Electronic Supplementary Information (ESI)

Multi-layered polymeric nanoparticles for pH-responsive and sequenced release of theranostic agents

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Section 1: Experimental Section

Materials: PLGA (lactide:glycolide: 75:25, Mw: 4,000-15,000) and Pluronic F127 (PF127) were purchased from Sigma (St. Louis, MO, USA). Polyvinyl Alcohol (PVA, Mw: 100 kDa) was purchased from Fisher Scientific (Pittsburgh, PA, USA). The CCK-8 cell proliferation reagent was purchased from Dojindo Molecular Technologies (Rockville, MD, USA). Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). The F-12K and DMEM cell culture media were purchased from ATCC (Manassas, VA, USA). Doxorubicin was purchased from LC laboratories (Woburn, MA, USA). Irinotecan was purchased from Selleck Chemicals (Houston, TX, USA). All other chemicals were purchased from Sigma unless specifically mentioned otherwise.

Preparation of NPs: S-NPs (with or without drug) were prepared using a single-emulsion method. Briefly, 10 mg of PLGA, 10 mg of PF127, and the desired amount of Cur were dissolved in 1 ml of dichloromethane (DCM) and this solution together with 10 ml of 2% polyvinyl alcohol (PVA) solution (in DI water)[1-4] was transferred into a centrifuge tube where the two immiscible solutions were emulsified by sonication for 2 min using a Branson 450 sonifier. After rotary evaporation of the resultant emulsion to remove organic solvent (DCM), the S-NPs were collected by centrifugation at 13,000 rpm for 10 min at room temperature and washed twice with DI water. D-NPs were prepared using a double-emulsion method, for which 10 mg of PLGA together with 10 mg of PF127 was dissolved in 2 ml of DCM. After adding 0.4 ml DI water either with or without Dox, the immiscible solutions were emulsified by sonication for 1 min. This first emulsion was mixed with 100 µl Cur solution (in DCM). Then, 4 ml of 2% PVA solution (in DI water) was added and the mixture emulsified by sonication for 2 min. After rotary evaporation to remove organic solvent, the NPs were collected by centrifugation at 13,000 rpm for 10 min at room temperature and washed twice with DI water. T-NPs were prepared using a triple-emulsion

method. First, 10 mg of PLGA, 10 mg of PF127, and the desired amount of Cur were dissolved in 0.5 ml of DCM. This solution together with 2 ml of 2% PVA solution (in DI water) was transferred into a centrifuge tube where the two immiscible solutions were emulsified by sonication for 1 min. The first emulsion solution was mixed with 100 µl Dox solution (in DI water). Second, 4 ml of DCM with PLGA and PF127 were added and emulsified by sonication for 2 min. The second emulsion was then mixed with 100 µl Cpt solution (in DCM). Third, 10 ml of % PVA solution (in DI water) were added and the mixture emulsified by sonication for 2 min. After rotary evaporation to remove organic solvent, the NPs were collected by centrifugation at 13,000 rpm for 10 min at room temperature and washed twice with DI water. Q-NPs were prepared using a quadruple-emulsion method. First, 10 mg of PLGA and 10 mg of PF127 were dissolved in 1 ml of DCM. After adding 0.2 ml DI water either with or without Dox, the immiscible solutions were emulsified by sonication for 1 min. This first emulsion was mixed with 100 µl Cur solution (in DCM). Second, 2 ml of 2% PVA solution (in DI water) were added and the mixture emulsified by sonication for 2 min. The second emulsion was mixed with 100 µl Icg solution (in DI water). Third, 4 ml of DCM with PLGA and PF127 were added and the mixture emulsified by sonication for 2 min. The third emulsion was mixed with 100 µl Cpt solution (in DCM). Fourth, 15 ml of % PVA solution (in DI water) were added and the mixture emulsified by sonication for 2 min. After rotary evaporation to remove organic solvent, the NPs were collected by centrifugation at 13,000 rpm for 10 min at room temperature and washed twice with DI water.

Characterization of NPs: The morphology of NPs was characterized using both transmission (TEM) and scanning (SEM) electron microscopy. For TEM, the NPs were negatively stained with uranyl acetate solution (2%, w/w) and examined using an FEI (Moorestown, NJ, USA) Tecnai G2 Spirit transmission electron microscope. SEM experiments were conducted by depositing 10 µL of aqueous suspension of the NPs on a freshly cleaved mica grid and allowing

them to dry for 60 minutes in air. A thin film of Au was then sputtered onto the NPs on the substrate. Samples were imaged with an FEI NOVA nano400 scanning electron microscope.

The NP size (diameter, nm), polydispersity index (PDI), and surface charge (or zeta potential) were determined using a Brookhaven (Holtsville, NY, USA) 90 Plus/BI-MAS dynamic light scattering (DLS) instrument by dispersing the NPs at 1 mg/ml in DI water.

Encapsulation efficiency and in vitro release: Encapsulation efficiency (EE) of agents using the NPs was calculated by the following equation:

$$EE=A/B \times 100\% \quad (1)$$

where "A" represents the amount of drug retained in NPs and "B" is the initial amount of drug fed for encapsulation. The amount of Cpt, Cur, Dox, and Icg in the NPs was determined using a Beckman Coulter (Indianapolis, IN, USA) DU 800 UV-Vis spectrophotometer based on their absorbance at 370 nm, 420 nm, 500 nm, and 780 nm, respectively.

