Supplementary Information

A nanodrug to combat cisplatin-resistance by protecting cisplatin with *p*-sulfonatocalix[4]arene and regulating glutathione S-transferases with loaded 5-fluorouracil

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General Methods

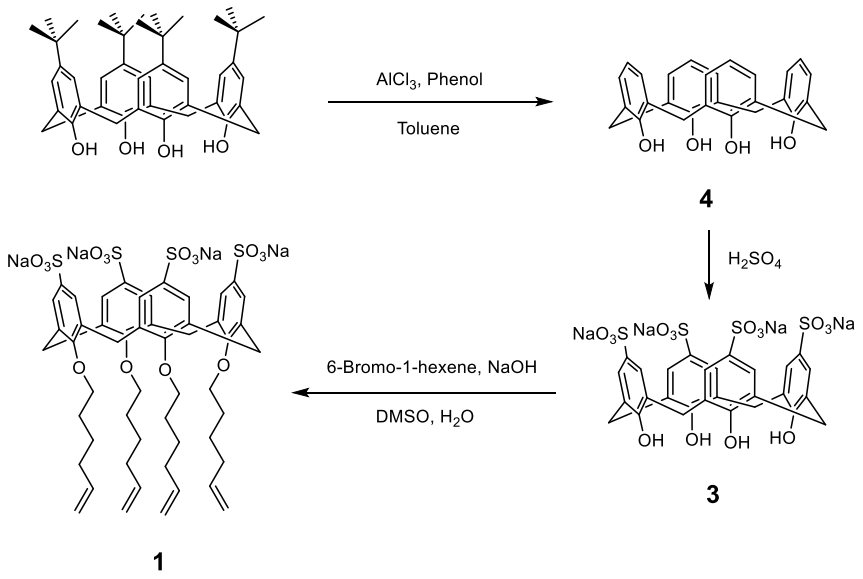
Routine NMR spectra were obtained using a Bruker AV II-400. ^{1}H NMR chemical shifts were measured relative to D_2O as the internal reference (D_2O: δ 4.79 ppm; $CDCl_3$: δ 7.26 ppm). Mass spectrometry (MS) was performed using Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry MALDI-TOF-MS. Particle size and zeta potential were measured using a dynamic light scattering (DLS) analyzer (Malvern Zetasizer Nano ZS90). Fluorescence intensity was measured using a Shimadzu RF-5301PC fluorescence spectrometer. Transmission electron microscopy (TEM) studies were performed using a TecnaiG2F20S-TWIN instrument operating at 120 kV. TEM specimens were prepared by gently placing a carbon-coated copper grid on the surfaces of the samples. The TEM grid was then removed, stained with an aqueous solution of 2% phosphotungstic acid, dried for 0.5 h at room temperature, then subjected to TEM observation. The content of Pt of Pt-cCAV$_{5\text{-FU}}$ was measured by inductively coupled plasma atomic emission spectrometry (ICP-AES, HK-2000). The UV–Vis spectrometry of 5-FU was monitored on a Shimadzu UV-2600 instrument. Gel permeation chromatography (GPC) was performed on a HW-2000 GPC workstation using water as the mobile phase with a flow rate of 0.6 mL min^{-1} at 35°C, and the molecular weights were reported relative to parameter a (0.71) and k (0.011). Human non-small cell lung cancer cells (A549) and cisplatin-resistant human non-small cell lung cancer cells (A549/CDDP) were obtained from the Chinese Academy of Science Cell Bank for Type Culture Collection (Shanghai, China) and used for all cell experiments. The cell lines were grown in Roswell Park Memorial Institute media (RPMI-1640, Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, BI, Israel) and 1% (v/v) penicillin/streptomycin (Gibco, USA) in an incubator with 5% CO_2 at 37°C. Cell toxicity was evaluated by measuring the percentage of cell viability via the MTT assay. Cell viability was measured using a Thermo Varioskan

Flash microplate reader (USA). Cell uptake was measured by flow cytometry (Becton Dickinson, USA). Fluorescence imaging of cells was performed using a confocal laser scanning microscope (CLSM, Leica TCP SP5).

Chemicals: Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification. All solvents for reactions were freshly distilled prior to use. Deionized water was used in all aqueous experiments. 4-Tert-butylcalix[4]arene, 4-hydroxybenzenesulfonic acid sodium salt, ethane-1,2-diyl bis(3-mercaptopropanoate), and 6-bromo-1-hexene were purchased from Tansoole (Shanghai, China). PPh_3AuNTf_2 was purchased from J&K (Beijing, China). Cisplatin, 5-fluorouracil (5-FU), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), chlorpromazine, and genistein were purchased from Aladdin (Shanghai, China). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine (DiI), hochest33323, the BCA Protein Assay Kit, and the Genomic DNA Mini Preparation Kit were purchased from Beyotime (Shanghai, China). Monoclonal antibody against GST-π was purchased from Abcam (Cambridge, UK). Goat anti-mouse secondary antibody conjugated with horseradish peroxidase was purchased from zsBio (Beijing, China). Phloxine B, amiloride, and the Glutathione-S-Transferase (GST) Assay Kit were purchased from Sigma-Aldrich (USA).

Synthesis:

Scheme S1. Synthesis of compound **1**

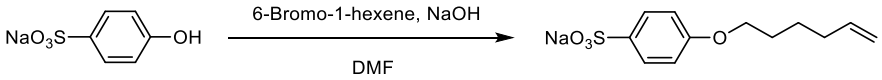


Compound 4.[1] 15 g (23.1 mmol) of *p*-tertbutylcalix[4]arene and 13.2 g of phenol were dissolved in 330 mL of toluene. 24 g (180 mmol) of $AlCl_3$ was added, and the mixture was stirred at 60°C for 6 h. After cooling, 300 mL of HCl (3%, v/v) was added, and the reaction mixture was stirred for 30 min. The aqueous phase was washed with toluene once, and the organic phases were dried with Na_2SO_4 before toluene removal via rotary evaporation. 100 mL of CH_3OH was added to the semisolid residue. After filtration and drying, a white residue was obtained, which was recrystallized from $CHCl_3/CH_3OH$ yielding 9.2 g (21.7 mmol, 93%) of **4**. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 10.20 (s, 4H), 7.04–7.06 (m, 8H), 6.71–6.75 (m, 4H), 4.25 (s, 4H), 3.55 (s, 4H).

Compound 3.[2] **4** was sulfonated by 98% concentrated sulfuric acid. 1 g (2.356 mmol) of **4** was dissolved in 6 mL sulfuric acid and heated at 80°C for 4 h, then the components were added to water without insoluble substances. When reduced to room temperature, the system was quenched with 20 mL saturated salt water droplets in an ice-salt bath, then refluxed for 10 min, placed in a 4°C refrigerator overnight, and the raw product recrystallized in H_2O/CH_3OH to yield **3** (1.1g, 82%). ^{1}H NMR (400 MHz, D_2O, δ): 7.56 (s, 8H), 4.00 (s, 8H).

Compound 1.[3] NaOH (100 mg, 2.5 mmol) was dissolved in 1 mL water and added to a reactor containing **3** (100 mg, 0.12 mmol) with stirring until completely dissolved. 6-bromo-1-hexene (407 mg, 2.5 mmol) was added to 4 mL DMSO and added to the reaction dropwise. The appropriate amount of water was added to clarify the system. After a 24-h reaction at 50°C, a large number of insoluble substances were precipitated and filtered. The product was recrystallized in H_2O/CH_3OH to yield **1** (150 mg, 64%). ^{1}H NMR (400 MHz, D_2O, δ): 7.27 (s, 8H), 5.75–5.85 (m, 4H), 4.92–5.03 (m, 8H), 4.34 (d, *J* = 11.12 Hz, 4H), 3.39 (s, 8H), 3.30 (d, *J* = 13.32 Hz, 4H), 2.09 (d, *J* = 6.68 Hz, 8H), 1.94 (s, 8H), 1.44 (s, 8H).

