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Supplementary Information

Oxaliplatin(IV) prodrug-based supramolecular self-delivery nanocarrier for targeted colorectal cancer treatment

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Materials and methods

Materials

All solvents and chemicals were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from PAA. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was bought from Alfa Aesar. HCT116 cells were obtained from ATCC.

Characterizations

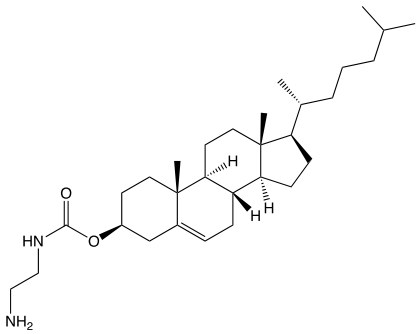
^{1}H NMR spectra were probed on 300 MHz Bruker spectrometer using $CDCl_3$, deuterated DMSO or D_2O as the solvent. ThermoFinnigan LCQ quadrupole ion trap mass spectrometer was used to record electronic spray ionization (ESI) mass spectra. High-resolution mass spectrometry (HR-MS) was performed on a Waters Q-tof Premier MS spectrometer. Fourier transform infrared (FTIR) spectra were recorded as KBr-pellet on a SHIMADZU IR Prestige-21 spectrophotometer. High performance liquid chromatography (HPLC) analysis was performed with Shimadzu LC2010 using the Waters XSelectTM HSS C18 (5 μm, 4.6 × 250 mm) column with UV detection at 240 nm. Acetonitrile/water binary system containing 0.5% trifluoroacetic acid was used as the mobile phase with a flow rate at 1 mL min^{-1}. The analysis was performed with gradient elution method starting with 95% water to 5% water and back to 95% water at 30 °C over a period of 40 min. Transmission electron microscope (TEM) images were collected on a JEM-1400 (JEOL) operated at 100 kV. Zeta potential and dynamic light scattering (DLS) measurements were measured using Zetasizer, Malvern Instrument Ltd. UV-Vis spectra were obtained on a Shimadzu UV-3600 UV-Vis-NIR spectrophotometer (1 mm quartz cell used). Inductively coupled plasma mass spectrometry (ICP-MS DRCe Elan, Perkin Elmer) was used for quantitative determination of platinum. Confocal laser scanning microscopy (CLSM) images were acquired by a Carl ZEISS LSM 800. Flow cytometry was recorded on a BD LSR Rortessa X20 (3 lasers).

Experimental procedures

Synthesis procedures

Schematic synthetic procedures of chol-NH_2, oxliPt(IV)-chol, CD-spermine and CD-NH_2 are shown in Schemes S1−S4.

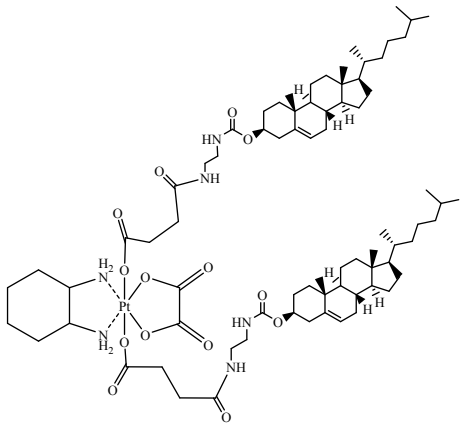
Synthesis of chol-NH_2



Chol-NH_2 was synthesized according to a modified procedure.[1] Triethylamine (0.259 mL, 1.86 mmol) was added to *N*-Boc-ethylenediamine (149 mg, 0.93 mmol) in DCM (10 mL), and the mixture was stirred at 0 °C. Following which, cholesteryl chloroformate (500 mg, 1.11 mmol) in DCM (10 mL) was added dropwise into the reaction solution over 30 min. The reaction was left to stir for 18 h. DCM was then added, and the solution was washed with dilute HCl and brine. The organic layer was collected, dried over sodium sulfate and filtered. The Boc-protected product was purified with column chromatography, with the product eluting at 20% ethyl acetate/hexane. Deprotection was performed by dissolving the obtained product (200 mg, 0.35 mmol) in TFA (0.27 mL, 3.50 mmol) and DCM (6 mL) and stirred at 25°C for 6 h. The solvent was removed, affording a colorless oil used without further purification. ^{1}H-NMR (300 MHz) δ (ppm) = 7.87 (bs, 3H), 5.37 (s, 1H), 5.30 (s, 1H), 4.44 (bs, 1H), 3.48– 3.23 (m, 4H), 2.30–2.19 (m, 2H), 2.02–1.81 (m, 6H), 1.56–0.85 (m, 37H). LC-MS m/z $[M + H]^+$ calcd. For $[C_{30}H_{52}N_2O_2]^+$ 472.76; found 473.18.

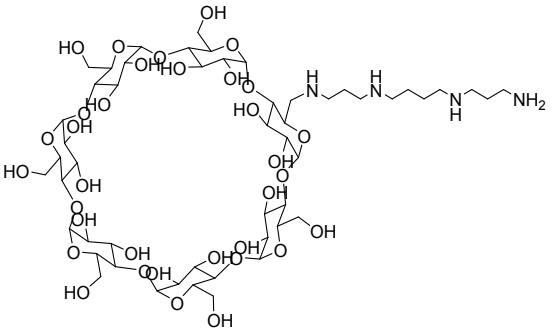
Synthesis of oxliPt(IV)-chol

The compound oxliPt(IV)-COOH was prepared according to the procedures reported in literature.[2]



