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Improved *in vivo* tumor therapy *via* host–guest complexation

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Electronic Supporting Information (11 Pages)

1.	*Materials and methods*	S2
2.	*Synthesis, characterization and decomposition of **HMNPs***	S2
3.	*Synthesis of N[1]-propylethane-1,2-diamine functionalized **HMNPs***	S3
4.	*Drug loading and release*	S4
5.	*The studies of host–guest interactions between **WP5** and model compound*	S5
6.	*Anticancer effect in vitro*	S7
7.	*Blood analysis and histology examinations*	S7
8.	*Anticancer effect in vivo*	S9
9.	*References*	S10

1. Materials and methods

N-[3-(Trimethoxysilyl)propyl]ethylenediamine, magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), ammonia chloride, ammonium hydroxide aqueous solution (28%), *N*-ethylethylenediamine (**MC**), and tetraethylorthosilicate (TEOS) were obtained from Aladdin Chemical Reagent Co. Ltd. China. All chemical agents were of analytical grade and used directly without further purification. Water soluble pillar[5]arene **WP5**[S1] was prepared according to literature procedures.

^{1}H NMR spectra were collected on a Bruker Advance DMX-500 spectrometer with internal standard TMS. SEM was performed by using a HITACHI S-4800 microscope at an accelerating voltage of 5 kV. TEM observations were performed by a Philips CM200UT microscope at a typical accelerating voltage of 160 kV. XRD was carried out by means of a Rigaku D/max-2550pc instrument with monochromatized Cu *K*a radiation and a scanning step of 0.02. FT-IR spectra were taken with potassium bromide pellets on a TENSOR 27 spectrometer. UV−Vis spectra were taken on a Shimadzu UV-2550 UV−Vis spectrophotometer. Dynamic light scattering measurements were performed on a goniometer ALV/CGS-3 using a UNIPHASE He-Ne laser operating at 632.8 nm.

*2. Synthesis of **HMNPs***

Monodispersed silica colloidal spheres with an average diameter of 200 nm were synthesized in accordance with the Stöber method firstly.[S2] Then magnesium chloride (0.75 mmol), ammonia chloride (10 mmol), and ammonia solution (1 mL, 28%) were dissolved in deionized water (20 mL). Silica colloidal spheres (100 mg) were dispersed homogeneously in deionized water (20 mL). These two solutions were mixed until homogeneous and then transferred into a Teflon-lined stainless-steel autoclave (50 mL) and sealed to heat at 160 °C. After reaction for 12 h, the autoclave was cooled to ambient temperature naturally. The obtained hollow mesoporous nanoparticles **HMNPs** were washed with D. I. water and ethanol in sequence, and then dried in vacuum at 60 °C overnight.

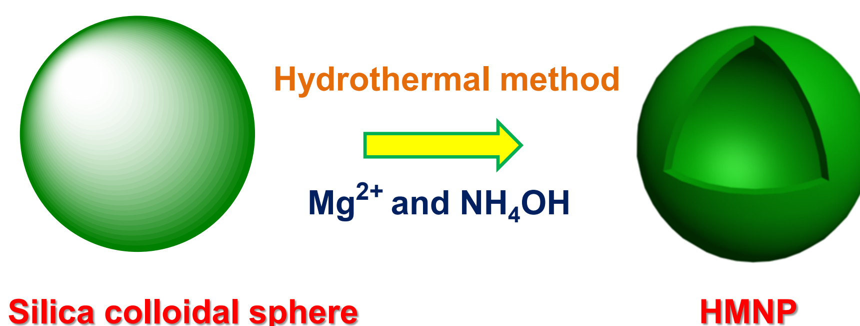


Fig. S1 Schematic illustration of the synthesis of hollow mesoporous nanoparticles **HMNPs**.