Scheme S2 Synthesis of compound **5**



Compound 5.[4] To a stirred solution of sodium 4-hydroxybenzenesulfonate (1.96 g, 0.010 mol) and NaOH (0.44 g, 0.011 mol) in DMF (15 mL), 6-bromo-1-hexene (2.61 g, 0.016 mol) was added. The mixture was stirred at 75°C for 20 h, and then concentrated under reduced pressure to 1/3rd of its original volume and filtrated to get the title compound **5** as a white solid (0.75 g, 34%). ^{1}H NMR (400 MHz, D_2O, δ): 7.76 (d, *J* = 8.88 Hz, 2H), 7.05 (d, *J* = 5.16 Hz, 2H), 5.85–5.96 (m, 1H), 5.03–5.10 (m, 2H), 4.09 (t, *J* = 6.52 Hz, 2H), 2.09-2.14 (m, *J* = 7.2 Hz, 2H), 1.74–1.81 (m, 2H), 1.50–1.57 (m, 2H).

Preparation of cCAM: **1** was dissolved in water to a concentration of 2 mM, and 2 equivalents of **2** (the linker: ethane-1,2-diyl bis (3-mercaptopropanoate)) were dissolved in DMF and added into the solution, with the volume of DMF being less than 2.5% of the solution volume. 0.08 equivalent of the catalyst PPh_3AuNTf_2 was dissolved in THF and added into the solution, with the volume of THF being

less than 2.5% of the solution volume. The solution was stirred for 20 h at 50 rpm and 50°C. After 48 h of dialysis, the nanoparticles were purified to obtain cCAM and then lyophilized.

Preparation of Pt-cCAV and Pt-cCAV$_{5\text{-FU}}$. In the dark, **1** was dissolved in water to a concentration of 2 mM, and 1 equivalent of cisplatin was added. Then, 2 equivalents of **2** (the linker: ethane-1,2-diyl bis (3-mercaptopropanoate)) were dissolved in DMF and added into the solution, with the volume of DMF being less than 2.5% of the solution volume. 0.08 equivalent of the catalyst PPh_3AuNTf_2 was dissolved in THF and added into the solution, with the volume of THF being less than 2.5% of the solution volume. The solution was stirred for 20 h, avoiding light, at 50 rpm and 50°C. After 48 h of dialysis, the nanoparticles were purified to obtain Pt-cCAV and then lyophilized. The crosslinking degree was estimated by calculating the percentage of consumed double bonds. The Pt-cCAV$_{5\text{-FU}}$ was obtained with the same procedure, except for employing a solution containing 5-FU instead of pure water.

The calculation for cisplatin loading capacity of Pt-cCAV and Pt-cCAV$_{\mathbf{5\text{-FU}}}$ was carried out by re-dissolved the lyophilized powder of Pt-cCAV or Pt-cCAV$_{\mathbf{5\text{-FU}}}$ in water, the content of Pt element in the powder was obtained by ICP-AES. The encapsulation amount of cisplatin in unit w/w% can be calculated.

Pt-cCAV$_{5\text{-FU}}$ was prepared in 5-FU aqueous solution at a set concentration and volume. During the dialysis purification, the unencapsulated amount of 5-FU was calculated using a 5-FU UV-Vis absorption standard curve (**Figure** S3). The encapsulation efficiency of 5-FU was equal to the encapsulation percentage EN%

$$EN\% = (1-Cf/Ct)\times 100\%$$

Cf was the amount of free drug, and Ct was the total amount of added drug. In addition, the loading capacity LC% can be calculated in w/w%

$$LC\% = (Ct-Cf)/Cp$$

Cp was the total amount of Pt-cCAV$_{5\text{-}FU}$ lyophilized powder. The mass ratio and molar ratio of cisplatin to 5-FU in Pt-cCAV$_{5\text{-}FU}$ can be obtained by combining the data of cisplatin loading capacity of Pt-cCAV$_{5\text{-}FU}$

The content of cisplatin ($W_{cisplatin}$) and 5-FU in the lyophilized powder of Pt-cCAV$_{5\text{-}FU}$ can be calculated according to the procedure introduced above and the quality of pure carrier ($W_{carrier}$) of Pt-cCAV$_{5\text{-}FU}$ can be deduced. Given an 81% crosslinking degree (**Figure** S4), the approximate pure carrier composition of **1:2** = 1 : 0.81 × 2. The molar loading ratio of cisplatin to **1** (*x*) can thus be calculated by the following formula (cisplatin has MW 300.05, **1** has MW 1161.24, and **2** has MW 238.32):

$$(300.05 \times x) / (1 \times 1161.24 + 0.8 \times 2 \times 238.32) = W_{cisplatin}/W_{carrier}$$

For example, with a cisplatin loading capacity of 13% (w/w%) and 5-FU encapsulation efficiency of 19%, the molar loading ratio of cisplatin to **1** was calculated as 77%.

Phloxine B leakage assay: The structure of the Pt-CAV was validated by the well-established Phloxine B leakage assay, where Phloxine B was trapped inside the large vesicles at a high concentration that gave significant self-quenching; after the addition of Titron X-100 aqueous solution to lyse the vesicles, all entrapped contents were released, leading to a sharp increase of fluorescence at 575 nm. Under dark conditions, **1** was added to a solution of Phloxine B (0.5 mg mL^{-1}) at a concentration of 2 mM, followed by adding 1 equivalent of cisplatin. After gently stirring at room temperature, a portion (0.5 mL) of the obtained solution was passed through a Sephadex G-50 column

using Milli-Q water as the eluent to remove the extravesicular Phloxine B. The nanoparticle fractions were combined and diluted to 5.0 mL with the same deionized water. The fluorescence at 575 nm (λ_{ex}=530 nm) was recorded. A 50-μL solution of Triton X-100 (1%, v/v) was added to lyse the nanoparticles, and the fluorescence (λ_{em} = 575 nm) was measured again. As shown in **Figure** S2B, the sharp increase of fluorescence at 575 nm suggested the formation of vesicular structures, while little increase in fluorescence hints at the formation of a micelle-like structure.

***In vitro* stability assay.** The lyophilized crosslinked vesicles (Pt-cCAV) was dissolved in water to a concentration of 1 mg mL^{-1}. The non-crosslinked vesicle (Pt-CAV) solution was prepared at the same concentration. The above two groups were diluted seven times exponentially. The particle size of each dilution was measured by DLS.

The FBS stability of crosslinked vesicles (Pt-cCAV) and non-crosslinked vesicle (Pt-CAV) was investigated by incubation with 10% (V/V) FBS. Briefly, 9 mL of Pt-cCAV solution (1 mg mL^{-1}) and Pt-CAV solution (1 mg mL^{-1}) were mixed with 1 mL FBS, respectively. The particle size at 0 h and after 1h of incubation at 37 ^{o}C was recorded by DLS to evaluate their stability.

***In vitro* cytotoxicity**: The MTT assay was used to estimate the anticancer activities of all tested groups against A549 and A549/CDDP cells. Briefly, cells were seeded in 96-well plates (5×10^3 cells per well) and cultured at 37°C/5% CO_2. After 24 h, culture media was removed, and fresh media (200 μL) containing various concentrations of all tested groups was added for 48 h. After treatment, culture media was removed, and fresh media (200 μL) containing MTT solution (40 μL, 5 mg mL^{-1}) was added to each well, and the plates were incubated at 37°C for another 4 h. After incubation, the culture media was removed, and formazan crystals were then dissolved in 150 μL dimethyl sulfoxide per well and added. The absorbance of the solution was measured at 490 nm using a microplate reader (Varioscan

Flash). Cell viability was calculated according to the following formula: cell viability (%) = A_{490} (sample)/A_{490} (control) × 100%. The IC_{50} was calculated by Calcusyn software. All nanodrugs used in *in vitro* cytotoxicity were converted into its payloads to calculated the drug concentration.

