Electronic Supplementary Information

Facile Green Synthesis of Calcium Carbonate/Folate Porous Hollow Spheres for Targeted pH-Responsive Release of Anticancer Drugs

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1 Materials and Methods

1.1 Materials

Calcium chloride ($CaCl_2$), sodium carbonate (Na_2CO_3), and Folic acid were purchased from Aladdin Industrial Corp.. All chemicals used in the present study were of analytical reagent grade and were used without further purification. Double distilled water (DD water) was used in the preparation and characterization of the FA-CaHMS. Ultrapure water was used in the biological assays.

1.2 Preparation of $CaCO_3$/FA HSs

The $CaCO_3$/FA HSs was prepared as follows. Briefly, 12.5 mL $CaCl_2$ aqueous solution (5 mM) was added slowly dropwise into the aqueous solution of FA (2.5 mM, 12.5 mL) under vigorous stirring. The pH of the reaction system was adjusted to 7.00 and the system was stirred for 2 h at 30℃ to allow the completely interaction between Ca^{2+} and FA. Then, 25 mL of Na_2CO_3 aqueous solution (2.5 mM) was added slowly dropwise into the system and stirred for 10 min. Subsequently, the reaction system was incubated for 24 h at 30℃. Finally, the product was collected by centrifugationand washed with DD water and absolute ethanol. The as-prepared product was dried under vacuum and denoted as $CaCO_3$/FA HSs. For comparison, the preparation was also performed with the FA: Ca^{2+} ratios of 1:1, 1:3, 1:5, and 2:1 under similar conditions. In addition, the $CaCO_3$ was also prepared with the pH values of 9.0 and 11.0 under nearly identical conditions as the typical experiment.

1.3 Characterization

The size and morphology of the as-prepared products were characterized by scanning electron microscopy (SEM, JSM-6390LV, JEOL). High-resolution transmission electron microscopy (HR-TEM) investigations were conducted on a JEOL JEM-2100 transmission electron microscope with the acceleration voltage of 200 kV. The crystal phases of the

products were determined by powder X-ray diffraction (XRD) using a D8ADVANCE X-ray diffractometer (Bruker axs Com., Germany) with graphite monochromatized Cu Kα radiation (λ = 0.15406 nm) in the 2θ range of 20-70°. The Fourier transform infrared spectroscopy (FT-IR) spectra were recorded on a Bio-Rad FTS-40 FT-IR spectrometer in the wavenumber range of 4000–400 cm^{-1}. The thermogravimetry-differential scanning calorimetry (TG-DSC) analysis was performed in the temperature range 25-900℃ with a linear heating rate of 10℃/min on an NETZSCH STA 449C instrument. The size distribution analysis of the samples was performed using the log normal function from 100 microspheres in an arbitrarily chosen area. The specific surface area and pore size distribution were analyzed through the Brunauer–Emmett–Teller (BET) determination at liquid nitrogen temperature using N_2 as an adsorbent (Gemini 2380, Micromeritics, USA).

1.4 DOX loading and the incorporation efficiency

8 mg of $CaCO_3$/FA HSs were added into 8 mL of DOX aqueous solution (0.1 mg/mL) and shaken in an orbital shaker for 24 h at 30℃ to load the DOX. Subsequently, the dispersion was centrifuged, rinsed with DD water for several times until the supernatant changed to colorless. All the supernatants were collected together. The obtained precipitate was dried and denoted as $CaCO_3$/FA/DOX. For the determination of the loading efficiency, the amounts of the free DOX in the supernatants were quantified by UV-Vis absorbance at 500 nm according to the DOX standard curve. The incorporation efficiency can be expressed both as loading content (%) and entrapment (%) represented by Equation (1) and (2), respectively. The reported data are the mean values of sextuplicate determinations.

$$Loading\ content\ (\%\ w/w) = \frac{mass\ of\ DOX\ in\ CaCO_3/FA/DOX \times 100}{mass\ of\ CaCO_3/FA/DOX} \quad (1)$$

$$Entrapment\ (\%\ w/w) = \frac{mass\ of\ DOX\ in\ CaCO_3/FA/DOX \times 100}{mass\ of\ DOX\ used\ in\ formulation} \quad (2)$$

