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Electronic Supporting Information

Ultra-low Background Signaling Cascade Amplifier for In Vivo Fluorescence Imaging of Hydroxyl Radical Production Induced by Testosterone

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Materials and Instruments

^1H and ^{13}C NMR spectra were recorded on an Invoa-400 (Invoa 400) spectrometer and referenced to solvent signals. The number-average molecular weight (M_n) and molecular distribution (polydispersity index, $\text{PDI} = M_w/M_n$) of polymers were determined at room temperature (RT) using a Waters GPC liquid chromatograph (Waters, USA) equipped with four TSK HXL series of polystyrene divinylbenzene gel columns (300×7.8 mm). Calibration was established with polystyrene standards from Polymer Laboratories. THF was used as solvent with a flow rate of 1 mL min^{-1} . Scanning electron microscopy (SEM) images were obtained on an S-4800 scanning electron microscope (Hitachi) with a working voltage of 5 kV. A drop of the micelle aqueous solution (0.05 mg mL^{-1}) was deposited onto a silicon slice and allowed to dry at RT before measurement. The mean size of the micelles was determined by dynamic light scattering (DLS) using a Malvern Nano S instrument (Malvern, UK).

The detailed synthesis and structural characterizations of (1)–(6)

Synthesis of 2, 2-Bis(hydroxyl methyl)propionate (1)

In a 250 mL round-bottom flask, 2, 2-bis(hydroxyl methyl)-propionic acid (2.24 g, 16.72 mmol), KOH (1.01 g, 18.04 mmol) and DMF (20 mL) was added. The mixture was stirred at $100 \text{ }^\circ\text{C}$ for 2 h, then propargyl bromide (2.13 g, 18.04 mmol) was added dropwise over 30 min. After 72 hours of reaction, the reaction mixture was filtered, the solvent was evaporated under reduced pressure, and the residues were dissolved in 50 mL of DCM and extracted three times with saturated salt water ($3 \times 10 \text{ mL}$). The organic phase was concentrated to yield crude product, which was purified by column chromatography (eluent: ethyl acetate/petroleum ether = 1/5, v/v). Yield: 1.3 g (45.14%). ^1H NMR (400 MHz, CDCl_3): $\delta = 4.76$ (d, 2H, CHCCH_2CO), 3.91 (d, 2H, CH_2OH), 3.72 (d, 2H, CH_2OH), 2.51 (t, 1H, CHCCH_2CO), 1.11 (s, 3H, CH_3CC). ^{13}C NMR (400 MHz, CDCl_3): $\delta = 175.01, 75.20, 67.82, 52.43, 49.29, 16.95$.

Synthesis of 5-Methyl-5-propargylxycarbonyl-1,3-dioxane-2-one (MPC) (2)

Compound 4 (1.18 g, 6.88 mmol) was mixed with ethyl chloroformate (1.48 g, 13.76 mmol) and THF (20 mL) in a sealed vessel that was purged with nitrogen and cooled in an ice bath. After stirring an hour, triethylamine (1.39 g, 13.76 mmol) was added drop wise over 30-min period under nitrogen atmosphere. The reaction was conducted at 0 °C with stirring for 3 h, then at 25 °C under stirring overnight. The solution was then subject to filtered, evaporated to dryness, and the as-obtained product was precipitated in a mixture of ethyl acetate and diethyl ether (1:1) as white crystals. Yield: 1.24 g (91.05%). ¹H NMR (400 MHz, CDCl₃): δ = 4.79-4.80 (d, 2H, CHCCH₂CO), 4.71-4.74 (d, 2H, CH₂OCO), 4.22-4.25 (d, 2H, CH₂OCO), 2.54-2.55 (t, 1H, CHCCH₂CO), 1.37 (s, 3H, CH₃CC). ¹³C NMR (100 MHz, CDCl₃): δ = 170.31, 147.21, 76.35, 75.94, 72.70, 53.47, 40.17, 17.39.

Synthesis of PEG-*b*-poly(MPC) (3)

The ring-opening polymerization of MPC was carried out under an inert atmosphere of nitrogen using standard Schlenk-line technique. In a typical experiment, MPC (0.594 g, 3 mmol), PEG_{5k} (0.601 g, 0.12 mmol), TU (0.055 g, 0.15 mmol), DBU (0.005 g, 0.03 mmol) and dried DCM (10 mL) were placed in a dried Schlenk tube fitted with a rubber septum. The solution was further degassed through three freeze-pump-thaw cycles. The resulting mixture was stirred at room temperature for 7 h, followed by precipitation in ice-cold diethyl ether and centrifugation. The resulting product was collected by filtration and dried under vacuum to yield a white powder. Yield: 1.1 g (92.0%). ¹H NMR (400 MHz, CDCl₃): δ = 4.73 (d, OCH₂CCH), 4.28-4.32 (m, OC(O)OCH₂), 3.65 (s, OCH₂CH₂O), 3.38 (s, CH₃O), 2.55 (s, CH₂CCH), 2.19 (s, OH), 1.29 (s, CH₃). GPC (THF, RI): M_n (PDI) = 7263 g mol⁻¹ (1.04).

Synthesis of (Z)-3,4-bis(phenylselanyl)but-3-en-1-ol (4)

1,2-diphenyldiselane (2.35 g, 7.48 mmol), but-3-yn-1-ol (0.27 g, 3.74 mmol) and THF (8.0 mL) were placed in a dried Schlenk tube under protection of nitrogen. The reaction was stirred under room temperature for 20 h. The reaction was quenched with water (30 mL), and then washed with ethyl acetate (3 × 30 mL). The organic layer was washed with water (3 × 10

mL), dried over MgSO_4 and filtered. The organic layer was dried over anhydrous MgSO_4 , filtered, concentrated and finally purified by silica gel column chromatograph using ethyl acetate/petroleum ether (1/10 v/v) as the eluent, affording (4) (0.67g, yield: 46.6%). ^1H NMR (400 MHz, CDCl_3): δ =7.53-7.59(m, 4H, ArH), 7.29-7.32 (m, 6H, ArH), 7.10 (s, 1H, ArSeCHC), 3.71(t, 2H, CH_2OH), 2.54 (t, 2H, $\text{CCH}_2\text{CH}_2\text{OH}$), 1.26(s, 1H, OH); ^{13}C NMR (100 MHz, CDCl_3): δ =132.91, 132.90, 131.97, 131.09, 130.75, 129.50, 129.40, 129.37, 129.10, 128.94, 127.66, 127.62, 127.03, 61.11, 42.83.

