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Electronic Supplementary Information (ESI) for:

Folic acid conjugated ferritins as photosensitizer carriers for photodynamic therapy

Zipeng Zhen[a,b], Wei Tang[a,b], Weizhong Zhang[a,b], Jin Xie[a,b,*]

[a] Department of Chemistry, University of Georgia, Athens, Georgia 30602, United States
[b] Bio-Imaging Research Center, University of Georgia, Athens, Georgia 30602, United States
[*] E-mail: jinxie@uga.edu

Materials and methods:

Cell culture

4T1 (murine breast cancer) cell line was from ATCC, and grown in RMPI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 1% streptomycin (MediaTech, USA). For cell culture, 4T1 cells were incubated at 37 °C in 5% CO_2.

Ferritin purification, folic acid coupling and $ZnF_{16}Pc$ loading

The protocols for producing ferritins and loading $ZnF_{16}Pc$ onto them are similar to what were published by us previously.[14] The photosensitizer loading was achieved by adding $ZnF_{16}Pc$ in DMSO into a FRT/FA-FRT solution in 0.01 M PBS (pH 7.4). After that, the mixture was incubated at room temperature for 45 min. The raw products were subjected to purification by going through a NAP-5 column to remove the unloaded $ZnF_{16}Pc$. The $ZnF_{16}Pc$ content was determined spectroscopically by comparing with a standard absorbance curve of $ZnF_{16}Pc$. The FRT/FA-FRT concentration was determined by Bradford protein assay. The loading rate was computed and expressed in weight percent (wt%). Coupling folic acid to ferritins was conducted following a published protein conjugation protocol with minor changes.[25] Typically, 5 mg of folic acid, 50 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), and 50 mg of N-hydroxysuccinimide (NHS) were dissolved in 50 mL of a bicarbonate buffer (50 mM $NaHCO_3$, pH 6) at room temperature (25 °C). A 10 mL aqueous solution (0.5 mg/mL) was subsequently added, and the mixture gently agitated for 2 h at room temperature. The raw product was purified by dialysis (MWCO = 100k) against PBS (pH 7.4) at 4 °C. The PBS was replenished every 12 hours, for at least 4 times. The purified FA-FRTs were concentrated using a centrifugal filter unit (Millipore, 100k). Bradford protein assay was used to determine the concentration of FA-FRTs.

For IRDye800 labelling, ferritins or FA-FRTs were incubated with IRDye800-NHS for 30 min at room temperature and then purified through a NAP-5 column. A starting molar ratio of 2:1 between the dye and the protein nanocage was used. The coupling efficiency was assessed spectroscopically by comparing with a predetermined IRDye800

absorbance standard curve (780 nm). It was determined that the final conjugates have on average one IRDye800 per ferritin particle.

Animal models

All the animal studies were performed following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia. For bio-safety evaluation, a total of 18 normal BALB/c mice were used. The animals were randomly divided into 3 groups, with 6 mice per group. Ferritins were administered either intraperitoneally (i.p.) at 50 mg/kg or intravenously (i.v.) at 15 mg/kg to two groups of the animals. A third group was monitored for controls. The animals were observed daily and their body weights were monitored for two weeks. 4T1 tumour models were established by subcutaneous injection of $\sim10^6$ 4T1 cancer cells to the hind limbs of 4-5 week BALB/c mic. The imaging or therapy studies were conducted 1 week after the inoculation when the tumours reached a size of ~100 mm^3.

***In vivo* tumour targeting**

For tumour targeting, IRDye800-labeled P@FA-FRTs (0.75 mg $ZnF_{16}Pc$/kg) were i.v. injected to 4T1 tumour bearing animals (average tumour size $\sim77.78 \pm 11.84$ mm^3, n = 5). Whole-body fluorescence images were acquired on a Maestro II imaging system (PerkinElmer) using an NIR emission filter (750 – 940 nm) up to 24 h post injection. The fluorescence images were unmixed by the vendor provided software. ROIs were circled around tumor areas, and the average optical intensities (in total scaled counts/cm^2/s) were recorded and compared. After the 24 h imaging, the animals were euthanized. The tumours as well as major organs were harvested for histology studies.

***In vivo* therapy studies**

The therapy studies were also performed in 4T1 tumour models. For the treatment group, the animals were i.v. injected with P@FA-FRTs (1.5 mg $ZnF_{16}Pc$/kg). The tumours were irradiated at 24 h by a 671 nm laser (300 mW/cm^2, over a ~1 cm diameter beam) for 15 min. The two control groups received (1) P@FA-FRTs without irradiation, and (2) PBS only. Tumour sizes were measured every other day by a calliper and computed following the formula: size (mm^3) = length (mm) × width $(mm)^2/2$.

