Supplementary Information Available

Fluorescent light-up nanoparticle probe with aggregation-induced emission characteristics and tumor-acidity responsiveness for targeted imaging and selective inhibition of cancer cells

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Experimental Section

1. Materials

2,3-Dimethylmaleic anhydride (DMA) and succinic anhydride (SA) were provided by Alfa Aesar (Ward Hill, Massachusetts, USA). Polyethylenimine (PEI, branched, M_n 60 kDa, 50 wt% in water), lithium wires, naphthalene, 4-bromobenzyl bromide, sodium azide, dichloro-bis(triphenylphosphine)palladium(II), $ZnCl_2$·TMEDA, triphenylphosphine, diethylamine, dimethyl sulfoxide (DMSO), carbon disulfide (CS_2), penicillin-streptomycin solution and other chemicals and solvents were all purchased from Sigma-Aldrich (St. Louis, USA) and used as received without further purification. Hexane and tetrahydrofuran (THF) were distilled from sodium benzophenoneketyl immediately prior to use. Dichloromethane (DCM) and chloroform were distilled over calcium hydride. Fetal bovine serum (FBS) was provided by Gibco (Lige Technologies, Switzerland). Ultrapure grade 10×phosphate-buffered saline (PBS) buffer with pH = 7.4 was purchased from 1st BASE Singapore. Milli-Q water was supplied by Milli-Q Plus System (Millipore Corporation,

Breford, USA). MCF-7 breast cancer cells and NIH/3T3 fibroblast cells were provided by American Type Culture Collection.

2. Characterization

^{1}H and ^{13}C NMR spectra were measured on a Bruker ARX 400 NMR spectrometer. High resolution mass spectra (HRMS) were recorded on a Finnigan MAT TSQ 7000 Mass Spectrometer. UV-vis absorption spectra were taken on a Milton Ray Spectronic 3000 array spectrophotometer. Photoluminescence (PL) spectra were recorded on a Perkin-Elmer LS 55 spectrofluorometer. The parameters with Ex slit of 15.0 nm and Em slit of 5.0 nm were used in this study. Average particle size and size distribution of the samples were measured by laser light scattering (LLS) with a particle size analyzer (90 Plus, Brookhaven Instruments Co. USA) at a fixed angle of 90° at room temperature. The zeta potential of the samples was determined by Malvern Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd., Worcestershire, UK) at room temperature. The morphology of the probes was investigated by transmission electron microscopy (TEM, JEM-2010F, JEOL, Japan).

3. Synthesis of 1,1-dimethyl-2,5-bis[4-(azidomethyl)phenyl]-3,4-diphenylsilole (1)

The compound was prepared according to our published procedures.[1] ^{1}H NMR (400 MHz, $CDCl_3$), δ (TMS, ppm): 7.06 (d, 4H), 7.01 (m, 6H), 6.92 (d, 4H), 6.78 (m, 4H), 4.24 (s, 4H), 0.47 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (TMS, ppm): 154.3, 141.3, 139.9, 138.5, 132.4, 129.9, 129.1, 127.9, 127.5, 126.3, 54.6, -3.9. HRMS (MALDI-TOF): *m*/*z* 524.2200 (M^+, calcd 524.2145).

4. Synthesis of 1,1-dimethyl-2,5-bis[4-(isothiocyanatomethyl)phenyl]-3,4-diphenylsilole (2)

To a solution of **1** (0.26 g, 0.5 mmol) in 5 mL of $CHCl_3$, CS_2 (0.69 ml, 30 mmol) and PPh_3 (0.39 g, 1.5 mmol) were added at room temperature. The mixture was refluxed for 2 h. After solvent evaporation under reduced pressure, the crude product was purified by a

silica-gel column using hexane/chloroform (1:1 v/v) as eluent. The product was obtained as a yellow solid in 67% yield (0.19 g). ^{1}H NMR (400 MHz, $CDCl_3$), δ (TMS, ppm): 7.05 (d, 4H), 7.00 (m, 6H), 6.92 (d, 4H), 6.78 (m, 4H), 4.62 (s, 4H), 0.47 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (TMS, ppm): 154.5, 141.2, 139.9, 138.4, 131.2, 129.9, 129.2, 127.5, 126.5, 126.4, 48.4, −3.9. HRMS (MALDI-TOF): *m*/*z* 556.1462 (M^+, calcd 556.1463).

