Enhanced Bioreduction-Responsive Diselenide-Based Dimeric Prodrug Nanoparticles for Triple Negative Breast Cancer Therapy

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Synthesis of PTXD-Se:

The synthesis routes of PTXD-Se were generally described in Scheme S1. Characterization of related compounds were shown in Figure S1-12.

Synthesis of compound 2

PTX (427 mg, 0.5 mmol), DMAP (6 mg, 0.05 mmol) and Et_3N (118 μl, 0.5 mmol) were dissolved in anhydrous DCM (5 ml). Then 5 ml anhydrous DCM solution of 4-nitrophenyl chloroformate (PNP) was added dropwise at room temperature. After 4 h, the reaction was finished as monitored by TLC. The mixture was subject to chromatography (Hexane: EtOAc 1:1). The product was obtained as white solid (371.1 mg, yield 73%). ^{1}H NMR spectrum and peak assignments was shown in Figure S1.

Synthesis of compound 3

Et_3N (51 μl, 0.36 mmol) and compound 20 (136 mg, 0.72 mmol) were dissolved in anhydrous DCM (5 ml). Then 5 ml anhydrous DCM solution of 2 (371.1 mg, 0.36 mmol) was added dropwise. The reaction was stirred overnight, then finished as monitored by TLC. The mixture was concentrated under vacuum and purified by column chromatography (Hexane: EtOAc 1:3) to give a white solid (386 mg, yield 86%). ^{1}H NMR spectrum and peak assignments was shown in Figure S1.

Synthesis of compound 4

Compound 3 (544.8 mg, 0.5 mmol) was dissolved in 2 ml of DCM/TFA (1:1 v/v) in an ice bath. After 5 min, the reaction was finished as monitored by TLC. The mixture was diluted with DCM (100 ml) and washed with 5% $NaHCO_3$ solution (5 × 20 ml), and then with DI water (3 × 20 ml). The organic phase was dried with anhydrous Na_2SO_4. After the solvent was removed under vacuum, compound 4 was acquired as white solid (476.8 mg, yield 97%).

Synthesis of compound 6

Compound 6 was synthesized as reported previously. Briefly, selenium (4.4 g, 0.056 mol) was added in small portions to a heated (65 ℃) and well stirred mixture of KOH (85%, 3.96 g, 0.06 mol), water (20 ml) and 55% hydrazine monohydrate (1.56 ml, 0.028 mol). The resulting brownish solution was heated at 90 ℃ for 2 h, then cooled to 50 ℃. 2-chloroethanol (3.8 ml, 0.056 mol) was added dropwish and heating continued for another 3 h. Then greenish-yellow solution was allowed to cool to room temperature and stirred overnight until the TLC showed the reaction had proceeded to completion. The reaction mixture was filtered through Celite to remove unreacted Se. The filtrate was extracted with DCM (5 × 40 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated. The crude product was purified by chromatography (Hexane: EtOAc 1:1) and got bright orange oil (2.2 g, 32%). ^{1}H NMR (400 MHz, $CDCl_3$), δ 3.93 (t, 4H, J = 3.9 Hz), 3.11 (t, 4H, J = 3.1 Hz). MS-EI (m/z): [M]+ calcd for $C_4H_{10}O_2Se_2$, 249.9; observed, 249.9. ^{1}H NMR spectrum and peak assignments was shown in Figure S2.

Synthesis of compound 8

An aqueous solution (150 ml) of 3-chloro-1-propanol (9.45 g, 100 mmol) was refluxed with sodium azide (9.75 g, 150 mmol, 1.5 equiv.) for 22 h. The product was extracted with 100 mL methylene chloride three times, dried over sodium sulfate and concentrated as colorless oil (9.31 g, 92 % yield). ^{1}H NMR (400 MHz, $CDCl_3$), δ 3.76 (t, 2H, J = 5.7 Hz), 3.46 (t, 2H, J = 6.5 Hz), 1.84 (m, 2H). MS-EI (m/z): [M]+ calcd for $C_3H_7N_3O$, 101.1; observed, 101.1. ^{1}H NMR spectrum and peak assignments was shown in Figure S3.

Synthesis of compound 9

Triphosgene (388 mg, 1.3 mmol) was added into 5 ml anhydrous DCM solution of compound 8 (330mg, 3.25mmol) in 0 ℃. After stirring at room temperature overnight, solvent was removed

under vacuum. The as prepared chloroformate was redissolved in 2 ml anhydrous DCM and added into an anhydrous DCM solution of compound 6 (2.43 g, 9.8 mmol) and DMAP (1.197 g, 9.8 mmol). After stiring for another 12 h, the mixture was concentrated under vacuum, extracted with 50 ml EtOAc, and washed with water (3 × 40 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and got a yellow oil. The crude product was purified with chromatography (Hexane: EtOAc 1:1) and acquired a yellow oil (720 mg, 59%). ^{1}H NMR (400 MHz, $CDCl_3$), δ 4.41 (t, 2H, J = 4.4 Hz), 4.25 (t, 2H, J = 4.3 Hz), 3.89 (t, 2H, J = 3.9 Hz), 3.43 (t, 2H, J = 3.4 Hz), 2.96 (t, 2H, J = 3.0 Hz), 2.90 (t, 2H, J = 2.9 Hz), 1.95 (m, 2H, J = 2.0 Hz). MS-EI (m/z): [M]+ calcd for $C_8H_{15}N_3O_4Se_2$, 376.9; observed, 376.9. ^{1}H NMR spectrum and peak assignments was shown in Figure S4.

Synthesis of compound 10

Compound 9 (188 mg, 0.5 mmol), DMAP (6 mg, 0.05 mmol) and Et_3N (118 µl, 0.5 mmol) were dissolved in anhydrous DCM (5 ml). Then 5 ml anhydrous DCM solution of 4-nitrophenyl chloroformate (PNP) was added dropwise at room temperature. After 6 h, the mixture was concentrated under vacuum and subject to chromatography (Hexane: DCM 1:4). The product was obtained as yellow solid (230 mg, yield 85%). ^{1}H NMR (400 MHz, CDCl3), δ 8.25 (d, 2H, J = 8.2 Hz), 7.37 (d, 2H, J = 47.4 Hz), 4.52 (t, 2H, J = 4.5 Hz), 4.39 (t, 2H, J = 4.4 Hz), 4.21 (t, 2H, J = 4.2 Hz), 3.40 (t, 2H, J = 3.4 Hz), 3.03 (t, 2H, J = 3.0 Hz), 2.97 (t, 2H, J = 3.0 Hz), 1.91 (m, 2H, J = 1.9 Hz). MS-EI (m/z): [M]+ calcd for $C_{15}H_{18}N_4O_8Se_2$, 540.2; observed, 540.2. ^{1}H NMR spectrum and peak assignments was shown in Figure S5.

Synthesis of compound 12

Compound 11 (2 g, 11.9 mmol) and imidazole (2.02 g, 29.7 mmol) were dissolved in 10 ml anhydrous DMF. Then 5 ml anhydrous DMF of TBSCl (4.5 g, 29.7 mmol) was added slowly in

an ice bath. The reaction was allowed to reach room temperature and stirred overnight. The mixture was concentrated under vacuum and purified by chromatography (Hexane: EtOAc 40:1). The product was obtained as transparent oil (4.4 g, yield 94%). ^{1}H NMR (400 MHz, CDCl3), δ 8.12 (s, 1H), 6.75 (s, 2H), 4.79 (s, 4H), 2.25(s, 3H), 0.92 (s, 18H), 0.09 (s, 12H). MS-EI (m/z): [M]+ calcd for $C_{21}H_{40}O_3Si_2$, 396.7; observed, 396.7. ^{1}H NMR spectrum and peak assignments was shown in Figure S6.

Synthesis of compound 13

Compound 12 (1.53 g, 3.9 mmol), Et_3N (1.92 ml, 11.7 mmol) were dissolved in 10 ml anhydrous THF. Then PNP (1.01 g, 5.07 mmol) was dissolved in 5 ml anhydrous THF and added dropwise at 0 ℃. The reaction was allowed to reach room temperature and stirred for another 2 h. The white suspension was filtered. And the filtrate was concentrated and purified by chromatography (Hexane: EtOAc 8:1). The product was obtained as white solid (1.95 g, yield 90%). The product was obtained as transparent oil (4.4 g, yield 94%). ^{1}H NMR (400 MHz, $CDCl_3$), δ 8.31 (d, 2H, J = 8.3), 7.48 (s, 2H, J = 7.5), 7.22 (s, 2H), 4.72(s, 4H), 2.38 (s, 3H), 0.92 (s, 18H), 0.09 (s, 12H). MS-EI (m/z): [M]+ calcd for $C_{28}H_{43}NO_7Si_2$, 561.2; observed, 561.2. ^{1}H NMR spectrum and peak assignments was shown in Figure S7.

Synthesis of compound 14

Compound 13 (662 mg, 1.2 mmol) was dissolved in anhydrous DCM. Then compound 20 (264.1 mg, 1.4 mmol) was added quickly. The reaction was stirred at room temperature for 4 h and monitored by TLC (Hexane: EtOAc 4:1). The solvent was removed under vacuum and product was purified by chromatography (Hexane: EtOAc 8:1). The product was obtained as white solid (531.3 mg, 73 %). ^{1}H NMR (400 MHz, $CDCl_3$), δ 7.19 (s, 2H), 4.51(s, 4H), 3.16 (s, 2H), 3.03 (s, 2H), 2.89 (m, 4H), 2.33 (m, 3H), 1.49 (s, 9H), 0.92 (s, 18H), 0.09 (s, 12H). MS-EI (m/z): [M]+

calcd for $C_{31}H_{58}N_2O_6Si_2$, 610.4; observed, 610.4. ^{1}H NMR spectrum and peak assignments was shown in Figure S8.

