

Nanoparticles of Metal-Organic Cages Designed to Encapsulate Platinum-Based Anticancer Agents

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Experimental Section

General information. *c,c,t*-[Pt(NH₃)₂Cl₂(ethyl carbamate)(succinate)] was prepared as previously reported.¹ All reagents were purchased from Strem, Aldrich or Alfa and used without further purification. Methoxy polyethylene glycol-*block*-polyglutamic acid (MPEG_{5k}-PGA₃₀) (**3**) was purchased from Polypeptide Therapeutic Solutions SL (Valencia, Spain). All reactions were carried out under normal atmospheric conditions. Deuterated solvents were purchased from Cambridge Isotope Laboratory (Andover, MA). ¹H and ¹³C NMR spectra were recorded on a Bruker AMX 400 NMR in the Department of Chemistry and Biochemistry at Kent State University. Chemical shifts in ¹H and ¹³C{¹H} NMR spectra were internally referenced to solvent signals (¹H NMR: acetone at δ = 2.05 ppm; DMSO at δ = 2.50 ppm; ¹³C NMR: DMSO at δ = 40.45 ppm). UV-Vis spectra were recorded on a VWR UV-1600PC scanning spectrophotometer. Fluorescence spectra were obtained on a Cary Eclipse fluorescence spectrophotometer. Graphite furnace atomic absorption spectroscopic (GFAAS) measurements were taken on a PerkinElmer PinAAcle 900Z spectrometer. Fluorescence images were acquired using an Olympus IX70 inverted epifluorescence microscope equipped with a digital CCD camera (QImaging). Images were processed and intensities were quantified with ImageJ software (NIH). Dynamic light scattering and zeta-potential analysis were carried out using a Horiba SZ-100 particle analyzer. 3D computational modeling was performed using Schrödinger Macromodel Suite based on MMFF force field. Flow cytometry was carried out on a BD

Bioscience Accuri C6 flow cytometer. Cryo-TEM specimens were prepared using thin-film plunge freezing in a FEI Vitrobot at Liquid Crystal Institute at Kent State University. The vitrified specimens were mounted onto a Gatan 626.DH cryo-holder and transferred into a FEI Tecnai F20 TEM. Cryo-TEM observation was performed using low-dose mode. The detailed experimental setup and procedure are reported previously.²

Synthesis of the metal-organic cage (1). Synthesis of the MOC was modified from the previous report.³ Pt(en)(NO₃)₂ (13.6 mg, 36 μmol) and 2,4,6-Tri(4-pyridyl)-1,3,5-triazine (7.6 mg, 24 μmol) were mixed in a microwave reaction vial (2-5 mL), followed by the addition of 4-mL H₂O and 4-μL of 65% HNO₃. The reaction vial was then sealed and placed in a Biotage Initiator Microwave Synthesizer. The mixture was stirred at 900 rpm and heated using a multi-step sequence (a. 70 °C, 100 W for 15 min; b. 150 °C, 150 W for 60 min; c. 170 °C 150 W for 60 min). The mixture was cooled to R.T., and then the solvent was evaporated under reduced pressure. To the solid was added 1 mL of water. A volume of 6 μL of 65% HNO₃ was added to the clear aqueous solution, and the product was formed as a white precipitate. Product was collected by centrifugation, washed with 1-mL 1% HNO₃ and acetone, and dried in vacuum. Yield: 15 mg, 72%. ¹H NMR (400 MHz, D₂O) δ 8.98 (d, *J* = 4.7 Hz, 24H, α-Pyridine), 8.46 (d, *J* = 4.8 Hz, 24H, β-Pyridine), 2.72 (s, 24H, en).

Synthesis of the fluorescein-conjugated Pt(IV) prodrug (2) As shown in Figure S2, the fluorescein-conjugated Pt(IV) prodrug was prepared via the amide-bond-formation reaction using *c,c,t*-[Pt(NH₃)₂Cl₂(ethyl carbamate)(succinate)] (**6**) and an amino-fluorescein derivative (**7**). Both **6** and **7** were prepared according to the reported procedure.^{1,4} To a 1.6-mL DMF solution of the Pt(IV) precursor (**6**) (83 mg, 0.079 mmol) was added a 1.6-mL DMF solution of HATU (33 mg, 0.088 mmol). The mixture was stirred at R.T. for 1 h, and a fluorescein derivative (**7**) (40 mg, 0.089 mmol) was then added to the mixture, followed by stirring at R.T. for an additional 1.5 h. Finally, DIPEA (28 μL, 0.16 mmol) was added to the mixture, and the solution was stirred for 22 h. A 32-mL portion of a saturated aqueous NaCl solution was added to the reaction mixture and an orange precipitation was obtained. The precipitate was washed with 1.5 mL water twice. The resulting orange solid **1** was dried in the lyophilizer overnight. Yield: 47 mg (63 %). ¹H NMR (400 MHz, DMSO-d₆): δ: 10.3-9.6 (m, 4H, CONH), 8.3 (s, 1H, Ar¹-H), 8.3 (m, 1H, phenol OH), 8.0 (m, 1H, phenol OH), 7.8(d, 1H, Ar^{2,3}-H), 7.2(m, 1H, (d, 1H, Ar^{2,3}-H), 6.8-6.4 (m, 12H, Ar-H

and two NH₃), 3.6 (m, 2H), 3.3-3.2 (q, 2H), 3.0-2.9 (q, 2H), 2.5-2.5(m, 2H succinate), 2.4-2.3 (m, 2H succinate), 1.0 (t, 3H, CH₃); ¹³C NMR (400 MHz, DMSO-d₆): δ: 181.2, 180.4, 172.4, 169.0, 164.2, 161.6, 160.0, 159.0, 152.5, 152.4, 147.6, 141.7, 130.3, 129.5, 127.0, 124.5, 117.1, 113.3, 110.8, 110.2, 102.7, 83.7, 43.9, 38.3, 36.3, 36.0, 32.0, 31.7, 15.9. HRMS (Positive mode) for C₃₀H₃₄Cl₂N₆O₁₀PtS: *m/z* [M + H]⁺ calcd: 936.1157; obsd: 936.1159.