For in vitro drug release study, drug-laden NPs (20-30 mg) were reconstituted in PBS (5 ml, pH 5 or 7.4) and transferred into dialysis bags (MWCO: 20 kDa) that were placed in 30 ml of the same PBS at 37 °C and stirred at 110 rpm using a mini-stir bar. At appropriate time points, 100 µl of the dialysate was collected, and the dialysate was replenished with the same amount of fresh PBS. The concentration of the released drugs in the removed dialysate was determined using UV-Vis spectrophotometer based on their absorbance.

Culture of cancer cells: Human prostate adenocarcinoma PC-3 cancer cells (ATCC, Manassas, VA, USA) were cultured in F-12K medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator. Human breast adenocarcinoma MDA-MB-231 cancer cells (ATCC, Manassas, VA, USA) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified

5% CO_2 incubator.

Cellular uptake and intracellular distribution of NPs: To study cellular uptake of NPs, different drug formulations were cultured with the MDA-MB-231 and PC-3 cells for 5 h. For the intracellular distribution, the PC-3 cells were further treated with medium containing 75 nM LysoTracker Green DND-99 to stain late endosomes/lysosomes. Finally, the cells were mounted onto a glass slide with anti-fade mounting medium from Vector Laboratories (Burlingame, CA, USA) for further examination using an Olympus FluoView™ FV1000 Confocal Microscope.

In vitro cytotoxicity study: For the in vitro cell viability assay, PC-3 and MDA-MB-231 cells were seeded and cultured with various drug formulations overnight in 96-well plates. The total drug content in all the groups was kept at the same. To determine cell viability on 24 h, fresh medium with 10% CCK-8 reagent was added to each well and incubated for 4 h at 37 °C and the absorbance at 450 nm was quantified using a Perkin Elmer VICTOR™ X4 multi-label plate reader. Cell viability was calculated as the ratio of the cell number determined for each group to that of control with no treatment.

2.10 Statistical analysis: All data are reported as the mean ± standard deviation (SD) of results from at least three independent runs conducted at three different times. Student's two-tailed *t*-test assuming equal variance was performed to determine the *p* value for assessing statistical significance ($p < 0.05$).

References

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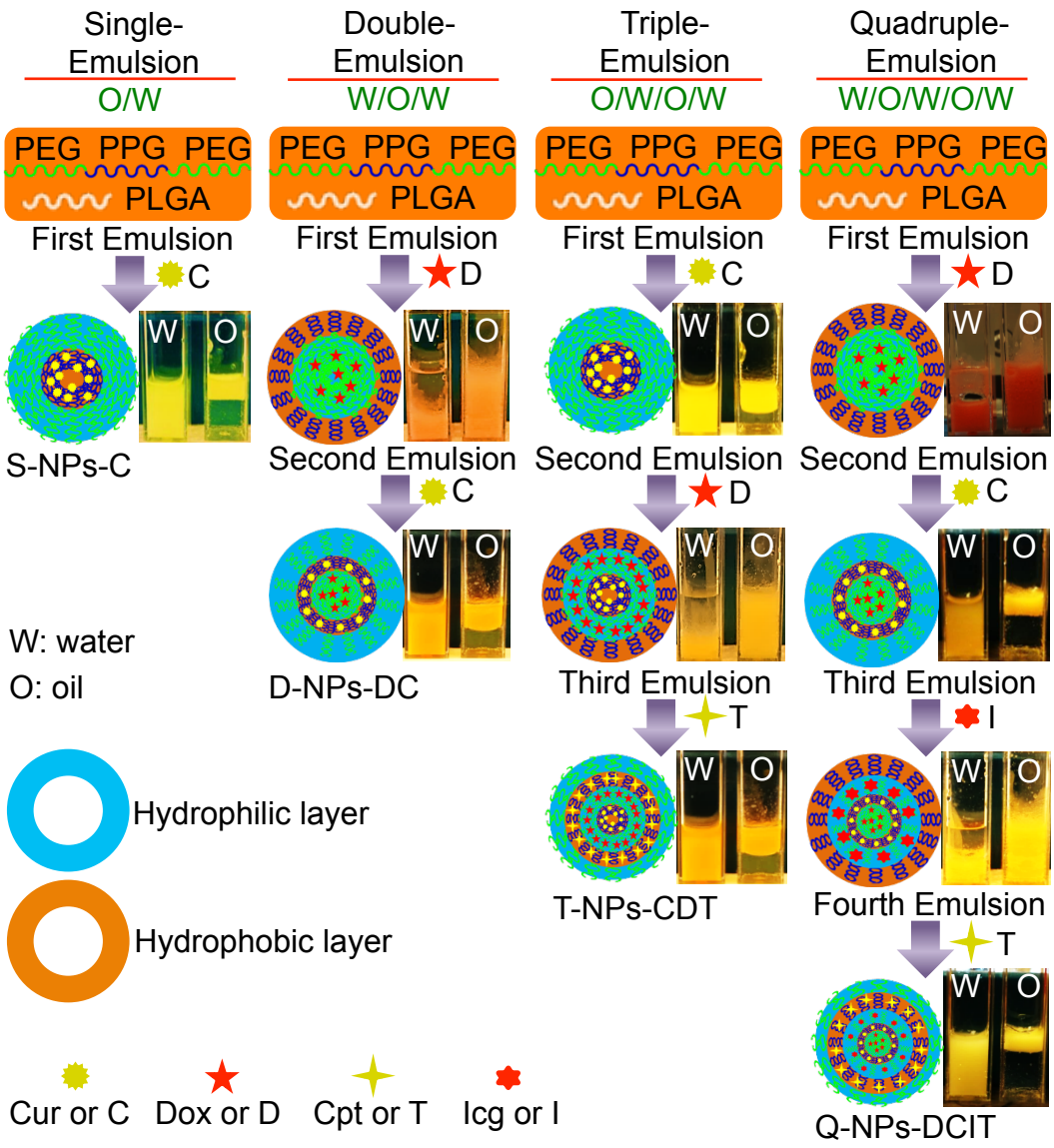
3. M. F. Zambaux, F. Bonneaux, R. Gref, P. Maincent, E. Dellacherie, M. J. Alonso, P. Labrude and C. Vigneron, *J. Control. Release*, 1998, **50**, 31-40.
4. Y. Y. Yang, T. S. Chung and N. P. Ng, *Biomaterials*, 2001, **22**, 231-241.

