Electronic Supplementary Information

Redox-responsive Fluorescent AIE Bioconjugate with Aggregation Enhanced Retention Features for Targeted Imaging Reinforcement and Selective Suppression of Cancer Cells

Ling Yang,[a] Wen Fang,[a] Yuxiao Ye,[b] Zhengke Wang,*[a] Qiaoling Hu,[a] and Ben Zhong Tang*[c]

[a]MOE Key Laboratory of Macromolecular Synthesis and Functionalization, Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China.

[b]School of Materials Science and Engineering, New South Wales University, Sydney 2052, Australia.

[c]Department of Chemistry, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong 999077, China.

Experimental Section

Synthesis of disulfide-linked carboxyl chitosan (CS-ss-COOH)

The terminal carboxyl groups of 3,3'-dithiodipropionic acid (DTDP) were conjugated to the amino groups of CS to synthesize CS-ss-COOH.[1] Briefly, 500 mg of CS (primary amino group, 2.33 mmol) was dissolved in hydrochloric acid solvent (HCl, 50 mL, 0.1 mol L^{-1}). Separately, DTDP (5.2 mmol) was dissolved in 40 mL of methanol containing EDC (6.0 mmol) and NHS (6.0 mmol). The reaction proceeded under stirring for 30 min at 0 °C to form active esters of DTDP. The DTDP solution in methanol was then added into the CS solution dropwise. The pH of the mixture was adjusted to 4~5 with sodium hydroxide solution (NaOH, 1 mol L^{-1}) and then the solution stirred overnight at room temperature. Evaporation of organic solvents was then conducted under reduced pressure at 40 °C. To ensure the removal of unreacted DTDP, the reaction was subjected to a 5-fold dilution with deionized (DI) water and filtered. The mixture was dialyzed against DI water using a dialysis membrane [molecular weight cutoff (MWCO): 14,000 Da] for 72 h and lyophilized to obtain CS-ss-COOH. The final yield of CS-ss-COOH was around 80%.

Synthesis of TCSC

CS-ss-COOH (0.1 g) was added into a two-necked flask, evacuated under vacuum and flushed with dry nitrogen three times. DMSO (10 mL) was added into the flask, and the mixture was stirred at 60 °C for 24 h. TPE-ITC (18.5 mg) was added into the mixture, and stirred for 72 h. The TCSC product was washed with THF (10 mL) 3 times and ethanol (10 mL) twice, dialyzed by deionized water for 3 days, and lyophilized. The product was obtained as a yellow solid in 72% yield.[2]

Characterization of TCSC

^{1}H NMR spectrum was recorded on a Bruker Avance 600 MHz spectrometer. Fluorescence (FL) spectra were recorded on a PerkinElmer LS 55 Spectrofluorometer.

UV-vis spectra were taken on a UV-2550 spectrophotometer. Transmission electron microscopy (TEM) was measured on a JEM 1200EX transmission electron microscope. Average particle size and size distribution of the samples were measured by dynamic light scattering (DLS) with a particle size analyzer (BI-90Plus, Brookhaven Instruments Co. USA) at a fixed angle of 90° at room temperature.

Cell culture

Human cervical carcinoma cells (HeLa), human liver carcinoma cells (HepG2) and human breast adenocarcinoma cells (MCF-7) were used as cancer cells. Human fibroblast cells (FIB) were used as normal cells. Each type of cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a humidified environment containing 5% CO_2, respectively. The cells were precultured prior to the experiments until 90% confluence was reached.

Cytotoxicity evaluation

The cytotoxicity of TCSC against cancer cells (HeLa, HepG2, MCF-7) and normal cells (FIB) were evaluated by MTT assay, respectively. Briefly, the cells were seeded in 96-well plates at a density of 2.5×10^4 cells mL^{-1}, respectively. After 24 h incubation, the culture medium was replaced by a TCSC DMEM solution with concentration of 0.1 mg mL^{-1} and cultured at 37 °C for an additional 1 d, 3 d, and 5 d. The cells were washed with PBS buffer three times, and 200 μL of a freshly prepared MTT (0.5 mg mL^{-1}) solution in the culture medium was added into each well. They were then cultured at 37 °C for 4 h. The culture medium was discarded, and DMSO (200 μL) was used to dissolve purple formazan crystals. A BIORAD microplate model 550 was used to evaluate cell survival by measuring the absorbance at 570 nm. Triplicate experiments were performed each time, and cells incubated in non-TCSC DMEM culture medium were set as blank control groups for each cell type.

