Electronic Supplementary Information

A smart pH-responsive nano-carrier as a drug delivery system for targeted delivery of ursolic acid: suppresses cancer growth and metastasis by modulating P53/MMP-9/PTEN/CD44 mediated multiple signaling pathways

Authors:

Kai Jiang [a, †], Ting Chi [a, †], Tao Li [a], Guirong Zheng [a], Lulu Fan [a], Yajun Liu [a], Xiufen Chen [a], Sijia Chen [a], Lee Jia [a], Jingwei Shao [a, *]

Affiliations:

a. Cancer Metastasis Alert and Prevention Center, Fujian Provincial Key Laboratory of Cancer Metastasis Chemoprevention and Chemotherapy, Fuzhou University, Fuzhou 350002, China

†. These authors contributed equally to the work.

*** Corresponding author**: Jingwei Shao

Address: 2 Xueyuan Road, Sunshine Technology Building, 6FL, Fuzhou University, Fuzhou, Fujian 350116, China

E-mail: shaojingwei@fzu.edu.cn (J.W. Shao); shaojw12@163.com (J.W. Shao)

Telephone: + 86-13600802402

Methods

Materials

N-cetyltrimethylammonium bromide (CTAB,99%), Tetraethoxysilane (TEOS), 2-cyanopropyltriethoxysilane (CPTES, 99%), N-Hydroxysulfosuccinimide sodium salt (NHS), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride(EDC), Folic acid, propidium iodide (PI) and 4, 6-diamidino-2-phenylindole(DAPI) were purchased from Aladdin Reagent Inc.. Chitosan was purchased from Shanghai Kabo Reagent Co. Ltd. (Shanghai, China). Ursolic acid (UA, purity > 90%) was purchased from Xi'an Ocean Biological Engineering Co.. Anti-PARP, anti-PTEN, anti-Bcl-2, anti-p53, anti-CD44, anti-MMP-9 and anti-β-action antibodies were obtained from Cell Signaling Technology, Inc..

Synthesis of carboxyl functionalized MSN

Carboxyl functionalized mesoporous silica nanoparticles (M-C) were synthesized by co-condensation method with a little modification [33]. Typically, cetyltrimethylammonium bromide (CTAB, 1.2 g) was mixed in distilled water (180 mL) and ethylene glycol (EG, 20 mL) with ammonia aqueous solution (5.5 mL, 25%) and the solution was vigorously stirred for about 30 min at 60 ℃. After rapidly adding tetraethyl or thosilicate (TEOS, 2.0 mL) and 0.4 mL 2-cyanopropyltriethoxysilane (CPTES, 0.4 mL) into the mixture, vigorous stirring continued for another 2 h at 60 ℃. Afterwards the solution was kept statically for 20 h at room temperature, followed by centrifugation (13000 rpm, 30 min), and then rinsed three times with Distillation-Distillation H_2O (ddH_2O) and ethanol, respectively. Thereafter, the dried product was dispersed into 9 M H_2SO_4 solution at 100 ℃ to prepare carboxyl functionalized MSN. Finally, acidic ethanol was configured (about 9 mL HCl in 100 mL ethanol),which was used to remove the surfactant templates CTAB through extraction at 65 ℃ for 24 h, and then the above mixture was separated by centrifugation at 15000 rpm to get the pure product. The freeze-dried product of the carboxyl functionalized mesoporous silica nanoparticles was used for further experiments.

Synthesis of biofunctionalized chitosan

To attach folic acid to chitosan (CS-FA), folic acid was dissolved in anhydrous DMSO solution containing NHS and EDC, and stirred for about 1h at room temperature to make the folic acid completely dissolve. Subsequently, the above solution was slowly added to the solution of 1% (w/v) CS in acetate buffer (pH 4.7). Then, the above mixture was vigorously stirred for 16 h in the dark at room temperature and adjusted to pH of 9 by dropwise addition of aqueous NaOH. Finally, the above solution was purified via dialysis (14000 MWCO) first against phosphate buffer solution (pH 7.4) for 3 days, dialysis against water for 3 days.