High-resolution mass spectrometry (HRMS) measurements. High resolution mass spectra of the metal-organic cage, fluorescein, and the host guest complex (*cf.* Figure S5) were obtained with an Orbitrap-based mass spectrometer (Exactive Plus, Thermo Scientific, Bremen, Germany) equipped with a heated electrospray ionization (ESI) source (HESI II probe, Thermo Scientific, Bremen, Germany). Precursors and products were dissolved in LC-MS grade methanol (Optima, Fisher Scientific, Pittsburgh, PA, USA) and water (75:25) at a concentration of ~64 μM. These solutions were introduced into the ESI source in via flow injection. A carrier flow of methanol and water (75:25) at a flow rate of 50 μL/min was supplied with a syringe pump (Fusion 100T, Chemyx, Inc., Stafford, TX, USA). A Rheodyne six-port valve (Model MXT715-000, IDEX Health and Science, Rohnert Park, CA) equipped with a 5-μL sample loop was used to inject the sample in the continuous flow of carrier solution. The ESI source was operated at 3.5 kV with a sheath-gas flow rate of 10 (manufacturers units). To prevent degradation of the metal-organic cage during the ionization and ion-transfer processes, the heater on the source was turned off and the inlet of the mass spectrometer was held at 100 °C. Mass spectra were recorded in the positive-ionization mode with a scan range of *m/z* 300 - 1500, a mass resolving power setting of 140,000, and an automatic gain control (AGC) target value of 1×10⁶ ions. To ensure very high mass accuracy, found to be better than 1 mmu, the instrument was mass calibrated daily and a lock mass of *m/z* 371.10124, due to polysiloxane, was used throughout. All mass-spectral data were collected and processed with the Xcalibur software (ver. 3.0, Thermo Scientific, San Jose, CA, USA).

Cell lines and cell culture. The HeLa human cancer cell line was obtained from ATCC. Unless otherwise specified, cells were incubated at 37 °C in 5% CO₂ and grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were passed every 3 to 4 days and restarted from a frozen stock upon reaching pass number 20.

Preparation of the nanoparticles of the MOC loaded with the fluorescein-conjugated Pt(IV) prodrug (2). To the 1-mL PBS solution of MPEG_{5k}-PGA₃₀ (1 mg) and the fluorescein-Pt(IV) prodrug (150 μ M) was added the aqueous solution containing 150 nmol of the MOC under shaking at 900 rpm at R.T. The mixture was shaken for 10 min further at R.T. The resulted red solution was filtered using a 0.2- μ m syringe filter. The filtrate was further purified using centrifugal filtration (MWCO = 30 kDa). The solution of the nanoparticles was concentrated to a volume of 0.4 mL. The solution was reconstituted to 0.5 mL by adding PBS. The prodrug (2) content was determined to be 89 μ M using the absorption at 503 nm by UV-vis spectroscopy (see Figure S12). The total Pt content was determined to be 779 μ M using GFAAS. Each MOC molecule contains six Pt centers. By subtracting the Pt content contributed by 2, the final concentration of 1 in the nanoparticles was obtained as 115 μ M.

MTT assays. Cytotoxicity profiles of cisplatin, 1, 2, and the nanoparticles against HeLa cell line were evaluated using an MTT assay. Cells were seeded on a 96 well plate (2000 cells per well) in 100 μ L DMEM, and incubated for 24 h at 37 °C. The following day, solutions of the platinum compounds were freshly prepared in DMEM media and quantitated by GFAAS. The cells were then treated with 50 μ L of the platinum compounds, separately at varying concentrations, and incubated for 72 h at 37 °C. The cells were then treated with 30 μ L of fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) and incubated for 3 h at 37 °C. The medium was removed, 200 μ L of DMSO was added to the cells, and the absorbance of the purple formazan was recorded at 565 nm using a BioTek Elx800 microplate reader. Each experiment was performed in triplicate for each cell line.

Annexin V APC-Propidium Iodide (PI) flow cytometric analysis. HeLa cells were incubated with or without the nanoparticles ([2] = 8 μ M) for 72 h at 37 °C. Cells were harvested from adherent cultures by trypsinization. Following centrifugation at 1500 rpm for 5 min at 4 °C, cells were washed with PBS and then fixed with 70% ethanol in PBS. The manufacture's protocol (Invitrogen) was followed to carry out this experiment. Briefly, untreated and treated cells (1×10^5) were suspended in $1 \times$ annexin binding buffer (100 μ L), then 5 μ L APC conjugated Annexin V and 5 μ L PI were added to each sample and incubated on ice for 15 min, after which, more binding buffer (500 μ L) was added while gently mixing. The samples were kept on ice

prior to analysis with the Accuri C6 flow cytometer. Cell populations were analyzed using the FlowJo software (Tree Star).

Job plots from UV-vis spectroscopy. A series of solutions containing the MOC (**1**) and fluorescein were prepared such that the sum of the total concentration remained constant (10 μ M). The molar fraction of 11 samples was varied from 0.1 to 1.0, and $\text{Abs}(504\text{nm}) - \epsilon(\text{fluorescein}) \times [\text{fluorescein}] - \epsilon(\mathbf{1}) \times [\mathbf{1}]$ was plotted against the molar fraction of fluorescein, where $\text{Abs}(504\text{nm})$ is the absorption of the sample at 504 nm, $\epsilon(\text{fluorescein})$ is the extinction coefficient of fluorescein measured at 504 nm, $\epsilon(\mathbf{1})$ is the extinction coefficient of the MOC(**1**) measured at 504 nm, and $[\text{fluorescein}]$ and $[\mathbf{1}]$ are the concentration of fluorescein and the MOC(**1**) within the sample.

Fluorescence titration studies. To a 2-mL PBS solution of fluorescein or its derivatives (4–10 μ M) was titrated with the MOC (**1**). The fluorescence data were recorded at R.T. The Scatchard plots were extracted from the corresponding fluorescence data.