Intracellular distribution and FL intensity of TCSC NPs

The HeLa, HepG2, MCF-7 and FIB cells were seeded in 8-well plates at a density of 2.5×10^4 cells mL^{-1}, respectively. After 24 h incubation, the culture medium was replaced by a TCSC DMEM solution with concentrations of 0.1 mg mL^{-1} and cultured at 37 °C for an additional 24 h. After being washed with PBS buffer three times to remove the free particles, the cells were stained with MitoTracker® Red (100 nM) for 0.5 h at 37 °C in dark. CLSM images of the stained cells were taken on a confocal laser scanning microscopy (ZEISS LSM 780). TCSC probe and MitoTracker® Red were excited at 405 and 561 nm, respectively. Average FL intensity per area for FIB, HeLa, HepG2, MCF-7 cells were then analyzed by Image J software. At least triplicate statistics were performed each time to compute the average.

Cellular imaging and tracing

Two portions of living HeLa and FIB cells (1×10^5 cells mL^{-1}) were grown overnight on a cover slide in the 35 mm petri dishes, respectively. The living cells were stained with a TCSC solution (0.1 mg mL^{-1}). After certain period of incubation, the dishes were washed with PBS buffer for three times, and fluorescent images of the stained cells were taken on a fluorescence microscope (Olympus 1X81 Japan), excitation: 330–380 nm, exposure time: 200 ms.

Quantitative analysis of TCSC NPs cellular uptake and intracellular FL intensity

Firstly, a series of TCSC DMEM solutions with varied concentration were tested by UV-2550 spectrophotometer to obtain the ultraviolet standard curve. Secondly, HeLa and FIB cells were seeded in 24-well plates. To realize lower TCSC uptake for HeLa cells, we seed HeLa cells in a density (5×10^4 cells mL^{-1}) lower than FIB cells (6.5×10^4 cells mL^{-1}) , respectively. After 24 h incubation, all the culture medium was replaced by a TCSC DMEM solution (0.1 mg mL^{-1}) and cultured at 37 °C for an additional period. After incubation, the culture supernatants of each well were collected and measured by

UV-2550 spectrophotometer. The engulfed amounts of TCSC NPs were determined with standard curve method in ultraviolet spectrophotometry. Relative ratio of cellular uptake was expressed by the ratio of cellular uptake amount for HeLa and FIB, Cell $Uptake_{HeLa}$ / Cell $Uptake_{FIB}$, with the same length of incubation time. Finally, the cells were trypsinized, washed and collected for the measurement of FL intensity. Relative FL Intensity was expressed by the ratio of FL intensity for HeLa and FIB, FL $Intensity_{HeLa}$ / FL $Intensity_{FIB}$ with the same length of incubation time.

Cell apoptosis detection

HeLa and FIB cells were seeded in 24-well plates at a density of 1×10^5 cells mL^{-1}, respectively. After 24 h incubation, the culture medium was replaced by TCSC DMEM solution (0.1 mg mL^{-1}) and cultured at 37 °C for an additional 1 d, 3 d, and 5 d. The cells were washed with PBS buffer three times, and then trypsinized, centrifuged and gently washed with PBS buffer before staining. The collected cells were incubated with FITC Annexin V® in a buffer containing propidium iodide (PI) and analyzed by flow cytometry (BD FACSCalibur Flow). HeLa and FIB cells without TCSC solution treatment were cultured as blank control groups and triplicate experiments were performed each time. Untreated cells were primarily FITC Annexin V® and PI negative, indicating that they were viable and not undergoing apoptosis. The relative fold of apoptosis was expressed by the ratio of the percentage of apoptotic cells (Q2+Q3) incubated with TCSC to that of the corresponding blank control groups. Analogously, the relative fold of necrosis was the ratio of the percentage of apoptotic and dead cells (Q1+Q2+Q3).