Synthesis of compound 15

Compound 14 (531.3 mg, 0.9 mmol) was stirred with 100 mg amberlyst-15 in a mixture of DCM/MeOH (2:1) overnight. After the reaction completed, the mixture was filtered to removed amberlyst-15. The suspension was subject to flash column (Hexane: EtOAc 4:1 to EtOAc) giving a white solid (299 mg, 90%). ^{1}H NMR (400 MHz, $CDCl_3$), δ 7.19 (s, 2H), 4.51(s, 4H), 3.47 (m, 4H), 3.16 (s, 2H), 3.03 (s, 2H), 2.33 (m, 3H), 1.42 (s, 9H). MS-EI (m/z): [M]+ calcd for $C_{19}H_{30}N_2O_6$, 382.5; observed, 382.5. ^{1}H NMR spectrum and peak assignments was shown in Figure S9.

Synthesis of compound 16

PNP (158 mg, 0.78 mmol) was dissolved in 2 ml anhydrous DCM. Then a mixture of compound 15 (100 mg, 0.26 mmol), Et_3N (109 µl, 0.78 mmol) and a catalytic amount of DMAP was added. The reaction was stirred at room temperature for 4 h and monitored by TLC (hexane: EtOAc 1:1). After completion, the solvent was removed under vacuum and the product was purified by chromatography (hexane: EtOAc 2:1) giving compound 16 as a white powder (59.3 mg, yield 32%). ^{1}H NMR (400 MHz, $CDCl_3$), δ 8.25(d, 4H, J = 8.2), 7.79(d, 4H, J = 8.2), 7.25 (s, 2H), 5.25(s, 4H), 3.48(m, 6H), 3.28 (s, 4H), 1.25 (s, 9H). MS-EI (m/z): [M]+ calcd for $C_{33}H_{36}N_4O_{14}$, 712.7; observed, 712.7. ^{1}H NMR spectrum and peak assignments was shown in Figure S10.

Synthesis of compound 17

Compound 4 (292.8 mg, 0.3 mmol), Et_3N (42 µl, 0.3 mmol) and a catalytic amount of DMAP were dissolved in 5 ml anhydrous DCM. Compound 16 (54 mg, 0.075 mmol) was dissolved in 2 ml anhydrous DCM and added dropwise. The reaction was stirred for 4 h and monitored by TLC

(DCM: MeOH 10:1). After completion, the mixture was concentrated and purified by chromatography (DCM: MeOH 20:1). The product was obtained as white powder (64 mg, 30%).

Synthesis of compound 19

Compound 17 (64 mg, 0.027 mmol) was dissolved in 2 ml of DCM/TFA (1:1 v/v) in an ice bath. After 5 min, the reaction was finished as monitored by TLC. The mixture was then diluted with DCM (100 ml) and washed with 5% $NaHCO_3$ solution (5 × 20 ml), and then with water (3 × 20 ml). The organic phase was dried with anhydrous Na_2SO_4. After the solvent was removed under vacuum, imtermediate product compound 18 was acquired as white solid (64 mg, 36%).

Compound 18 (42 mg, 0.018 mmol), Et_3N (5 µl, 0.036 mmol) and a catalytic amount of DMAP were dissolved in 5 ml anhydrous DCM. Compound 10 (20 mg, 0.036 mmol) was added quickly and the solution was stirred for 4h. The reaction was monitored by TLC (DCM: MeOH 20:1). After completion, the product was purified by chromatography (DCM: MeOH 20:1), giving a white powder (25.6 mg, 54%). Q-TOF results implied the successful synthesis of compound 19 (Figure S11). Q-TOF (m/z): [M]+ calcd for $C_{129}H_{153}N_{11}O_{41}Se_2$, 2671.56; observed, 2671.8556.

Synthesis of compound 20

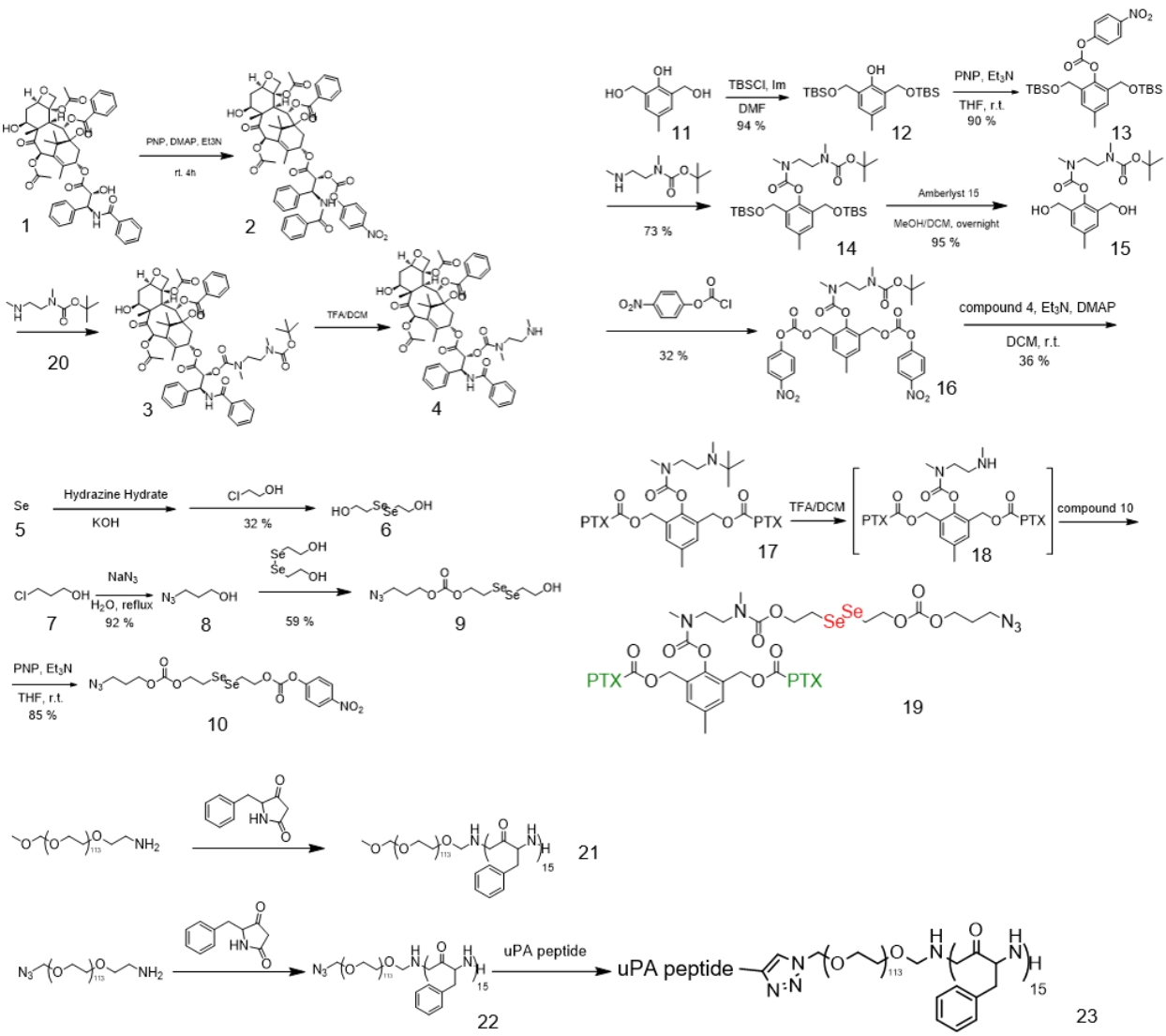
$(Boc)_2O$ (2.552 g, 11.7 mmol) was dissolved in 10 ml anhydrous THF and slowly added into 10 ml anhydrous THF of N,N'-dimethylethylenediamine (0.132 g, 1.5 mmol) in 2 h. The mixture was stirred for another 22 h and the solvent was removed under vacuum. Water (20 ml) was added to the residue and the insoluble solid was filtered off. The filtrate was extracted by DCM (3 × 20 ml) and washed by water (3 × 20 ml) and brine (20 ml). The organic phase was dried over Na2SO4 and DCM was removed to obtain transparent oil (169 mg, 60%). ^1H NMR (400 MHz, $CDCl_3$), δ 3.32(t, 2H, J = 2.7), 2.87(s, 3H), 2.72 (t, 2H, J = 2.7), 2.45(s, 3H), 1.45(s, 9H). MS-EI (m/z): [M]+

calcd for $C_9H_{20}N_2O_2$, 188.2; observed, 188.2. ^1H NMR spectrum and peak assignments was shown in Figure S12.

