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Supporting Information

Taking advantage of disadvantage: employ high aqueous instability of amorphous calcium carbonate to realize burst drug release within cancer cells

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This version of the ESI, uploaded 22/07/2020, replaces the previous version originally published on 14/02/2017. The original contained incorrect TEM images due to errors in figure preparations (Figures S1C, E and S2B), which have now been corrected.

Experimental Section

Materials: Ammonium carbonate ($(NH_4)_2CO_3$) and anhydrous calcium chloride ($CaCl_2$) were purchased Sinopharm Chemical reagent Co., LTD (Shanghai, China). Doxorubicin hydrochloride (DOX) was a gift from Hisun Pharm Co., Ltd. (Hangzhou, China). Polyethylene glycol monostearate (PEG2000−SA, Mw = 2000) was supplied by TCI Development Co., Ltd. (Shanghai, China). Oleic acid (OA) was purchased from Aladdin (Shanghai, China). Hoechst 33342, Lyso tracker Blue and Fluo-4 AM were obtained from Thermo Fisher Scientific (MA, USA). All other chemicals and reagents otherwise stated were from Sinopharm Chemical reagent Co., LTD and of analytical grade.

Preparation of nanoparticles: The synthesis of ACC was performed in accordance with previous report with minor modifications.[1] Briefly, $CaCl_2$ (200 mg) was dissolved in absolute ethanol (100 mL). The mixture were transferred into a glass bottle and covered by parafilm with several pores. The bottle was then left in a desiccator along with two glass bottles of $(NH_4)_2CO_3$ at 25 °C. After vapor diffusion reaction for 2-3 days, the products were centrifuged (Allegra 64R, Beckman Coulter, USA, 8,000 rpm, 10 min), rinsed several times and then re-dispersed in proper amount of absolute ethanol. Drug (DOX) loaded ACC-DOX were prepared in the same way except that DOX (0.4 mL, 10 mg/mL, aqueous solution) was added to the ethanol solution at the same time with $CaCl_2$.

The ACC were then dispersed into absolute ethanol solution containing tenth weight of OA and stirred at 37 °C overnight. After that, the mixture was centrifuged at 3,000 rpm for 10 min to precipitate OA-ACC. The collected nanoparticles were rinsed several times with absolute ethanol and re-dispersed in cyclohexane. OA-ACC-DOX were prepared with the same protocol.

The solvent evaporation method was utilized to prepare PEG/OA-ACC. Briefly, OA-ACC (0.2 mL, 5 mg/mL, cyclohexane) were mixed with OA (0.1 mL 1 mg/mL, ethanol) and PEG-SA (1 mL, 5 mg/mL, ethanol) in 10 mL of ethanol solution. After being ultrasonicated at 400 W at room temperature for 20 times (work 2 s and stand 3 s) by a Lab ultrasonic cell pulverizer (JY92-II, Ningbo Scientz Biotechnology Co., Ltd, China), the mixture was subjected to rotary evaporation under vacuum at 50 °C for 30 min, followed by vacuum drying in desiccator for 6 h to form a dried film on the bottle wall and thoroughly remove the organic solvent. The film was hydrated by water at 37 °C for 30 min and centrifuged at 3000 rpm for 10 min to finally obtain the PEG/OA-ACC (supernatant). DOX loaded PEG/OA-ACC-DOX were prepared with the same protocol. For PEG/OA-ACC-DOX, the resulted formulation was further dialysis against distilled water (Milipore, USA) for 4 h (MWCO: 7 kDa, 2 L × 6) with gentle agitation to remove unloaded drugs.

CCC-DOX were prepared according to previous reported method with modifications.[2] Briefly, 555 mg $CaCl_2$ and 4 mg DOX were charged into 5 mL water in an ice bath with gentle agitation to obtain a clear solution. 5 mL of 1 M $(NH4)_2CO_3$ aqueous solution was quickly added into the mixture under vigorously stirring and the reaction was lasted for 10 min. After the addition of 10 mL of distilled water and discarding the large particles which were precipitated without centrifugation, the

suspension was centrifuged (8000 rpm, 10 min) and re-dispersed in ethanol. The preparation of OA-CCC and PEG/OA-CCC was the same as mentioned above.

