Electronic Supplementary Information (ESI)

Self-assembly of Stimuli-Responsive Imine-linked Calix[4]arene Nanocapsules for Targeted Camptothecin Delivery

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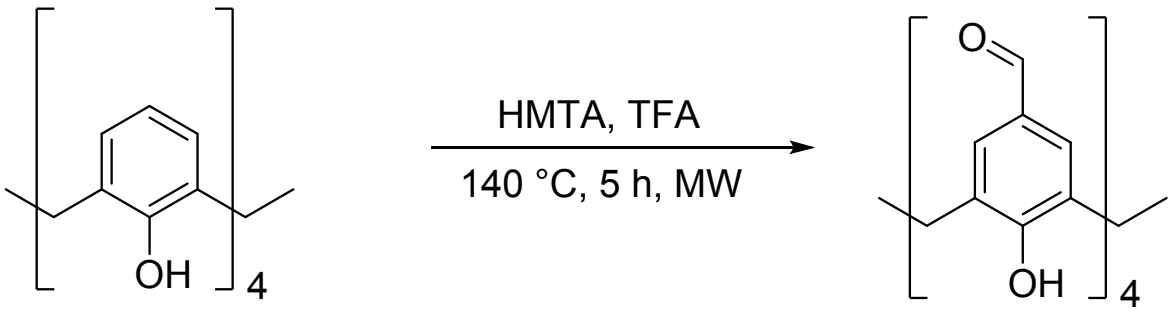
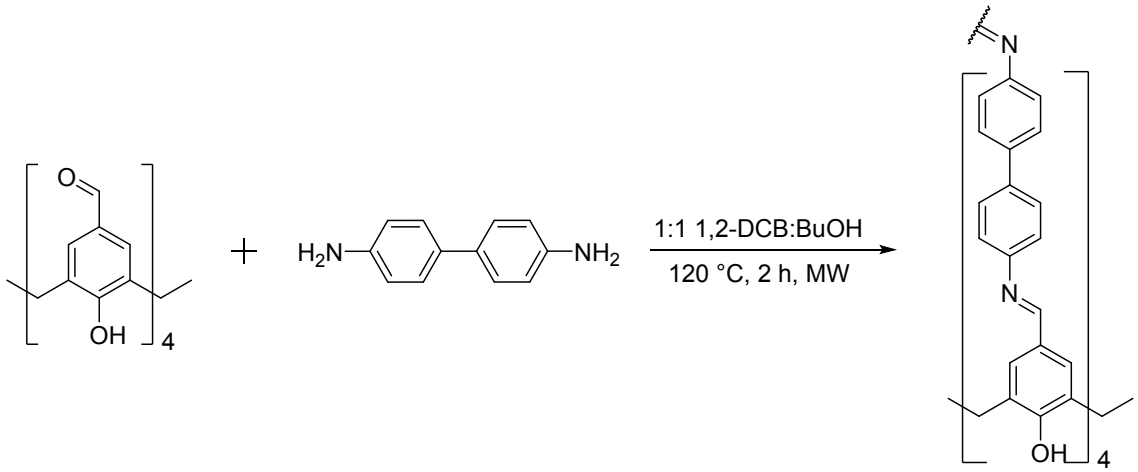
General. All chemicals and solvents were purchased from Sigma-Aldrich and used without further purification. Deionized water was used from Millipore Gradient Milli-Q water purification system. Routine nuclear magnetic resonance (NMR) spectra were recorded at 25 °C on a Bruker Avance spectrometer, with working frequencies of 500 MHz for ^{1}H, and 125 MHz for ^{13}C nuclei, respectively. All chemical shifts are reported in ppm relative to the signals corresponding to the residual non-deuterated solvent.

Materials characterization. FTIR studies were carried out on Agilent 670-IR spectrometer in the attenuated total reflectance (ATR) mode. TGA experiments were performed on a TA SDT Q600 with a heating rate of 10 °C/min over a temperature range of 22–1000 °C. Powder X-ray diffraction (PXRD) measurements were carried out on Bruker D8 Advance X-ray diffractometer with Cu K_{α} (λ = 1.5405 Å) radiation source operating at 40 kV and 30 mA. The patterns were recorded with divergent slit of 1/16° over the 2θ range of 1–50° with step size = 0.01°. SEM images were obtained from FEI Quanta 450FEG. TEM images were obtained from a FEI-Titan 300 operating at 200 kV. Dynamic light scattering (DLS) measurements were performed on a Malvern Zetasizer NanoSeries to obtain the hydrodynamic diameter of the nanoparticles. UV-Vis studies were carried out on Cary 5000 UV-Vis-NIR spectrophotometer. All UV-Vis spectra were recorded at room temperature using a quartz cell with 10 mm path length over the range 200–800 nm and corrected against an appropriate background spectrum. Emission spectra at room temperature were recorded on a Perkin Elmer LS55 Fluorescence Spectrometer using an excitation wavelength of 370 nm, which corresponds to the maximum absorption of camptothecin (CPT). Phase contrast and fluorescence images were observed on the Olympus FV1000MPE confocal scanning microscope using a 405 nm laser at 10–12 % power.



