Supplementary information

Nanobubbles loaded with carbon quantum dots for ultrasonic fluorescence dual detection

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Materials and methods

Materials

Carbon quantum dots were obtained from Janus New-Materials Co., Ltd. (Nanjing, China). Perfluoropentane was obtained from Aladdin Chemistry Co. DOTAP (1,2-dioleoyl l-3-trim ethylammonium-propane (chloride salt)) was obtained from AVT Pharmaceutical Technology Co., Ltd. (Shanghai, China). Cholesterol was obtained from Sigma Aldrich (Shanghai) Trading Co., Ltd. (San Francisco, USA). Phosphate-buffered saline (PBS) was purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). Penicillin–streptomycin solution was acquired from HyClone Laboratories (Logan, UT, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute-1640 (RPMI-1640) were purchased from Gibco (Thermo Fisher Scientific, USA). The experimental water was made using a Milli-Q ultrapure water system (Millipore, Ireland). All other chemicals and reagents were of analytical grade or higher.

Cells and animals

4T1 cells and Hepa 1-6 cells were grown in RPMI-1640 or DMEM culture media supplemented with 10% fetal bovine serum and 100 IU/mL penicillin and 100 μg/mL streptomycin. Both cell lines were maintained in a 5% CO_2 incubator at 37 ℃.

Female BALB/c mice ((20±3) g, 6 weeks old) and C57BL/6 mice ((20±3) g, 6 weeks old) were supplied by the Experimental Animal Center of Shandong University. All the animals were kept and used for the experiment in accordance with the ethics and regulation of animal experiments of Pharmaceutical Sciences, Shandong University, China.

Preparing and evaluating the Lip and CQDs-Lip

DOTAP and cholesterol were dissolved in chloroform in a certain proportion and dosage for rotary evaporation, and a film formed after complete volatilization. PBS solution was added, and liposomes were evenly formed by magnetic stirring for 1 h in a water bath at 40 ℃. Then, 5 μL

perfluoropentane was added and ultrasonicated in an ultrasonic cleaning instrument or ultrasonic crushing instrument at different powers for different times to obtain liposome nanobubbles. The particle size of the nanobubbles was measured in a particle size potential analyzer.

Based on the above results, the optimum process was selected to prepare the nanobubbles. By adding different doses of CQDs (10 mg/mL) and stirring at room temperature for 30 min in the dark, liposome nanobubbles loaded with CQDs were obtained. The nanobubble solution was dialyzed for 12 h at room temperature in the dark. The zeta potential of the CQDs-Lip was detected in a particle size potential analyzer.

Screening the ratio of liposomes to cholesterol

DOTAP and cholesterol were dissolved in different proportions in chloroform, and liposomes were prepared according to the above method. Then, 5 μL perfluoropentane was added and ultrasonicated at 20% power for 1 s in an ultrasonic crushing instrument to obtain liposome nanobubbles. The particle size was detected in a particle size potential analyzer.

Screening the perfluoropentane dosage

Liposomes were prepared with a controlled ratio of DOTAP to cholesterol. Different dosages of perfluoropentane were added to the ultrasonic crushing instrument at 20% power for 1 s to obtain liposome nanobubbles. The particle size was detected in a particle size potential analyzer.

Screening the liposome-forming solvent

The ratio of DOTAP to cholesterol was kept unchanged, and PBS solution and the prepared glycerol:PBS= 1:9 (v/v) solution were used as the forming solvent to prepare nanobubbles. The particle size was detected in a Zetasizer Nano ZS particle size potential analyzer.

Testing the influence of temperature on the nanobubbles

According to the above results, the optimum process was selected to prepare the nanobubbles. The particle size of the same nanobubble was detected at different temperatures to explore the influence of the detection temperature on the particle size of the DOTAP liposome nanobubbles.

Establishment of the breast cancer model

Breast cancer was implanted in the mice after the mice were acclimated to the environment. 4T1 cells in the logarithmic growth phase were digested, counted, centrifuged and resuspended in serum-free RPMI 1640 medium. The concentration of 4T1 cells was controlled at 10 million/mL. Each mouse was implanted with 1 million 4T1 cells (i.e., 100 μL of the cell suspension) on the fat pad of the second pair of the right breasts of the mouse, and the growth of tumors was observed every other day.

Establishment of the liver cancer model

Hepa 1-6 hepatoma cells in the exponential growth stage were prepared as a cell suspension

with PBS, and the cell concentration was adjusted to 10 million cells/mL C57BL/6 mice weighing approximately 20 g were injected with 0.2 ml of cell suspension into the caudal vein to construct a liver cancer metastasis model.

In vivo ultrasound imaging

The tumor-bearing mice were anesthetized by intraperitoneal injection of 4% chloral hydrate. The tumors in the mice were imaged by an ultrasonic detector. One hundred microliters of nanobubble solution was injected into the tail vein, and the time was recorded. After 5 min, ultrasound was used to detect the tumor and the imaging results were recorded. The experiment was repeated 3 times.

Assay of biodistribution in mice

To investigate the drug biodistribution and tumor targeting efficacy in vivo, Hepa 1-6 tumor-bearing mice were randomly grouped (n=3) and anesthetized with 4% chloral hydrate. The mice were intravenously injected with 0.2 ml Lip-CQDs. After 5 min, real-time images were acquired via the Xenogen IVIS Lumina system.

Table S1 Size, PDI, and potential of nanobubbles (*n*=3, mean±SD)

Sample	Size (nm)	PDI	Zeta potential (mV)
1	222.8	0.272	45.8
2	223.7	0.173	45.1
3	227.3	0.289	47.6
Mean±SD	224.6±2.4	0.245±0.063	46.2±1.3

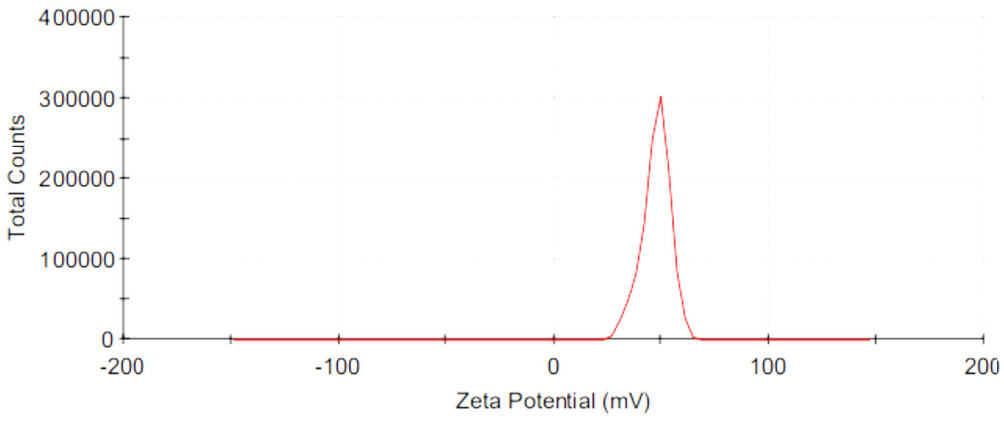

Fig. S1 Zeta potential distribution of nanobubbles.