Immunological gadolinium doped mesoporous carbon nanoparticles for tumor-targeted MRI and photothermal-immune co-therapy

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1. MATERIALS AND METHODS

1.1 Purification of Extracellular vesicle

Extracellular vesicle were purified using differential ultracentrifugation method [33, 34]. Firstly, FBS used for cell incubation was centrifuged at 100,000 g to wipe out the existing exosomes. 4T1 cells were incubated in exosome-free DME/F-12 medium for 48 h. Then, the cell culture mediums were centrifuged at 1000 g for 10 min, 10,000 g for 30 min and 100,000 g for 1 h to get the pellet. After washed with PBS, the purified extracellular vesicle was obtained by another centrifugation at 100,000 g for 1 h.

1.2 Cell Culture and Animals

NIH-3T3 mouse embryonic fibroblast cells and 4T1 mouse breast cancer cells were obtained from Chinese Academy of Sciences Cell Bank (Shanghai, China). The above cell lines were cultured in DMEM and DMEM/F-12 medium containing 10 % FBS and 100 U/mL penicillin – streptomycin, respectively. The cells culture condition was incubating in 5 % CO_2 atmosphere at 37 ℃.

18-20 g of female BALB/c mice were purchased from Xuzhou Medical University. All involving experimental animals were approved by the Animal Care Committee of Xuzhou Medical University and carried out in compliance with the Guidelines.

1.3 Evaluation of the Tumor Inhibition In Vivo

The established 4T1 tumor-bearing mice were randomly allocated into 6 groups and were received with PBS, NIR, EV@Gd-MCNs-R837, Gd-MCNs + NIR, Gd-MCNs-R837 + NIR and EV@Gd-MCNs-R837 + NIR treatments (n = 3), respectively. For the NIR, Gd-MCNs + NIR, Gd-MCNs-R837 + NIR and EV@Gd-MCNs-R837 + NIR groups, the tumors of mice were irradiated under an 808 nm laser (1 W/cm^2) for 5 min at 4 h post-injection. The tumor volumes were measured every other day (tumor volume (mm^3) = length × $width^2$/2). At the final stage of the experiment, mice were sacrificed, and the tumors were extracted.

1.4 Long-Term Immune Memory Effect

The established 4T1 tumor-bearing mice were randomly allocated into 6 groups and were received with PBS, NIR, EV@Gd-MCNs-R837, Gd-MCNs+NIR, Gd-MCNs-

R837+NIR and EV@Gd-MCNs-R837+NIR treatments on day −35 (n = 3). Four hours later, mice in the NIR, Gd-MCNs + NIR, Gd-MCNs-R837 + NIR and EV@Gd-MCNs-R837 + NIR groups were irradiated using an 808 nm laser (1 W/cm^2) for 5 min. Surgical excision was performed to remove the remaining tumors one week after the treatment. Four weeks later, mice were reinoculated with 4T1 cancer cells and the volumes of distant tumors were monitored every other day.

For cytokine production analysis, the blood samples were obtained from mice receiving different treatments. Blood samples were harvested on day 3 after different treatments. The serum secretion of TNF-α and IL-6 was detected using an ELISA kit (Solarbio) based on the manufacturer's protocol. The spleen was dissected 7 days after treatment. Tumors were also harvested on days 1 and 7 after different treatments. The spleen and tumors were then fixed, embedded in paraffin, sectioned, and immunostained with anti-mouse CD11c-Alexa Fluor 647 on day 1 after treatment, or anti-mouse CD8-Alexa Fluor 647 antibody and anti-mouse CD3-APC/Cy7 on day 7 after treatment.

During the treatment, if the tumor volume of the mice was more than 2000 mm^3 or serious complications occurred, the mice would be euthanized according to the requirements of the Animal Ethics Committee.

1.5 Biosafety study

Firstly, MTT assay was introduced to evaluate the cytotoxicity of EV@Gd-MCNs-R837. In brief, 5×10^3 4T1 cells or NIH-3T3 cells were cultured into a 96-well plate for 24 h, and then exposed to different concentrations of EV@Gd-MCNs-R837 (0, 20, 40, 60, 80, 100, 150, 200 μg/mL) for another 24 h. Then, the cells were processed by MTT assay to evaluate the cytotoxicity of EV@Gd-MCNs-R837.