Preparation of chitosan or chitosan–folic acid conjugate modified M–C

Chitosan modified carboxyl functionalized mesoporous silica nanoparticles were synthesized. The carboxylic group of mesoporous silica nanoparticles was firstly activated. The details were described as follows: 300 mg M-C were dispersed in 50 mL phosphate buffer solution (pH 7.0). Subsequently, EDC/NHS mixture (EDC= 0.2 M, NHS= 0.2 M) in 25 mL of phosphate buffer solution (pH 5.0) was put into the above solution and then the mixture was stirred for 4 h at room temperature. Independently, 0.1 g of chitosan was dissolved in an aqueous solution of acetic acid (2%, 5 mL) and stirred for 24 h at room temperature to get a transparent solution of chitosan (2% w/v). Finally, 2% chitosan solution was added dropwise to the activated M-C solution and the mixture was stirred 12 h at ambient temperature. The resulting chitosan end-capped nanoparticles (M-CS) were collected by centrifugation and washed with deionized water before freeze-drying. Chitosan-folic acid conjugate modified carboxyl functionalized mesoporous silica nanoparticles (M-CS-FA) and folic acid modified carboxyl functionalized mesoporous silica nanoparticles (M-FA) were prepared by the same method.

Cell culture

The human epithelial carcinoma cell line (HeLa) and human hepato cellular liver carcinoma cell line (HepG2) were purchased from the cell Bank of Shanghai Institute of cell Biology. HeLa, FR positive tumor cell and HepG2, FR negative tumor cell, were cultured in RPMI-1640 medium containing heat-inactivated fetal bovine serum

(FBS, 10%), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified incubator at 37 ℃ and 5% CO_2.

Preparation of FITC labeled M-CS-FA

Not all amino groups in NH_2-CS-FA reacted with MSN, thus there remaining free NH_2 moieties were utilized for labelling with FITC. Briefly, 50 mg powered M-CS-FA were dissolved in 5 mL deionized water, followed by the addition of 5 mL FITC ethanol solution (0.3 mg/mL). After stirring in the dark for 6 h, the nanoparticles were collected by centrifugation and washed three times with ethanol until the supernatants were colorless. The FITC labelled nanoparticles were used for HeLa and HepG2 cells uptake experiments by confocal microscopy observations.

Cell adhesion, invasion and wound healing assay

To explore the anti-adhesion effect of UA@M-CS-FA, fluorescence-based analysis was carried out using 24-well plate. HUVECs were cultured to confluence in 24-well culture plates, pretreated with IL-1β (1 ng/mL) for 4 h. And HeLa and HepG2 cells were labeled with Rhodamine 123, then co-cultured with the HUVECs monolayers in each well, followed by treatment with PBS, 2 μg/mL of free UA, UA@M-C, UA@M-CS, UA@M-CS-FA. After 1 h incubation, cells were washed with PBS to remove the nonadhered cells for three times. Five fields were selected randomly per filter in each group and images were taken under a fluorescence microscope (Zeiss, Germany). The adhesion rate was calculated according to the following equation:

adhesion (%)= (the number of adhered cells in samples/the number of adhered cells in the control group) × 100%.

To assess cell invasion, HeLa cells were seeded into transwell chambers (Costar, Coring Incorporated, USA)，which had been pre-coated with Matrigel and bottom chamber of serum RPMI 1640 medium in a 24-well plate was in the presence of free medium, UA, UA@M-C, UA@M-CS, UA@M-CS-FA with final concentration of 2 μg/mL UA. After 24 h incubation, cells were fixed in methanol for 20 min. And then the transwell chambers were gently removed. The invaded cells in the bottom chamber were stained with crystal violet (0.5%) and then washed with PBS twice. The

invaded cells were counted and photographed under a light microscope (Zeiss Co., Germany) at × 200 magnification.

For wound healing assay, HeLa cells was cultured in 12-well plates at a density of 4 x 10^5 cells/mL for 1 mL (4 x 10^5 cells/well) overnight. At 80% cell confluence, a scratch wound using a sterile 200 μL pipette tip was performed and then HeLa cells were treated with free medium, UA, UA@M-C, UA@M-CS, UA@M-CS-FA (2 μg/mL). The wound width was measured under a light microscope (Zeiss Co., Germany) at 0 and 24 h after drug treatment to assess the migration ability of HeLa cells. The data were performed in duplicate and analyzed using Image J.