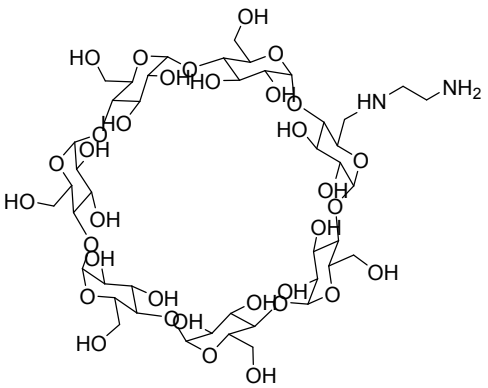
For the synthesis of oxliPt(IV)-chol, a two-necked round bottle flask containing oxliPt(IV)-COOH (57 mg, 90.3 μmol) and 1,1'-carbonyldiimidazole (44 mg, 0.271 mmol) was flushed with nitrogen to maintain an inert environment. Anhydrous DMF (2 mL) was added and the reaction solution was stirred at 60 °C for 10 min. After which, the solution was cooled to room temperature followed by the addition of chol-NH_2 (205 mg, 0.44 mmol) and triethylamine (88 mg, 0.87 mmol). After stirring overnight, solvent was removed. The obtained residue was dissolved in acetone and then precipitated with diethyl ether to give the final product. ^{1}H-NMR (300 MHz) δ (ppm) = 8.33 (m, 2H), 8.12 (m, 2H), 7.87 (m, 2H), 7.67 (m, 1H), 7.03 (t, 2H), 5.33 (m, 2H), 4.33– 4.30 (m, 4H), 3.05– 2.67 (m, 12H), 2.32–1.78 (m, 24H), 1.54–0.89 (m, 73H). LC-MS m/z $[M + 3H]^{3+}$ calcd. For $[C_{76}H_{124}N_6O_{14}Pt]^+$ 514.29; found 513.33.

Synthesis of CD-spermine



CD-Tos was prepared according to procedures reported in literature.[3] Spermine (1.53 g, 7.76 mmol) in anhydrous DMF (3 mL) was added to a solution of CD-Tos (1.0 g, 0.78 mmol) in anhydrous DMF (5 mL). The reaction was stirred under inert condition at 80 °C overnight. The resulting solution was then cooled to room temperature and the solvent was evaporated. The crude solid obtained was dissolved in deionized (DI) H_2O. The product was obtained by precipitation with acetone and collected by filtration. ^{1}H-NMR (300 MHz) δ (ppm) = 5.10–5.08 (m, 7H), 3.97–3.30 (m, 47H), 2.76–2.61 (m, 18H), 2.23 (m, 12H), 1.73–1.71 (m, 10H). HRMS m/z $[M + H]^+$ calcd. For $[C_{52}H_{94}N_4O_{34}]^+$ 1319.57; found 1319.58.

Synthesis of CD-NH_2



The compound CD-NH_2 was prepared according to procedures reported in literature.[4] Ethylenediamine (0.311 mL, 23.3 mmol) was added to a solution of CD-Tos (300 mg, 0.23 mmol) in anhydrous DMF (3 mL). The reaction was stirred under inert conditions at 80 °C overnight. The resulting solution was then cooled to room temperature and the solvent was

evaporated. The crude solid obtained was dissolved in DI H_2O. The product was obtained by precipitation with acetone and collected by filtration.

Reduction of oxliPt(IV)-chol prodrug and its chelation with 5'-GMP

To study the reduction products of the prodrug, oxliPt(IV)-chol (1 mM) was dissolved in sodium ascorbate aqueous solution (2.5 mM) and left to stir overnight. The reaction mixture was then diluted with methanol and analyzed by HPLC and LC-MS.

For the chelation study of Pt species with 5'-GMP, a solution of oxliPt(IV)-chol, 5'-GMP, and sodium ascorbate at final concentrations of 1 mM, 5 mM and 5 mM, respectively, was prepared and incubated at 37 °C for 12 h. The reaction mixture was then diluted with methanol and analyzed by HPLC and LC-MS. For comparison, a solution of oxliPt(II) and 5'-GMP at final concentrations of 1 mM and 5 mM, respectively, was prepared and incubated at 37 °C for 12 h. The solution was then diluted to suitable concentration for HPLC and LC-MS measurements.

CMC determination of pre-formed nanoparticles

Stock solutions of oxliPt(IV)-chol and CD-spermine were prepared in DMSO and distilled water, respectively. oxliPt(IV)-chol was titrated into a solution of CD-spermine (5 μM) in 1 cm cuvette to achieve different ratios. After each addition, the mixture solution was stabilized for 5 min before the UV-vis curve was recorded. The transmittance at 240 nm was plotted as a function of the concentration of oxliPt(IV)-chol.

Preparation of prodrug nanocarrier

The prodrug nanocarrier was prepared by a nanoprecipitation method. Briefly, stock solution of oxliPt(IV)-chol in DMSO was added dropwise to CD-spermine or CD-NH_2 dissolved in DI water. The mixture was sonicated at room temperature for 30 min, and CD-spermine : oxliPt(IV)-chol or CD-NH_2: oxliPt(IV)-chol nanoparticles were formed spontaneously. The prepared nanoparticles were then dialyzed (MWCO = 2000) against deionized water to remove DMSO.

To prepare FITC-labelled nanoparticles for imaging purposes, the above procedure was carried out with the addition of FITC-labelled CD-spermine. FITC-labelled CD-spermine was obtained by reacting FITC isomer I with CD-spermine in DMSO for 2 h, dialyzed against distilled H_2O for 2-3 days and finally lyophilized. Stock solutions of oxliPt(IV)-chol in DMSO, CD-spermine (or CD-NH_2) and FITC-labelled CD-spermine in H_2O were mixed in the molar ratio of 1 : 1.4 : 0.6 and then sonicated at room temperature for 30 min. The prepared nanoparticles were then dialyzed (MWCO = 2000) against distilled water. The amount of FITC dye adsorbed on the nanoparticles was indirectly calculated using FITC calibration curve based on the amount of free FITC in dialysate solution.