Nanoparticle uptake inhibition by various inhibitors: A549 cells (1×10^5 cells per well) were seeded in 6-well plates and cultured for 24 h. Then the cells were treated with medium containing genistein (200 μM), chlorpromazine (10 μg mL^{-1}), or amiloride (50 μM) at 37°C respectively with DiI-labeled Pt-$cCAV_{5\text{-}FU}$ at a Pt concentration of 50 μM ([DiI] = 5 μg mL^{-1}, λ_{ex} = 549 nm, λ_{em} = 565 nm) for 4 h. The uptake with no inhibitors at 37°C was set as a positive control, and the group treated at 4°C was set as a negative control. The cells were then washed with PBS three times and then harvested to lyse with cell lysis buffer. The fluorescence intensities of cell lysates at 565 nm were measured.

Flow cytometry of cellular uptake of nanoparticles: A549 and A549/CDDP cells (1×10^5 cells per well) were seeded in 6-well culture plates and allowed to incubate at 37°C/5% CO_2 for 36 h. Subsequently, the culture medium was replaced by 2 mL of fresh medium containing equal amounts of DiI-labeled Pt-$cCAV_{5\text{-}FU}$ at a Pt concentration of 50 μM ([DiI] = 5 μg mL^{-1}, λ_{ex} = 549 nm, λ_{em} = 565 nm), while untreated cells were set as the control. At time intervals of 2, 6, or 12 h, cells were washed with PBS three times, collected, and analyzed by flow cytometry.

Confocal laser scanning microscopic imaging: A549 cells (5×10^4 cells mL^{-1}) were seeded in Ø = 35 mm glass Petri dishes and incubated at 37°C/5% CO_2 for 24 h. Subsequently, the cells were cultured with DiI-labeled Pt-$cCAV_{5\text{-}FU}$ at a Pt concentration of 50 μM ([DiI] = 5 μg mL^{-1}, λ_{ex} = 549 nm, λ_{em} = 565 nm) at 37°C for 1, 2, or 4 h, respectively. The culture medium was removed, and the cells were washed three times with PBS. Then the nuclei were stained by Hoechst 33342 (λ_{ex} = 350 nm, λ_{em} =460

nm) for 15 min. The medium was removed and washed three times with PBS, and another 1 mL PBS was added. The cellular uptake was observed under CLSM.

Analysis of Pt-DNA adduct formation: A549 and A549/CDDP cells were grown at a concentration of 10^6 cells in 100 mm^2 cell round culture dishes for 24 h. Then cells were incubated with fresh medium containing Pt-cCAV or Pt-$cCAV_{5\text{-}FU}$ at platinum concentrations of 50 μM, respectively. After 12 h, the culture medium was removed, and the cells were washed three times with PBS and harvested. The intracellular genomic DNA was extracted using a Genomic DNA Mini Preparation Kit. The DNA concentration was determined by UV-Vis spectroscopy at 260 nm. After digestion, the amount of platinum was analyzed by ICP-AES.

Western blot analysis of GST-*π*: A549/CDDP cells were seeded in 6-well plates (1×10^5 cells per well) and cultured at 37°C/5% CO_2. Then the cells were harvested after incubation with cisplatin, Pt-cCAV, or Pt-$cCAV_{5\text{-}FU}$ at platinum concentrations of 50 μM, respectively, for 12 h. The control group had no drug added. Then cells were lysed with radio immunoprecipitation assay (RIPA) buffer containing a protease inhibitor and centrifuged at 10000 rpm for 30 min at 4°C. After centrifugation, supernatants were collected, and the protein concentration was determined using a BCA Protein Assay Kit. Then 40 μg of total protein was separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for 1 h and then incubated with monoclonal antibody against GST-π (1:200) overnight at 4°C. Then the membranes were washed three times for 5 min each with 15 mL of Tris-buffered saline Tween (TBST) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000). An enhanced ECL Western Blotting Detection Kit was used to

detect the luminescence by a ChemiDoc XR+UV illuminator (Bio-Rad, USA). β-actin was used as the loading control.

GST activity test: A549/CDDP cells were seeded in 6-well plates (1×10^5 cells per well) and cultured at 37°C/5% CO_2. Then the cells were incubated with cisplatin, Pt-cCAV, and Pt-cCAV$_{5\text{-FU}}$ at platinum concentrations of 50 μM respectively for 12 h; the control group had no drug added. Then the culture medium was removed, cells were washed with PBS three times, and the cells were collected with a rubber policeman. After 10 min of centrifugation at 4°C and 1000 rpm, the cells were re-suspended in 40 μL cold buffer (100 mM potassium phosphate, pH 7.0, including 2 mM EDTA) containing protease inhibitor. The cells were lysed on ice, and the supernatant was used for analysis after centrifugation at 10,000 rpm for 15 min at 4°C. The Glutathione-S-Transferase (GST) Assay Kit was used to detect enzyme activity, and the protein content was measured using a BCA Protein Assay Kit.

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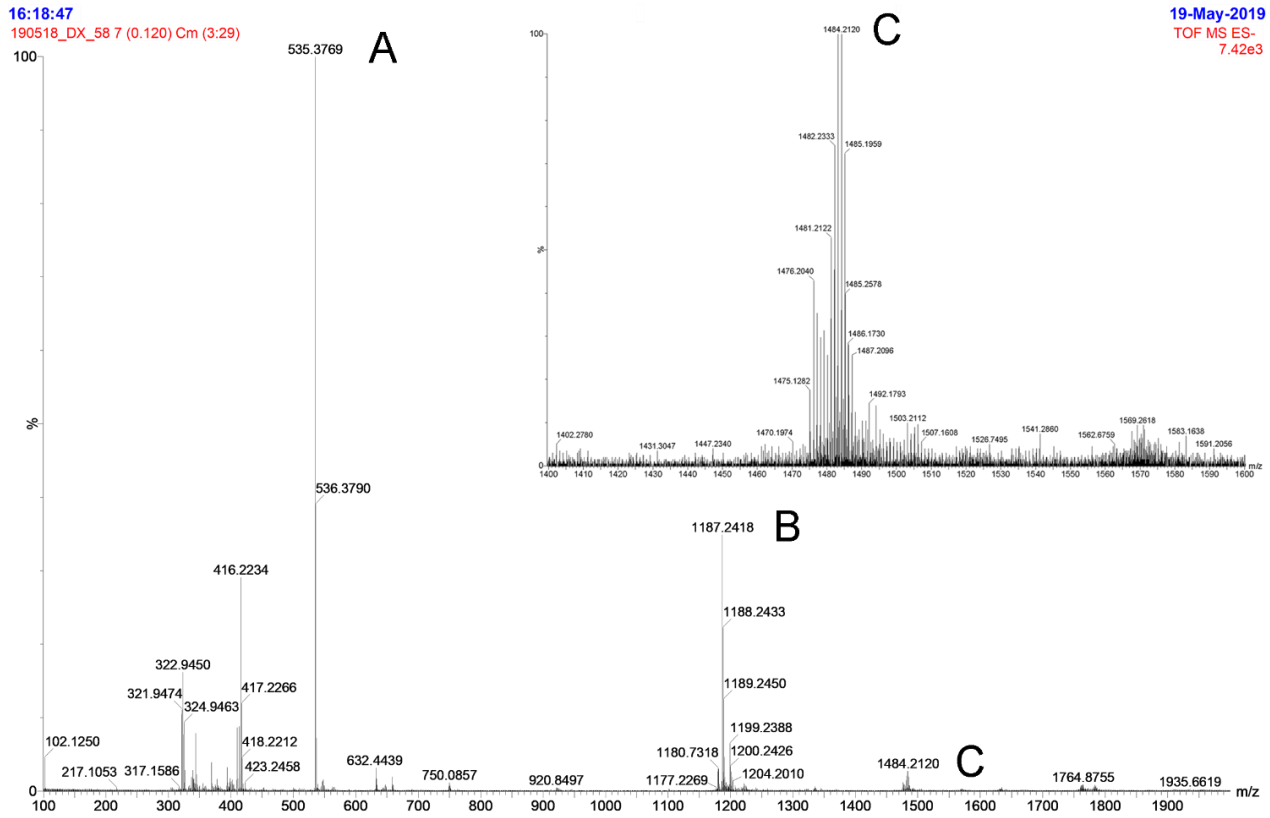

Figure S1 The MALDI-TOF-MS spectrum (negative mode) of cisplatin and **1** at a ratio of 1:1. Peaks are assigned as: **A**, 535.1455 *m/z* [**1**-4Na^++2H^+]$^{2-}$; **B**, 1187.1662 *m/z* [**1**-4Na^++3K^+]$^-$; **C**, 1484.1237 *m/z* [**1**-4Na^+ + 3K^+ + Cisplatin]$^-$.

