# Electronic Supplementary Information for

## POEGMA-Based Disulfide-Containing Fluorescent Probes for Imitating and Tracing Noninternalization-Based Intracellular Drug Delivery

*Yunyang Ling,[†] Jing Ren,[†] Tao Li, Yibing Zhao, and Chuanliu Wu[*]*

*The MOE Key Laboratory of Spectrochemical Analysis and Instrumentation, State Key Laboratory of Physical Chemistry of Solid Surfaces, Department of Chemistry, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen, 361005, P.R. China*

[†]These authors contributed equally

*To whom correspondence should be addressed, Email: chlwu@xmu.edu.cn

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**Materials and methods**

Ethyl 2-bromoisobutyrate, S-(4-azidobutyl) ethanethioate, DL-dithiothreitol (DTT), Rhodamine B isothiocyanate (RITC), 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), N,N-diisopropylethylamine (DIPEA) and 6-maleimidocaproic acid were purchased from Sigma Aldrich. Cupric chloride ($CuCl_2$), cuprous chloride (CuCl), oligoethyleneglycol monomethylether methacrylate ($OEGMA_{500}$, average $M_n$ = 500), 2-aminoethyl methacrylate hydrochloride (AEMA), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) , trifluoroacetic acid (TFA) were purchased from Alfa Aesar. 2,2-Bipyridine, 2,4-dimethylpyrrole, 4-(prop-2-ynyloxy)benzaldehyde, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), and boron trifluoride etherate was purchased from J&K Scientific Ltd. Sodium ascorbate and dialysis cassettes (MWCO 3500) were purchased from Sangon. Tide Quencher$^{TM}$ 2 (TQ2) was purchased from AAT Bioquest. Hela, U87 and MCF7 cells were purchased from CoBioer Biosciences CO., LTD (Nanjing, China). Dulbecco's Modified Eagle Medium (DMEM) with high glucose, DMEM (high glucose, no glutamine, no phenol red) and phosphate buffered saline (PBS) were obtained from Thermo Scientific (Beijing, China). 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), L-cysteine (Cys) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Polymers were synthesized using atom-transfer radical polymerization and then purified by dialysis. $^1$H NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer using the residual proton resonance of the solvent as the internal standard. Molecular weight of the polymer was calculated from $^1$H NMR spectra and also estimated by gel permeation chromatography (GPC) using PMMA standard with a refractive index detector. UV-Vis absorption spectra were recorded by a HITACHI U-3900H spectrophotometer. Fluorescence spectra were recorded by a

HITACHI F-7000 spectrofluorimeter.

**Synthesis and characterization of Poly(AEMA-co-OEGMA)**

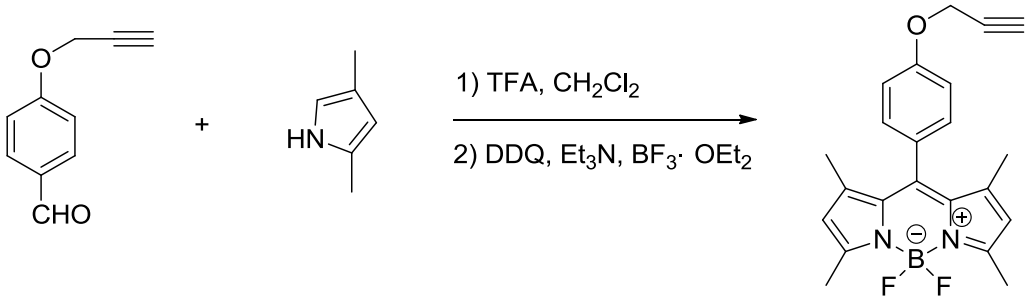
60 μL ethyl 2-bromoisobutyrate (initiator) and 940 μL DMF were mixed and sonicated to get a homogenous solution. 2, 2'-Bipyridine (171.8 mg), cupric chloride ($CuCl_2$, 51.1 mg) and cuprous chloride (CuCl, 5.0 mg) were added to a flask. The solid mixture was degassed and back-filled with purging nitrogen repeatedly. To a 10-mL-Schlenk flask, 6 mL deionized water was added followed by three freeze-pump-thaw cycles to remove oxygen and filled with purging nitrogen. Then, 5 mL deionized water was transferred from the Schlenk flask with a degassed syringe and added to the above degassed solid mixture. The mixture was sonicated for 3 minutes to form a dark-brown solution. 2-Aminoethyl methacrylate (AEMA, 146.8 mg, 0.8 mmol) was dissolved in 2 mL deionized water in a 10-mL-Schlenk flask, to which oligoethyleneglycol monomethylether methacrylate (OEGMA, average $M_n$ 500, 0.926 mL, 2.0 mmol) and 25 μL of the initiator solution (0.01 mmol) were added. The reaction mixture was stirred to be homogenous followed by five freeze-pump-thaw cycles to remove the dissolved oxygen. After that, 1 mL solution of metal catalyst was transferred with a degassed syringe to the mixture and the polymerization reaction proceeded at 30 $^{o}$C under nitrogen atmosphere for about 6 h. The obtained polymer was further purified by dialysis and characterized by $^{1}$H NMR and gel permeation chromatography (GPC). The polymer was dissolved in water and stored in refrigerator under –20 ℃ to prevent degradation. $^{1}$H NMR (400 MHz, $D_2O$, $\delta_{ppm}$): 0.70–1.20 ($C\underline{H}_3$-C-), 1.60–2.20 (-$C\underline{H}_2$C-), 3.35 (-O$C\underline{H}_3$), 3.60-3.76 ($CH_3O$-$C\underline{H}_2$-$C\underline{H}_2$-), 4.16 (-COO-$C\underline{H}_2$-). $M_n$ ($^{1}$H NMR): 76000, $M_n$ (GPC): 75000, $M_w/M_n$: 1.16 (GPC). The number of amino groups estimated from $^{1}$H NMR: 57.

**Synthesis of Poly(AEMA-co-OEGMA)-SPDP**

10 mg Poly(AEMA-co-OEGMA) (0.13 μmol, containing 7.4 μmol amino groups) was dissolved in 4 mL 0.2 M PBS (pH=7.4, containing 30% DMF) and SPDP (2.7 mg, 8.6 μmol in 100 μL DMF). The mixture was stirred overnight at room temperature. The obtained product was further purified by dialysis and characterized by $^1$H NMR. $^1$H NMR (400 MHz, $D_2O$, $\delta_{ppm}$): 3.33 (-$OCH_3$), 7.33, 7.86, 8.47 (pyridine, 4H). Pyridyldithio groups: 19 (33.9% of the amino groups were converted to pyridyldithio groups).

**Synthesis of BODIPY-SH**

**BODIPY-H1.**

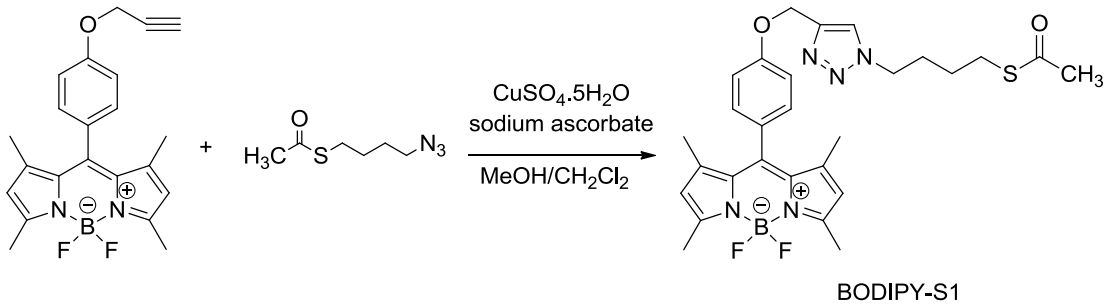



BODIPY-H1

BODIPY-H1 was synthesized according to the general procedure reported by Zhang C. *et al.* (Journal of the American Chemical Society, 2013, 135(28):10566-10578). Under $N_2$ atmosphere, a mixture of 4-(prop-2-ynyloxy)benzaldehyde (1 g, 6.2 mmol) and 2, 4-dimethylpyrrole (1.2 g, 12.6 mmol ) in dry $CH_2Cl_2$ (300 mL) was stirred at room temperature. While the flask was kept in ice bath, TFA ( 0.1 mL ) was added. Then, the mixture was stirred at room temperature overnight. 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 1.52g, 6.2 mmol) was dissolved in THF (40 mL) and the solution was added into the mixture and stirred for 7 h. With ice bath cooling, triethylamine (6 mL) was added dropwise, and the mixture was stirred for 0.5 h. Then, boron trifluoride etherate (6

mL) was added dropwise and stirred overnight. The solution was concentrated under reduced pressure and the residue was added into water, the mixture was stirred for 24 h. The solution was then extracted with $CH_2Cl_2$ and the organic layer was collected and dried over anhydrous $MgSO_4$. The solvent was evaporated under reduced pressure. The crude product was further purified using column chromatography (silica gel, petroleum ether : ethyl acetate = 8 : 1, *v*/*v*) to give BODIPY-H1 as red powder (yield: 43.4 %). $^1$H NMR (400 MHz, $CD_3OD$-$d_4$, $\delta_{ppm}$): 1.43 (s, 6H, 2×$CH_3$), 2.56 (s, 6H, 2×$CH_3$), 4.77 (d, 2H, -$OCH_2$-), 5.98 (s, 2H, Pyr-H), 7.11 (d, 2H, Ph-CH), 7.22 (d, 2H, Ph-CH). HRMS: m/z calcd: 401.16 [M+$Na^+$]; found: 401.16032.