5. Synthesis of 3-{4-[5-(4-(isothiocyanatomethyl)phenyl)-1,1-dimethyl-3,4-diphenyl-silolyl]benzyl}-1,1-diethyl-thiourea (3, Net-TPS-NCS)

To a solution of **2** (110 mg, 0.2 mmol) in 6 mL of DCM, diethylamine (0.02 mL, 0.2 mmol) was added in room temperature under nitrogen. The mixture was stirred overnight. After solvent evaporation under reduced pressure, the crude product was purified by a silica-gel column using hexane/ethyl acetate (1:10 v/v) as eluent. A yellow product was obtained in 63% yield (80 mg). ^{1}H NMR (400 MHz, $CDCl_3$), δ (TMS, ppm): 7.06 (m, 4H), 6.99 (m, 6H), 6.92 (m, 4H), 6.79 (m, 4H), 5.53 (s, 1H), 4.80 (d, 2H), 4.60 (s, 2H), 3.67 (m, 4H), 1.21 (t, 6H), 0.47 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (TMS, ppm): 180.9, 155.3, 154.7, 142.0, 141.5, 140.6, 139.6, 139.3, 139.1, 136.1, 131.7, 130.5, 129.9, 129.8, 128.1, 128.0, 127.1, 126.9, 50.5, 49.0, 45.8, 13.4, −3.2. HRMS (MALDI-TOF): *m*/*z* 629.2356 (M^+, calcd 629.2355).

6. Synthesis of Net-TPS-PEI

PEI (10 mg, 0.167 μmol) was added into a flask, which was followed by addition of DMSO (2 mL) to dissolve it. Net-TPS-NCS (0.55 mg, 0.87 μmol) was dissolved in THF (1 mL), which was then added into the flask. The resultant mixture was stirred for 24 h, which was then precipitated into diethyl ether, filtered, and dialyzed against Milli-Q water using a 14 kDa molecular weight cutoff dialysis membrane for 24 h. The product (denoted as Net-TPS-PEI) was obtained by lyophilisation. The numbers of Net-TPS-NCS that were

conjugated to the PEI were determined by UV-vis absorption measurement and NMR analyses.

7. Synthesis of Net-TPS-PEI-DMA

Net-TPS-PEI (3.5 mg, 0.056 μmol) and DMA (8.8 mg, 70 μmol) were dissolved in DMSO (3 mL), then triethylamine (30 μL) and pyridine (10 μL) was added under N_2 atmosphere. The mixture was allowed for reaction at room temperature for 24 h. The resulting solution was precipitated into diethyl ether, filtered and dialyzed against pH 9.0 borate buffer (1 mM) for 24 h. The product (denoted as Net-TPS-PEI-DMA) was obtained by lyophilization. The numbers of DMA that were conjugated to the Net-TPS-PEI were determined by the NMR spectra.

8. Synthesis of Net-TPS-PEI-SA

Net-TPS-PEI-SA was synthesized by following the same procedure as that for the preparation of Net-TPS-PEI-DMA.

9. Quantum yield measurement

The quantum yields of Net-TPS-NCS at pH 6.5 and 7.4, Net-TPS-PEI-DMA at pH 7.4, Net-TPS-PEI-DMA at pH 6.5 upon addition of poly(acrylic acid) were determined using quinine sulfate in 0.1 M H_2SO_4 as the standard.[2] The absorbance of both sample and standard was adjusted to be 0.1. The parameters with Ex slit of 15.0 nm and Em slit of 5.0 nm were used for emission measurements.