CX4-CHO (1) synthesis. CX4-CHO was prepared according to a previously published procedure[1] with minor modifications. In a 35 mL microwave reaction vessel calix[4]arene-25,26,27,28-tetrol (0.2 g, 0.471 mmol, 1 eq) and hexamethylenetetramine (1.32 g, 9.423 mmol, 20 eq) were dissolved in trifluoroacetic acid (15 mL), and the reaction mixture was heated at 140 °C for 5 h under microwave irradiation. A mixture of 40 mL 1M HCl and 40 mL $CHCl_3$ was added to the crude reaction mixture and stirred overnight at room temperature. The aqueous phase was extracted with $CHCl_3$, and the collected organic phases were washed with water. The solvent was evaporated at reduced pressure and triturated from dichloromethane to give a dark yellow solid (70 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.62 (s, 4H, CHO), 8.32 (s, 4H, OH), 7.63 (s, 8H, CH), 4.41 (b, 8H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 190.9 (CHO), 161.9 (C-OH), 130.7 (CH), 128.1 (C-CH_2), 31.9 (CH_2).



CX4-NC synthesis. 25.0 mg of CX4-CHO was dissolved in 2.5 mL of 1,2-dichlorobenzene and 2.5 mL of 1-butanol, and the mixture was added to 17.0 mg of benzidine in a 8-mL microwave reactor tube. 100 µL of 6M acetic acid was added as a catalyst. The reaction was performed in CEM Discover SP microwave reactor at 120 °C for 2 h with high stirring. The contents of the reaction vessel were centrifuged and repeatedly washed with ethanol. The solid was dried in a vacuum oven at 40 °C overnight.

***In situ* drug loading.** 6.0 mg of CX4-CHO was dissolved in 1.2 mL of a 1:1 mixture of 1,2-dichlorobenzene and 1-butanl, and the mixture was added to 4.0 mg of benzidine and 0.84 mg of CPT in a 8-mL microwave reactor tube. 50 µL of 6M acetic acid was added slowly as a catalyst.

The mixture was heated in a microwave at 120 °C for 2 h with high stirring. The contents of the reaction vessel were centrifuged and repeatedly washed with 20 % DMSO, water, and ethanol to remove surface-adsorbed CPT molecules, and dried in vacuum oven at 40 °C overnight. The amount of CPT encapsulated was quantified using a previously reported method.[2,3] In brief, 0.300 mg of CPT-encapsulated polymer was suspended in 300 µL of DMSO and the mixture was briefly sonicated. After stirring the mixtures for 10 minutes, an aliquot of 10 µL was mixed with 2.0 mL PBS, 20 µL 1N HCl and 20 µL sodium dodecyl sulfate (SDS). The fluorescence was read (370 nm excitation, 428 nm emission) in a 1-cm quartz cuvette. The quantity of CPT encapsulated was determined using a calibration curve with various concentration of free CPT in DMSO in the same quantification mixture of PBS, HCl and SDS.

Post-synthetic drug loading. 2.7 mg of CPT was dissolved in 300 µL of DMSO and diluted with water to a total volume of 14 mL. **CX4-NC** (4.5 mg) was suspended in the above drug solution, sonicated for 30 s and stirred at 500 rpm for 7 days. The mixture was centrifuged and the solid was repeatedly washed with 20 % DMSO, water and methanol to remove surface-adsorbed CPT molecules, and dried in vacuum oven at 40 °C overnight. The amount of CPT encapsulated was studied in the same way as described above for in situ loaded drug.

Drug release experiment. CPT release was studied at acidic pH of 5.4 in 0.1 M acetate buffer to mimic the tumor environment.[2] 0.700 mg of CPT-loaded capsules were dispersed in 4 mL of the buffer, briefly sonicated and stirred at 350 rpm. At various pre-determined time points, 2 mL of the mixture was centrifuged and the supernatant was mixed with 20 µL 1N HCl, 20 µL SDS and 10 µL DMSO. The fluorescence was read (370 nm excitation, 428 nm emission) in a 1-cm quartz cuvette. Whenever signal reached saturation point, the sample was further diluted in the same mixture of acetate buffer, HCl, SDS and DMSO and fluorescence was re-read. For every time point, 2 mL of the buffer removed was replenished with fresh one. CPT release in PBS and in presence of glutathione was studied in the same way as described above for release at acidic pH.

Cell culture. Human breast cancer cell line MCF-7 was cultured at 37 °C and 5 % CO_2 in 50 mL tissue cultures flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % FBS and 2% penicillin-streptomycin antibiotic. Cells were detached with trypsin and counted using Countess II FL Automated Cell Counter (ThermoFisher Scientific) to obtain a final concentration of 50,000 cells/mL.

***In vitro* cell viability assay.** 96-well plates were seeded with cells (~5,000 cells per well in 100 µL of DMEM) and incubated at 37 °C for 24 hours. The medium was removed and replaced with fresh DMEM (control) or various concentrations of CPT, free **CX4-NC** or CPT-loaded **CX4-NC** (all in 10 % DMSO) and incubated at 37 ºC for 48 hours. Thereafter, cells were washed with PBS and incubated with 20 µL of CellTiter-Blue® (CTB, Promega) reagent per well for six hours at 37 ºC. The fluorescence of the resorufin product ($\lambda_{ex}/_{em}$ 560/620) was measured. Untreated wells were used as a control.

Confocal microscopy. MCF-7 cells were seeded in CELLview™ cell culture dishes (Greiner Bio One) at a density of 50,000 cells/mL and incubated at 37 °C and 5 % CO_2 for 72 h. The medium

was removed and replaced with 10 % DMSO (control) or 10 μg/mL free **CX4-NC** or CPT-loaded **CPT-CX4-NC** (10 % DMSO), and incubated for various time periods (3 h, 24 h, 48 h) under the same conditions. The cells were washed with PBS and DMEM several times before being imaged in DMEM.