**BODIPY-S1.**

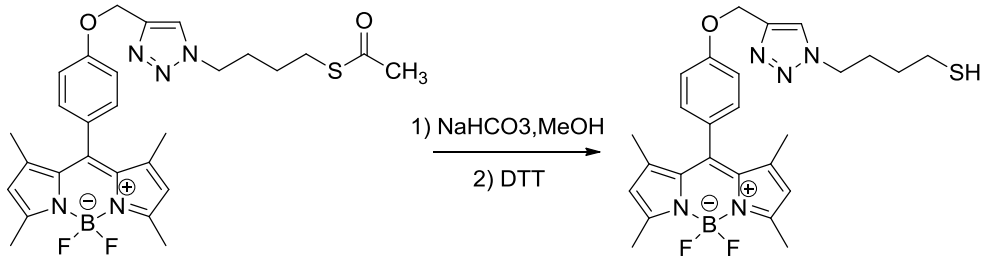



BODIPY-S1

15 mg (0.04 mmol) BODIPY-H1, 6.9 mg (0.04 mmol) S-(4-azidobutyl) ethanethioate and 100 mg (0.40 mmol) $CuSO_4 \cdot 5H_2O$ were added to a flask under $N_2$ atmosphere. Methanol : dichloromethane = 8 : 1 (*v*/*v*) was added as solvent. 314 mg (0.16 mmol) sodium ascorbate was added quickly. The reaction mixture was kept in dark place and stirred under $N_2$ atmosphere for 4 h. After removing deposition by centrifugation, the solvent was evaporated under reduced pressure. The crude product was further purified using column chromatography (silica gel, petroleum ether : ethyl acetate = 3 : 1, *v*/*v*) to give BODIPY-S1 as oily liquid (yield: 52.0%). $^1$H NMR (400 MHz, $CD_3OD$-$d_4$, $\delta_{ppm}$): 1.43 (s, 6H, 2×$CH_3$), 1.65 (m, 2H, -$CH_2$-C$\underline{H}_2$-$CH_2$-S-), 2.02 (m, 2H, -N-$CH_2$ -C$\underline{H}_2$-), 2.35 (s, 3H, $CH_3$), 2.56 (s, 6H, 2×$CH_3$), 2.92 (t, 2H, -$CH_2$ -C$\underline{H}_2$ -S-), 4.43 (t, 2H, -N-C$\underline{H}_2$ -$CH_2$-), 5.27 (s, 2H, -$OCH_2$-), 5.99

(s, 2H, Pyr-H), 7.11 (d, 2H, Ph-CH), 7.22 (d, 2H, Ph-CH), 7.69 (s, 1H, Triazole-H). ESI-MS: m/z calcd: 574.23 [M+$Na^+$]; found: 574.2.

**BODIPY-SH.**




BODIPY-S1 BODIPY-SH

15 mg (0.027 mmol) BODIPY-S1 was dissolved in methanol, and sodium bicarbonate was added to form a saturated solution. The mixture was stirred overnight at room temperature. After removing deposition by centrifugation, the solvent was evaporated under reduced pressure. 50 mL dichloromethane was added, and the solution was washed by water to remove sodium bicarbonate. Dichloromethane was evaporated under reduced pressure. Under alkaline conditions, BODIPY-SH dimer was formed, and DTT was added to get BODIPY-SH. The product was purified by HPLC (Figure S6). HRMS: m/z calcd: 510.22 [M+$H^+$]; found: 510.23113 (Figure S7).

**Synthesis of poly(AEMA-co-OEGMA)-BODIPY (probe 1)**

10 mg Poly(AEMA-co-OEGMA)-SPDP (0.12 μmol, containing 2.3 μmol pyridyldithio groups) was dissolved in 2 mL 50 mM PBS (pH=7.4), and 4.0 mg BODIPY-SH (4.7 μmol) in 2 mL acetonitrile was added dropwise. The mixture was stirred overnight at room temperature. The obtained polymer was further purified by dialysis and using column chromatography (sephadex LH-20, water). About 4.0 BODIPY molecules were grafted, estimated by UV-Vis absorption (Figure S8).

**Synthesis of poly(AEMA-co-OEGMA)-BODIPY-TQ2 (probe 2)**

4.0 mg Poly(AEMA-co-OEGMA)-BODIPY (0.05 μmol, containing 1.8 μmol amino groups) was dissolved in 2 mL 50 mM PBS (pH=7.4), and 1.0 mg TQ2 (2.1 μmol) in 2 mL methanol was added dropwise. The mixture was stirred overnight at room temperature. The obtained polymer was further purified by dialysis and using column chromatography (sephadex LH-20, water). About 3.2 TQ2 molecules were grafted, estimated by UV-Vis absorption (Figure S9).

**Synthesis of poly(AEMA-co-OEGMA)-BODIPY-RhB (probe 3)**

4.0 mg Poly(AEMA-co-OEGMA)-BODIPY (0.05 μmol, containing 1.8 μmol amino groups) was dissolved in 2 mL 50 mM PBS (pH=7.4), and 1.1 mg RITC (2.1 μmol) in 2 mL methanol was added dropwise. The mixture was stirred overnight at room temperature. The obtained polymer was further purified by dialysis and using column chromatography (sephadex LH-20, water). About 5.0 RhB molecules were grafted, estimated by UV-Vis absorption (Figure S10).

**Synthesis of poly(AEMA-co-OEGMA)-maleimide-BODIPY (non-cleavable, probe 4)**

6-Maleimidocaproic acid (3.2 mg, 15 μmol) and HATU (5.7 mg, 15 μmol) were dissolved in 500 μL DMF, into which 2 drops of DIPEA were added. The mixture was stirred for 90 min at room temperature. Then, 10 mg Poly(AEMA-co-OEGMA) (0.13 μmol, containing 7.4 μmol amino groups) in 200 μL $H_2O$ was added into the mixture and stirred for 2 h at room temperature. The obtained polymer-maleimide conjugate (Poly(AEMA-co-OEGMA)-maleimide) was then purified by dialysis and characterized by $^1$H NMR. $^1$H NMR (400 MHz, $D_2O$, $\delta_{ppm}$): 4.11 (OEGMA, -COO-$CH_2$-, 2H), 6.24, 6.26 (maleimide, 2H). The number of maleimide groups estimated from $^1$H NMR spectrum (Figure S18): 13.

8 mg Poly(AEMA-co-OEGMA)-maleimide (0.11 μmol, containing 1.4 μmol maleimide groups)

was dissolved in 2 mL 50 mM PBS (pH=7.4), and 1.4 mg BODIPY-SH (2.8 μmol) in 2 mL acetonitrile was added dropwise. The mixture was stirred overnight at room temperature. The obtained polymer-BODIPY conjugate was purified by dialysis and using column chromatography (sephadex LH-20, water). About 3.1 BODIPY molecules were grafted, estimated by UV-Vis absorption (Figure S19).

**Fluorescence dequenching of probes 1–3**

To examine the fluorescence dequenching of probes **1–3** in GSH buffer (or in Cys or DTT buffer), in a typical experiment, the fluorescence of probes **1–3** (final concentration: 15 nM) in 1 mL phosphate buffer (100 mM, pH 7.4) containing 10 mM GSH (or Cys/DTT) and 0.1% Triton X-100 was recorded at predefined times using a fluorimeter ($\lambda_{ex}$: 490 nm,$\lambda_{em}$: 511 nm). To examine the fluorescence dequenching in 50% FBS or 50% DMSO, the probes (final concentration: 15 nM) were dissolved in 1.0 mL phosphate buffer/DMSO (or phosphate buffer/FBS) mixture containing 10 mM GSH (or DTT), and then the fluorescence was monitored at predefined times ($\lambda_{ex}$: 490 nm,$\lambda_{em}$: 511 nm). The experiment was performed in triplicate.

**Cell culture**

Hela, U87 and MCF7 cells were maintained in DMEM medium (high glucose) supplemented with 10% FBS, and 1% penicillin/streptomycin (penicillin: 10000 $U \cdot mL^{-1}$, streptomycin: 10000 $U \cdot mL^{-1}$) at 37 ℃ in a humidified atmosphere containing 5% $CO_2$. Cells were passaged at about 80% cell confluency using a 0.25% trypsin solution.