Synthesis of outer shell polymer $mPEG_{5K}$-pPhe(n) and uPA-PEG-pPhe(15)

L-phenylalanine N-carboxyanhydride (Phe-NCA) was synthesized as previously reported[6b]. $mPEG_{5K}$-pPhe(n) of various polymerization degrees were synthesized *via* ring-opening polymerization (ROP) reaction. In brief, $mPEG_{5K}$-NH_2 (50 mg, 0.01 mmol) and different equivalent of Phe-NCA (5 eq., 10 eq., 15 eq., 20 eq.) were dissolved in 5 ml anhydrous DMSO and stirred at 50 ℃ under nitrogen protection for 48 h. The polymers were purified by repeated precipitation from DMSO into diethylether and vacuum-dried to obtain white solid. $mPEG_{5K}$-pPhe(15) and uPA-PEG-pPhe(15) were characterized *via* NMR and IR (Figure S13-14).

uPA-PEG-pPhe(15) was synthesized in two steps. First, N_3-PEG_{5K}-pPhe(15) was synthesized in the same above procedure with N_3-PEG_{5K}-NH_2 as initiator. Then uPA peptide was coupled to the terminus of N_3-PEG_{5K}-pPhe(15) *via* click reaction. Briefly, N_3-PEG_{5K}-pPhe and uPA peptide (2 eq.) were dissolved in DMF under nitrogen protection. A freshly prepared solution of CuI (0.5 eq.), sodium ascorbate (20 eq.) and DIPEA (1 eq.) were added into the mixture and stirred at room temperature for 24 h. uPA-PEG-pPhe(15) was purified by dialysis using a membrane (Mw: 5000) against 10 mM EDTA for 24 h, DI water for 24 h, and followed by lyophilization. The disappearance of N_3 group in IR spectrum implied the successful synthesis of uPA-PEG-pPhe(15) (Figure S14).



Scheme S1. Synthesis route of PTXD-Se, mPEG5k-pPhe(15) and uPA-PEG5k-pPhe(15)