10. Cell culture

MCF-7 breast cancer cells, human hepatocelluar carcinoma cells (HepG2), human cervical cancer cells (HeLa), human lung cancer cells (NCI-H446), 4T1 breast cancer cells and NIH/3T3 fibroblast 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. Adipose-derived stem

cells were isolation from the abdominal and inguinal adipose tissue of 8-12 weeks old male FVB mice, which were subsequently cultured and expanded in 10 cm^2 plate in complete growth medium containing α-Minimum Essential Medium supplemented with 20% FBS and 100 U/mL of penicillin-streptomycin at 37 ^oC in a humidified environment containing 5% CO_2. Before experiment, the cells were precultured until confluence was reached.

11. Cell imaging

MCF-7 cells were cultured in a LAB-TEK chamber (Chambered Coverglass System, Rochester, USA) at 37 °C. After 80% confluence, the medium was removed and the adherent cells were washed twice with 1 × PBS buffer. The Net-TPS-PEI-DMA (5 μM based on TPS) in the culture medium at pH 6.5 and 7.4 were then added to the chamber, respectively. After incubation for 1 h at 37 ^oC, the cells were washed three times with 1 × PBS buffer and then fixed with 3.7% formaldehyde in 1 × PBS for 30 min at room temperature. The nuclei were stained with propidium iodide (PI) for 40 min. The cell monolayer was then washed twice with 1 × PBS buffer and imaged by CLSM (Zeiss LSM 410, Jena, Germany) with imaging software Olympus Fluoview FV1000. The fluorescent signals from the nanoparticle probes were collected upon excitation at 405 nm (1 mW) with a 505 nm longpass barrier filter. MCF-7 cancer cells were also incubated with Net-TPS-PEI-DMA (5 μM based on TPS) at 4 ^oC in the culture medium at pH 7.4 and 6.5, respectively, which was followed by CLSM imaging.

12. Flow cytometry study

Three groups of MCF-7 cancer cells were precultured in culture flask to achieve desired confluence, respectively. One group was used as the control without treatment. For the rest two groups, Net-TPS-PEI-DMA (5 μM based on TPS) in the culture medium at pH 7.4 and 6.5 was added, respectively. After incubation for 1 h at 37 ^oC, the control and sample groups were treated with 1 × trypsin and washed with DMEM medium by

contrifugation. Flow cytometry measurements were conducted using Cyan-LX (DakoCytomation). The mean fluorescence was determined by counting 10,000 events (λ_{ex} = 405 nm, 450/25 nm bandpass filter).

13. *In vivo* tumor imaging

All animal studies were performed in compliance with the guidelines set by Tianjin Committee of Use and Care of Laboratory Animals and the overall project protocols were approved by the Animal Ethics Committee of Nankai University. 4T1 breast cancer cell suspension containing 5–6 × 10^6 cells (0.1 mL) were injected subcutaneously to female Balb/c mice at the right flank. When the tumor volume reached a mean size of about 300 mm^3, the mice were intravenously injected with 200 μL of Net-TPS-PEI-DMA nanoparticle probes at the concentration of 20 μM based on TPS. After 2 h and 6 h post-injection, respectively, the mice were sacrificed and tumors as well as muscles in mouse legs were dissected. The tissues were subsequently fixed in 4% paraformaldehyde for 2 h, incubated in 20% sucrose/PBS overnight and embedded in Optimal Cutting Temperature (OCT) compound (Tissue-Tek). Sections (6 μm) were immunostained with monoclonal antibody against platelet/endothelial cell adhesion molecule 1 (PECAM-1; PharMingen). Alexa Fluor 488-conjugated antirabbit antibody was used as secondary antibody (Molecular Probes). The cell nuclei were stained with TOPRO-3 (Molecular Probes). The tissue slices were imaged by CLSM with excitation wavelength at 405 nm, 488 nm, and 633 nm for Net-TPS-PEI-DMA, PECAM-1, and TOPRO-3, respectively.

