Supporting Information

Hydrogen Sulfide-Responsive Self-Assembled Nanogel

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1. General methods and instrumentation

All the reagents and solvents were purchased from commercial sources and used without further purification unless otherwise indicated. Silica gel flash column chromatography was performed on an automated CombiFlash® Rf 200 system with RediSep Rf prepacked silica columns. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian spectrometer (600 MHz). Chemical shifts (δ) are given in parts per million (ppm) relative to internal standards: $CHCl_3$ (δ_H = 7.26), $CDCl_3$ (δ_C = 77.0 for the central line of triplet) and $(CH_3)_2SO$ (δ_H = 2.50). The splitting patterns are reported as s (singlet), d (doublet), t (triplet), m (multiplet), dd (double of doublets) and br (broad). Coupling constants (*J*) are given in Hz. The ESI−MS experiments for compound characterization were conducted on an Agilent 6230 ESI-TOFMS high-resolution mass spectrometer. Nanogels were formulated by sonication (Misonix Sonicator, S-4000). DLS analyses and zeta potential measurement were conducted on a Malvern Zetasizer Nano ZS. TEM observations were carried out on a JEOL JEM-2010. Fluorescence measurements were conducted on a Horiba Jobin Yvon Fluorolog and a Hitachi F-2700 fluorescence spectrophotometer. Ultracentrifugation was performed on a Hitachi himac CS120GXII micro ultracentrifuge.

2. Abbreviations

SC-Dex = H_2S-responsive cholesterol-modified dextran, CBS = cystathionine β-synthase, CSE = cystathionine γ-lyase, CHP = cholesterol-modified pullulan, DCM = dichloromethane, DMAP = 4-dimethylaminopyridine, THF = tetrahydrofuran, DMSO = dimethyl sulfoxide, DMF = dimethylformamide, TBDPSCl = *tert*-butylchlorodiphenylsilane, TBAF = tetrabutylammonium fluoride, CDI = 1,1′-carbonyldiimidazole, NMR = nuclear magnetic resonance, TEM = transmission electron microscopy, NG = nanogel, DLS = dynamic light scattering, GSH = glutathione, FITC-BSA = fluorescein isothiocyanate labelled bovine serum albumin, RhB = rhodamine B.

3. Synthesis of SC-Dex

Compound 1

Compound **1** was prepared according to previously reported procedure. The spectral data was in agreement with previously reported data.

Compound 2

Compound **1** (165.0 mg, 0.92 mmol) and imidazole (99.7 mg, 1.46 mmol) were dissolved in DMF (5 mL) and added the solution of TBDPSCl (211.7 mg, 0.77 mmol) in DMF (10 mL) dropwise over 1 h. The reaction mixture was further stirred at room temperature for 4 h and then partitioned between EtOAc and brine. The organic layer was washed with brine three times, dried over $MgSO_4$, filtered and purified by silica gel chromatography (100% hexane to EtOAc/hexane = 7:3) to afford compound **2** (113.5 mg, 35%).

$C_{24}H_{27}N_3O_2Si$; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.75–7.70 (4 H, m), 7.49–7.39 (7 H, m), 7.32 (1 H, d, *J* = 6.0 Hz), 7.20 (1 H, t, *J* = 6.0 Hz), 4.88 (2 H, s), 4.77 (2 H, s), 2.20 (1 H, br), 1.13 (9 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 135.6, 134.9, 134.8, 134.2, 133.2, 129.9, 128.0, 127.9, 127.8, 125.8, 62.4, 61.6, 26.9, 19.3.

Compound 3

Compound **2** (71.4 mg, 0.17 mmol) and cholesteryl chloroformate (228.6 mg, 0.51 mmol) were dissolved in THF (3 mL). The solution was added DMAP (6.2 mg, 0.051 mmol) and then pyridine

(80 μL, 0.98 mmol) slowly. The reaction mixture was stirred at 60 ºC for 3 h, cooled, and concentrated under reduced pressure. The residue was diluted with EtOAc and washed with H_2O then brine. The organic phase was dried over $MgSO_4$, filtered and purified by silica gel chromatography (100% hexane to EtOAc/hexane = 1:9).

The purified intermediate was dissolved in THF (2 mL) and added TBAF trihydrate (108.4 mg, 0.34 mmol). The reaction mixture was stirred at room temperature for 2 h and then concentrated under reduced pressure. The residue was purified by silica gel chromatography (100% hexane to EtOAc/hexane = 3:7) to afford compound **3** as white solid (79.3 mg, 76%).