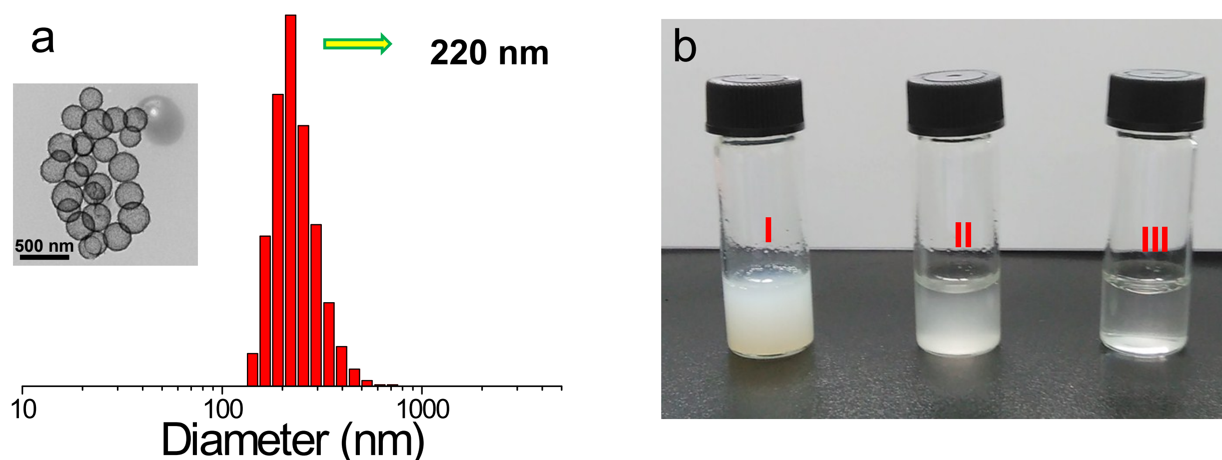


Fig. S2 (a) DLS study and TEM image (inset) of **HMNPs**. (b) Optical photograph of **HMNPs** exposed to a PBS buffer medium at pH 4.5 with different time periods: I, fresh; II, 4 days; III, 15 days.

3. Synthesis of N^1-propylethane-1,2-diamine functionalized ***HMNPs***

100 mg of **HMNPs** were dispersed in a mixture of isopropanol (40 mL) and *N*-[3-(trimethoxysilyl)propyl]ethylenediamine (500 μL) heated up to 80 °C for 2 hours to functionalize the silica surface with N^1-propylethane-1,2-diamine groups. The N^1-propylethane-1,2-diamine-functionalized **HMNPs** were collected by centrifugation, washed with ethanol 5 times, and finally dried under vacuum at room temperature for 12 h.

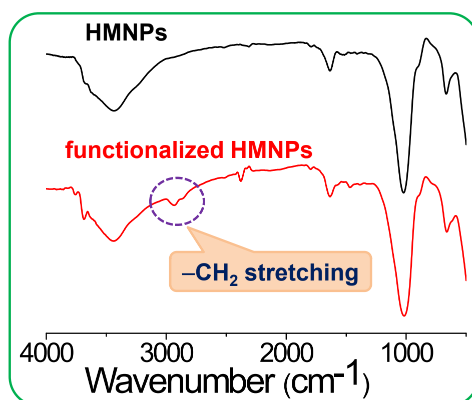


Fig. S3. FT-IR spectrum of **HMNPs** compared with N^1-propylethane-1,2-diamine-functionalized **HMNPs**.

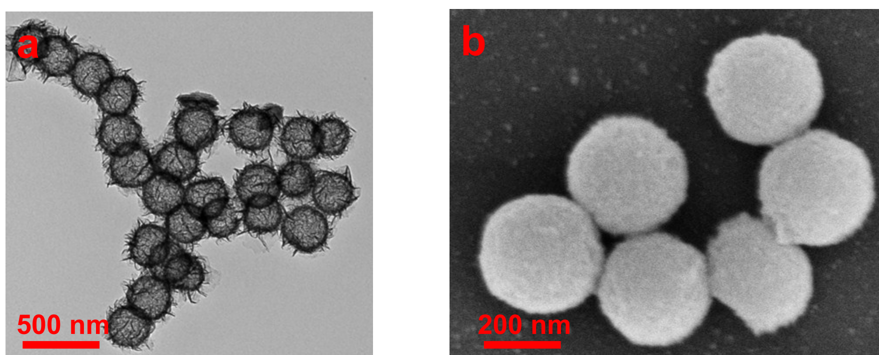


Fig. S4 TEM (a) and SEM (b) images of N^1-propylethane-1,2-diamine-functionalized **HMNPs**.