Synthesis of (Z)-3,4-bis(phenylselanyl)but-3-en-1-yl 3-bromopropanoate (5)

(Z)-3,4-bis(phenylselanyl)but-3-en-1-yl 3-bromopropanoate was synthesized by esterification of (4) with 3-Bromopropionyl chloride. Typically, triethylamine (6.07g, 60 mmol), (Z)-3,4-bis(phenylselanyl)but-3-en-1-ol (15.28 g, 40 mmol), and dry THF (200 mL) were charged into a 500 mL round-bottom flask, cooled to 0 °C in an ice water bath, and then 3-Bromopropionyl chloride (6.84 g, 40 mmol) in 100 mL dry THF was added dropwise over a period of 1 h under vigorous magnetic stirring. After the addition was completed, the reaction mixture was stirred at room temperature overnight. After filtration and evaporating all the solvents, the residues were diluted with ethyl acetate and washed twice with water and brine, respectively. The organic layer was dried over anhydrous MgSO_4 , filtered, concentrated and finally purified by silica gel column chromatograph using ethyl acetate/petroleum ether (1/10 v/v) as the eluent, affording (5) as a yellowish liquid (7.2g, yield: 34.8%). ^1H NMR (400 MHz, CDCl_3): δ = 7.51-7.59 (m, 4H, ArH) , 7.29-7.34(m, 6H, ArH), 7.09(s, 1H, ArSeCHC), 4.23(t, 2H, $\text{C}(\text{O})\text{OCH}_2$), 3.51 (t, 2H, BrCH_2), 2.86(t, 2H, $\text{BrCH}_2\text{CH}_2\text{C}(\text{O})\text{O}$), 2.62(t, 2H, $\text{CH}_2\text{CH}_2\text{C}$); ^{13}C NMR (100 MHz, CDCl_3): δ =165.48, 128.19, 127.98, 125.87, 125.02, 124.60, 124.26, 122.94, 122.80, 98.41, 34.08, 32.92, 20.99.

Synthesis of (Z)-3,4-bis(phenylselanyl)but-3-en-1-yl 3-azidopropanoate (2Se-N₃) (6)

Compound 5 (6.21 g, 12.0 mmol) and sodium azide (1.56 g, 24.0 mmol) were dissolved in DMF (15 mL), and the mixture was stirred at room temperature for 24 hours. The solution

was added DCM (100 mL) and reversed extraction with water three times (3×100 mL). The organic phase was dried over anhydrous MgSO_4 , filtered and evaporated to dryness. The product was collected as a yellow solid powder. Yield: 5.18 g (90.1%). ^1H NMR (400 MHz, CDCl_3): δ = 7.52-7.58 (m, 4H, ArH), 7.25-7.33(m, 6H, ArH), 7.09(s, 1H, ArSeCHC), 4.23(t, 2H, C(O)OCH₂), 3.51 (t, 2H, CH₂C(O)), 2.62(t, 2H, CH₂CSe), 2.51(t, 2H, N₃CH₂); ^{13}C NMR (100 MHz, CDCl_3): δ = 170.63, 132.93, 132.78, 132.65, 129.40, 129.39, 127.73, 127.61, 63.22, 46.72, 38.82, 33.92.

Synthesis of squarylium dye (SQ-dye)

3-Iodopropionic acid (32.0 g, 0.16 mol) was suspended in 2,3,3-trimethylindolenine (25 mL, 0.15 mol). The suspension was stirred at 100 °C for 15 hours under an argon atmosphere, and then allowed to cool to room temperature, and the resulting solid was collected by filtration. The solid was washed with chloroform and dried under reduced pressure to afford 1-(β -Carboxyethyl)-2,3,3-trimethylindolenium iodide as a pink solid. The results solid (0.72 g, 2.00 mmol) and squaric acid (104 mg, 0.91 mmol) were dissolved in toluene (10 mL), 1-butanol (10 mL), and pyridine (5 mL). The flask was fitted with a Dean-Stark trap, and the mixture was refluxed for 12 hours under an argon atmosphere, then allowed to cool to room temperature. The solvent was removed under reduced pressure. The resulting residue was dissolved in 2 N HCl aq. (10 mL) and MeOH (10 mL), and the solution was refluxed for 12 hours to cleave the n-butyl ester allowed to cool to room temperature. The resulting solid was collected by filtration and washed with acetone to afford SQ-dye as a green powder (205.2 mg, yield 38 %). ^1H NMR (400 MHz, DMSO-d_6): δ = 12.59 (s, 1H), 7.52 (d, 2H, J = 7.3 Hz), 7.36-7.34 (m, 4H), 7.15-7.10 (m, 2H), 5.81 (s, 2H), 4.33 (t, 4H, J = 6.8 Hz), 2.70 (t, 4H, J = 6.8 Hz), 1.68 (s, 12H). ^{13}C NMR (101 MHz, DMSO-d_6): δ = 197.99, 171.66, 141.83, 140.93, 129.43, 129.01, 123.57, 115.65, 54.34, 54.34, 43.58, 43.58, 40.21, 40.00, 39.79, 39.58, 39.37, 39.17, 38.96, 31.17, 31.17, 21.95, 21.95, 14.39, 14.39.

Preparation of Micelles and Amylose Loaded Micelles

Micelles of PEG-*b*-poly(MPC) and PMPC-2Se were prepared by a dialysis method. The amphiphilic block copolymer (25.0 mg) was dissolved in DMF (1.5 mL), and then DI water (10 mL) was slowly added with vigorous stirring. After vigorous stirring for another 2 h at RT, the resulting solution was got and submitted to dialyze against DI water that was renewed regularly for every 4 hour to remove DMF (MWCO 1 kDa). The final micelle concentration was adjusted by adding DI water to 0.5 mg mL⁻¹.

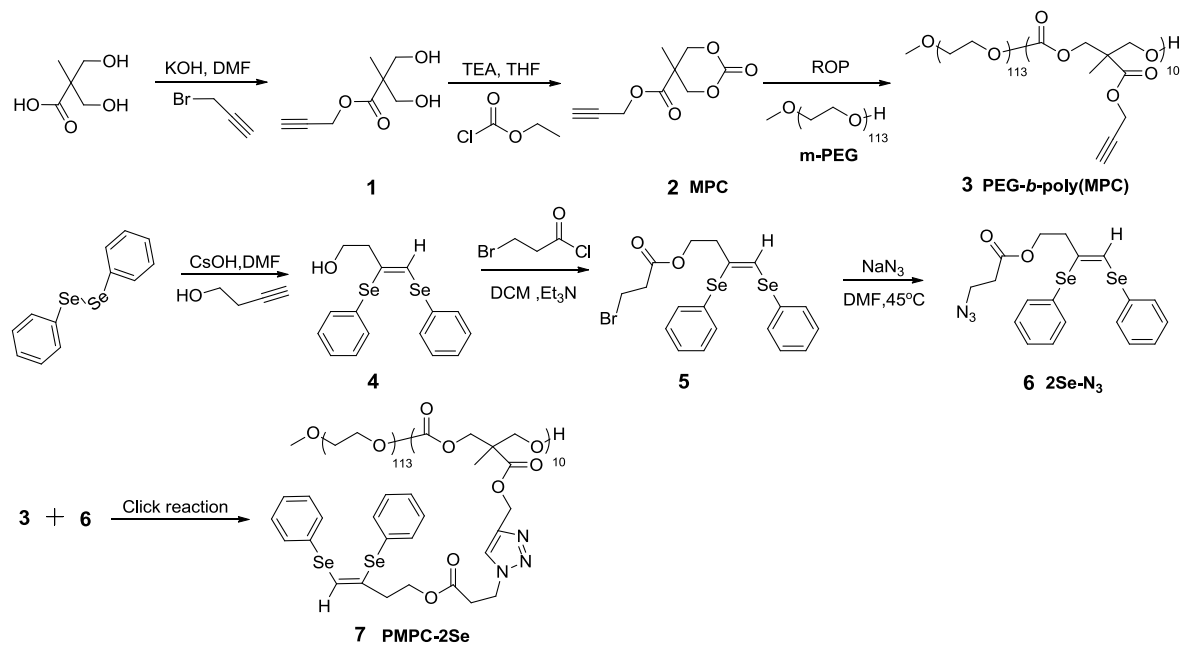
Measurement of the Critical Micellar Concentration (CMC)

The CMC of PEG-*b*-poly(MPC) and PMPC-2Se amphiphiles were determined by a dye solubilization method. Nile Red (NR) in THF (0.1 mg mL⁻¹, 30 μL) was added to a vial using a pipettor. After THF was evaporated, micellar solution (0.1 to 5 × 10⁻⁴ mg mL⁻¹, 2 mL) was added and stirred for 12 h. The fluorescence intensity of the resulting solutions was measured under the excitation wavelength of NR (550 nm).