Immunofluorescence staining

The cryogenic slides with 8 μm thickness were fixed with cold acetone for 30 min and washed by running water for 5 min. Subsequently, phycoerythrin-labeled anti-CD31 antibody (ab25644) was incubated with the slides at 4 °C overnight. After gently rinsing with PBS, the slides were mounted. The images were acquired on an Olympus IX71 microscope.

Hematoxylin and eosin staining

H&E staining was performed according to a protocol provided by the vendor (BBC Biochemical). Briefly, 6 μm paraffin-embedded slides were prepared. After treated with 100% xylene for 3 times (3 min each time), the slides were hydrated with a gradient concentrations of alcohol (100, 95, and 70%), each for 3 min. The hematoxylin staining was then performed for 3 min, and the slides were washed with running water for 3 min. The eosin staining was performed for 1 min. The slides were washed, dehydrated, treated with xylene, and then mounted with Canada balsam. The images were acquired on a Nikon Eclipse 90i microscope.

Statistical methods

Quantitative data were expressed as mean ± s.e.m. A two-tailed Student's t-test was used for statistically comparing the treatment group and the control group. $P < 0.05$ was considered statistically significant.

Supplementary data:

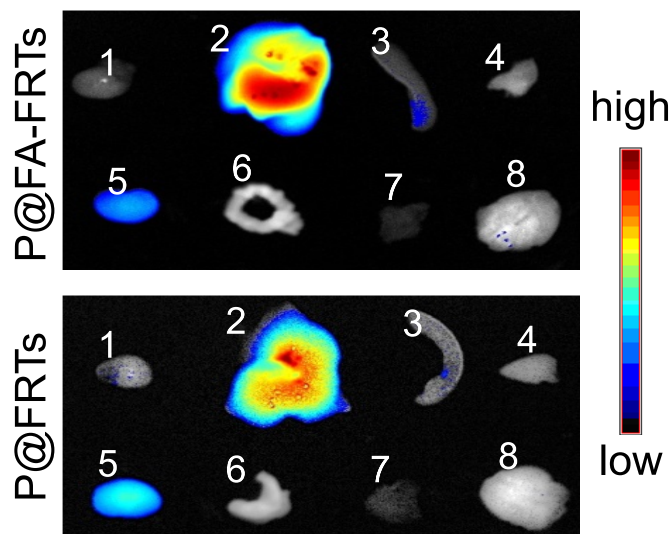

Figure S1. *Ex vivo* imaging results of 4T1 tumour-bearing BALB/c mice administrated with IRDye800-labeled P@FA-FRTs and FRTs. The organs were arranged in the following order: 1, Heart; 2, Liver; 3, Spleen; 4, Skin; 5, Kidney; 6, Intestine; 7, Muscle; 8, Brain.

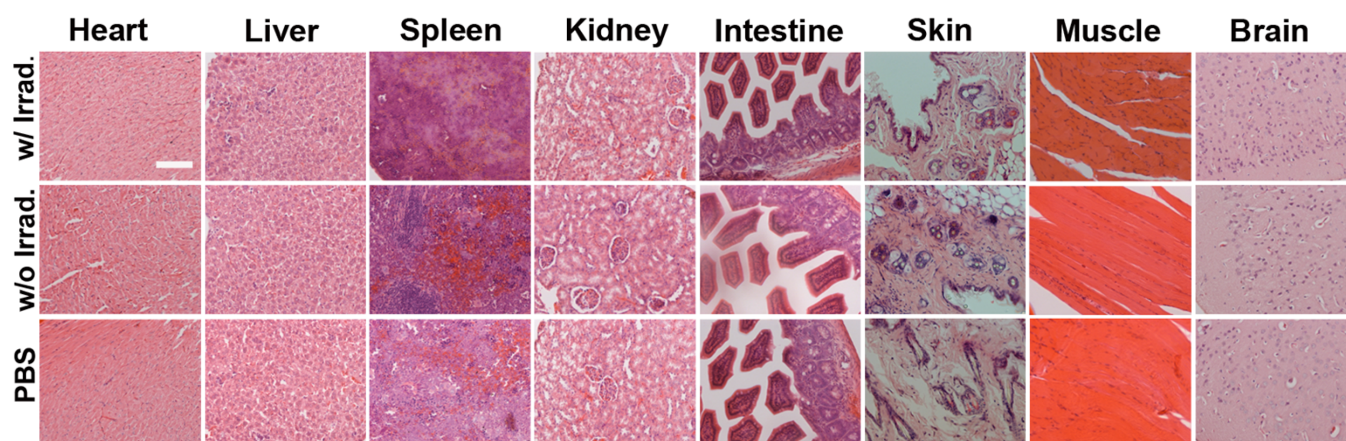

Figure S2. H&E staining results from other main organs. No obvious pathological abnormalities were found in these organs from all groups. Scale bar, 100 μm.