$C_{36}H_{53}N_3O_4$; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.36 (1 H, d, *J* = 6.0 Hz), 7.33 (1 H, d, *J* = 6.0 Hz), 7.16 (1 H, t, *J* = 6.0 Hz), 5.41–5.36 (1 H, m), 5.21 (2 H, s), 4.74 (2 H, s), 4.52–4.45 (1 H, m), 2.67 (1 H, br), 2.44–2.32 (2 H, m), 2.04–1.90 (3 H, m), 1.90–1.78 (2 H, m), 1.69–0.81 (33 H, m), 0.67 (3 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 154.3, 139.3, 136.6, 135.0, 130.1, 129.7, 129.2, 126.0, 123.0, 78.3, 65.7, 61.5, 56.7, 56.2, 50.0, 42.3, 39.6, 38.0, 36.9, 36.5, 36.2, 35.8, 31.9, 31.8, 28.3, 28.1, 27.7, 23.9, 22.9, 22.6, 21.1, 19.3, 18.8, 11.9; HRMS (ESI) calcd for $C_{36}H_{53}N_3NaO_4$: 614.3934, found: m/z 614.3927 $[M + Na]^+$.

Compound 4

Compound **3** (101.5 mg, 0.17 mmol) was dissolved in DCM (3 mL) and added CDI (82.4 mg, 0.50 mmol). The reaction mixture was stirred at room temperature for 3 h and then concentrated under reduced pressure. The residue purified by silica gel chromatography (100% hexane to EtOAc/hexane = 3:7) to afford compound **4** (122.4 mg, quant.).

$C_{40}H_{55}N_5O_5$; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.15 (1 H, s), 7.51–7.45 (2 H, m), 7.43 (1 H, s), 7.26 (1 H, t, *J* = 6.0 Hz), 7.06 (1 H, s), 5.52 (2 H, s), 5.41–5.36 (1 H, m), 5.27 (2 H, s), 4.54–4.47 (1 H, m), 2.44–2.33 (2 H, m), 2.03–1.91 (3 H, m), 1.90–1.76 (2 H, m), 1.69–0.81 (33 H, m), 0.67 (3 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 154.0, 148.3, 139.1, 138.1, 137.1 131.9, 131.8, 130.7, 130.1, 128.3, 126.2, 122.9, 117.0, 78.2, 66.0, 65.3, 56.6, 56.0, 49.9, 42.2, 39.6, 39.4, 37.9, 36.4, 36.1, 35.7, 31.8, 31.7, 28.2, 27.9, 27.5, 23.8, 22.8, 22.5, 19.2, 18.6, 11.8; HRMS (ESI) calcd for $C_{40}H_{56}N_5O_5$: 686.4281, found: m/z 686.4280 $[M + H]^+$.

SC-Dex

The solution of dextran (40 kDa, 100 mg, 0.62 mmol of anhydrous glucose unit) in DMSO (1.988 mL) was added the solution of compound **3** in DMSO (562 μL of 33 mM solution, 0.019 mmol), the solution of DMAP in DMSO (350 μL of 64 mM solution, 0.022 mmol) and pyridine (100 μL). The reaction mixture was stirred at 40 °C for 24 h and then precipitated out in Et_2O/EtOH (9 : 1). The precipitate was collected by centrifugation at 20000 rpm at 20 °C for 10 min and then redissolved in DMSO. The resulting DMSO solution was dialyzed against ddH_2O using 3.5 k regenerated cellulose membrane (Spectrum) and lyophilized to afford SC-Dex. The degree of chlosteryl substitution was determined by ^{1}H NMR analysis of SC-Dex in d_6-DMSO/D_2O (9 : 1).

4. ^{1}H NMR analysis of SC-Dex degradation

SC-Dex (5.55 mg) was dissolved in d_6-DMSO (1 mL). 50 μL of deuterated phosphate buffer (100 mM, pH 7.4) with or without 50 mM of H_2S was added into 450 μL of SC-Dex solution. The resulting mixture was incubated at 37 °C. ^{1}H NMR spectra were acquired at different time points.

5. Nanogel formulation

SC-Dex was suspended in ddH_2O (2 mg mL^{-1}). The solution was probe sonicated (S-4000, Misonix Sonicator) for 15 min in an ice bath and then centrifuged at 13400 rpm for 5 min. The supernatant was passed through a 0.22 μm PES syringe filter (Olympus) and lyophilized to afford SC-Dex NGs. DLS and TEM were used to characterize size and size distribution of NGs.

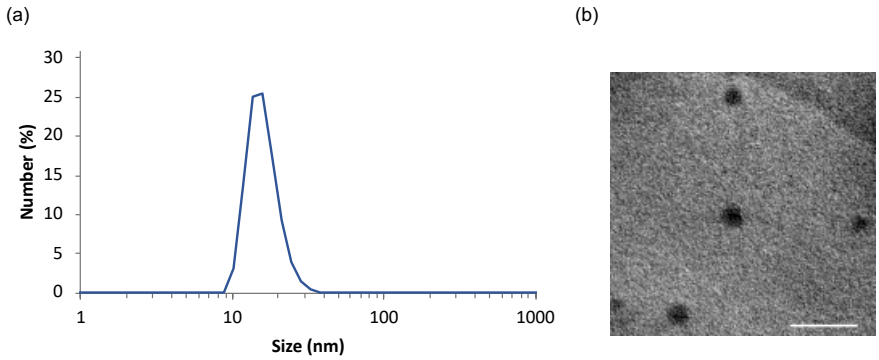


Figure S1. (a) Size distribution of SC-Dex NGs measured by DLS. (b) TEM image of SC-Dex NGs (scale bar = 100 nm).