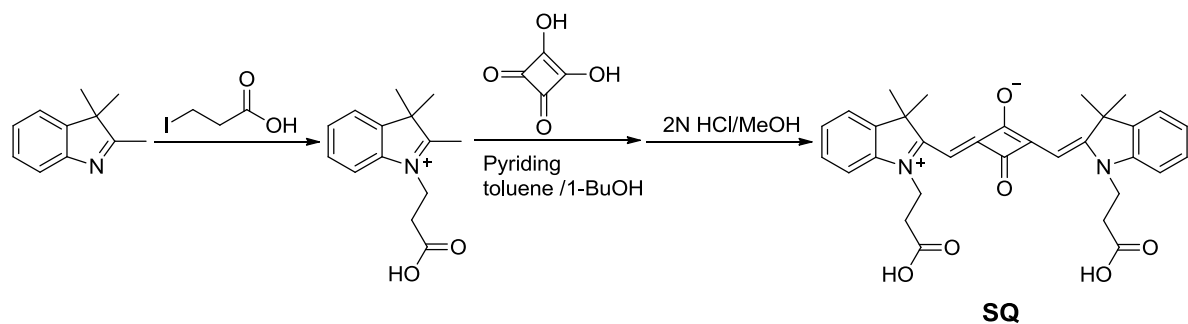
Cell culture and tumor xenografts model construction

LNCaP cells were obtained from the college of life science of Inner Mongolia University (Hohhot, China). Those cell was cultured using high-glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 1% penicillin-streptomycin (10,000 U/mL, 10,000 μg mL⁻¹, Invitrogen) and 10% fetal bovine serum (GIBCO) in an atmosphere of 5% CO₂ and 95% air at 37 °C.

Eight-week-old BALB/c nude mice (male) purchased from SJA Co., Ltd. (Changsha, China) were used as in vivo imaging mode. Preparation of tumor samples follow the previous procedure: a total of 3×10⁶ LNCaP cells diluted in 100 μL of serum-free DMEM medium were injected subcutaneously into the right flank of 6 to 8-week-old BALB/c nude mice to inoculate tumors. After 20 days, the mice were used for imaging.



Scheme S1. Synthesis of (3) PEG-*b*-poly(MPC), (6) 2Se-N₃ and (7) amphiphilic copolymer PMPC-2Se.



Scheme S2. Reaction scheme for synthesis of fluorescent dye SQ

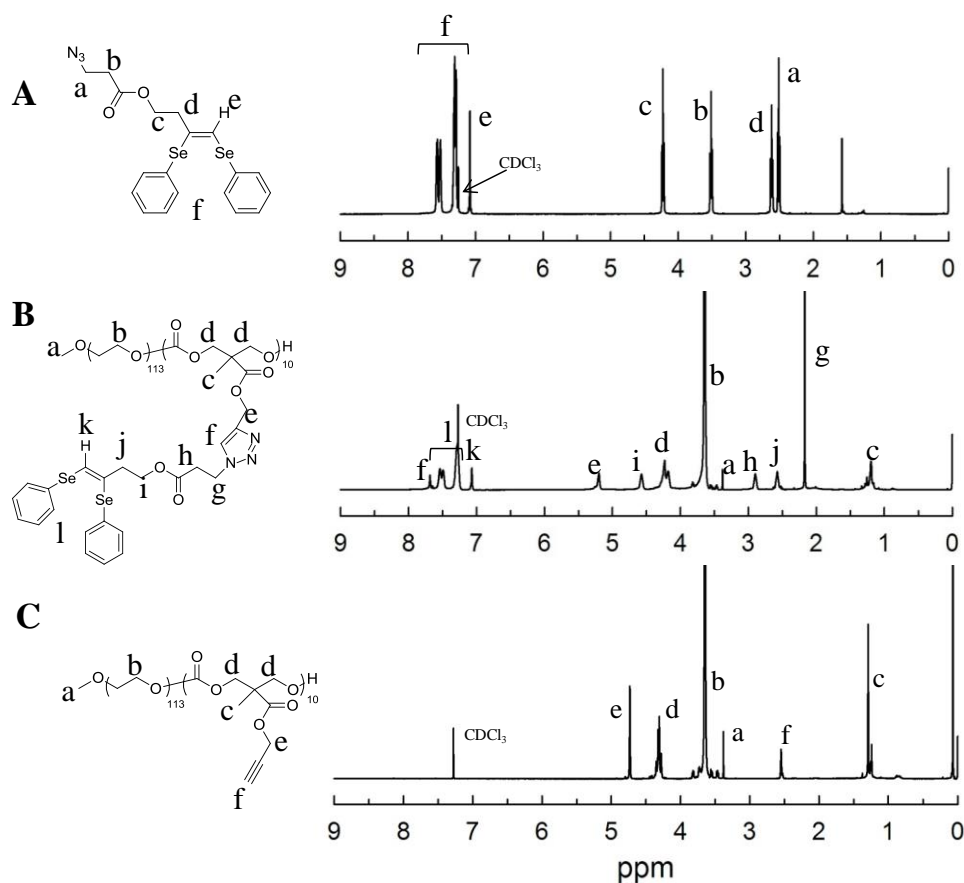


Figure S1. ^1H NMR spectra of 2Se-N₃ (A), PMPC-2Se (B) and PEG-*b*-poly(MPC) (C).

Table S1. Molecular Characteristics of Amphiphiles PEG-*b*-poly(MPC) and PMPC-2Se

| Entry | M_w/M_n^a | $M_{n,\text{GPC}}^a$ | $M_{n,\text{NMR}}^b$ | CMC ^c (mg mL ⁻¹) |
|--------------------------|-------------|----------------------|----------------------|-----------------------------------------|
| PEG- <i>b</i> -poly(MPC) | 1.0483 | 7263 | 6984 | 0.0116 |
| PMPC-2Se | 1.1109 | 13109 | 11800 | 0.0123 |

^a Both molecular weight ($M_{n,\text{GPC}}$) and the polydispersity (M_w/M_n) of the amphiphiles were determined by GPC.
^b $M_{n,\text{NMR}}$ was determined by ^1H NMR.
^c CMC: the critical micellar concentration of the amphiphiles was determined by fluorescence spectroscopy (Figure S3).

