Electronic Supplementary Material (ESI) for Journal of Materials Chemistry

Supporting Information

Biopolymer-directed Synthesis of High-surface-area Magnetite Colloidal Nanocrystal Cluster for Dual Drug Delivery in Prostate Cancer

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Section 1. Materials and Methods

1. Materials

Iron(III) chloride hexahydrate ($FeCl_3 \bullet 6H_2O$), ammonium acetate (NH_4OAc), ethylene glycol (EG), and anhydrous ethanol were purchased from Shanghai Chemical Reagents Company (China). Poly(γ-glutamic acid) (M_w = 1000 kDa, Microbial fermentation) was purchased from Dingshunyin Biotechnology Company (China). Agarose (Mw = 15-20 kDa, low EEO), and chitosan (M_w = 200 kDa, 90% of deacetylation) were purchased from Gene Technology Company (China). Casein (M_w = 19-24 kDa, consisting of α, β, γ, and κ caseins) and soybean (M_w = 10-15 kDa) proteins were purchased from Aladdin Chemicals (China). Docetaxel (DOC) was purchased from Knowshine Pharmachemicals Inc. (China) and used as received. Ceramide (CER) was purchased from Sigma. RPMI-1640, fetal bovine serum (FBS), penicillin G, streptomycin and trypsinase were obtained from Beyotime Institute of Biotechnology (China). [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) was purchased from Sigma. All chemicals were used as received without further purification.

2. Methods

2.1 Synthesis of magnetite nanocrystal clusters (MCNCs) [1,2]

The MCNCs were prepared through the modified solvothermal reaction. Typically, 0.68 g of $FeCl_3 \bullet 6H_2O$ and 1.93g of NH_4CH_3COOH were dissolved in 35 mL of ethylene glycol to form a homogeneous solution by ultrasonication for 10 min. The mixture was transferred into a 50 mL of three-necked flask and stirred at 300 rpm at 160 ^oC. 0.5 g of the biopolymer was then added as stabilizer, and the feeding amount was the same for each biopolymer. The reaction proceeded for 1 h in air. During the process, the added biopolymer could be dissolved completely, and the mixture changed gradually from the yellow color to the dark brownish color. And then the dispersion was instantly transferred into a Teflon-lined stainless-steel autoclave (50 mL capacity). The autoclave was kept in oven at 200 ^oC for 24 h. After reaction, when being cooled to room temperature, the dark brownish precipitates in solution were isolated by magnet and washed several times with ethanol and deionized water for removing the residue in products.

2.2 Drug loading in MCNCs [3,4]

Nanoprecipitation method was applied to load DOC and CER into MCNCs. 12 mg of MCNCs were dispersed in 4.5 mL of ethanol and the solution mechanically stirred for 1 h. 0.5 ml of ethanol solution containing 8 mg of DOC and 1 mg of CER was stepwise dropped into the MCNCs solution and the mixture was further stirred for 2 h. Afterwards, 3 mL of deionized water was added dropwise in 30 min. The mixture stirred for another 10 h until the ethanol and water were both evaporated completely, therefore leading to penetration of the drug molecules to inside of MCNCs. The dual-drugs-loaded MCNCs were washed repeatedly with PBS (pH = 7.4) and

deionized water to eliminate weakly surface-adsorbed DOC and CER, dried under vacuum, and stored in a freezer at -20 ^{o}C. As a control, the single-drug-loaded MCNCs were prepared for DOC or CER with the similar way.

2.3 Cell culture

The PC3 cell lines were purchased from the Cell Bank of Chinese Academy of Sciences and maintained in a 5% CO_2 incubator at 37 ºC with a concentration of 50 mg/mL in RPMI 1640 containing 10% of fetal bovine serum and 100 μg/mL of penicillin. They were subcultured twice weekly.

2.4 Cell viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. 200 μL of cells were seeded in a 96-well plate at a density of 4×10^3 cells/well and were subsequently incubated for 24 h to allow attachment. All of DOC-, CER- and DOC+CER-loaded MCNCs were added to the culture medium, respectively, and as a control, free MCNCs, DOC, CER and dual-drugs mixture were added simultaneously. After incubation for 24 h, 48 h and 72 h, to the wells was added 20 μL of MTT solution (5 mg/mL in PBS) and continued for another 4 h. MTT internalization was terminated by aspiration of the media, and the cells were lysed with addition of 150 μL of DMSO. The absorbance of the suspension was measured at 570 nm on an ELISA reader.