Cell cycle analysis

Flow cytometry was used to analyze cell cycle. Briefly, cells were treated with 20 μg/mL UA, UA@M-C, UA@M-CS, UA@M-CS-FA for 24 h, respectively. Cells were harvested and washed twice with PBS, and fixed in 70% ice-cold ethanol for 24h. After centrifugation, cells were washed with cold PBS twice, and followed by staining for DNA with a mixture solution containing 1% Triton X-100, 0.01% RNase, and 0.05% PI for 30 min at 37 ℃ in the dark. Cell cycle was imaged by the FACS Aria III flow cytometer.

DAPI staining

HeLa cells and HepG2 cells treated with 20 μg/mL UA, UA@M-C, UA@M-CS, UA@M-CS-FA for 24 h, were harvested, washed twice with PBS, and then were stained for cell nucleus with 1 μg/mL DAPI for 30 min at 37 ℃ in the dark. Finally, the cells were observed via fluorescence microscope.

Animals assay

All studies involving animals were performed according to the NSFC regulation concerning the care and use of experimental animals and approved by our Animal Care and Use Committee to reduce the suffering and use of animals. To evaluate *in vivo* antitumor activity of UA@M-CS-FA, the tumor volume was measured in a double-blinded manner. HeLa cells (1 x 10^6) in 200 μL PBS were subcutaneously injected at the right armpit of the 6-week-old male nude mice. After tumor volume reached approximately 40 mm^3, the tumor nude mice were divided into four groups

randomly. The mice were treated with PBS, free UA, UA@M-CS and UA@M-CS-FA (11 mg/kg, 200 μL), through tail vein injection at day 1, 4, 7, 10, 13, 16, 19, 22, 25 post initial treatment. Tumor volume was calculated according to the following equations: $V= (L \times W^2)/2$ (Where L and W represent the length and width of a tumor, respectively). At the end of treatment for 25 days, all animals were sacrificed，the histologic sections were stained by hematoxylin and eosin (H&E), and tumor tissues were collected for assessment of apoptosis by TUNEL assay. The tunel staining was performed in according with the instructions in the protocol for cell sections (Meilun, Dalian, China). Images were acquired via upright microscope (ZEISS Primo Star).

To establish the lung metastasis model, nude mice were inoculated with HeLa cells (1×10^6) in 200 μL PBS through the tail vein. Tumor cell-injected mice were randomly divided into four groups. They were administered with PBS, free UA, UA@M-CS and UA@M-CS-FA (2 mg/kg, 200 μL), through tail vein injection at day 1, 4, 7, 10, 13, 16, 19, 22, 25 post initial treatment. After 25 days' treatment, all animals were sacrificed, all lungs and primary tumors were collected, imaged and used for histological examination. To evaluate the in vivo anti-metastasis effects, we counted the macroscopic metastatic nodules in each lung. Hematoxylin and eosin (H&E) were used to stain the histologic sections lungs and tumors. To explore the expression of CD44 of frozen sections, immunohistochemistry (IHC) was performed according to the methods of a commercial kit (Meilune, Dalian, China). To quantitatively examine the number of CD44 positive cells, three fields from each tissue section were randomly selected, photographed and analyzed with the MIQAS analysis system (Biological technology Corp., Shanghai, China).

A

Nanoparticles	Size (nm)	Zeta Potential(mV)	PDI
MSN	110.10 ± 4.55	-19.03 ± 0.72	0.185 ± 0.090
M-C	122.93 ± 1.79	-29.53 ± 0.70	0.179 ± 0.033
M-CS	184.70 ± 8.48	+14.86 ± 0.52	0.269 ± 0.114
M-CS-FA	199.83 ± 3.87	-23.14 ± 2.01	0.285 ± 0.094