Fourier Transform Infrared Spectroscopy (FTIR) and X-ray Diffraction (XRD) measurements: ACC and OA-ACC in organic solvents were oven dried to obtain nanoparticle powder. Aqueous PEG/OA-ACC solution was centrifuged (20000 rpm, 10 min) to precipitate PEG/OA-ACC and then oven dried to obtain powder. The comparative FTIR (FT/IR-4100, JASCO, Japan) of different nanoparticles was investigated to study the chemical composition and interaction. The polymorph of different particles were recorded by XRD (Rotaflex RU-200, Rigaku, Japan). The polymorph of ACC within PEG/OA-ACC was examined by testing the extractive OA-ACC from PEG/OA-ACC, since PEG-SA with particular crystal form will influence the results. The extraction was performed as follows: the precipitated PEG/OA-ACC were dispersed in proper amount of absolute ethanol with gentle agitation for 10 min, followed by centrifugation (3000 rpm) for another 10 min. The resulted precipitate (OA-ACC) was collected, oven dried and subjected to XRD analyzation. Other similar nanoparticles were treated with the same protocol unless otherwise stated.

Morphology, particle size and zeta potential measurements: The morphology and particle size of different nanoparticles were observed by transmission electron microscopy (TEM). Briefly, a drop of nanoparticle solution (ACC, ACC-DOX or CCC-DOX, 0.1 mg/mL, ethanol; OA-ACC or OA-ACC-DOX, 0.1 mg/mL, cyclohexane; PEG/OA-ACC, PEG-OA-ACC-DOX or PEG/OA-CCC-DOX, 0.5 mg/mL, water) was deposited on a lacy carbon-coated cupper grid. The excess solution was removed by a piece of filter paper. The sample was air dried and observed under a TEM system (JEM-1200, JEOL, Japan) at an accelerating voltage of 80 kV. Moreover, the comparative particle size and zeta potential were further determined by Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK).

Stability Studies: The ACC and PEG/OA-ACC were dispersed in water to achieve the final concentration of 0.1 mg/mL and 0.5 mg/mL, respectively. Their particle size and morphology were recorded by Zetasizer Nano ZS90 and TEM, respectively. On the other hand, ACC and PEG/OA-ACC incubated with water were isolated from the water using centrifugation (20000 rpm, 10 min) at scheduled time intervals (0.5, 1 and 2 h), followed by desiccation and then subjected to comparative FTIR and XRD analysis on crystal transition.

In addition, drug loaded ACC-DOX and PEG/OA-ACC-DOX were dispersed into water, their ultraviolet (UV) spectra between 400-750 nm were recorded by an ultraviolet-visible spectrophotometer (TU-1810, Purkinje, China). The UV spectrum of ACC-DOX in ethanol was employed as a control.

ACC and PEG/OA-ACC were incubated with fetal bovine serum (FBS, Gibco BRL, USA) at 37 °C for 2 h. Samples were measured at different time intervals and their changes in particle size were recorded.

Drug release: The drug release profile of ACC-DOX, CCC-DOX and PEG/OA-ACC-DOX was investigated by dialysis method. Briefly, samples were placed into individual dialysis bag (MWCO: 7 KDa) and immersed in plastic tube containing 25 mL of phosphate buffer with pH of 7.4, 6.5 and 5.5, respectively. The plastic tubes were fixed

in a thermostatic shaker (HZQ-C; Haerbin Dongming Medical Instrument Factory, China) at 37 °C with a stirring speed of 100 rpm. At predetermined time intervals, buffer solution within the tubes was removed for analysis and replaced with equal volume of fresh medium. The drug concentration was determined by fluorescence spectrophotometer (F-2500, Hitachi Co., Japan) with excitation wavelength, emission wavelength and slit openings set at 505, 605, and 5 nm, respectively. [3]

Drug localization and cellular uptake assay: MCF-7 (human breast carcinoma) cell line was purchased from Institute of Biochemistry and Cell Biology (Shanghai, China) and cultured in dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) FBS (Gibco BRL, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 95% air/5% CO_2 incubator at 37 °C. All experiments were performed on cells in the logarithmic phase of growth.