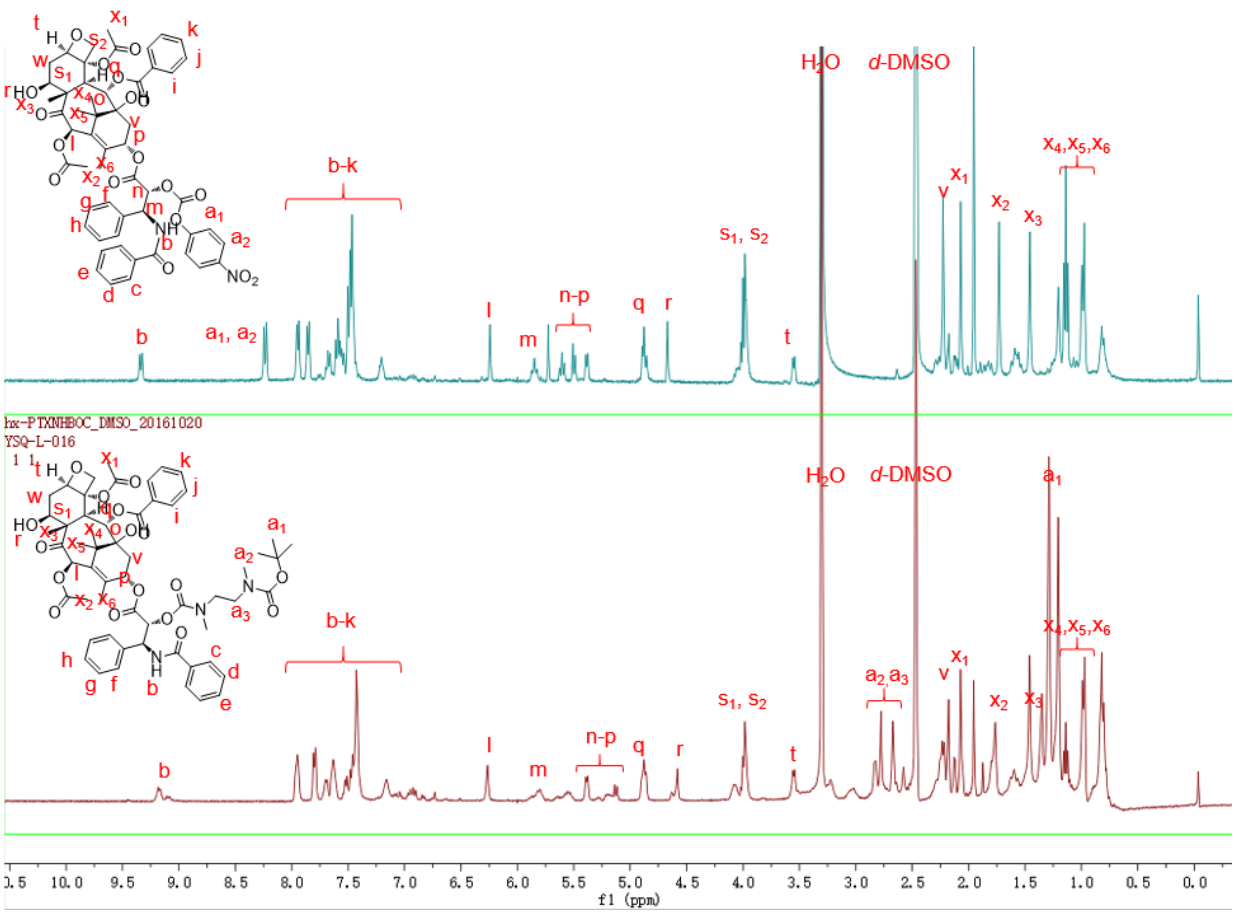


Figure S1. ^{1}H NMR of compound 2, 3

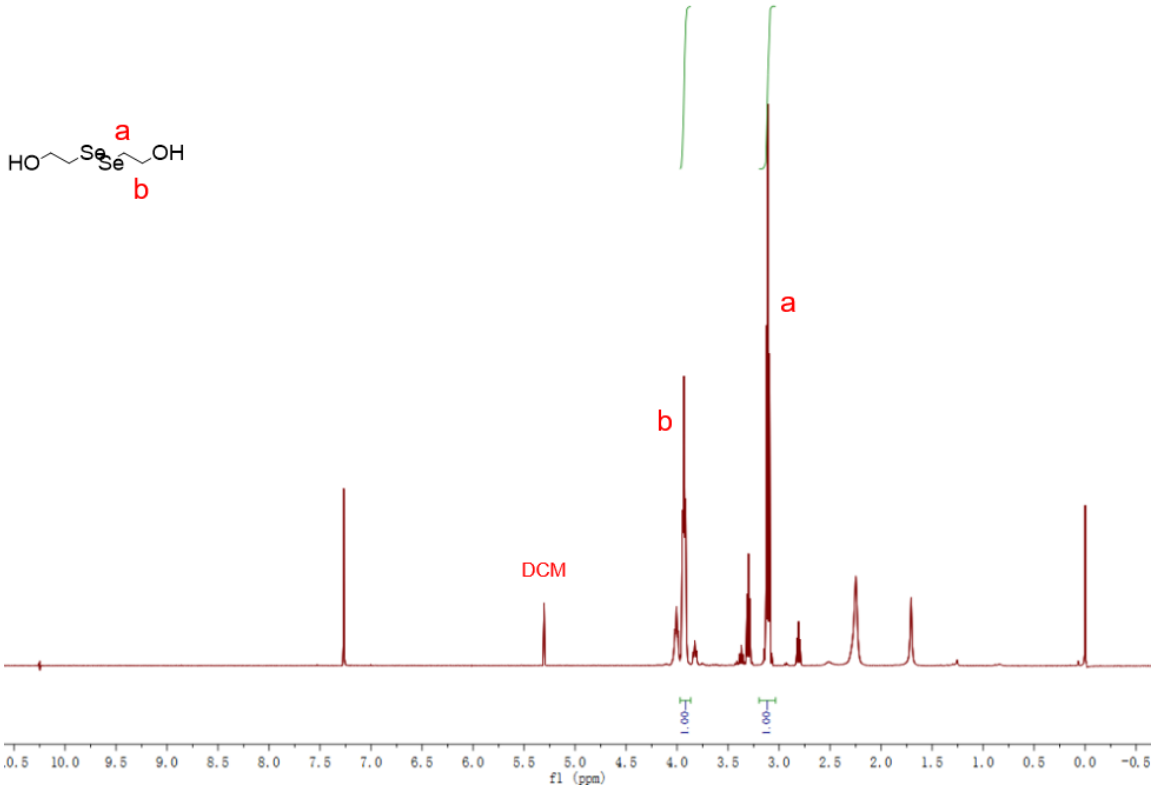


Figure S2. ^{1}H NMR of compound 6

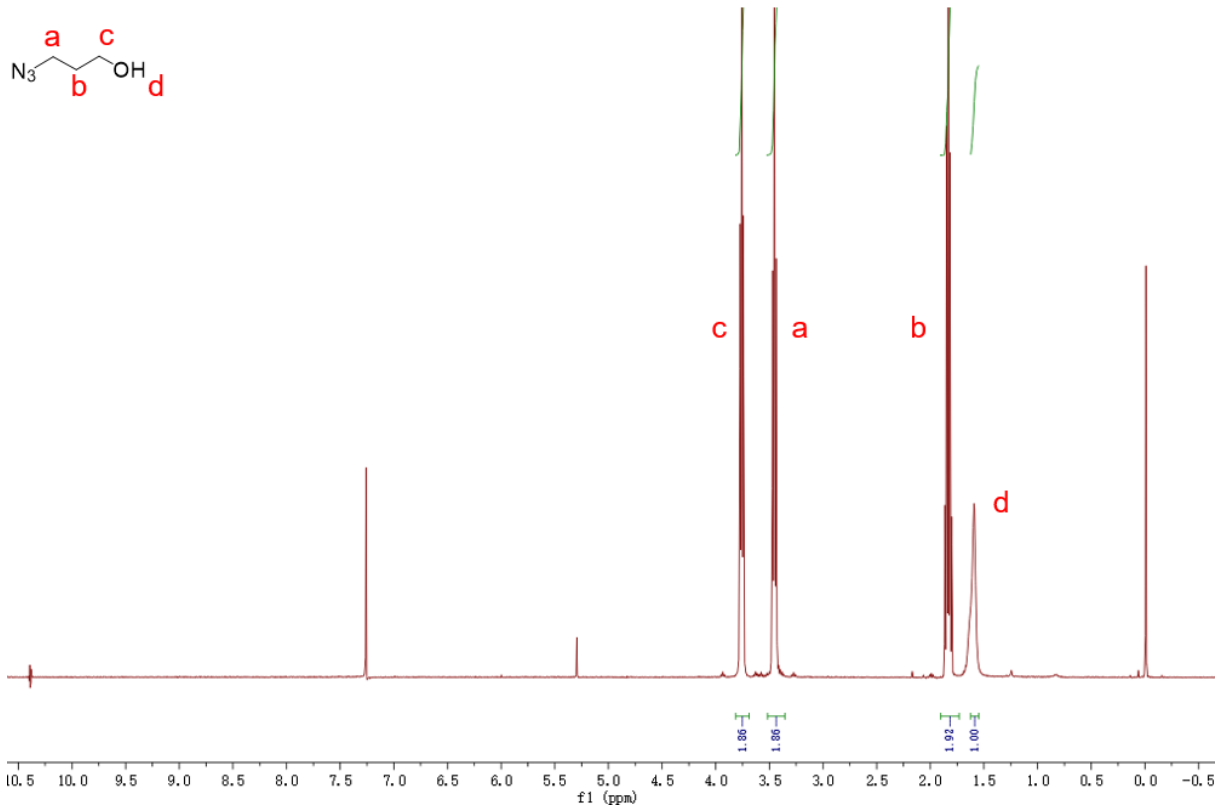


Figure S3. ^{1}H NMR of compound 8

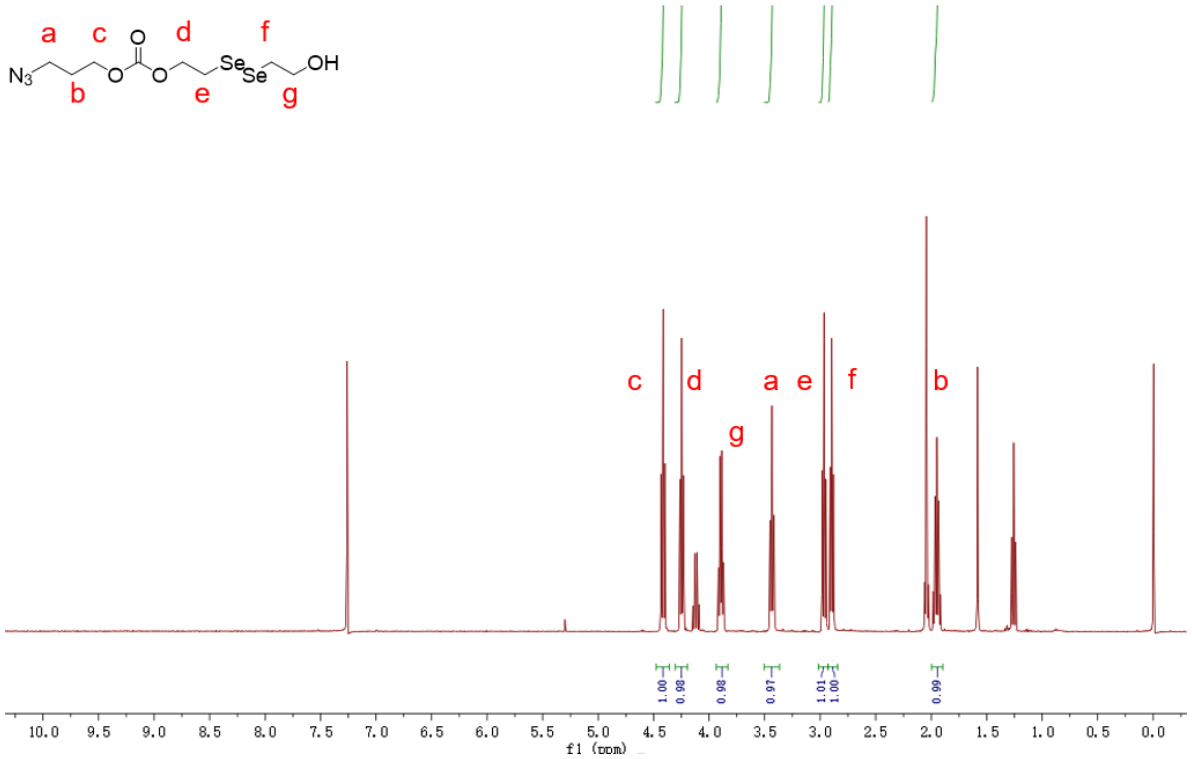


