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Remodeling tumor microenvironment to improve drug permeation and antitumor effects by co-delivering quercetin and doxorubicin

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Materials and methods

***Materials*:** DiR and DAPI were offered by Invitrogen Co. (Carlsbad, CA, USA). Quercetin (Que), doxorubicin (Dox) and all other chemicals and reagents otherwise stated were from Sinopharm Chemical reagent Co., LTD and of analytical grade.

Cell culture: HepG2/ADR (human hepatic carcinoma resistant to Dox) and NIH3T3 (mouse embryonic fibroblast) cell lines were purchased from Institute of Biochemistry and Cell Biology (Shanghai, China). All cells were cultured in dulbecco's modified eagle medium (DMEM, Sigma, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Beyotime Biotechnology, China) in a humidified atmosphere of 95% air/5% CO_2 incubator (Thermo Forma 311, Thermo Scientific, USA) at 37 °C. All experiments were performed on cells in the logarithmic phase of growth. [1]

Multi-cellular tumor spheroid model (MCTS): A 96-well plate (Corning, USA) was firstly covered with autoclaved agarose solution (1.5%, w/v) at 50 μL/well and then cooled to room temperature. Mixed HepG2/ADR and NIH3T3 cells (1:1) were seeded at a density of 2×10^3 cells per well and incubated for 4 days to grow into MCTS. The formation of MCTS was monitored using optical microscope (TE2000-S, Nikon, Japan). [2]

Animal model: New Zealand rabbit and female BALB/c nude mice (4-5 weeks, 16 g) were purchased from Shanghai Laboratory Animal Center (SLAC, China) and maintained at 22 ± 2 °C with access to food and water ad libitum. All animal experiments were approved by the Animal Care and Use Committee of Zhengzhou University in accordance with the guidelines for the care and use of laboratory animals. HepG2/ADR tumor co-bearing tumor xenograft model was established according to previous report with minor modifications. [2] Briefly, the suspensions of HepG2/ADR (1×10^7) cells in 100 μL of phosphate buffer saline (PBS, 0.01 M, pH 7.4) were inoculated subcutaneously in the flank of nude mice. Tumor sizes were measured using a Vernier caliper, and tumor volumes were calculated as $V = a^2 \times b/2$ mm^3 (a: minor axis; b: major axis).

Preparation of HA/ZIF/DQ: The nanosized ZIF as drug-loaded core (ZIF/DQ) was synthesized according to previous report. [3] In detail, $Zn(NO_3)_2 \cdot 6H_2O$ (0.2 g), Dox (0.15 g) and Que (0.1 g) were dissolved in 0.8 mL methanol, and 2-methylimidazole (4 g) was dissolved in another 8 mL methanol. A 2-methylimidazole solution was added into the $Zn(NO_3)_2$ solution dropwise and then stirred for 10 min. The solvent was removed by centrifugation. The product was washed more than three times with methanol and dried at 60 °C under vacuum.

For the preparation of HA/ZIF/DQ, HA was adsorbed on the surface of ZIF/DQ through a mechanical mixing method. [4] Briefly, pre-formed ZIF/DQ was dispersed in a HA solution of 10 mg/mL using deionized water as the solvent. The mixture was sonicated for 10 min and stirred at room temperature for 48 h to form a HA/ZIF/DQ suspension. The product was collected by centrifugation, followed by washing more

than three times with deionized water and drying at 37 °C under vacuum.

Characterizations of nanoparticles: The particle size and zeta potential were further determined by Particle/Zeta Analyzer (Litesizer 500, Anton Paar, Austria). Moreover, the morphology and particle size of different nanoparticles were observed by transmission electron microscopy (TEM, JEM-1200, JEOL, Japan) at an accelerating voltage of 80 kV.

The freshly prepared HA/ZIF/DQ was diluted with phosphate buffered saline (PBS, pH 7.4, 1:10, v/v) and mouse plasma Afterwards, the change in particle size was monitored for 7 days to estimate the colloidal stability.

The hemolysis of the nanoparticles at different concentrations (0.1, 0.25, 0.5, 0.75, 1 mg/mL) were determined by incubating nanoparticles with 2% red blood cells suspension of New Zealand rabbit. After 1 h of incubation at 37 °C, the cells were removed using centrifugation (3000 rpm, 10 min) and the UV absorption at 545 nm of the supernatant was determined by UV spectrophotometer (EU-2600D, Onlab, Shanghai, China).

The drug release profile of HA/ZIF/DQ was investigated by dialysis method. Briefly, samples were placed into individual dialysis bag (MWCO: 7 KDa) and immersed in plastic tube containing 25 mL of phosphate buffer with pH of 7.4 and 5.5, respectively. The plastic tubes were fixed in a thermostatic shaker (HZQ-C; Haerbin Dongming Medical Instrument Factory, China) at 37 °C with a stirring speed of 100 rpm. At predetermined time intervals, buffer solution within the tubes was removed for analysis and replaced with equal volume of fresh medium. The Dox concentration was determined by fluorescence spectrophotometer (F-2500, Hitachi Co., Japan) with excitation wavelength, emission wavelength and slit openings set at 505, 605, and 5 nm, respectively. [5]

Cellular uptake: HepG2/ADR cells were seeded onto 35-mm glass-bottom culture dishes (Corning, USA) at a density of 1×10^5 cells per well and cultured overnight for 50-60 % confluence. Afterwards, the media were replaced by fresh serum-free medium containing HA/ZIF/DQ. After 4, 12, and 24 h of incubation, the culture media were removed, and the cells were rinsed with PBS thrice. Subsequently, the cells were fixed with 4 % paraformaldehyde (15 min), followed by staining with DAPI (10 μg/mL) for 15 min. Samples were observed and imaged under confocal laser scanning microscopy (CLSM, BX61W1-FV1000, Olympus, Japan).