1.5 Cell culture

In this study, HeLa human cervical carcinoma cells (ATCC No. CCL-2) and V79-4 Chinese hamster lung cells (ATCC No. CCL-93) were used as model cancer cell and normal cell respectively. The cells were cultured in Eagle's minimum essential medium (EMEM)

supplemented with heat-inactivated FBS (10%), Penicillin (100 units/mL), Streptomycin (100 μg/mL), amphotericin B (fungizone, 0.25 μg/mL) and sodium bicarbonate (2.2 mg/mL) in a humidified incubator at fully humidified atmosphere at 37°C, 5% CO_2 and 95% room air.

1.6 Cytotoxic effects of FA, pure DOX, and $CaCO_3$/FA/DOX on HeLa and V79-4 Cells

100 μL of culture medium containing HeLa or V79-4 cells with initial density of 2.5×10^4 cells/mL were seeded separately in the wells of sterile 96-well flat bottom culture microplates and incubated for 24 h. Subsequently, 100 μL of FA, pure DOX, $CaCO_3$/FA, and $CaCO_3$/FA/DOX with the concentrations of 0.0244, 0.0488, 0.0977, 0.1953, 0.3906, and 0.7813 μg/mL in culture medium were added into the wells and incubated for 72 h. The treatment of cells with culture medium rather than samples was prepared as the control. The cytotoxic effects of the FA, pure DOX, $CaCO_3$/FA, and $CaCO_3$/FA/DOX on HeLa and V79-4 cells were determined by MTT colorimetric assay. Briefly, after 72 h treatment, 20 μL of freshly prepared MTT (3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl tetrazolium bromide, 5 mg/mL in PBS) was added to each well and incubated at 37°C, under 5% CO_2 for 5 h. Then, the supernatant was carefully discarded and 150 μL of DMSO were added to each well to dissolve the dark blue crystals completely. The absorbance of the solution in each well at the wavelength 570 nm was determined by a microplate reader. The extent of cell proliferation was reflected by the average value of absorbance while the cytotoxic effects of the FA, pure DOX, $CaCO_3$/FA, and $CaCO_3$/FA/DOX were calculated by Equation 3. The data were reported as mean ± standard deviation (SD) based on the measurements of the triplicate samples.

$$Percentage\ of\ inhibition\ (\%) = \left(1 - \frac{O.D._{570nm}\ of\ treatment\ group}{O.D._{570nm}\ of\ control\ group}\right) \times 100 \qquad (3)$$

1.7 Determination of $[Ca^{2+}]$

To determine the $[Ca^{2+}]$, $CaCO_3$/FA were incubated in EMEM medium and the cultivation systems of HeLa and V79-4 cells under standard cell culture conditions, respectively. After incubated for 3 days, the mixtures were ultracentrifuged to remove any possible solid samples completely. Subsequently, the supernatants were diluted with 2% HNO_3 and analyzed using the inductively coupled plasma-mass spectrometer (ELAN DRC-e, Perkin–Elmer Sciex) to

determine the [Ca^{2+}] in the supernatants. For comparison, the [Ca^{2+}] in the pure EMEM medium was also determined.

1.8 Transmission electron microscopy of cells

HeLa cells were seeded on culture flasks and treated with $CaCO_3$/FA HSs for 12 h. After treatment, cells were collected, fixed with glutaraldehyde (2.5%) and washed three times with PBS. Subsequently, post-fixation with osmium tetroxide (1%) was performed followed by washed with PBS, dehydration with ascending series of alcohol before embedding in Spurr. Ultrathin sections with 70 nm were cut by Leica Reichert ultracut and doubly stained with uranyl acetate and lead citrate. Images were acquired using transmission electron microscope.

