Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Developing Chemically Modified Redox-responsive Protein As Smart Therapeutics

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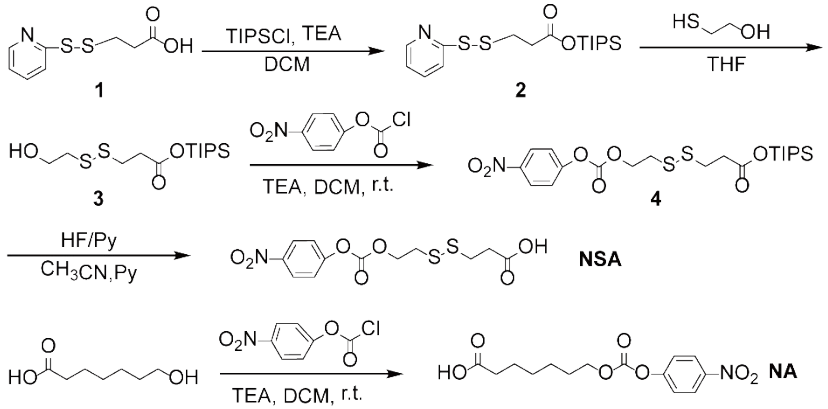
General

All chemical used for lipid synthesis and protein modification were purchased from Aladdin, TCI or Sigma-Aldrich and used as received. RNase A was purchased from Sigma, the ribonuclease activity was assayed according to previous report,[1] the intracellular GSH level was determined using DTNB assay according to previous report.[2-4] The lipids were synthesized and characterized according to our previous reports.[5]

Synthesis of NSA and NA



Scheme S1. Synthetic route of NSA and NA for protein modification.

Synthesis of **2**: 3-(pyridine-2-yldisulfanyl) propanoic acid **1** was synthesized following an early report [6]: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (d, *J* = 4.7 Hz, 1H), 7.67 (d, *J* = 8.1 Hz, 1H), 7.64 – 7.56 (m, 1H), 7.09 – 7.02 (m, 1H), 3.01 (t, *J* = 7.0 Hz, 2H), 2.77 (t, *J* = 7.1 Hz, 2H), 1.27 (dt, *J* = 14.8, 7.5 Hz, 3H), 1.04 (d, *J* = 7.5 Hz, 21H).To a solution of **1** (6.8g, 31.6 mmol) and triethylamine (TEA, 4.6 mL, 38.00 mmol) in CH_2Cl_2 (20 mL) was added chlorotriisopropylsilane (TIPSCl, 6.09g, 31.6 mmol) dropwise at 0 ^{o}C. After stirring at room temperature for 4 h, the reaction mixture was dried by rotary evaporator, then diluted with ethyl acetate (100 mL). The organic layer was separated, washed by saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under reduced pressure. Flash chromatography of the residue on silica gel using Petroleum ether /EtOAc (10/1) as the eluent gave **2** (11 g, 98 % yield) as colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (d, *J* = 4.7 Hz, 1H), 7.67 (d, *J* = 8.1 Hz, 1H), 7.64 – 7.56 (m, 1H), 7.09 – 7.02 (m, 1H), 3.01 (t, *J* = 7.0 Hz, 2H), 2.77 (t, *J* = 7.1 Hz, 2H), 1.27 (dt, *J* = 14.8, 7.5 Hz, 3H), 1.04 (d, *J* = 7.5 Hz, 21H). ESI-MS Calcd for $C_{17}H_{30}NO_2S_2Si$ $[M+H]^+$: 372.1, Found: 372.1.

Synthesis of **3**: To a solution of **2** (5 g,13.461 mmol) in THF (15 mL) was added 2-mercaptoethanol (1.26 g,16.15 mmol) dropwise, the mixture stirring at room temperature overnight. The reaction mixture was concentrated in vacuum. The residue was purified on a silica gel column using petroleum/EtOAc (4/1) as the eluent to give **3** (3.87 g, 85%) as a colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.89 (s, 2H), 2.94 (t, *J* = 6.9 Hz, 2H), 2.87 (t, *J* = 5.6 Hz,

2H), 2.80 (t, *J* = 6.8 Hz, 2H), 2.12 (s, 1H), 1.29 (dd, *J* = 14.6, 7.5 Hz, 3H), 1.08 (d, *J* = 7.3 Hz, 19H). ^{13}C NMR (75 MHz, DMSO) δ 171.16, 59.45, 41.03, 34.78, 33.39, 17.58, 11.32. ESI-MS Calcd for $C_{14}H_{30}O_3S_2SiNa$ $[M+Na]^+$: 361.1, Found: 361.0.