6. Detection of hydrophobic nanodomains of SC-Dex NGs

The pyrene stock solution (1×10^{-4} M) was prepared by dissolving pyrene in EtOH. The solution of varying amount of SC-Dex NGs in ddH_2O (495 μL) were added the pyrene stock solution (5 μL) to give the final pyrene concentration of 1×10^{-6} M. The resulting solutions were stirred vigorously for 24 h. The ratio of the emission intensities at 374 nm (I_1) and 385 nm (I_3) was plotted against the concentration of SC-Dex NG.

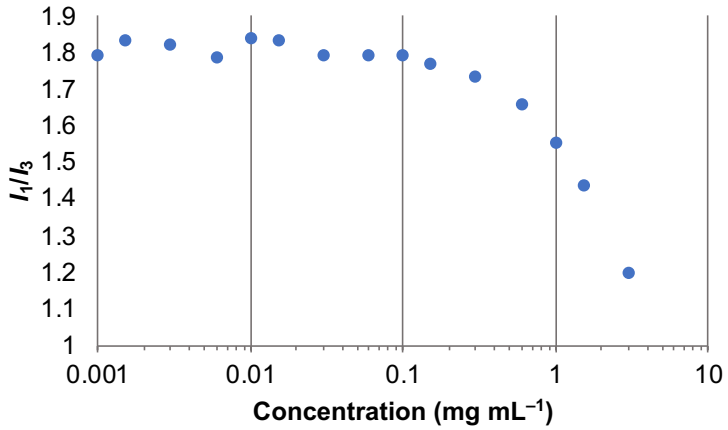


Figure S2. I_1/I_3 ratio of pyrene with varying concentration of SC-Dex NGs in ddH_2O.

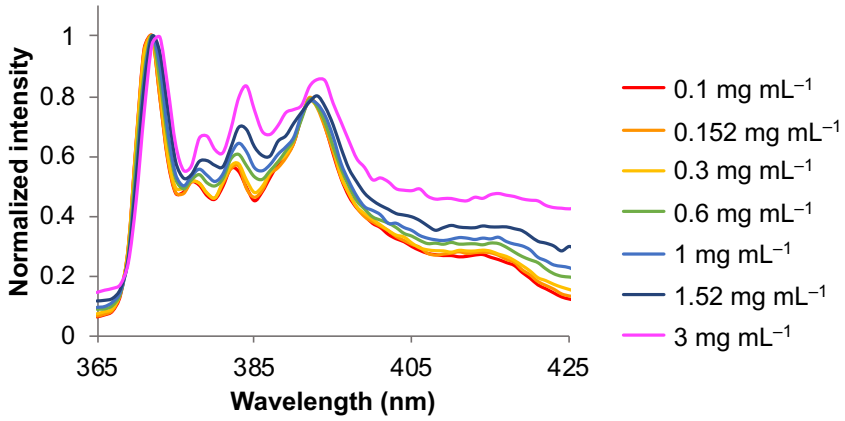


Figure S3. Fluorescence spectra of pyrene with varying concentration of SC-Dex NGs in ddH_2O.

7. Cytotoxicity studies

RAW267.4 cells (RIKEN Bio Resource) were seeded on a 96 well plate at the density of 5 000 cells per well. After 18 h incubation at 37 ºC, the cells were added varying concentrations of SC-

Dex NGs in triplicates and incubated at the same condition for another 24 h. The cells were then added cell-counting kit (Dojindo). The cell viability was quantified by measuring the absorbance at 450 nm using microplate reader.

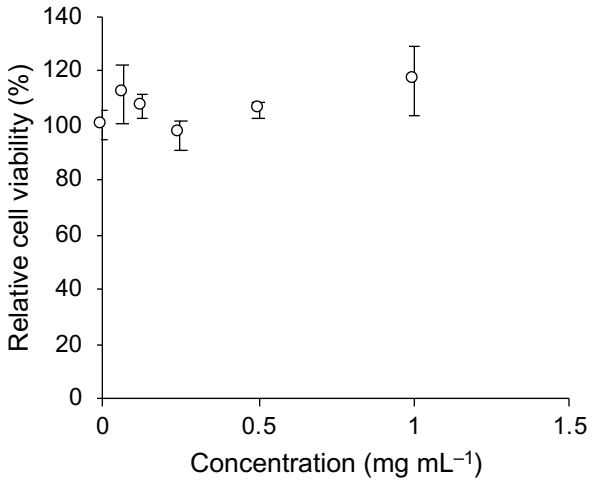


Figure S4. Cell viabilities of RAW267.4 cells after 24 h incubation with different concentrations of SC-Dex NGs.

