Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Supramolecular imaging of spermine in cancer cells

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1. Materials and methods

1.1 Materials

All the reagents and solvents were commercially available and used as received unless otherwise specified purification. Sodium dodecyl benzene sulfonate (SDBS), lucigenin (LCG), Nile Red (NiR), spermine and chloroauric acid ($HAuCl_4$) were purchased from Sigma.

The HEPES buffer solution of pH 7.4 was prepared by dissolving 2.38 g of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) in approximate 900 mL double-distilled water. Titrate to pH 7.4 at the lab temperature of 25 °C with NaOH and make up volume to 1000 ml with double-distilled water. The pH value of the buffer solution was then verified on a pH-meter calibrated with three standard buffer solutions.

Fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), trypsin-EDTA (0.25%) and penicillin streptomycin (Pen-Strep) were purchased from Thermo Fisher Scientific. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), sterilized phosphate buffer saline (PBS) solution (pH 7.4, 10 mM) and 4% paraformaldehyde was purchased from Beijing Solarbio Science & Technology Co. Ltd. Lactate dehydrogenase (LDH) assay kit was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000] (FDP) was purchased from Avanti Polar Lipids, Inc. 293FT (human embryonic kidney cell line) and A549 (human lung cancer cell line) were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HCT116 (human colorectal cancer cell line) was purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd.

1.2 Apparatus

^{1}H and ^{13}C NMR data were recorded on a Bruker AV400 spectrometer. Mass spectra were performed on a Varian 7.0T FTICR-MS (MALDI-TOF and ESI). Steady-state fluorescence spectra were recorded in a conventional quartz cell (light path 10 mm) on a Cary Eclipse fluorescence spectrophotometer equipped with a Cary single-cell peltier accessory. UV-Vis

spectra were recorded in a conventional quartz cell (light path 10 mm) on a Cary 100 UV-Vis spectrophotometer equipped with a Cary dual-cell peltier accessory. The isothermal titration calorimetry (ITC) measurements were done by a fully computer-operated isothermal calorimetry instrument (PEAQ-ITC, Malvern). Dynamic light scattering (DLS) measurements and Zeta potential measurements were examined on a laser light scattering spectrometer (NanoBrook 173plus) equipped with a digital correlator at 659 nm at a scattering angle of 90°. MTT assay and LDH assay were measured on Thermo Scientific Multiskan FC plate reader with 405, 450, 492, 570 and 620 nm filters and an oscillator. Fluorescence microscopy images were obtained by a confocal laser scanning microscopy (CLSM) (Leica TCS SP3, Germany).

1.3 Preparation of LCG@SC5A12C, NiR@SC5A12C and LCG@SC5A12C/FDP

SC5A12C was dissolved in pH = 7.4 HEPES buffer at a concentration of 10 mM. The sample was sonicated at 80 °C for 2 h, subsequently cooled to room temperature, forming the SC5A12C nanocarrier. A certain amount of SC5A12C solution, LCG solution or NiR solution and pH = 7.4 HEPES buffer were mixed until LCG@SC5A12C was acquired with final concentrations of 2 mM for LCG or 120 μM for NiR and 6 mM for SC5A12C. For LCG@SC5A12C/FDP, a solution of 250 μM FDP in chloroform was dried under vacuum. Then a certain amount of SC5A12C solution was added and the samples were sonicated at 80 °C for 2 h, subsequently cooled to room temperature. LCG solution and pH = 7.4 HEPES buffer were added until finally got LCG@SC5A12C/FDP with concentrations of 2 mM for LCG, 6 mM for SC5A12C, and 30 μM for FDP.

1.4 Measurements of host-guest binding affinities by fluorescence titrations

The binding affinities of SC5A12C with LCG and spermine were obtained through fluorescence direct and competitive titrations. All fluorescence titrations were measured in pH = 7.4 HEPES buffer (10 mM) at 25 °C. Data of titrations were fitted in a nonlinear manner, and the fitting functions can be downloaded from the website of Prof. Nau's group (http://www.jacobs-university.de/ses/wnau).

1.5 Measurements of the binding affinity between SC5A12C and spermine by ITC

All titrations were measured in pH = 7.4 HEPES buffer (10 mM) at 25 °C. A constant volume (2 μL per injection) of the guest solution (350 μM) in a 40-μL syringe was injected into the reaction cell (280 μL) charged with the SC5A12C solution (35 μM) within 4 s under stirring (750 rpm). Nineteen successive injections were made for each titration. The dilution heat was measured by injecting the guest solution into a pure HEPES buffer without SC5A12C. The net reaction heat was calculated by subtracting the dilution heat from the apparent reaction heat. The net reaction heat was fitted by computer simulation using the "One Set of Sites" model (MicroCal PEAQ-ITC Analysis Software). The association constants were calculated from the reciprocal of the dissociation constants (provided by the software).

1.6 Surface tension measurements for CAC of SC5A12C determination

Surface tension measurements were done by Shiyanjia Lab (www.shiyanjia.com).

1.7 "Diluting-concentration" method for CAC of SC5A12C determination

We tried to determine the CAC of SC5A12C using the "diluting-concentration" method reported by Cao and co-workers.[1] A certain amount of 200 μM SC5A12C aqueous solution and 500 μM $HAuCl_4$ aqueous solution were mixed in 10 mL deionized water and vigorously stirred at room temperature, followed by the dropwise addition of $NaBH_4$ aqueous solution (the SC5A12C:$HAuCl_4$:$NaBH_4$ molar ratio was typically 1:1:6). The solution turned red and stirring was continued for another 1 h. Diluting to a series of concentrations of solution and concentrating by rotary evaporation after an overnight standing. The size of the gold nanoparticle probe was determined by measuring the maximum absorption wavelength using an UV-Vis spectrometer.

1.8 In vitro cytotoxicity assay of SC5A12C

1.8.1 Cell culture

All cells were incubated in a 5% CO_2 humidified incubator at 37 °C using DMEM with 10% FBS and 1% Pen-Strep as medium. The cells were sub-cultured when the density reached 70-80%.

1.8.2 Cell viability assay

The MTT assay was used to evaluate the cytotoxicity of the SC5A12C nanocarrier against three kind of cells. All cells (5×10^3 cells per well, respectively) were seeded and cultured in 96-well plates. After 24 h of incubation at 37 °C, the cells were treated with either SC5A12C or SDBS of various concentrations, ranging from 0 μM to 150 μM or 0 μM to 750 μM, respectively. The SDBS concentration of each group equals five times of that of SC5A12C. After 24 h, culture medium was removed and replaced with fresh medium containing 0.5 mg/mL of MTT, and further incubated for 4 h. Thereafter, the medium was removed and 150 μL DMSO was added, followed by gentle shaking for 10 min. The absorption was measured at 492 nm. The relative cell viability was calculated as:

$$\text{cell viability} = (OD492_{(samples)} - OD492_{(blank)})/(OD492_{(control)} - OD492_{(blank)}) \times 100\%$$

where $OD492_{(control)}$ was obtained from the sample without SC5A12C or SDBS treatment, and $OD492_{(samples)}$ was obtained from the sample after treatment of SC5A12C or SDBS and $OD492_{(blank)}$ was obtained by untreated DMEM medium. Each value was averaged from three independent experiments. Data are presented as mean ± standard deviation (s.d.) ($n = 3$).