To evaluate the biosafety of EV@Gd-MCNs-R837*in vivo*, healthy Balb/c mice were intravenously injected with EV@Gd-MCNs-R837 (10 mg/kg). The blood sample of each mouse was collected at determined time points post-injection (1d, 7d and 21d). Blood samples from mice with saline injection was harvested as control. Then, the index of WBC, RBC, PLT, HGB, MCV, MCH, MCHC, HCT, ALB, AST, ALT, TP, Urea and creatinine in the collected blood samples were detection by blood

biochemistry examination and blood route test. Furthermore, major organs were also collected from the sacrificed mice for H&E staining. The images were observed by an optical microscope.

1.6 Statistical analysis

All results were presented as error bars which represent ± standard deviation (SD). The t-tests were used to evaluate the statistical significance of the differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

2. SUPPLEMENTARY FIGURES

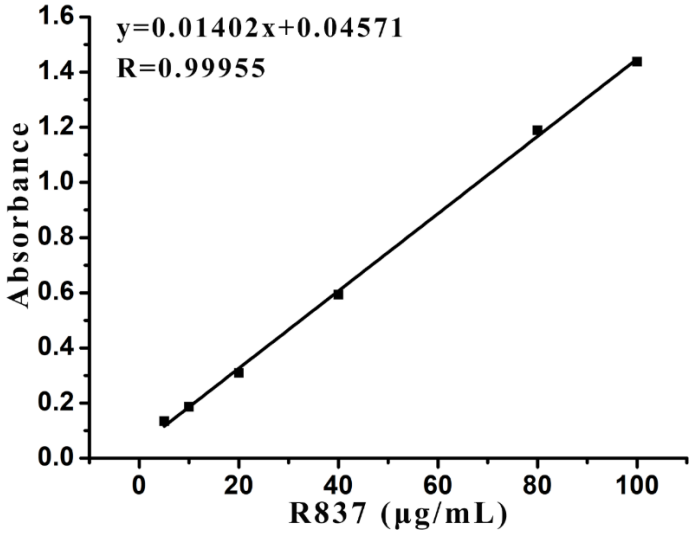

Figure S1. Linear equation of R837 with different concentrations in UV-vis absorbance.

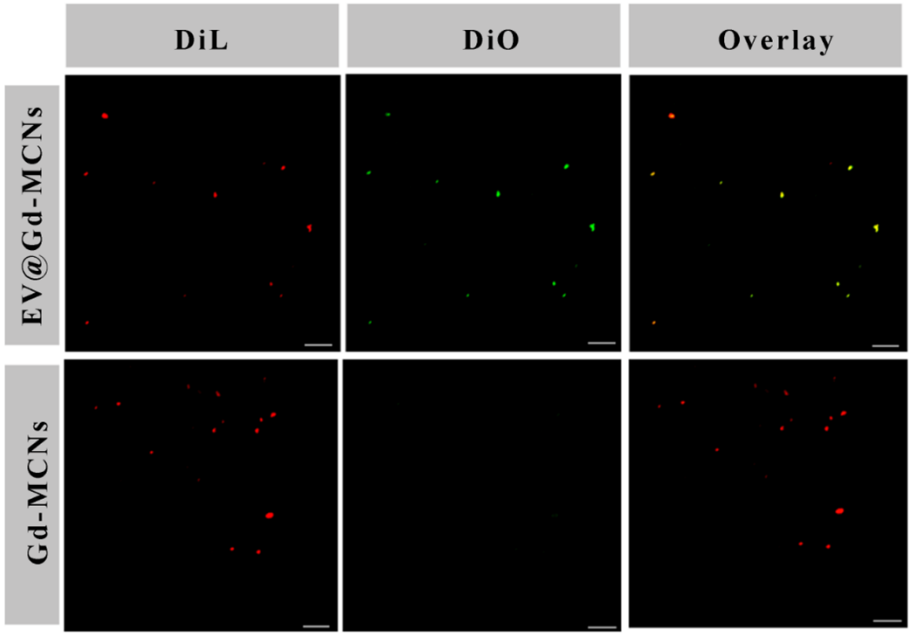

Figure S2. The Colocalization of DiL (red) and DiO (green) in Gd-MCNs and EV@Gd-MCNs by confocal microscopy.