8. Responsiveness study of SC-Dex NGs

SC-Dex NGs (0.5 mg mL^{-1}) were incubated in 50 mM phosphate buffer (pH 7.4), 50 mM phosphate buffer with 3 mM H_2S (pH 7.4) or 50 mM phosphate buffer with 10 mM GSH (pH 7.4). at 37 ^oC, and DLS analyses were performed at different time points. For TEM observation, the NG solution was concentrated and desalted by centrifugation (15 min, 13400 rpm) using Amicon Ultra-0.5 Centrifugal Filter (10 K, Millipore) after 48 h of incubation. The concentrated samples were diluted to approximately 1 mg mL^{-1} using ddH_2O. 5 μL of concentrated sample was dripped on a TEM copper grid, dried under vacuum, stained with 1.5% ammonium molybdate for 10 sec and imaged by TEM (JEM-2010, JEOL, Japan).

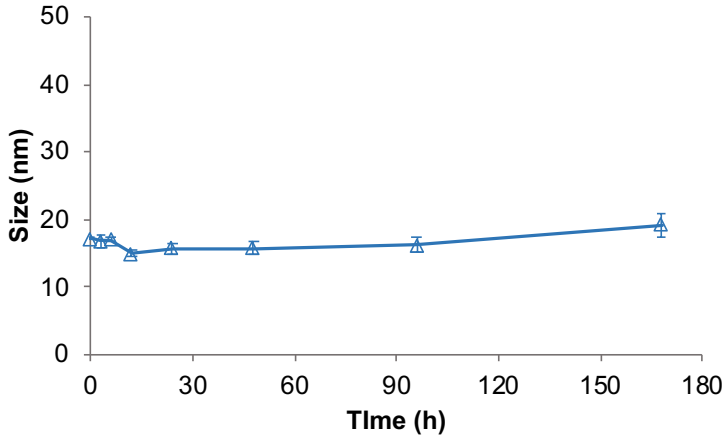


Figure S5. Size change of SC-Dex NGs in 50 mM phosphate buffer (pH 7.4) for 7 days.

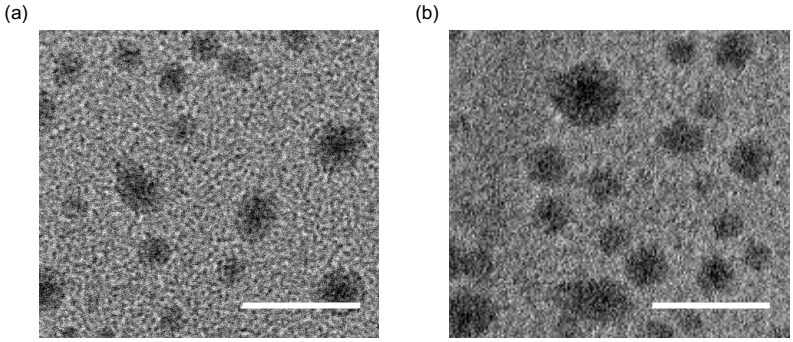


Figure S6. TEM images of SC-Dex NGs after incubation in (a) 50 mM phosphate buffer (pH 7.4) for 48 h and (b) 50 mM phosphate buffer with 10 mM GSH (pH 7.4) for 48 h (scale bar = 100 nm).

9. Encapsulation of FITC-BSA into RhB-labeled SC-Dex NGs

FITC-BSA was prepared using BSA (Sigma Aldrich) and FITC labeling kit (DOJINDO). RhB-labeled SC-Dex was prepared following the procedures reported in a previous literature.[1] RhB-labeled SC-Dex NGs were formulated following the same procedure stated previously.

RhB-labeled SC-Dex NGs (0.1 mg mL^{-1}) and FITC-BSA (0.01 mg mL^{-1}) were incubated in PBS at 37 °C for 24 h. The solution was then centrifuged at 120,000 g and 4 °C for 90 min to separate free FITC-BSA from the pellet, NG–protein complexes. The pellet was taken, and its fluorescence spectrum was recorded at an excitation wavelength of 495 nm to confirm the successful encapsulation of FITC-BSA into RhB-labeled SC-Dex NGs. The size and zeta potential of NG–protein complexes were characterized using Malvern Zetasizer Nano ZS. The complexation ratio

was calculated to be 50% by comparing the fluorescence intensity of fed FITC-BSA and free FITC-BSA, indicating that the weight ratio of SC-Dex NG and FITC-BSA within the complex is 20 : 1.