Synthesis of **4**: To a solution of **3** (1g, 2.96 mmol) in DCM (10 mL) was added triethylamine (0.5 mL, 0.36 g) dropwise with stirring. After stirring for 30 minutes, was added 4-Nitrophenyl chloridocarbonate (0.76 mg, 3.55 mmol) dropwise at 0 °C. The mixture stirred for 4 h, and extracted with CH_2Cl_2. The combined organic layer was dried over $MgSO_4$ and and concentrated under reduced pressure. Flash chromatography of the residue on silica gel using Petroleum ether /EtOAc (10/1) as the eluent gave **4**(1.2 g, 80%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (d, *J* = 8.8 Hz, 2H), 7.40 (d, *J* = 8.7 Hz, 2H), 4.54 (t, *J* = 6.5 Hz, 2H), 3.03 (t, *J* = 7.1 Hz, 2H), 2.96 (d, *J* = 6.9 Hz, 2H), 2.80 (t, *J* = 6.8 Hz, 2H), 1.31 – 1.24 (m, 3H), 1.07 (d, *J* = 7.3 Hz, 18H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.62, 155.55, 152.44, 145.60, 125.47, 121.93, 67.00, 36.66, 35.63, 33.93, 17.90, 12.02. MALDI-FTICR-MS Calcd for $C_{21}H_{33}NO_7S_2SiNa$ $[M+Na]^+$: 526.13654, Found: 526.13627.

Synthesis of **NSA**: To a solution of **4** (1 g, 1.98 mmol) in pyridine (3 mL) and acetonitrile (3 mL) was added HF/pyridine (70% weight, 5 mL) at 0 °C with stirring. After stirring for 30 minutes, the reaction was quenched by adding EtOAc (50 mL). The organic layer was separated, washed by water, saturated aqueous CuSO4 and brine, dried over $MgSO_4$, and concentrated. The crude product was purified on a silica gel column (eluent: hexanes/EtOAc = 1/1) to give 5 (412.2 mg, 60% yield) as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.28 (d, *J* = 8.8 Hz, 1H), 7.39 (d, *J* = 8.8 Hz, 1H), 4.54 (t, *J* = 6.4 Hz, 1H), 3.03 (t, *J* = 6.4 Hz, 1H), 2.97 (t, *J* = 6.9 Hz, 1H), 2.82 (t, *J* = 6.9 Hz, 1H).^{13}C NMR (75 MHz, $CDCl_3$) δ 177.74, 155.51, 152.44, 145.60, 125.47, 121.92, 66.94, 36.72, 33.90, 32.96. ESI-MS Calcd for $C_{12}H_{13}NO_7S_2Na$ $[M+Na]^+$: 370.0, Found: 370.0.

NA is synthesized in a similar procedure to that of **NSA**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.46 – 8.08 (m, 2H), 7.46 – 7.28 (m, 1H), 4.60 – 3.97 (m, 3H), 2.77 – 2.36 (m, 2H), 1.97 – 1.17 (m, 8H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.33, 158.36, 152.85, 145.95, 125.46, 121.95, 68.25, 33.68, 29.32, 28.64, 26.31, 22.17. ESI-MS Calcd for $C_{14}H_{17}NO_7Na$ $[M+Na]^+$: 334.1, Found: 334.1.

Chemical conjugation of RNase A with NSA and NA

RNase A-NSA and RNase A-NA were prepared by reacting RNase A with an excess amount of NSA or NA. Briefly, 2 mg RNase A was dissolved in 450 μL 0.1 M $NaHCO_3$ buffer solution (pH = 8.5). To above solution was added 20 μL DMSO solution containing 12 mg NSA or NA dropwise. The reaction mixtures were then stirred at room temperature for additional 8 h, followed by ultrafiltration purification using Amicon® Ultra Centrifugal Filters (MWCO = 10000, Millipore, MA). The fluorescent labeled FITC-RNase A-NSA was prepared by reacting RNase A-NSA with

twice weight amount of NHS-FITC for 2 h in 0.1 M $NaHCO_3$ buffer solution (pH = 8.5), the mixtures were diluted by 50 mM Tris (pH = 8.0) using Desalt column and protein were concentrated and stored at -20 ^oC.