2 Results

2.1 Characterization of $CaCO_3$/FA HSs

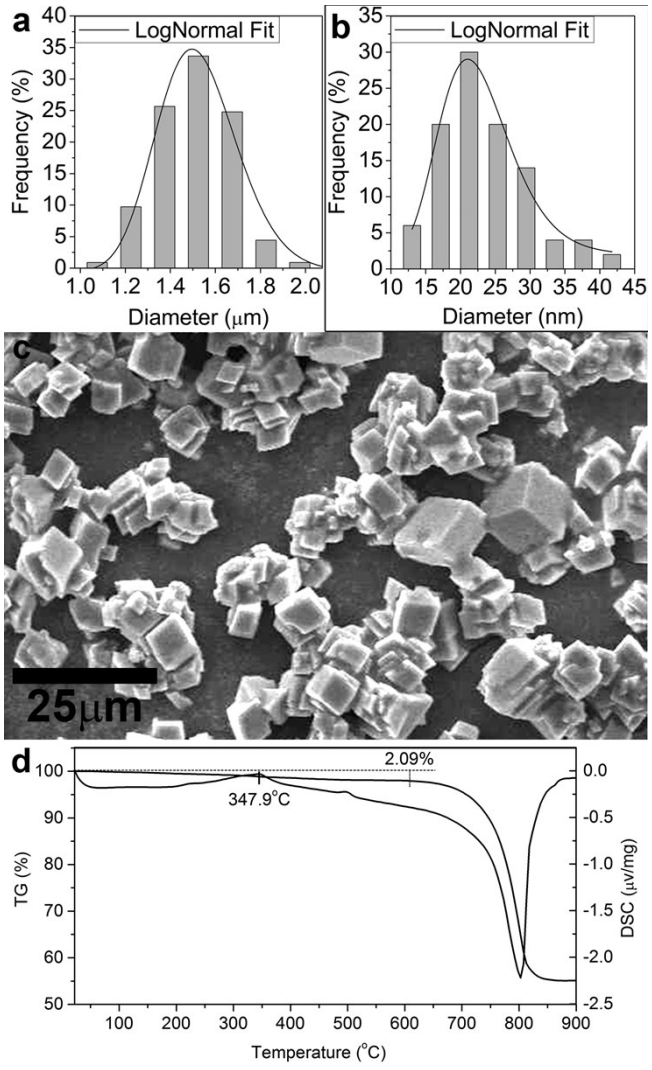

Fig. S1 (a) Diameter distribution histogram of $CaCO_3$/FA HSs. (b) Size distribution histogram of the nanoclusters in the $CaCO_3$/FA HSs. (c) SEM image of the control product obtained in

the absence of FA under the similar conditions to the typical experiment. (d) TG-DSC curve of $CaCO_3$/FA HSs.