Suplementary Figure & Table

Table S1. Integration information from the ^{1}H NMR spectrum of TCSC

Protons of different units	δ (ppm)	Integration	Relative percent of units
-$CHNH_2$	3.0-3.3	1	100%
-CH_2CH_2-	2.5-2.9	1.43	17.88%
-Ar(Batch A)	7.0-7.25	1.64	8.63%
-Ar(Batch B)	7.0-7.25	0.98	5.16%
-Ar(Batch C)	7.0-7.25	0.41	2.16%

Setting the total repeating sugar ring units in chitosan as 100%; degree of substitution (D.S.) values and degree of labeling (D.L.) values were calculated based on the data shown in Table S1 using the following equations:

D.S. = [(Integration(-CH_2CH_2-)/8)/(1/1)]*100%

D.L. = [(Integration(-Ar)/19)/(1/1)] *100%

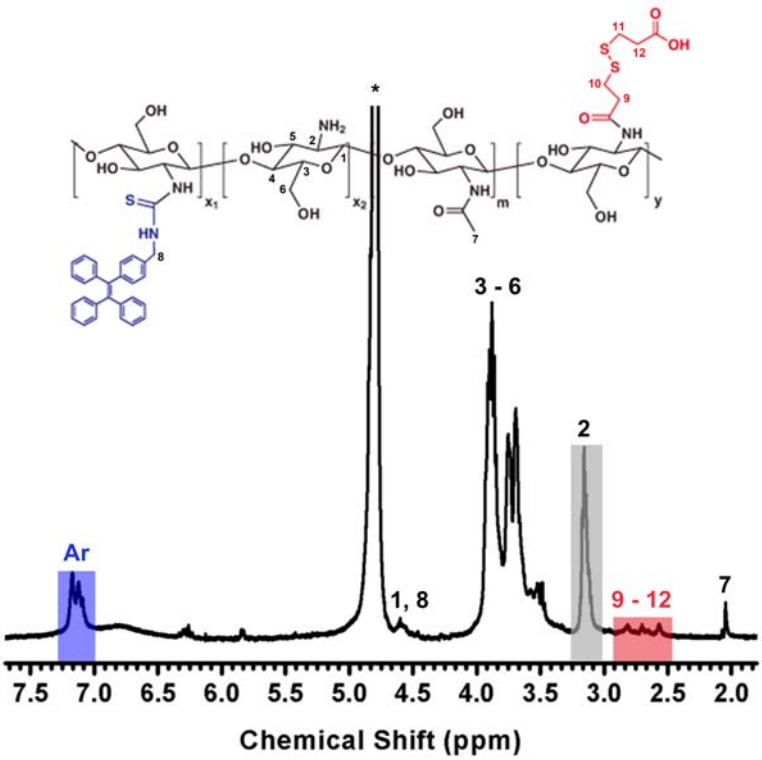

Fig. S1. ^{1}H NMR spectra of TCSC in D_2O

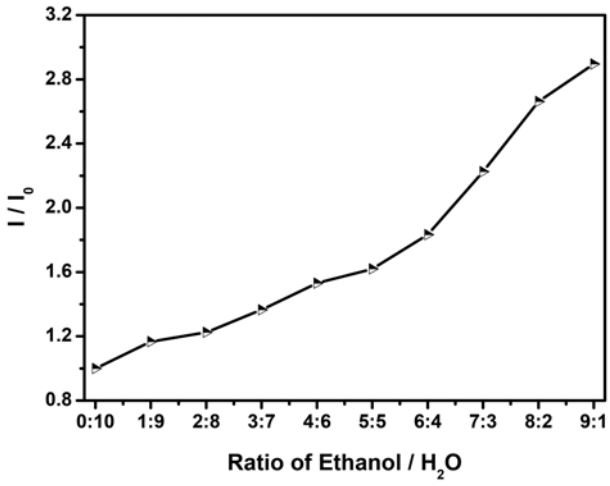

Fig. S2. The relationship between I/I_0 and the ratio of Ethanol / H_2O, where I_0 and I are the fluorescence intensities of TPE-NSCS in the absence and presence of ethanol, respectively.