Section 2: Supplementary Scheme, Figures, and Tables



Scheme 1 A schematic illustration of the procedures for preparing multi-layered polymeric nanoparticles (NPs) with up to four emulsions together with pictures of the NP samples in cuvettes after each emulsion showing their solubility in water (W) versus oil (O, i.e., dichloromethane or DCM in this study). A continuous homogeneous appearance of the sample in the cuvettes indicates miscible or soluble while phase separation with a two-layered appearance in the cuvettes indicates immiscible or insoluble. Successful assembly of a layer-by-layered configuration can be examined by the solubility of the sample in water or oil dependent on the

hydrophilicity of the surface layer of the nano-assembly. During each emulsion, one hydrophobic or hydrophilic agent was added into the system for encapsulation to obtained S-NPs-C, D-NPs-DC, T-NPs-CDT, and Q-NPs-DCIT for the single, double, triple, and quadruple-emulsion methods, respectively. Cur or C: curcumin (hydrophobic); Dox or D: doxorubicin hydrochloride (hydrophilic); Cpt or T: irinotecan (hydrophobic); Icg or I: indocyanine green (hydrophilic); PEG: polyethylene glycol; PPG: polypropylene glycol; and PLGA: poly ($_{D,L}$-lactide-*co*-glycolide).

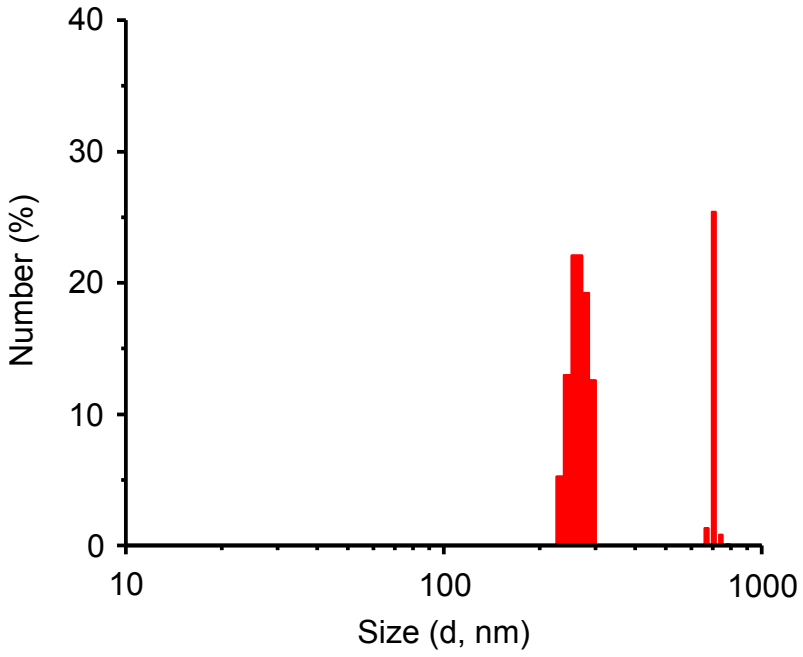


Fig. S1 Size distribution of NPs made of PLGA alone using the double-emulsion method in DI water determined by dynamic light scattering (DLS): two peaks show up because the PLGA NPs of 262 nm tend to form aggregates of 707 nm, probably due to the hydrophobic nature of their surface that drives the smaller PLGA nanoparticles to stick together in the hydrophilic environment of DI water to minimize interfacial energy.