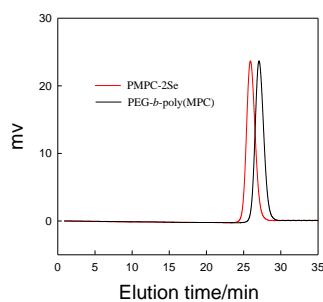


Figure S2. GPC traces of PEG-*b*-poly(MPC) (dark line) and PMPC-2Se (red line)

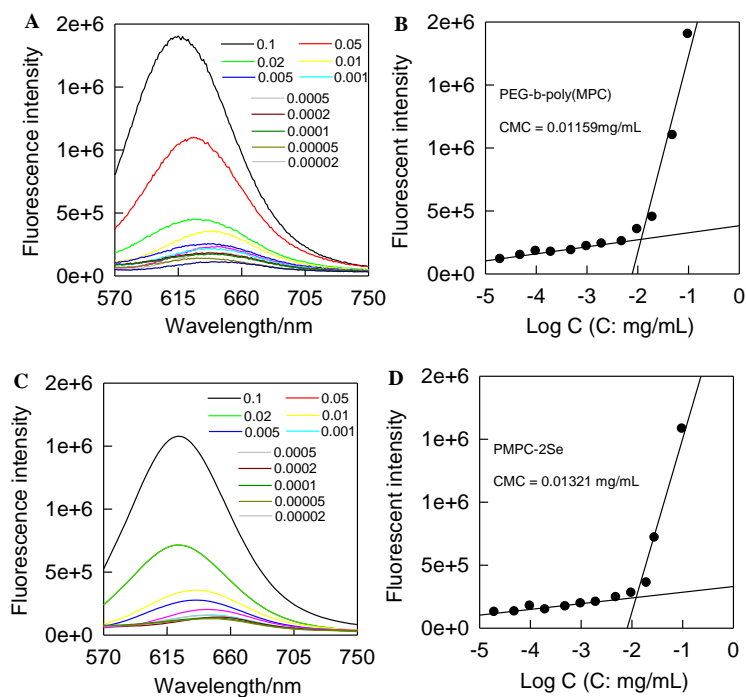


Figure S3. Fluorescence emission spectra of Nile Red in (A) PEG-*b*-poly(MPC) micelles and (C) PMPC-2Se micelles of varying concentrations (mg mL^{-1}) and the relevant emission intensity at 630 nm versus the log of concentration for (B) PEG-*b*-poly(MPC) micelles and (D) PMPC-2Se micelles.

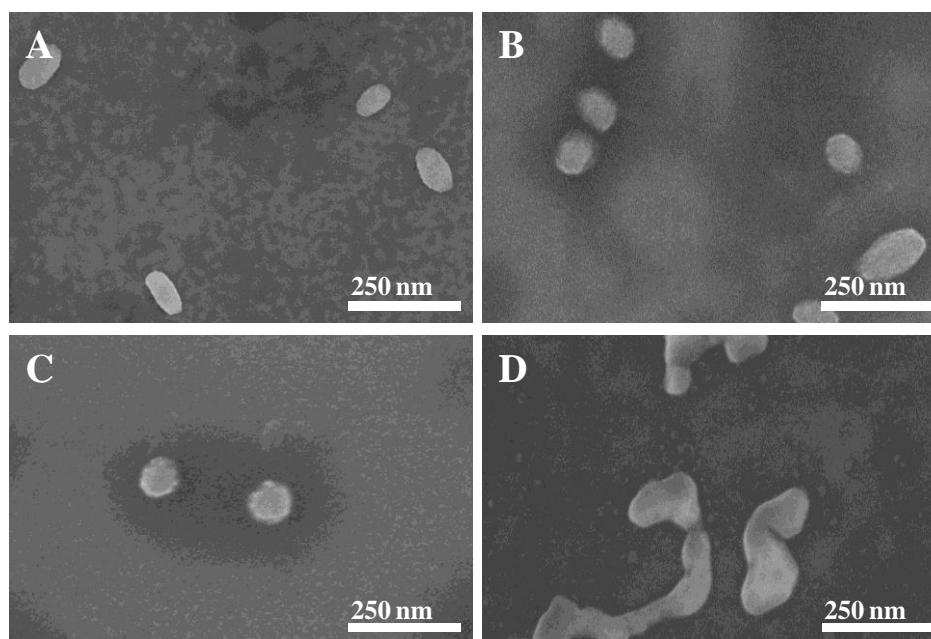


Figure S4. SEM photographs of the micelles: (A) PEG-*b*-poly(MPC), (B) PMPC-2Se, (C) PEG-*b*-poly(MPC) treated by OH, and (D) PMPC-2Se treated by OH.

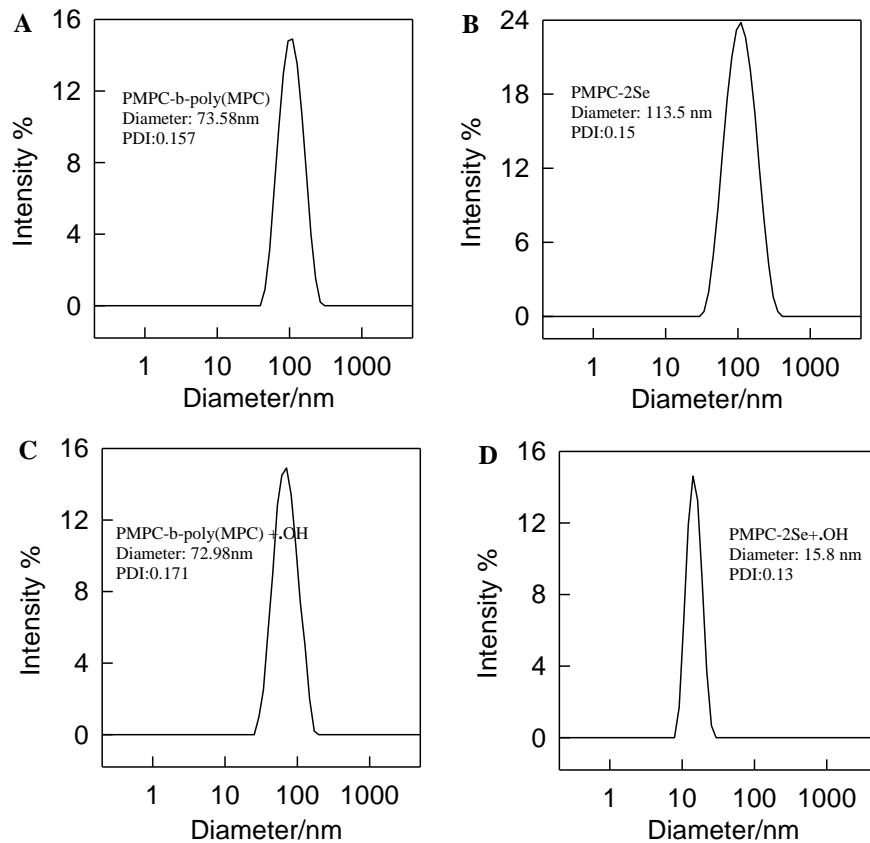


Figure S5. Mean size distributions of the micelles determined by DLS: PEG-*b*-poly(MPC) (A), P PMPC-2Se (B), PEG-*b*-poly(MPC) treated by OH (C) and PMPC-2Se after 60 min treated by OH (D).