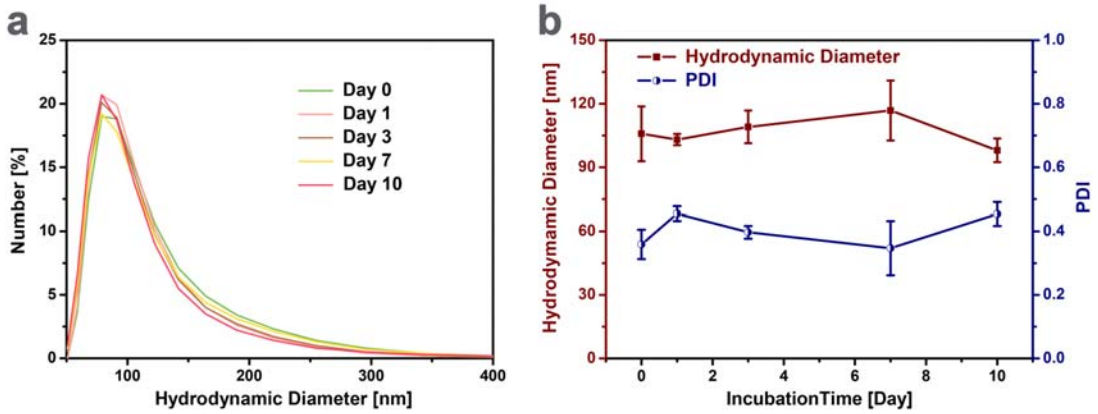

Fig. S3. Excellent water dispersability and nano-size stability of TCSC. (a) Hydrodynamic diameter distribution; (b) Number mean diameter and PDI fluctuation of 0.1 mg mL^{-1} TCSC in DMEM with different incubation time.

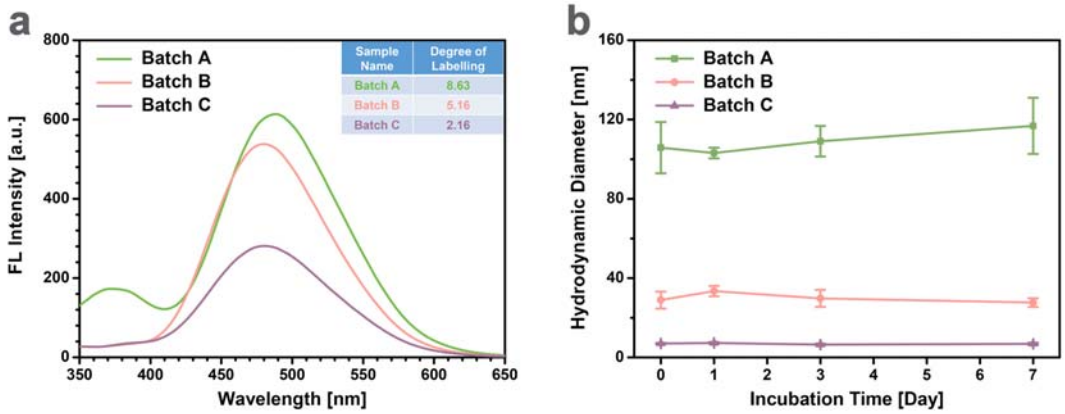

Fig. S4. The AIE properties and nano-size stability of TCSC with varied degree of labelling. (a) Fluorescence spectrum of the TCSC with different degree of labelling. Inset: degree of labelling of different samples; (b) Number mean diameter fluctuation of varied TCSC samples (0.1 mg mL^{-1}) in DMEM with different incubation time.