Drug release study

To study the drug release behavior in physiological conditions and cellular environments, CD-spermine : oxliPt(IV)-chol (1 mM) was dissolved in phosphate buffer solution (PBS, pH 7.4, 2 mL). A dialysis bag (MWCO = 2000 Da) used to contain the solution was immersed into PBS (78 mL) comprising either 0 mM or 5 mM sodium ascorbate. The release was conducted at 37

°C. The dialysate (1 mL) was withdrawn at predefined time and equivalent volume of incubation medium was added to ensure the total volume of solution was maintained. The amount of Pt in the collected samples was measured by ICP-MS. The percentage of drug released is expressed as the amount of accumulative drug in the incubation medium to the total drug in the original nanoparticles.

Cell culture

HCT116 cells were cultured in DMEM medium supplemented with 10% (v/v) FBS, 0.03% *L*-glutamine and 1% penicillin-streptomycin under 5% CO_2 atmosphere at 37 °C. DMEM was replaced every two days.

***In vitro* cytotoxicity assay**

HCT116 cells were seeded into 96-well plate with an initial cell seeding density of 1 x 10^4 cells/well. The cells were then incubated with various drug formulations of oxliPt(II) and CD-spermine : oxliPt(IV)-chol at final equivalent Pt concentrations ranging from 1 to 100 μM. After incubation at 37 °C for 24 h and 48 h, the incubation medium was removed. Fresh culture medium containing MTT (5 mg/mL, 100 μL) was added into each well, and the cells were incubated at 37 °C for another 4 h. DMSO (100 μL) was added to dissolve the formazan crystals. The optical density (OD) was read at 490 nm using a microplate reader (infinite M200, TECAN). The control was taken as the culture medium in the absence of samples. The normalized cell viability in % was calculated according to cell viability = $\frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}}$ x 100%. The results were obtained from 5 replicates for each sample and expressed as means.

Determination of cellular platinum contents

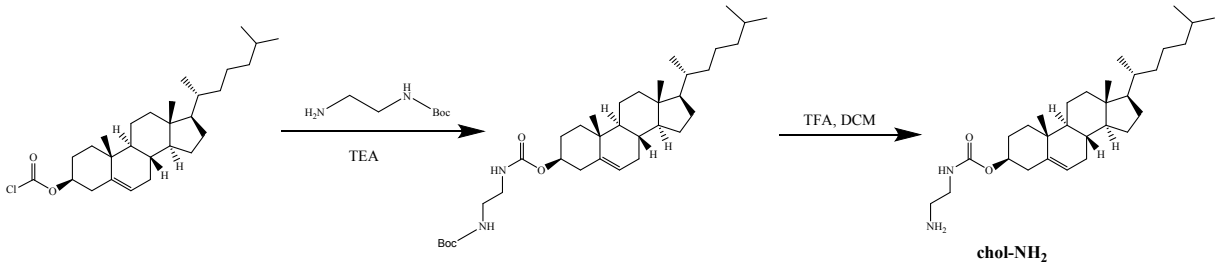
HCT116 cells were seeded in 24-well plates at a density of 10^5 cells per well. Fresh culture medium containing oxliPt(II) or CD-spermine : oxliPt(IV)-chol was incubated with the cells at 37 °C for 2 h or 12 h. The platinum concentration of each sample was regulated at 25 μM. After the stipulated incubation period, the cells were washed three times with PBS, harvested by trypsination and collected by ultra-centrifugation. Thereafter, 1 mL cell lysis buffer (Invitrogen cell extraction buffer) was used to lyse the cell pellets. The cell lysis solution (100 μL) of each sample was used in the measurement of Pt content by ICP-MS. The cell lysis solution (25 μL) was used for the determination of the protein content by bicinchoninic acid (BCA) protein assay kit (Pierce Thermo Scientific). The amount of Pt content was expressed as nanograms of Pt per milligram of total proteins.

Cellular uptake study

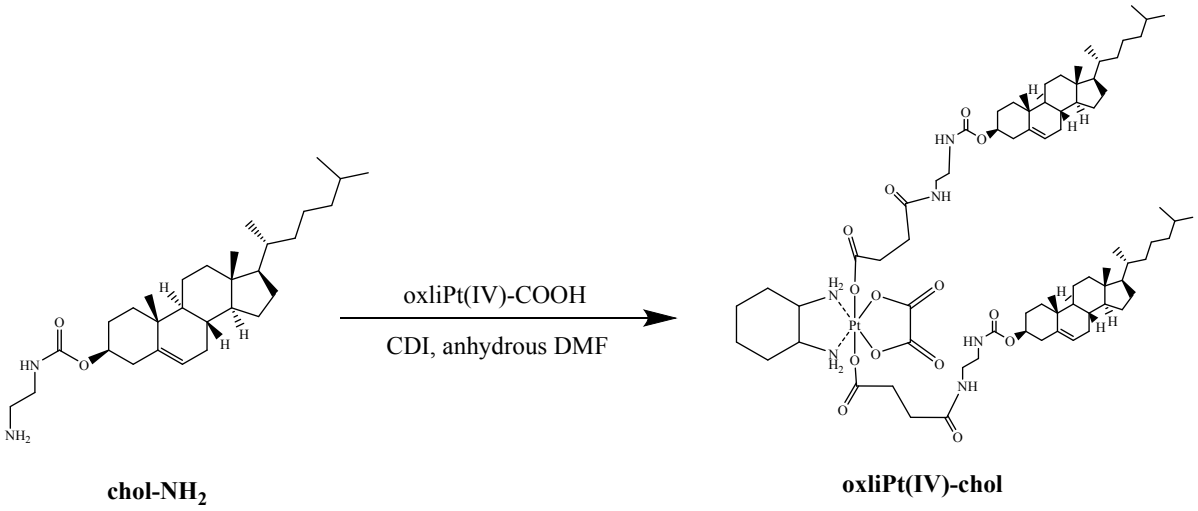
HCT116 cells were seeded on glass cover slip and allowed to grow for 24 h. The cells were then incubated with cell culture medium containing 10 μM FITC-labelled CD-spermine : oxliPt(IV)-chol or CD-NH_2 : oxliPt(IV)-chol for 30 min and 4 h. After which, the cells were washed with PBS three times, and Hochest 33342 was used to stain the nucleus before cells fixation. After the treatment, cells were imaged by CLSM (ZEISS LSM 800). Fluorescence was examined under excitation at 405 nm for Hochest 33342 and 488 nm for FITC.