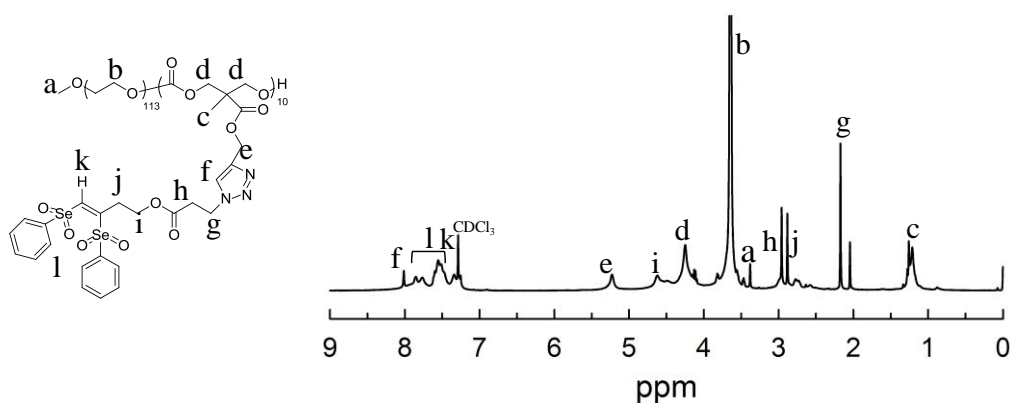


Figure S6. ^1H NMR of PMPC-2Se micelles (0.5 mg mL^{-1}) treated with OH (0.5 mM). (The product was allowed to dialyze in aqueous solution and lyophilize for ^1H NMR detection.)

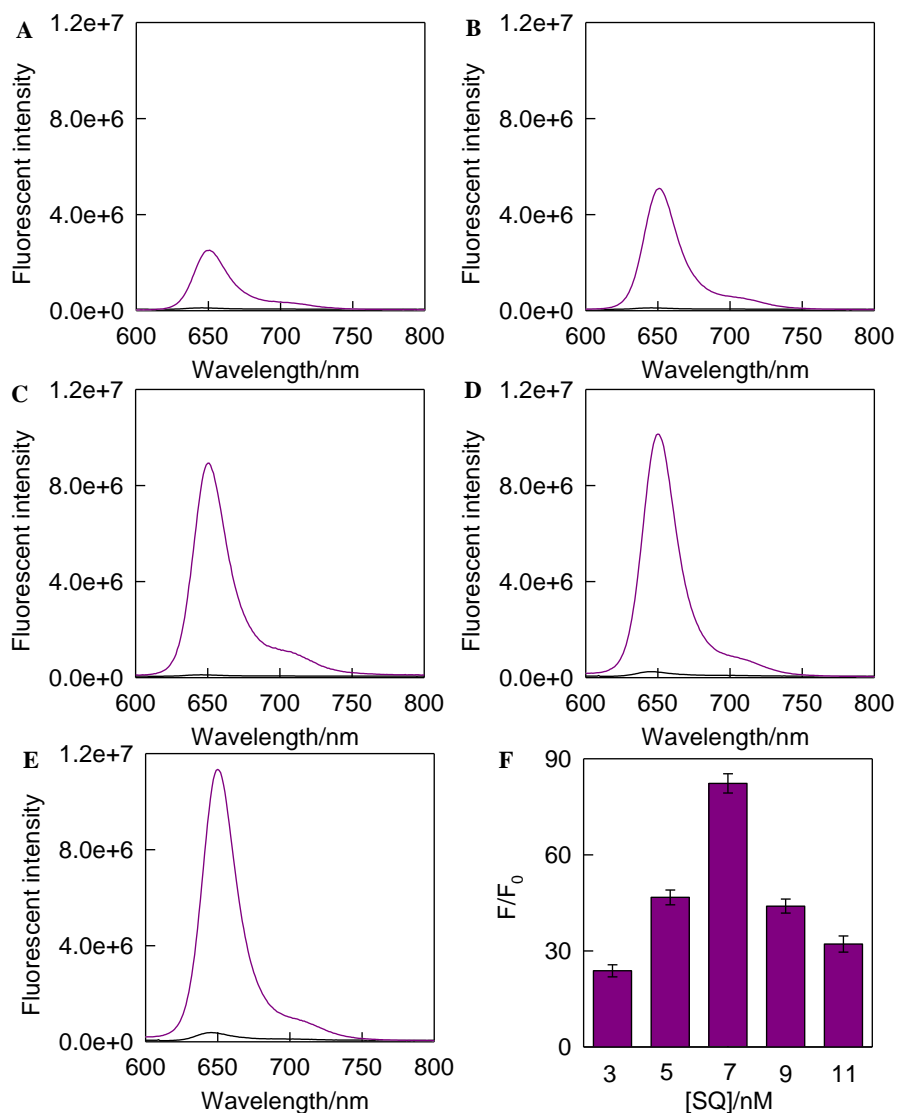


Figure S7. (A-E) Fluorescence emission spectra of SQ dye (3, 5, 7, 9, 11 nM) wrapped in PMPC-2Se micelles (0.1 mg mL^{-1}) (dark line) and released SQ dye (purple line) from the micelles of PMPC-2Se@SQ treated by OH under the condition of PBS buffer solution (20 mM, pH=7.4). (F) The fluorescence ratio (F/F_0) of PMPC-2Se@SQ probe as a function of enwrapped SQ dye concentrations in PBS (20 mM, pH=7.4) solution. error bars were obtained through the detection of three parallel samples. $\lambda_{\text{ex}}/\lambda_{\text{em}} = 590 \text{ nm}/649 \text{ nm}$.

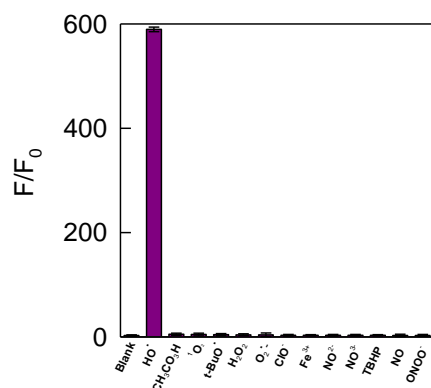


Figure S8. The selectivity of PMPC-2Se@SQ probe (0.5 mg mL^{-1}) toward various reactive oxygen species (ROS).

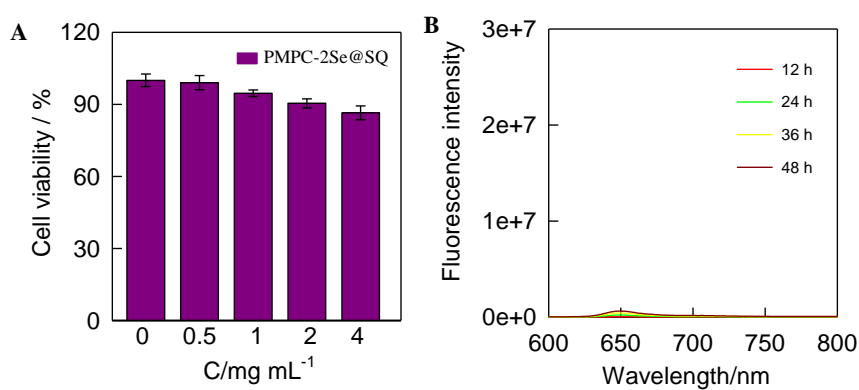


Figure S9. (A) Cell viability of normal LNCaP cells after 24 h treated with different concentrations of PMPC-2Se@SQ at 37°C . (B) Effect of human cell lysate-contained biological fluids on PMPC-2Se@SQ ($0.05 \text{ }\mu\text{g mL}^{-1}$) at different times (12, 24, 36, and 48 h).