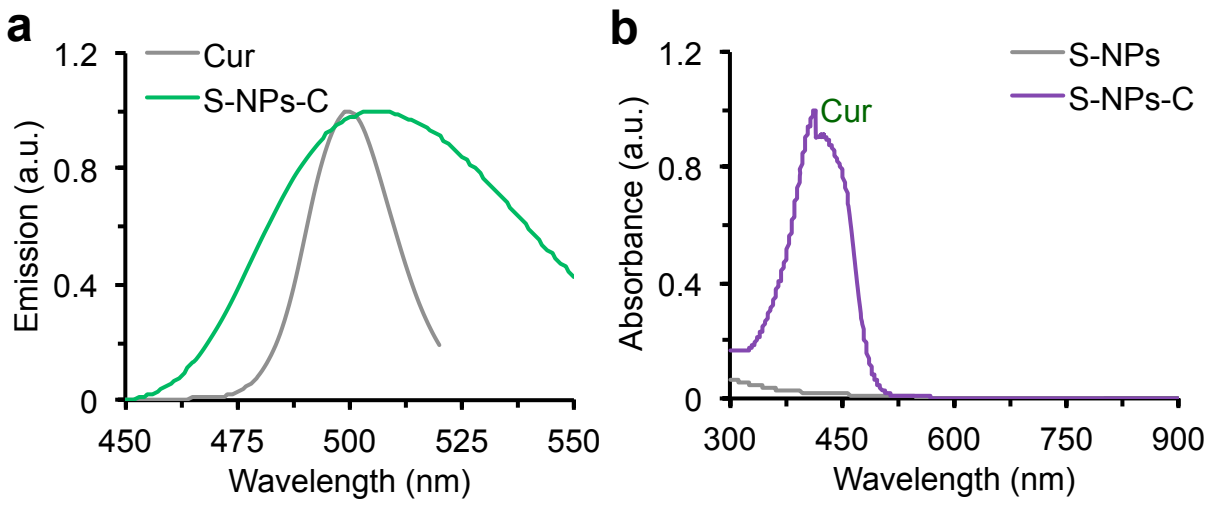


Fig. S2 Encapsulation of curcumin (Cur or C) in S-NPs-C using single-emulsion method. (a) Fluorescence spectra of emission (excitation: 420 nm) of free Cur and S-NPs-C. (b) UV-Vis absorbance of S-NPs and S-NPs-C (in DI water). The data show successful encapsulation of Cur in the S-NPs-C produced using the single-emulsion method.