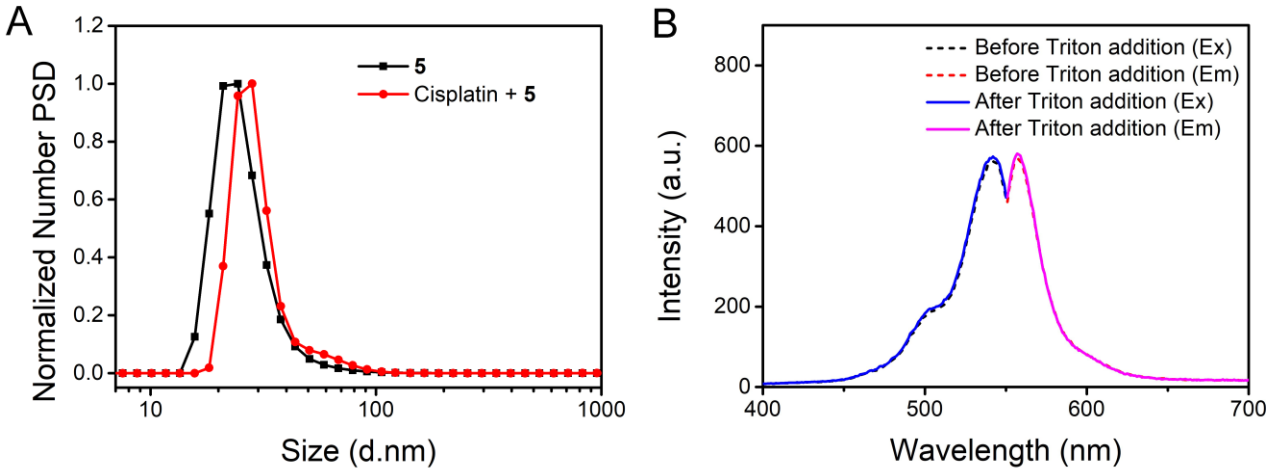

Figure S2 Size distributions of hydrodynamic diameters of **5** and [cisplatin + **5]** (A). Fluorescence leakage experiment of [cisplatin + **5**] (molar ratio = 1:4); little increase in fluorescence hints at the formation of micelle-like structures (B). [**5**] = 8 mM, [cisplatin]=2 mM.

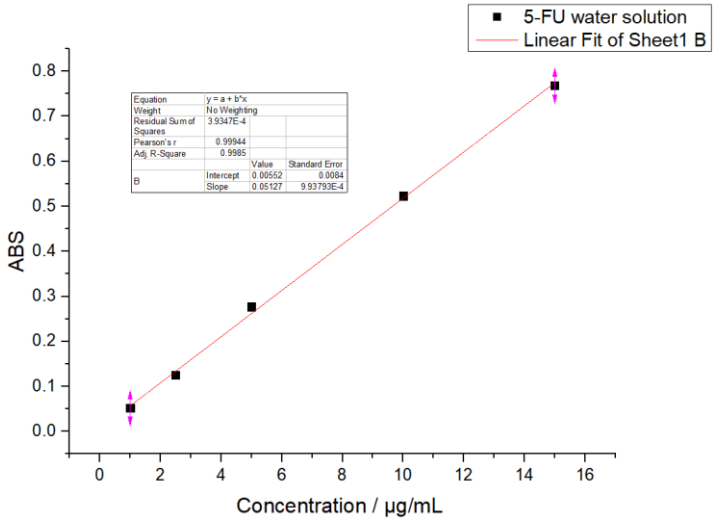

Figure S3 UV-Vis absorbance standard curve of a 5-FU water solution

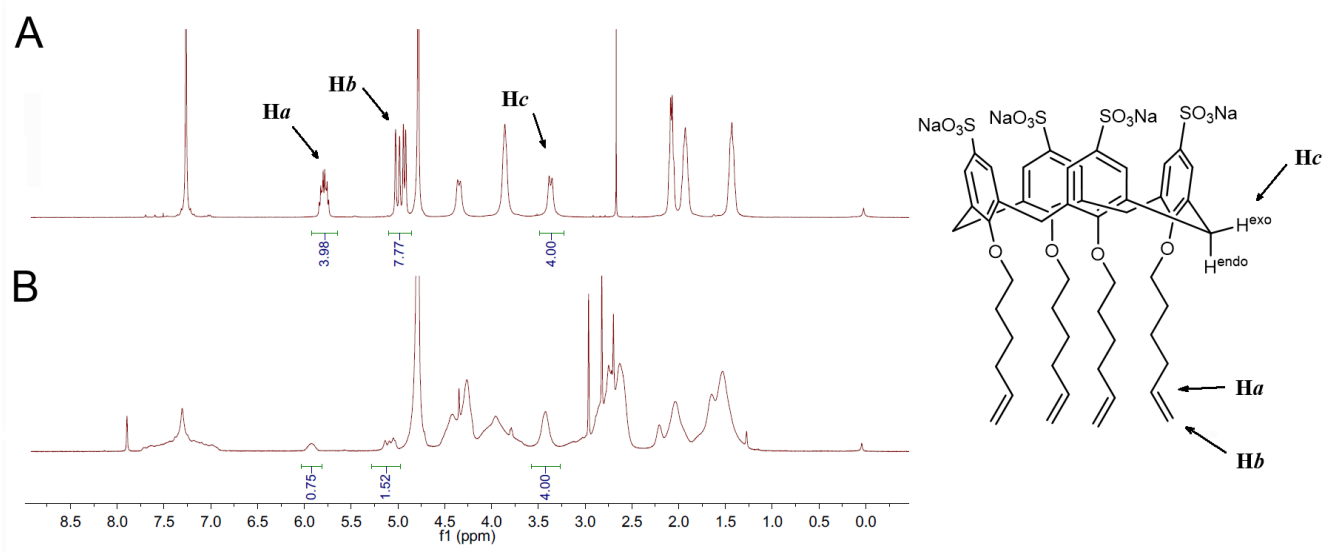

Figure S4 ^{1}H NMR spectra of **1** (A) and Pt-cCAV (B) in D_2O. The crosslinking degree was calculated to be about 81%.