**Stability of 2 and 3 in serum-free medium**

Probe **2** and **3** (25 nM) in 1.0 mL serum-free incomplete medium (high glucose, no glutamine, no

phenol red) supplemented with 1% penicillin/streptomycin were incubated at 37 ℃, 5% $CO_2$. Fluorescence was then analyzed using an ELISA reader (PerkinElmer Enspire®) (BODIPY: $\lambda_{ex}$ = 490 nm, $\lambda_{em}$ = 510 nm) at predefined times. The measured intensity was blank corrected, and three independent experiments were performed.

**Fluorescence analysis of probe 2 and 3 in the presence of Hela cells**

Hela cells were seeded into 24-well cell culture dishes at a total of $10^5$ cells/well. After 24 h at 37 ℃, 5% $CO_2$, cells were grown to about 80% confluence. The medium was then removed and the cells were washed twice with PBS. After addition of 1 mL serum-free incomplete medium containing 25 nM fluorescence probe (**2** and **3**) or incomplete medium containing 25 nM fluorescence probe (**2** and **3**), the fluorescence was recorded using an ELISA reader at predefined times. The measured intensity was blank corrected, and three independent experiments were performed.

Hela cells were seeded as described above. To measure the effect of inhibitors on the thiol−disulfide exchange of fluorescence probes, cells were maintained in 1 mL incomplete medium containing 100 μM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and 25 nM fluorescence probe (**2** and **3**). Fluorescence was then analyzed using an ELISA reader at predefined times. DTNB were not cytotoxic at the concentration used. The measured intensity was blank corrected, and three independent experiments were performed.

**Confocal cell imaging**

Cells were plated into glass bottom cell culture dishes at a total of $10^5$ cells/well. After 24 h at 37 ℃, 5% $CO_2$, the cells were grown to about 80% confluence. The medium was then removed and the cells were washed twice with PBS. Cells were then incubated with 1 mL serum-free complete

medium containing 125 nM fluorescence probe (**2** and **3**) at 37 ℃, 5% $CO_2$ for desired lengths of time. After that, medium was removed and cells were washed three times with PBS and were then imaged in a Leica TCS SP5X Confocal Microscope System at different detection channels. (BODIPY channel: $\lambda_{ex}$ = 488 nm, $\lambda_{em}$ = 520–560 nm; RhB channel: $\lambda_{ex}$ = 543 nm, $\lambda_{em}$ = 600–670 nm).

Cells were seeded as described above. Upon medium removal, the cells were washed twice with PBS, cells were incubated with 1 mL of 125 nM fluorescence probe (**2** and **3**) in serum-free complete medium containing 100 μM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) or 200 μM Cys at 37 ℃, 5% $CO_2$ for the desired lengths of time. After that, medium was removed and cells were washed three times with PBS and then were imaged in a Leica TCS SP5X Confocal Microscope System.

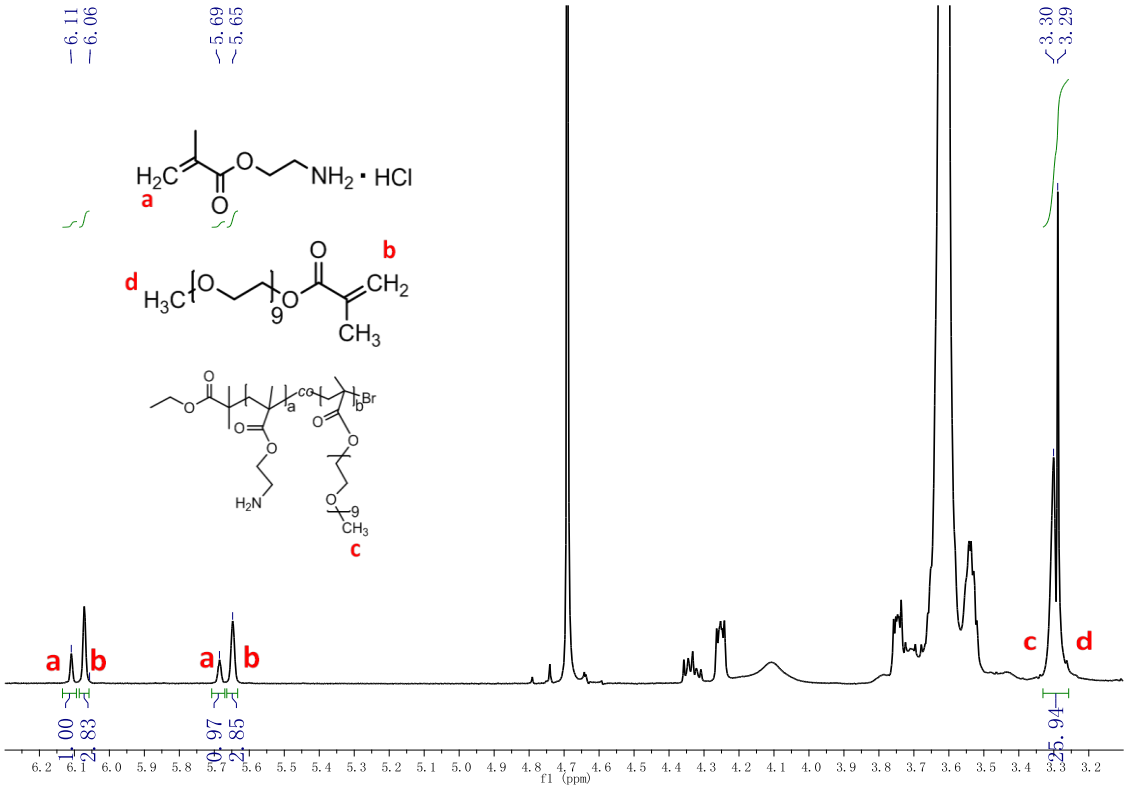



**Figure S1.** $^{1}$H NMR of Poly(AEMA-co-OEGMA) (400 MHz, $D_2O$, $\delta_{ppm}$) before the dialysis. Based on the intensity of peaks b, c and d, a monomor conversion of 67% (for OEGMA) can be calculated. The conversion of AEMA monomer was considered to be similar to that of OEGMA monomer (based on the relative intensity of peaks a and b). The number of amino groups and the molecular weight of the polymer were both calculated from the monomer conversion.

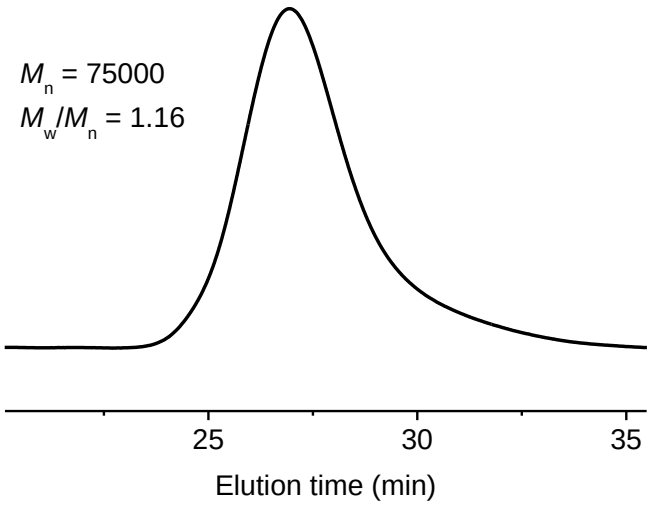



**Figure S2.** The GPC curve of poly(AEMA-co-OEGMA), eluting in HAc/NaAc (at pH 4), flow rate: 0.5 mL/min, column temperature: 30 ℃.