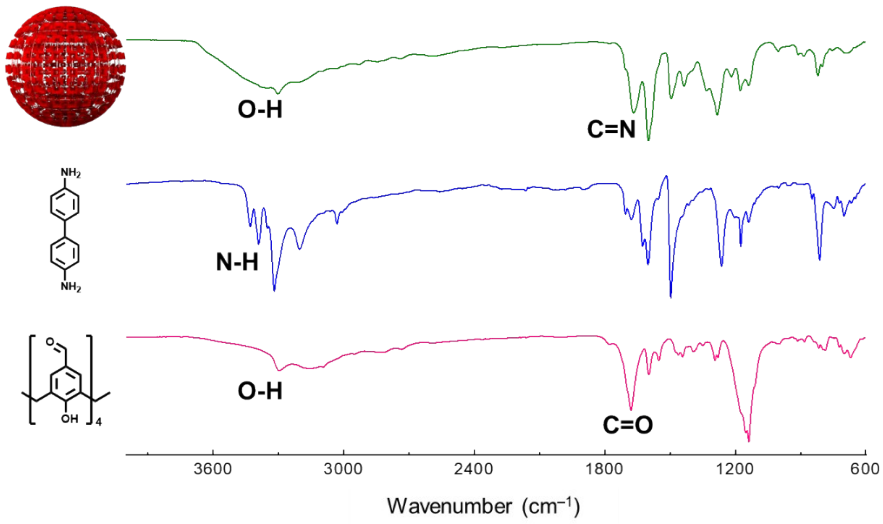


Figure S1. FTIR spectra of **CX4-NC** and corresponding monomers, CX4-CHO (**1**) and benzidine (**2**).

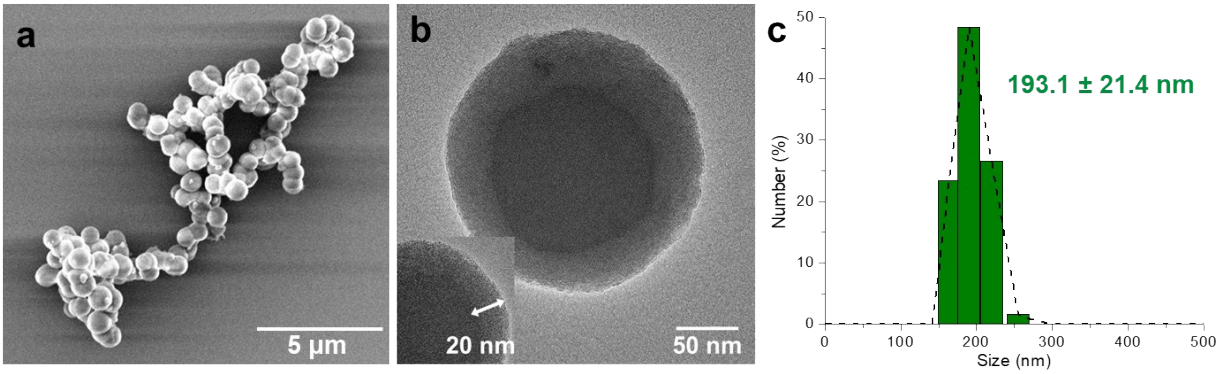


Figure S2. Characterization of as-synthesized **CX4-NC**. SEM micrograph (a), TEM micrograph (b) and size distribution measured by DLS (c). Inset shows the shell thickness of **CX4-NC**.

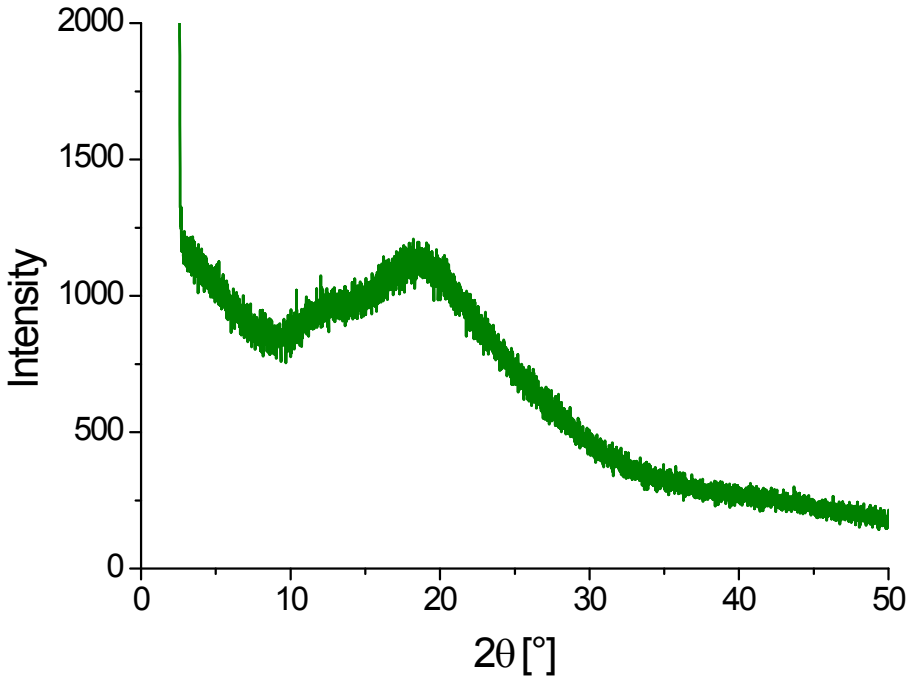


Figure S3. Powder X-ray diffraction (PXRD) pattern of **CX4-NC**.

