# Supporting Information

## Designed Preparation of Polyacrylic Acid/Calcium Carbonate Nanoparticles with High Doxorubicin Payload for Liver Cancer Chemo-Therapy

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### EXPERIMENTAL SECTION

**Chemicals.** Calcium chioride anhydrous ($CaCl_2$, ≥96.0 %), isopropyl alcohol (IPA) were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd and used without further purification. Polyacrylic acid sodium salt (PAAS, Mw = 5100) and doxorubicin (DOX) were obtained from Sigma-Aldrich. Deionized (DI) water was used in all experiments.

**Characterization.** Fourier transform infrared (FTIR) spectra were obtained on a Magna560 FTIR spectrometer (Nicolet, USA). Transmission electron microscopy (TEM) was performed on a JEOL-2100F transmission electron microscope under 200 kV accelerating voltage. UV–

Vis absorption spectroscopy was obtained on U-3010 spectrophotometer (Hitachi, Japan). Fluorescence spectra were performed with Eclipse fluorescence spectrophotometer (Varian, USA). Scanning electron microscopy (SEM) images and Energy dispersive X-ray spectroscopy (EDS) were obtained using an XL30 ESEM-FEG field-emission scanning electron microscope (FEI Co.). Confocal laser scanning microscopy (CLSM) was operated on Olympus Fluoview FV1000. $N_2$ adsorption–desorption isotherms and BJH pore size distributions were obtained on a Micromeritics ASAP 2010 instrument. Thermogravimetric (TG) analyses were performed on a Perkin-Elmer TG-7 analyzer heated from 30 to 1000 $^oC$ at the heating rate of 10 °C/min. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was measured with Leeman ICP-AES Prodigy. Particle size distribution was obtained using a Mastersizer 2000 laser particle size analyzer. XPS (X-ray photoelectron spectroscopy) was carried out on an ECSALAB 250 instrument with non-monochromatized AlKa radiation. X-ray powder diffraction (XRD) patterns were performed on a D8 Focus diffractometer (Bruker) with Cu Kα radiation.

**Synthesis of PAAS and polyacrylic acid (PAA)-Ca NPs.** In brief, 0.1 g PAAS was mixed with 10 mL DI water in a 250 mL of flask under magnetic stirring at ambient temperature. After 30 min, 190 mL IPA was dropwise added into the mixture solution by continuously stirring for 30 min. The PAAS NPs were obtained. For the fabrication of PAA-Ca NPs, 40 mL DI water solution of $CaCl_2$ (0.6 M) was slowly dripped into the 200 mL as-prepared PAAS NPs under stirring for 2 h. Then, the obtained PAA-Ca NPs were centrifuged and kept at room temperature for further experiments.

**Synthesis of PAA/calcium carbonate ($CaCO_3$) NPs.** PAA/$CaCO_3$ NPs were obtained by

thermal treatment of the PAA-Ca precursors at 400 $^{o}$C for 4 h under high-purity $CO_2$ atmosphere (2 $^{o}$C min$^{-1}$ to 400 $^{o}$C), the product was washed several times with DI water in order to remove impurities to obtain the PAA/$CaCO_3$ NPs.

**DOX loading into PAA/$CaCO_3$ NPs.** 1 mg of PAA/$CaCO_3$ NPs were dispersed in 1 mL DI water, then 120 μL DOX (10 mg mL$^{-1}$) solution was slowly added into PAA/$CaCO_3$ NPs solution. The mixture was shaken for 24 h at room temperature to reach the equilibrium state. The solution was centrifuged at 8000 rpm for 10 min and washed three times with DI water to collect the DOX-loaded PAA/$CaCO_3$ NPs. To calculate the amount of DOX loaded into the PAA/$CaCO_3$ NPs, the content of original DOX and the supernatant were determined by UV-Vis measurements at 480 nm. The DOX loading efficiency (LE) can be calculated by Equation as follows:

$$LE\ (\%) = [m(\text{original DOX}) - m(\text{DOX in supernatant})] / m(\text{original DOX}) \times 100\ \%$$

***In vitro*** **release of DOX-loaded PAA/$CaCO_3$ NPs.** *In vitro* release of DOX from DOX-loaded PAA/$CaCO_3$ NPs was performed using dialysis method. A certain amount of as-prepared DOX-loaded PAA/$CaCO_3$ NPs were washed by DI water, divided into two parts and dispersed in phosphate-buffered saline (PBS, 3 mL, pH = 5.3 and 7.4). Both of the release mediums were transfered into pretreated semi-permeable dialysis bags and then immersed into 6 mL PBS solution (pH = 5.3 and 7.4) at 37 $^{o}$C. The amount of released DOX was monitored at certain time intervals by fluorescence spectrophotometer with emission at 591 nm and excitation at 479 nm. The release test was performed in triplicate to calculate a mean value and standard deviation.