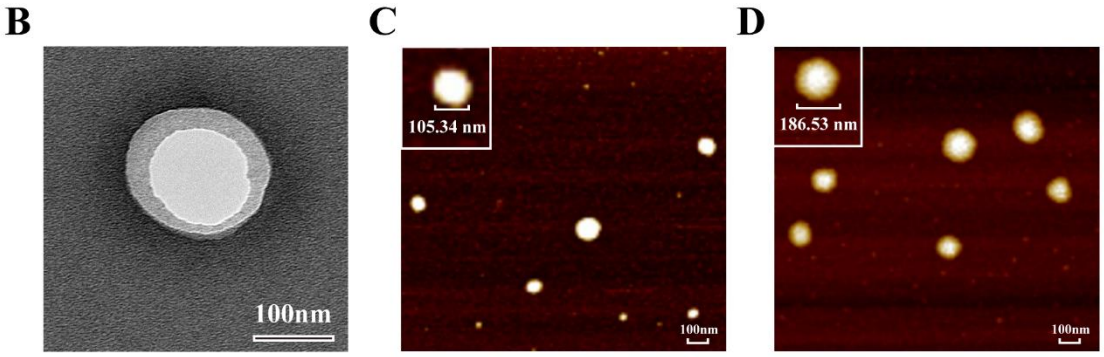


Fig. S1. Characterization of the nanoparticles. (A) The size, zeta potential and PDI of different nanoparticles. **(B)** TEM image of M-CS-FA