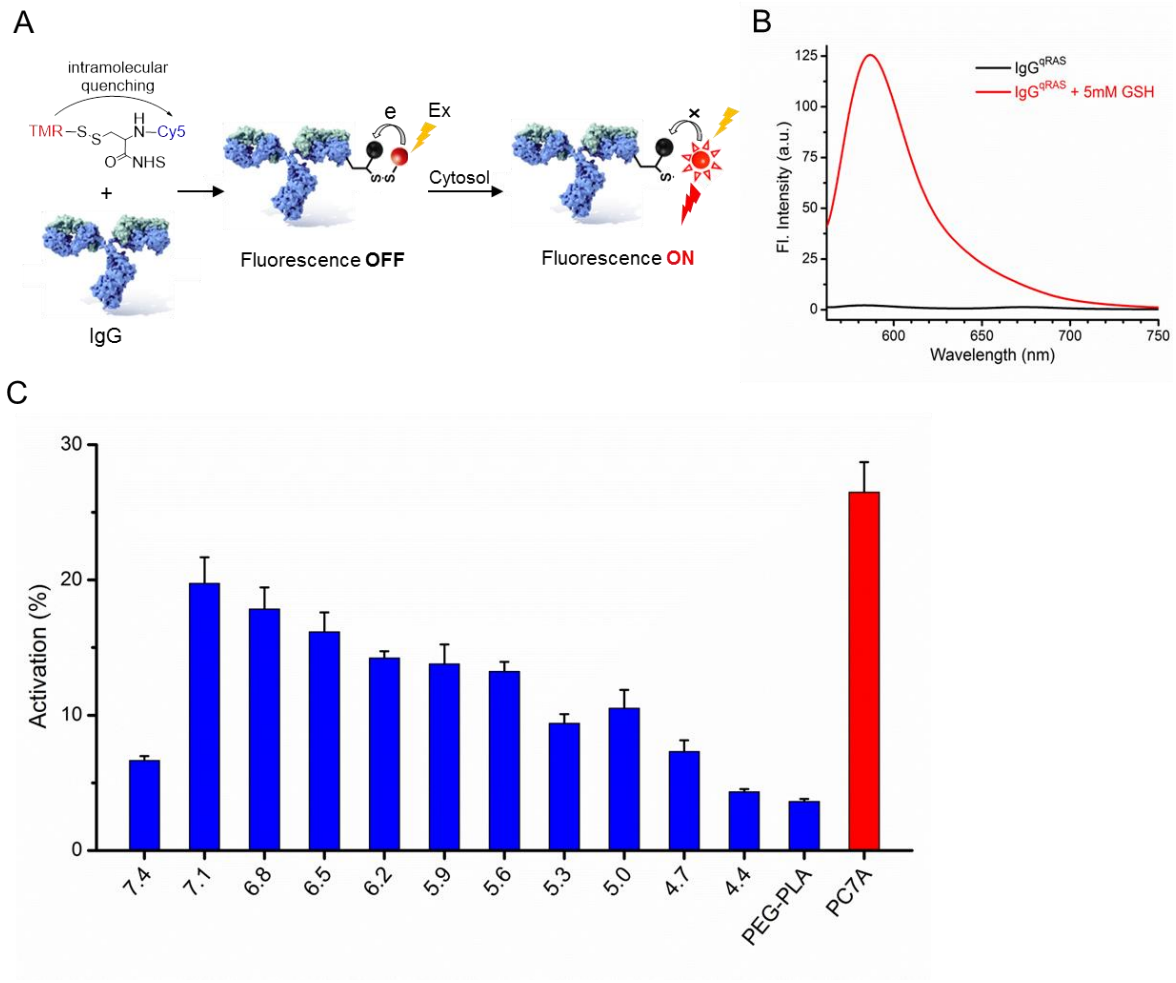

Figure S12. The high-throughput quantification of cytosolic delivery efficiency using qRAS-labeled IgG. A) Scheme of qRAS conjugation to IgG. B) The fluorescence emission spectra of IgG^{qRAS} before and after the addition of 5 mM GSH. The excite wavelength is 550 nm. C) The cytosolic delivery efficiency of the polymers at 24 h after co-incubation with IgG^{qRAS} for 40min.