Dialysis experiments. Micro-dialysis bags with 10 kDa MWCO membrane were filled with 1 mL of PBS solution of the nanoparticles of MOCs loaded with Pt(IV) prodrugs (10 μ M). The dialysis was carried out against 1 L of PBS (pH = 7.4) or acetate buffer (pH = 5.0) with 1 mM ascorbic acid at R.T., and water was replaced twice during the first two hours, and then once a day. At varying time points, the Pt content within the bags was analyzed by GFAAS.

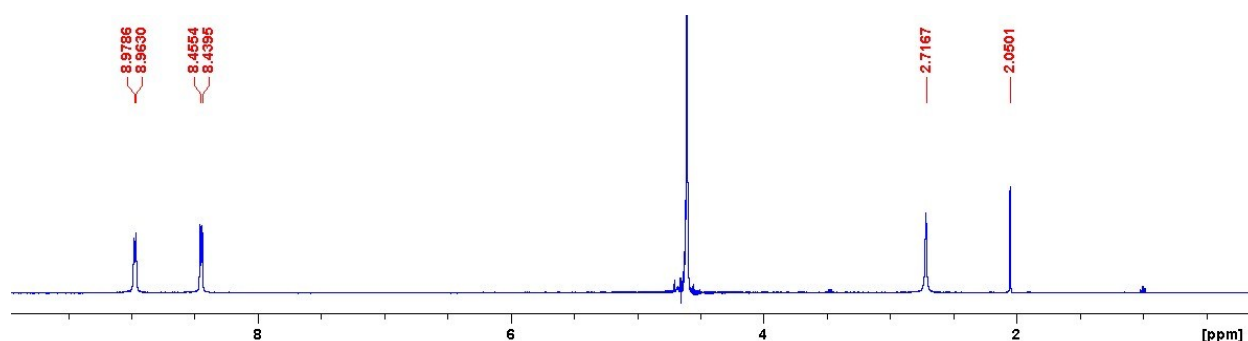


Figure S1. ¹H NMR spectrum of the cationic MOC (**1**) in D₂O.

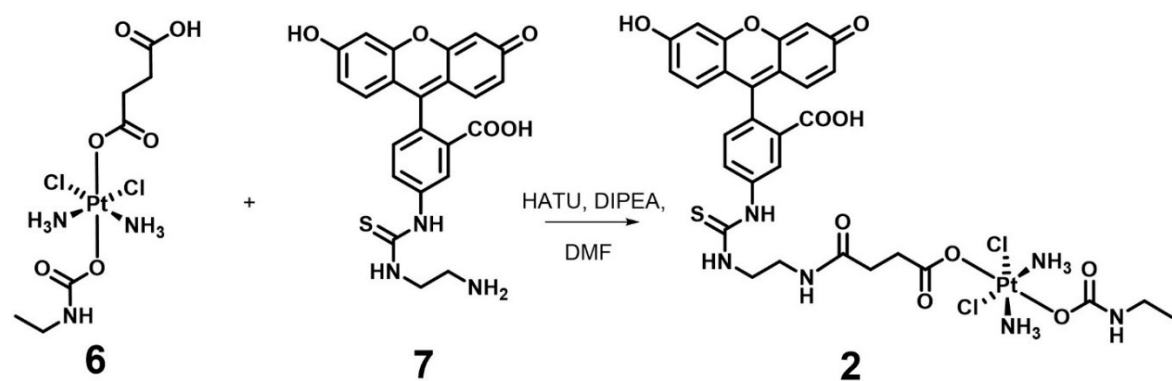


Figure S2.

Synthesis of the fluorescein-conjugated Pt(IV) prodrug (**2**) via the amide-bond-formation reaction using *c,c*-*t*-[Pt(NH₃)₂Cl₂(ethyl carbamate)(succinate)] (**6**) and an amino fluorescein derivative (**7**).