**Cell culture.** A liver cancer cell lines, Bel-7402 cells ($2.5\times10^4$ cells per well) were grown as

a monolayer in a humidified incubator at 37 $^{o}$C in a 95 % air/5 % $CO_2$ in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10 % fetal bovine serum.

***In vitro* cytotoxicity of PAA/$CaCO_3$ NPs against Bel-7402 cells.** The Bel-7402 cells ($2.5\times10^4$ cells per well) were planted in a 96-well plate and incubated for 24 h at 37 $^{o}$C in a humidified atmosphere with 5 % $CO_2$ to allow cells to attach. The medium was then replaced with culture serum-free medium. The *in vitro* cell toxicity was evaluated by standard 3-(4,5-dimethylthialzol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using Bel-7402 cells. The free DOX, DOX-loaded PAA/$CaCO_3$ NPs and PAA/$CaCO_3$ NPs with different concentrations (1.5625, 3.125, 6.25, 12.5, 25, 50, 100 μg/mL) were added into the cells, respectively. One row of the 96-well plate was used as a blank control with culture medium only. After 24 h co-incubation in 5 % $CO_2$ under 37 $^{o}$C, MTT solution (20 μL, 5 mg $mL^{-1}$) was added and further incubated for 4 h. Finally, the medium was replaced with DMSO (150 μL) to dissolve the MTT formazan crystals and the absorbance was determined at 490 nm by a microplate reader. We determined cell viability as a percentage by comparing the untreated cells with viable cells after treated with free DOX, DOX-loaded NPs and empty NPs.

**Fluorescence imaging in Bel-7402 cells.** The Bel-7402 cells were seed onto clean cover slips in 6-well culture plates in DMEM medium containing 10 % FBS overnight incubation in 5 % $CO_2$ at 37 $^{o}$C. DOX-loaded PAA/$CaCO_3$ NPs (30 μg/mL, 400μL) were added into the Bel-7402 cells at 37 $^{o}$C and incubated for 1.5, 3.5, 12 and 24 h. The medium was removed and the cell monolayer on the cover slip was repeatedly washed with PBS for three times to remove the remaining NPs and dead cells, fixed with frozen paraformaldehyde (400 μL in each well) for 10 min, and then rinsed with PBS three times again. Subsequently, the cells

were treated with DAPI for 15 min to stain the nuclei. The cells were washed with PBS three times to remove extra dye molecules and then sealed with a microscope glass slide. The observations were performed by using CLSM.

**Animal.** All animal experiments were performed in accordance with the principles and procedures described in "Regulations for the Administration of Affairs Concerning Laboratory Animals" and "The National Regulation of China for Care and Use of Laboratory Animals''. Kunming mice (≈20 g) were purchased from the Changchun Institute of Biological Products and all animal procedures were approved by the University Animal Care and Use Committee. Animals were housed under normal conditions with 12 h light and dark cycles and given access to food and water ad libitum.

**Anti-tumor performance of DOX-loaded PAA/$CaCO_3$ NPs *in vivo*.** The tumors were established by subcutaneous injection of mouse hepatoma H22 cells, which were allowed to grow for around 8 days to reach a size of around 250 $mm^3$. The twelve tumor-bearing mice were divided randomly into three groups (four animals in each group), which were treated by tail vein injection of DOX-loaded PAA/$CaCO_3$ NPs, the same dose of pure DOX and physiological saline were used as control. The DOX dose was equivalent to the total amount of 1 mg $kg^{-1}$ (the equivalent nanoparticle dose was 12 mg $kg^{-1}$) body weight. The body weights, tumor volumes, and survival rate of animals were monitored every the other day after treatment. The tumor sizes were measured by a vernier caliper and calculated as the volume = (tumor length) × $(\text{tumor width})^2/2$.[1] After administration for 11 days, animals of each group were randomly chosen and euthanized to retrieve tumors and organs. The excised tumors and organs were washed with DI water and fixed with 4 % (weight)

paraformaldehyde solution. The tissues were processed routinely and sections were stained with hematoxylin and eosin. Blood of the experimental and control groups were collected from the orbital sinus by quickly removing the eyeball from the socket with a pair of tissue forceps. Approximately 0.8 mL of blood from each mouse was collected before it was euthanatized for blood chemistry test and complete blood panel analysis.[2]