1.8.3 LDH assay

LDH assay was conducted according to the manufacturer's instructions. Briefly, 293FT cells (5×10^5 cells per well) were seeded and incubated in a 96-well plate. After 24 h of incubation at 37 °C, the cells were treated with various concentrations of SDBS ranging from 0 μM to 750 μM. Untreated cells were regarded as a control for background LDH release, and cells treated with lysis buffer was regarded as a control for maximal LDH release. For the measurement of LDH release, 10 μL supernatant of each well was transferred to another 96-well plate, followed by addition of 25 μL of LDH assay buffer and 5 μL of NAD buffer per well. The mixture was incubated for 15 min at 37 °C, then 25 μL phenylhydrazine chromogenic solution was added to each well, and the resulting mixture was further incubated for 15 min. Finally, 100 μL of basic chromogenic solution and 150 μL of distilled water was added to each well. The 96-well plate was let on stand for 5 min before the absorption was measured by microplate reader at 450 nm. The relative LDH release was calculated as:

LDH release = $(OD450_{(samples)} - OD450_{(blank)})/(OD450_{(control)} - OD450_{(blank)}) \times 100\%$

where $OD450_{(control)}$ was obtained from the sample after treatment of lysis buffer, and $OD450_{(samples)}$ was obtained from the sample after treatment of SDBS and $OD492_{(blank)}$ was obtained from the sample without any treatment.

1.8.4 Zeta potential measurements for cytotoxicity

293FT cells (5×10^5 cells per well) were seeded and incubated in 6-well plates. After 24 h of incubation at 37 °C, the cells were treated with various concentrations of SDBS ranging from 0 μM to 750 μM, and further incubated for 15 min. The cells were then harvested and suspended in 1.6 mL water for Zeta potential measurements.

1.9 Intracellular fluorescence imaging

For spermine-responsive fluorescence imaging, 293FT cells, A549 cells and HCT116 cells (1×10^5 cells per well, respectively) were seeded and cultured in confocal imaging chambers. After 24 h of incubation at 37 °C, the cells were treated with either LCG (50 μM) or LCG@SC5A12C (50/150 μM or 5/15 μM), and further incubated for 4 h. The cells were washed three times with PBS before imaging by CLSM.

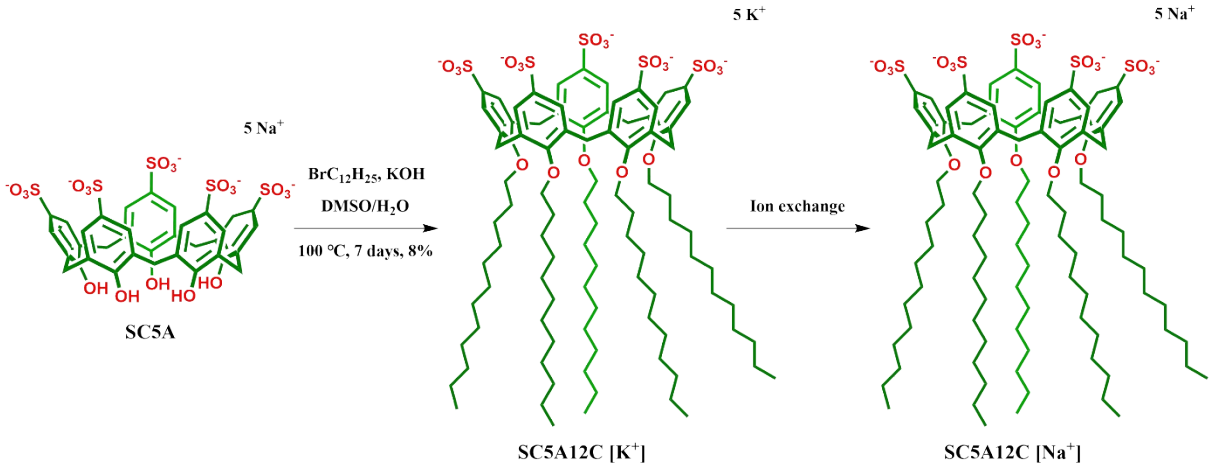
For the investigation of fluorescence imaging of LCG@SC5A12C/FDP, A549 cells and HCT116 cells (1×10^5 cells per well, respectively) were seeded and cultured in confocal imaging chambers. After 24 h of incubation at 37 °C, the cells were treated with either LCG@SC5A12C (50/150 μM) or LCG@SC5A12C/FDP (50/150/0.75 μM), and further incubated for 4 h. The cells were washed three times with PBS before imaging by CLSM.

For the calculation of average fluorescence intensity per cell, the CLSM images were analyzed by the imageJ program. Briefly, the CLSM images were binned in 8-bit type and the threshold were chosen manually. The average fluorescence intensity was calculated by dividing the total fluorescence intensity by total number of the cells (counted manually).

1.10 Cellular uptake of SC5A12C assembly quantification

Comparable numbers of A549, HCT116, and 293FT cells were cultivated in a 12-well plate. In the first row the cells were incubated with NiR@SC5A12C (3/150 μM). The second row was used as a reference without additive incubation. After 4 h incubation, each pore was washed with PBS for three times to remove residual NiR@SC5A12C. Following trypsinization, the homogenously suspended cells were counted to estimate the total number of cells on each pore. Three freeze-thaw cycles were then preformed with the cells for completely lysis and the supernatants were obtained by centrifugation at 8000 rpm for 10 min at 4 °C.[2] Fluorescence experiments were conducted after dilution to 3 mL. To determine the absolute uptake of SC5A12C, different concentrations of NiR@SC5A12C were added to the lysed reference solutions to construct calibration curves. The absolute concentrations of SC5A12C after uptake into cells were calculated according to the fluorescence intensities of NiR in diluted solutions, the number of cells, and the volumes of cells. The volumes of cells were estimated by employing spherical cell volumes derived from reported average cell diameters.[3, 4]