4. Drug loading and release

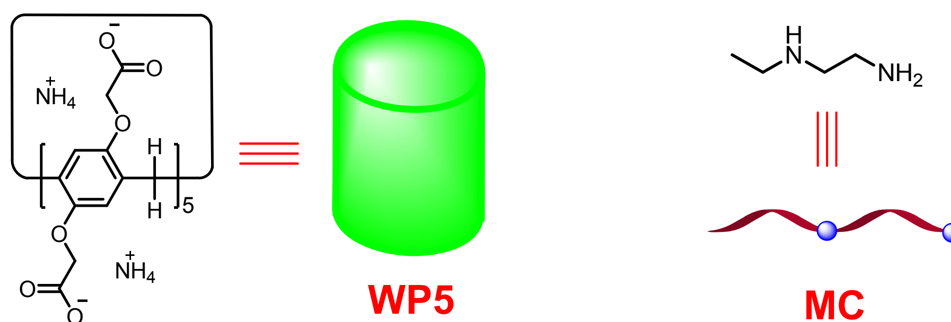
The loading of the drugs was carried out by the immersion of N^1-propylethane-1,2-diamine-functionalized **HMNPs** in a DOX solution with a certain concentration. A typical procedure for loading DOX in **HMNPs** is described as follows: **HMNPs** was suspended in PBS solutions (pH 4.5, 6.0, 7.4) with different concentrations of DOX under stirring for 24 h in the dark to reach the equilibrium state. After DOX was loaded, a solution of **WP5** (0.05 g) in distilled water (1 mL) was slowly added to the former solution and stirred for 12 h. The DOX-loaded **HMNPs** were isolated from the solution by centrifugation and washed three times with PBS (pH = 7.4) to remove the physically adsorbed DOX. The supernatant was sucked and properly diluted to determine the drug-loading amount by UV-Vis spectrophotometer.

The loading capacity of **HMNPs** was determined by the following equation:

$$\text{Drug loading capacity} = (W_{\text{initial DOX}} - W_{\text{DOX in supernatant}}) / W_{\textbf{HMNPs}}\ (\text{mg g}^{-1})$$

Where $W_{\text{initial DOX}}$ is the weight of DOX in the initial solution, $W_{\text{DOX in supernatant}}$ is the weight of DOX in supernatant, and $W_{\textbf{HMNPs}}$ is the weight of **HMNPs** added in DOX solution. *In vitro* DOX release from **HMNPs** was performed in the PBS buffer at pH values of 7.4, 6.0, and 4.5. The DOX-loaded **HMNPs** was then re-suspended in 5 mL of the PBS buffer at pH values of 7.4, 6.0, and 4.5, and stirred at 37 ºC for DOX release. At selected time intervals, the sample was collected by a permanent magnet and the supernatant was determined by a UV−Vis spectrophotometer at 480 nm. Then, 5 mL of fresh PBS buffer was added for further release.