MCF-7 cells were seeded onto 35-mm glass-bottom culture dishes (Corning, USA) at a density of 1×10^5 cells per well and cultured overnight for 50-60 % confluence. After that, the primary culture media were removed and replaced with equal volume of serum-free medium containing free DOX, PEG/OA-CCC-DOX or PEG/OA-ACC-DOX (final DOX concentration: 1 μg/mL). For cell observation, after predetermined co-incubation time, cells were washed several times by phosphate buffer saline (PBS, 0.01 M, pH 7.4) and fixed with 4 % paraformaldehyde (15 min), followed by staining with Hoechst 33342 (10 μg/mL) for 15 min. At last, all samples were observed and imaged using a confocal laser scanning microscopy (CLSM, BX61W1-FV1000, Olympus, Japan). Quantitative uptake of nanoparticles and cell sorting were further investigated by flow cytometry (FCM, FC500MCL, Beckman Coulter, USA).

Calcium release and intracellular distribution observation: Cells cultured under the same condition as described above were adopted. For calcium release experiment, the primary medium was removed and replaced with Hank's Balanced Salt Solution (HBSS) containing 5 μM of Fluo-4 AM. After incubating at 37 °C for 30 min, the Fluo-4 AM containing medium was discarded and cells were thoroughly washed with HBSS. Free DOX, PEG/OA-CCC-DOX or PEG/OA-ACC-DOX diluted with HBSS was added to the dishes. At pre-determined time points (15 min and 2 h), cells were subjected to CLSM observation (BX61W1-FV1000).

For intracellular distribution of PEG/OA-ACC-DOX, cells were firstly incubated with HBSS containing PEG/OA-ACC-DOX at 37 °C for 4 h. At the end of incubation, Fluo-4 AM and Lyso tracker Blue were added to incubate with cells for 30 min and 15 min, respectively. Afterwards, cells were rinsed with fresh HBSS and then subjected to CLSM (Nikon A1R, Nikon, Japan) observation. Images were obtained with excitation at 280 nm for endo-lysosome or nuclei (blue color), 494 nm for Ca^{2+} (green color) and 546 nm for DOX (red color).

Cytotoxicity: For the cell viability measurements, 200 μL of MCF-7 cells (1×10^4 cells/mL) in DMEM suspension was seeded in 96-well plates (Corning, USA) and allowed to culture overnight. The mediums were discarded and all wells were washed twice with fresh PBS. Subsequently, 200 μL of serum-free medium was added to each well, in which DOX or nanoparticles was diluted to achieve the designated concentrations. After proper incubation, the cell viability was assessed with standard

MTT assay. Briefly, the medium was replaced with an equal volume of fresh medium containing 5 mg/mL MTT and incubated for 4 h at 37 °C. Then MTT was removed, and cells were lysed with dimethyl sulfoxide (DMSO) with stirring for 15 min on a microtiter plate shaker. The cell viability was estimated according to the absorption values determined by a microplate reader (Bio-Rad, model 680, USA) at the wave length of 570 nm. [4]