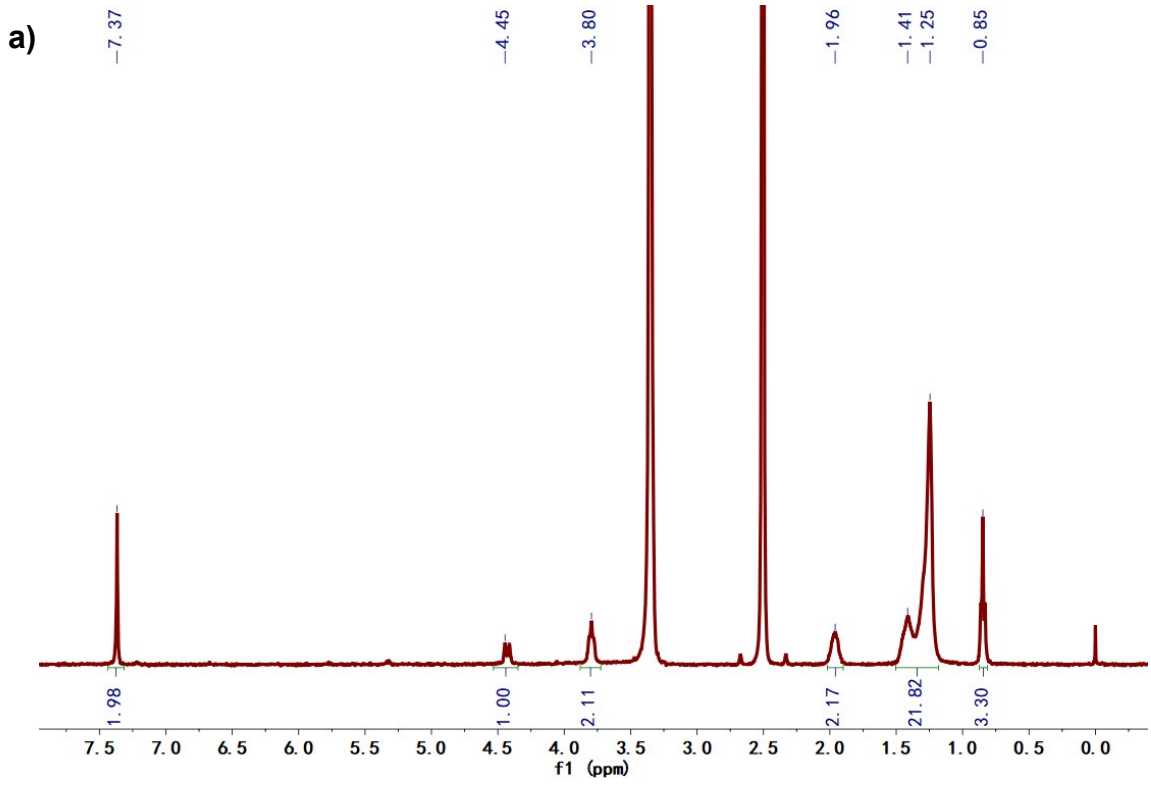
2. Synthesis of SC5A12C

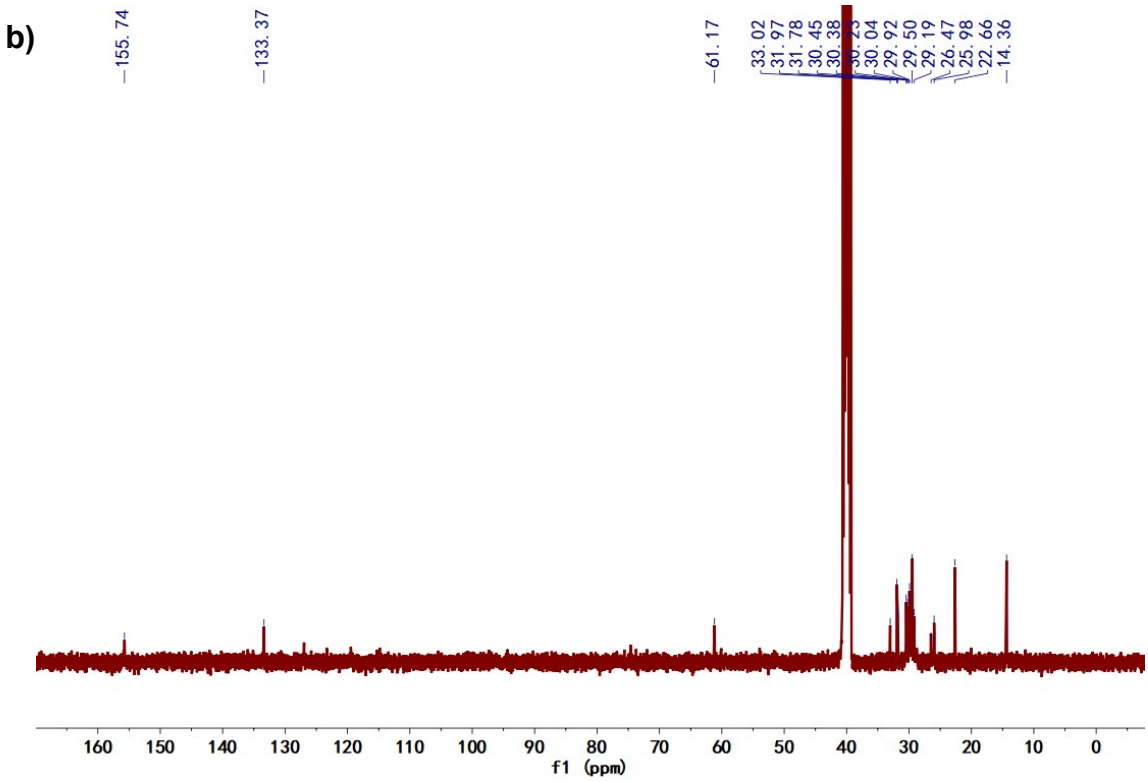


Scheme S1. Synthetic route of SC5A12C.

Synthesis of 5,11,17,23,29-pentasulfonato-31,32,33,34,35-pentakis(dodecyloxy)calix[5]arene (SC5A12C): SC5A (2.15 g, 1.92 mmol) was mixed with KOH (5.39 g, 96 mmol) in water (15 mL) and 1-bromododecane (23.93 g, 96 mmol) in dimethyl sulfoxide (60 mL), and the reaction mixture was heated to 100 °C for 7 days. After cooling to room temperature, the precipitate was

recovered by filtration and dissolved in water (10 mL). The insoluble material was removed by filtration, and the product was precipitated from the filtrate by diluting with ethanol. The crude product was dissolved in hot water and then guanidine hydrochloride was added under vigorous string. The precipitate was recovered by filtration after 30 min. The obtained white solid was dissolved in ethanol and then sodium ethylate was added under vigorous string. The product of sodium salt was recovered by filtration after 30 min. Yield: 0.29 g (8%). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.37 (s, 10H; ArH), 4.45 (d, J = 13.9 Hz, 5H; Ar-CH_2-Ar), 3.80 (s, 10H; -CH_2-O-Ar), 1.96 (s, 10H; -CH_2-CH_2-O-Ar), 1.49~1.15 (m, 90H; alkyl CH_2), 0.85 (s, 15H; CH_3-CH_2-). ^{13}C NMR (400 MHz, DMSO-d_6, δ): 155.74, 133.37, 61.17, 33.02, 31.97, 31.78, 30.45, 30.38, 30.23, 30.04, 29.92, 29.50, 29.19, 26.47, 25.98, 22.66, 14.36. ESI-FTMS *m/z*: $[M + 4H - 5K]^-$ calcd. for $C_{95}H_{149}O_{20}S_5$ 1770.9279, found 1770.9245.