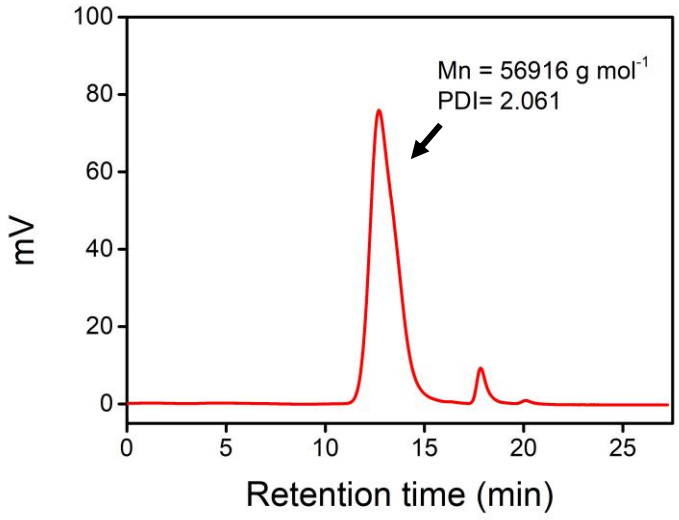

Figure S5. GPC curve of Pt-cCAV

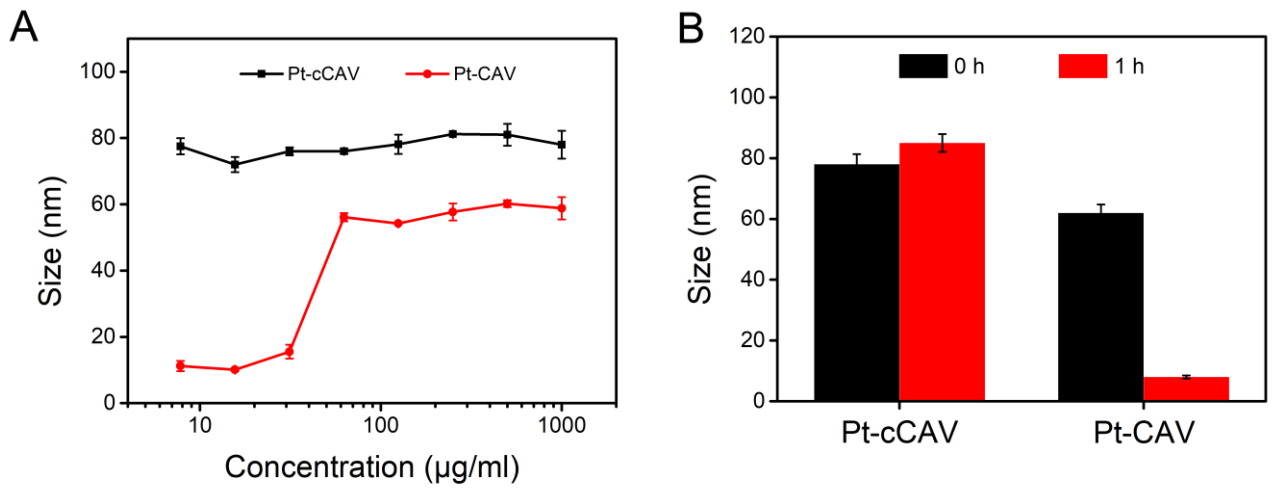

Figure S6 Particle sizes of Pt-cCAV and Pt-CAV as a function of their concentration in water (A). Particle sizes of Pt-cCAV and Pt-CAV after incubation with 10% FBS at 37°C for 1 h (B).

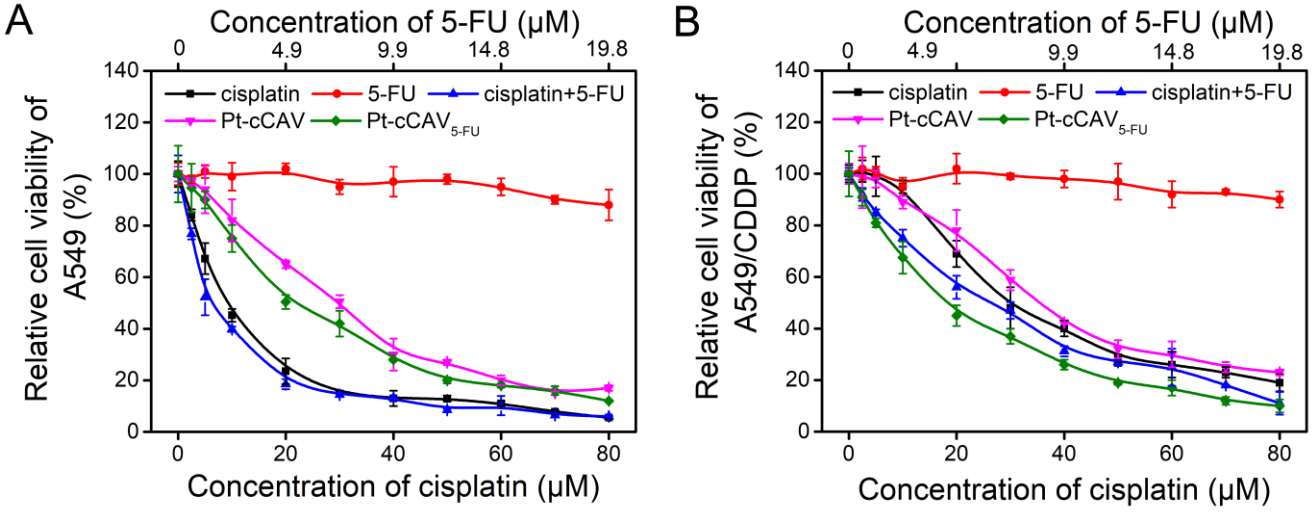

Figure S7 Cytotoxicity of cisplatin, 5-FU, [cisplatin + 5-FU], Pt-cCAV, and Pt-cCAV$_{5\text{-FU}}$ to A549 (A) and A549/CDDP (B) cells for 48 h.

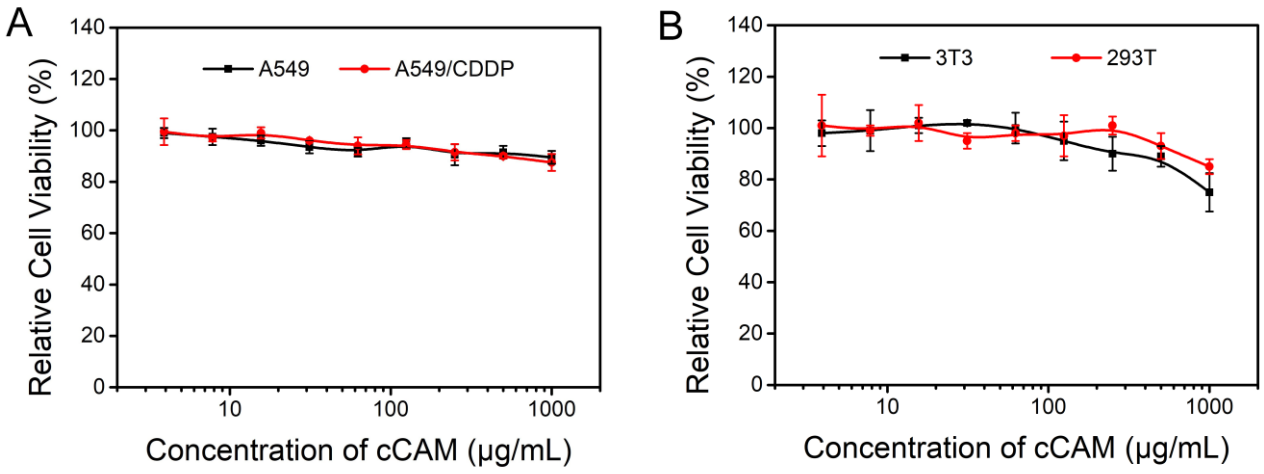

Figure S8 Cytotoxicity of cCAM to A549 and A549/CDDP cells for 48 h (A) and to 3T3 and 293T (B) cells for 48 h.

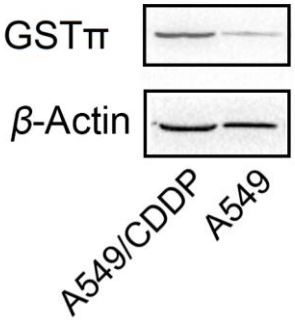

Figure S9 GST-π protein expression levels in A549 and A549/CDDP cells.