nanoparticles, about 175nm. **(C)** AFM image of M-C nanoparticles. **(D)** AFM image of M-CS-FA nanoparticles.

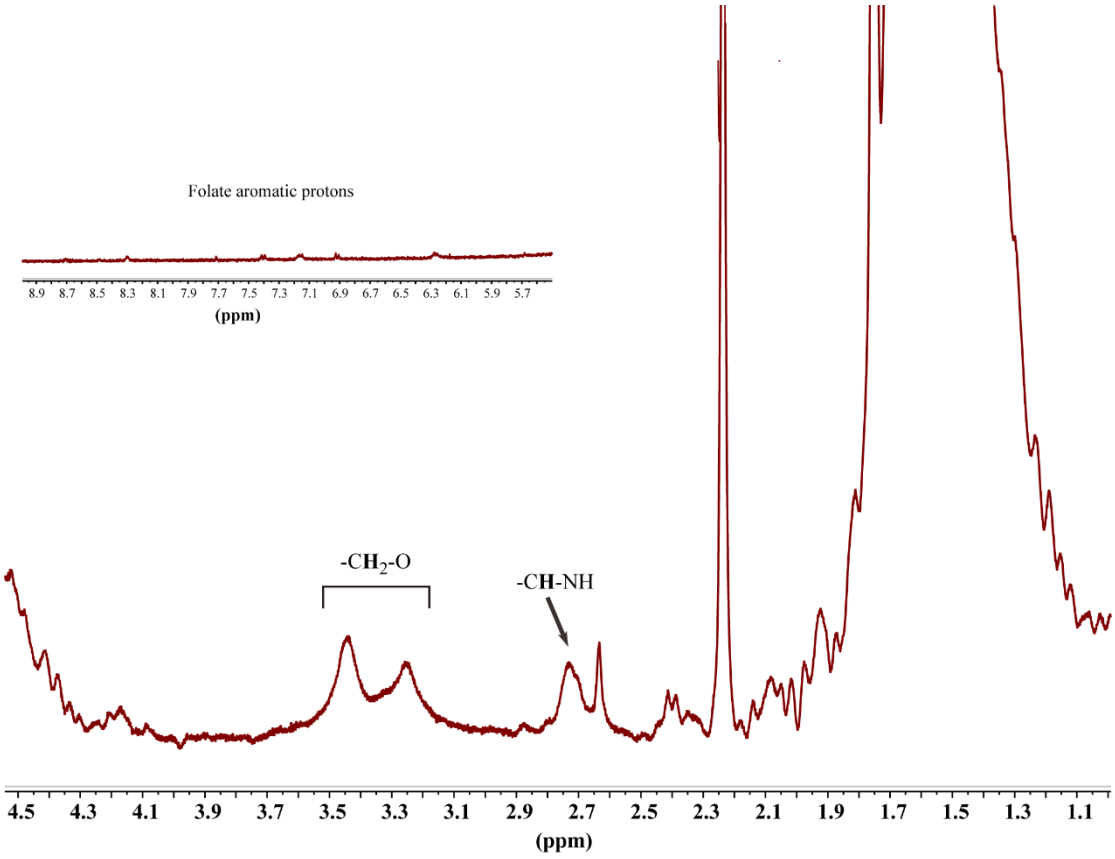
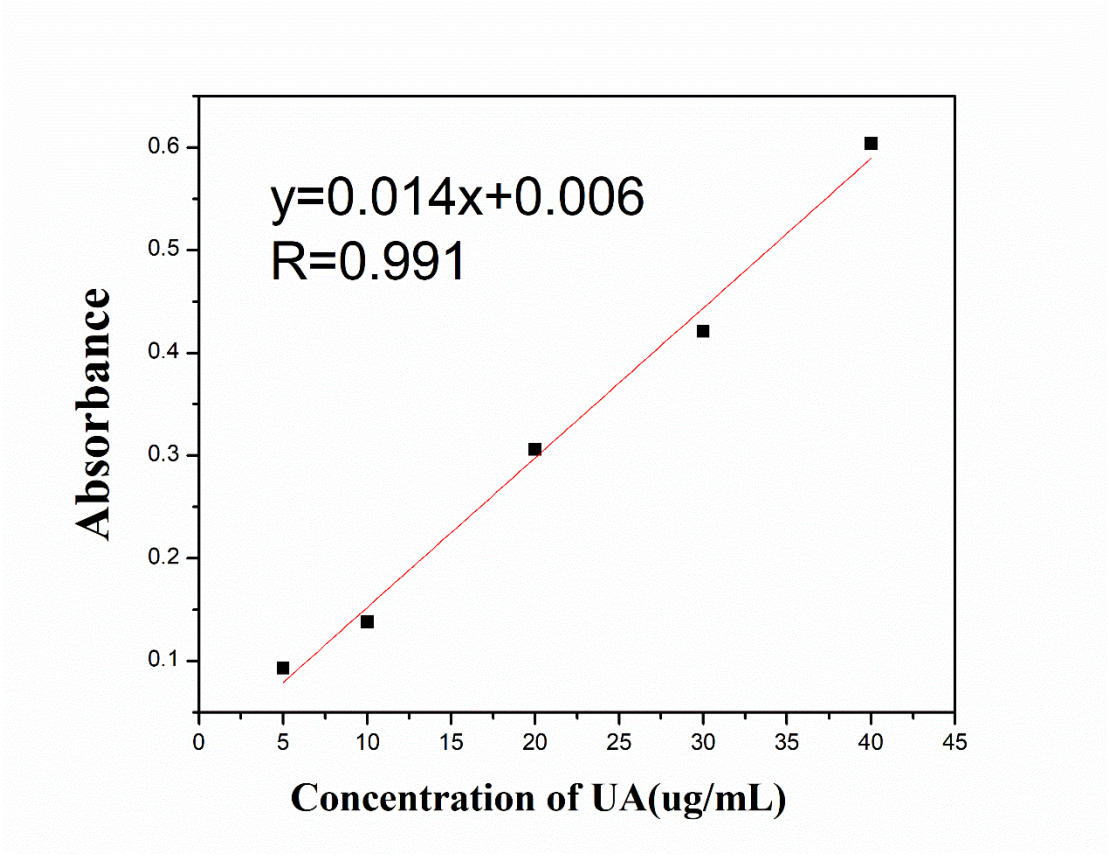