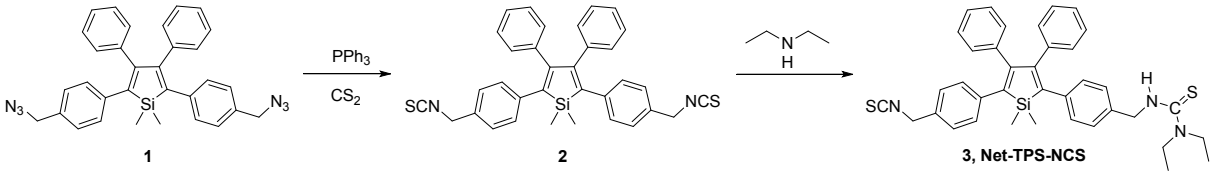
14. Cytotoxicity test

The cytotoxicities of Net-TPS-PEI and Net-TPS-PEI-DMA against cancer cells and normal cells, respectively, were evaluated by MTT assay. MCF-7 breast cancer cells, human hepatocelluar carcinoma cells (HepG2), human cervical cancer cells (HeLa) and human lung cancer cells (NCI-H446) were used as cancer cells. NIH/3T3 fibroblast cells

and adipose-derived stem cells were used as normal cells. Briefly, the cells were seeded in 96-well plates (Costar, IL, USA) at an intensity of 4×10^4 cells/mL, respectively. After 24 h incubation, the normal cells were exposed to a series of doses of Net-TPS-PEI or Net-TPS-PEI-DMA in culture medium at pH 7.4 at 37 °C. And the cancer cells were exposed to a series of doses of Net-TPS-PEI or Net-TPS-PEI-DMA in culture medium at pH 6.5 at 37 °C. After 24 h co-incubation, the wells were washed twice with 1 × PBS and 100 μL of freshly prepared MTT (0.5 mg/mL) solution in culture medium was added into each well. The MTT medium solution was carefully removed after 3 h incubation in the incubator. DMSO (100 μL) was then added into each well and the plate was gently shaken for 10 minutes at room temperature to dissolve all the precipitates formed. The absorbance of MTT at 570 nm was monitored by the microplate reader (Genios Tecan). Cell viability was expressed by the ratio of absorbance of the cells incubated with sample suspension to that of the cells incubated with culture medium only.

15. Western blot analysis

Protein levels of phosphate-Akt, Bcl-2, and cleaved caspase-3 were analyzed by Western blot after NCI-H446 cancer cells were co-cultured with Net-TPS-PEI-DMA at the concentration of 10 and 20 μM based on TPS, respectively. Briefly, cell lysates were prepared, electrotransferred, and subsequently immunoblotted with anti-phosphate-Akt (Sigmaaldrich), anti-Bcl-2 (Santa Cruz), and anti-cleaved caspase-3 (Santa Cruz). Detection was carried out with Western blotting reagent ECL (Amersham), and chemiluminescence was exposed by the filters of Kodak X-Omat films.



Scheme S1 Synthetic route towards Net-TPS-NCS.