For the flow cytometric study, HCT116 cells were seeded in a 6-well tissue culture plate (2 mL medium) and cultured for 24 h. Samples were added in the culture medium and incubated for 4 and 18 h. After the treatment, the cells were washed with PBS for two times and treated with trypsin (0.5 mL) for 2 min. Subsequently, fresh medium (0.5 mL) was added to each culture well, and the cells were collected *via* centrifugation and washed with PBS twice before resuspended in PBS (1 mL) and subjected to flow cytometry analysis.

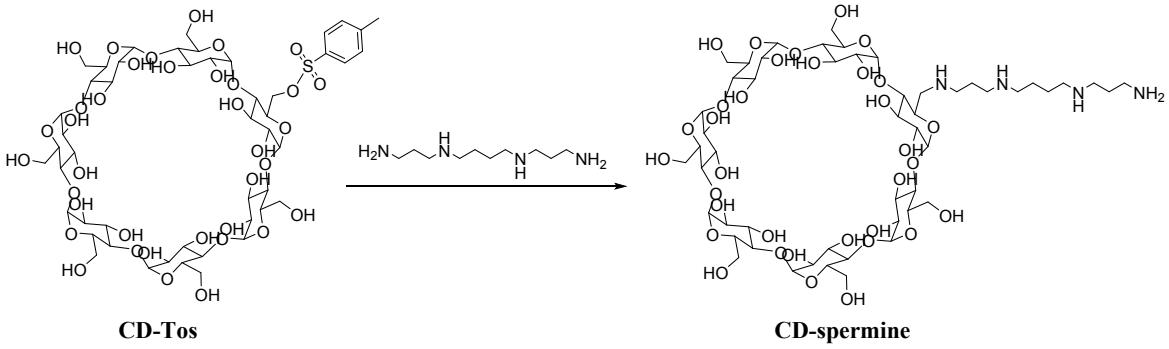
Experiments for the spermine competition assay were carried out as described above, except that cells were pretreated with spermine for 4 h before incubation with the nanoparticles.



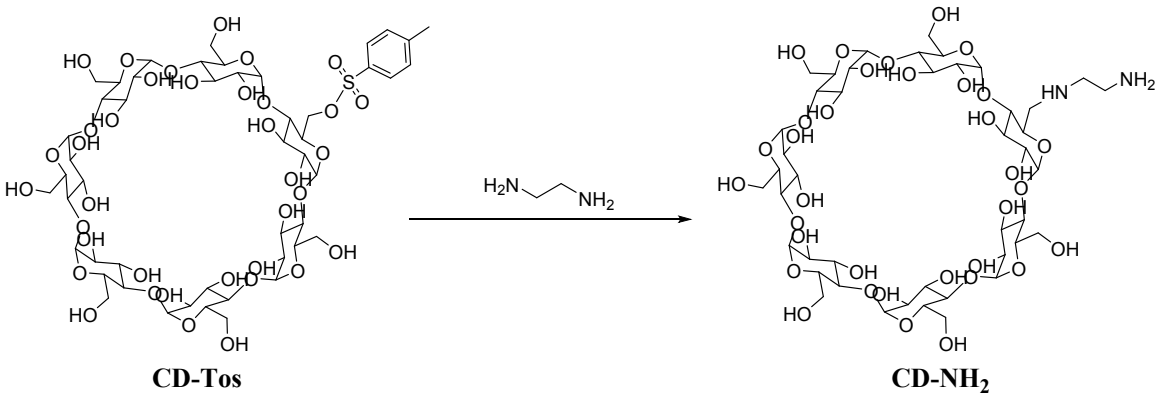
Scheme S1. Schematic illustration for the synthesis of chol-NH_2.



Scheme S2. Schematic illustration for the synthesis of oxliPt(IV)-chol.



Scheme S3. Schematic illustration for the synthesis of CD-spermine.



Scheme S4. Schematic illustration for the synthesis of CD-NH_2.