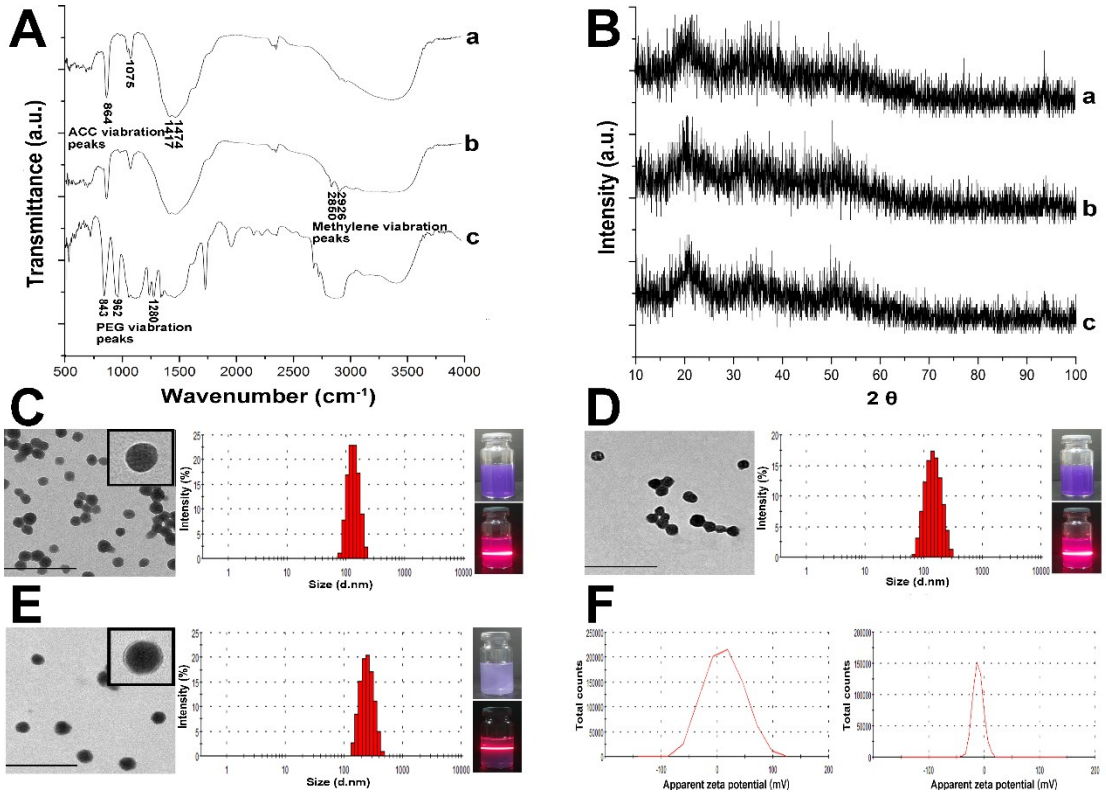

Figure S1. Characterization of drug loaded PEG/OA-ACC-DOX. A) FTIR spectra of ACC-DOX (a), OA-ACC-DOX (b) and PEG/OA-ACC-DOX (c). B) XRD pattern of ACC-DOX (a), OA-ACC-DOX (b) and extractive OA-ACC-DOX core of PEG/OA-ACC-DOX (c). Representative TEM (left), size distribution (middle), colloidal solution (right, upper) and Tyndall light scattering images (right, lower) of ACC-DOX (C), OA-ACC-DOX (D) and PEG/OA-ACC-DOX (E). Scale bars: 500 nm. F) Zeta potentials of ACC-DOX (left) and PEG/OA-ACC-DOX (right).