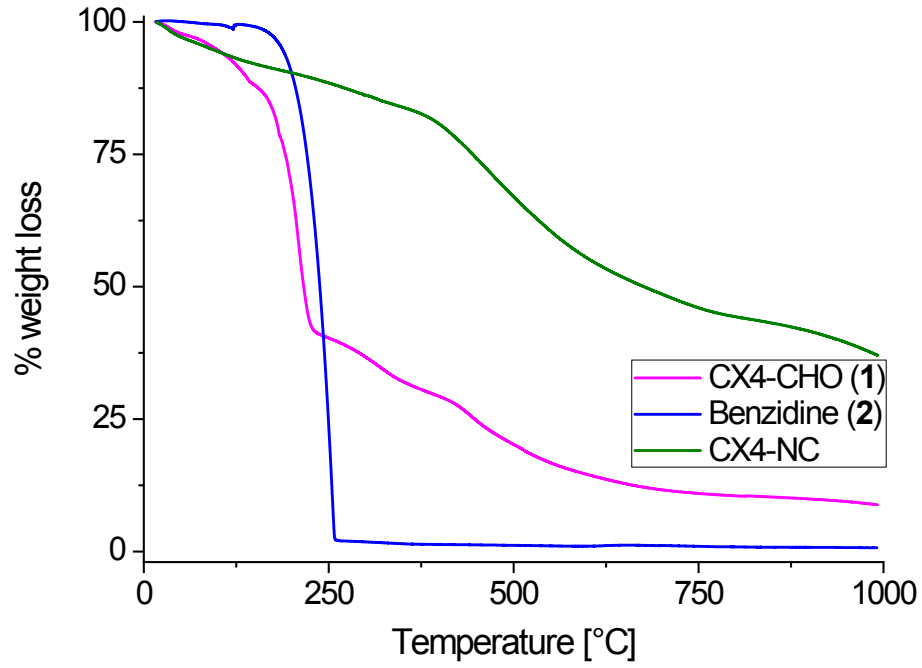


Figure S4. TGA plots of **CX4-NC** (green), benzidine (blue, **2**) and CX4-CHO (magenta, **1**).

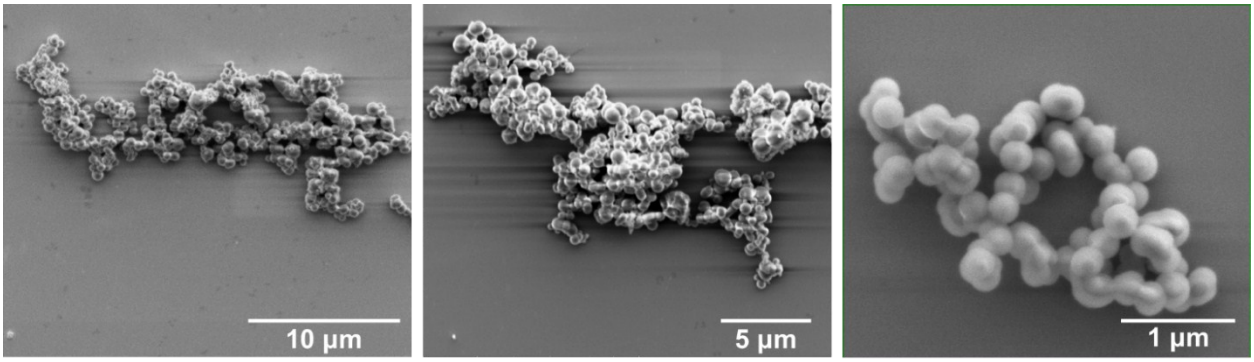


Figure S5. Evaluation of stability of **CX4-NC** in PBS. SEM images obtained after 48 h incubation in PBS at room temperature.