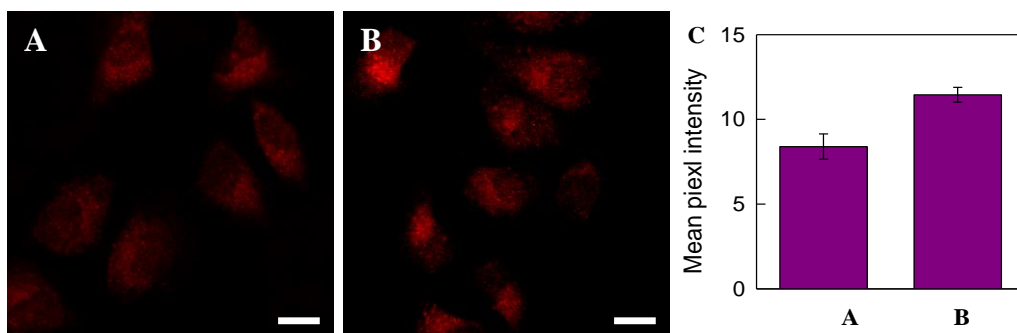
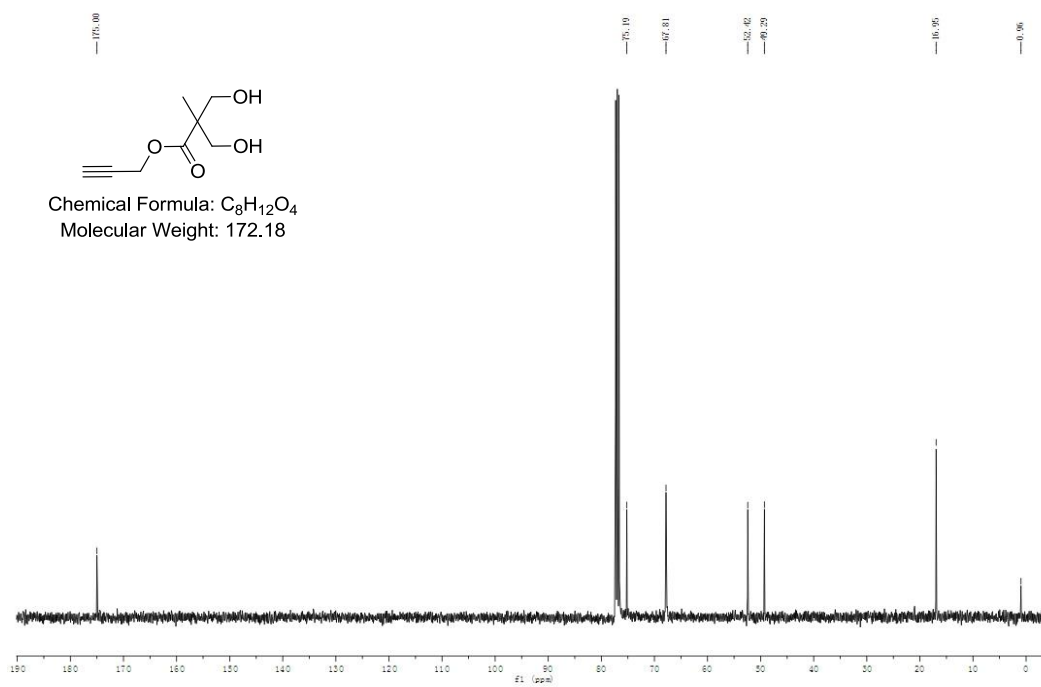
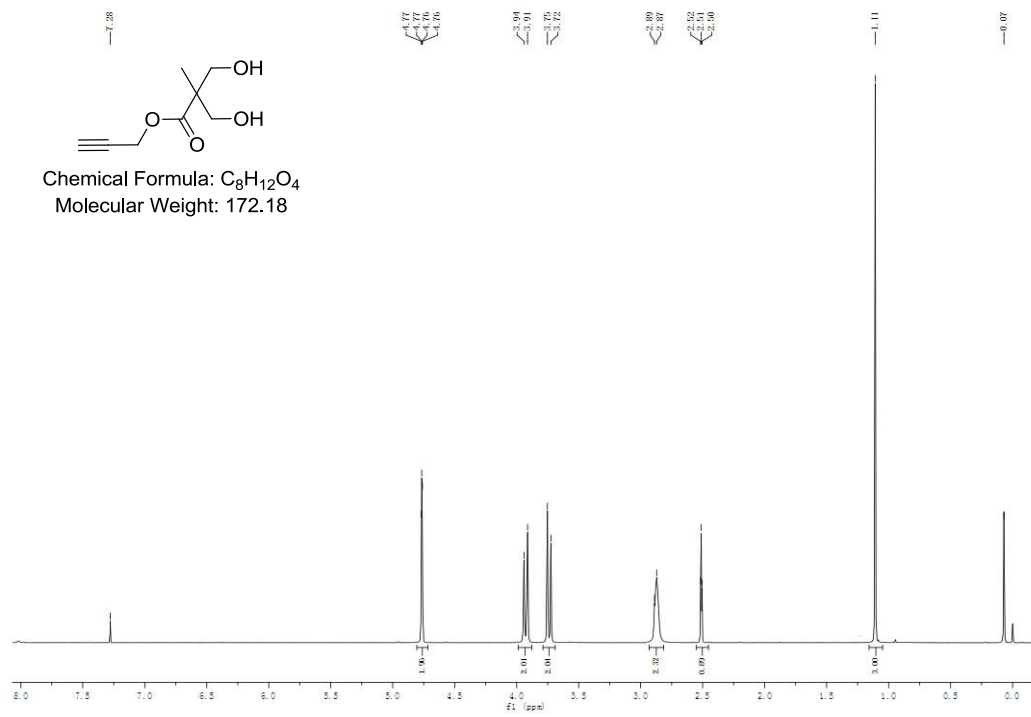
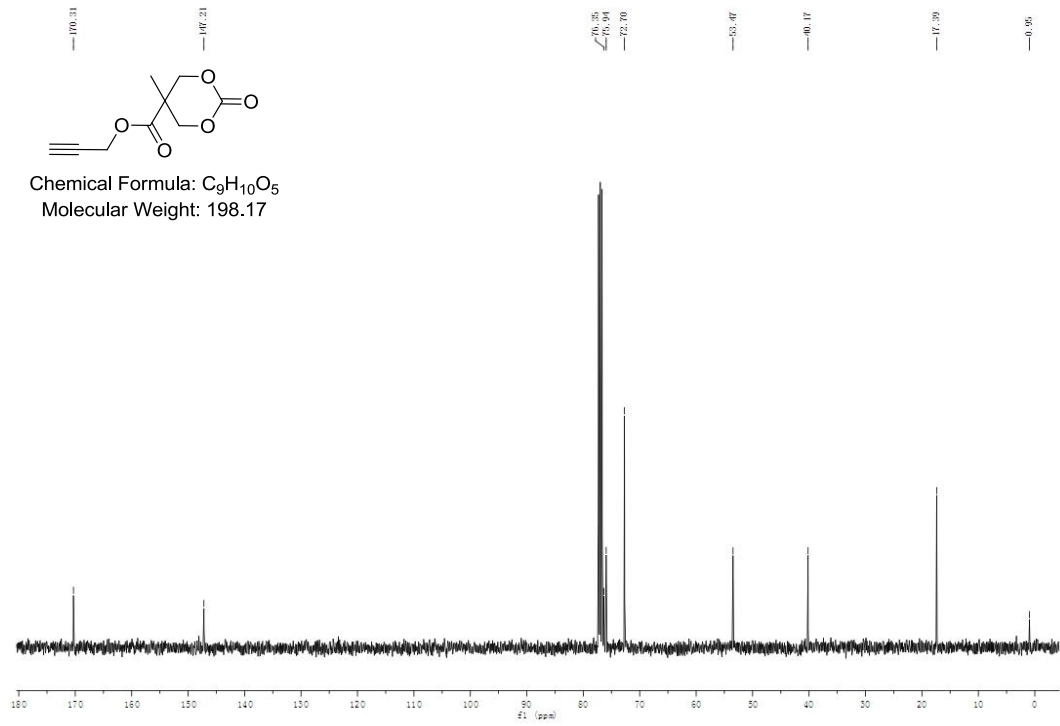