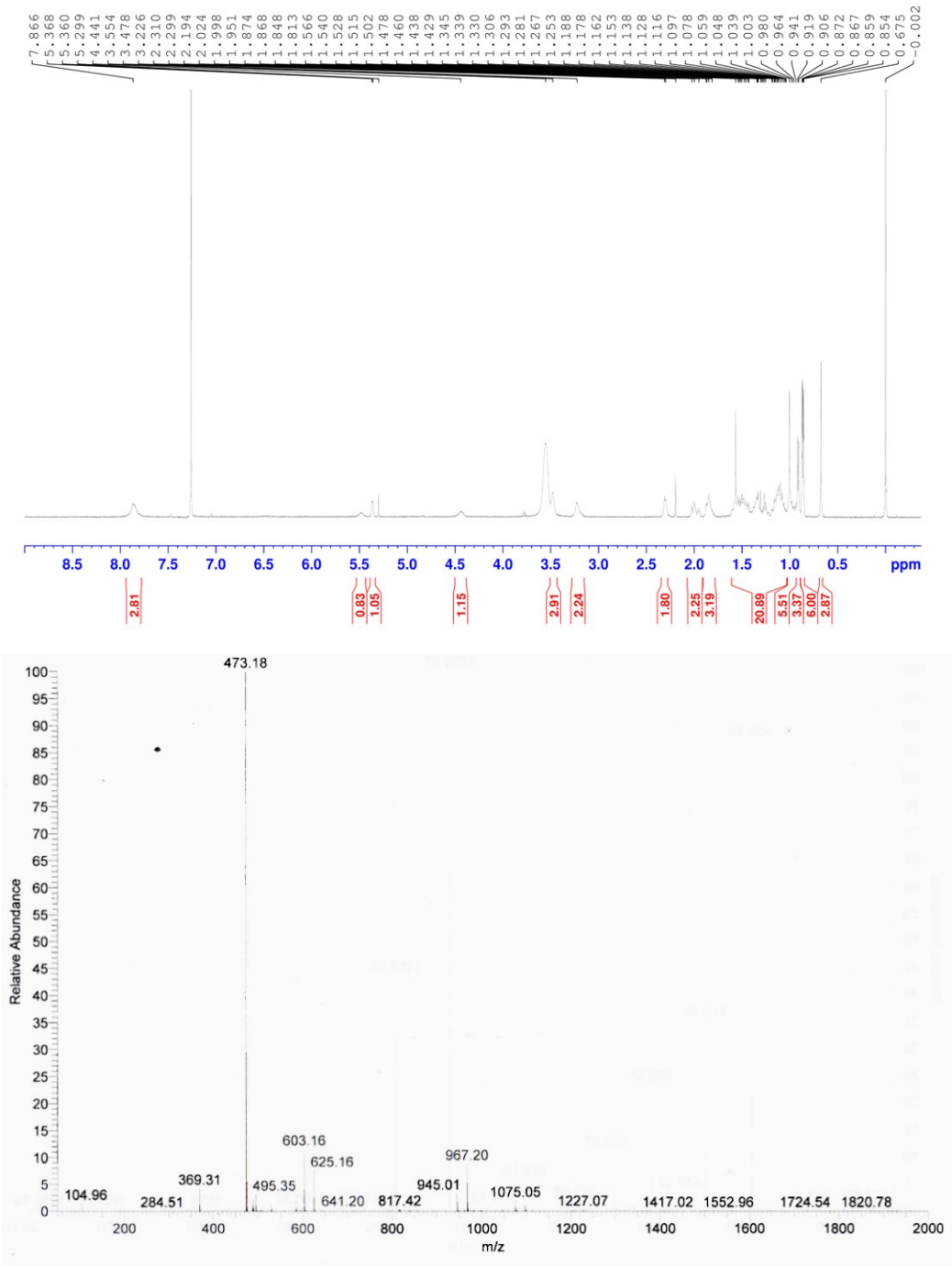
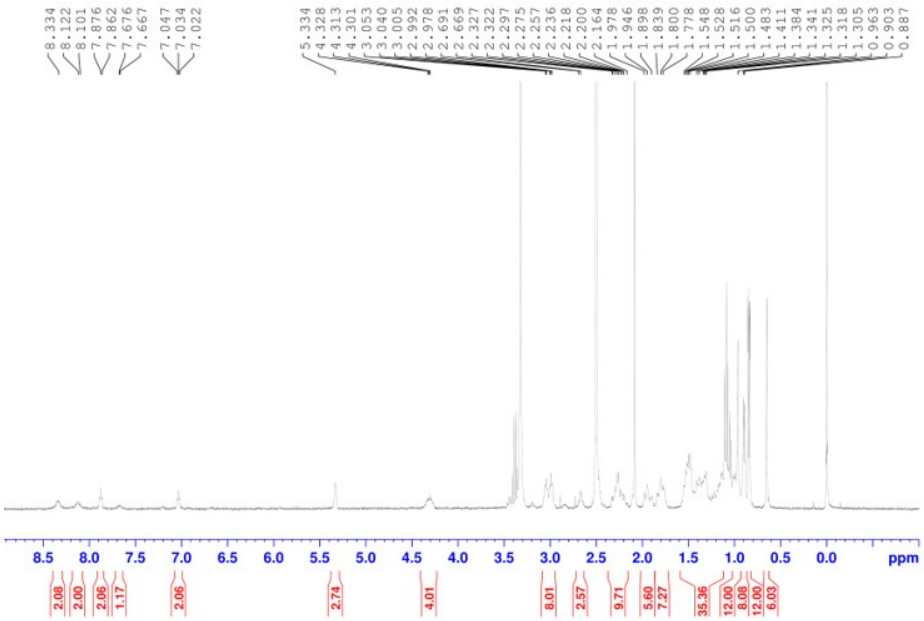
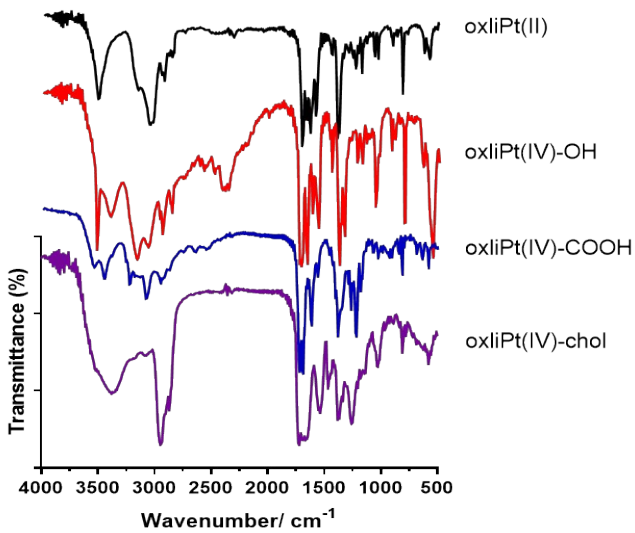


Figure S1. ^{1}H NMR (top) and HRMS (bottom) spectra of chol-NH_2.





	Calcd [%]			Found [%]		
Anal. Calcd. for	C	H	N	C	H	N
oxliPt(II)	24.18	3.53	7.05	20.59	3.85	6.09
oxliPt(IV)-OH	22.27	3.71	6.5	18.83	3.72	5.32
oxliPt(IV)-COOH	30.43	3.8	4.44	23.13	3.70	4.04
oxliPt(IV)-chol	59.26	8.06	5.46	48.64	7.57	4.91

Figure S2. ^{1}H NMR spectrum (top), FTIR spectrum (middle) and CHN elemental analysis (bottom) of oxliPt(IV)-chol.