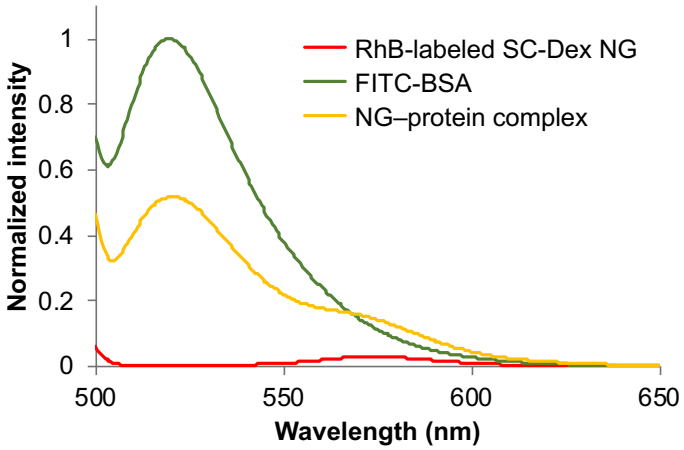


Figure S7. Fluorescence emission spectra of FITC-BSA, RhB-labeled SC-Dex NG and NG–protein complex at an excitation wavelength of 495 nm.

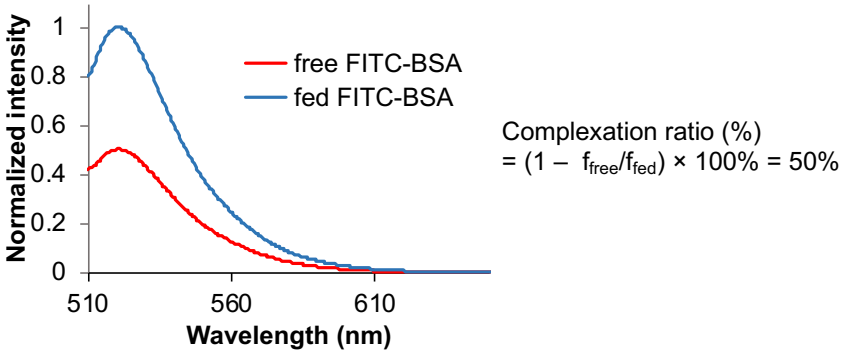


Figure S8. Fluorescence emission spectra of fed FITC-BSA and free FITC-BSA after protein encapsulation.

10. Release study of FITC-BSA from NG–protein complexes

NG–protein complexes (2 mL; NG, 0.1 mg mL^{-1}; FITC-BSA, 0.005 mg mL^{-1}) were incubated in PBS (pH 7.4) or PBS with 3 mM H_2S (pH 7.4) at 37 °C. At each time point, the incubation sample was centrifuged at 120,000 g and 4 °C for 90 min. The pellet was re-dispersed in the corresponding buffer and incubated at 37 °C until the next time point. The fluorescence spectra

of the supernatants were recorded at an excitation wavelength of 495 nm, and the emission intensity at 520 nm was used to calculate the amount of released FITC-BSA.