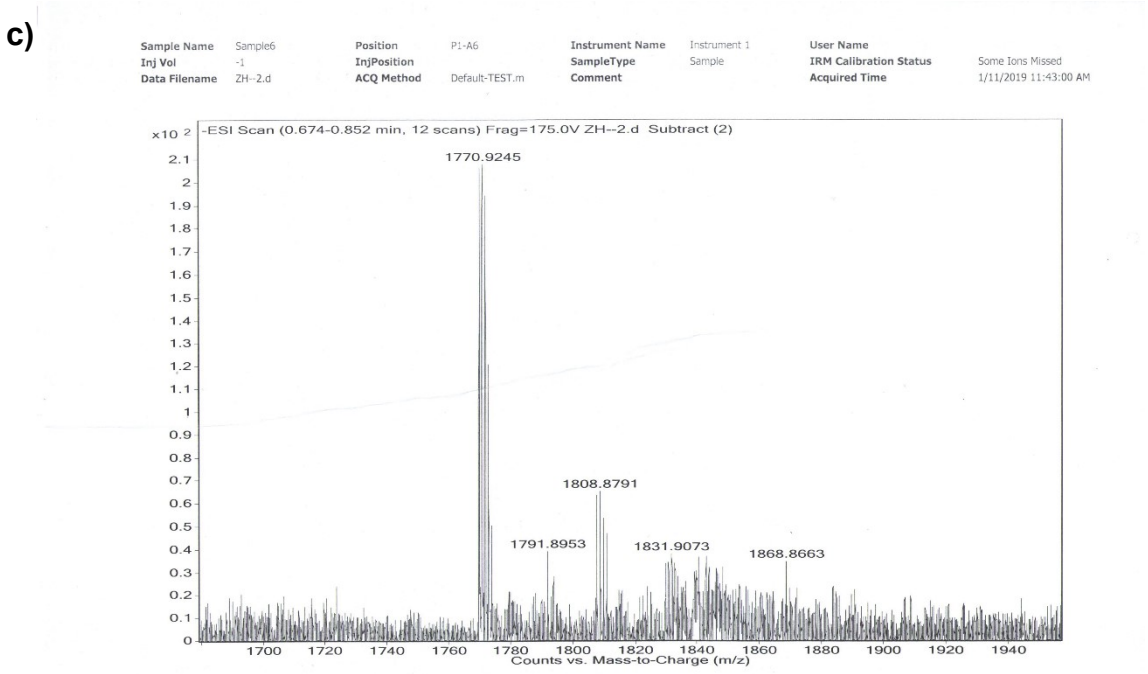
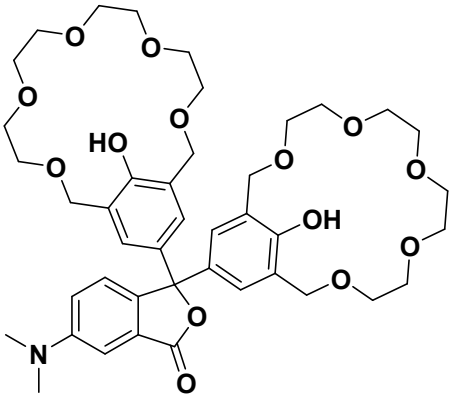
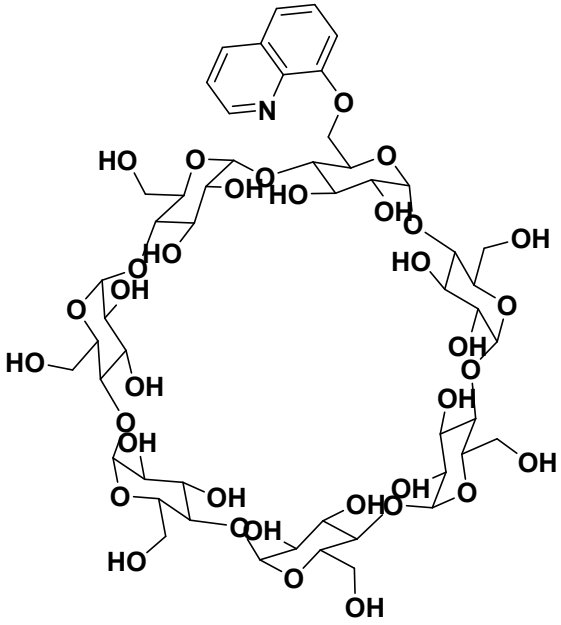
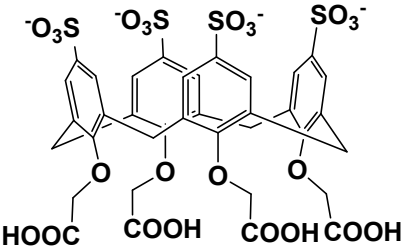
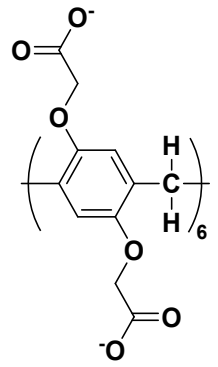
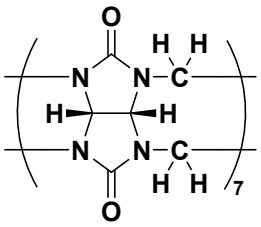


Figure S1. (a) ^{1}H NMR spectrum of SC5A12C in DMSO-d_6, 400 MHz, 298 K; (b) ^{13}C NMR spectrum of SC5A12C in DMSO-d_6, 100 MHz, 298 K; (c) ESI FT-ICR MS of SC5A12C.

3. Comparison of various macrocyclic host molecules on spermine complexation

Table S1. Representative macrocyclic receptors and their binding affinities to spermine

Structure	Binding affinity	Solvent	Ref.
	$2.57 \times 10^3\ M^{-1}$	Methanol	5
 Imprinted form	Not mentioned	Aqueous	6
	Not mentioned	pH 8.5 borate buffer	7

	$2.58 \times 10^7\ M^{-1}$	pH 7.4 20 mM PB buffer	8
	$3.26 \times 10^5\ M^{-1}$	pH 5.50 PBS buffer	9

4. Supporting results

4.1 CAC determination by the "diluting-concentration" method

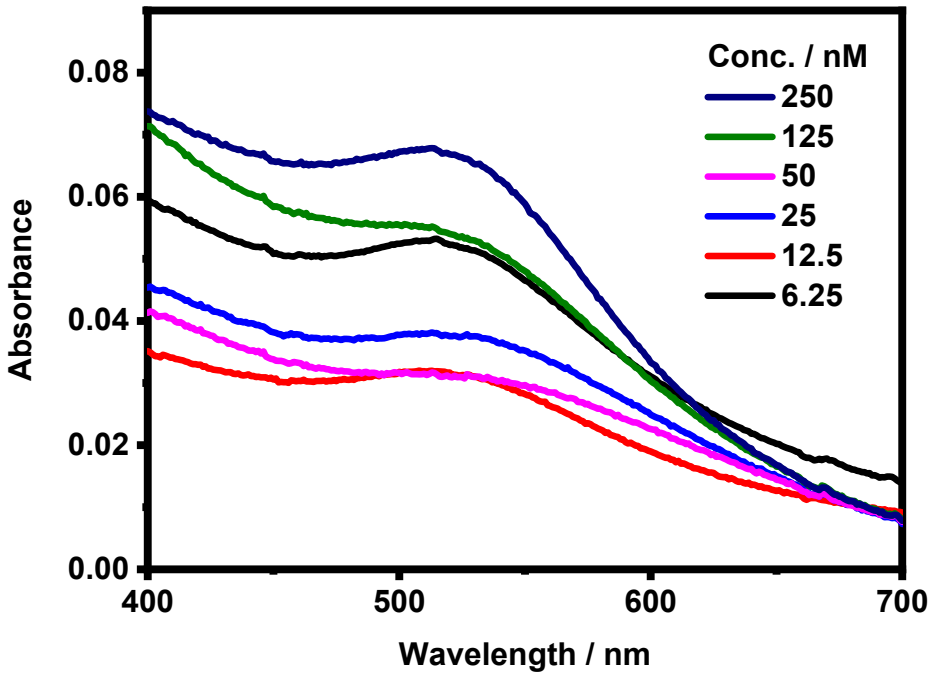


Figure S2. UV-Vis spectra of various concentrations of SC5A12C protected AuNPs aqueous solutions.