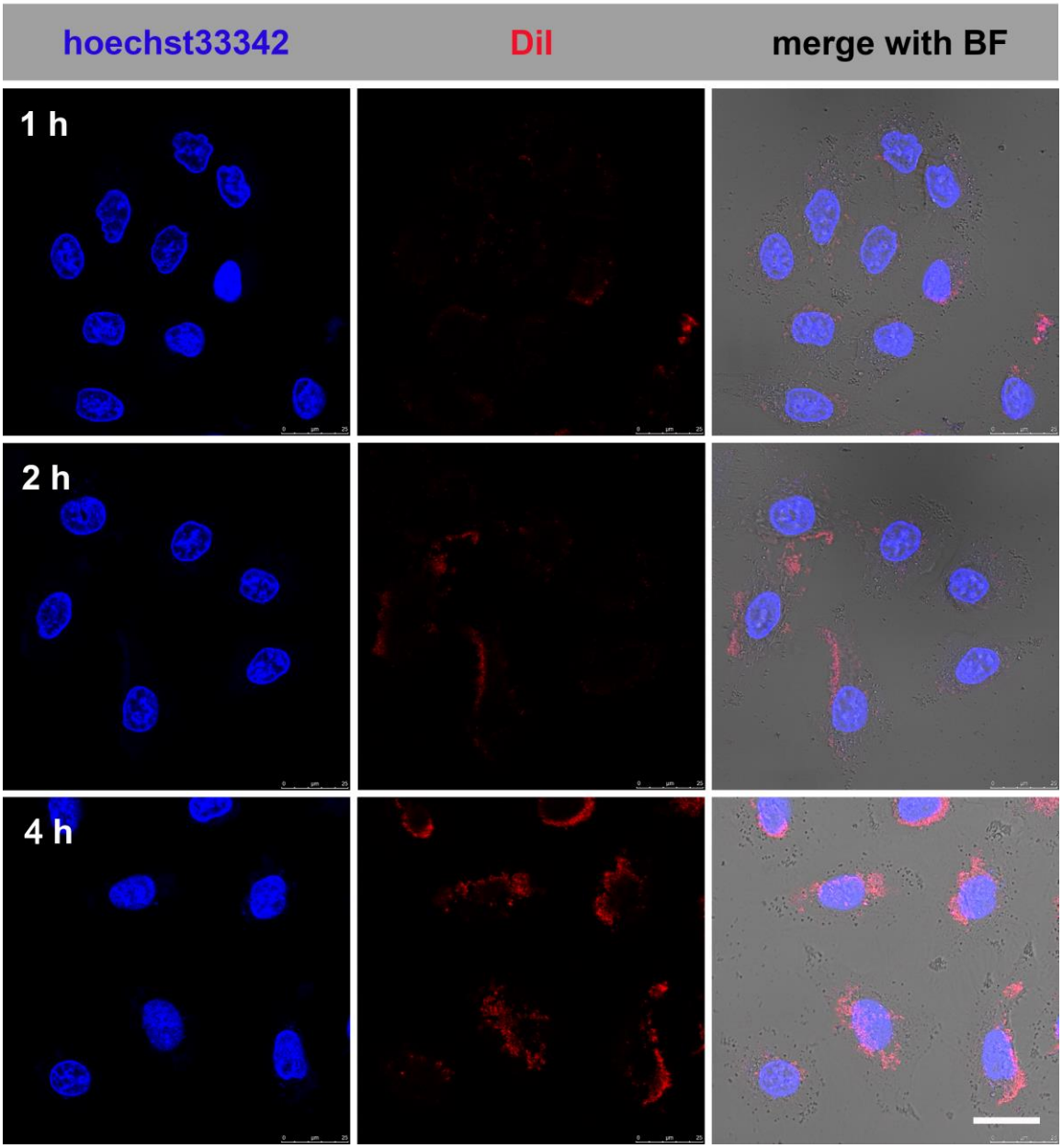

Figure S10 Uptake of DiI-labeled Pt-cCAV$_{5\text{-FU}}$ by A549 cells at 1, 2, and 4 h by CLSM.

Table S1 Effect of different ratios of cisplatin on particle size and polydispersity of Pt-CAV, and corresponding final cisplatin loading of Pt-cCAV

Cisplatin/**1** (molar ratio)	Particle size (nm)	Polydispersity index	Pt loading (w/w%)
0.25	31.2 ± 5.3	0.538 ± 0.112	—
0.5	37.7 ± 15.1	0.557 ± 0.171	—
0.75	40.9 ± 11.2	0.384 ± 0.085	—
1.0	58.8 ± 0.3	0.168 ± 0.031	12.4% ± 0.42
1.5	58.4 ± 0.5	0.191 ± 0.009	11.8% ± 0.94
2.0	57.3 ± 0.6	0.142 ± 0.011	12.4% ± 1.88

[**1**] = 2 mM; Pt loading was calculated according to: Pt loading = weight of encapsulated cisplatin/weight of Pt-cCAV × 100%

Table S2 Effect of different ratios of 5-FU on particle size and polydispersity of Pt-CAV$_{5\text{-FU}}$, and the encapsulation efficiency of 5-FU in Pt-cCAV$_{5\text{-FU}}$

5-FU/**1** (molar ratio)	Particle size (nm)	Polydispersity index	Encapsulation percentage (%)
0.25	59.1 ± 0.4	0.171 ± 0.043	9.7% ± 2.35
0.5	58.4 ± 0.2	0.135 ± 0.071	14.4% ± 1.14
0.75	58.4 ± 0.1	0.162 ± 0.012	16.3% ± 0.62
1.0	57.2 ± 1.1	0.158 ± 0.022	19.2% ± 0.88
1.5	66.2 ± 4.5	0.252 ± 0.074	19.6% ± 0.28
2.0	140.7 ± 33.1	0.681 ± 0.133	—

[**1**] = 2 mM, [cisplatin] = 2 mM, encapsulation percentage EN% = (1-Cf/Ct) × 100%; Cf is the amount of free drug, and Ct is the total amount of drugs added.