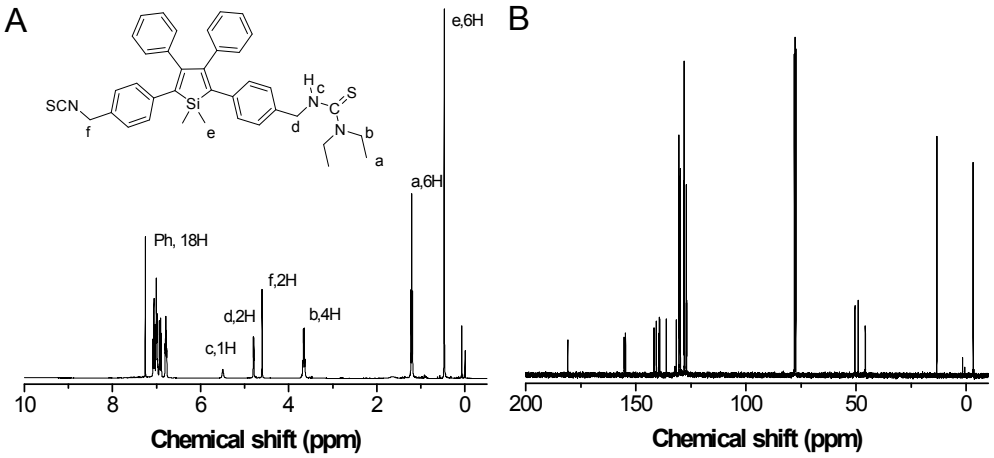


Fig. S1 (A) ^{1}H and (B) ^{13}C NMR spectra of Net-TPS-NCS in $CDCl_3$.

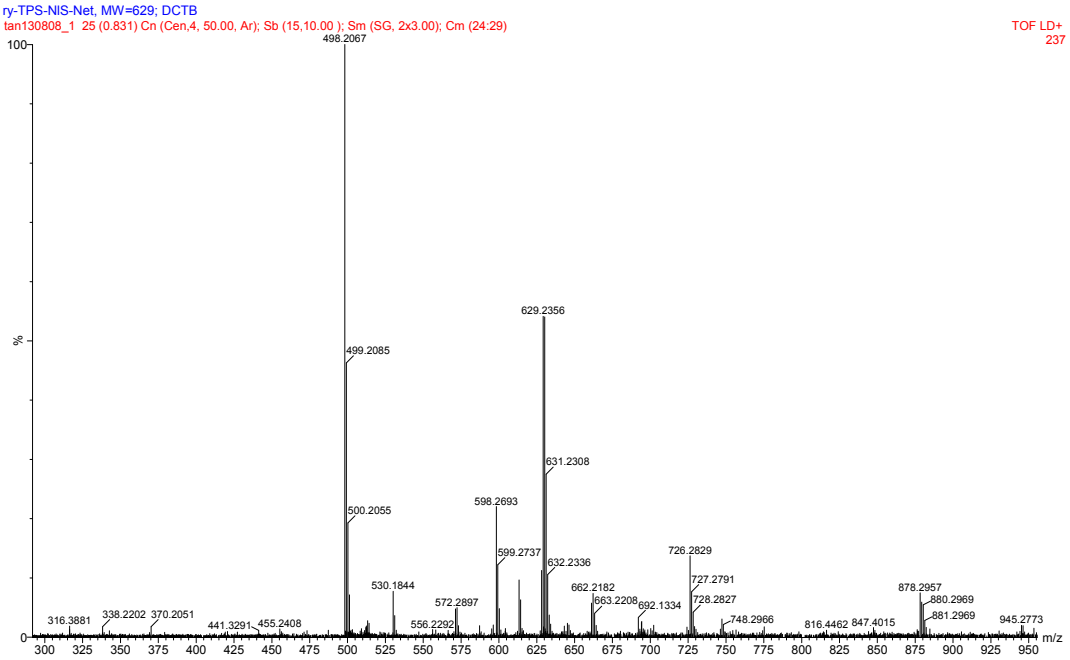


Fig. S2 HRMS spectrum of Net-TPS-NCS.