4.2 DLS characterization of the SC5A12C and SC5A12C/FDP assembly

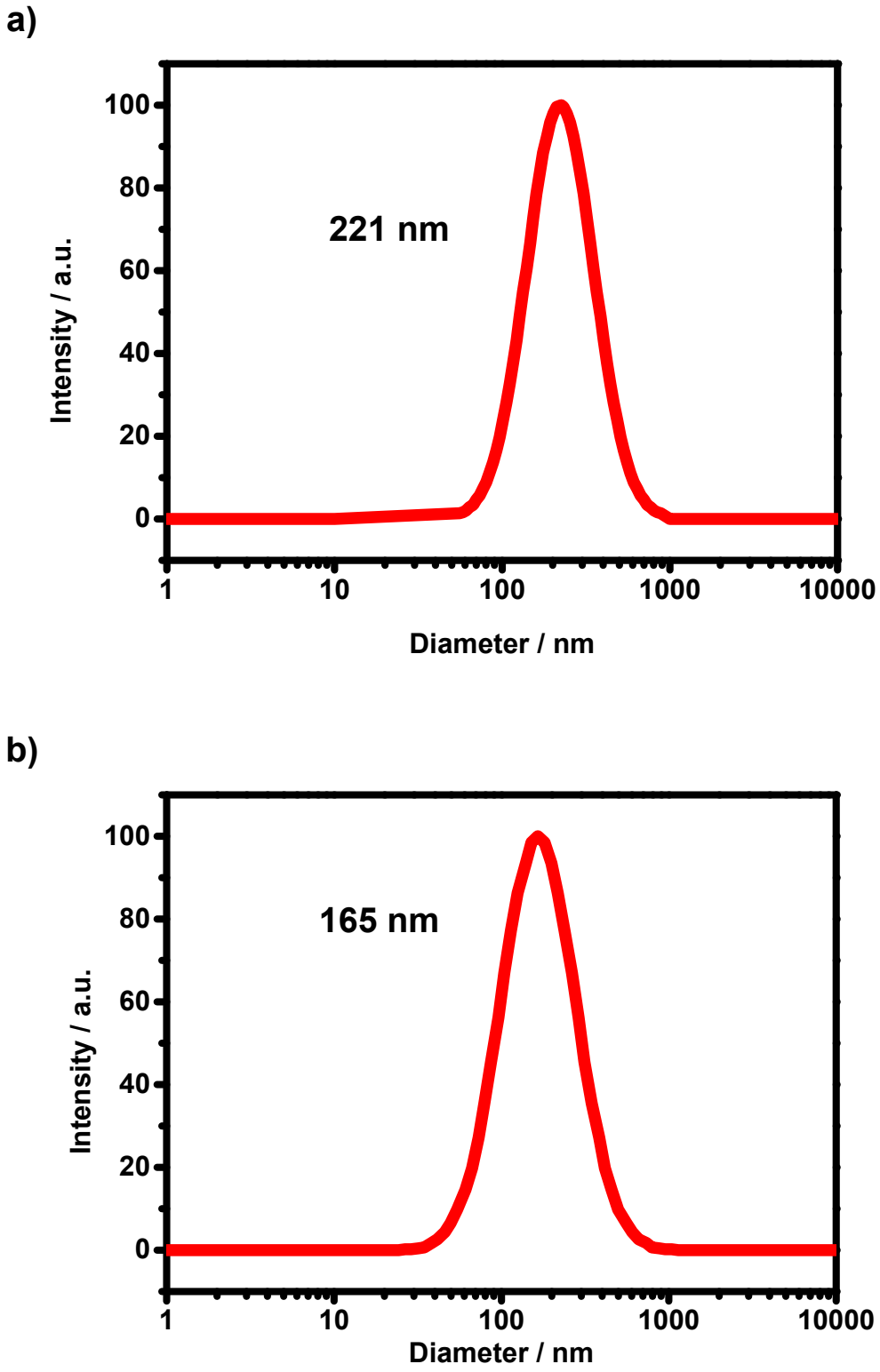
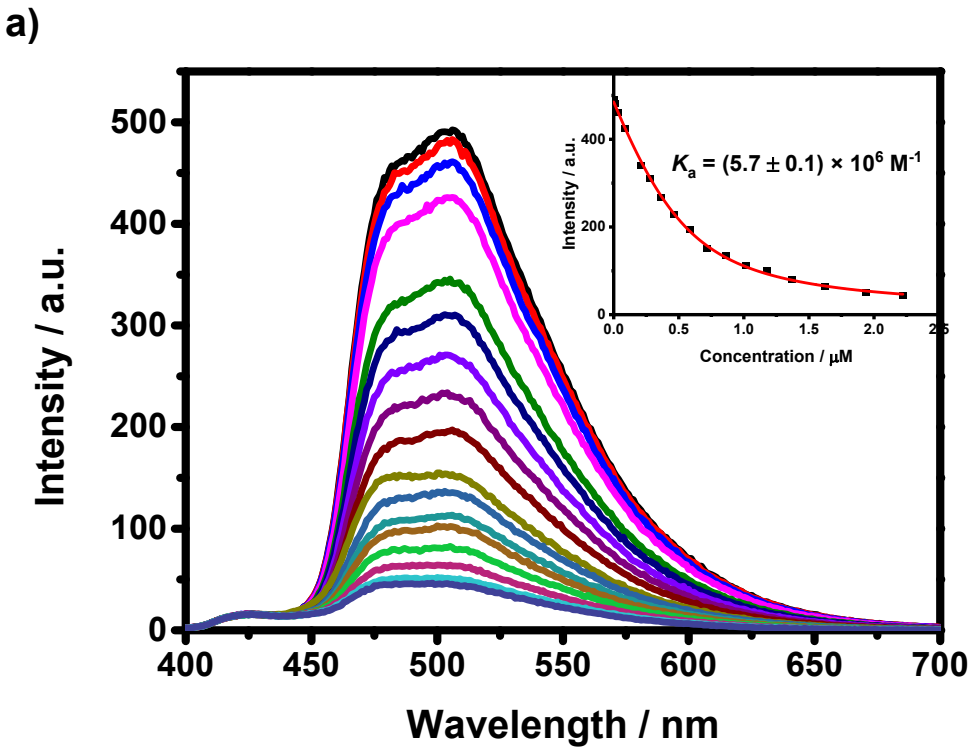


Figure S3. DLS data of assemblies of (a) SC5A12C (100 μM) and (b) SC5A12C/FDP (100/0.5

μM) in pH = 7.4 HEPES buffer.