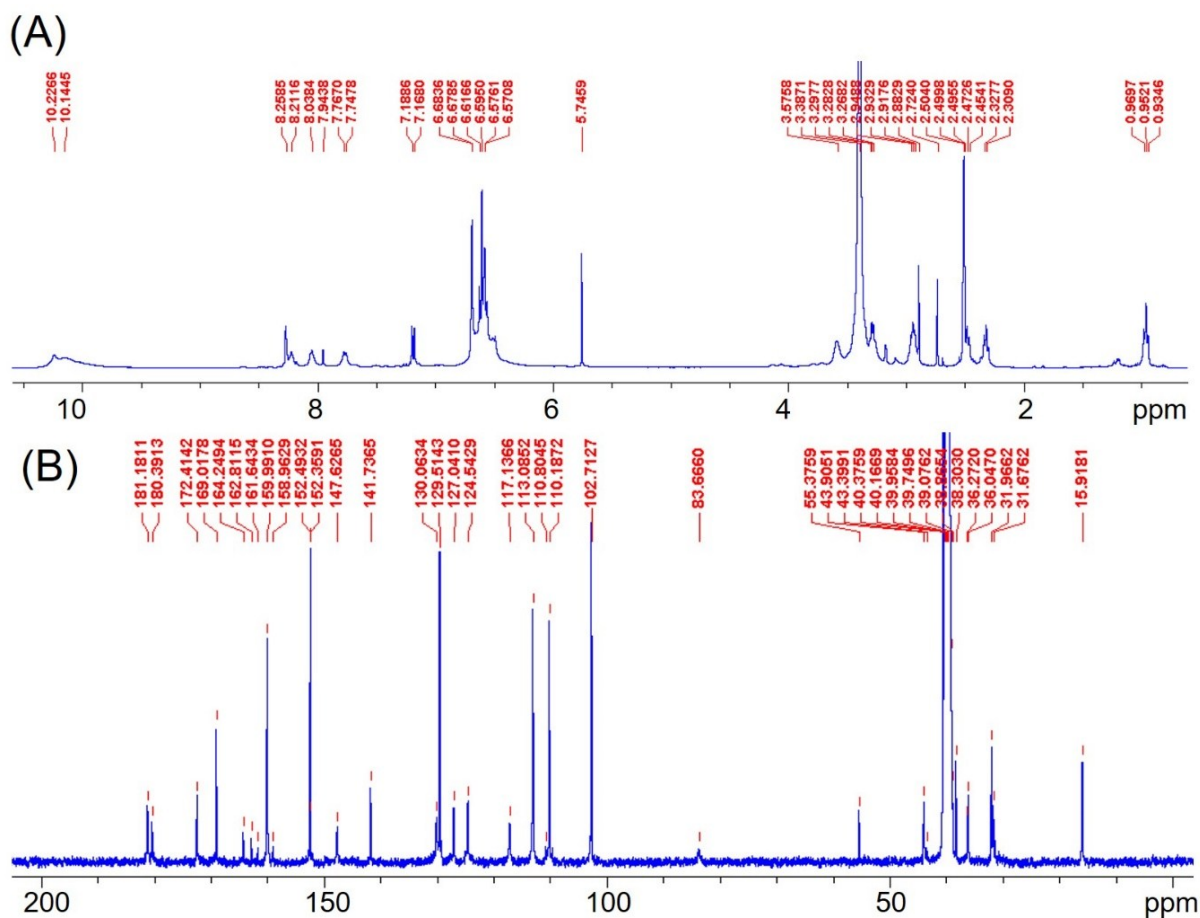


Figure S3. ¹H (A) and ¹³C (B) NMR spectra of the fluorescein-conjugated Pt(IV) prodrug in DMSO-d₆.

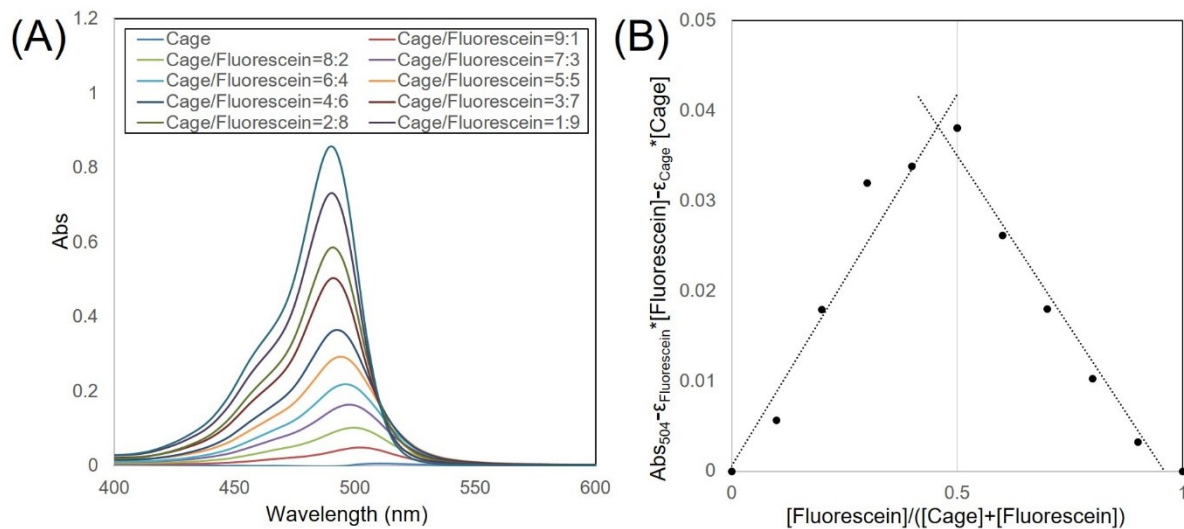


Figure S4. UV-vis (A) studies of the non-covalent binding between fluorescein and the MOC (**1**) in PBS and the corresponding Job plot (B).