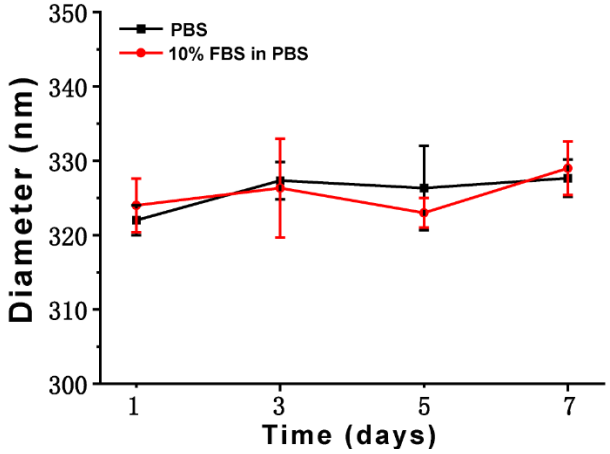

Figure S3. The changes of hydrodynamic diameter of EV@Gd-MCNs-R837 in PBS or PBS with 10 % FBS for 1, 3, 5 and 7 days.

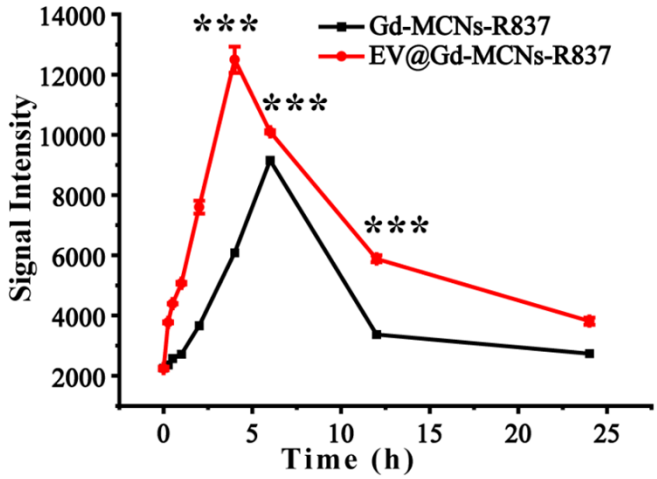

Figure S4. The corresponding MRI signal intensity of tumor for Figure 4f.