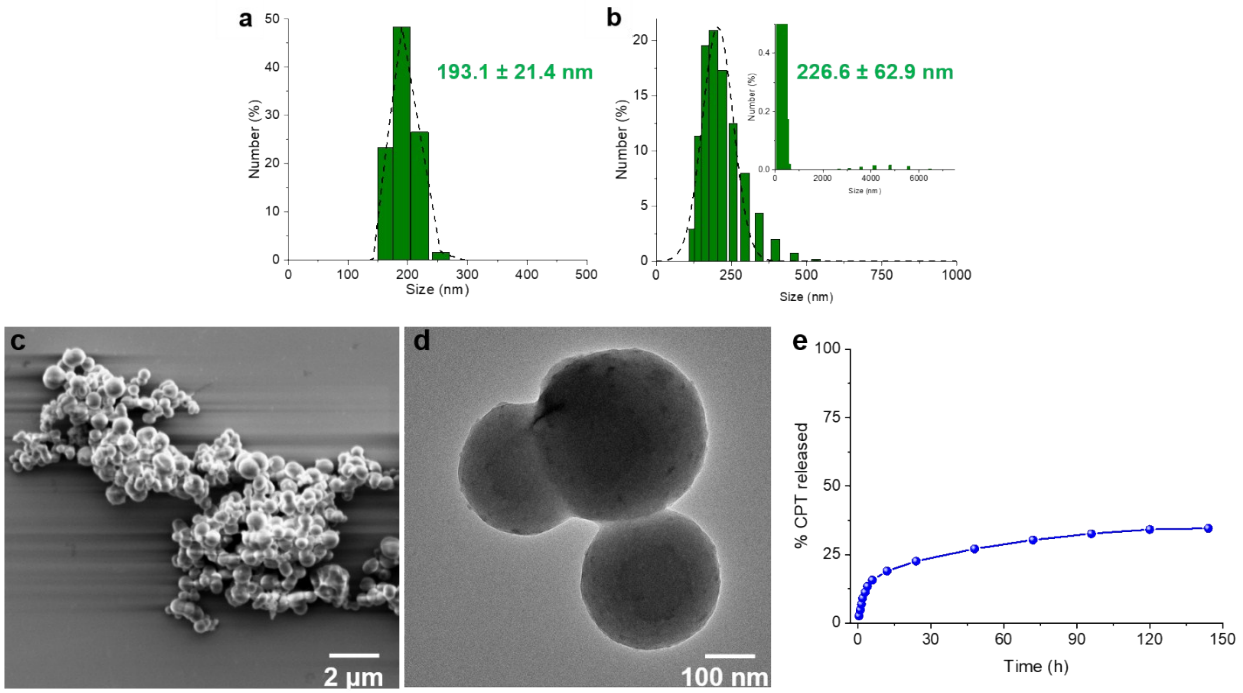


Figure S6. DLS measurements of as-synthesized **CX4-NC** dispersed in PBS (a) and following a *48h* incubation in PBS (b). Evaluation of stability of **CPT-CX4-NC** at 37 ^{0}C in PBS. (c) SEM and (d) TEM images of **CX4-NC** obtained after 48 h incubation. (e) Drug release profile of **CPT-CX4-NC** incubated in PBS at 37 ^{0}C.

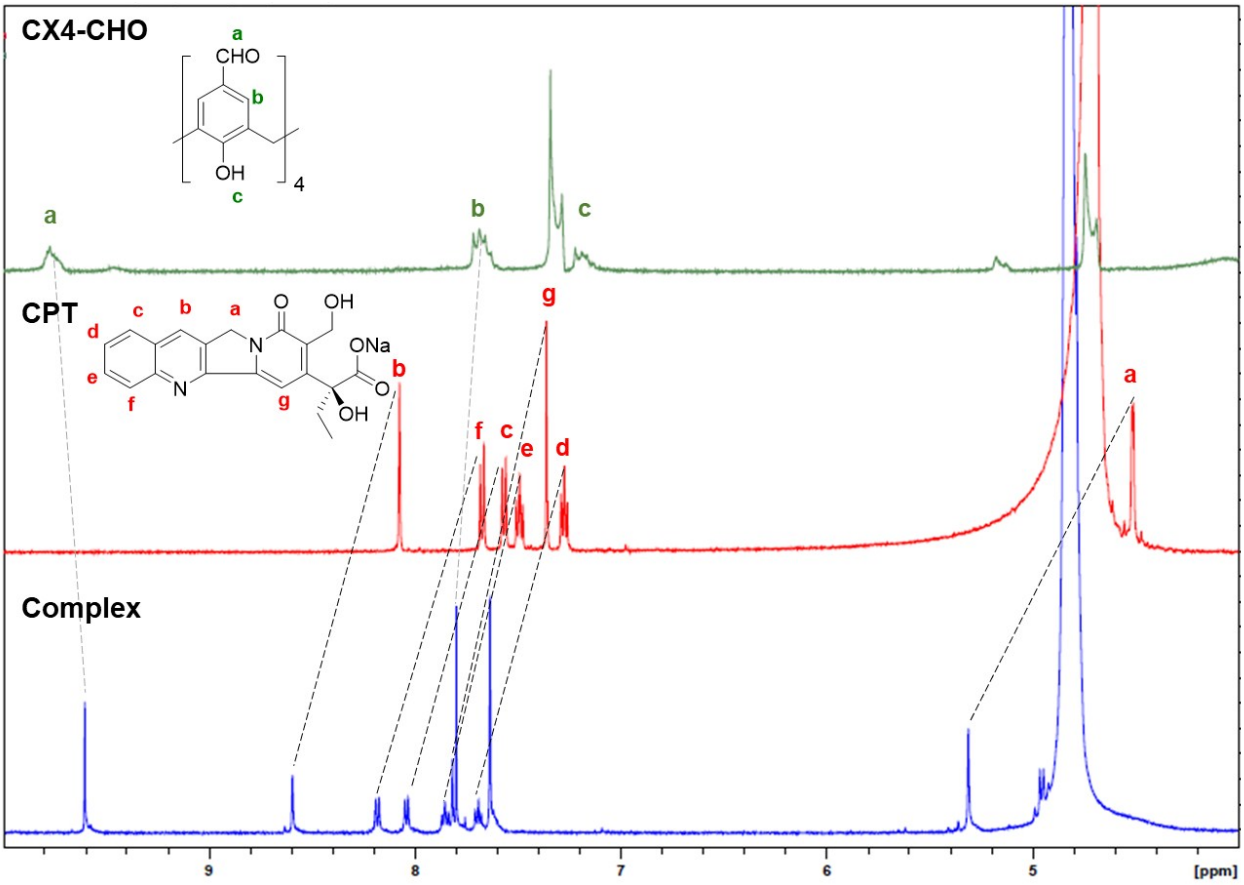


Figure S7. ^{1}H NMR spectra (500 MHz, CD_3OD:$CDCl_3$; 4:1) of individual CX4-CHO and CPT compared with the spectrum of a mixture of the two in a 1:1 molar ratio. In free CPT, aromatic protons appear at 7.27, 7.36, 7.49, 7.56, 7.67 and 8.10 ppm whereas in the complex these peaks shift to 7.69, 7.82, 7.84, 8.03, 8.18 and 8.59 ppm, respectively. Furthermore, CX4-CHO also exhibits changes in chemical shifts of its protons. Specifically, its aromatic proton shifts from 7.64 ppm to 7.82 ppm.