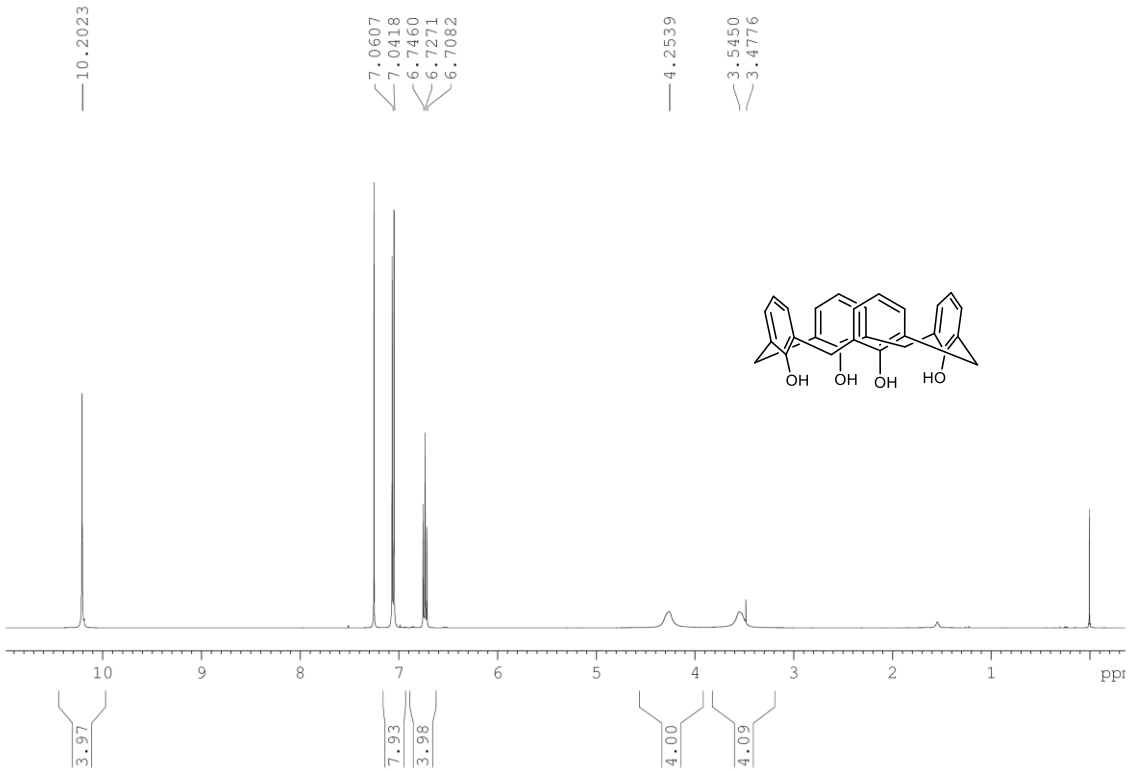

Figure S11 ^{1}H NMR spectrum of compound **4**

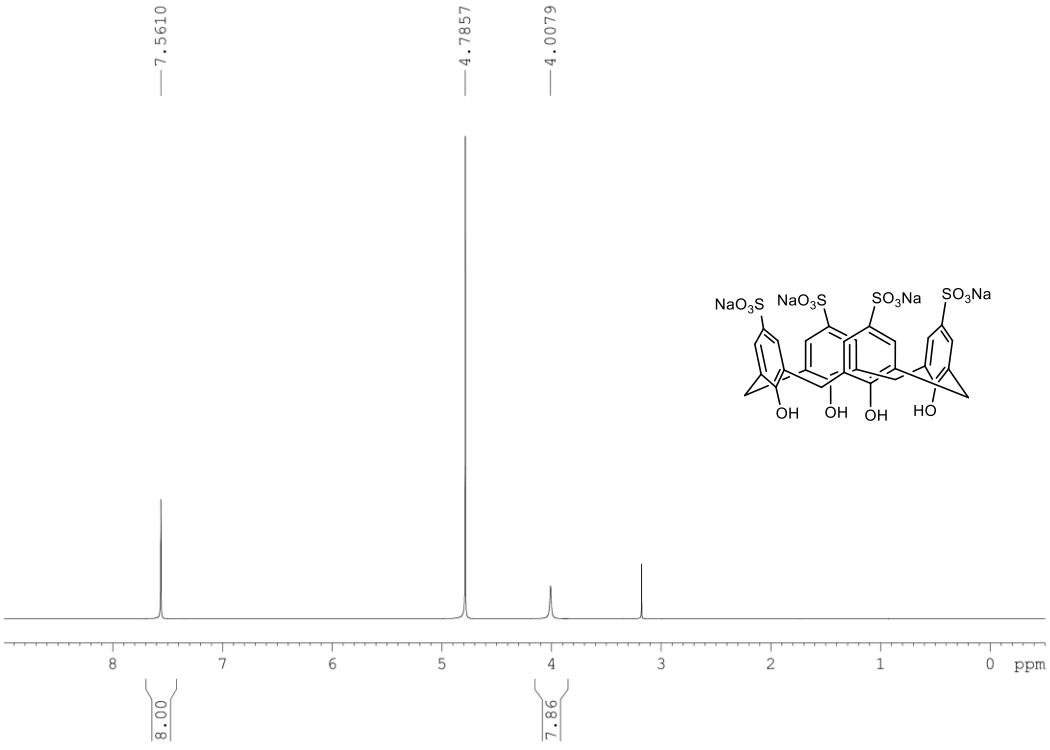

Figure S12 ^{1}H NMR spectrum of compound **3**

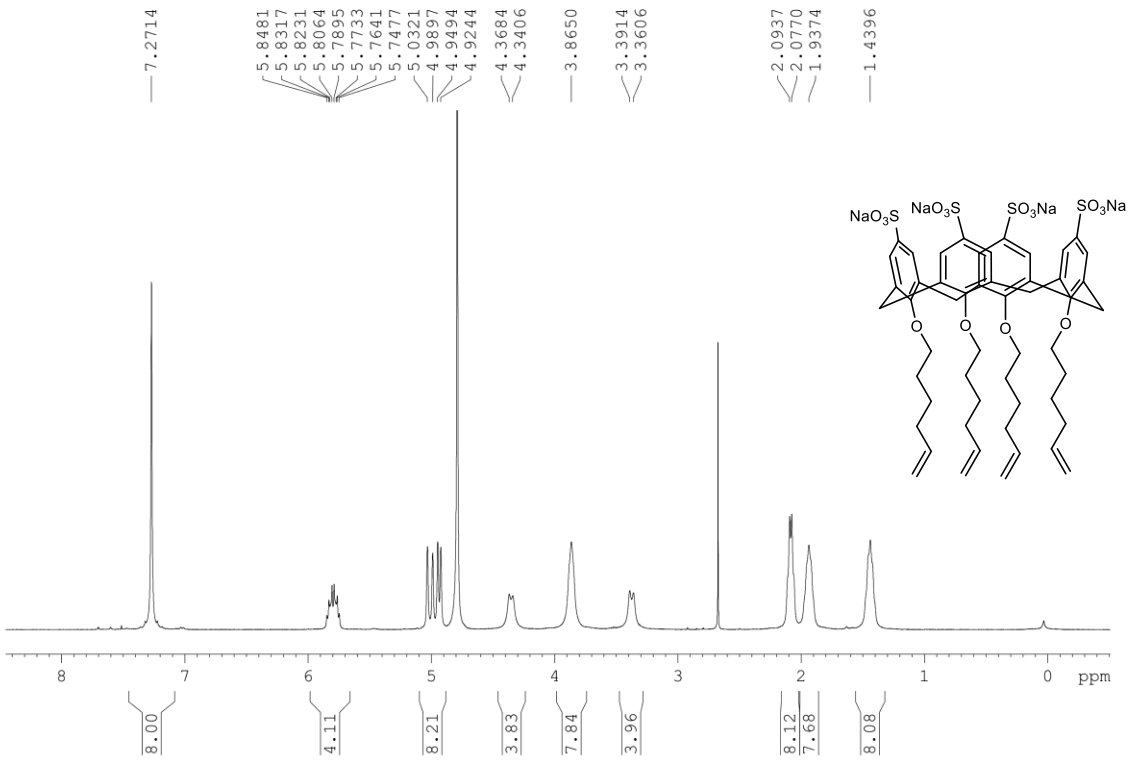

Figure S13 ^{1}H NMR spectrum of compound **1**

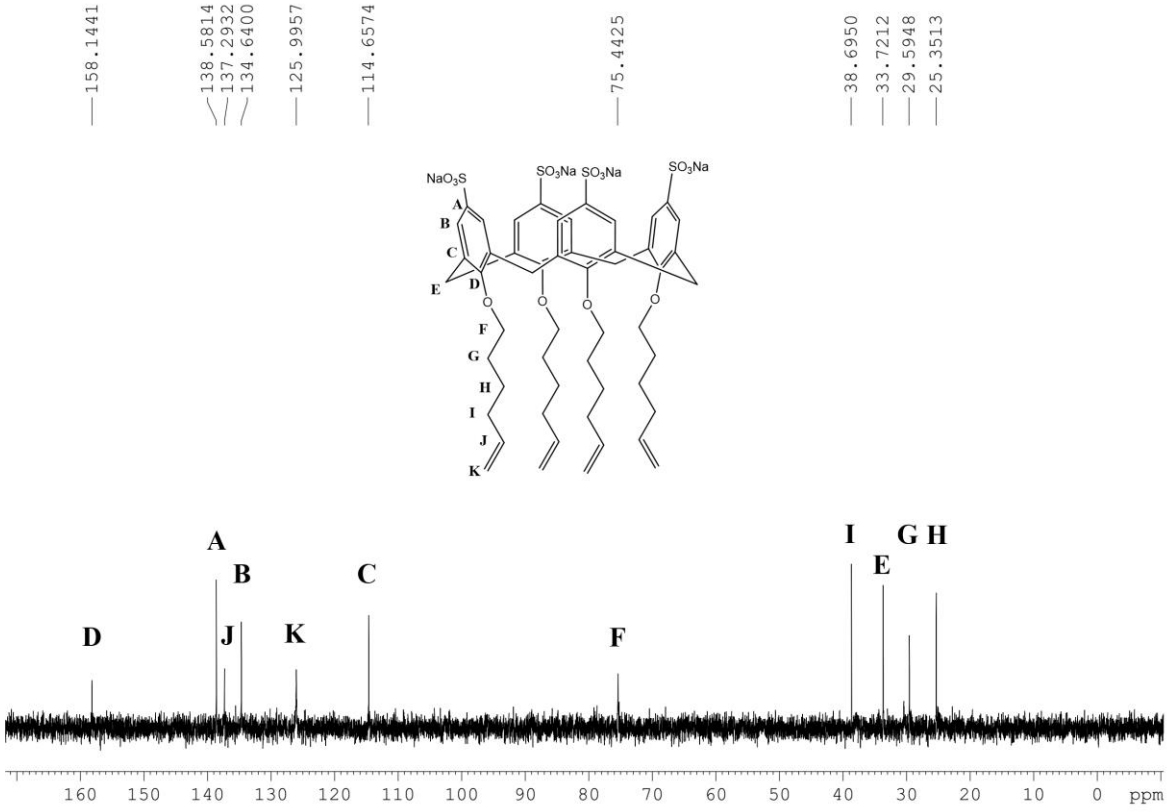

Figure S14 ^{13}C NMR spectrum of compound **1**