Activation of RNase A-NSA by GSH

RNase A-NSA or RNase A-NA were incubated with GSH at 37 ^oC for 2h, 4h, 6 h, respectively, followed by dialysis against DEPC water to remove excessive GSH and then concentrated by ultrafiltration purification using Amicon® Ultra Centrifugal Filters (MWCO = 10000, Millipore, MA). The proteins were then subjected to MALDI-TOF characterization and ribonuclease activity assay. To verify the selective RNase A-NSA activation by GSH, RNase A-NSA (14 mM) was incubated with several main reductive substances of cellular concentration for 6 h at 37 ^oC, followed by RNase A activity assay as described above. The concentrations of the reductive species were listed below: 0.1 mM Cys; 10 μM Hcy; 4 mM GSH; 1 mM AA; 250 μM Na_2S (for generation of H_2S); 200 μM NADH.

Cell culture

HeLa, SiHa, 3T3 and HEK-293T cells were purchased from National Infrastructure of Cell Line Resource (China) and cultured in high-glucose dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 ^oC in the presence of 5% CO_2. For the intracellular delivery, the cells were sub-cultured and seeded in 48-well or 96-well plate 24 h prior to experiment.

Intracellular delivery of RNase A-NSA

Cells were seeded in 96-well plate at a density of 1×10^4 per well and treated with different lipid/protein nanocomplexes for 6 h before changing fresh culture medium, the cells were incubated for additional 42 h before MTT assay to measure cell viability. For CLSM imaging study, HeLa cells were treated with PBPA-O16B (5.0 μg/mL)/FITC-RNase A-NSA (250 nM) nanocomplexes for 6 h, then the cells were washed with cold DPBS twice before fluorescence imaging. The endosome was stained using 100 nM LysoTracker@Red according to the manufacturer's instruction.

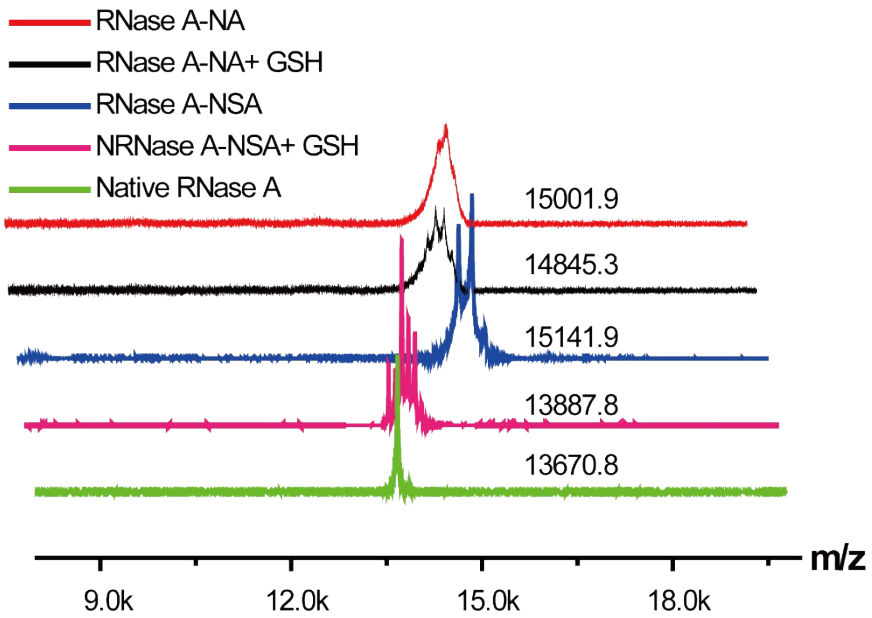


Fig. S1. Mass characterization of native RNase A and RNase A-NSA and RNase A with and without 4 mM GSH treatment.