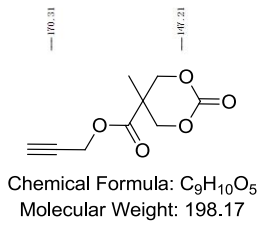
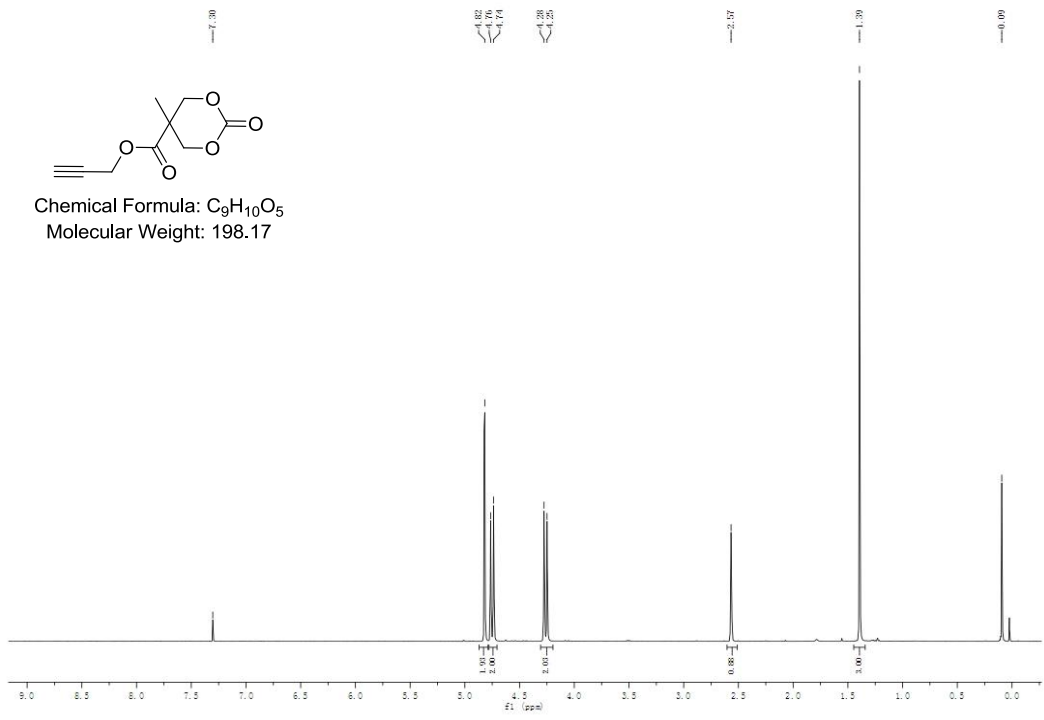
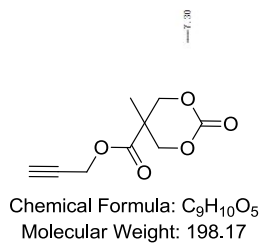