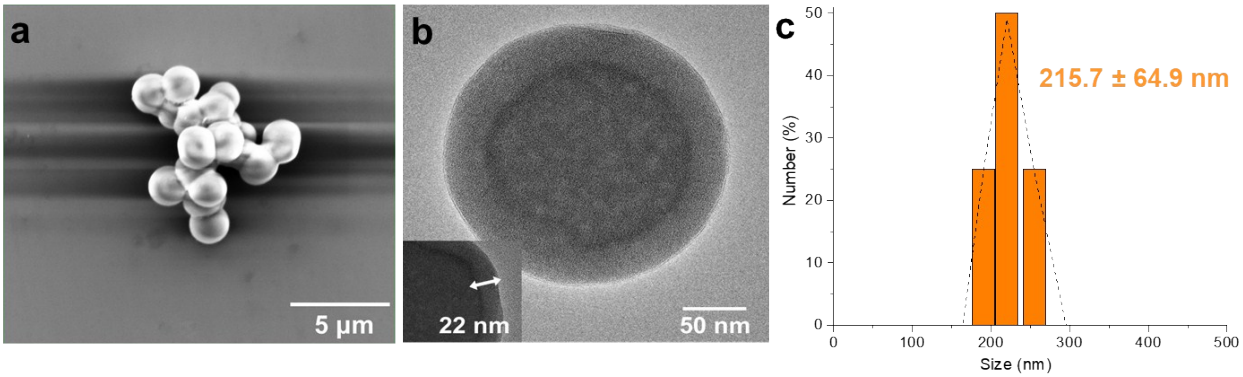


Figure S8. Characterization of **CX4-NC** after CPT loading. SEM micrograph (a), TEM micrograph (b) and size distribution measured by DLS (c) of post-synthetically CPT-loaded **CX4-NC**. Inset shows the shell thickness of **CPT-CX4-NC**.

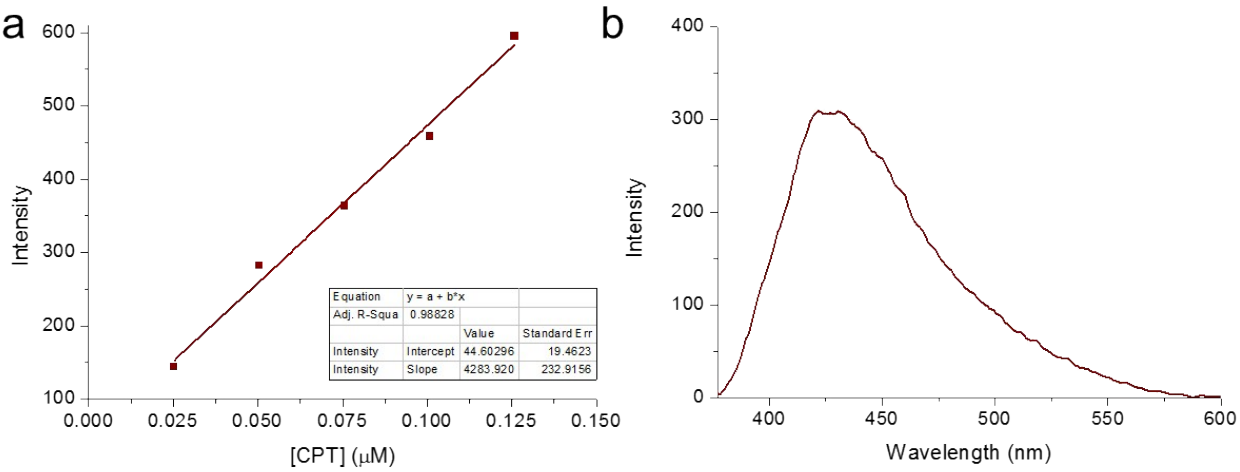


Figure S9. Calibration curve used to determine CPT loading in **CX4-NC** based on a reported method (a),[2,3] and fluorescence signal obtained in calculating CPT loading (b).

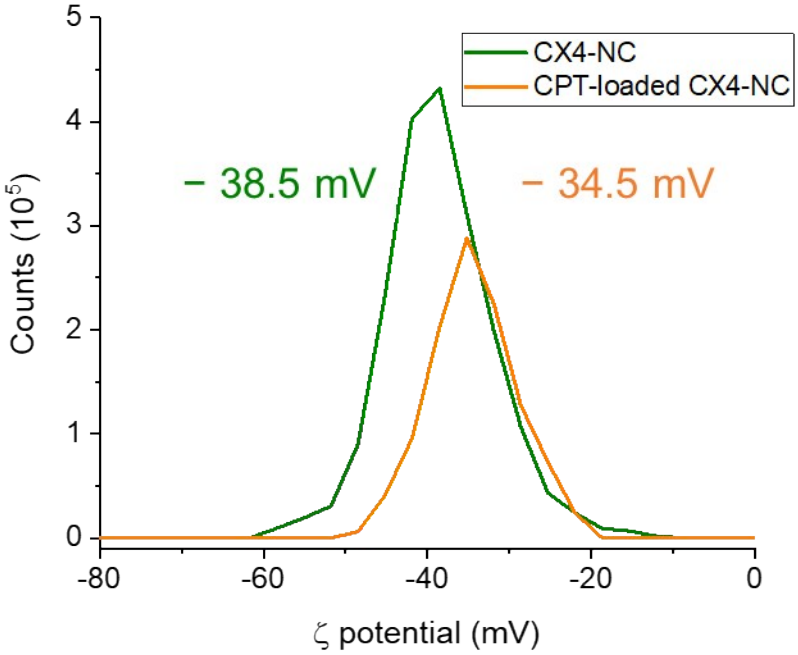


Figure S10. ζ potential for **CX4-NC** before and after CPT loading.