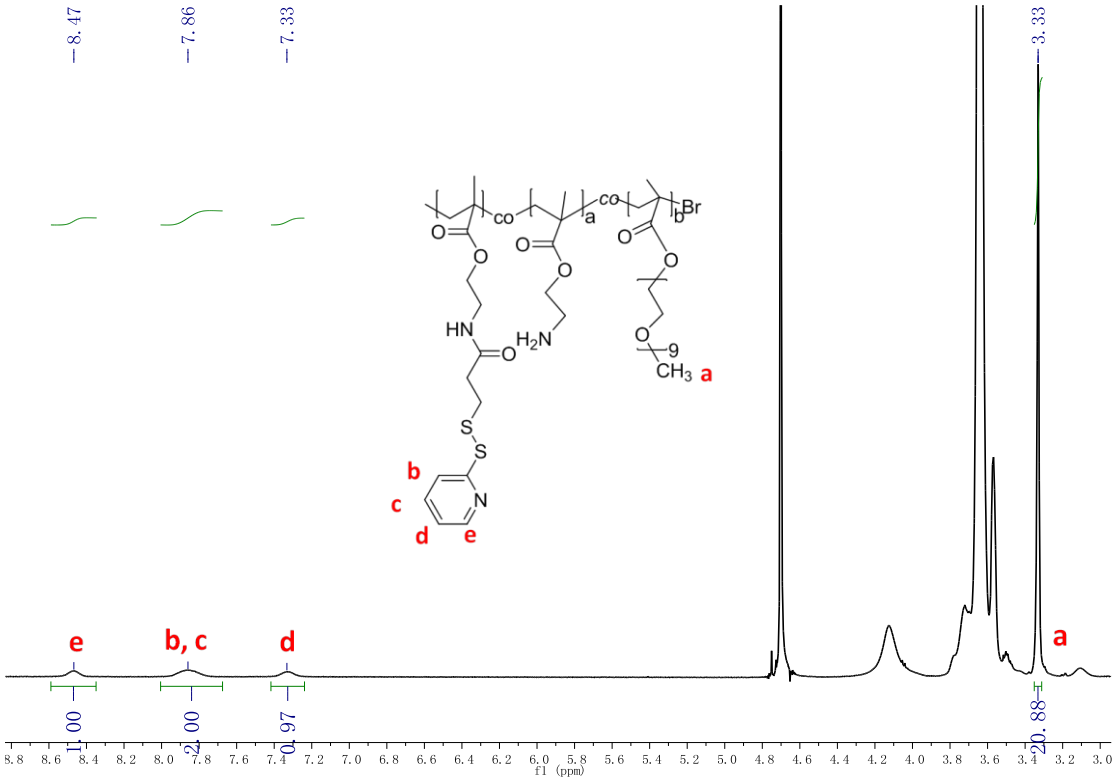



**Figure S3.** $^{1}$H NMR of poly(AEMA-co-OEGMA)-SPDP (400 MHz, $D_2O$, $\delta_{ppm}$).

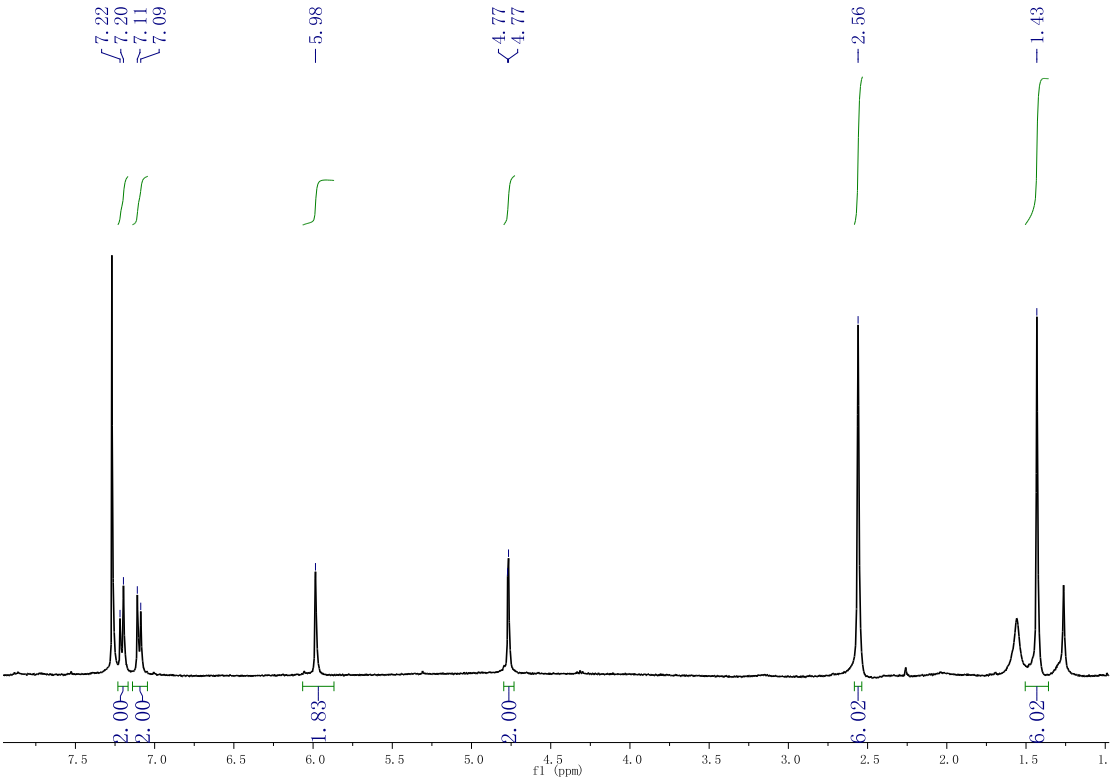



**Figure S4.** The $^{1}$H NMR spectrum of BODIPY-H1 (400 MHz, $CD_3OD$-$d_4$, $\delta_{ppm}$).

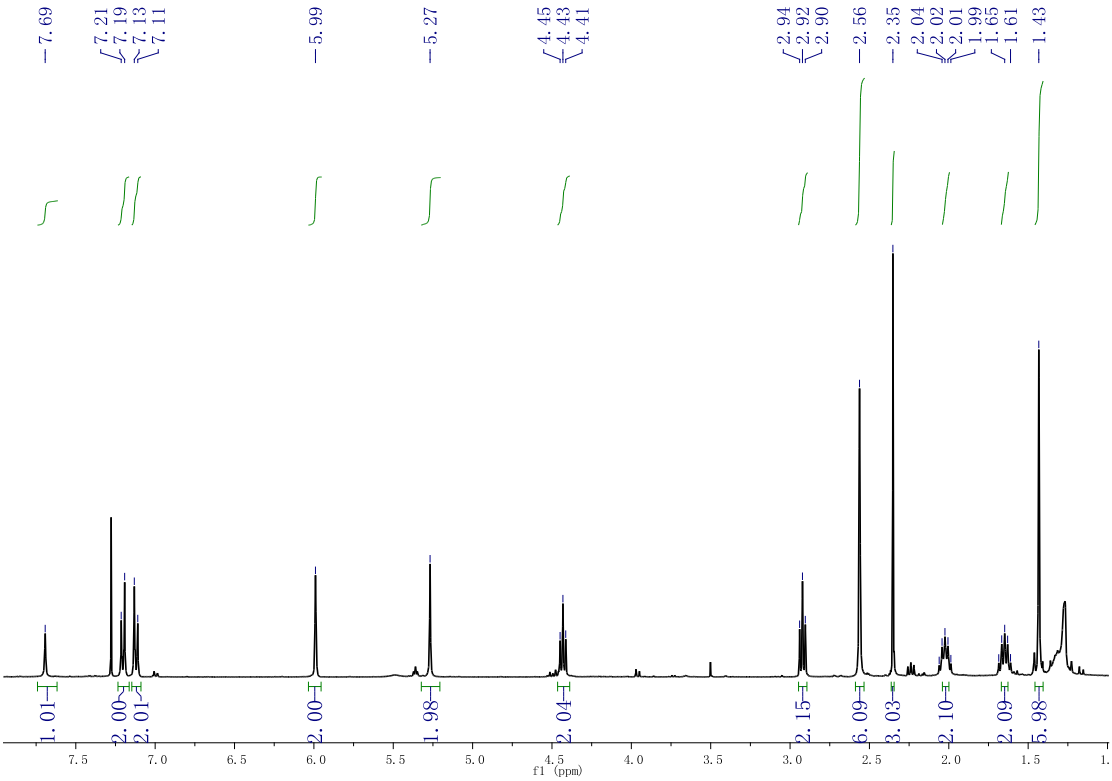



**Figure S5.** The $^{1}$H NMR spectrum of BODIPY-S1 (400 MHz, $CD_3OD$-$d_4$, $\delta_{ppm}$).

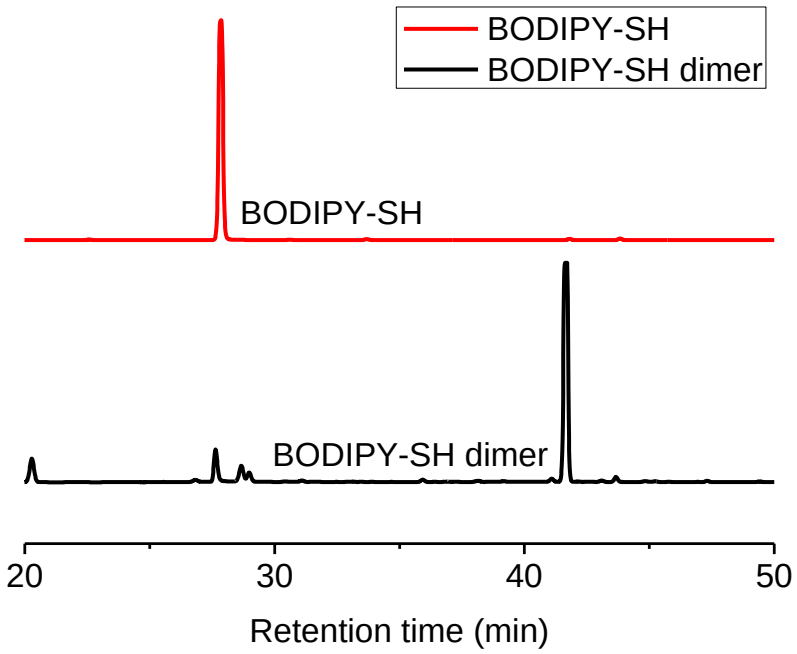