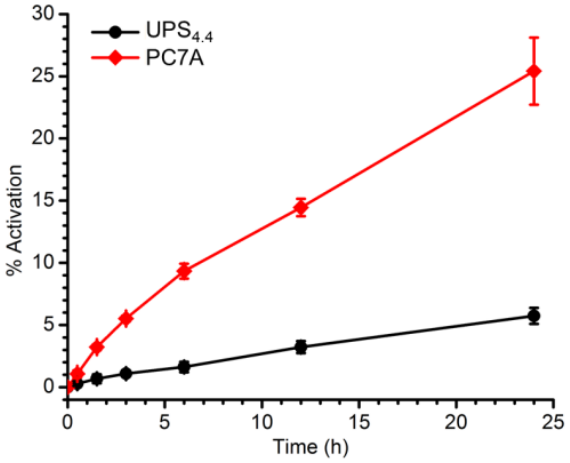

Figure S13. Kinetics of cytosolic delivery of OVA^{qRAS} by $UPS_{4.4}$ and PC7A in A549 cells (n = 3).

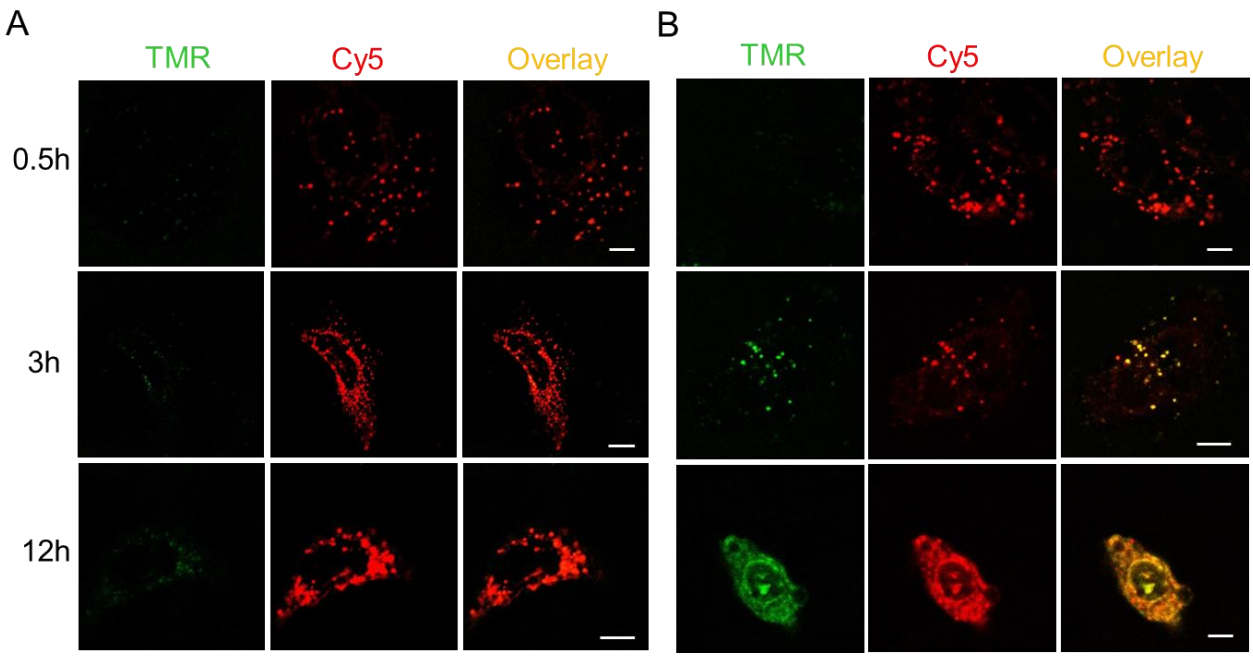

Figure S14. Subcellular imaging of cytosolic activation of OVA^{qRAS} by confocal microscopy. A549 cancer cells were co-incubated with OVA^{qRAS} (25 μg/mL) and $UPS_{4.4}$ (A) or PC7A (B) at 100 μg/mL for 40 min. Both the intracellular fluorescence images of TMR (Green) and Cy5 (Red) channel were taken at different times. Scale bars = 10 μm.