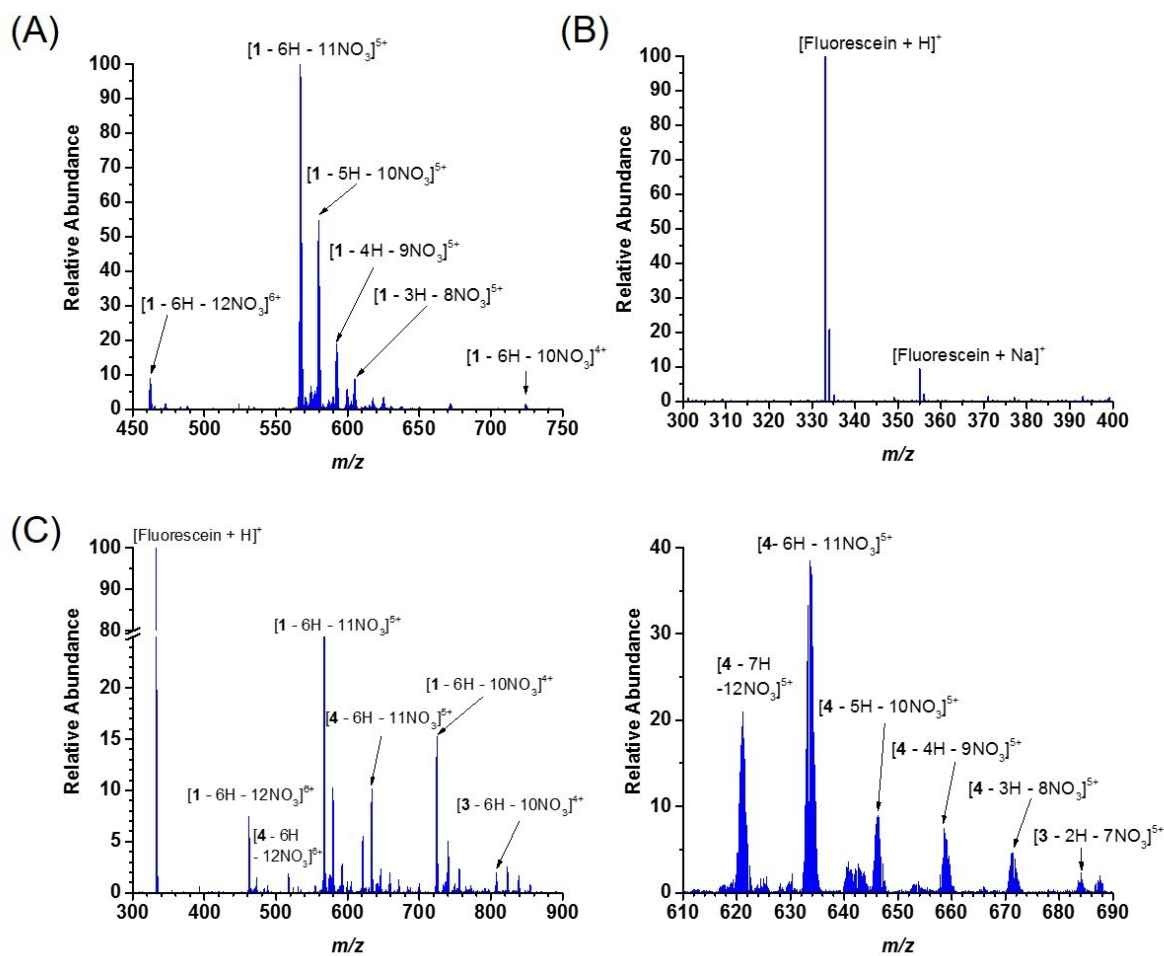


Figure S5. ESI-MS of the MOC (**1**) (A), fluorescein (B), and the host-guest complex (**4**)(C).

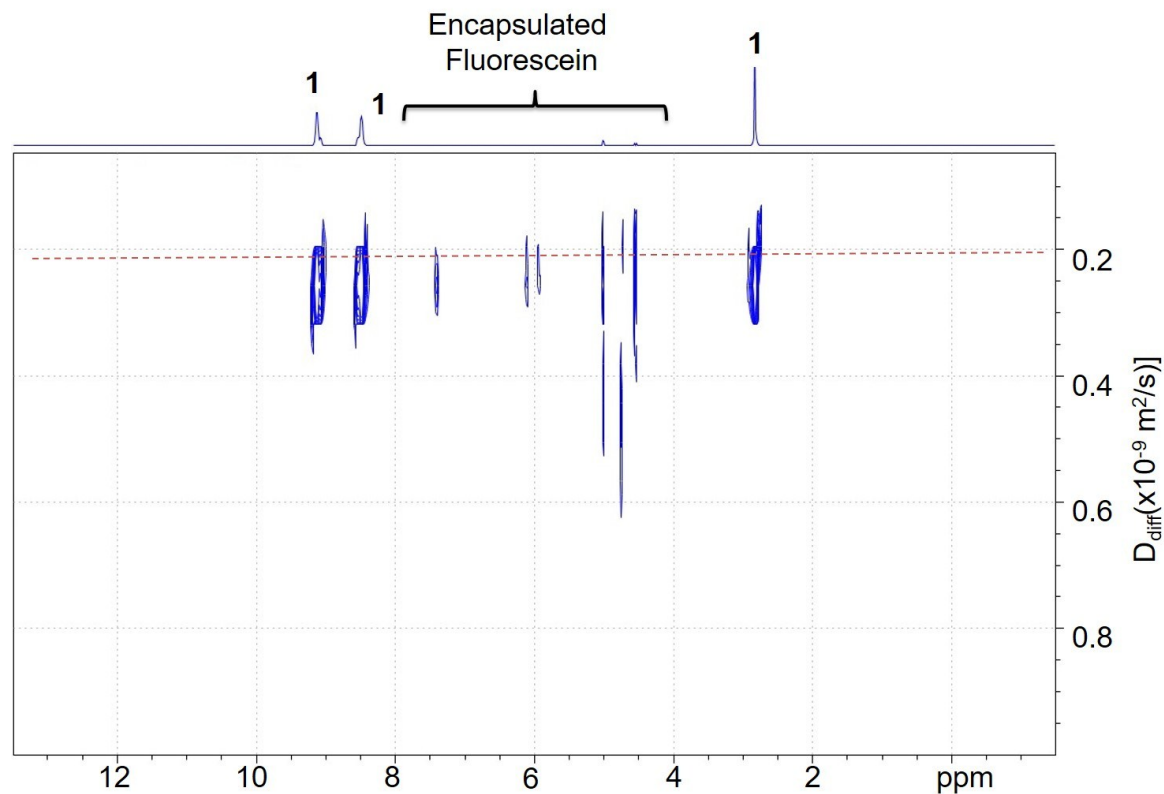


Figure S6. DOSY NMR spectrum of the host-guest complex (**4**) in D₂O.