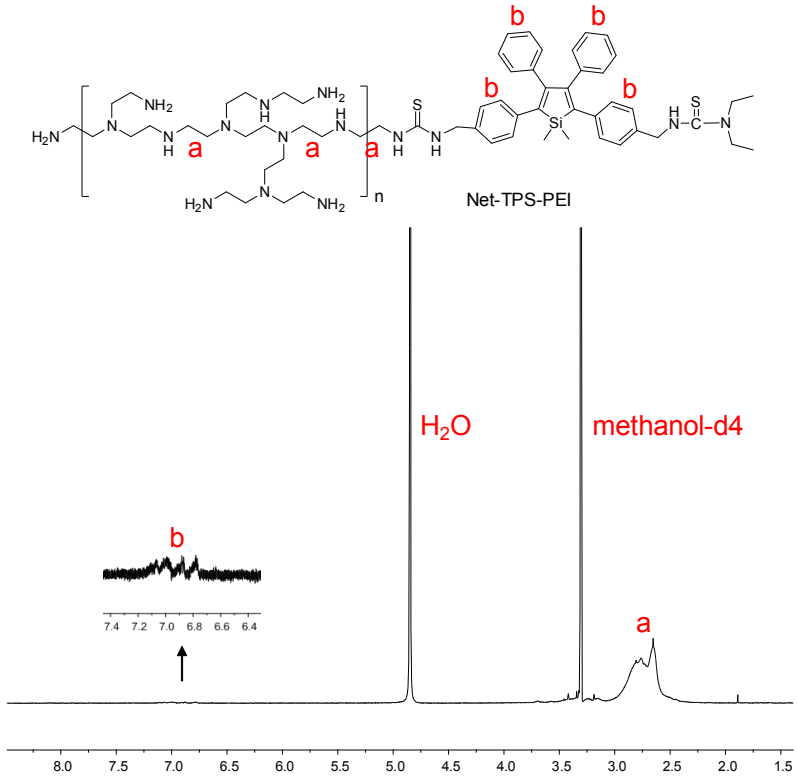


Fig. S3 ^{1}H NMR spectrum of Net-TPS-PEI in methanol-d_4.

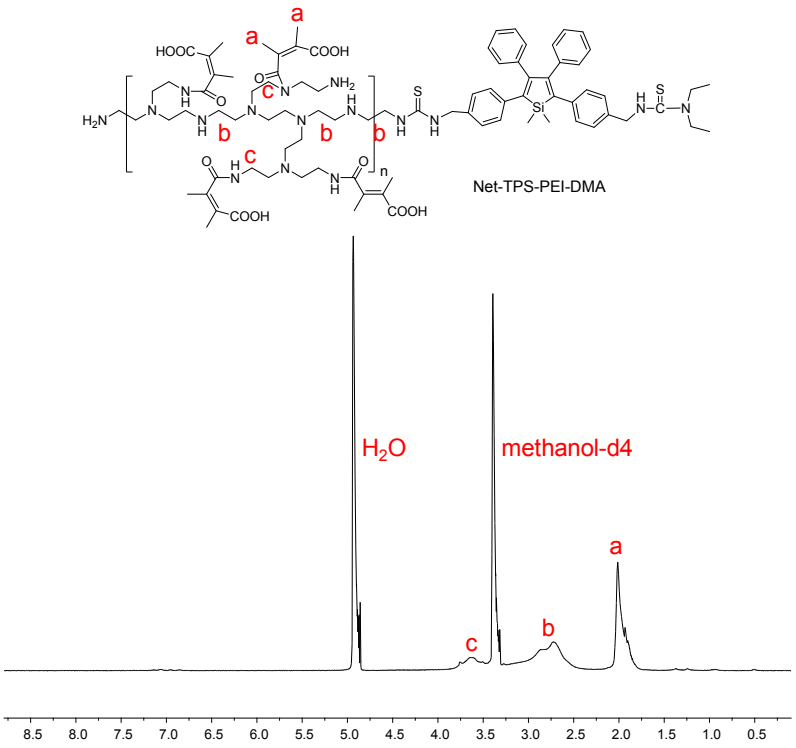


Fig. S4 ^{1}H NMR spectrum of Net-TPS-PEI-DMA in methanol-d_4.