**Figure S6.** HPLC analysis of BODIPY-SH and BODIPY-SH dimer, absorbance was recorded at 490 nm.

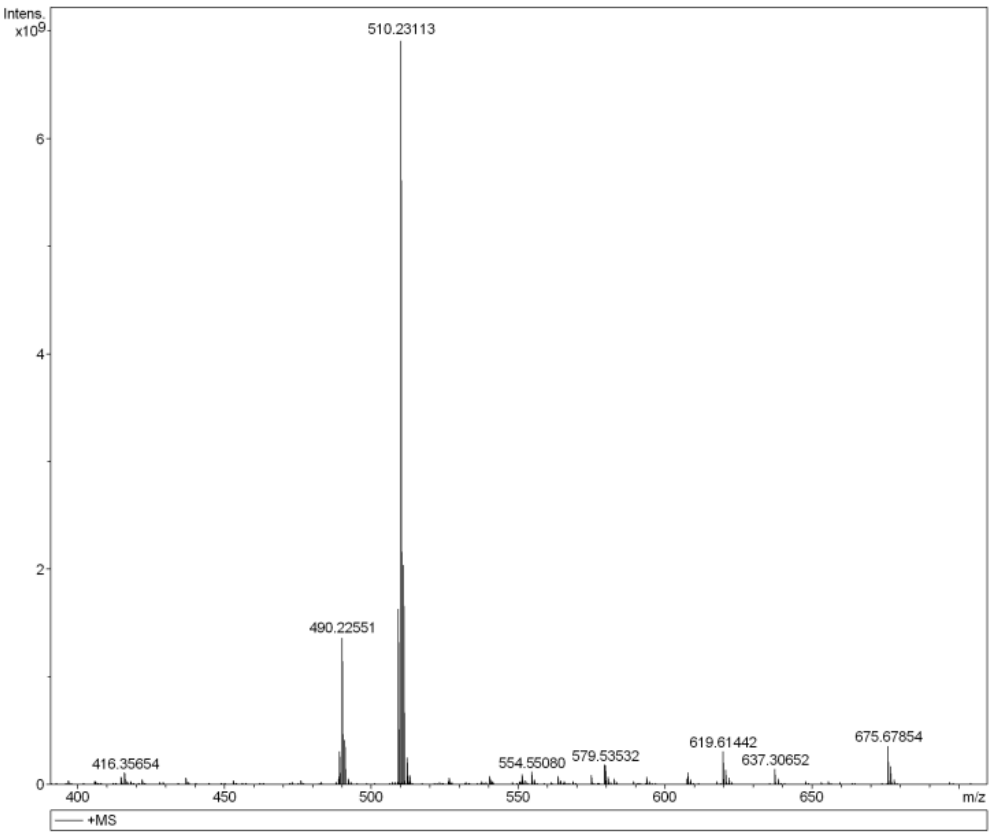



**Figure S7.** The high-resolution MS spectrum of BODIPY-SH.

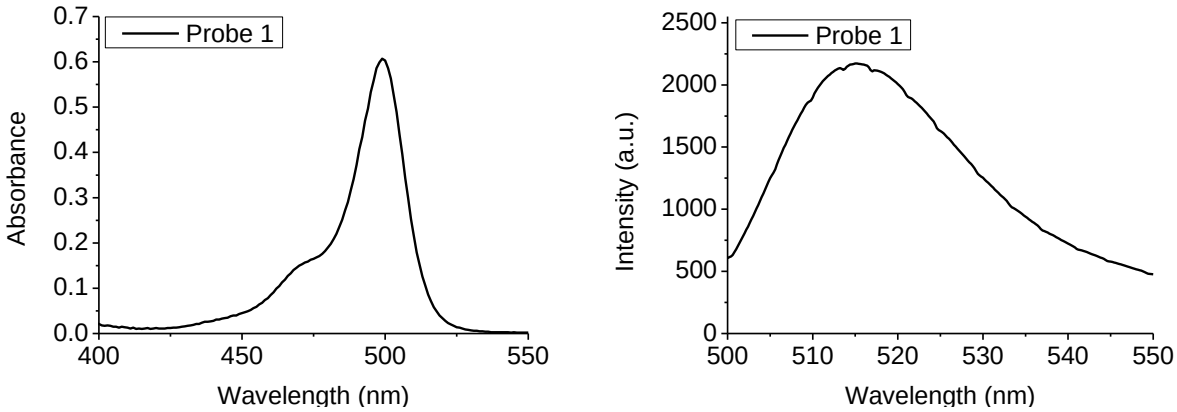



**Figure S8.** UV-Vis absorption (7.5 μM) and fluorescence spectra (15 nM) of probe **1** in water ($\lambda_{ex}$: 490 nm).

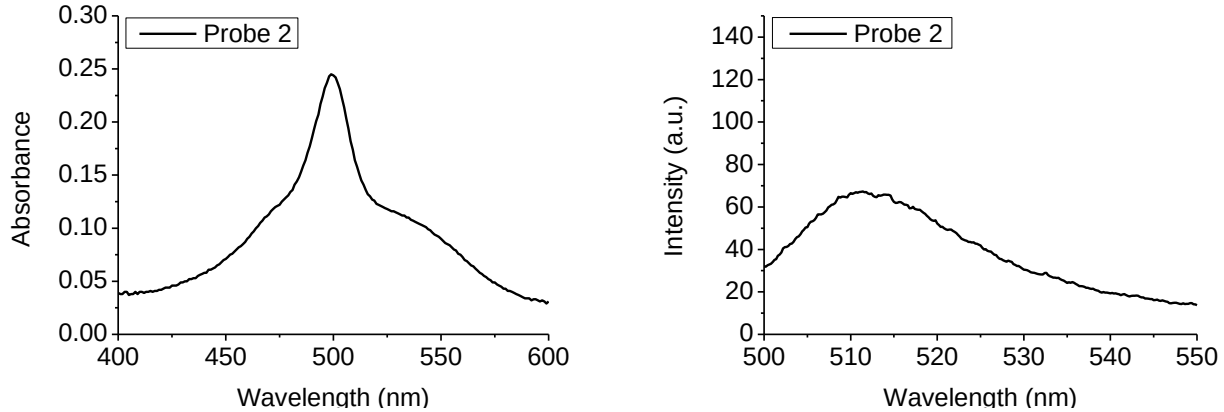



**Figure S9.** UV-Vis absorption (3.25 μM) and fluorescence spectra (15 nM) of probe **2** in water ($\lambda_{ex}$: 490 nm).