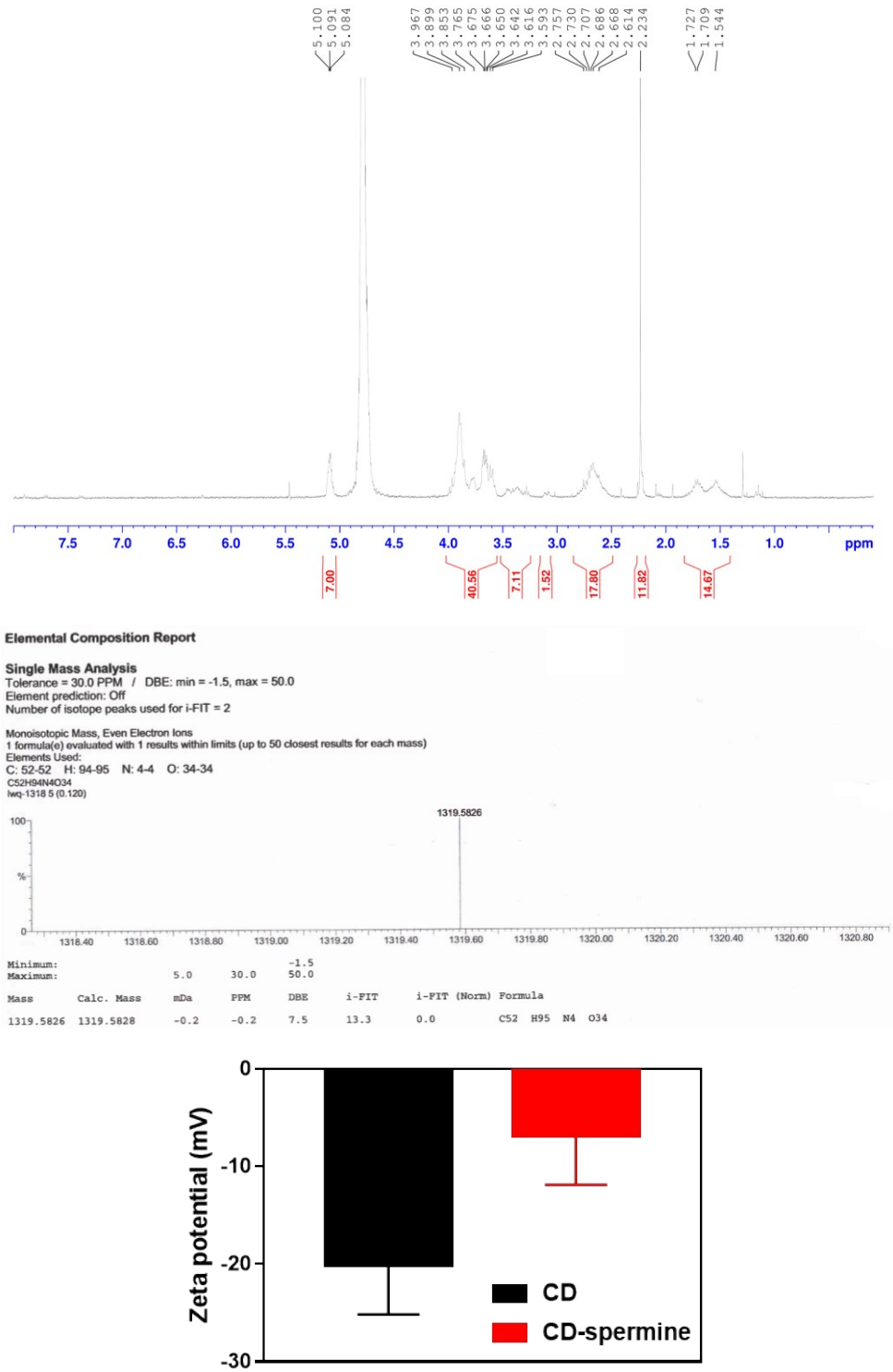


Figure S3. ^{1}H NMR spectrum (top), HRMS spectrum (middle) and zeta potential (bottom) of CD-spermine.