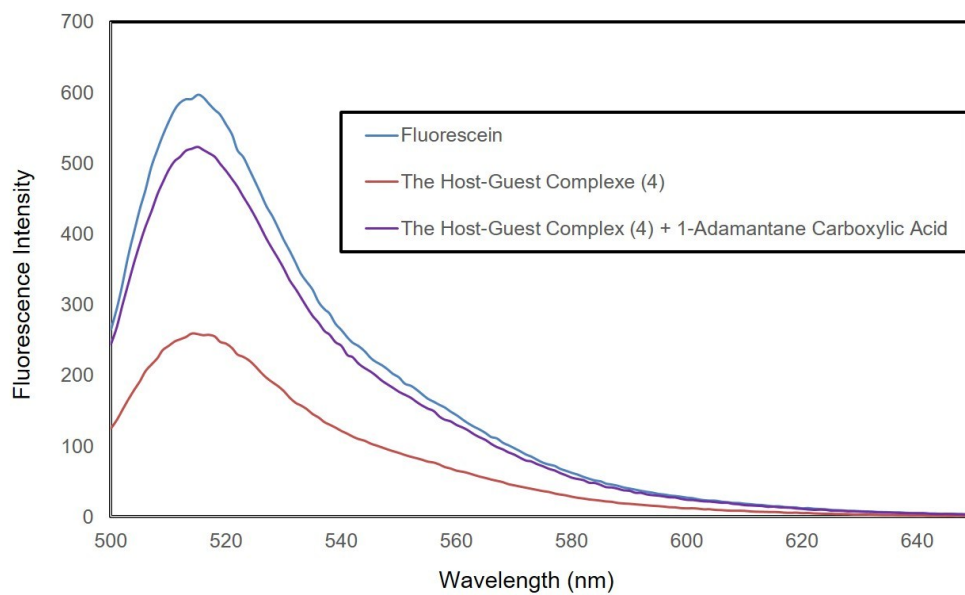


Figure S7. Fluorescence spectra of fluorescein (6 μ M), the host-guest complex (**4**) form by the MOC (**1**, 6 μ M) and fluorescein (6 μ M), and the host-guest complex (**4**, 6 μ M) upon addition of excess of 1-adamantane carboxylic acid (48 μ M).

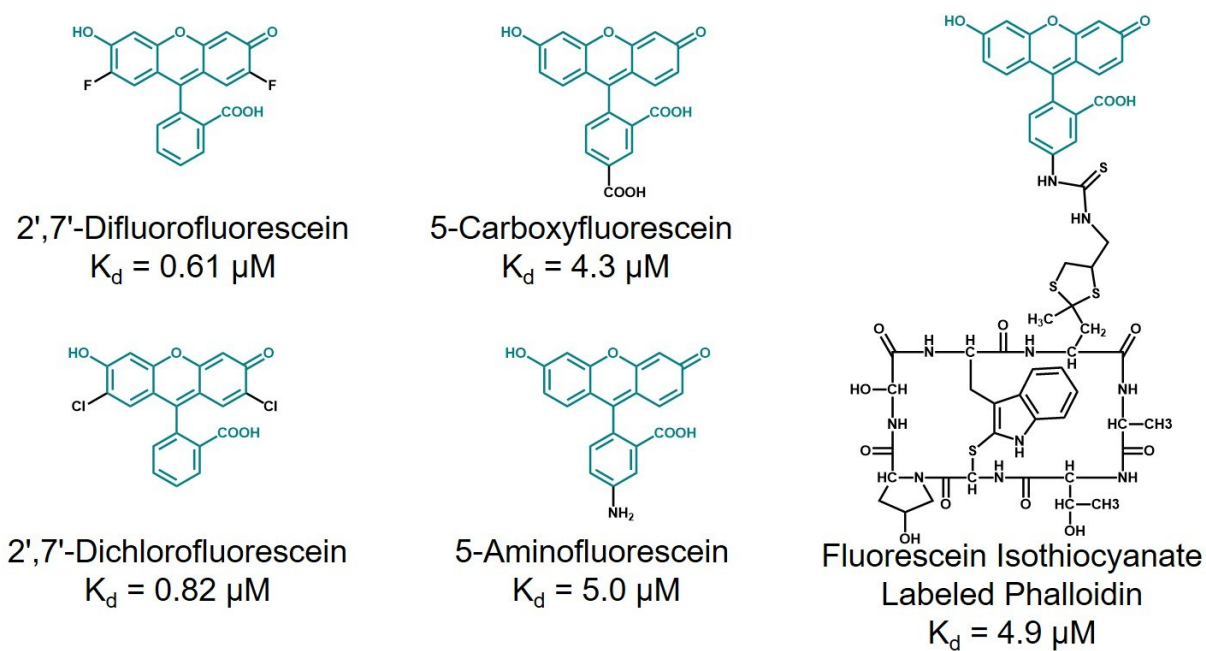


Figure S8. Variable fluorescein derivatives capable of binding to the MOC (1) and their binding constants determined by fluorescence titration.

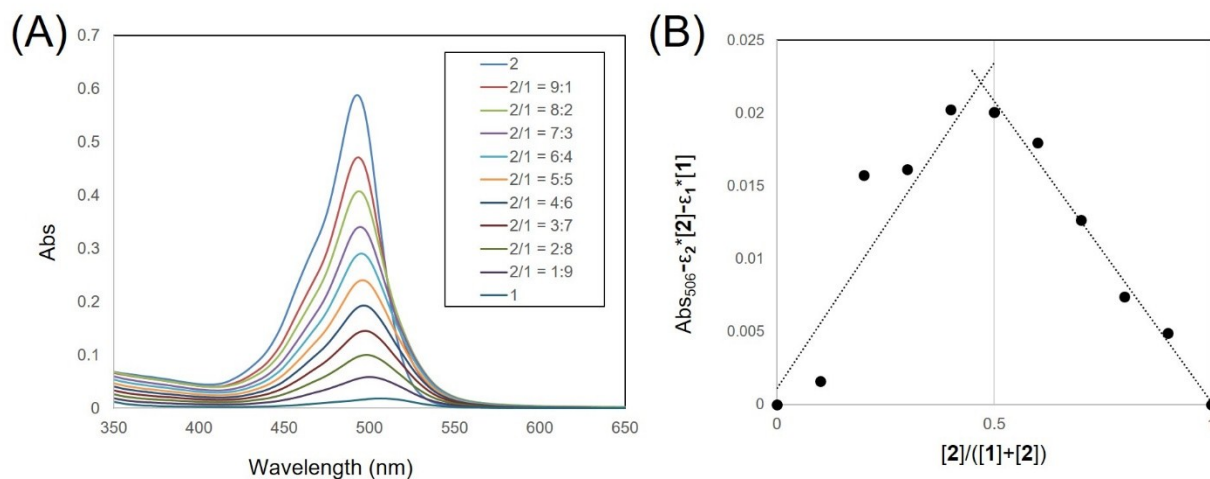


Figure S9. UV-vis (A) studies of the non-covalent binding between the Pt(IV) prodrug (2) and the MOC (1) in PBS and the corresponding Job plot (B).