Figure S4. ^{1}H NMR of compound 9

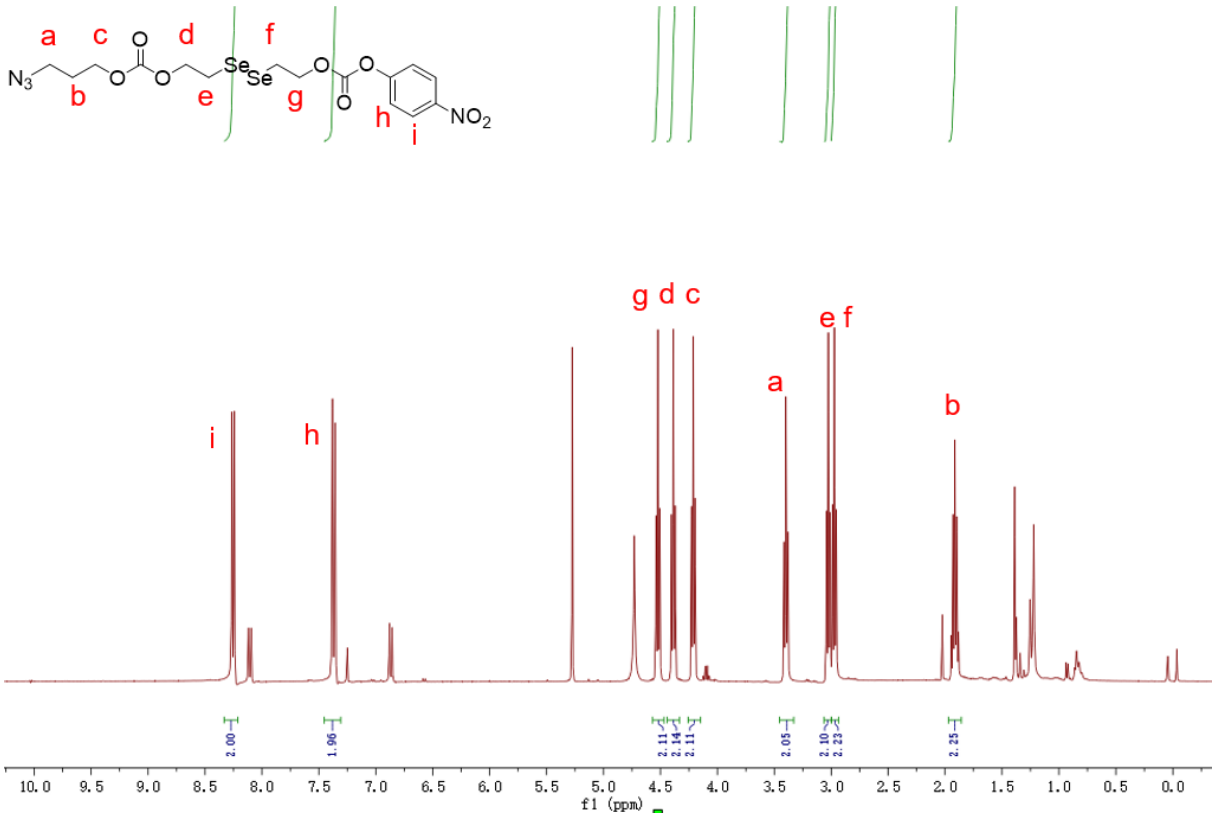


Figure S5. ^{1}H NMR of compound 10

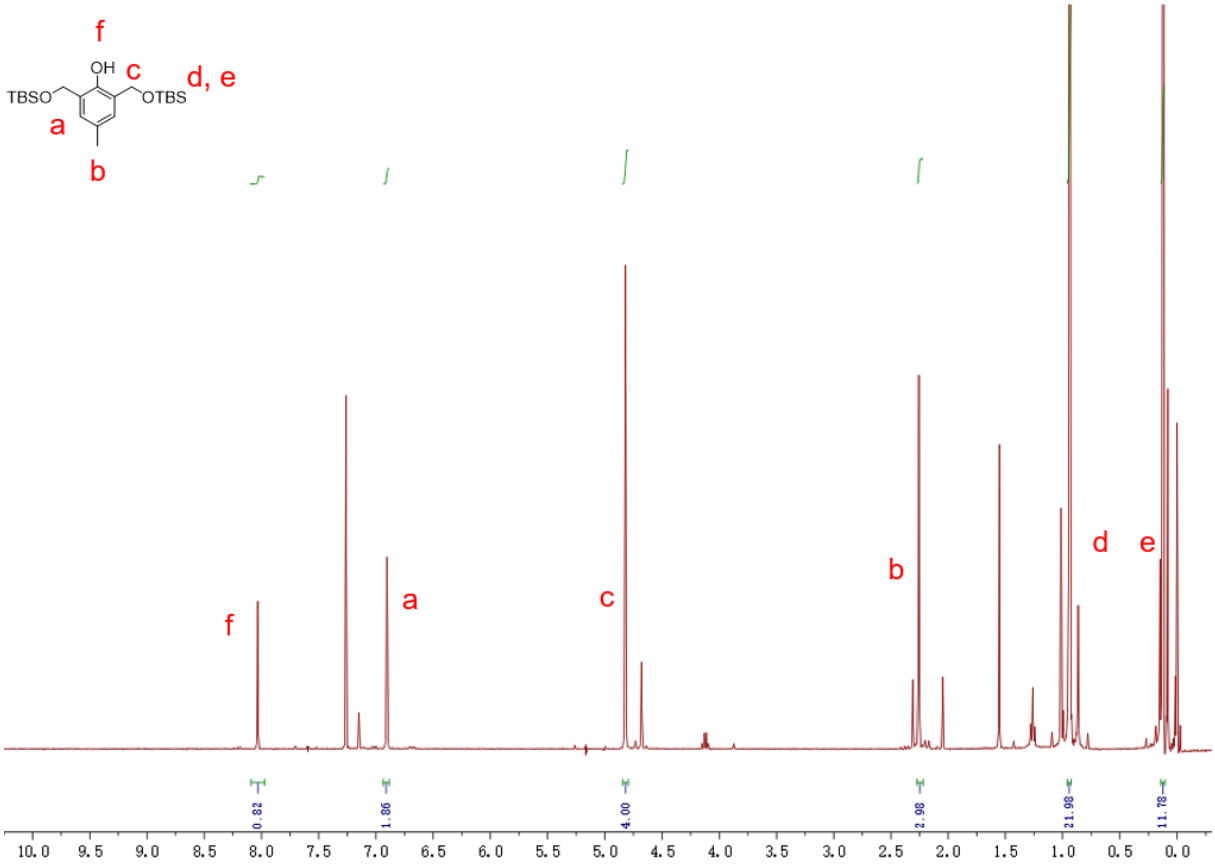


Figure S6. ^{1}H NMR of compound 12

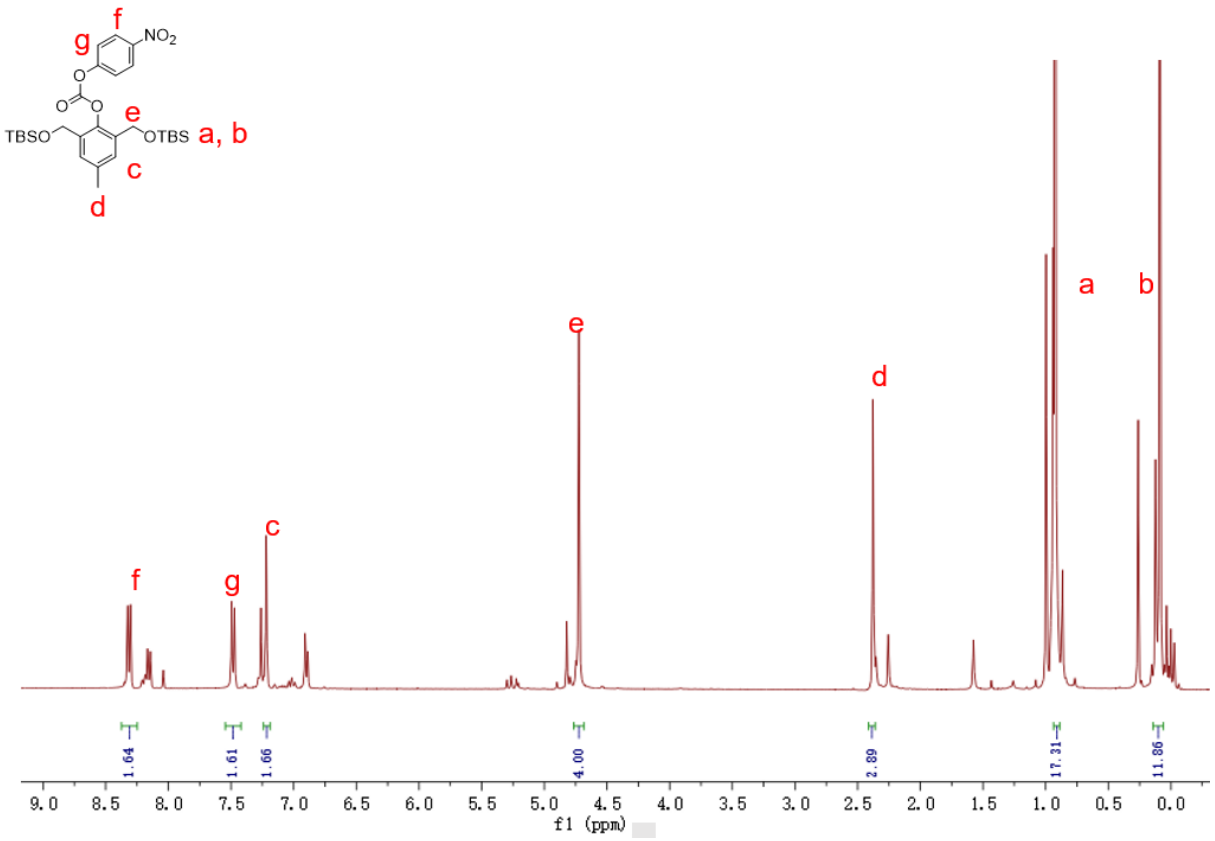


Figure S7. ^{1}H NMR of compound 13