Fig. S2. 1H NMR spectrum of folic acid-chitosan conjugates in an acetic acid /D_2O solution (1/3 v/v).



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172 **Fig. S3. The absorbance of UA molecules as a function of UA concentration.**

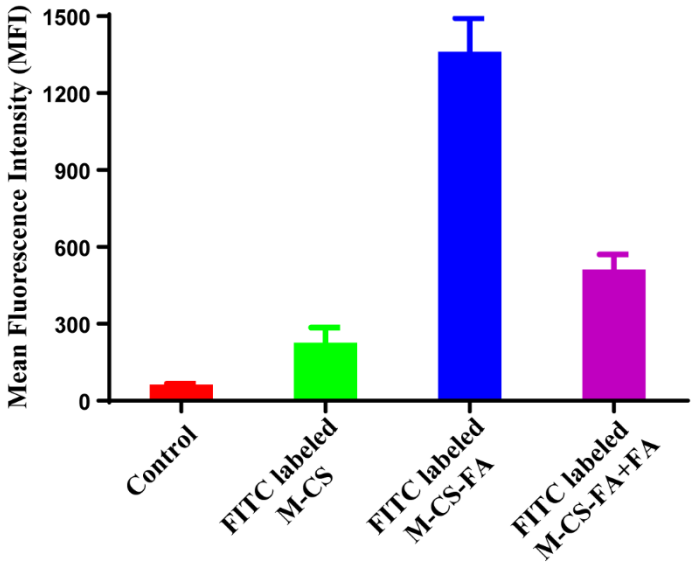

Fig. S4. Intracellular mean fluorescence intensity (MFI) of blank cells (Control), cells with FITC-labeled M-CS, cells with FITC-labeled M-CS-FA kept (M-CS-FA) and cell preincubated with FA before the addition of FITC-labeled M-CS-FA (M-CS-FA +FA) in HeLa cells were analyzed by FACS.

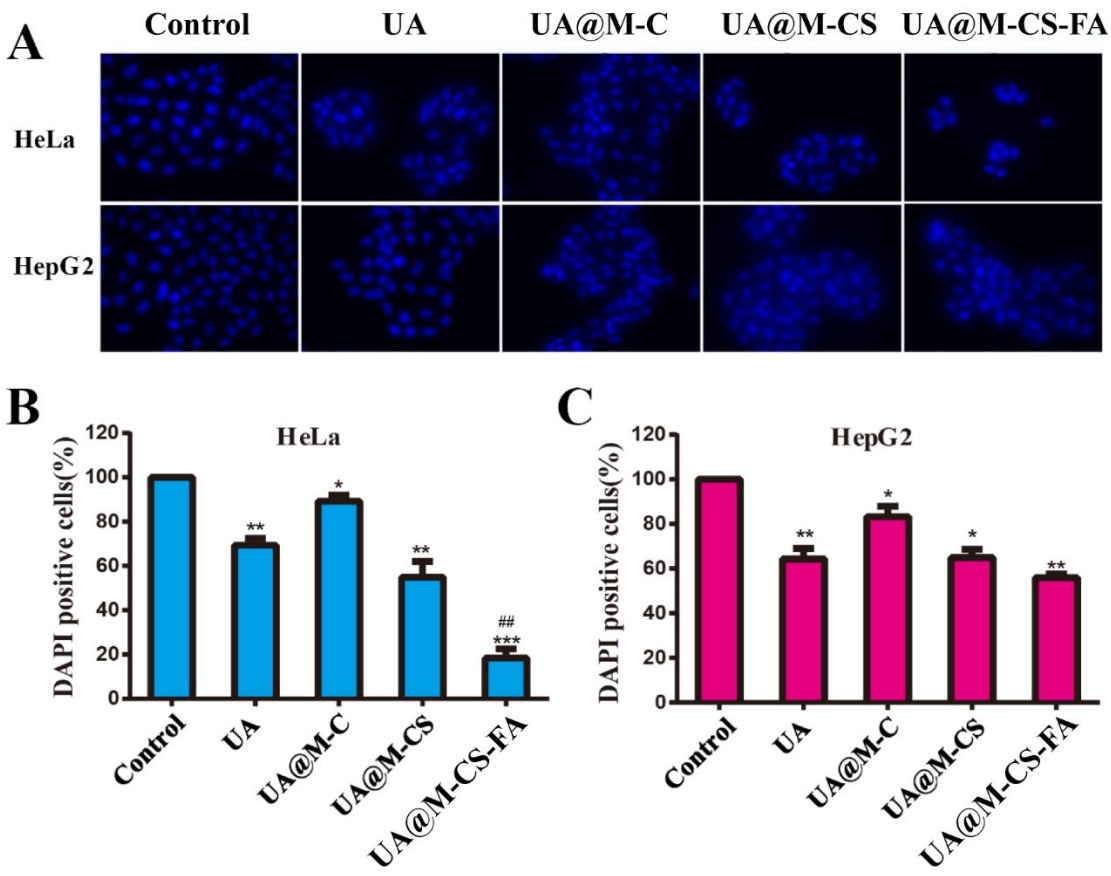

Fig. S5. DAPI staining of UA, UA@M-C, UA@M-CS, UA@M-CS-FA in HeLa and HepG2 cells. *p<0.05, **p<0.01, ***p<0.01 versus control group, ##<0.05 versus UA@M-CS group.