Figure S10. Confocal fluorescence micrographs of LNCaP cells under the treatment of DHE 1 μM (a commercial ROS fluorescent probe) (A) and PMPC-2Se@SQ (0.05 μg mL⁻¹) (B), respectively. Graph (C) showing relative fluorescence intensities of treatments from (A-B). Scale bar: 20 μm.

^1H NMR and ^{13}C NMR





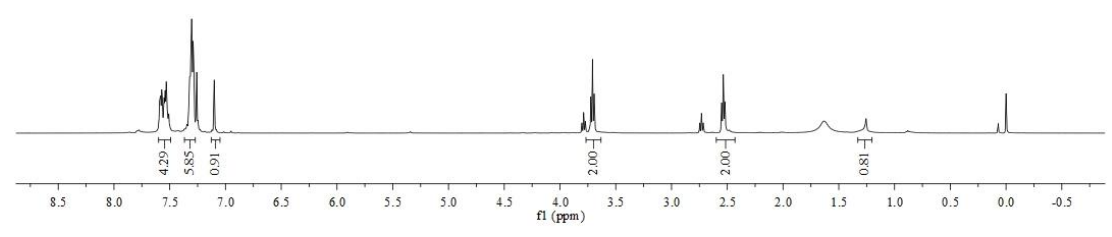
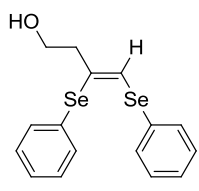
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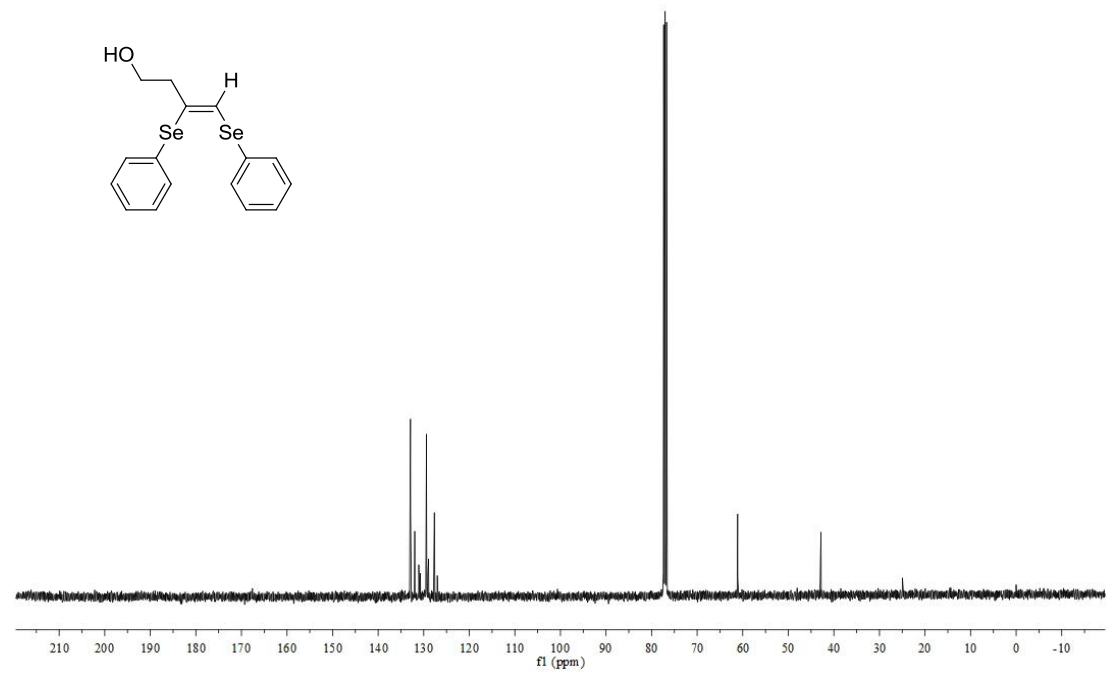
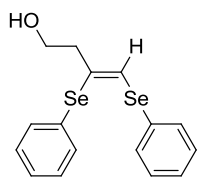
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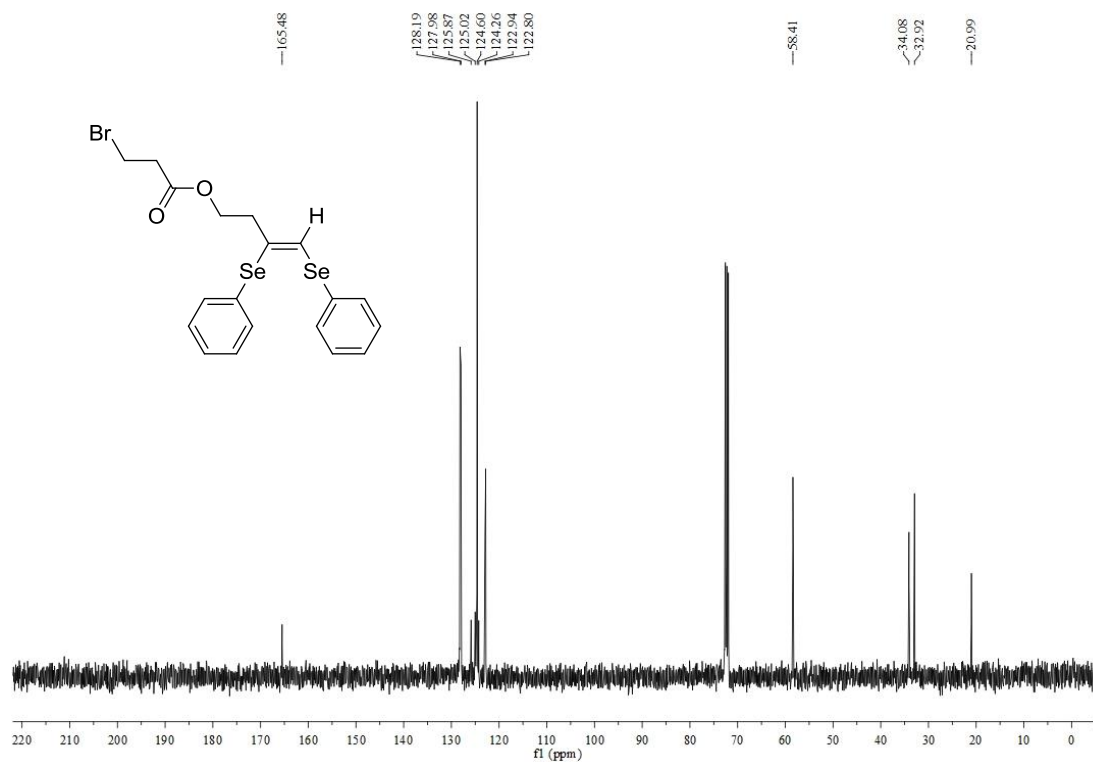
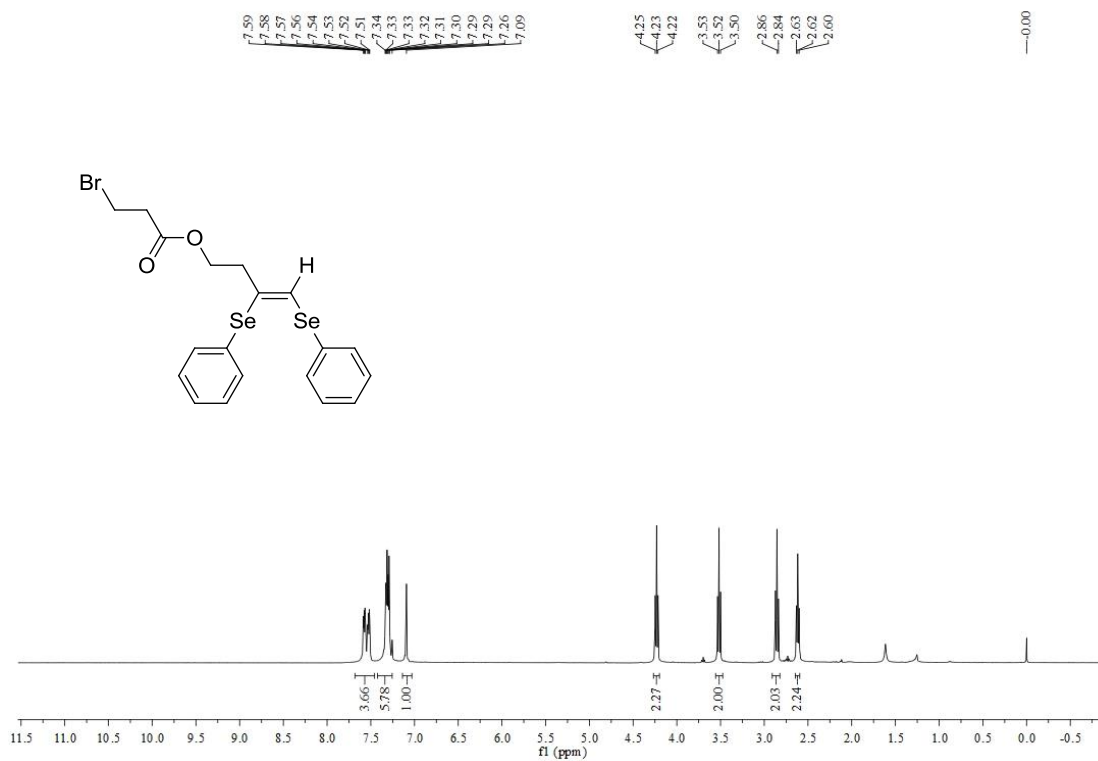


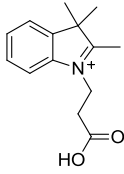
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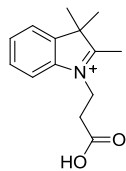
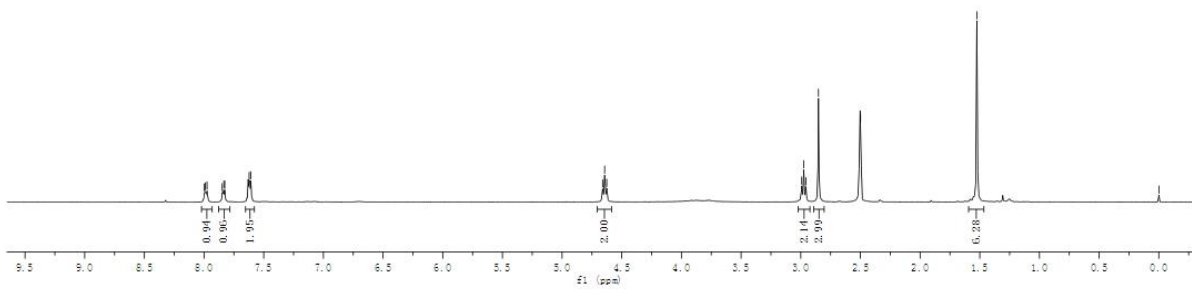
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Chemical Formula: $C_{14}H_{18}NO_2^+$
Molecular Weight: 232.30



Chemical Formula: $C_{14}H_{18}NO_2^+$
Molecular Weight: 232.30