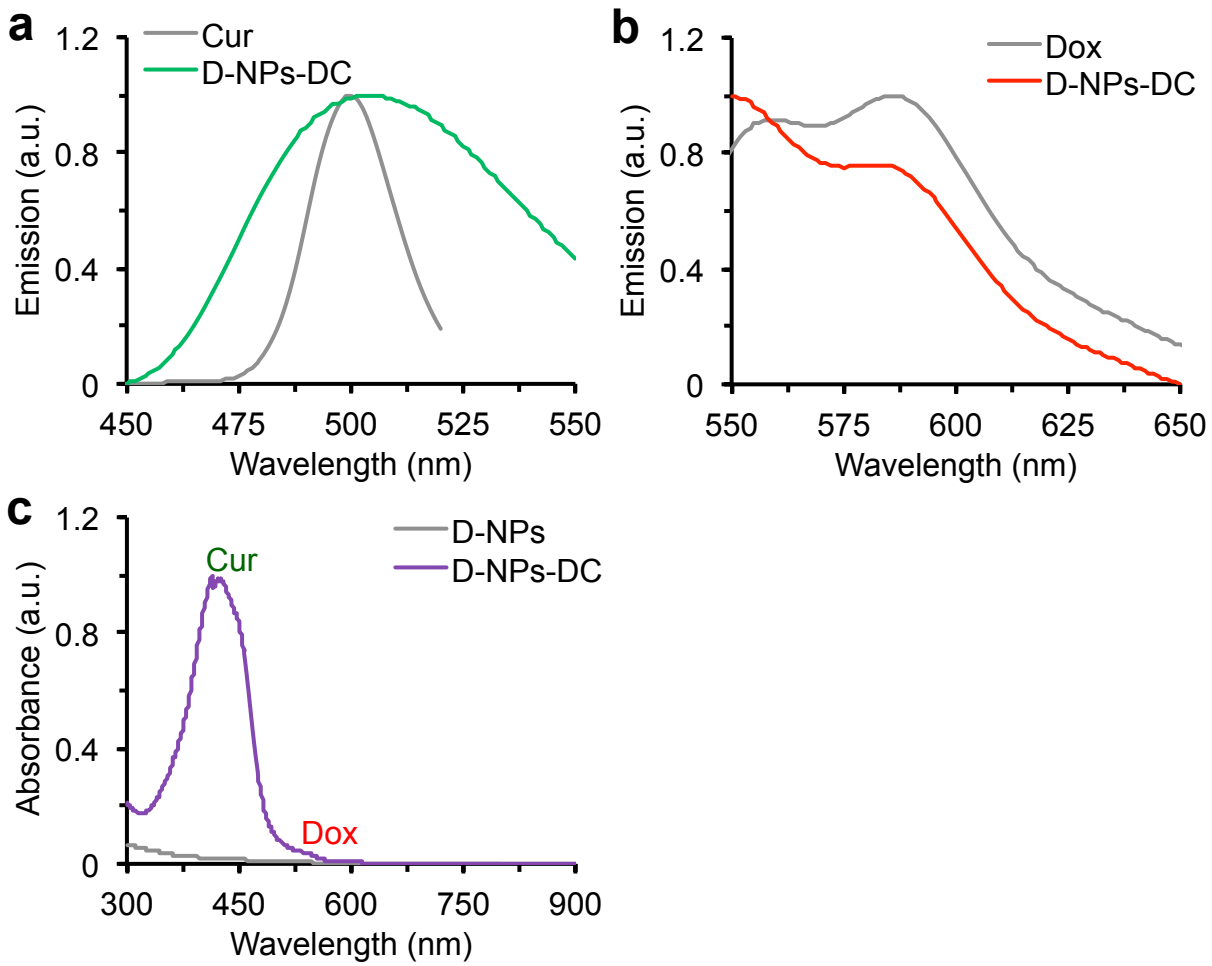


Fig. S3 Encapsulation of doxorubicin (Dox, or D) and curcumin (Cur or C) in D-NPs-DC using double-emulsion method. Fluorescence spectra of emission from (a) free Cur and D-NPs-DC (excitation: 420 nm) and (b) Dox and D-NPs-DC (excitation: 486 nm). (c) UV-Vis absorbance of D-NPs without encapsulating any agents and D-NPs-DC (in DI water). The data show successful encapsulation of Cur and Dox in the D-NPs-DC produced using the double-emulsion method.

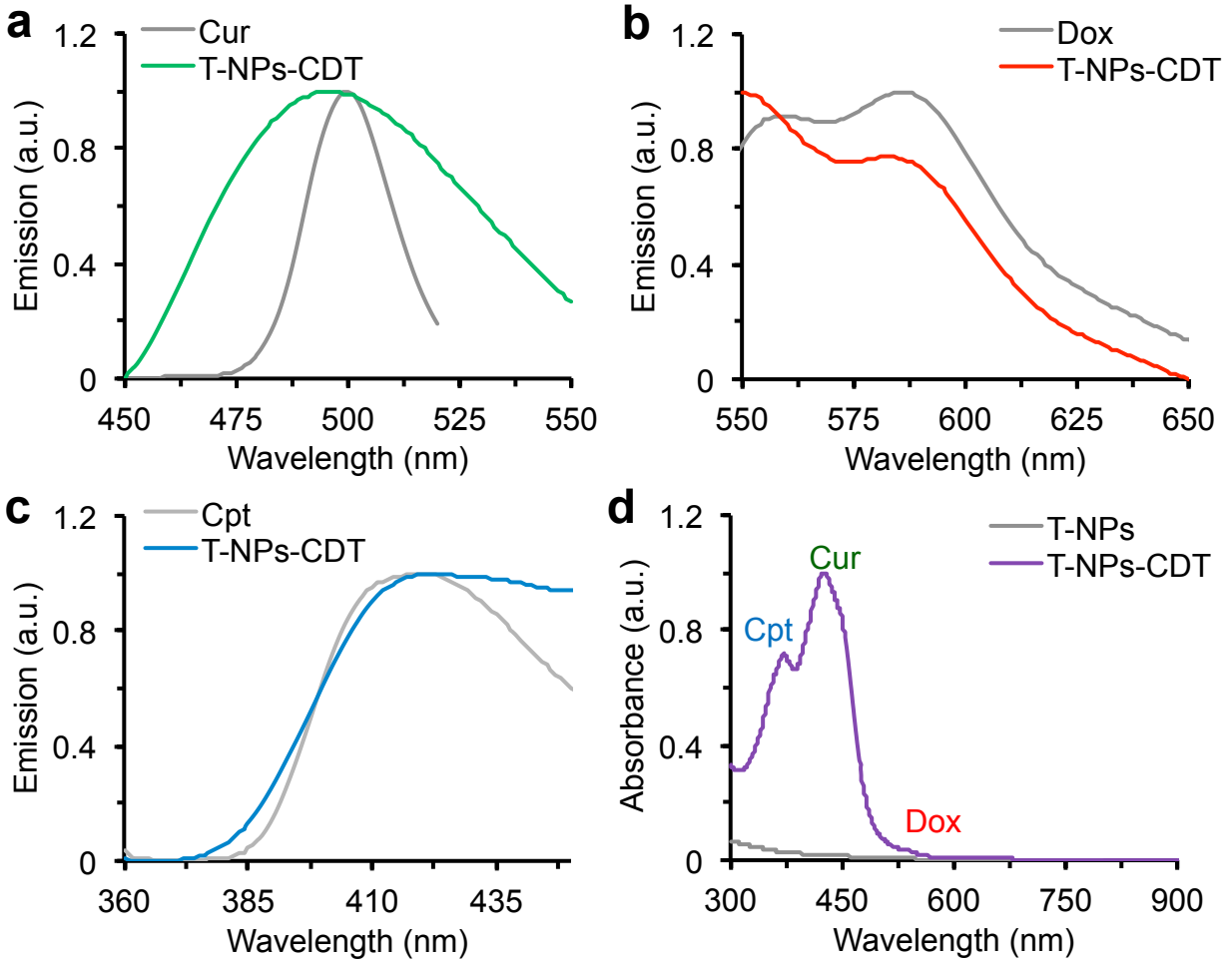


Fig. S4 Encapsulation of curcumin (Cur or C), doxorubicin (Dox, or D), and irinotecan (Cpt or T) in T-NPs-CDT using triple-emulsion method. Fluorescence spectra of emission from (a) free Cur and T-NPs-CDT (excitation: 420 nm), (b) free Dox and T-NPs-CDT (excitation: 486 nm), and (c) free Cpt and T-NPs-CDT (excitation: 350 nm). (d) UV-Vis absorbance of T-NPs without encapsulating any agents and T-NPs-CDT (in DI water). The data show successful encapsulation of Cur, Dox, and Cpt in the T-NPs-CDT produced using the triple-emulsion method.