*5. The studies of host–guest interactions between **WP5** and model compound **MC***



WP5 possesses both a hydrophobic cavity and ten charged substituents, thus providing a very efficient recognition motif for guests with multiple functional groups such as *N*-ethylethylenediamine **MC**. A detailed study of the inclusion property of **WP5** with **MC** displayed high affinity for **MC** in water. As shown in Fig. S5, a 1 : 1 mixture of **WP5** and **MC** in D_2O had significant chemical shift changes in the ^1H NMR signals compared with the free host and free guest under the same conditions. Only one set of peaks was found for the solution of **WP5** and **MC**, indicating fast-exchange complexation on the ^1H NMR time scale. The protons on **MC** moved upfield sharply, indicating host–guest complexation. Binding of **MC** made the shielding effect on all protons of **MC** a little diminished, causing the signals of these protons to move downfield. Adjusting the pH value to make the solution acidic protonates the carboxylate groups on both rims of the pillar[5]arene. The conversion of the water-soluble –COO⁻ groups of **WP5** to insoluble –COOH groups resulted in the decomplexation of **WP5⊃MC**.

Further investigation of the complexation of **WP5** with **MC** was carried out by isothermal titration calorimetry (ITC), which is a powerful tool for studying host−guest complexes because it not only gives the complexation association constants (K_a) but also yields their thermodynamic parameters (enthalpy and entropy changes). A 1:1 stoichiometry was established for the complexation between **WP5** and **MC** by ITC experiments (Fig. S6). The association constant (K_a) was determined in D_2O to be $(2.33 \pm 0.04) \times 10^6$ M^{-1}.

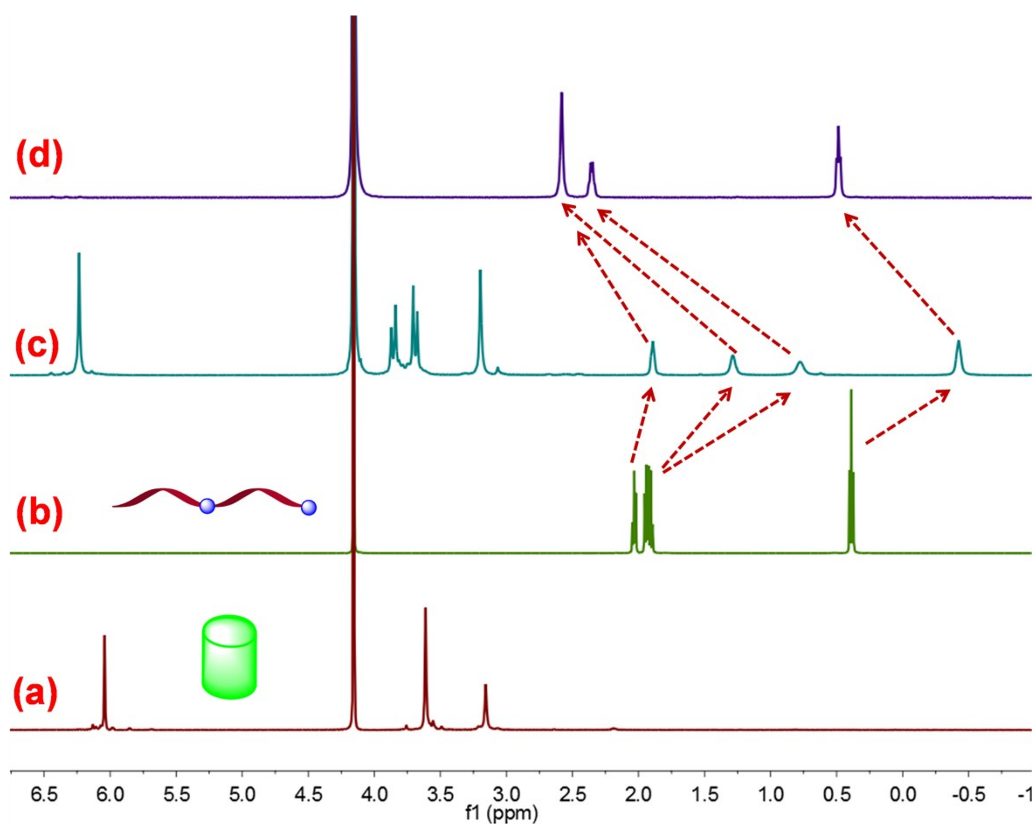


Fig. S5 ^{1}H NMR spectra (400 MHz, D_2O, room temperature): (a) **WP5**; (b) **MC**; (c) **WP5** and **MC** (pH = 7.0); (d) **WP5** and **MC** (pH = 4.0).