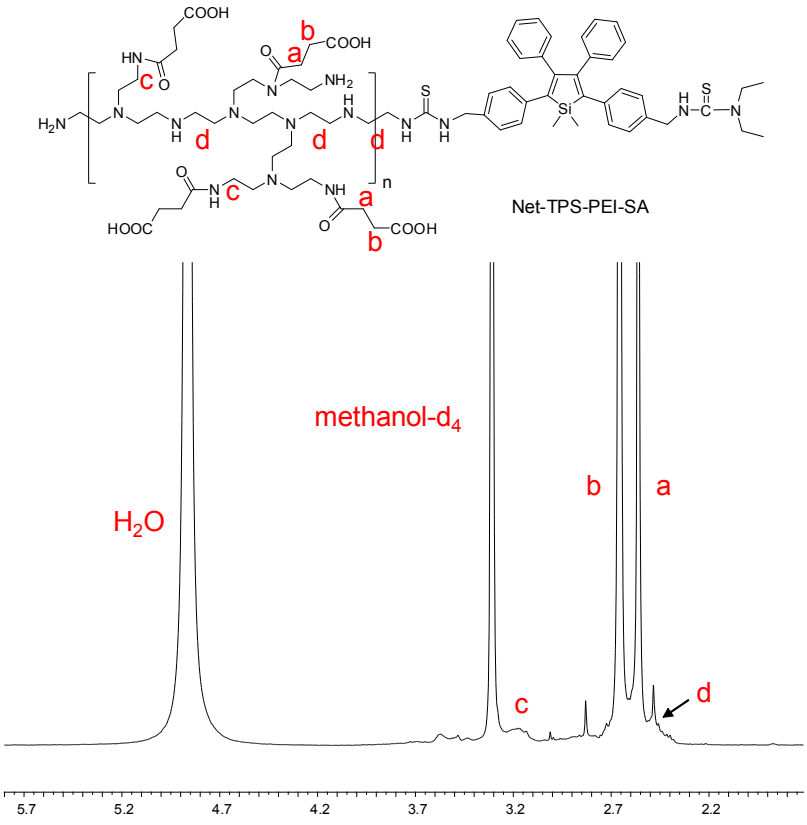


Fig. S5 ^{1}H NMR spectrum of Net-TPS-PEI-SA in methanol-d_4.

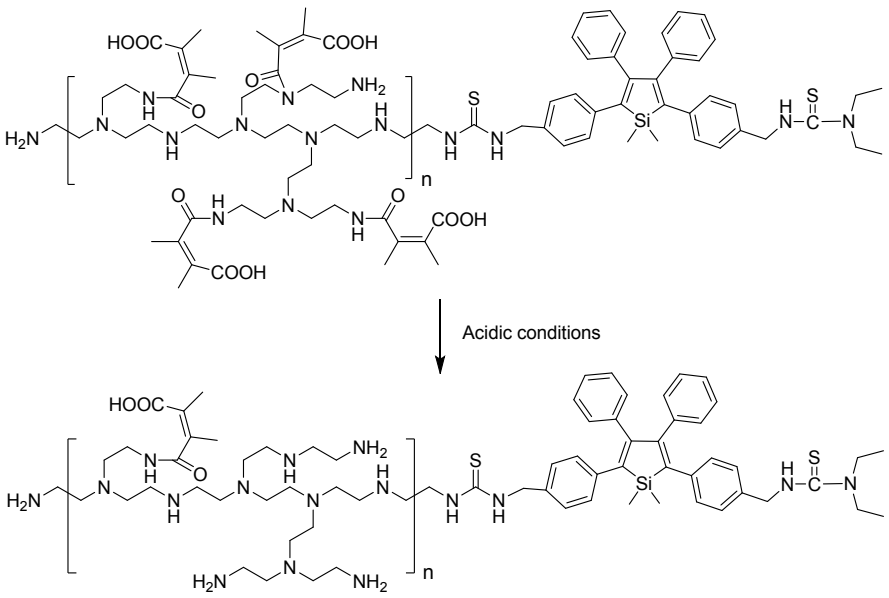


Fig. S6 Schematic illustration of the hydrolysis of Net-TPS-PEI-DMA in acidic conditions.