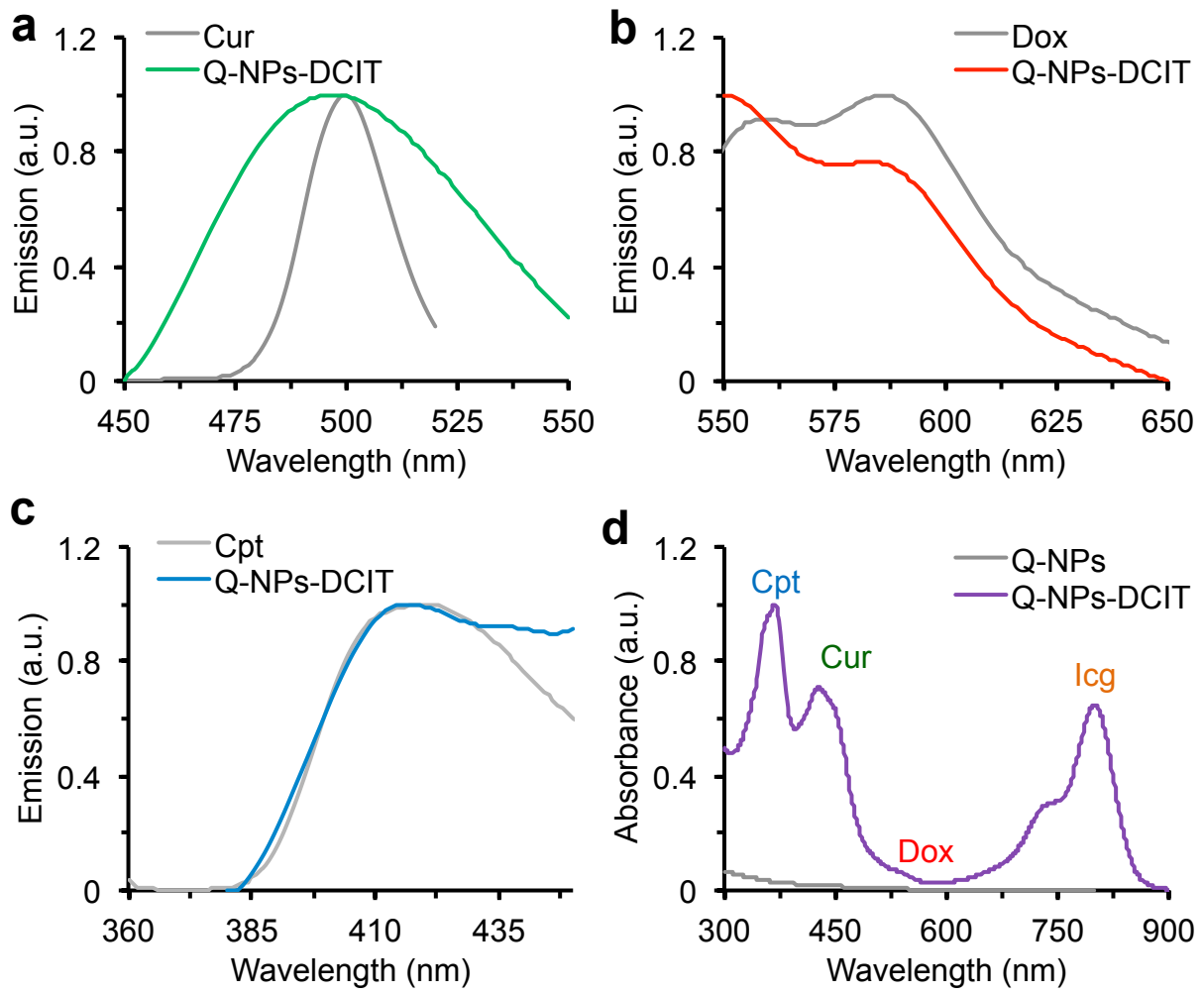


Fig. S5 Encapsulation of doxorubicin (Dox, or D), curcumin (Cur or C), indocyanine green (ICG or I), and irinotecan (Cpt or T) in Q-NPs-DCIT using quadruple-emulsion method. Fluorescence spectra of emission from (a) free Cur and Q-NPs-DCIT (excitation: 420 nm), (b) free Dox and Q-NPs-DCIT (excitation: 486 nm), and (c) free Cpt and Q-NPs-DCIT (excitation: 350 nm). (d) UV-Vis absorbance of Q-NPs without encapsulating any agents and Q-NPs-DCIT (in DI water). The data show successful encapsulation of Cur, Dox, Icg, and Cpt in the Q-NPs-DCIT produced using the quadruple-emulsion method.