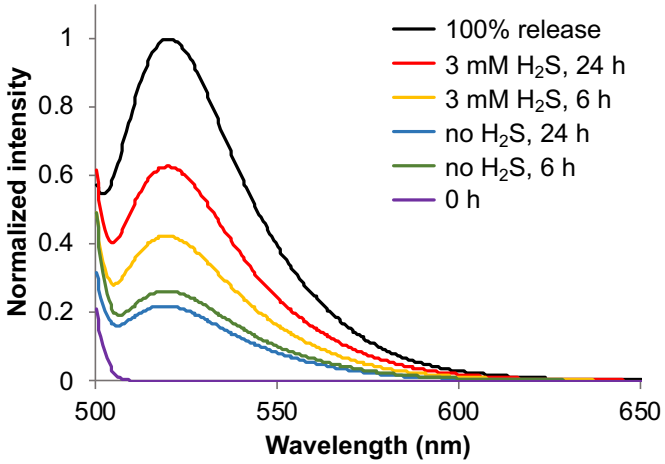
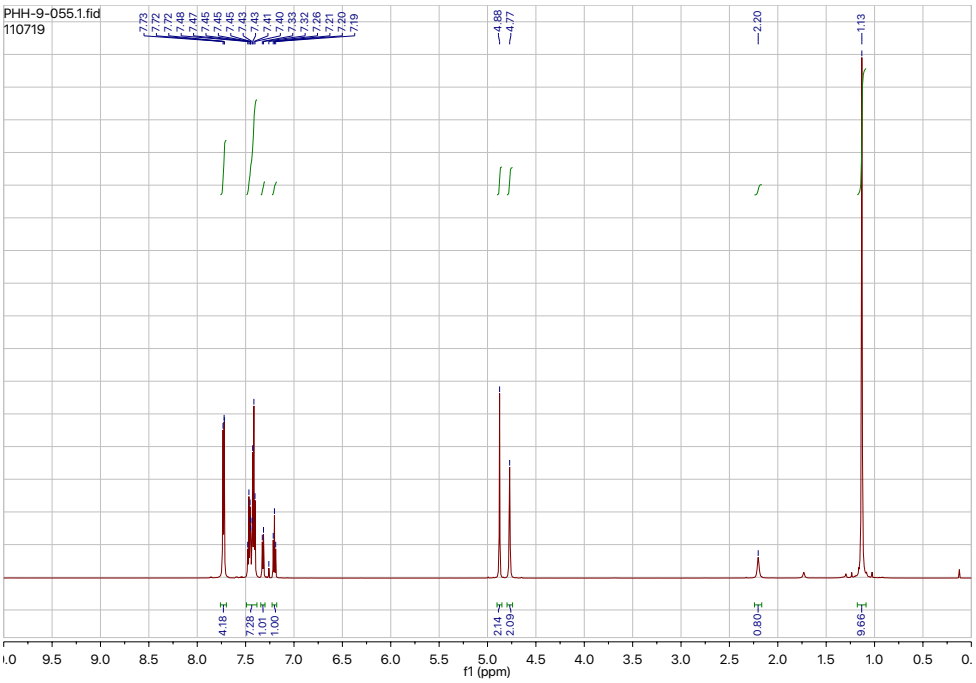
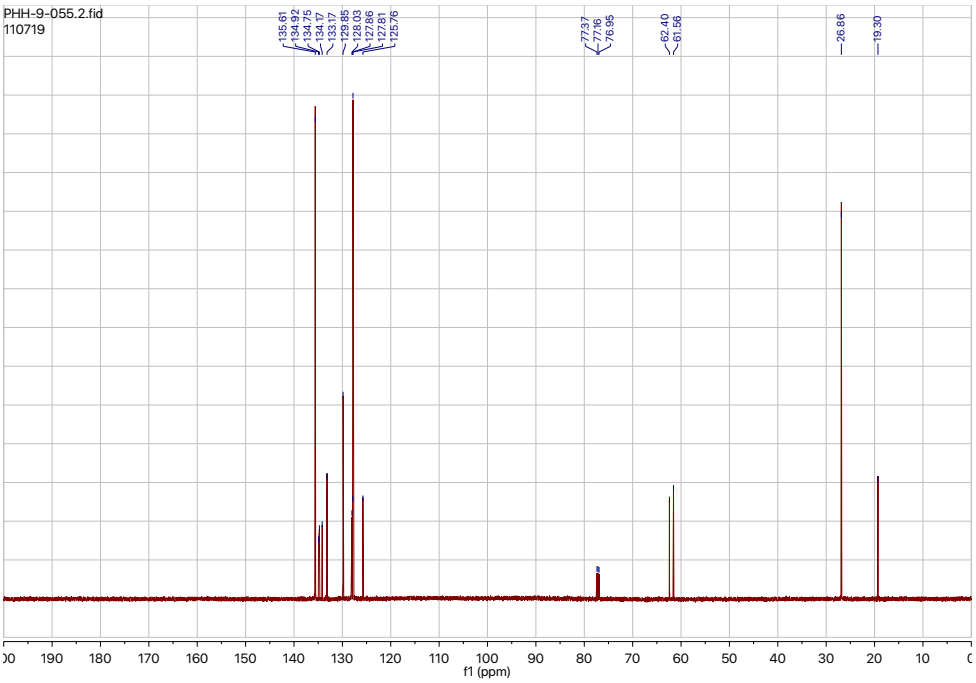


Figure S9. Fluorescence emission spectra of released FITC-BSA from different samples at an excitation wavelength of 495 nm.