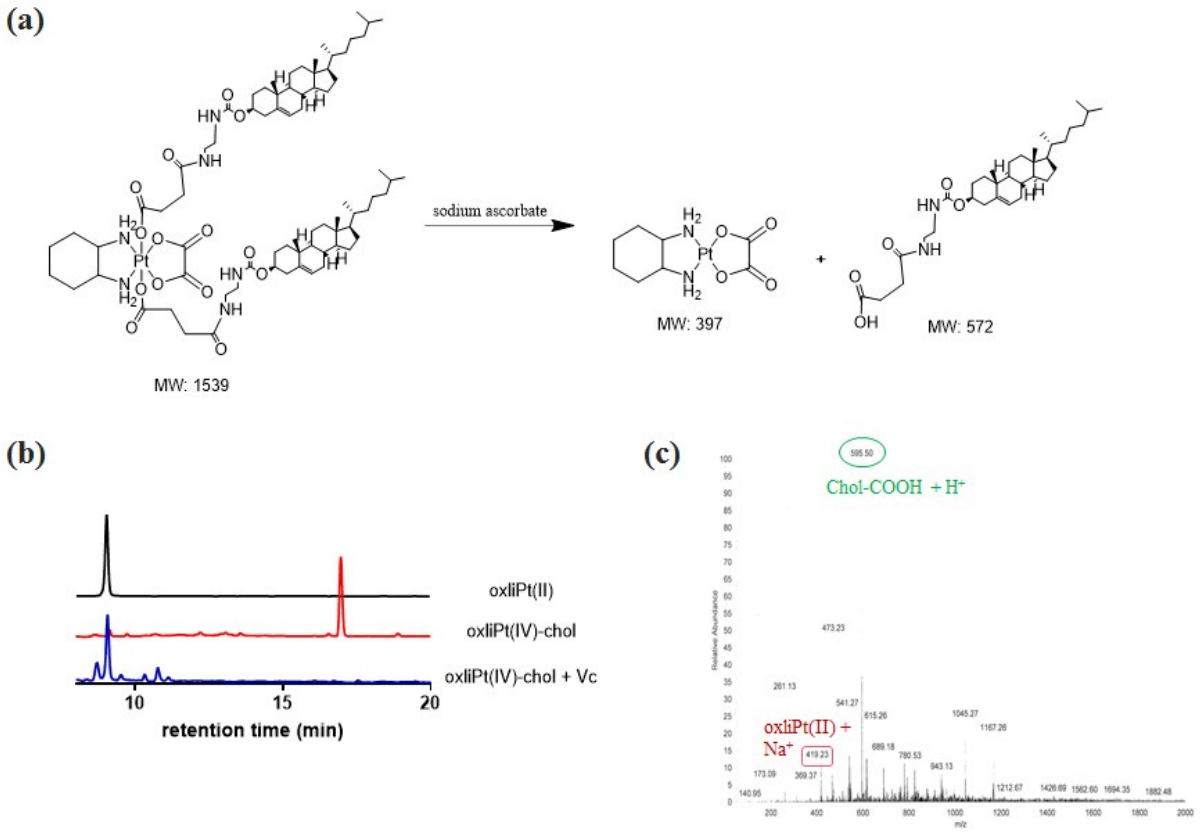
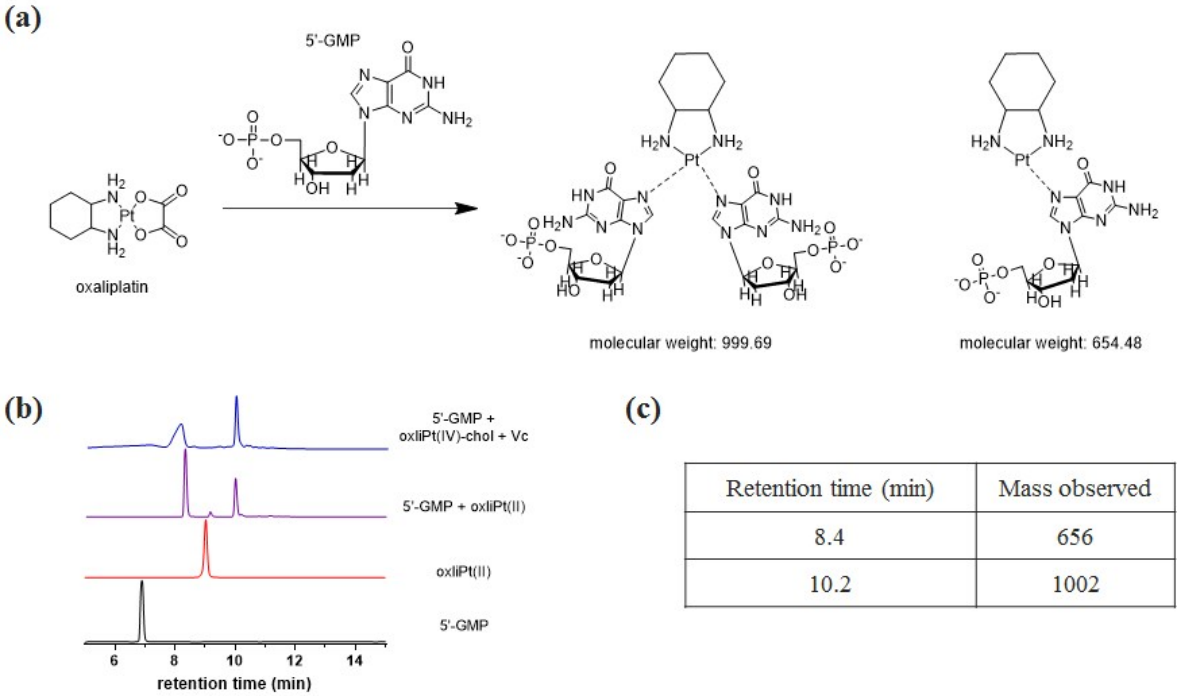


Figure S4. (a) Schematic illustration of possible reduction products of oxliPt(IV)-chol. (b) HPLC analysis of oxliPt(II), and oxliPt(IV)-chol with and without the treatment of sodium ascorbate. The retention time at 9.5 min corresponds to oxliPt(II), while the retention time at 17 min corresponds to oxliPt(IV)-chol. The absorbance channel was set at 240 nm. (c) LC-MS for the reaction mixture of oxliPt(IV)-chol and sodium ascorbate, revealing mass peaks corresponding to oxliPt(II) and chol-COOH.



Retention time (min)	Mass observed
8.4	656
10.2	1002

Figure S5. (a) Schematic illustration of possible Pt conjugates upon reduction of oxliPt(IV)-chol in the presence of 5'-GMP and sodium ascorbate. (b) HPLC chromatograms of 5'-GMP, oxliPt(II) and prodrug oxliPt(IV)-chol with and without the incubation with sodium ascorbate. (c) Tabulated mass of the chromatogram peaks observed at 8.4 and 10.2 min measured with ESI-MS.

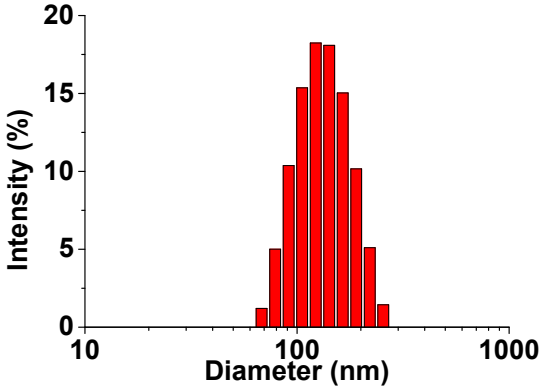


Figure S6. Hydrodynamic size distribution of CD-spermine : oxliPt(IV)-chol as measured by dynamic light scattering (DLS).