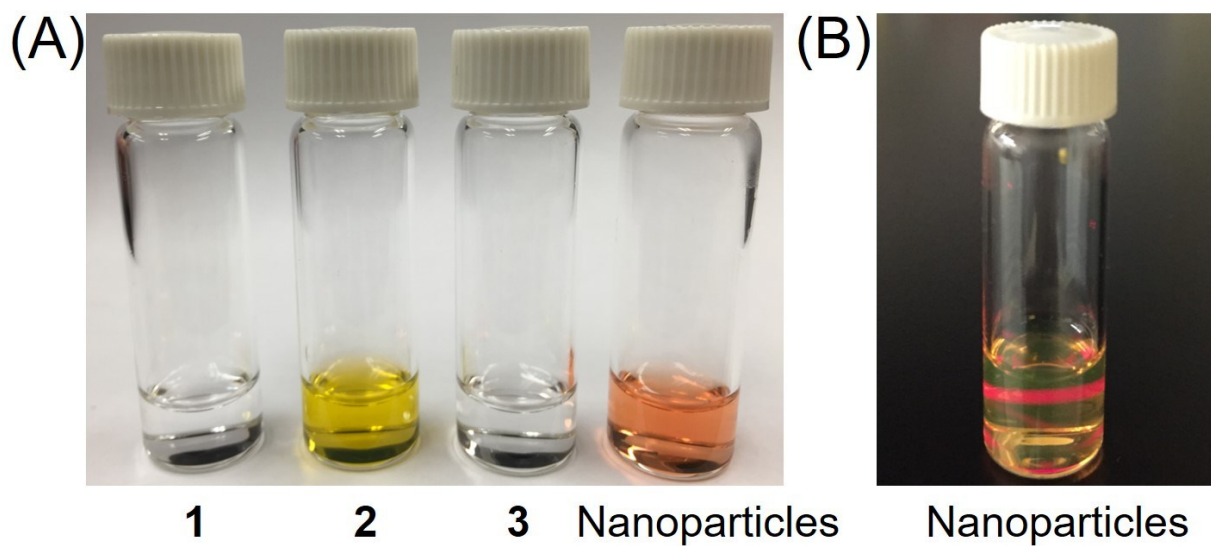


Figure S10. Representative images showing the chromatic change upon formation of the nanoparticles (A) and the corresponding Tyndall effect (B).

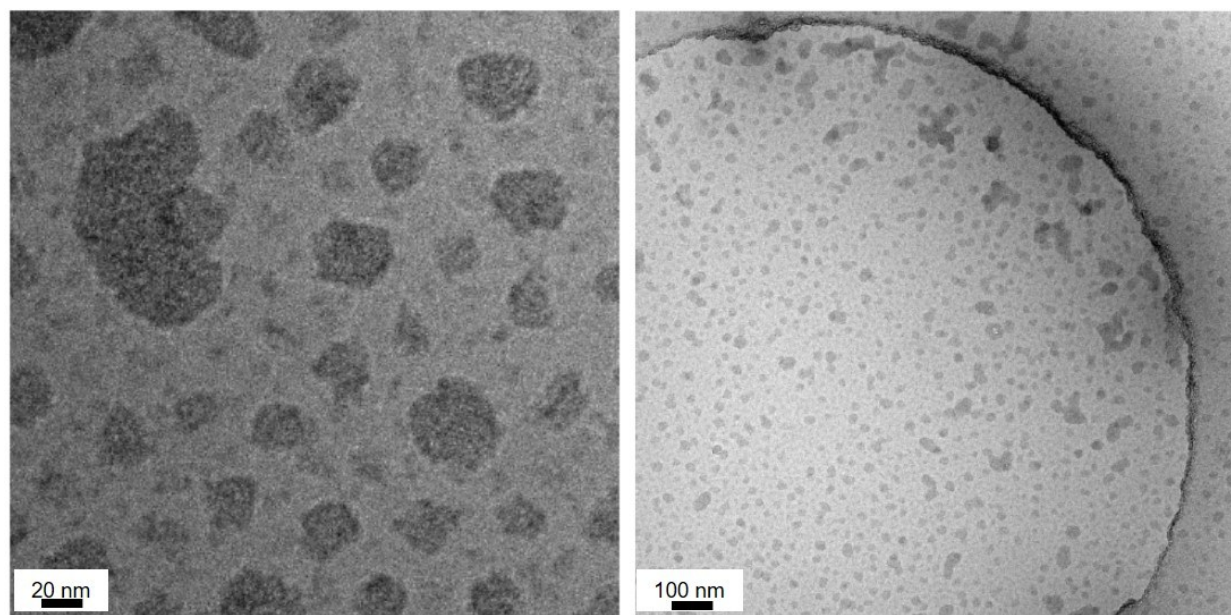


Figure S11. Representative cryo EM images of the nanoparticles of the MOCs loaded with Pt(IV) prodrugs