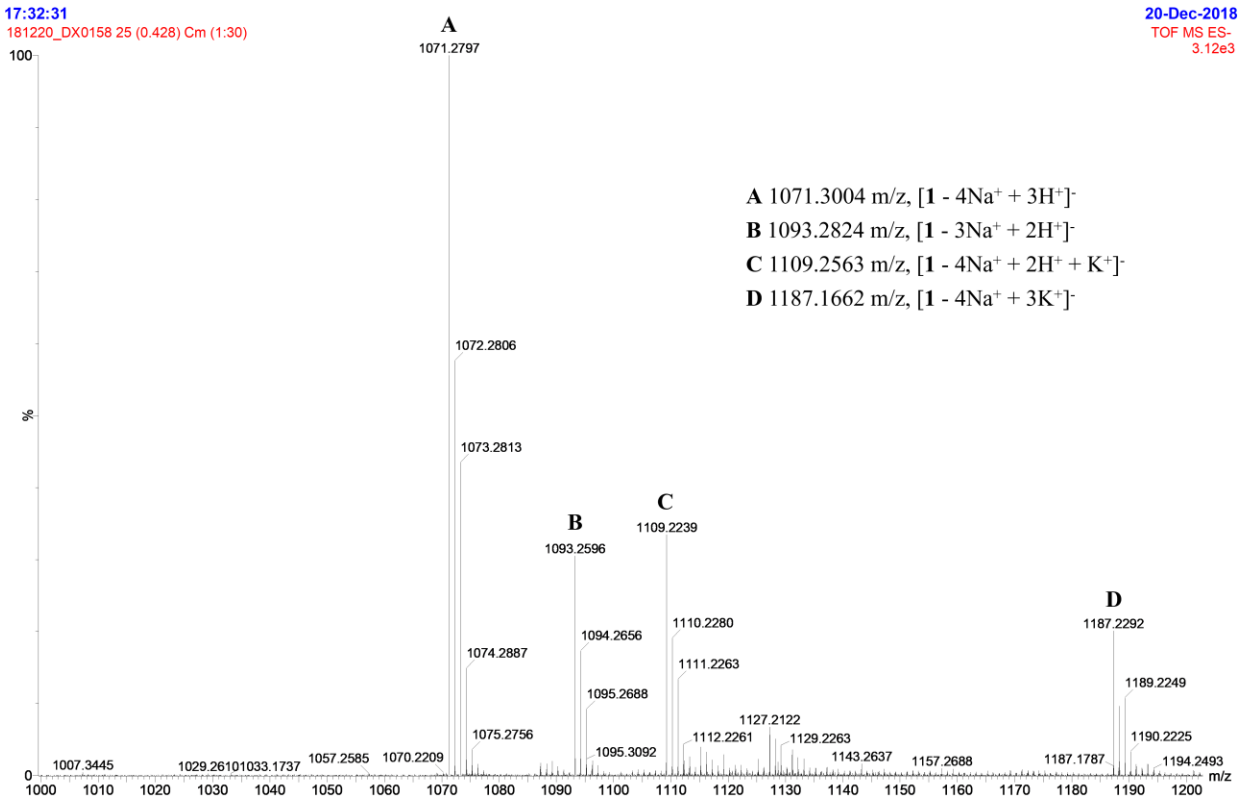

Figure S15 The MALDI-TOF-MS spectrum (negative mode) of compound **1 .** Peaks are assigned as: A, 1071.3004 *m/z* [**1**-4Na^++3H^+]$^-$; B, 1093.2834 *m/z* [**1**-3Na^++2H^+]$^-$, C, 1109.2563 *m/z* [**1**-4Na^++2H^++K^+]$^-$; D, 1187.1662 *m/z* [**1**-4Na^++3K^+]$^-$

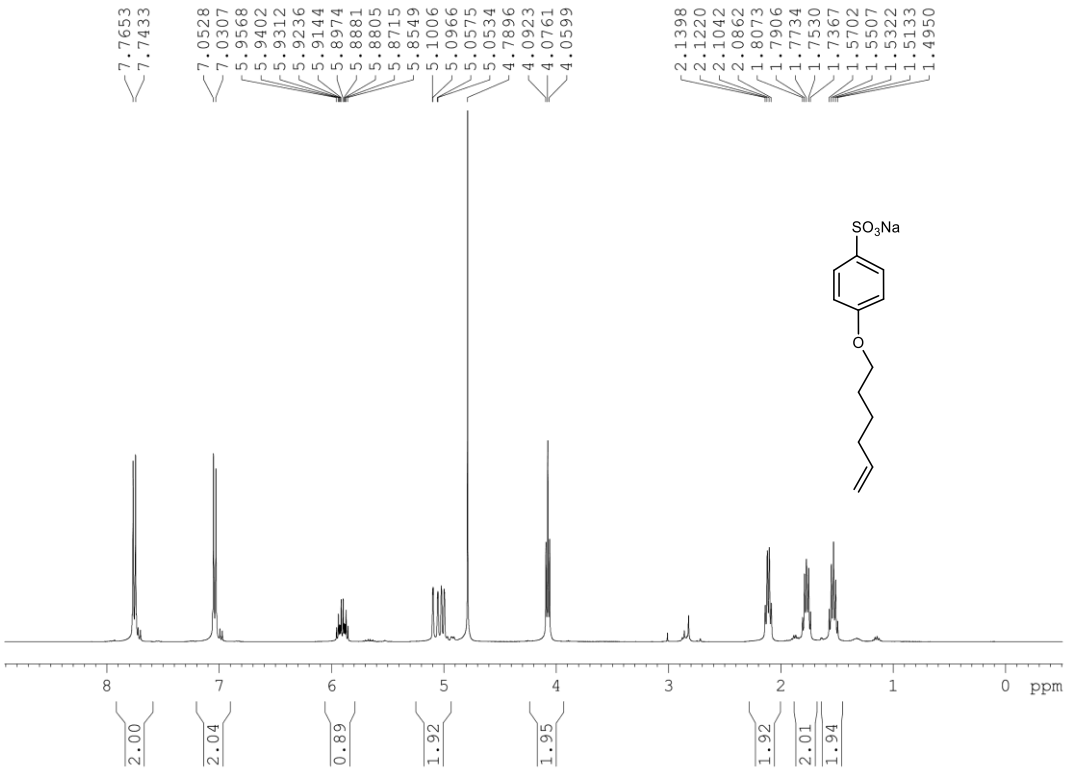

Figure S16 ^{1}H NMR spectrum of compound **5**