2.5 Annexin V staining apoptosis assays

Cell apoptosis was evaluated by AnnexinV-FITC apoptosis detection kit (Beyotime, China). After exposure to the samples for 24 h, the cells were harvested with 0.25% of trypsin and resuspended in 100 μL of PBS to achieve a concentration of 1×10^6 cells/mL. Following the protocol of manufacturer, 5 μL of Annexin V-FITC and 10 μL of propidium iodide (20 μg/mL) were added and incubated in the dark for 15 min at room temperature. Finally, 400 μL of the binding buffer was added to every reaction tube, for analyzing the cells by the Fluorescence Activating Cell Sorter (FACS) (Becton–Dickinson, USA). The data was treated by Cellqust software (Becton Dickinson, USA).

2.6 Western blot assays

After treatment the cells were washed with PBS and harvested. Cell lysates were isolated by the protein extraction buffer (containing 150 mM of NaCl, 10 mM of Tris (pH 7.2), 5 mM of EDTA, 0.1% of Triton X-100, 5% of glycerol, and 2% of SDS), and then were incubated at 4 ºC for 30 min. After centrifugation at 12,000 rpm for 30 min, the protein concentration in cell lysates was determined using Bradford assay. Proteins were denatured in the sample buffer containing 2-mercaptoethanol and bromophenol blue for 10 min at 95 ^{o}C. The equal amount of proteins (50 μg) was fractionated using 8% or 12% of SDS–PAGE and it was transferred onto PVDF membranes. Upon being blocked with 5% of non-fat milk, the membranes were incubated overnight at 4 ^{o}C with the primary antibodies (Bcl-2, Bax), washed with

PBS three times, and incubated with secondary antibodies at room temperature. The intensity of target proteins was detected using the enhanced chemiluminescence detection system.

2.7 Caspase-3 activity Assays

Caspase-3 activity was detected by Caspase-3 assay kit (Beyotime, China), according to the protocol of manufacturer. Briefly, the cells were seeded into 96-well plates at 1×10^5 cells/well. After being exposed to the samples for 24 h, the cells were washed with PBS. Cell lysates were isolated by the protein extraction buffer (containing 150 mM of NaCl, 10 mM of Tris, 5 mM of EDTA, 0.1% of Triton X-100, 5% of glycerol, and 2% of SDS), and then incubated at 4 ^{o}C for 30 min. For measurement of the activities of Caspase-3, the supernatants from the cell lysates were collected by centrifugation at 12,000 rpm for 30 min at 4 ^{o}C. 10 μL of Ac-DEVD-pNA (2 mM) and 90 μL of Caspase buffer were mixed to make the homogeneous reagent of Caspase-3. 10 μL of the above supernatant was diluted up to 100 μL using the Caspase-3 reagent and the solution was added to each well. The absorbance of pNA was measured at 570 nm on an ELISA reader. The activity unit of Caspase-3 was gained by calculating the amount of pNA.

3. Characterization

Transmission electron microscopy (TEM) images were obtained on an H-600 (Hitachi, Japan) transmission electron microscope at an accelerating voltage of 75 kV. High-resolution TEM (HR TEM) images were taken on a JEM-2010 (JEOL, Japan) transmission electron microscope at an accelerating voltage of 200 kV. Samples dispersed at an appropriate concentration were cast onto a carbon-coated copper grid. Scanning electron microscopy (SEM) measurement were performed using a TS-5136MM (TESCAN, Czech) scanning electron microscope at an accelerating voltage of 20 kV. Samples dispersed at an appropriate concentration were cast onto a glass sheet at room temperature and sputter-coated with gold. Powder X-ray diffraction (XRD) patterns were collected on a D8 advance (Bruker, Germany) diffraction meter with Cu Kα radiation at $\lambda = 0.154$ nm operating at 40 kV and 40 mA. Nitrogen sorption isotherms were obtained on an ASAP2020 (Micromeritics, USA) accelerated surface area analyzer at 77 K. Before measurements, the samples were degassed in vacuum at 200 ^{o}C for at least 6 h. The Brunauer-Emmett-Teller (BET) method was utilized to calculate the specific surface areas. By using the Barrett-Joyner-Halenda (BJH) model, the pore size distributions were derived from the desorption branches of isotherms, and the total pore volumes were estimated from the adsorbed volume at a relative pressure of 0.971. Magnetic characterization was carried out with a vibrating sample magnetometer on a Model 6000 physical property measurement system (Quantum Design, USA) at 300 K. Thermogravimetric (TG) analysis data was obtained with a Pyrisis-1 (Perkin Elmer, USA) thermal analysis system under a flowing nitrogen atmosphere at a heating rate of 20 ^{o}C/min from 100 to 600 ^{o}C.