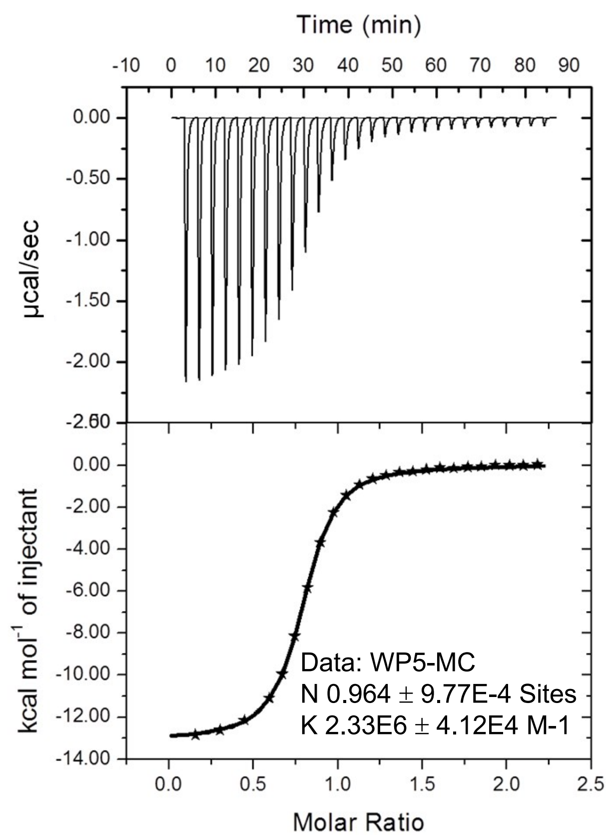


Fig. S6 ITC studies of host−guest interactions between **WP5** and **MC**.

6. *Anticancer effect in Vitro*

The anticancer assay of nanocarriers against HepG2 cell lines were assessed by the standard MTT assay. The HepG2 cell lines were plated into 96-well plates at 10000/well for 24 h to allow cell attachment. Then the cells were incubated with PBS, **HMNPs**, free DOX, DOX loaded **HMNPs**, and DOX loaded **WP5@HMNPs** at indicated concentrations, respectively. After 24 h, the medium were replaced with fresh DMEM containing MTT (5 mg mL^{-1}), and the cells were incubated for another 4 h. The supernatant of each well was drawn off carefully and then 150 μL of dimethyl sulfoxide (DMSO) was added to each well and mixed thoroughly by concentrating for 10 min. The absorbance from the plates was read at 570 nm with a microplate reader.

To understand the localization of **HMNPs**, HepG-2 cells were seeded at a density of 8×10^4 cells in 3.5-cm dishes with cover slips for 24 h and then cultured with free DOX and **HMNPs**-DOX (at equally DOX concentration with 20 μg mL^{-1}) for 3 h at 37 °C, followed by washing with PBS for three times to fully remove extracellular DOX. Then, the cells are stained with 300 nM of Lysotracker Green (Ex504 nm, Em 511 nm, Molecular Probes, USA) for 30 minutes and 5 μg mL^{-1} Hoechst 33342 (Ex 345 nm, Em 478 nm, Beyotime, China) for another 10 minutes. The cells were rinsed with PBS three times after staining and fixed with 4% paraformaldehyde for 20 minutes at room temperature, and subjected to confocal laser scanning microscope (CLSM). All images are captured under the same instrumental settings and analyzed with image analysis software.