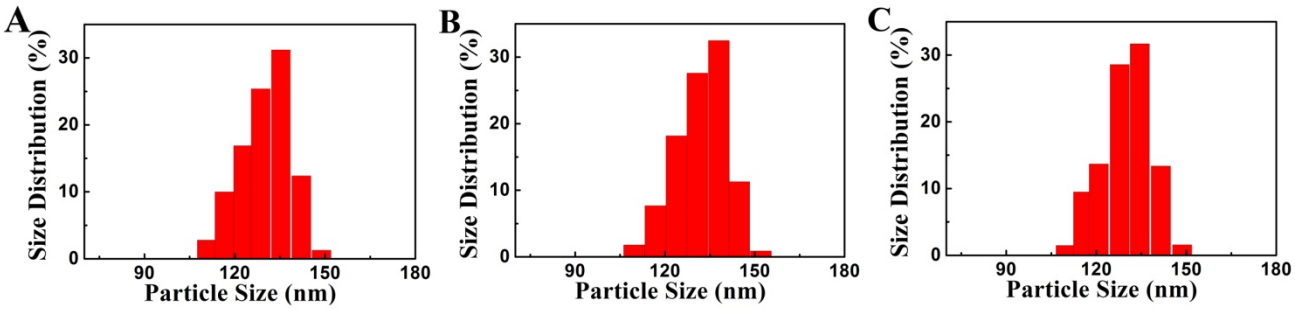


Figure S1. Size distribution of PAA/$CaCO_3$ NPs dispersed in DI water (A), PBS (pH = 7.4) (B) and serum solution (C).

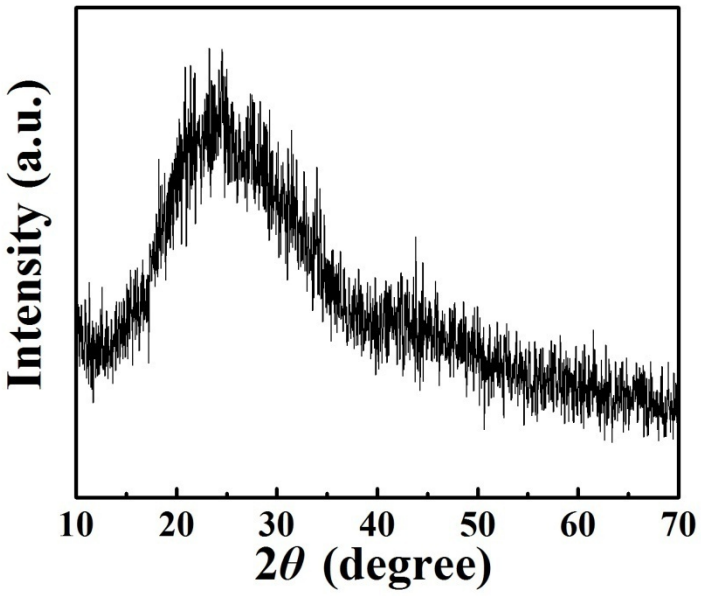


Figure S2. XRD pattern of PAA/$CaCO_3$ NPs.

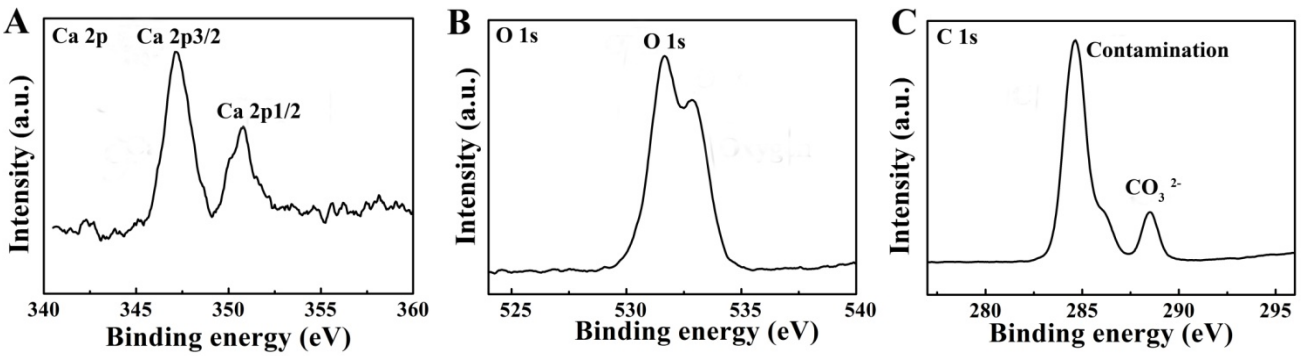


Figure S3. XPS spectra of PAA/$CaCO_3$ NPs.