The grain sizes of the primary magnetite nanocrystals from the MCNCs were estimated by using Scherrer's equation as previously reported.[5] In the PXRD pattern (Fig. 2A), the principle peaks indexed to 311 lattice plane were firstly identified and then their half peak widths could be obtained for calculating the crystalline grain sizes following the equation as below.

$$D=k\gamma/\beta\cos\theta$$

D—crystalline grain size (nm); k—Scherrer constant (0.89); γ—wavelength of the X-ray (nm); β—Full-Width Half-Maximum of a designated peak (rad); θ—Bragg diffraction angle (^{o})

References

1. B. Luo, S. Xu, A. Luo, W. R. Wang, S. L. Wang, J. Guo, Y. Lin, D. Y. Zhao and C. C. Wang, *ACS Nano*, 2011, **5**, 1428.
2. J. Liu, Z. K. Sun, Y. H. Deng, Y. Zou, C. Y. Li, X. H. Guo, L. Q. Xiong, Y. Gao, F. Y. Li and D. Y. Zhao, *Angew. Chem. Int. Ed.*, 2009, **48**, 5875.
3. S. Dhar, F. X. Gu, R. Langer, O. C. Farokhzad and S. J. Lippard, *Proc. Natl. Acad. Sci.*, 2008, **105**, 17356.
4. (a) S. Aryal, C. M. J. Hu and L. F. Zhang, *Small*, 2010, **6**, 1442; (b) B. Luo, S. Xu, W. F. Ma, W. R. Wang, S. L. Wang, J. Guo, W. L. Yang, J. H. Hu and C. C. Wang, *J. Mater. Chem.*, 2010, **20**, 7107.
5. K. Tanaka, S. Nakashima, K. Fujita and K. Hirao, *J. Phys.: Condens. Matter.*, 2003, **15**, 469.

Section 2. Photographs of the initial reaction changes

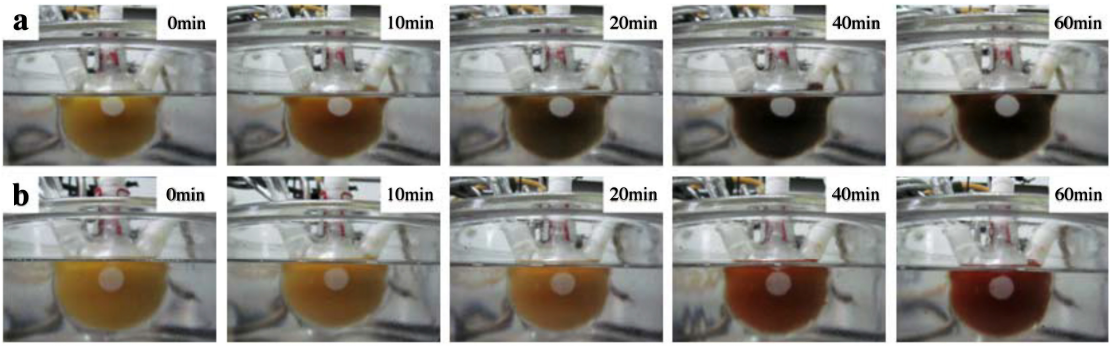

Fig. S1 Photographs recording the changes as the reaction proceeded at 160^{o}C in ethylene glycol in 60 min. Under otherwise identical conditions, the mixtures in the group (a) contained PGA, and as contrasted, the same reaction in the group (b) was without addition of any stabilizers.