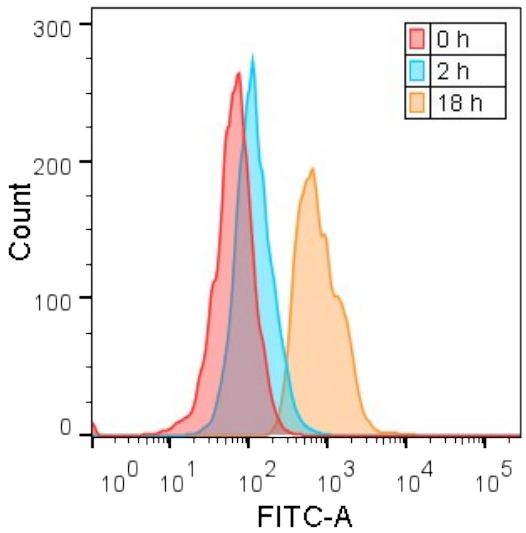


Figure S7. Cellular accumulation of CD-spermine : oxliPt(IV)-chol in HCT116 cells after 2 and 18 h incubation, measured by flow cytometry.

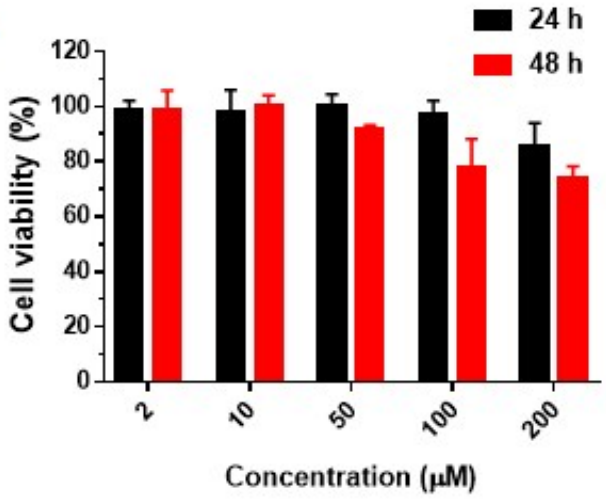


Figure S8. Cell viability of HCT116 cells after incubation with different concentrations of free CD-spermine for 24 and 48 h.

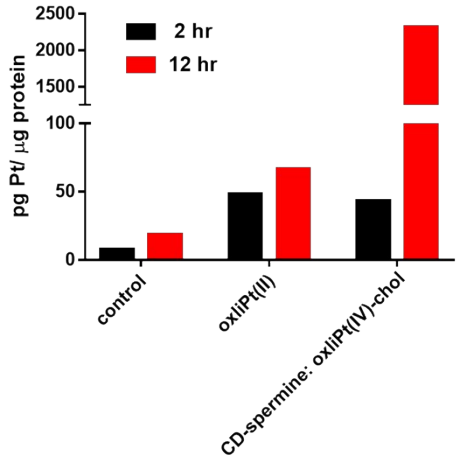


Figure S9. Cellular Pt accumulation in HCT116 cells after 2 and 12 h incubation with CD-spermine : oxliPt(IV)-chol, measured by ICP-MS.

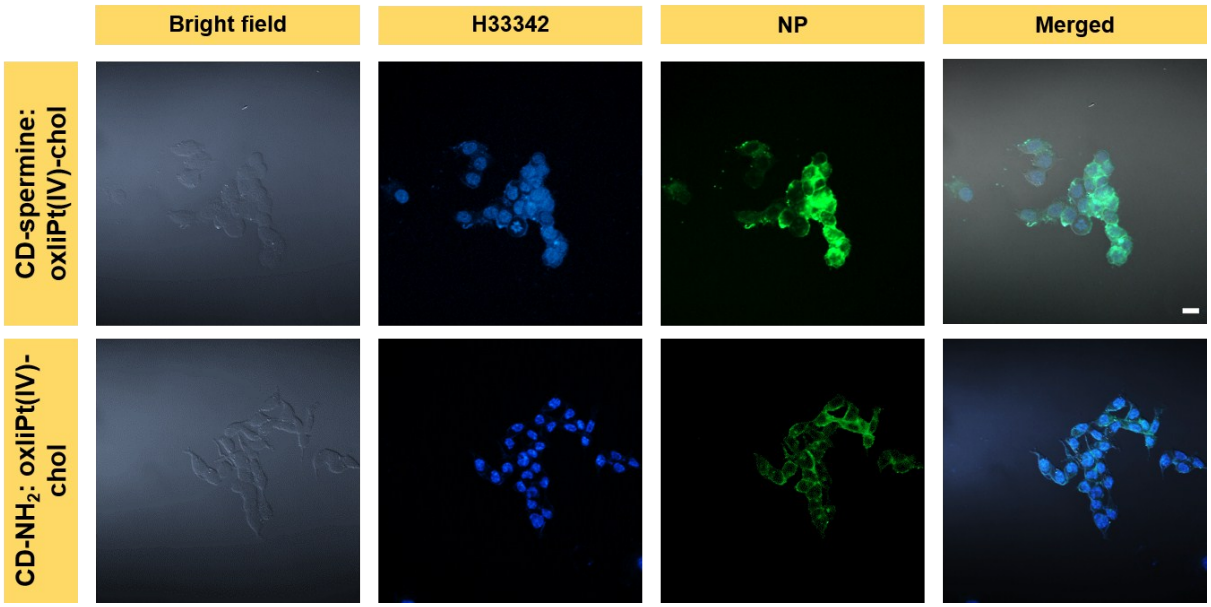


Figure S10. CLSM images of HCT116 cells incubated with 10 µM CD-spermine : oxliPt(IV)-chol or CD-NH_2 : oxliPt(IV)-chol nanoparticles for 4 h. Nuclei were stained with H33342. Scale bar: 20 µm.

References

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