In vitro drug release: Cells cultured under the same condition as described above were adopted. After that, the primary culture media were removed and replaced with equal volume of serum-free medium with different pHs (7.4 and 5.5) containing HA/ZIF/DQ. At 12 h post incubation, cells were washed several times by PBS, fixed with paraformaldehyde and stained with DAPI. Samples were subjected to CLSM observation.

In vivo tumor targeting ability: For *in vivo* imaging analysis, near-infrared (NIR)

probe DiR was loaded into HA/ZIF/DQ during the preparation procedure. Subsequently, DiR-loaded HA/ZIF/DQ was intravenously injected into the HepG2/ADR tumor-bearing nude mice (with or without intravenously HA pretreatment for 1 h) at a dose of 50 μg DiR/kg to investigate their biodistribution and tumor-targeting efficacy (n = 3). At 6 and 12 h post-injection, the NIR fluorescent images were captured at an excitation wavelength of 740 nm and an emission wavelength from 740-950 nm using an *in vivo* imaging system (Maestro *In-vivo* Imaging System, USA). After 24 h of living imaging, mice were sacrificed, the main organs and tumor tissues were excised for *ex vivo* imaging using the same imaging system.

In vitro cytotoxicity: For the cell viability measurements, 200 μL of HepG2/ADR cells (1×10^4 cells/mL) in DMEM suspension was seeded in 96-well plates (Corning, USA) and allowed to culture overnight. The mediums were discarded and all wells were washed twice with fresh PBS. Subsequently, 200 μL of serum-free medium was added to each well, in which different nanoparticles (HA/ZIF/D, HA/ZIF/Q and HA/ZIF/DQ) were diluted to achieve the designated concentrations. After proper incubation, the cell viability was assessed with standard MTT assay. [6] Briefly, the medium was replaced with an equal volume of fresh medium containing 5 mg/mL MTT and incubated for 4 h at 37 °C. Then MTT was removed, and cells were lysed with dimethyl sulfoxide (DMSO) with stirring for 15 min on a microtiter plate shaker. The cell viability was estimated according to the absorption values determined by a microplate reader (Bio-Rad, model 680, USA) at the wave length of 570 nm.

MCTS with diameters of 200-300 μm were divided into four groups and treated with fresh medium containing different nanoparticles. Untreated MCTS was employed as control. The spheroids were allowed to continue to incubate at 37 °C for 6 days. The diameter of the spheroids was recorded every day using an optical microscope.

In vivo tumor growth inhibition: HepG2/ADR tumor-bearing mice were divided into four groups and treated with saline (the control group), HA/ZIF/D, HA/ZIF/Q and HA/ZIF/DQ at the Dox/Que dosage of 5 mg/kg. Each sample was intravenously injected via tail vein every 2 days. The measurement of tumor size and mice body weight was repeated once every 2 days before injection over a 14-day therapeutic period and the tumor volume was calculated by the formula: $(W^2 \times L)/2$, where W and L is the shortest and longest diameter, respectively. Afterwards, the main organs (heart, liver, spleen, lung and kidney) and tumor were collected, weighed, washed with saline thrice and fixed in 10% formalin. Formalin-fixed main organs and tumors were embedded in paraffin blocks to prepare sections and then subjected to HE, Ki67, TUNEL, collagen (COL) or α-SMA staining, respectively. Tissue images were captured at 200× magnification with the corresponding microscope.

Drug penetration study: The MCTS was incubated with HA/ZIF/D and HA/ZIF/DQ at the DOX concentration of 1 μg/mL for 24 h, respectively. Then, the tumor spheroids were washed thrice with ice-cold PBS, fixed with paraformaldehyde for 30 min, and placed in cavity microscope slides. The images of the tumor spheroids were acquired by tomoscan using Z-stack imaging with 5 μm intervals from the top of

the spheroid to the middle by CLSM.

When the tumor volume reached 200 mm^3, the HeLa tumor-bearing nude mice were intravenously administrated by HA/ZIF/D and HA/ZIF/DQ at a dose of 5 mg DOX/kg, respectively. At 48 h post-injection, tumors were collected and washed by PBS, followed by cryotomy. The frozen tumor sections were stained by DAPI (Solarbio Life Science, China) according to the manufacturer's instructions, followed by observation under CLSM. [7]

Statistical analysis: Quantitative data were presented as mean ± standard deviation (S.D.) from triplicate experiments performed in a parallel manner with three repeats unless otherwise noted. Statistical significance was tested by two-tailed Student's t-test or one-way ANOVA $*P < 0.05$ or $**P < 0.01$was considered statistically significant.

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