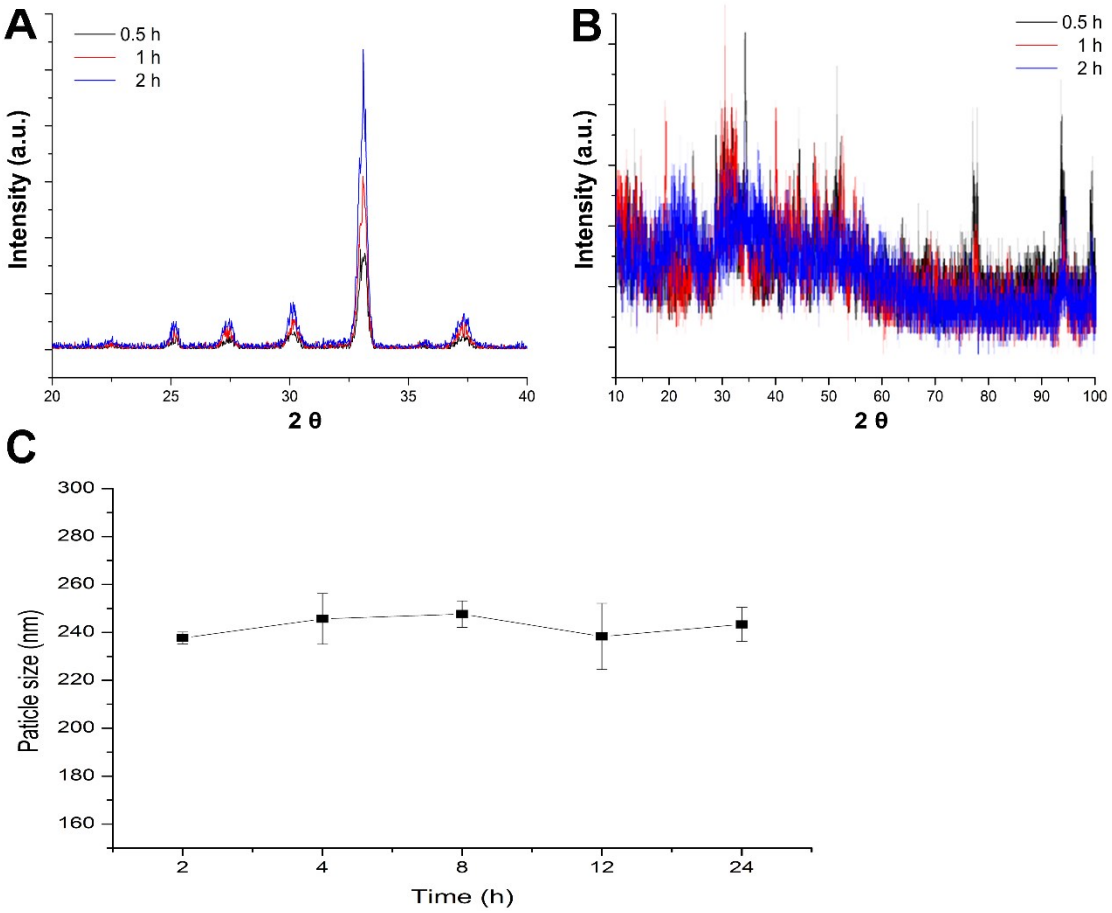

Figure S2. Comparative XRD patterns of ACC (A) and extractive OA-ACC core of PEG/OA-ACC (B) incubated with water for different time intervals (0.5, 1 and 2 h). (C) Stability tests of PEG/OA-ACC under physiological condition (PBS, pH 7.4) for 24 h. Results were expressed as mean ± S.D. (n = 3).

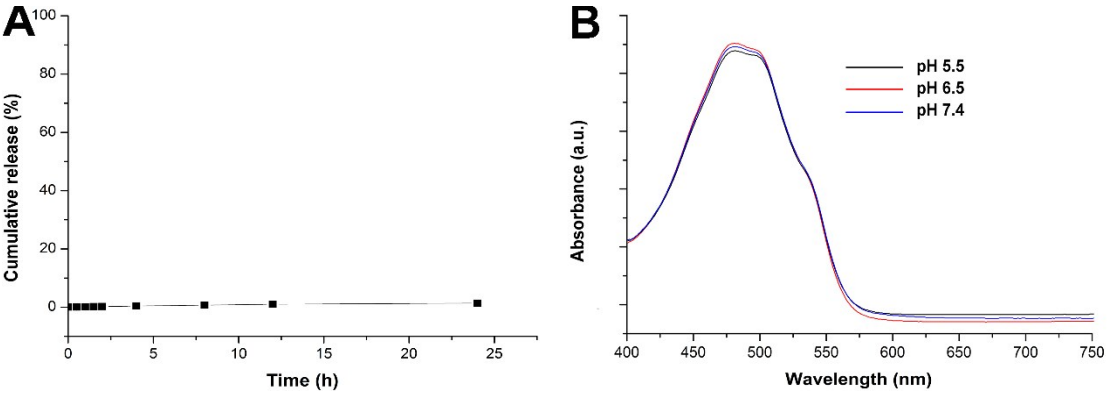

Figure S3. A) *In vitro* drug release of DOX from ACC-DOX in ethanol for 24 h. Results were expressed as mean ± S.D. (n = 3). B) UV spectra of the released DOX from ACC-DOX in various pH conditions (7.4, 6.5, and 5.5). When the ACC-DOX were charged into in aqueous solutions, the resulted solutions showed the featured absorption peak of DOX at 480 nm.