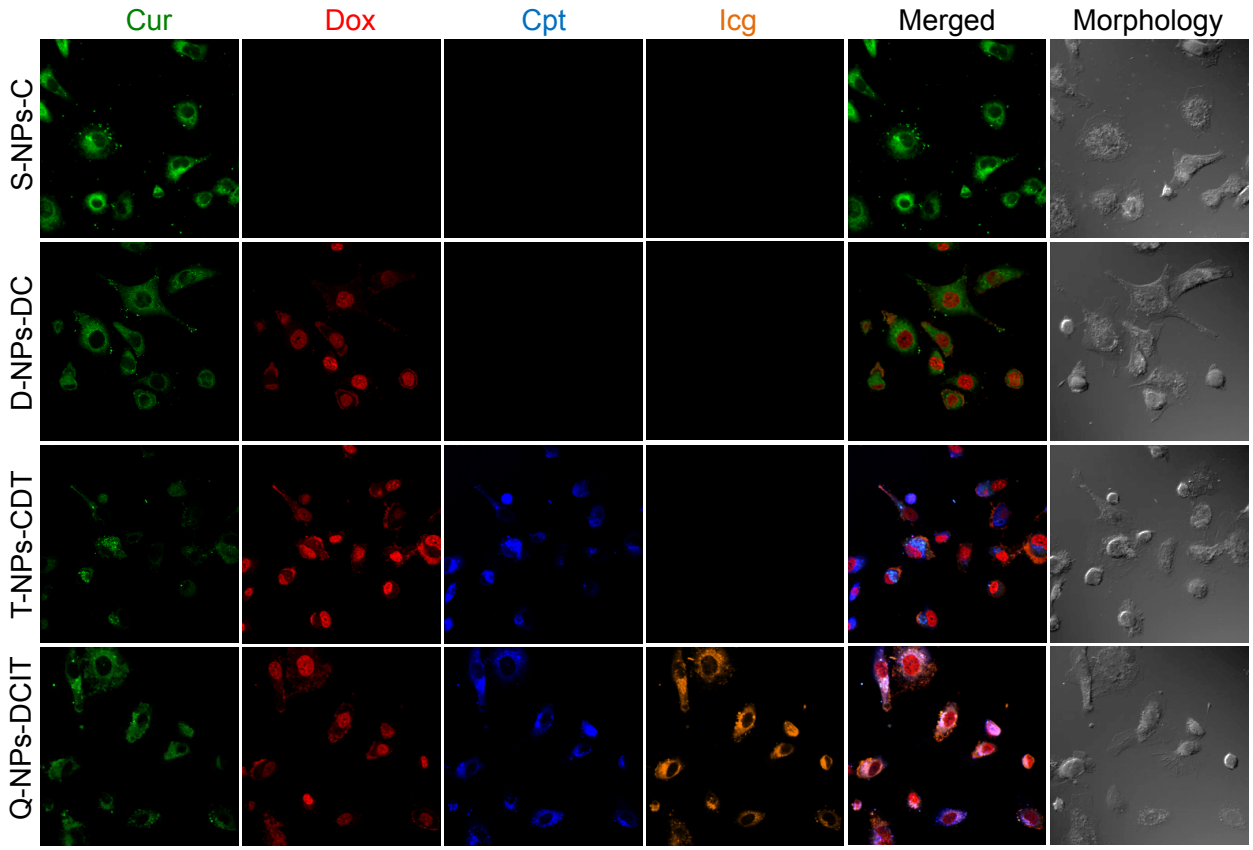


Fig. S6 Confocal micrographs of PC-3 cancer cells treated with S-NPs-C, D-NPs-DC, T-NPs-CDT and Q-NPs-DCIT showing the NPs could be used to simultaneously deliver multiple agents into cancer cells.