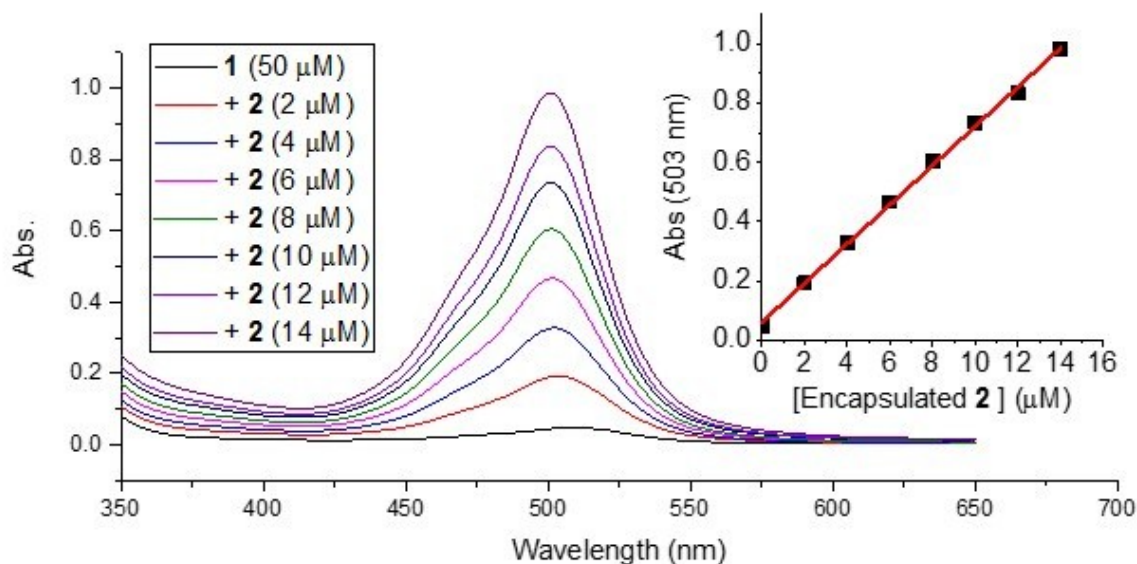


Figure S12. Calibration curve generated by the absorption at 503 nm of the encapsulated Pt(IV) prodrug (2) upon adding 2 to Cage 1 in PBS.

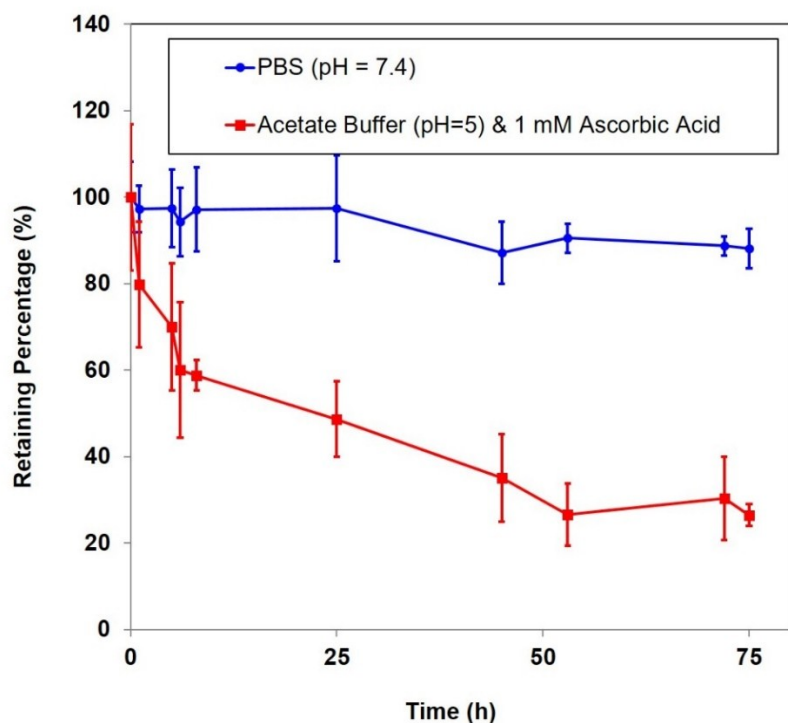


Figure S13. Release profiles of the nanoparticles ([2] = 10 μM) in PBS (pH = 7.4) (blue) and acetate buffer (pH = 5.0) with 1 mM ascorbic acid (red).

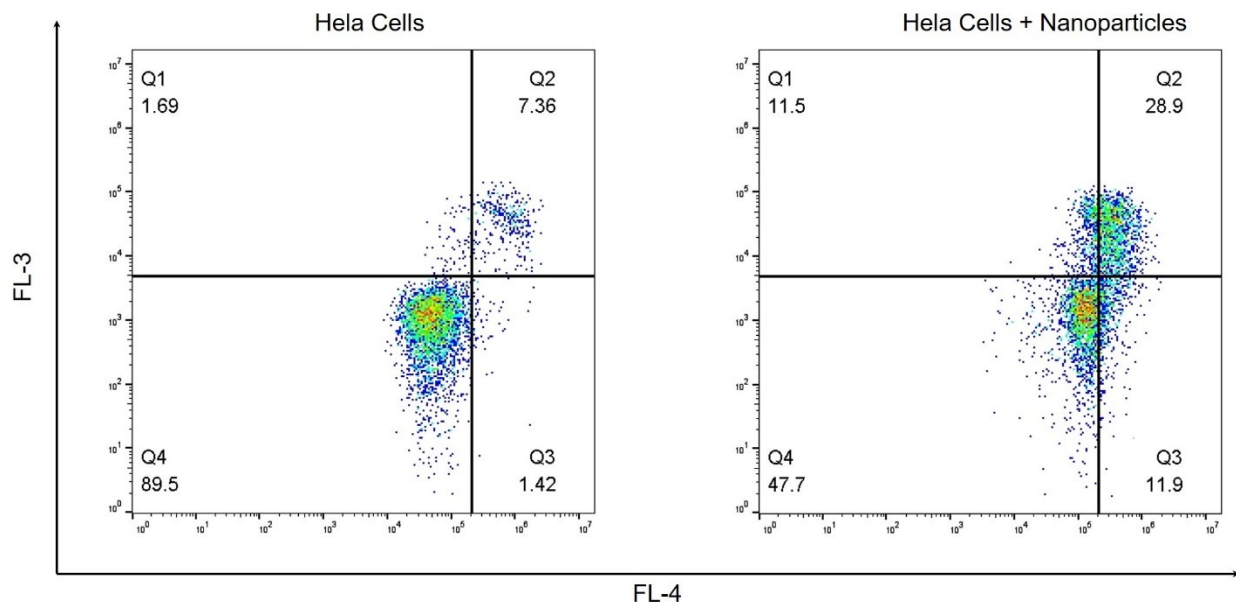


Figure S14. Annexin V-APC PI flow cytometric analysis of HeLa cells before (left) and after the treatment of the nanoparticles ($[2] = 8 \mu\text{M}$ for 72 h) (right).

Reference:

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