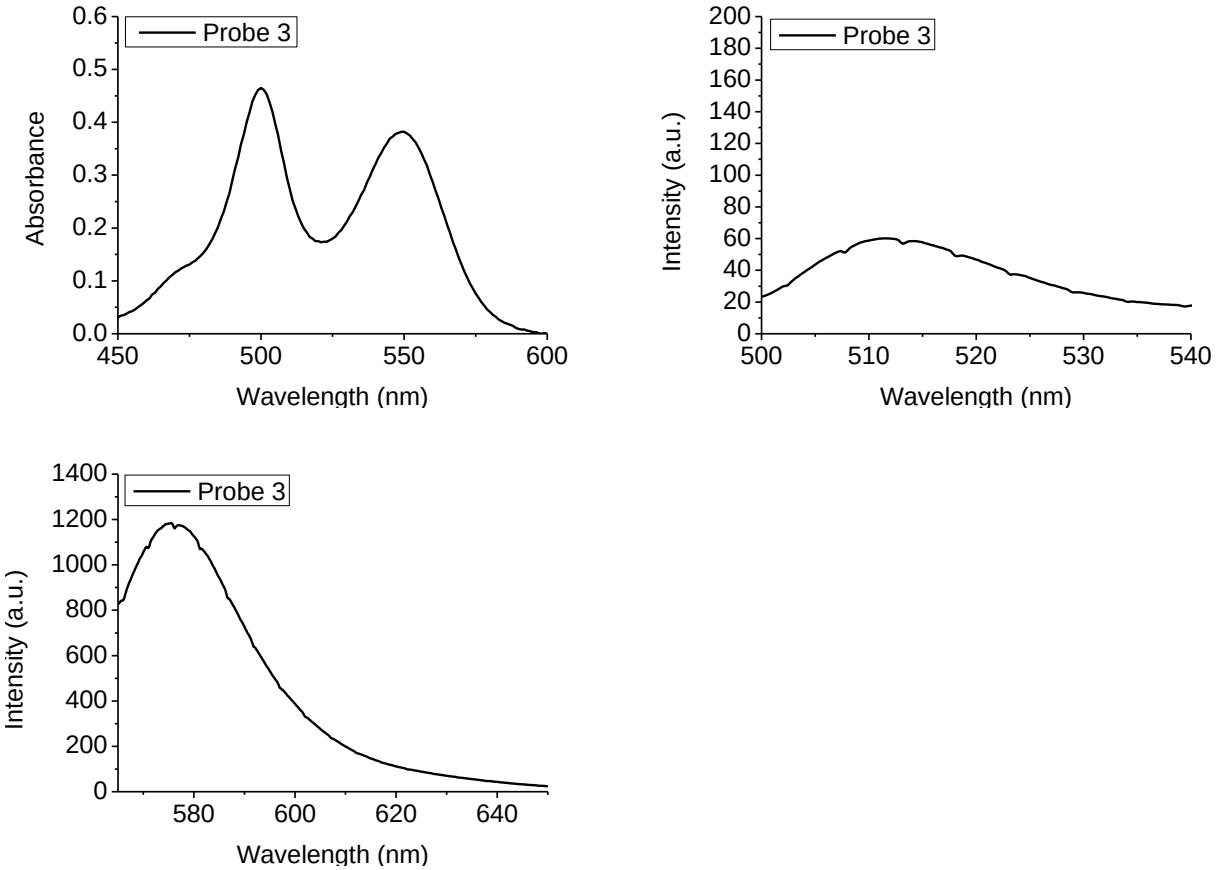



**Figure S10.** UV-Vis absorption (6 μM) and fluorescence spectra (15 nM) of probe **3** in water ($\lambda_{ex}$ : 490 nm (BODIPY), $\lambda_{ex}$ : 557 nm (RhB)).

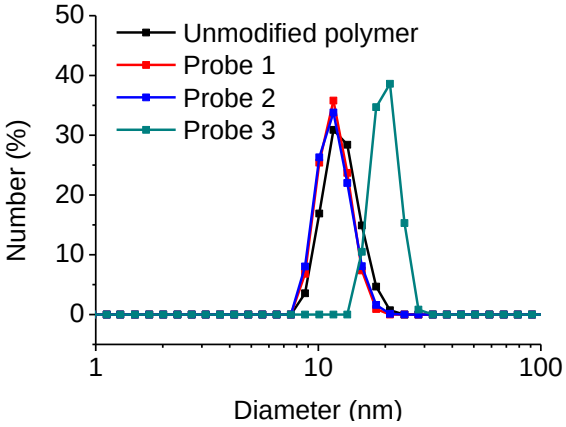



**Figure S11.** Size distribution of the unmodified polymer (pOEGMA-$NH_2$) and the probes **1**–**3** at 25 ℃ in water.

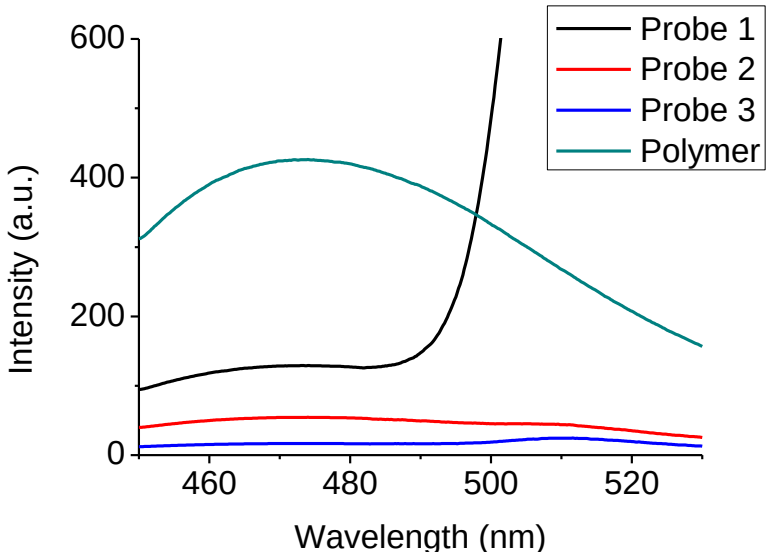



**Figure S12.** Fluorescence emission from fluorescamine after reacting with the unmodified polymer and the probes **1**–**3** (unmodified polymer: 0.019 μM, probes **1**–**3**: 0.35 μM, fluorescamine: 100 μM; $\lambda_{ex}$: 390 nm).

**Table S1.** The number of free amino groups in polymers and that determined using the fluorescamine assay.

| | pOEGMA-$NH_2$ | Probe **1** | Probe **2** | Probe **3** |
|---|---|---|---|---|
| Amino group (total) | 57 | 38 | 35 | 33 |
| Amino groups (measured) | 35.3 | 6.7 | 2.8 | 1.4 |
| Percentage | 61.9% | 17.6% | 8.0% | 4.2% |

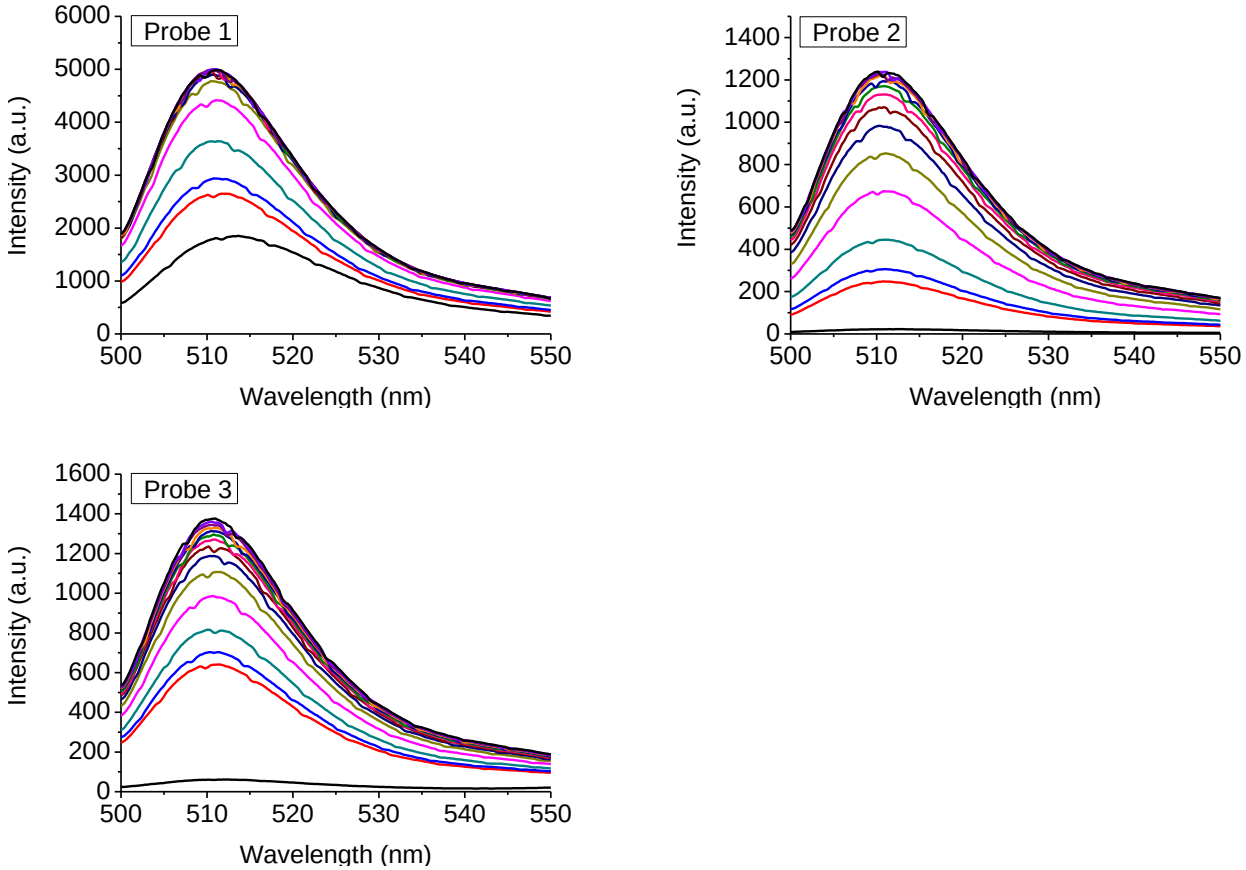
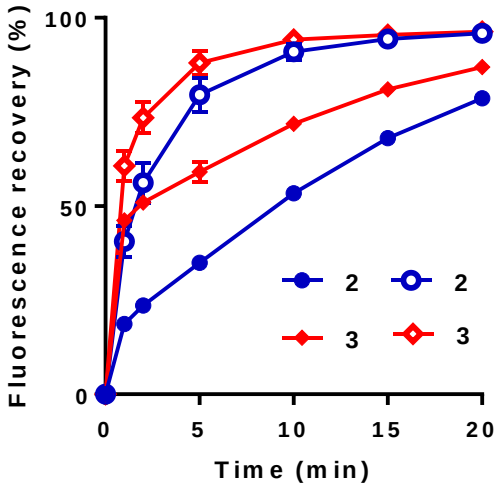



**Figure S13.** Fluorescence spectra of probe **1-3** in 10 mM DTT, pH 7.4, sampling interval: 5 min ($\lambda_{ex}$: 490 nm).




c

**Figure S14.** Percentages of fluorescence recovery of probes **2**–**3** in 10 mM DTT, hollow symbols: probes **2**–**3** in 50% DMSO.