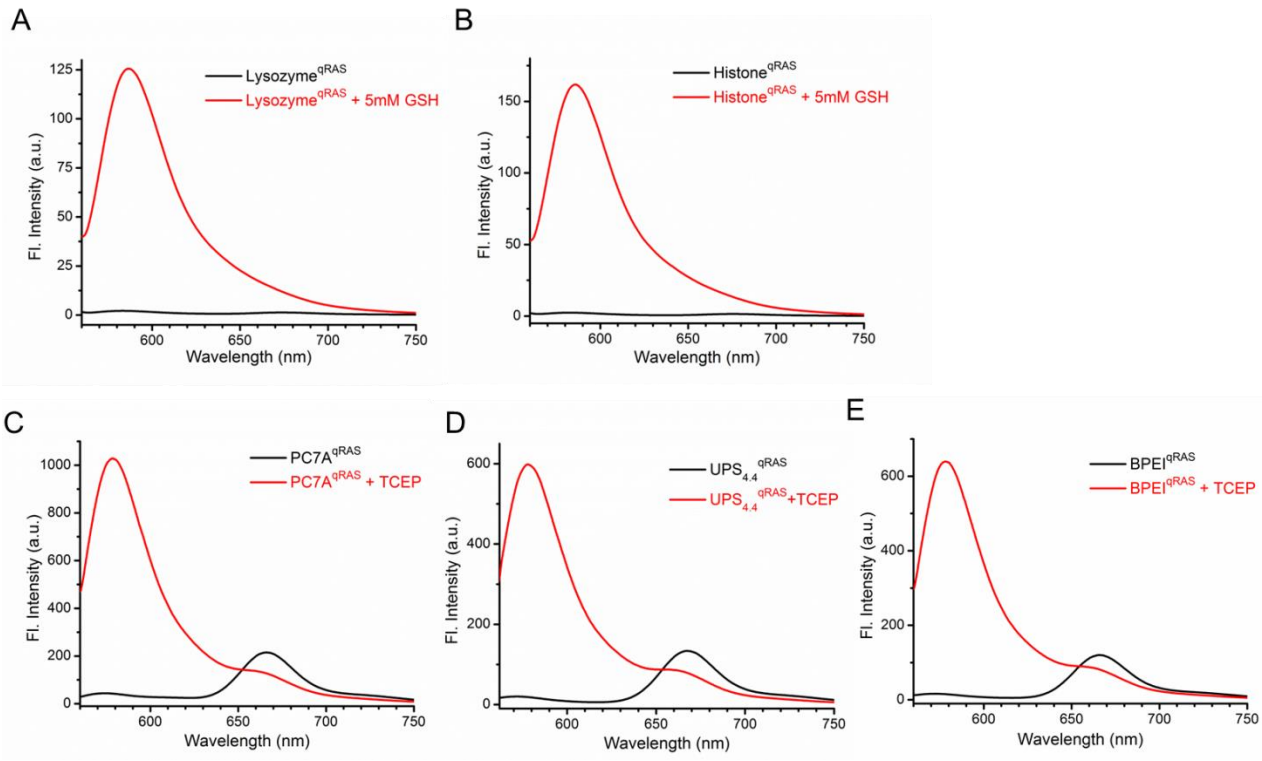

Figure S15. Demonstration of broad utility of qRAS in conjugation to various biomacromolecules. Lysozyme (A), histone (B), PC7A (C), $UPS_{4.4}$ (D) and polyethylenimine (E, Branched, 25K Da, BPEI) were used for qRAS conjugation, and demonstrated robust redox activation in response to a reducing reagent. Samples were excited at 550 nm.