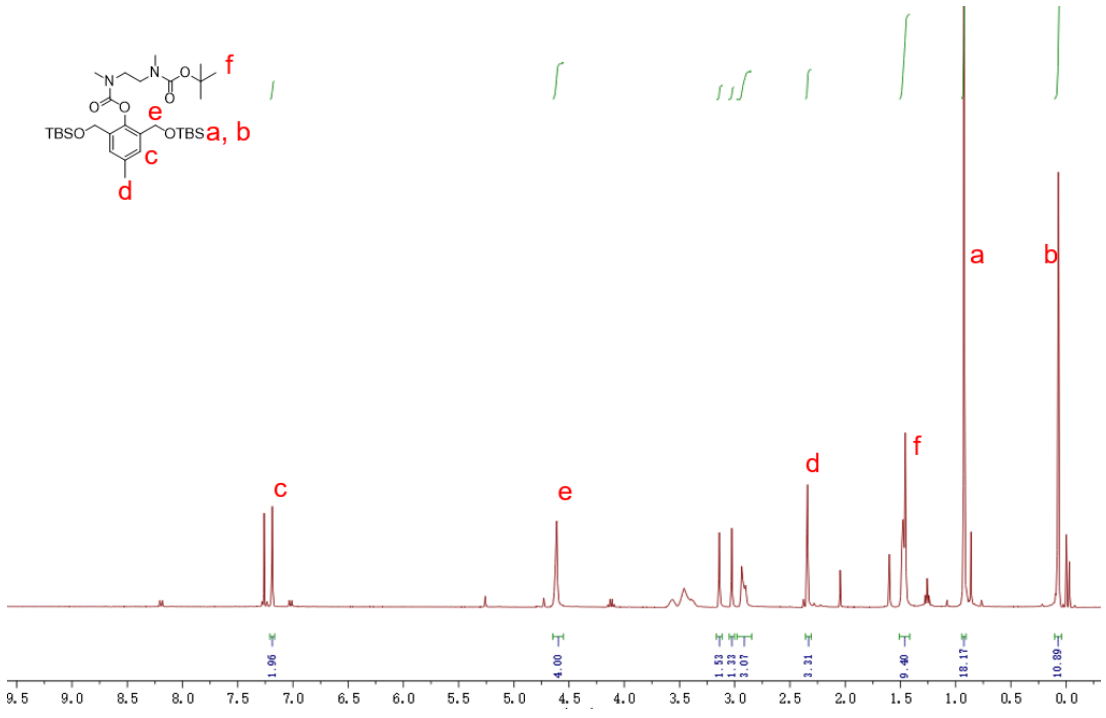


Figure S8. ^{1}H NMR of compound 14

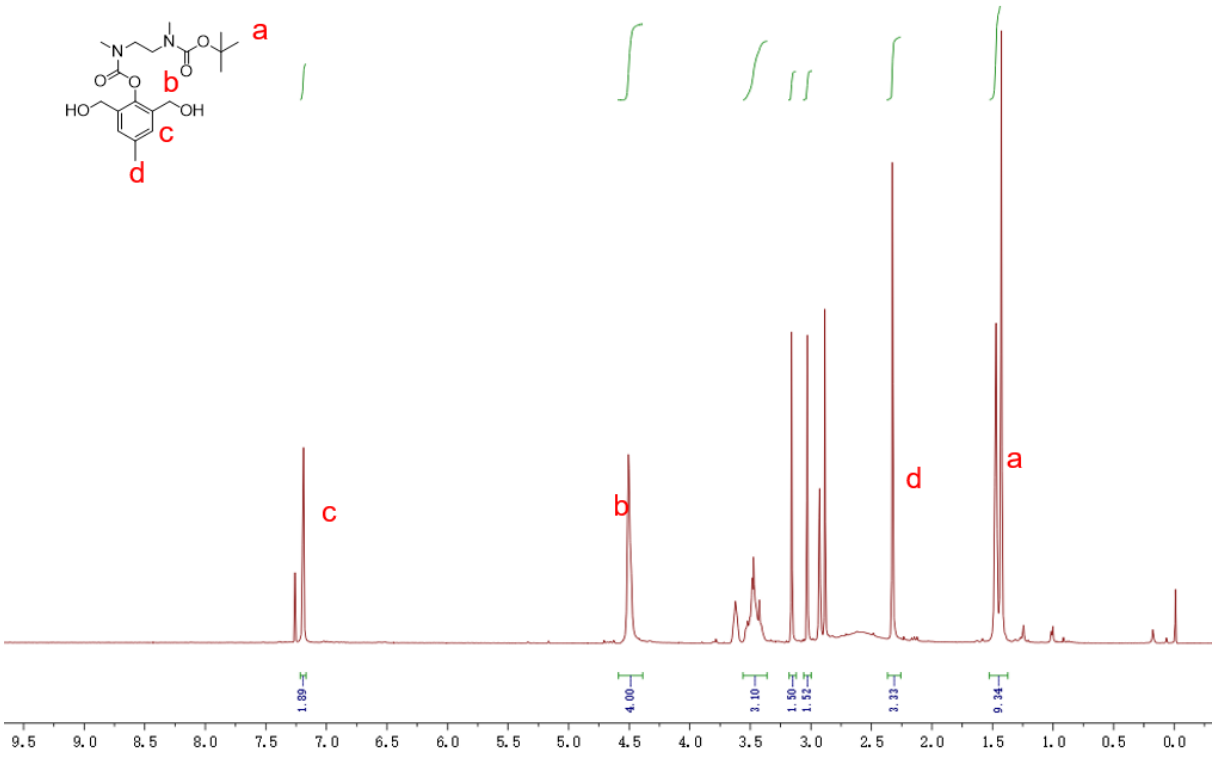


Figure S9. ^{1}H NMR of compound 15

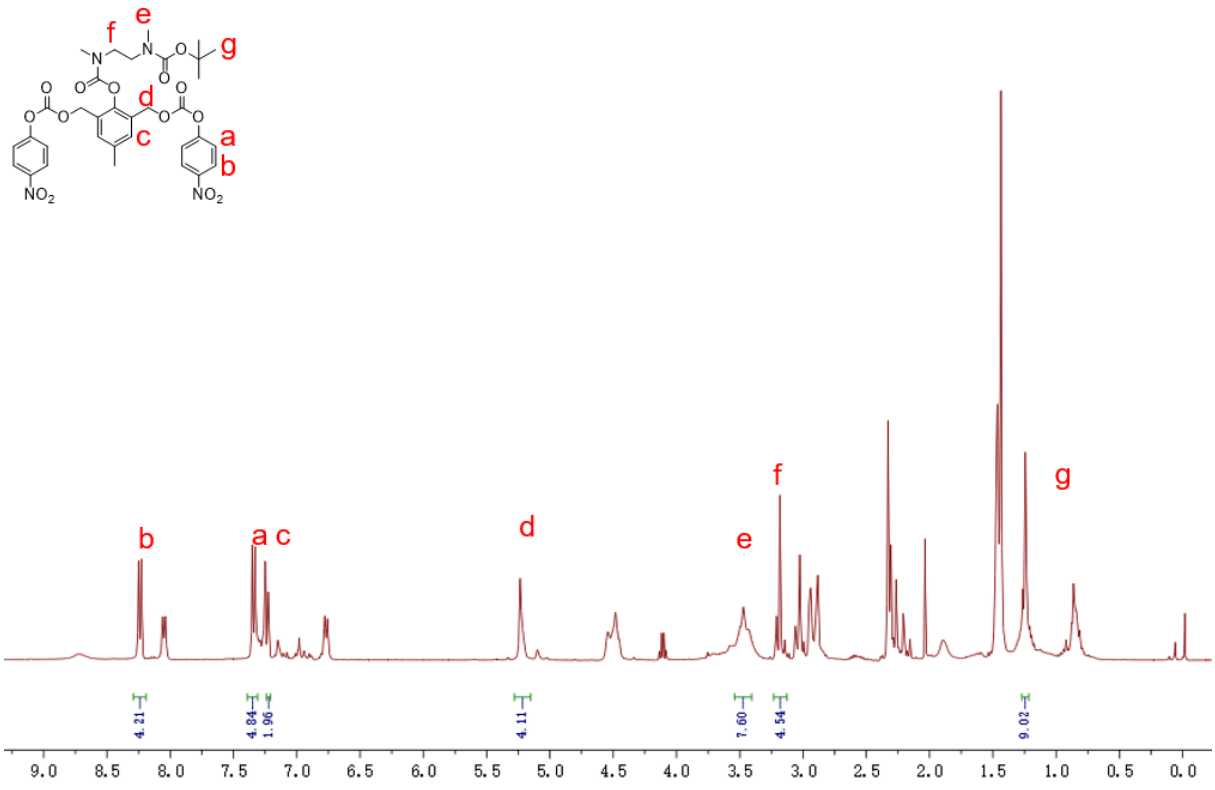


Figure S10. ^{1}H NMR of compound 16

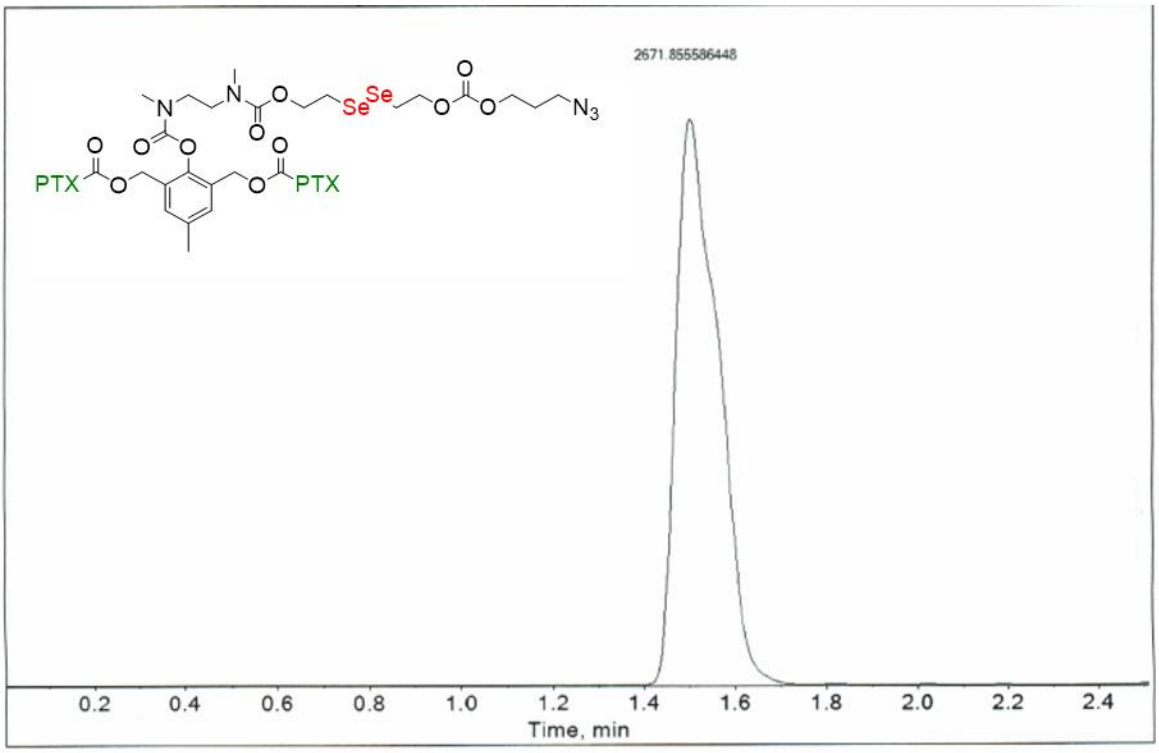


Figure S11. Q-TOF of compound 19