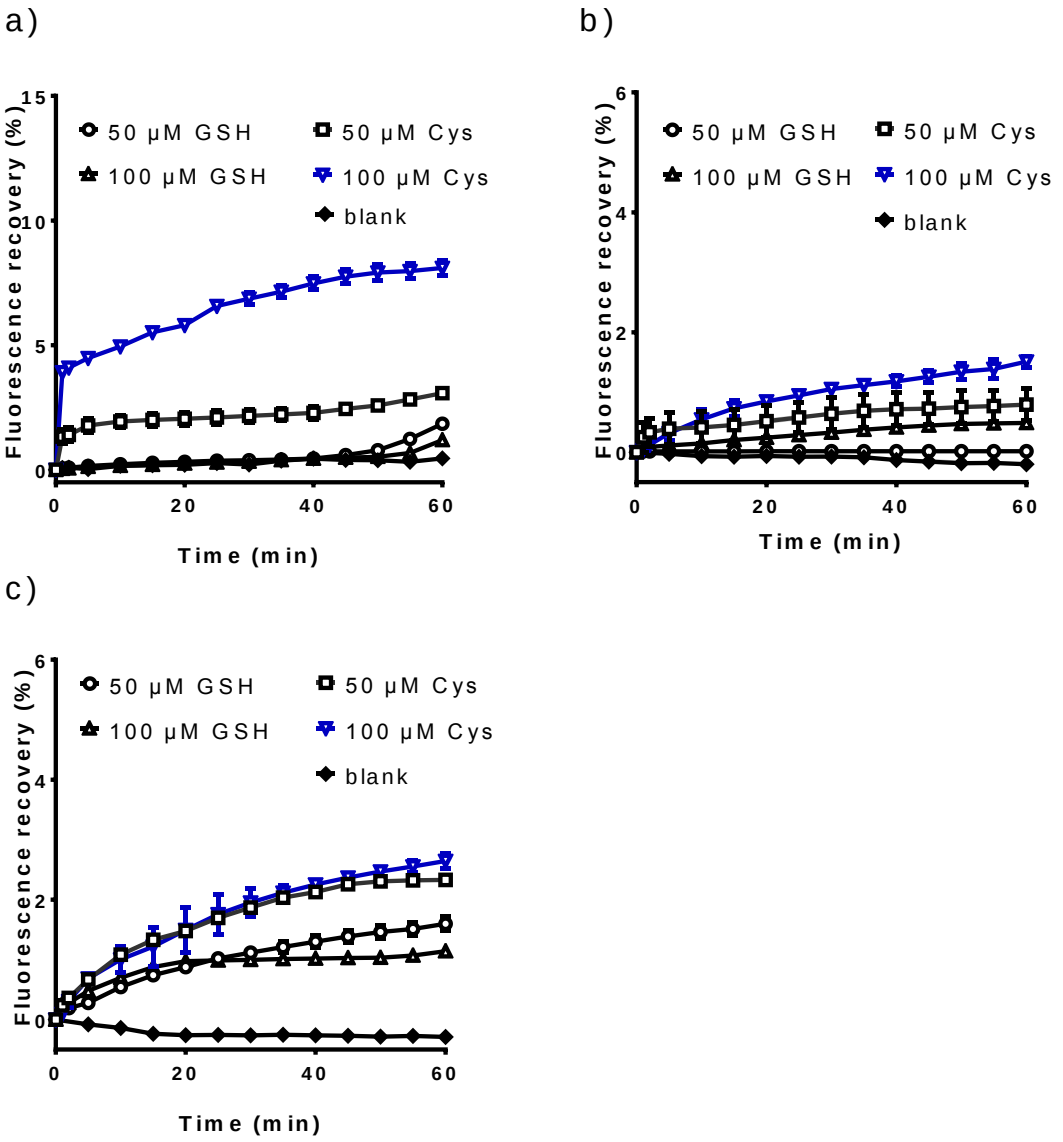



**Figure S15.** Fluorescence dequenching of the probes **1** (a), **2** (b), and **3** (c) in 50 μM and 100 μM GSH/Cys buffer.

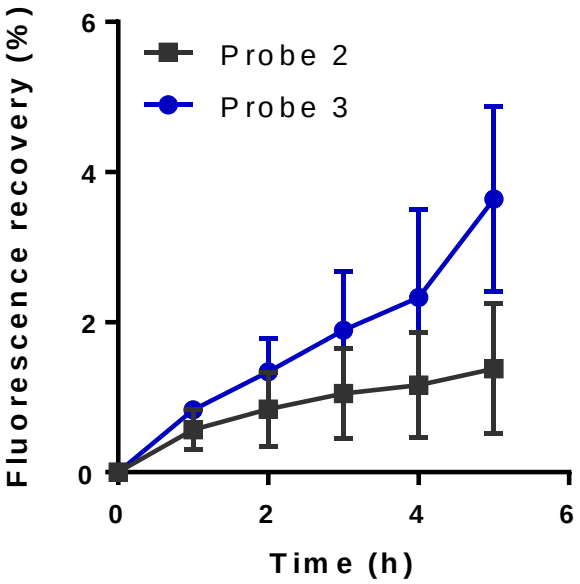
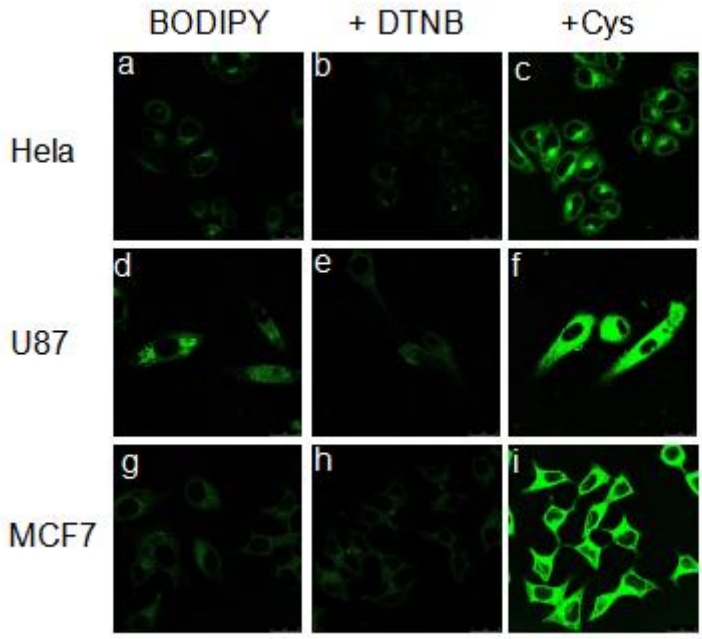



**Figure S16.** Kinetics of fluorescence recovery of probes **2** and **3** in 50% FBS (100 mM phosphate buffer, pH 7.4)

a)




b)

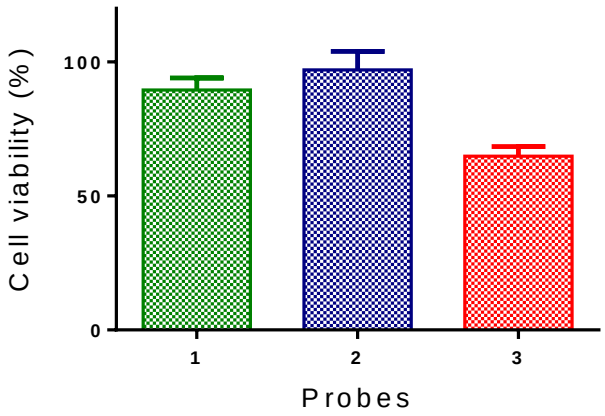



**Figure S17.** a) Representative confocal microscopy images of cancer cells (a–c: Hela cells; d–f: U87 cells; g–i: MCF7 cells) incubated with **2** (125 nM) over 4 h at 37 ℃. From left to right: cells alone (a, d and g); cells in the presence of 100 μM DTNB (b, e and h); cells in the presence of 200 μM Cys (c, f and i). b) Cell viability treated with probes **1**–**3** (125 nM) determined by MTT assay ( Hela cells, 24 h incubation at 37 ℃. Only the probe **3** exhibits moderate cytotoxicity.