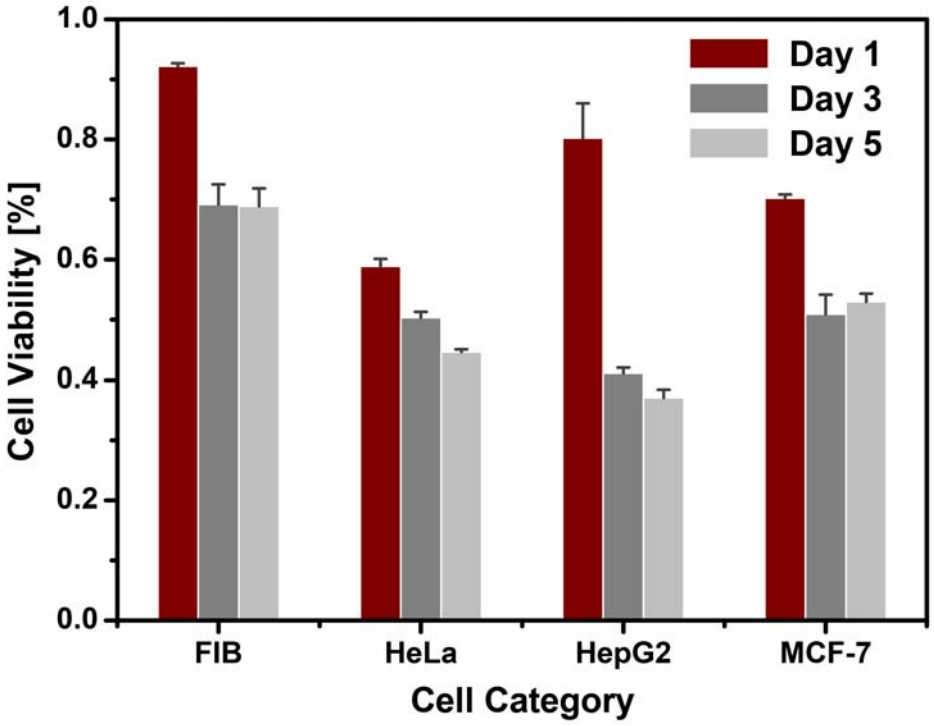

Fig. S5. Comparisons on cytotoxicity towards FIB, HeLa, HepG2, MCF-7 cells incubated in 0.1 mg mL^{-1} TCSC DMEM+10% FBS solutions. Triplicate experiments were performed each time, cells incubated with DMEM+10% FBS were set as blank control correspondingly.

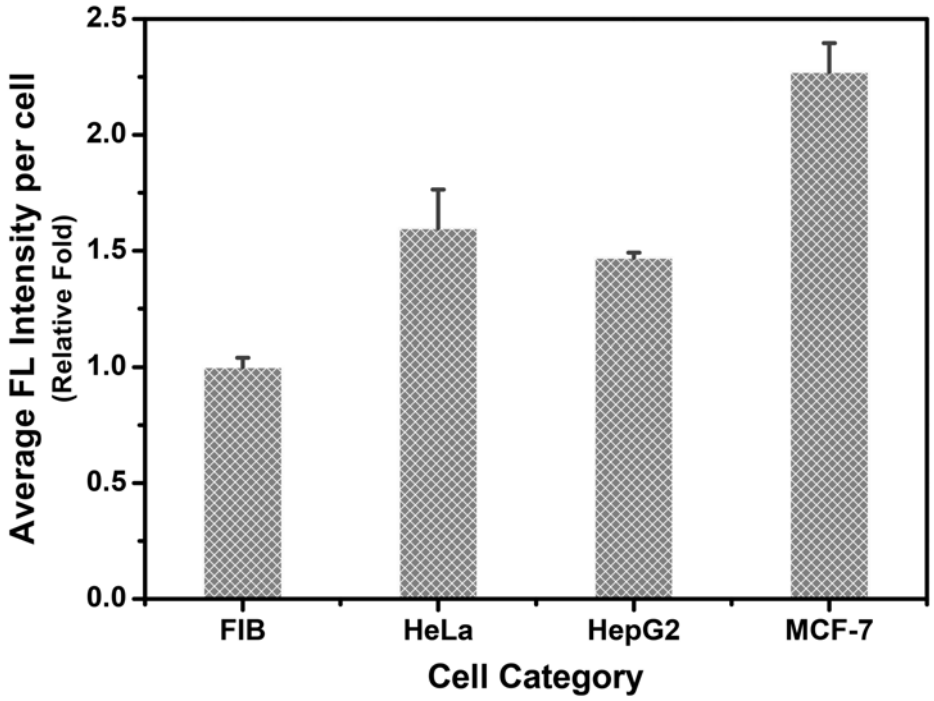

Fig. S6. Average FL intensity per area for FIB, HeLa, HepG2, MCF-7 cells incubated in 0.1 mg mL^{-1} TCSC DMEM+10% FBS solutions. Setting the average FL intensity in FIB cells as 100%.