4.3 Measurements of the binding affinities between SCnA12C and LCG/spermine by fluorescence titrations



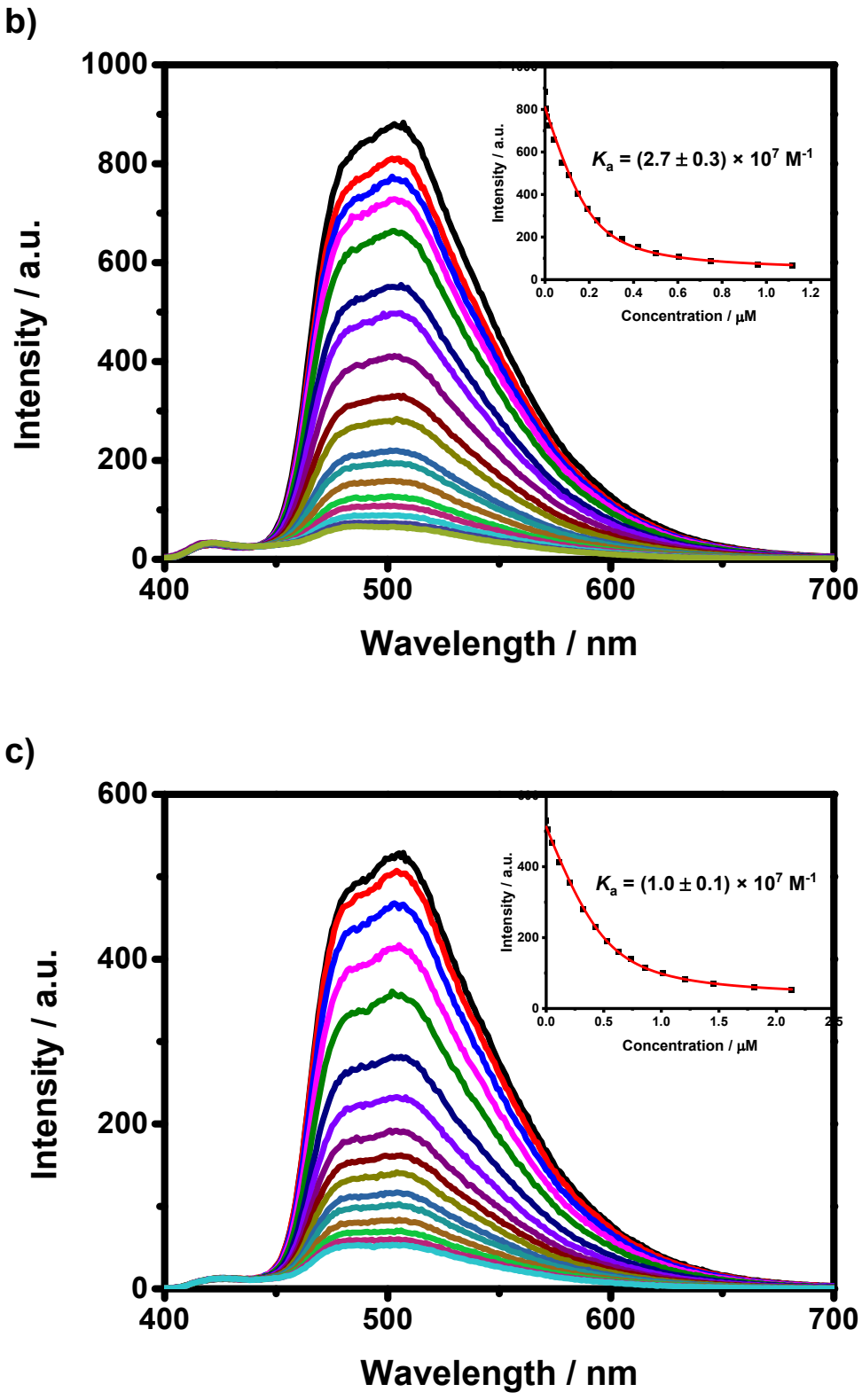
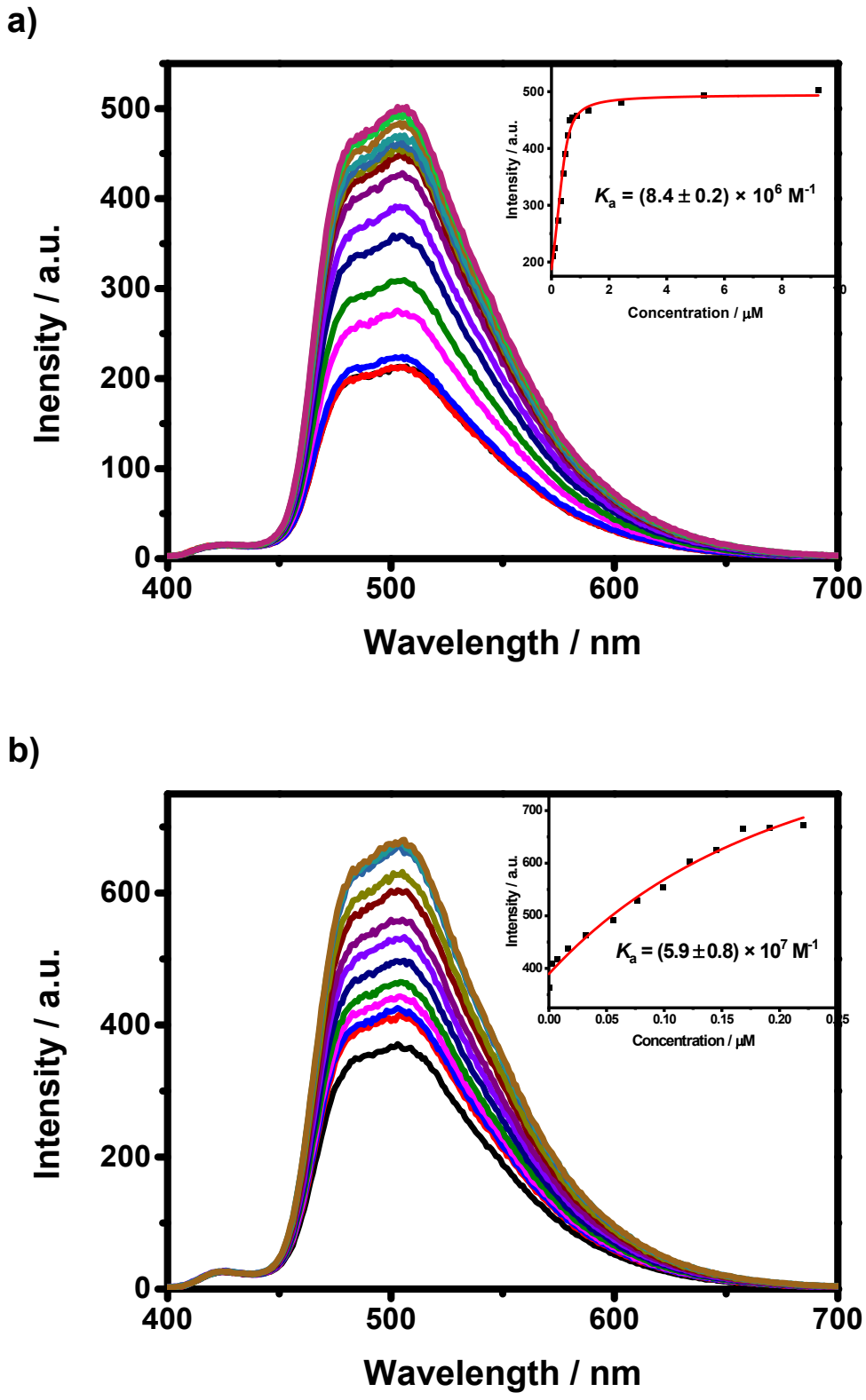


Figure S4. Fluorescence titrations of LCG (0.5 μM) with (a) SC4A12C, (b) SC5A12C (the concentration of LCG is 0.2 μM) and (c) SC6A12C in pH = 7.4 HEPES buffer. λ_{ex} = 368 nm. Inserts show the fitting results corresponding to the titration curves at λ_{em} = 507 nm according

to a 1:1 binding stoichiometry.



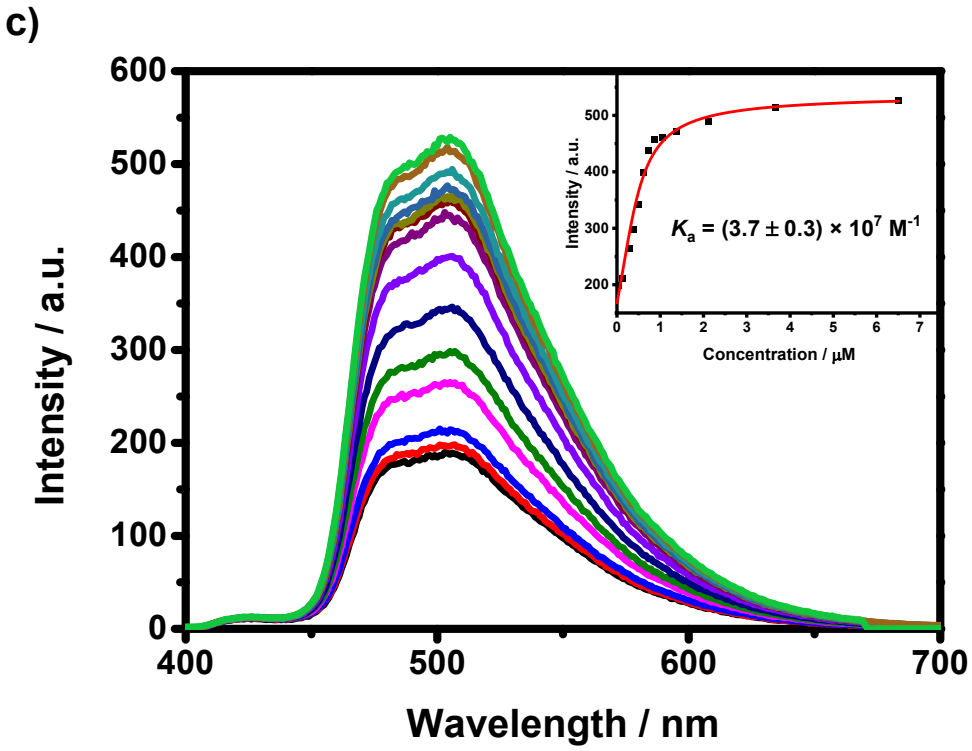


Figure S5. Competitive fluorescence titrations of (a) LCG@SC4A12C (0.5:0.5 μM), (b) LCG@SC5A12C (0.3:0.1 μM) and (c) LCG@SC6A12C (0.5:0.5 μM) with spermine in pH = 7.4 HEPES buffer. λ_{ex} = 368 nm. Inserts show the fitting results corresponding to the titration curves at λ_{em} = 507 nm according to a 1:1 competitive binding model.