Section 3. HR TEM images

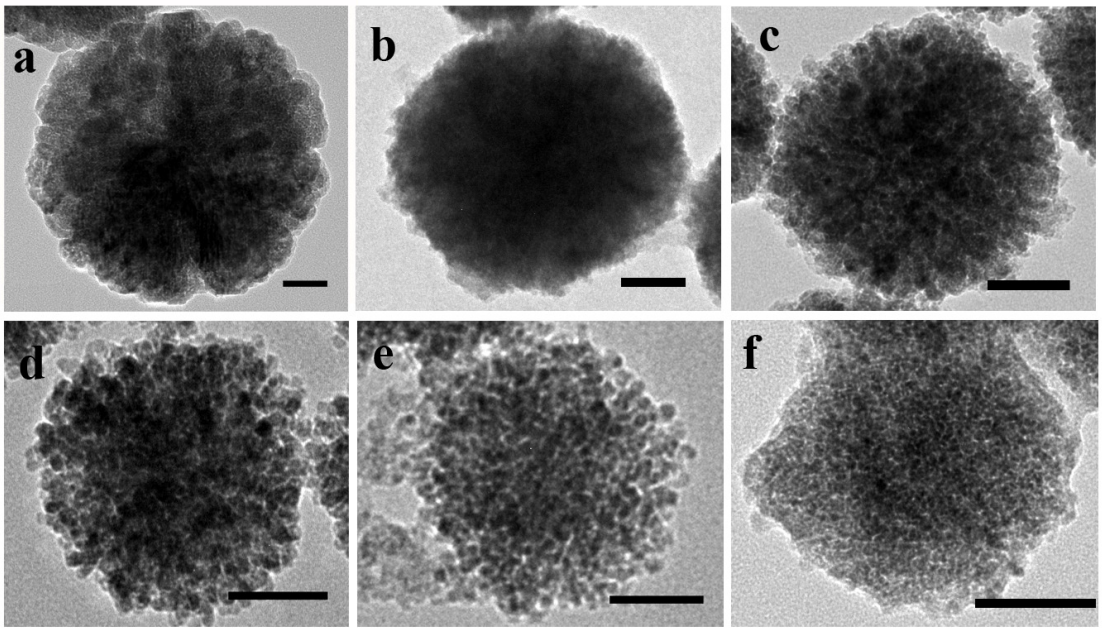

Fig. S2 HR TEM images of the MCNCs without (a) and with the stabilizers chitosan (b), agarose (c), PGA (d), casein (e), and soybean (f). All scale bars are 50 nm.