7. *Blood analysis and histology examinations.*

Healthy mice were injected with PBS, **HMNPs**, and DOX-loaded **WP5@HMNPs** (n = 5). Two weeks later, the mice were sacrificed by cervical dislocation and an approximate 0.8 mL portion of blood from each mouse was sampled by eyeball extirpation for blood chemistry tests and complete blood panel analysis. And their major viscera (heart, liver, spleen, lung and kidneys) were harvested and fixed in a 10% formalin solution. All the blood parameters were measured in the First Affiliated Hospital of Zhejiang University. For histological examination, organs from the treated groups and control group were fixed in 4% formalin and conducted with paraffin embedded sections for H&E staining. The slices were examined by using a digital microscope.

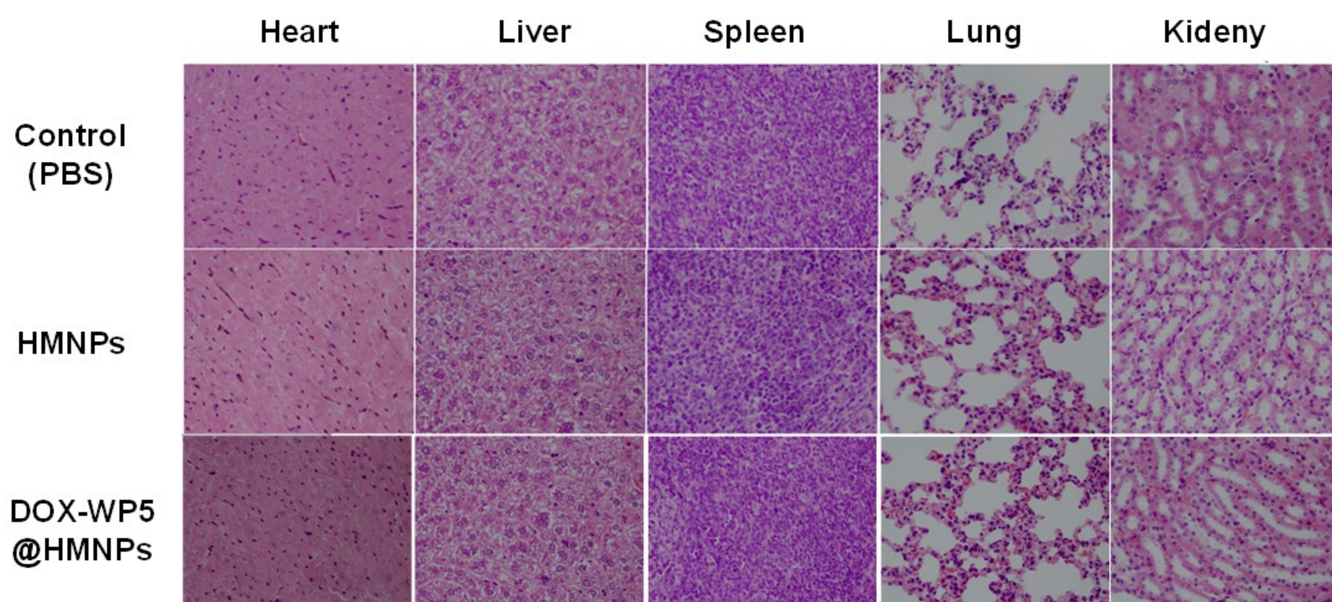


Fig. S7 Representative H&E stained images of major organs collected from a control untreated mouse, a **HMNPs** injected mouse, and a DOX-loaded **WP5@HMNPs** injected mouse.