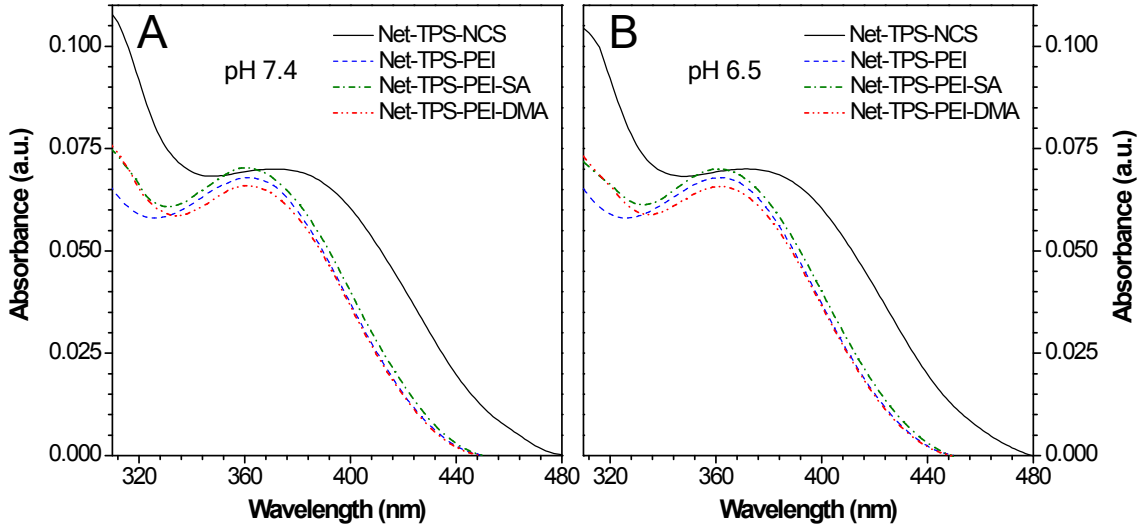


Fig. S7 Absorption spectra of Net-TPS-NCS, Net-TPS-PEI, Net-TPS-PEI-DMA and Net-TPS-PEI-SA in PBS buffer at (A) pH 7.4 and (B) 6.5, respectively.

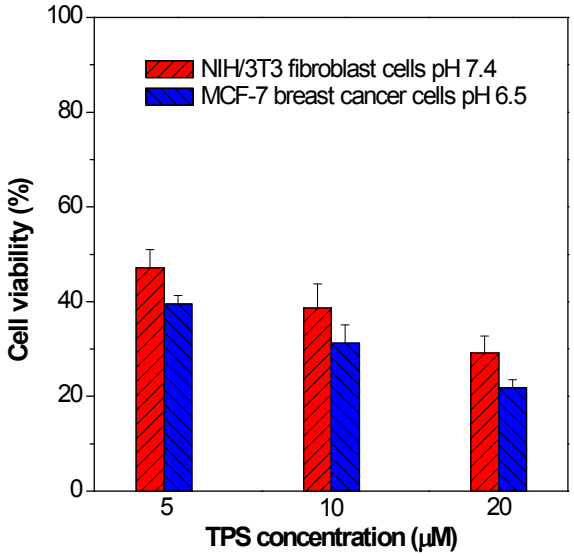


Fig. S8 Metabolic viabilities of NIH/3T3 cells and MCF-7 cancer cells after incubation with Net-TPS-PEI at pH 7.4 and 6.5 for 24 h, respectively.

References

1. Y. Yu, C. Feng, Y. Hong, J. Liu, S. Chen, K. M. Ng, K. Q. Luo and B. Z. Tang, *Adv. Mater.*, 2011, **23**, 3298.

2. W. H. Melhuish, *J. Phys. Chem.*, 1961, **65**, 3298.