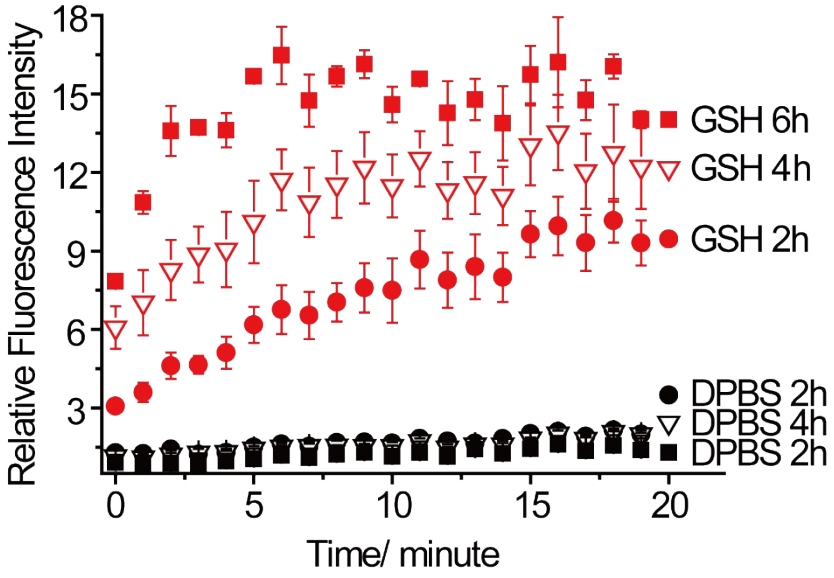


Fig. S2. Ribonuclease activity RNase A-NSA treated with 4 mM GSH at different time points. 14 mM RNase A-NSA was treated with GSH before enzyme activity assay.

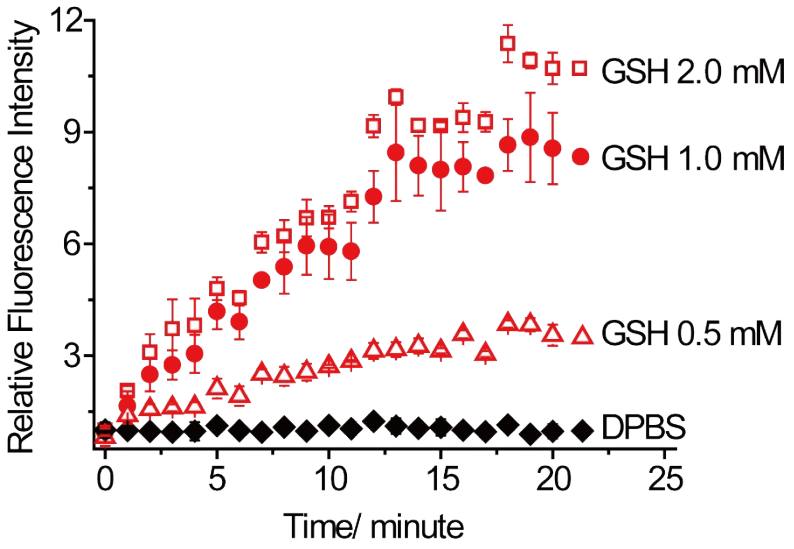
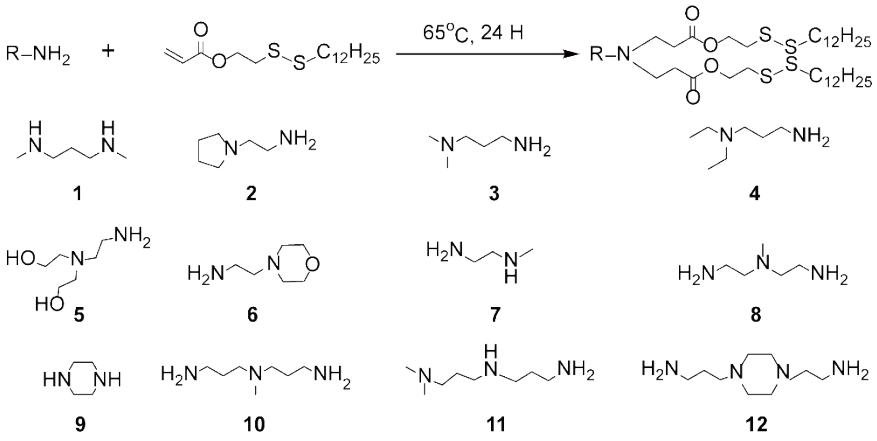
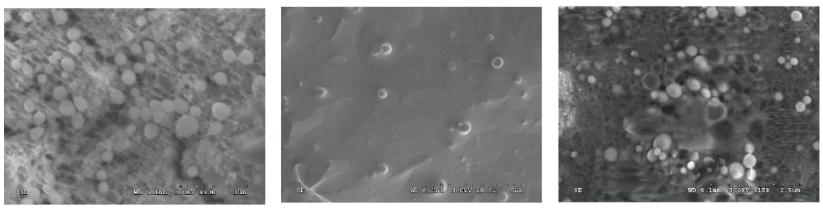