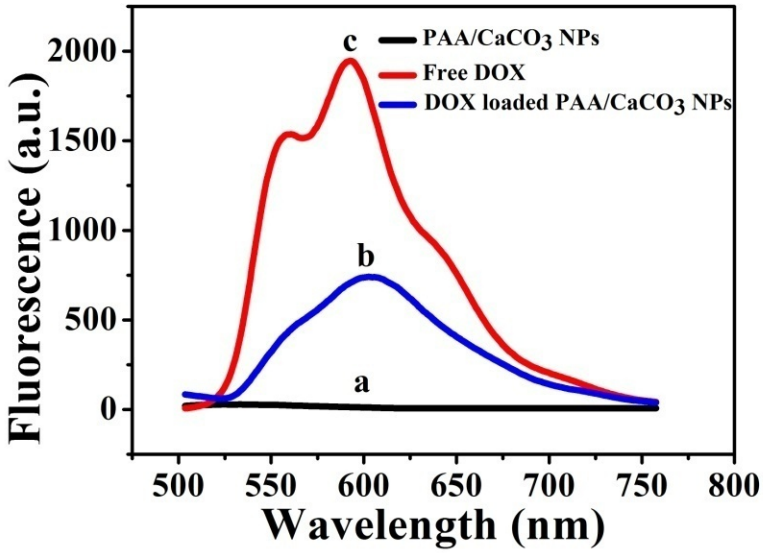


Figure S4. Fluorescence spectra of PAA/$CaCO_3$ NPs (a), DOX-loaded PAA/$CaCO_3$ NPs (b) and free DOX(c).

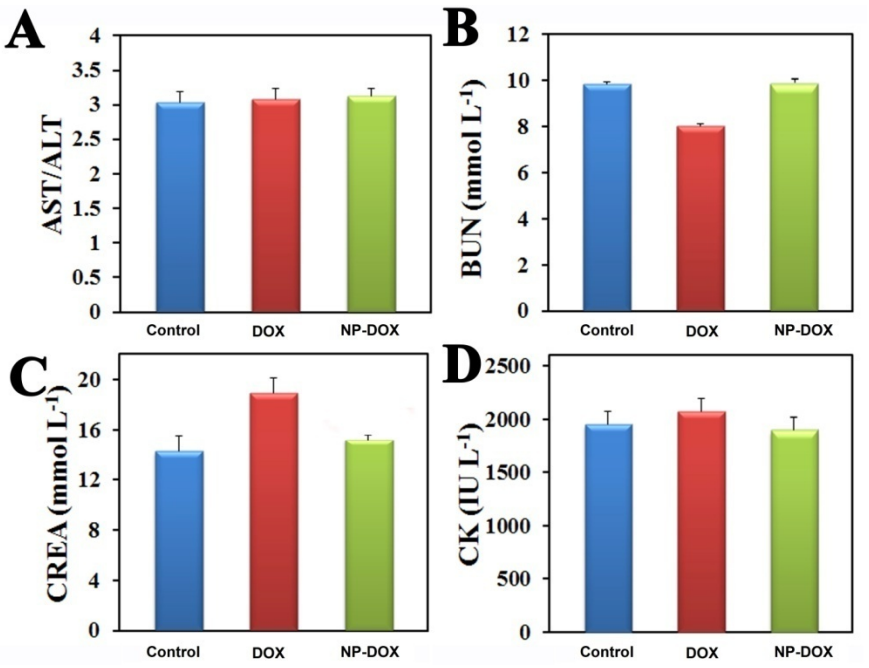


Figure S5. Blood analysis data for mice treated with free DOX, DOX-loaded PAA/$CaCO_3$ NPs and a control group.

1. X. M. Luo, C. Y. Xie, H. Wang, C. Y. Liu, S. L. Yan and X. H. Li, *Int. J.Pharm.*, 2012, **425**, 19.

2. L. Cheng, K. Yang, M.W. Shao, X. H. Lu and Z. Liu, *Nanomedicine*, 2011, **6**, 1327.