2.2 Size distribution analysis of the products prepared with different reaction periods

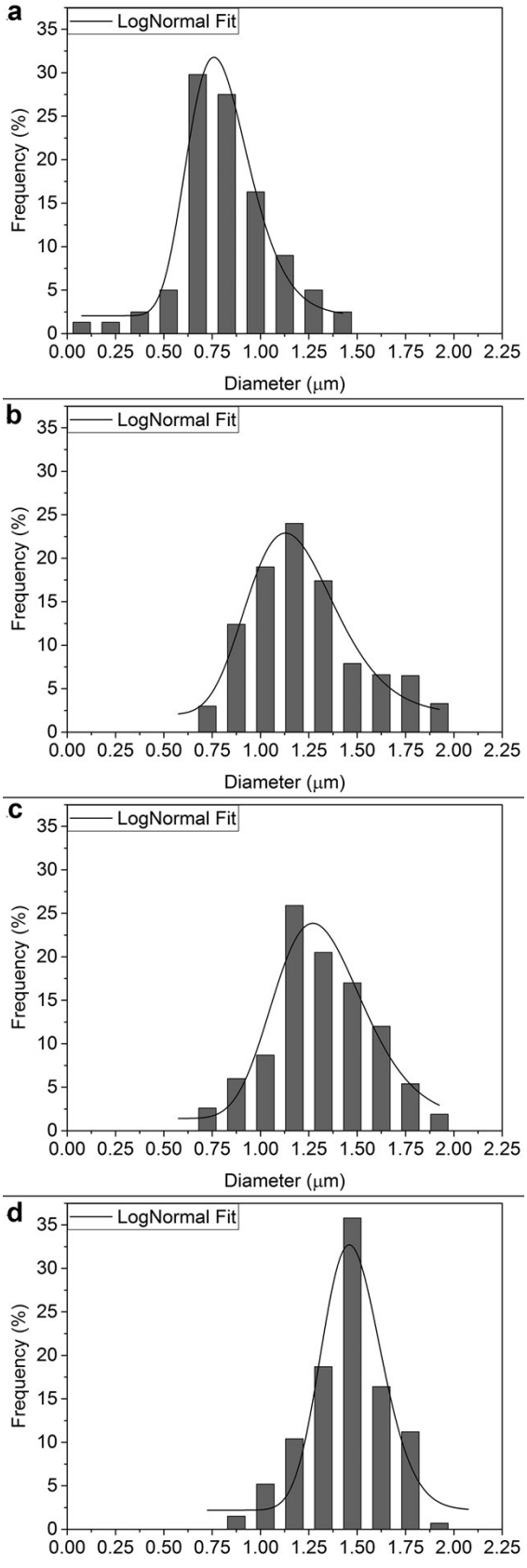

Fig. S2 Size distribution of $CaCO_3$/FA HSs obtained at different reaction periods: (a) 3 h, (b) 6 h, (c) 12 h, (d) 18 h.

2.3 SEM observations of the products prepared with different FA/Ca^{2+} and pH values

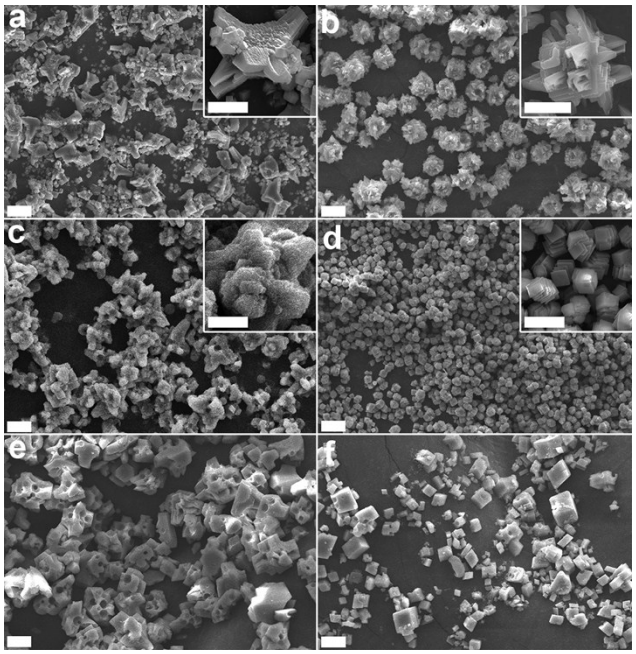

Fig. S3 SEM images of the products prepared with different FA/Ca^{2+} ratio and pH values. (a) (a) 1:1, (b) 1:3, (c)1:5, (d) 2:1, (e) pH 9.0; (b) pH 11.0. Scale bar: 10 μm. Inset: magnified SEM images. Scale bar: 5 μm.

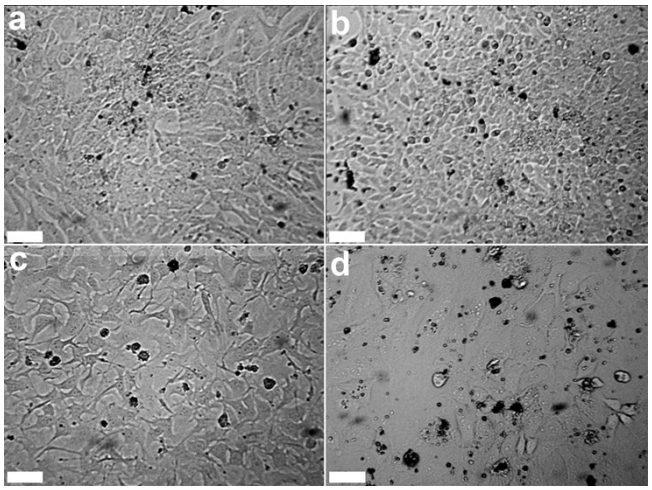

Fig. S4 Light micrographs of V79-4 cells of (a) control and (b) $CaCO_3$/FA/DOX treatment for 5 d. Light micrographs of HeLa cells of (c) control and (d) $CaCO_3$/FA/DOX treatment for 3 d. Scale bar: 200 μm.