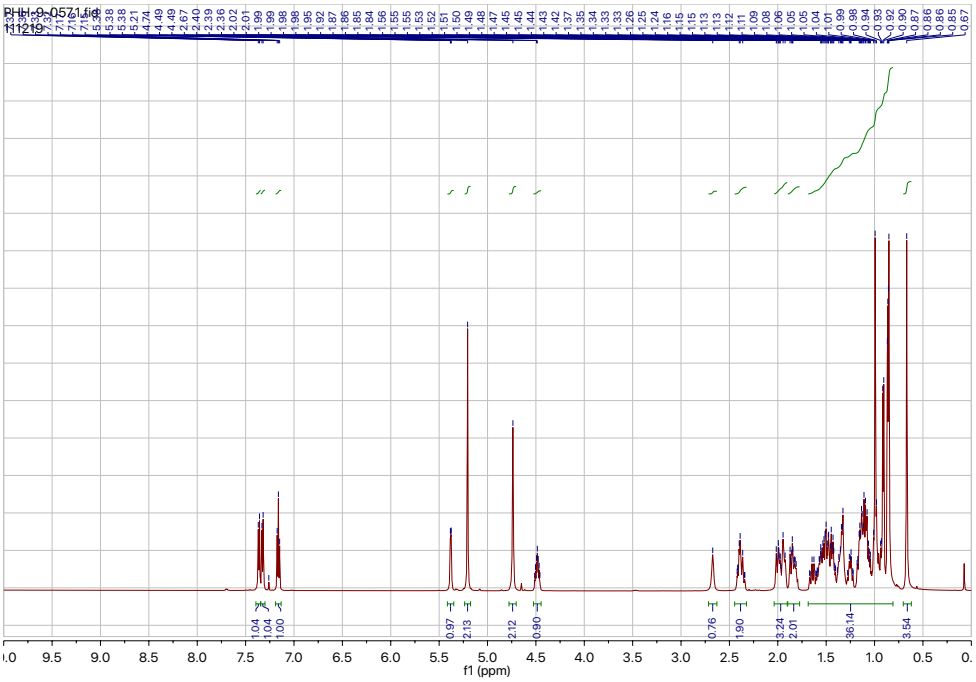
11. NMR spectra



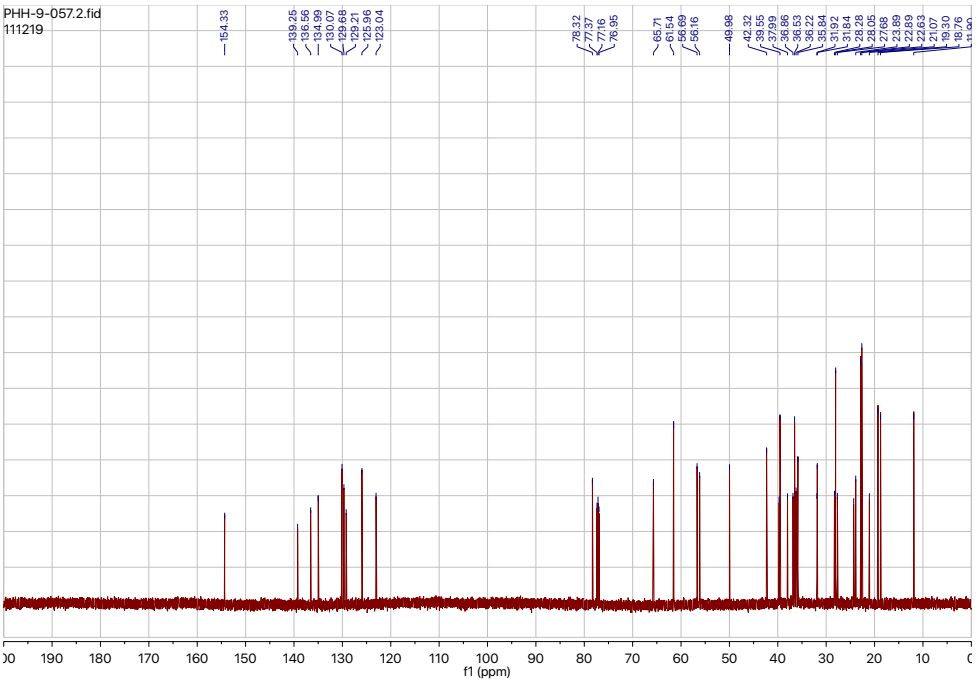
^{1}H NMR spectrum of compound **2** in $CDCl_3$



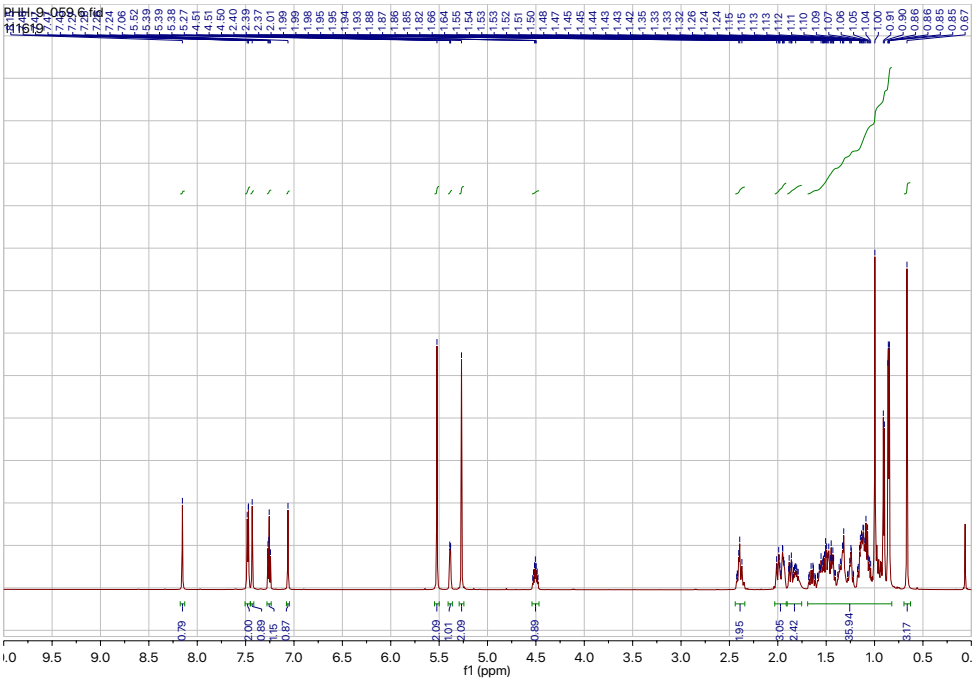
^{13}C NMR spectrum of compound **2** in $CDCl_3$