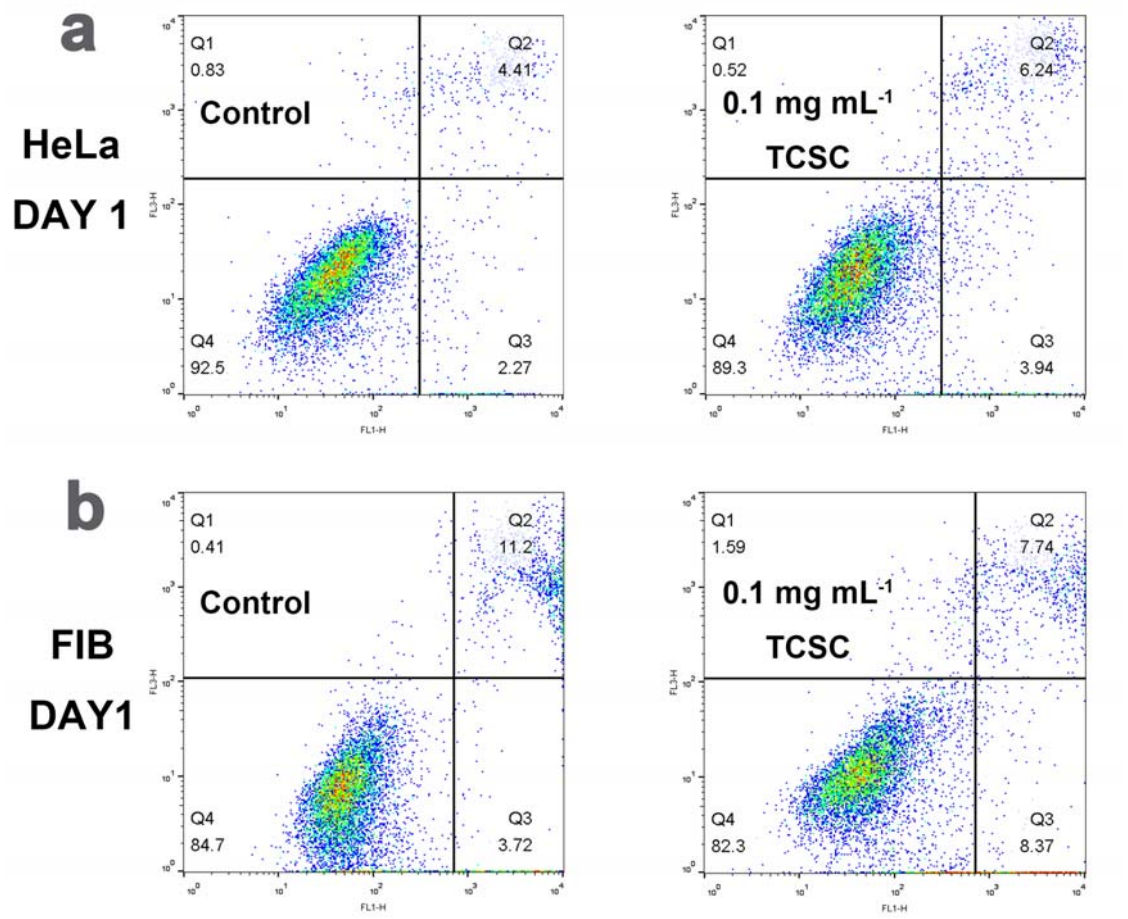

Fig. S7. Flow cytometric analysis of FITC Annexin V and propidium iodide (PI) staining for control cells stained without TCSC and experimental cells incubated in 0.1 mg mL^{-1} TCSC DMEM+10% FBS solutions. (Q1: FITC Annexin V and PI positive, death; Q2: FITC Annexin V and PI positive, end stage apoptosis; Q3: FITC Annexin V positive and PI negative, early apoptosis; Q4: Annexin V and PI negative, viable, or no measurable apoptosis).

Reference

1. M. Huo, Y. Liu, L. Wang, T. Yin, C. Qin, Y. Xiao, L. Yin, J. Liu and J. Zhou, *Mol. Pharm.*, 2016, **13**, 1750-1762.
2. Z. Wang, S. Chen, J. W. Lam, W. Qin, R. T. Kwok, N. Xie, Q. Hu and B. Z. Tang, *J. Am. Chem. Soc.*, 2013, **135**, 8238-8245.