Section 4. Colloidal stability tests

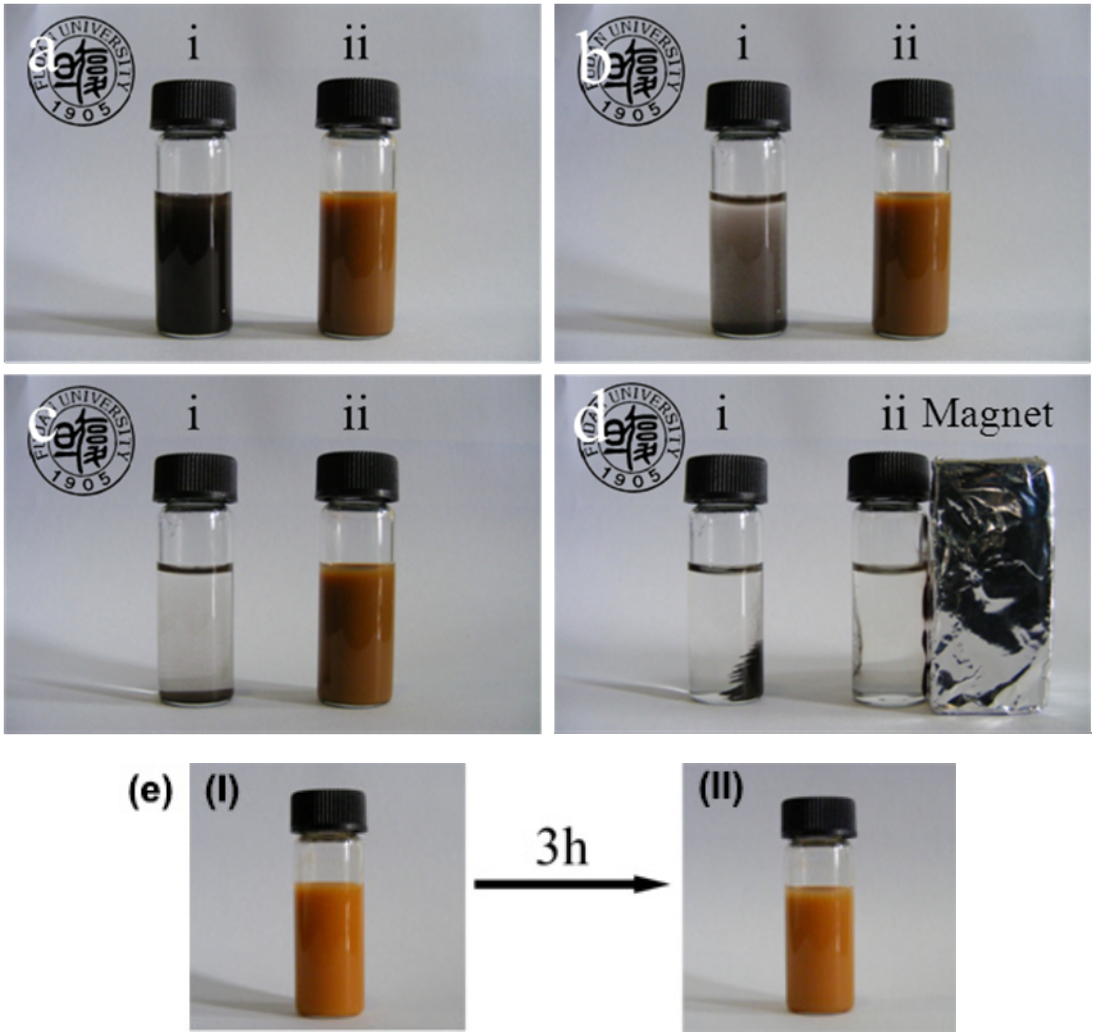

Fig. S3 Comparison of the colloidal stability of the bare MCNCs (i) and PGA-stabilized MCNCs (ii), which were dispersed in the PBS solution (0.2M, pH=7.4), respectively, for 1 min (a), 15min (b), and 3 h (c). Afterwards, the two samples were collected by an applied magnet for demonstrating the magnetic behavior (d). Also, we show the stability of dual-drug-loaded MCNCs in the PBS solution in 3 h (e).

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Section 5. Thermal gravimetric analysis