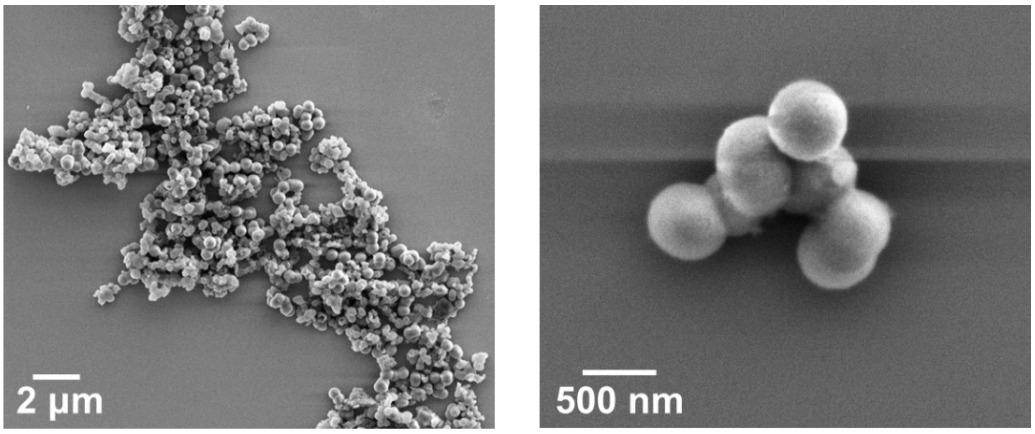


Figure S11. SEM images of **CX4-NC** after CPT release at physiological conditions.

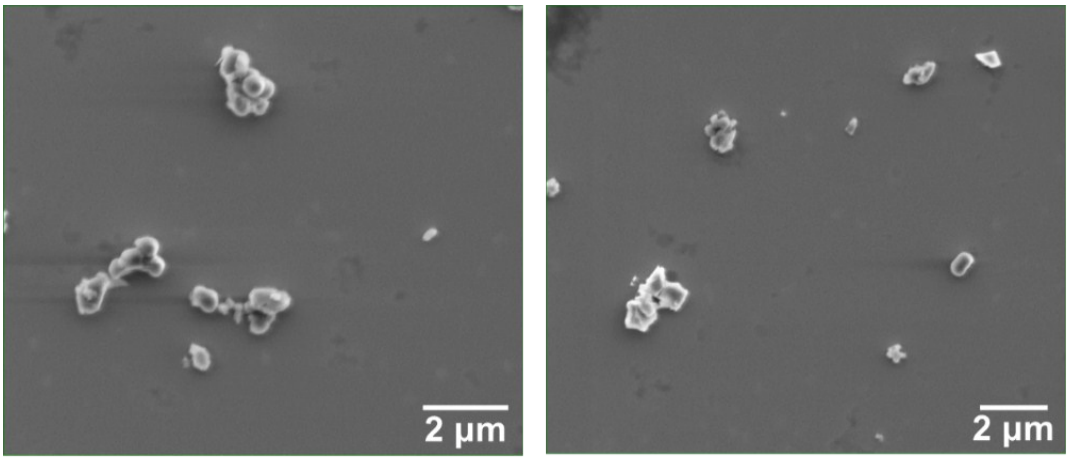


Figure S12. SEM images of **CX4-NC** after CPT release at acidic conditions.

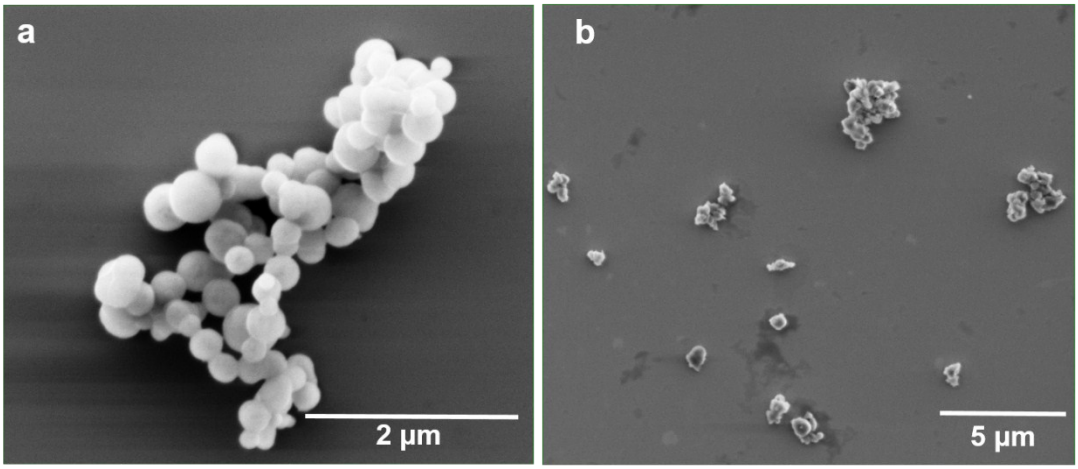


Figure S13. SEM images of **CX4-NC** at different stages of CPT release in presence of GSH. In early stages (a, t = 2 h), the morphology of capsules is preserved, but over time (b, t = 48 h) NCs disintegrate.