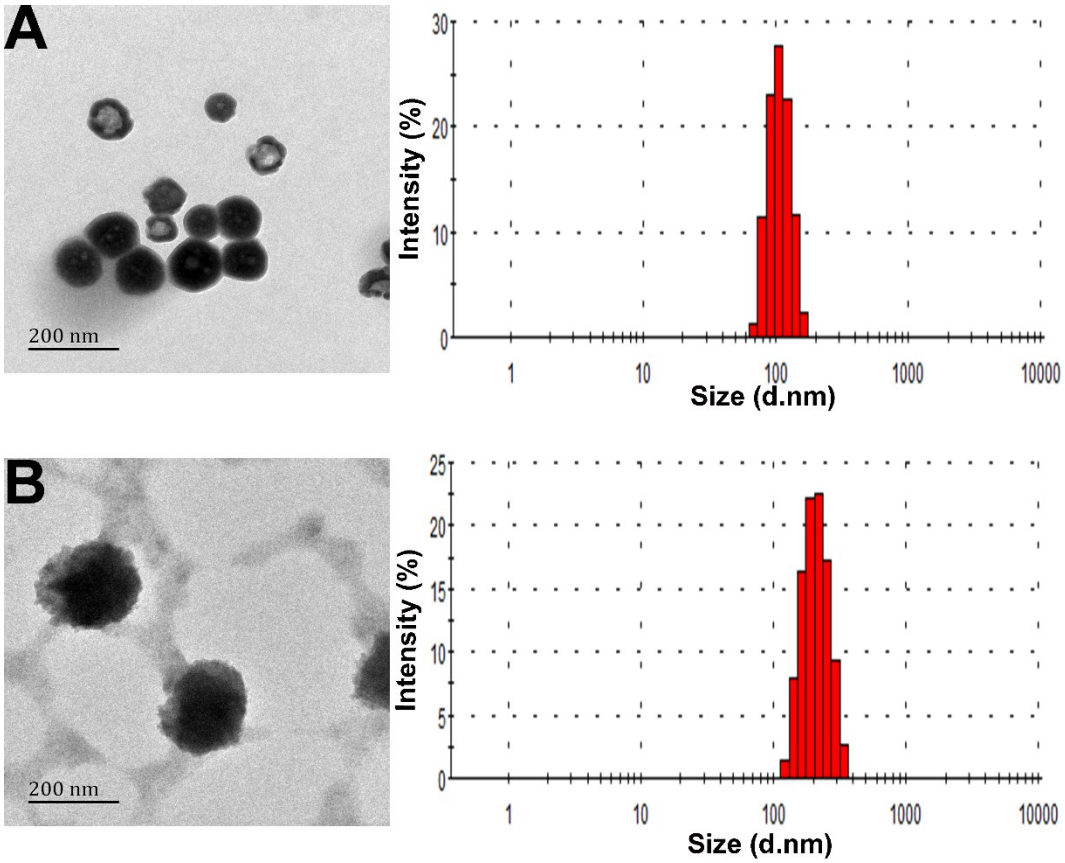

Figure S4. Representative TEM (left) and size distribution (right) images CCC-DOX (A) and PEG/OA-CCC-DOX (C). Scale bars: 200 nm.

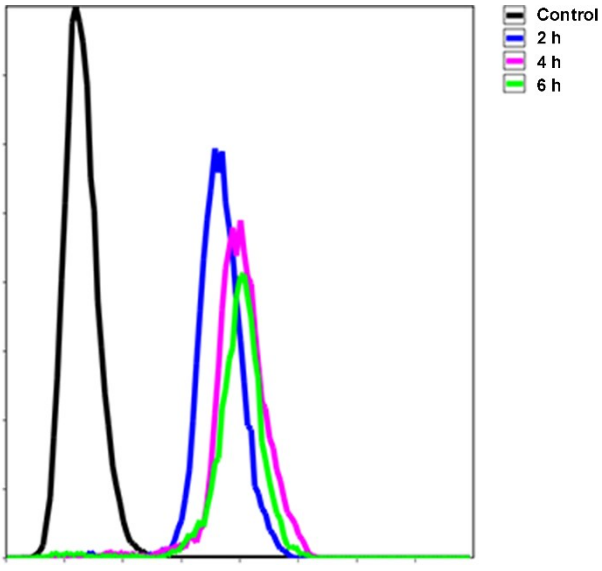

Figure S5. Time dependent (2, 4 and 6 h) cellular uptake of PEG/OA-ACC-DOX in MCF-7 cells determined by flow cytometry.

Reference

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