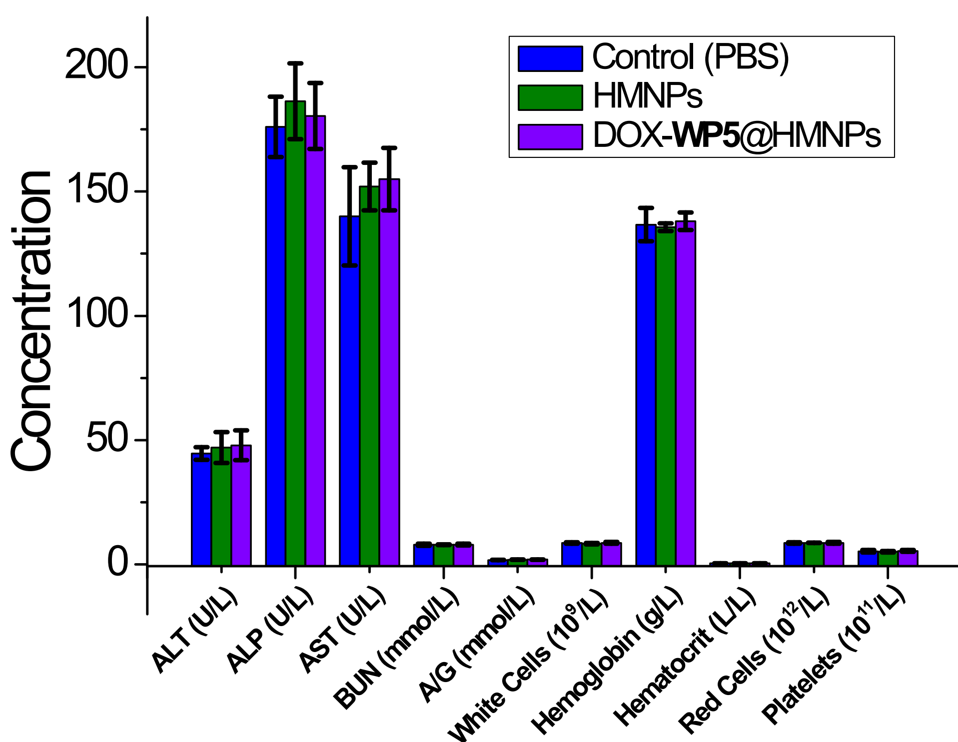


Fig. S8 Blood biochemical assay and hematology of PBS, **HMNPs**, and DOX-loaded **WP5@HMNPs** injected mice.

8. *Anticancer effect in Vivo*

In order to understand the anticancer effect of DOX loaded **WP5@HMNPs** *in vivo*, 100 μL of 5 × 10^6 HepG2 cells were inoculated by subcutaneous injection onto the back of each mouse. The mice were divided into six groups (n = 5) randomly when the tumor grew into the volume of 60 mm^3. Then the mice were injected by intravenous injection everyday with PBS, **HMNPs**, free DOX, DOX loaded **HNMPs**, and DOX loaded **WP5@HNMPs** at a DOX dose of 5 mg kg^{-1}, respectively. Body weight of mice and tumor volume were measured every 2 days, and the tumor volume was calculated as (tumor length) × (tumor width)^2/2. Relative tumor volumes were calculated as V/V_0 (V_0 was the tumor volume when the treatment was initiated).

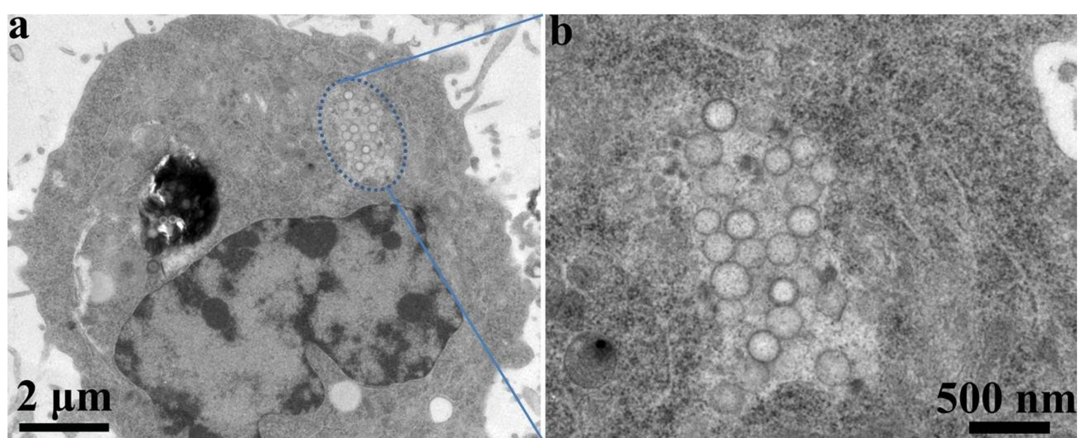


Fig. S9 TEM (a) and enlarged TEM (b) images of the slice of tumor tissue after injecting by intravenous injection with DOX loaded **WP5@HMNPs**.