4.4 Measurements of the binding affinity between SC5A12C and spermine by ITC

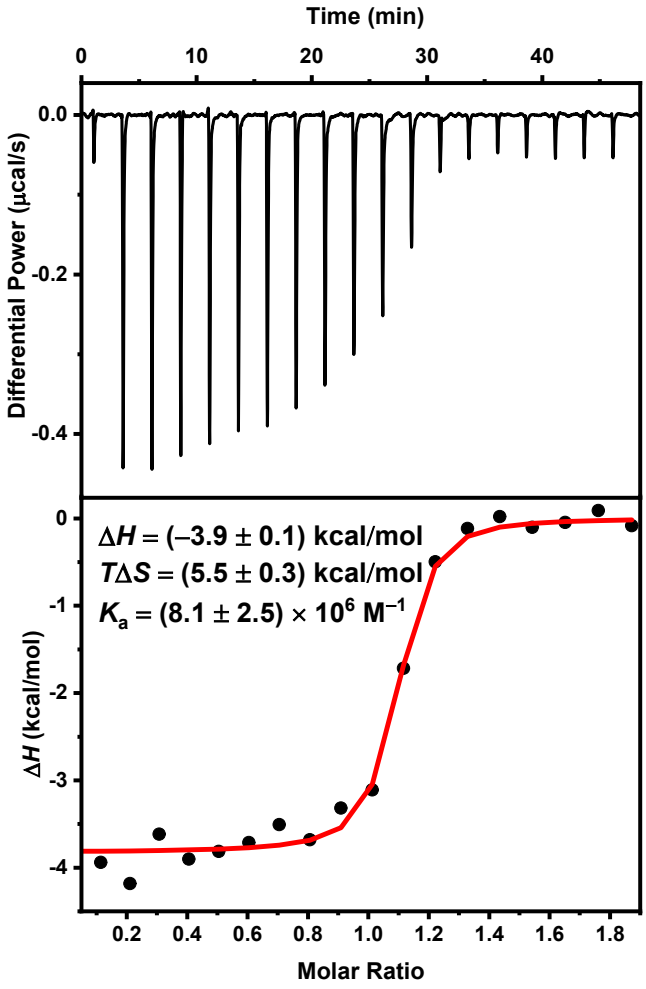


Figure S6. Microcalorimetric titration of SC5A12C with spermine in pH = 7.4 HEPES buffer. Upper: Raw data for sequential nineteen injections (2 µL per injection) of spermine solution (350 µM) injecting into the SC5A12C solution (35 µM). Lower: "Net" heat effects of the complexation for each injection, obtained by subtracting the dilution heat from the reaction heat. Data were fitted by computer simulation using the "One Set of Sites" model.

ITC measurement requires higher concentration of SC5A12C than fluorescence titration, thus increasing the amount of its counterion Na^+ in solution as well. Na^+ has weak interaction with sulfonatocalixarenes, which will be a competitor when investigating the interactions of calixarenes with other guests. The higher concentration of Na^+ is, the more pronounced decrease of measured binding affinity will be.[10] Therefore, the binding constant measured by ITC is smaller than that measured by fluorescence titration.

4.5 Cytotoxicity mechanism investigation using Zeta potential measurement and LDH assay

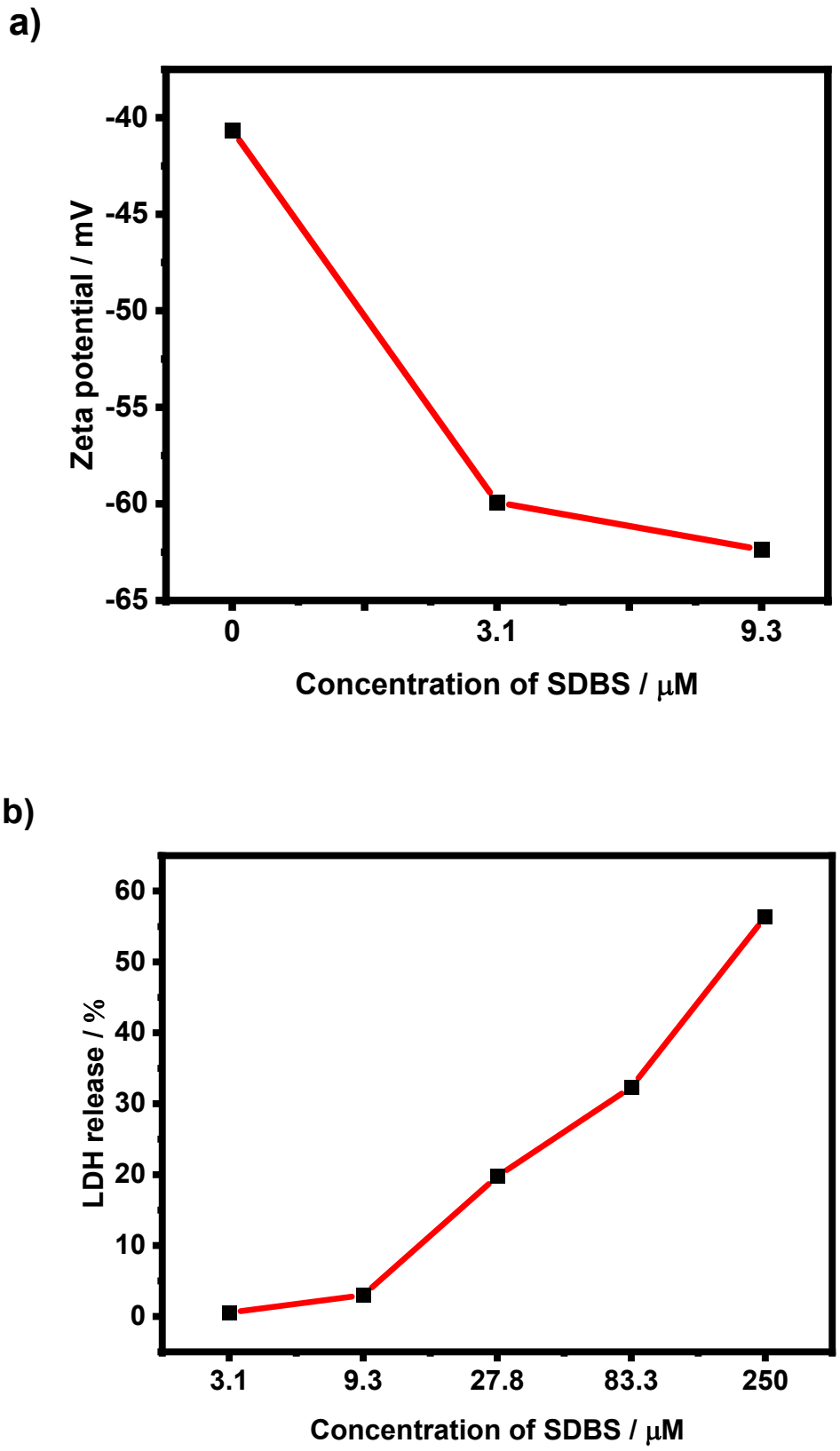


Figure S7. (a) Zeta potential of 293FT cell solution incubated with various concentrations of SDBS (b) Ratio of LDH release after treated with various concentrations of SDBS.