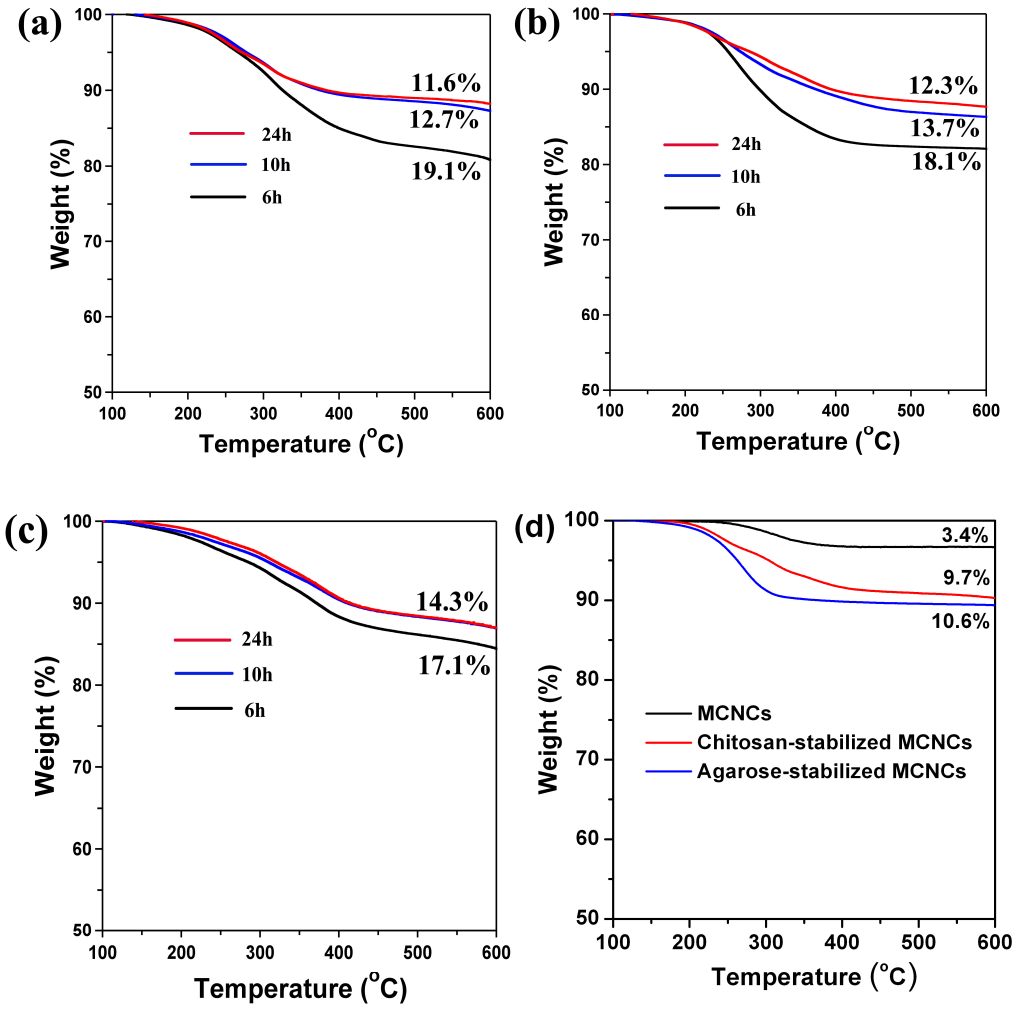

Fig. S4 TG curves of the resulting MCNCs synthesized with assistance of PGA (a), casein (b), and soybean (c). In each group, the samples for measurement were obtained after 6 h, 10 h, and 24 h, respectively. The Figure (d) shows the TG curves of the MCNCs synthesized without any biopolymers, chitosan-stabilized MCNCs and agarose-stabilized MCNCs, respectively, all of which were obtained after 24 h of reaction.

Section 6. N_2 isotherm sorption curves

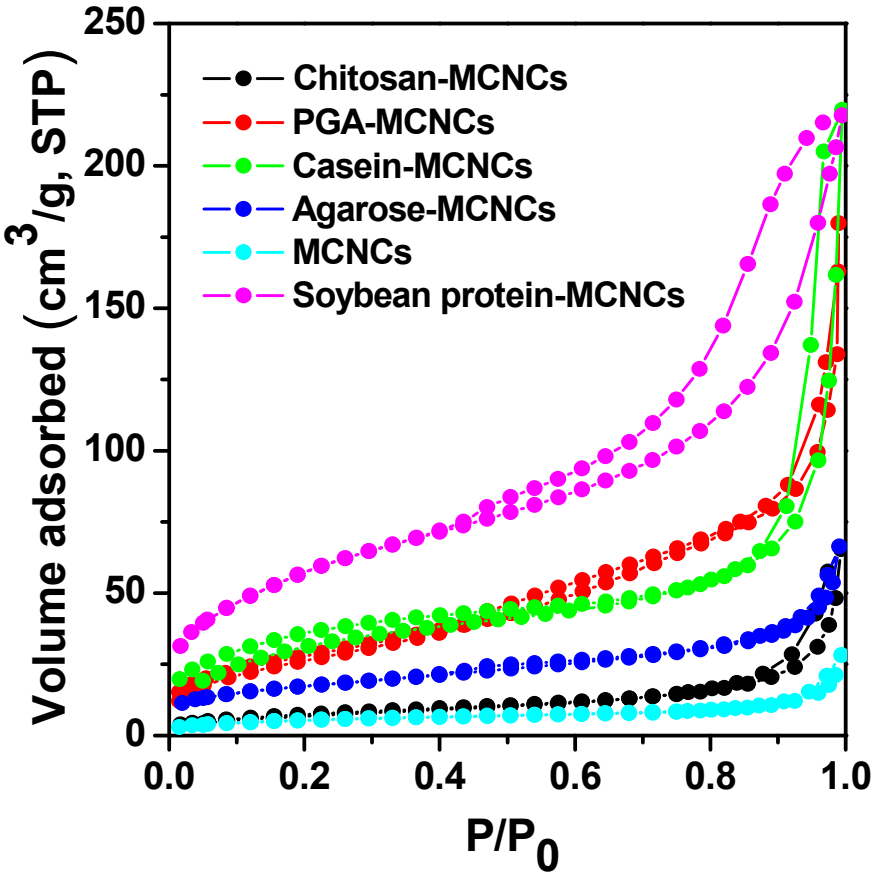

Fig. S5 Nitrogen adsorption-desorption isotherms of MCNCs stabilized by various polymers.