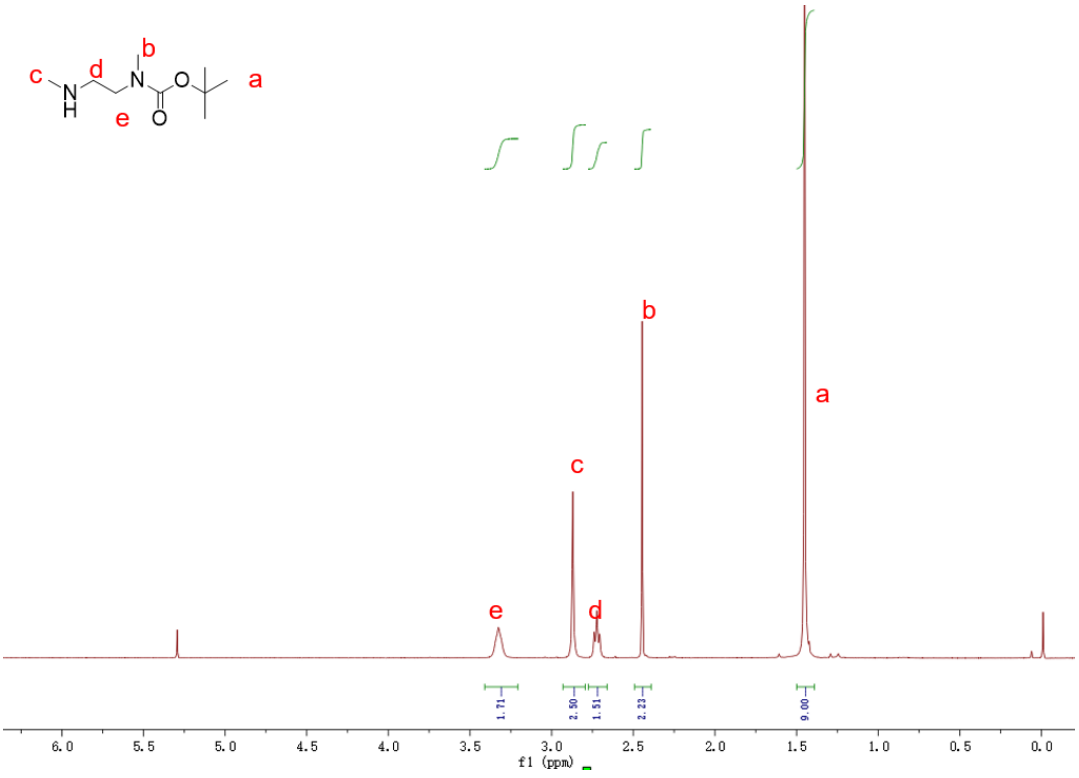


Figure S12. ^{1}H NMR of compound 20

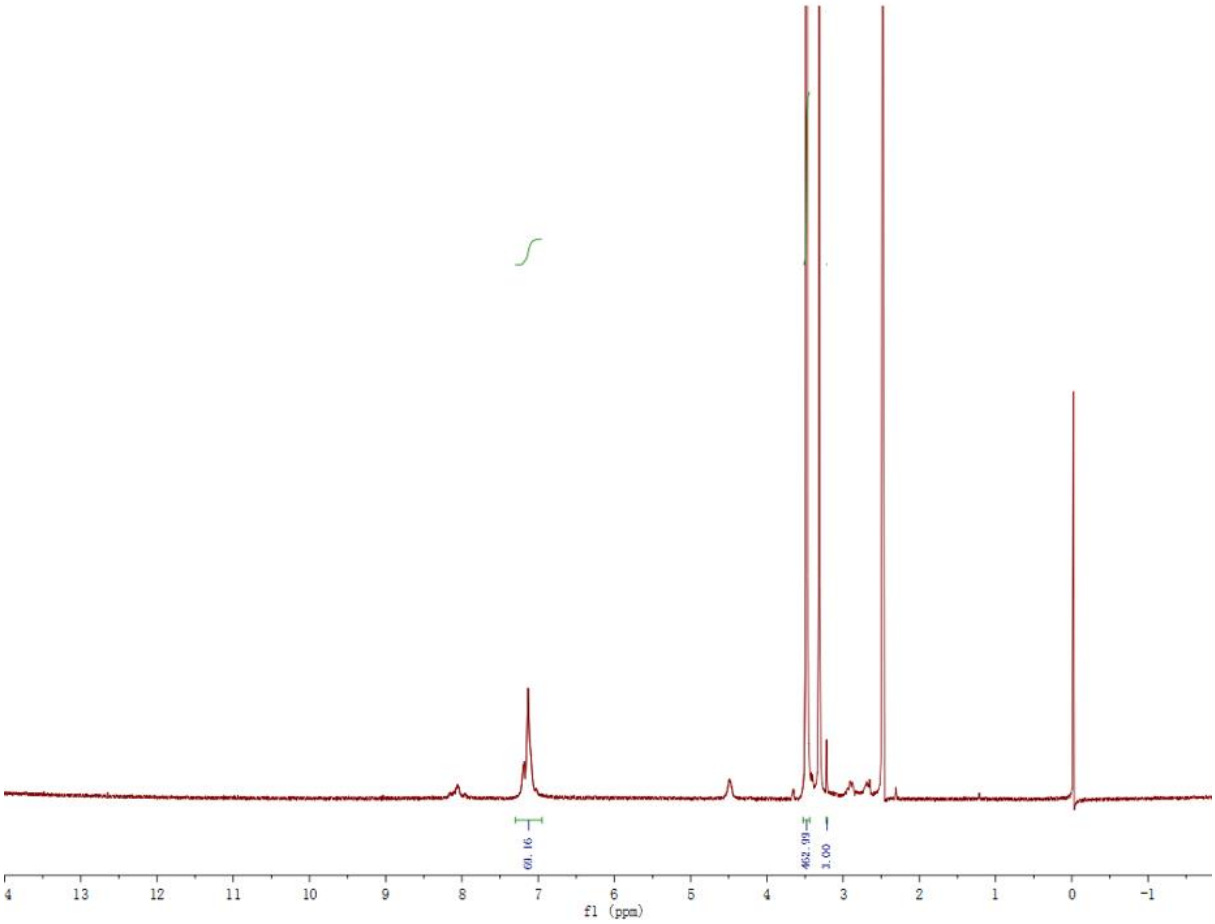


Figure S13. ^{1}H NMR of compound 21

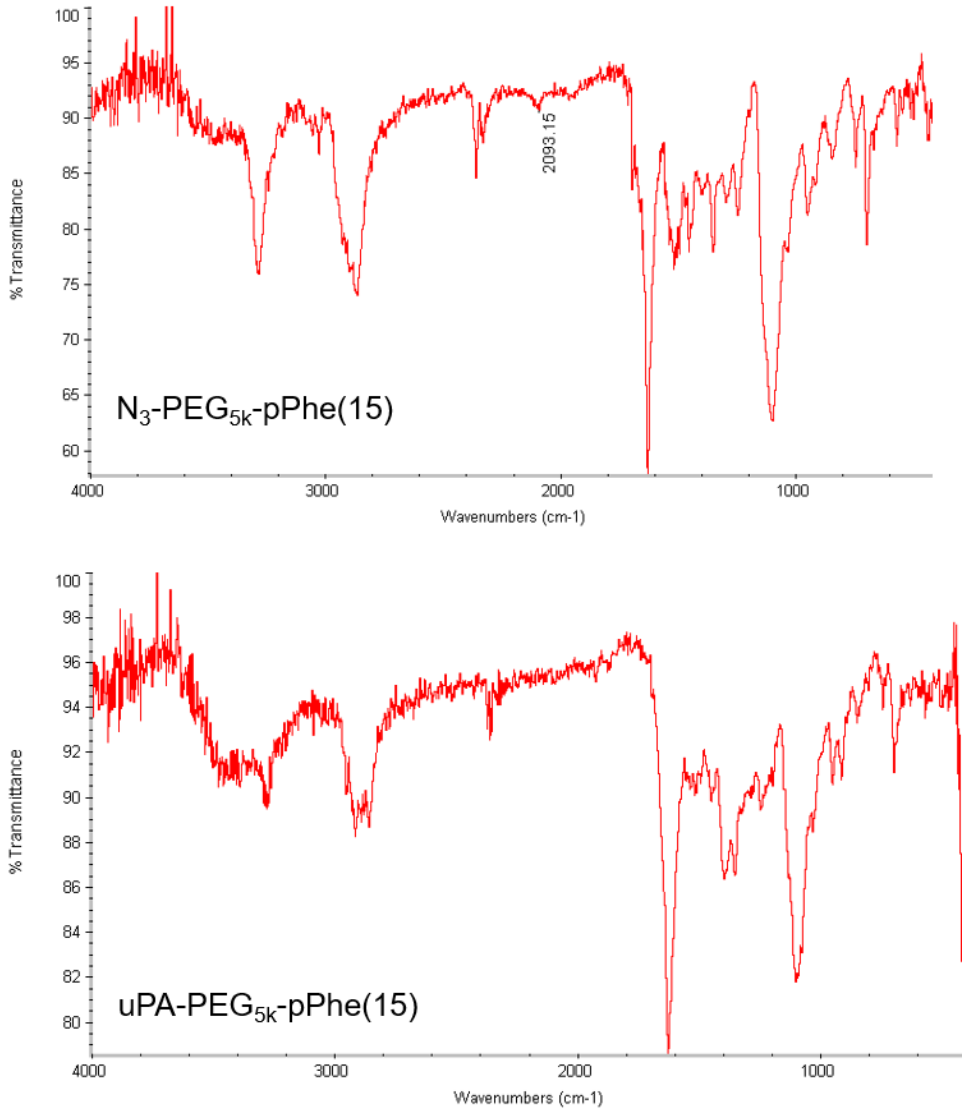


Figure S14. IR spectrum of N_3-PEG_{5k}-pPhe(15) and uPA-PEG_{5k}-pPhe(15)