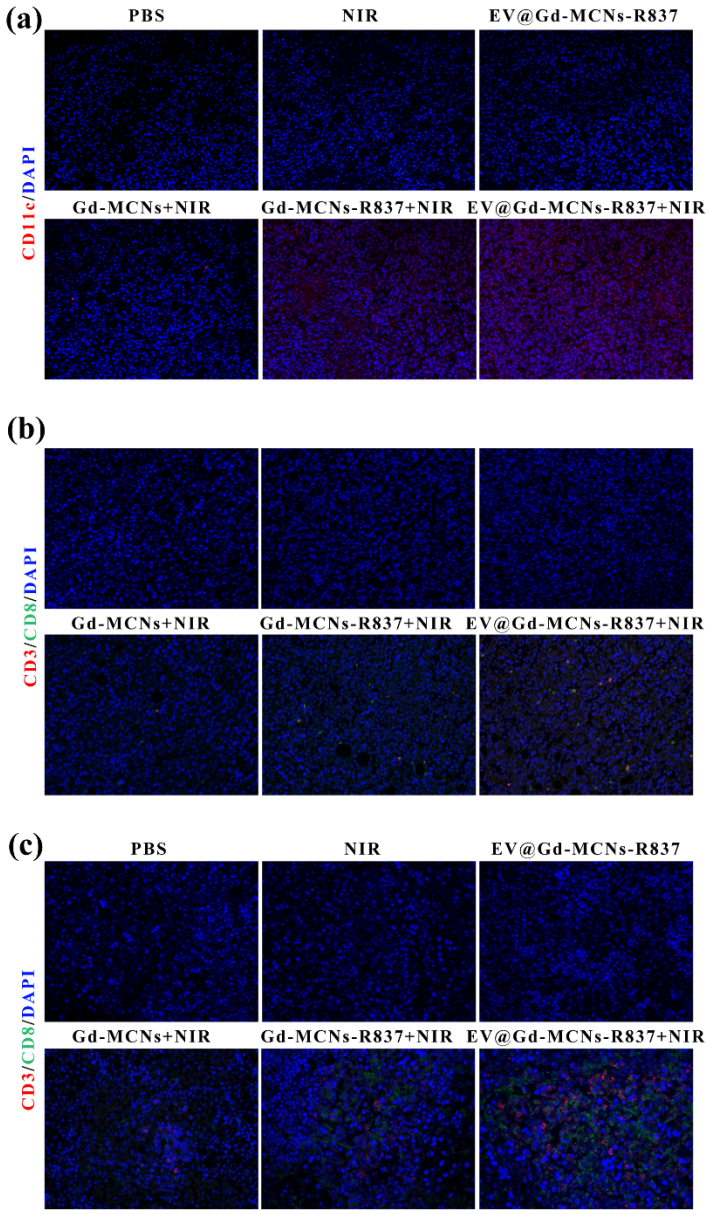

Figure S5. Immunofluorescence staining of different T cell markers (CD11c+, CD3+, CD8+) in **(a)** **(b)** tumor tissue sections and **(c)** spleen tissue sections e after different treatments.

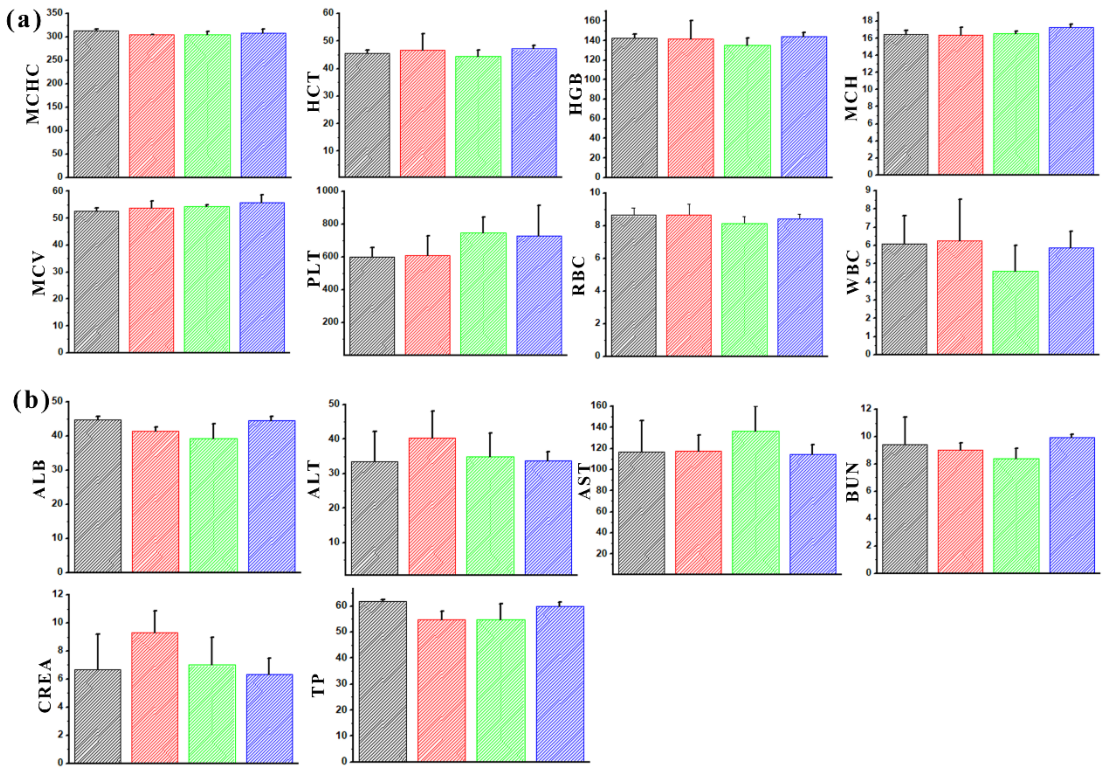

Figure S6. (a) Blood routine and **(b)** blood biochemical analyses of mouse serum during 21 day with EV@Gd-MCNs-R837 injection.

Table S1 The loading and encapsulation efficiency of R837 with different amount

R837 (mg/mL)	Loading efficiency (%)	Encapsulation efficiency (%)
0.25	15 ± 0.01 %	75 ± 0.14 %
0.50	29 ± 0.01 %	87 ± 0.27 %
0.75	37 ± 0.45 %	86 ± 1.15 %
1.00	42 ± 0.91 %	84 ± 1.77 %