Section 7. Drug loading estimation

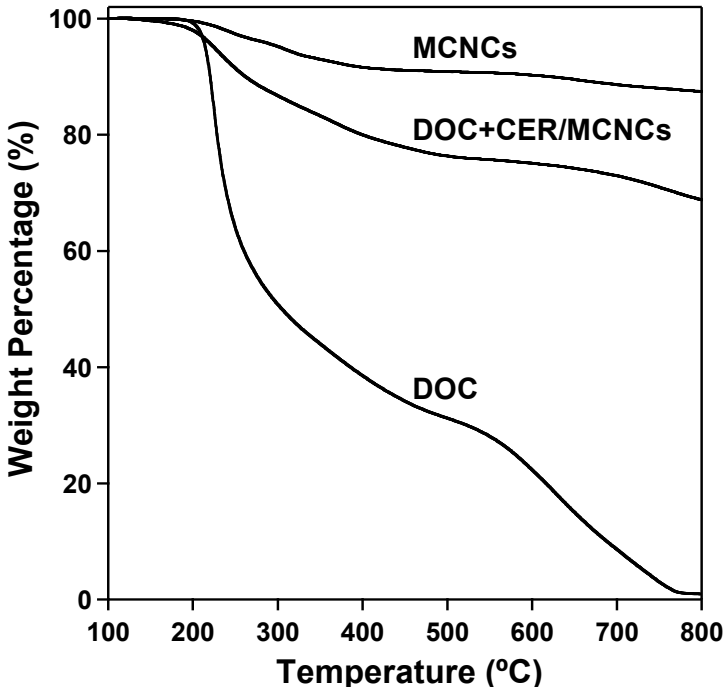

Fig. S6 TG curves of the MCNCs, DOC+CER-loaded MCNCs and DOC alone.

Section 8. Drug release in vitro

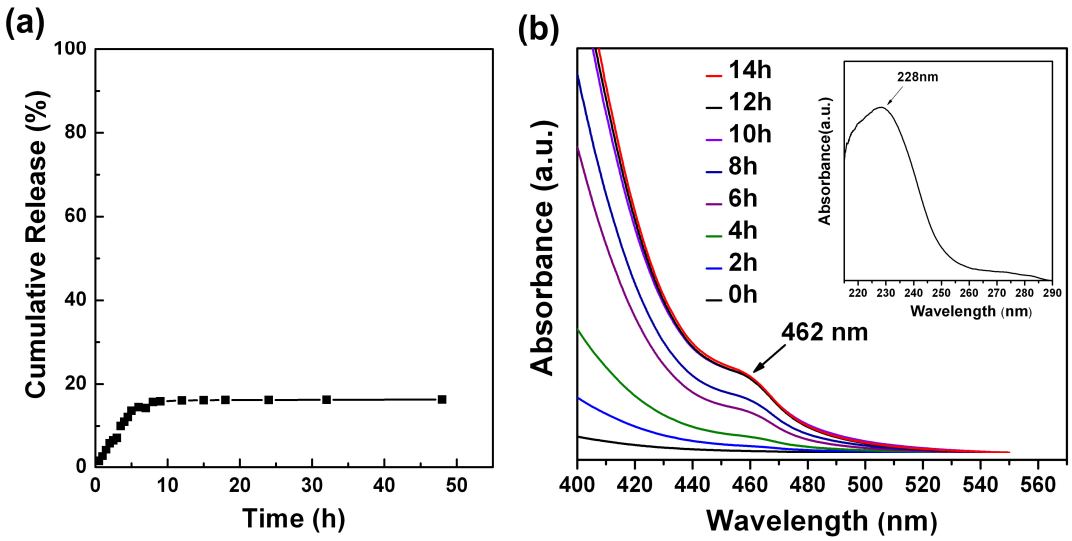

Fig. S7 (a) Drug release profile of the DOC+CER-loaded MCNCs as a function of incubation time at pH of 7.4 in PBS; (b) UV-vis spectrum of the solution of the DOC+CER-loaded MCNCs at pH of 5.0 (b) in the citrate buffer solution. The inset of (b) is an absorption curve of the free DOC in the aqueous solution.

Section 9. Hydrodynamic diameters

Table S1. DLS results of the various biopolymer-stabilized MCNCs dispersed in water.

Sample	Size/nm	PDI
Bare MCNCs	334.9	0.228
Chitosan-stabilized MCNCs	387.0	0.126
Agarose-stabilized MCNCs	395.3	0.139
PGA-stabilized MCNCs	334.6	0.089
Casein-stabilized MCNCs	306.2	0.105
Soybean-stabilized MCNCs	311.2	0.178