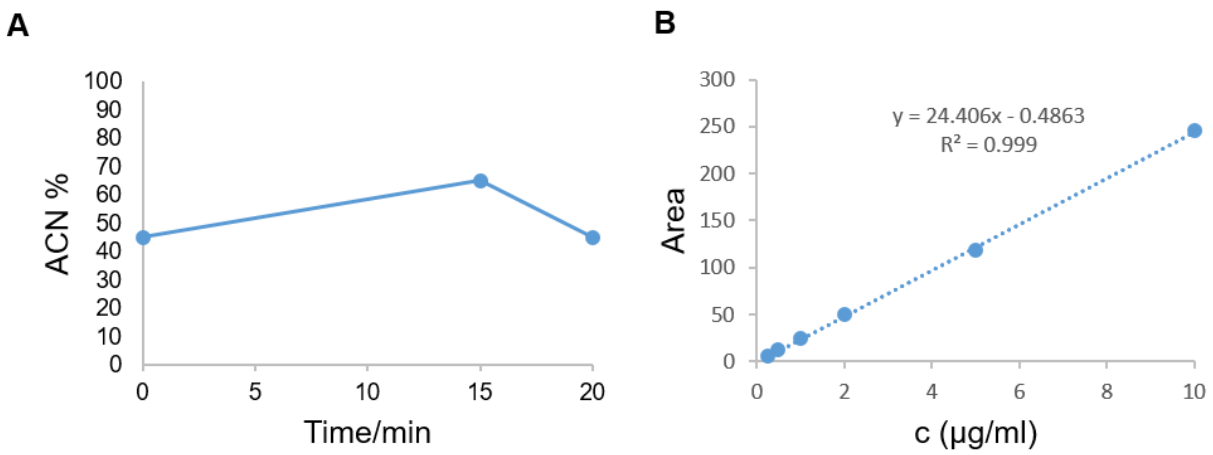


Figure S15. (A) HPLC method for PTX analysis. (B) HPLC Standard curve of PTX based on UV (227 nm) (R^2=0.999)

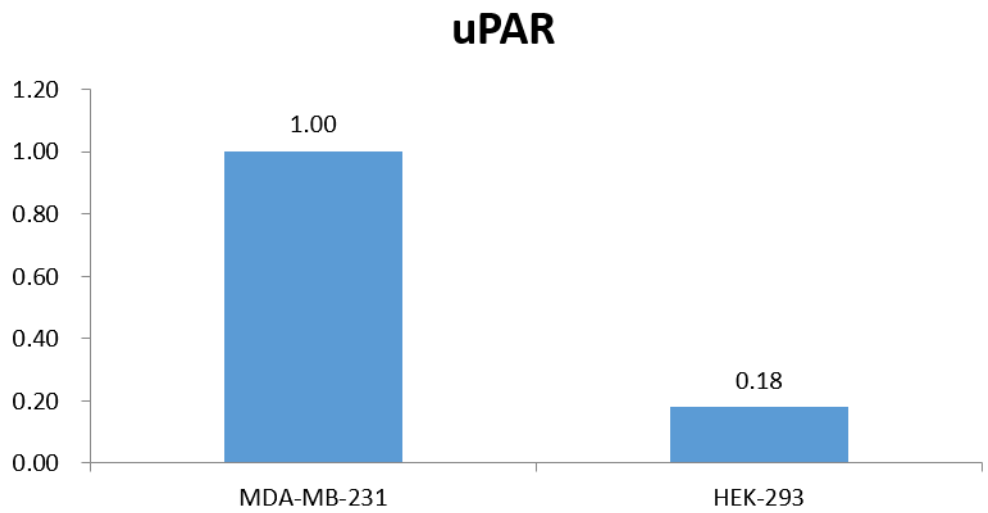


Figure S16. RNA expression levels of uPAR were evaluated by real-time RT-PCR in TNBC MDA-MB-231 cells and non-carcinomas HEK-293 cells.

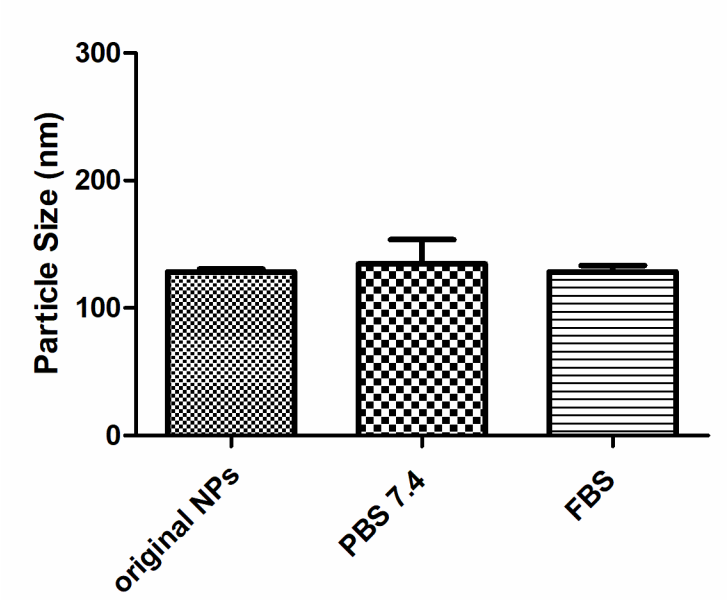
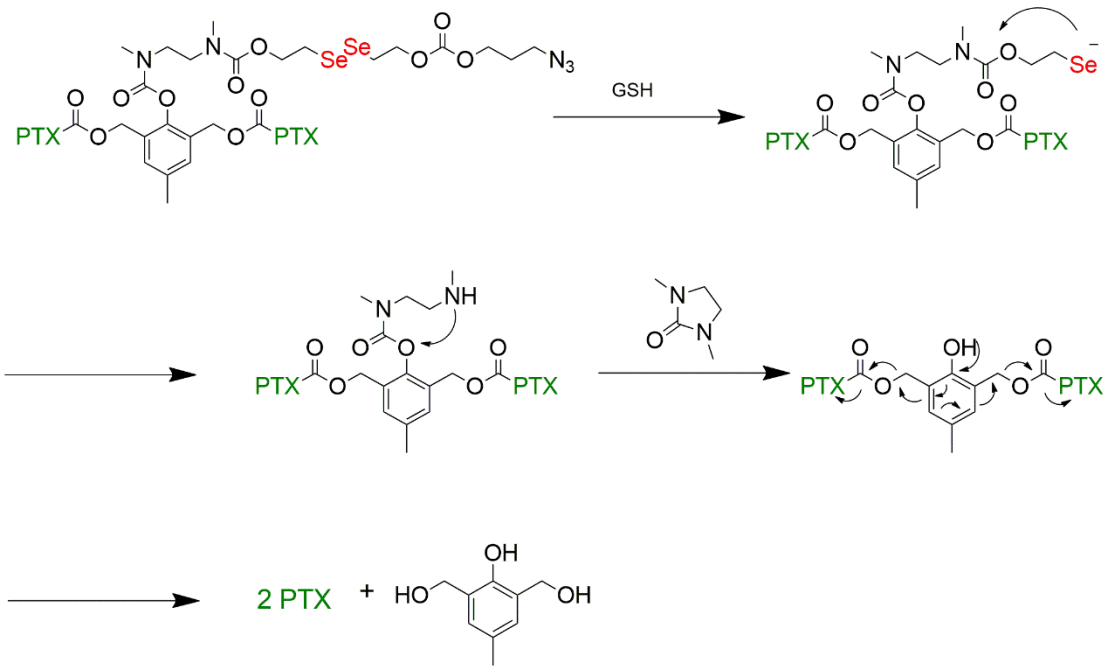


Figure S17. Particle size change of uPA-PTXD NPs incubated with PBS 7.4 and FBS for 12 h.



Scheme S2. Proposed release mechanism of GSH triggered drug release

Entry	600 rpm	Day 0		Day 7	
	PTXD:PEG5k-pPhe(5)	Size (nm)	PDI	Size (nm)	PDI
1	8:1	205.2	0.250	175.9	0.138
2	4:1	167.0	0.129	179.3	0.135
3	2:1	153.1	0.209	164.8	0.131
4	1:1	166.7	0.182	175.0	0.170
5	1:2	-	0.441	-	0.400
6	1:4	-	0.489	-	0.569
	PTXD:PEG5k-pPhe(10)	Size (nm)	PDI	Size (nm)	PDI
7	8:1	162.5	0.144	171.9	0.192
8	4:1	146.6	0.150	172.3	0.196
9	2:1	-	0.289	-	0.314
10	1:1	-	0.376	-	0.372
11	1:2	-	0.357	-	0.611
12	1:4	-	0.291	-	0.478
	PTXD:PEG5k-pPhe(15)	Size (nm)	PDI	Size (nm)	PDI
13	8:1	150.3	0.170	188.5	0.220
14	4:1	135.0	0.110	152.5	0.177
15	2:1	103.6	0.138	109.6	0.199
16	1:1	112.7	0.199	-	0.353
17	1:2	-	0.328	-	0.326
18	1:4	-	0.334	-	0.518
	PTXD:PEG5k-pPhe(20)	Size (nm)	PDI	Size (nm)	PDI
19	8:1	150.6	0.144	184.3	0.268
20	4:1	147.1	0.200	167.0	0.207
21	2:1	144.5	0.228	-	0.341
22	1:1	-	0.374	-	0.357
23	1:2	-	0.525	-	0.479
24	1:4	-	0.487	-	0.464

Table S1. Formulation of PTXD-Se with mPEG$_{5k}$-pPhe(n) *via* Nanoprecipitation