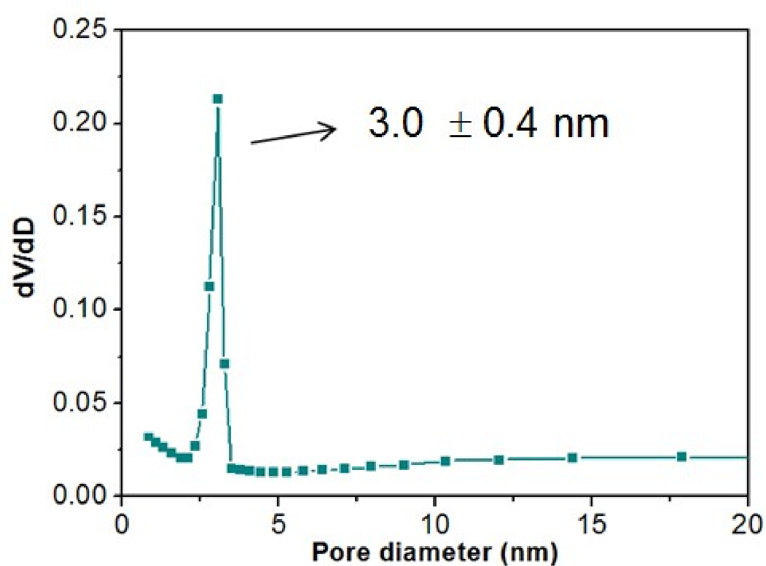


Fig. S10 Pore size distritution of **HMNPs**.

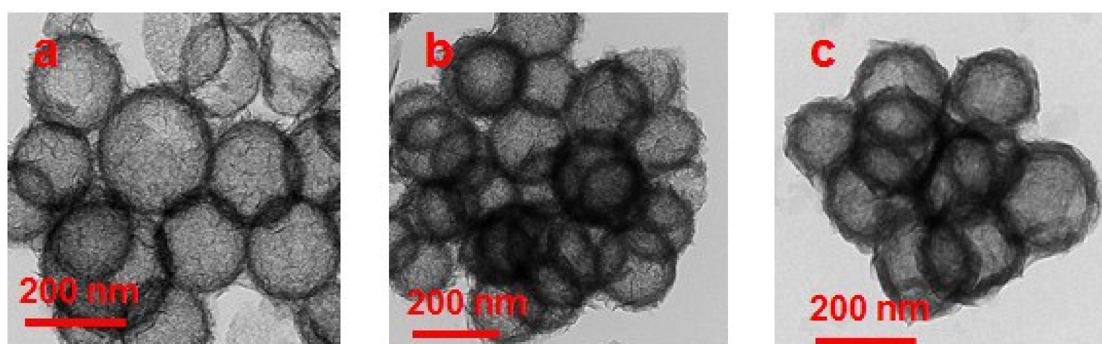


Fig. S11 TEM images of **HMNPs** exposed to a PBS buffer medium at pH 7.4 with different time periods: (a) 1 h; (b) 2 d; (c) 2 weeks;

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S1. T. Ogoshi, M. Hashizume, T.-a. Yamagishi and Y. Nakamoto, *Chem. Commun.*, 2010, **46**, 3708–3710.

S2. B. Wang, W. Meng, M. Bi, Y. Ni, Q. Cai and J. Wang, *Dalton Trans.*, 2013, **42**, 8918−8925.