4.6 Determination of cellular uptake of the SC5A12C assembly

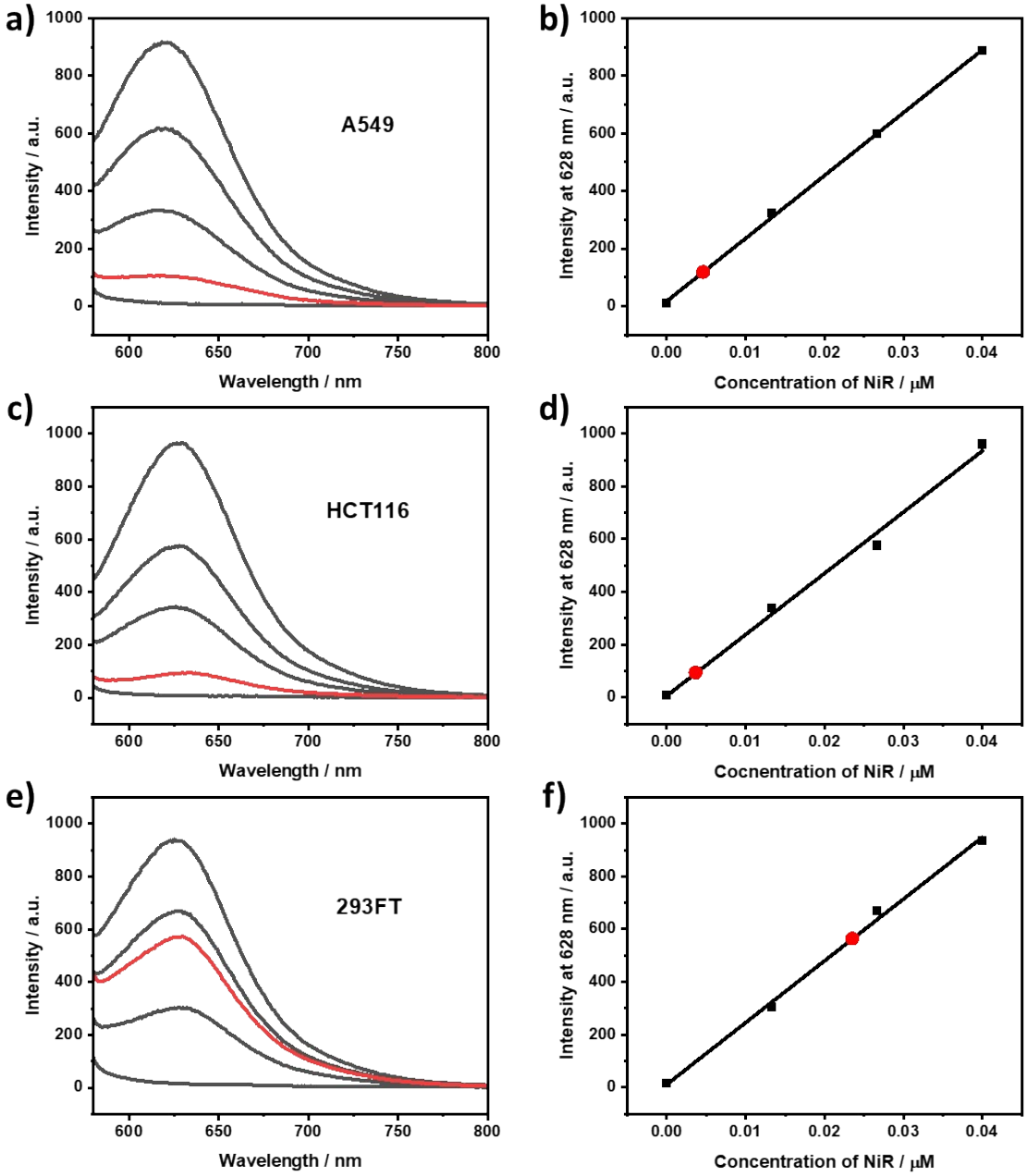


Figure S8. Fluorescence spectra of lysed (a) A549, (b) HCT116, and (c) 293FT cells after incubation with NiR@SC5A12C (red lines). The black spectra are for different concentrations of NiR@SC5A12C in the lysed reference solutions; (b)(d)(f) Calibration curves obtained from the fluorescence intensity at 628 nm of the black spectra in (a)(c)(e). The red data points mark the concentrations of NiR@SC5A12C in lysates. λ_{ex} = 368 nm.

Table S2. The quantification of cellular uptake of the SC5A12C assembly

	Diameter of a cell / μm	Number of cells	Calculated [NiR] after dilution / μM	Calculated [NiR] in cells / μM	Calculated [SC5A12C] in cells / μM
A549	14.9	$(9.5 \pm 1.6) \times 10^5$	$(3.8 \pm 0.1) \times 10^{-3}$	7.4 ± 1.3	$(3.8 \pm 0.5) \times 10^2$
HCT116	18.3	$(2.6 \pm 0.3) \times 10^5$	$(3.0 \pm 0.7) \times 10^{-3}$	10.6 ± 1.4	$(5.3 \pm 0.7) \times 10^2$
293FT	13.0	$(1.0 \pm 0.0) \times 10^6$	$(2.6 \pm 0.2) \times 10^{-2}$	63.8 ± 3.8	$(3.2 \pm 0.2) \times 10^3$

4.7 Imaging results of cells incubated with lower concentration of LCG@SC5A12C

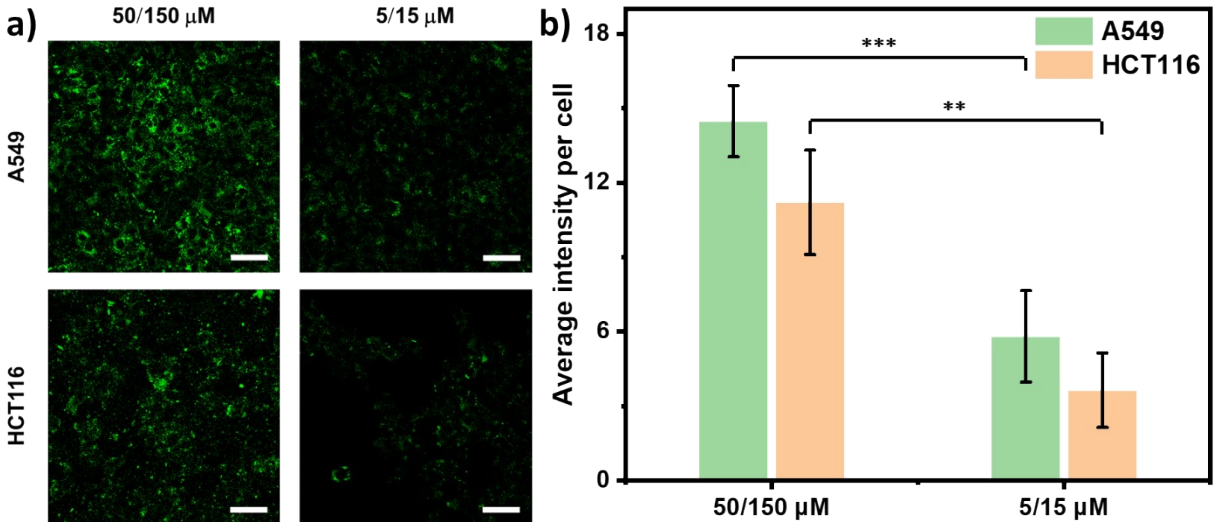


Figure S9. (a) CLSM images and (b) average fluorescence intensity per cell of A549 and HCT116 cells incubated with LCG@SC5A12C (50/150 μM or 5/15 μM) for 4 h. $\lambda_{ex} = 488$ nm. Scale bar: 50 μm. Significance levels are analyzed by one-way ANOVA with the Tukey's test, and expressed as **p < 0.01 and ***p < 0.001.

5. References

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