Fig. S3. Ribonuclease activity assay of RNase A-NSA treated with GSH at indicated concentration.



Scheme S2. The synthesis route and the chemical structure of amines used for RNase A delivery.

a)



PBPA-O16B | PBPA-O16B +RNase A-NA | PBPA-O16B +RNase A-NSA

b)

NPs	Size/ nm	Zeta potential/ mV
PBPA-O16B	195.20 ±7.79	0.80 ±0.48
PBPA-O16B+ RNase A-NSA	247.83 ±5.71	-7.98 ±0.87
PBPA-O16B+ RNase A-NA	237.37 ±10.92	-8.10 ±0.92

Fig. S4. (a) Diameter and zeta potential characterization of PBPA-O16B, PBPA-O16B/RNase A-NA and PBPA-O16B/RNase A-NA nanoparticles;(b) Cryo-SEM image of PBPA-O16B, PBPA-O16B/RNase A-NA and PBPA-O16B/RNase A-NA nanoparticles.

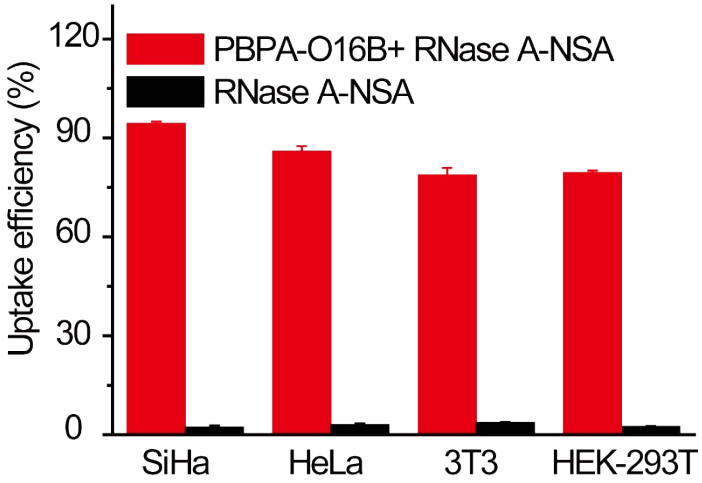


Fig. S5. Uptake efficiency of PBPA-O16B/RNase A-NSA nanoparticles on normal cell lines and cancer cell lines after treatment of 250 nM protein dose for 8 hours.

References

1 M. Wang, K. Alberti, S. Sun, C. L. Arellano and Q. Xu, *Angew. Chem.*, 2014, **53**, 2893-2898.

2 I. Rahman, A. Kode and S. K. Biswas, *Nat. Protoc.*, 2006, **1**, 3159-3165.

3 Y. S. Takeda, M. Wang, P. Deng, Q. Xu, *Bioeng. Transl. Med.,* 2016, **1**, 160-167.

4 M. Wang, J. A. Zuris, F. Meng, H. Rees, S. Sun, P. Deng, Y. Han, X. Gao, D. Pouli, Q. Wu, I. Georgakoudi, D. R. Liu, Q. Xu, *Proc. Natl. Acad. Sci. U. S. A.,* 2016, **113,** 2868-2873.

5 Y. Li, J. Bolinger, Y. Yu, Z. Glass, N. Shi, L. Yang, M. Wang, Q. Xu, *Bio. Sci.,* 2018, **7**, 596-606.

6 J. G. Sheppard, J. P. McAleer, P. Saralkar, W. J. Geldenhuys, T. E. Long, *Eur. J. Med. Chem.,* 2018, **143**, 1185-1195.