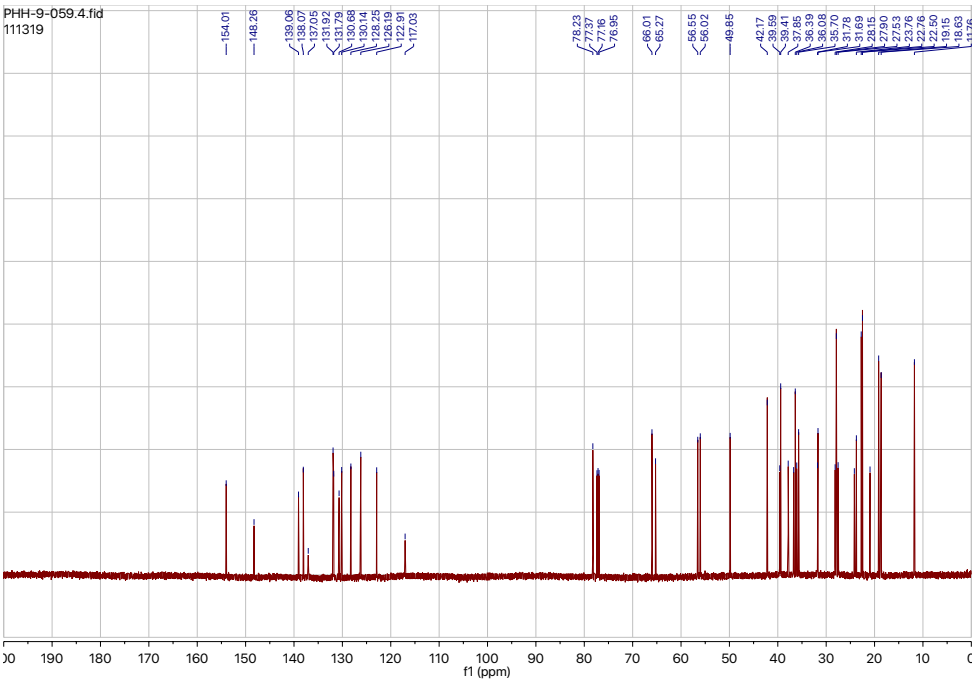
^{1}H NMR spectrum of compound **3** in $CDCl_3$



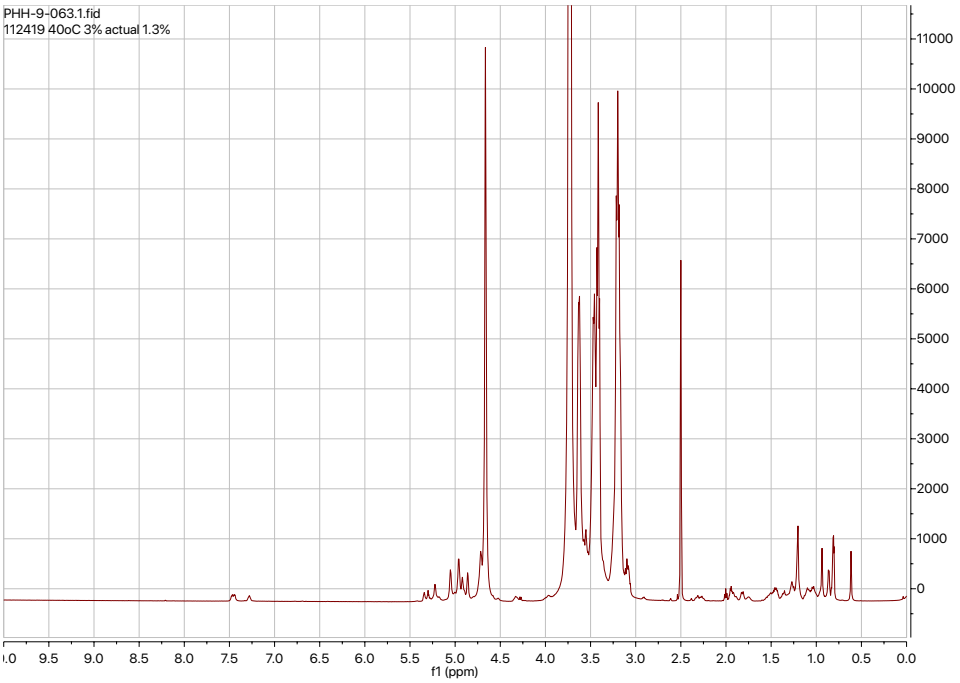
^{13}C NMR spectrum of compound **3** in $CDCl_3$



^{1}H NMR spectrum of compound **4** in $CDCl_3$



^{13}C NMR spectrum of compound **4** in $CDCl_3$



^{1}H NMR spectrum of SC-Dex in d_6-DMSO/D_2O (9 : 1)

12. Reference

(1) Kawasaki, R.; Sasaki, Y.; Akiyoshi, K. *Biochem. Biophys. Res. Commun.* 2017, 483, 147–152.