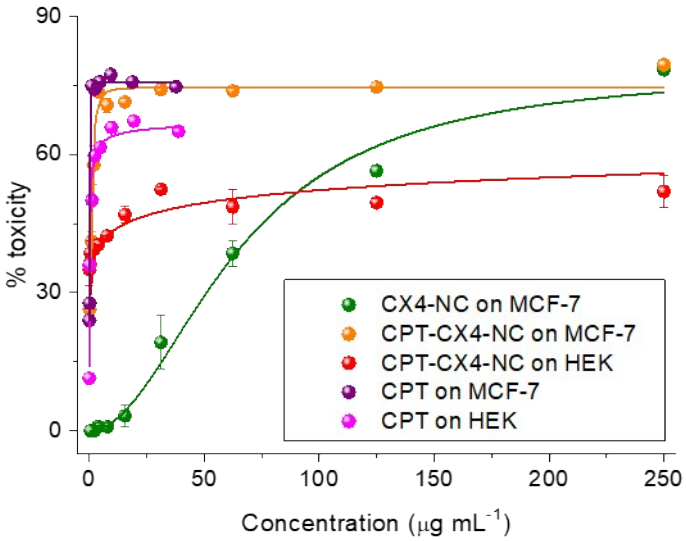


Figure S14. Tumor cytotoxicity observed for **CX4-NC, CPT-CX4-NC** and **CPT** alone on MCF-7 breast cancer cells and HEK noncancerous cells at concentrations ranging from 0.6 to 250 μg mL^{-1}.

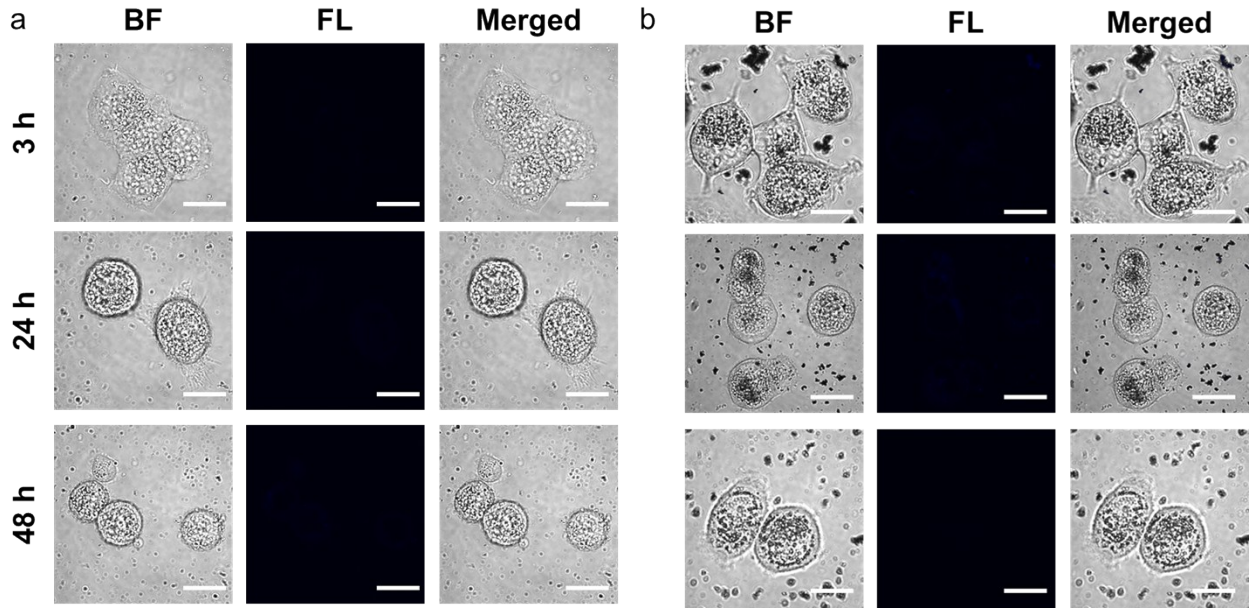


Figure S15. Confocal microscopy images of control (a; 10 % DMSO) and **CX4-NC** (b; 10 μg mL^{-1}) at various times of incubation (3 h, 24 h and 48 h) with MCF-7 cells. Scale bar 5 μm.

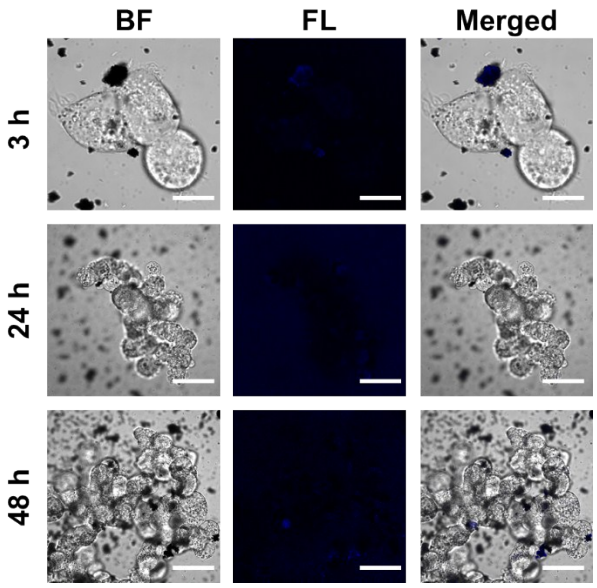


Figure S16. Confocal microscopy images of **CPT-CX4-NC** (10 μg mL^{-1}) incubated with HEK cells at various times of incubation (3 h, 24 h and 48 h). Scale bar 5 μm.

References

1 S. Pasquale, S. Sattin, E. C. Escudero-Adán, M. Martínez-Belmonte and J. de Mendoza, *Nat. Commun.*, 2012, **3**, 785.

2 A. J. Sawyer, J. K. Saucier-Sawyer, C. J. Booth, J. Liu, T. Patel, J. M. Piepmeier and W. M. Saltzman, *Drug Deliv. Transl. Res.*, 2011, **1**, 34–42.

3 K. T. Householder, D. M. Diperna, E. P. Chung, G. M. Wohlleb, H. D. Dhruv, M. E. Berens and R. W. Sirianni, *Int. J. Pharm.*, 2015, **479**, 374–380.