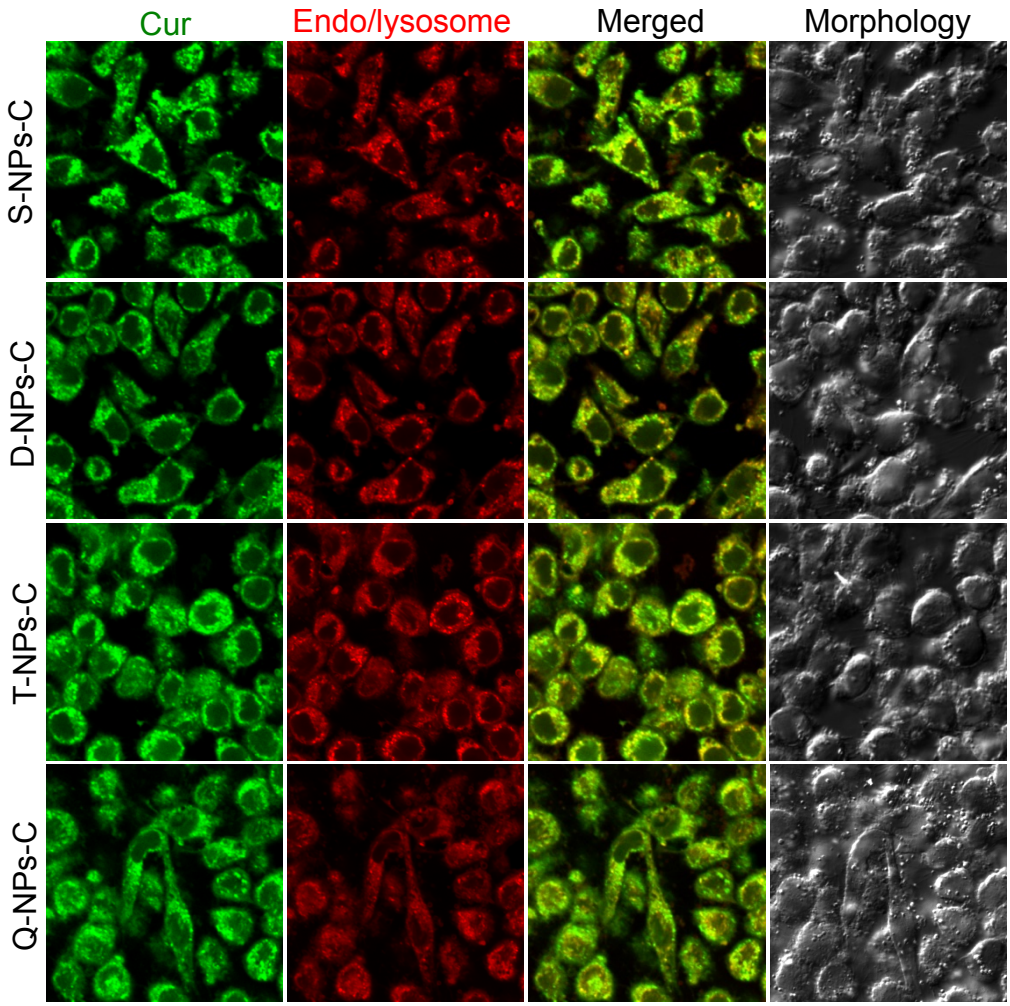


Fig. S7 Cellular uptake of NPs and their distribution inside PC-3 cancer cells observed by confocal microscopy. The cells were cultured on 35 mm dishes with a glass surface for 24 h, followed by incubating first with the respective NPs for 5 h at 37 °C and then LysoTracker Red to stain late endosomes/lysosomes for 30 min at 37 °C before examination using confocal microscopy.

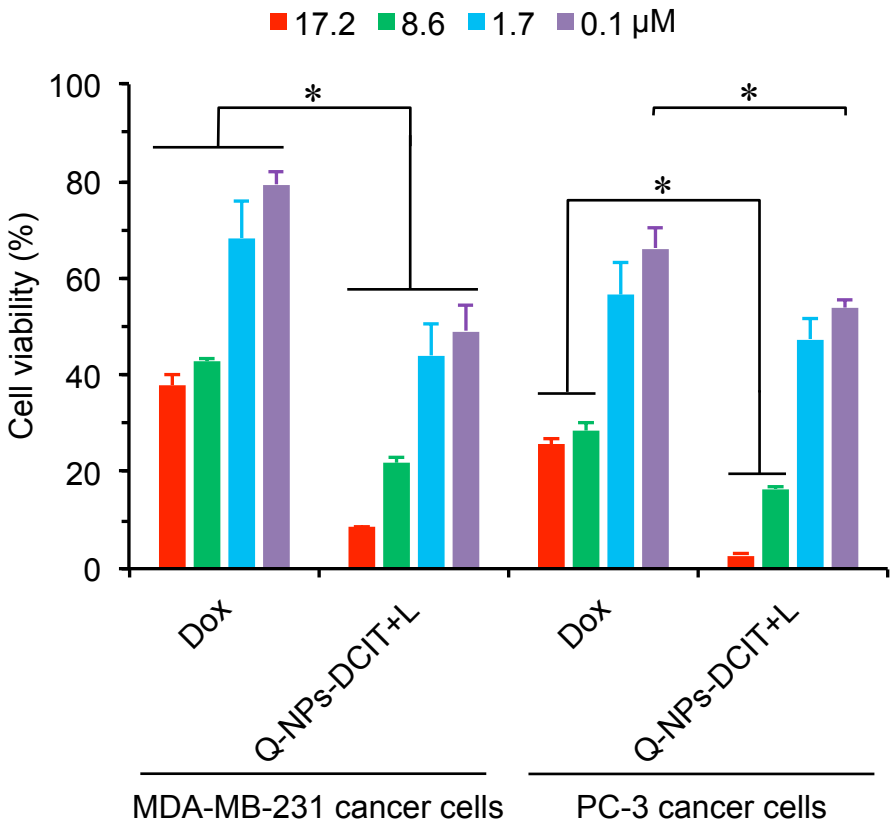


Figure S8. Cell viability of PC-3 and MDA-MB-231 cancer cells after treated with free Dox and Q-NPs-DCIT (with laser irradiation) at different concentrations for 24 h. The asterisk indicates $p < 0.05$ between the indicated groups.

Table S1. Encapsulation efficiency (EE) of different drugs using S-NPs, D-NPs, T-NPs and Q-NPs. N/A: not applicable

Polymer : Drug = 20 : 1	Encapsulation efficiency (EE), % in weight				Loading Content (LC), % in weight
	Cur	Dox	Cpt	Icg	
S-NPs-C	54.0 ± 1.3	N/A	N/A	N/A	5.7 ± 1.4
D-NPs-DC	50.3 ± 2.6	60.5 ± 1.8	N/A	N/A	10.9 ± 2.5
T-NPs-CDT	65.7 ± 1.6	54.3 ± 1.0	44.8 ± 0.5	N/A	8.4 ± 3.3
Q-NPs-DCIT	72.5 ± 1.1	71.2 ± 0.9	43.7 ± 0.6	47.0 ± 2.5	11.52 ± 3.4