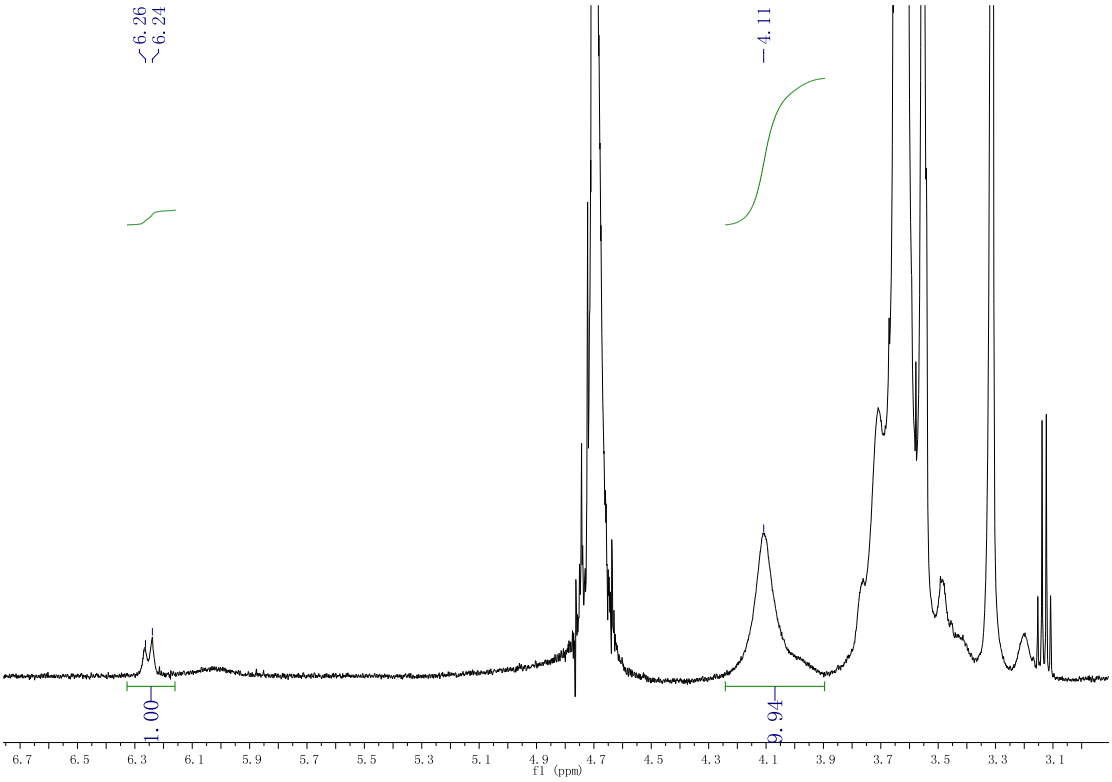



**Figure S18.** $^{1}$H NMR of poly(AEMA-co-OEGMA)-maleimide (400 MHz, $D_2O$, $\delta_{ppm}$).

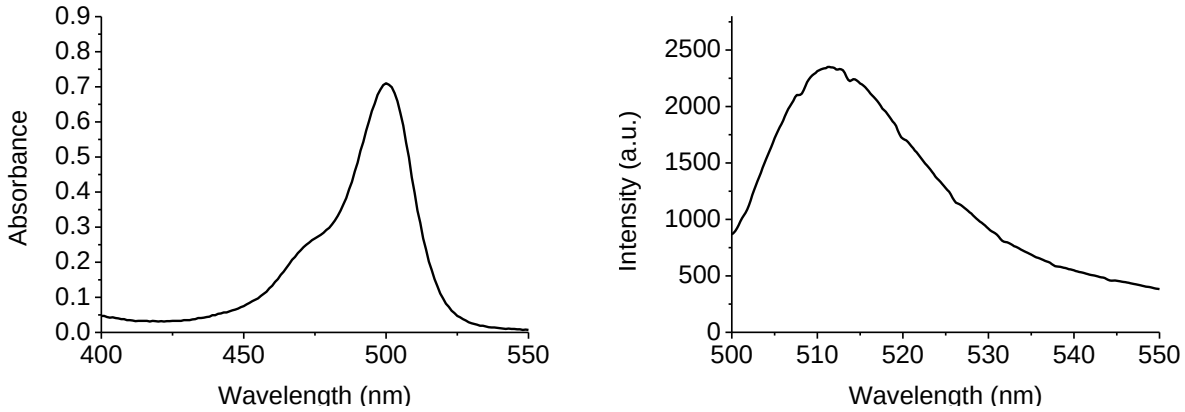



**Figure S19.** UV-Vis absorption (12 μM) and fluorescence (20 nM) spectra of non-cleavable conjugate in water ($\lambda_{ex}$: 490 nm).

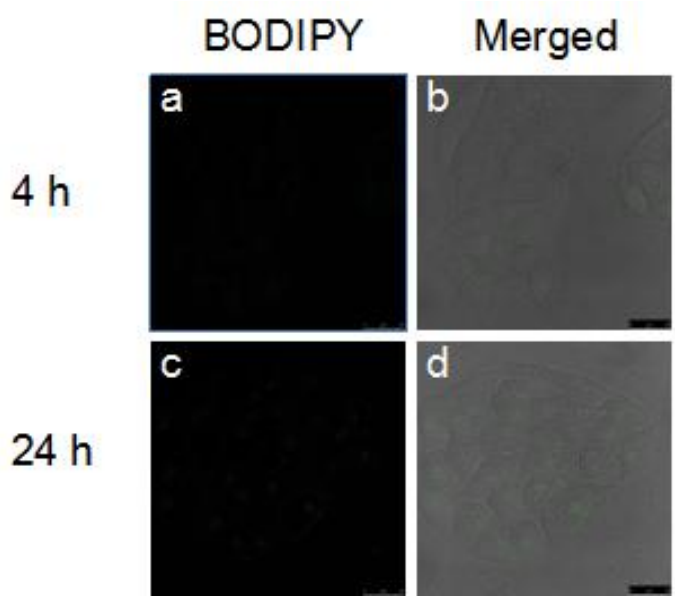



**Figure S20.** Representative confocal microscopy images of Hela cells incubated with the non-cleavable BODIPY conjugate at 37 ℃ (a and b: 4 h; c and d: 24 h).

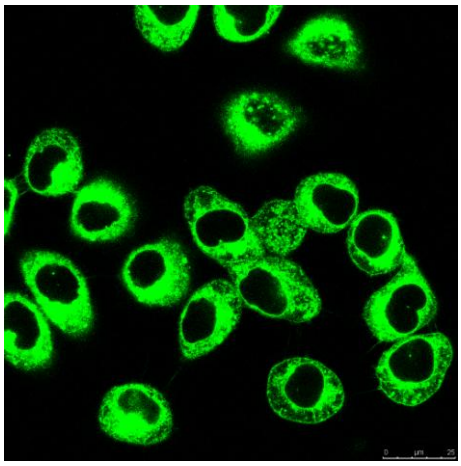


**Figure S21.** Representative confocal microscopy images of Hela cells incubated with free BODIPY-SH (0.5 μM, generated by incubating probe **2** with 0.5 mM DTT) over 4 h at 4 ℃.

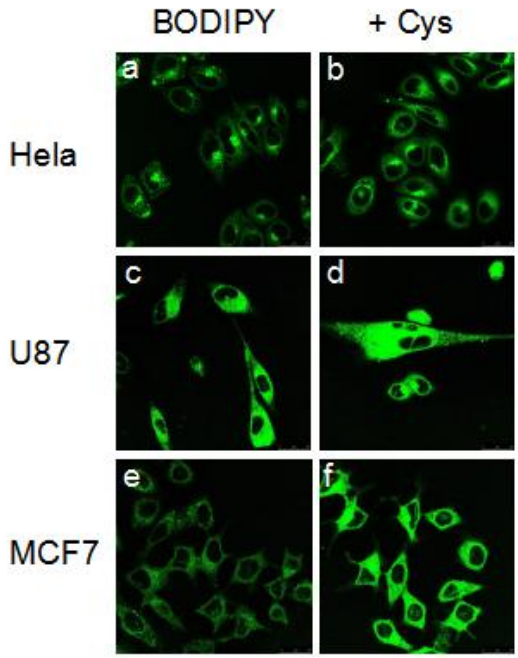



**Figure S22.** Representative confocal microscopy images of cancer cells (a–b: Hela cells; c–d: U87 cells; e–f: MCF7 cells) incubated with **3** (125 nM) over 4 h at 37 ℃. From left to right: cells alone (a, c and e); cells in the presence of 200 μM Cys (b, d and f).