Probing and Enhancing Ligand-Mediated Active Targeting of Tumors Using Sub-5 nm Ultrafine Iron Oxide Nanoparticles

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**SUPPORTING INFORMATION**

**Materials and chemicals.** All the chemicals were used without further purification. Ferric nitrite \((\text{FeNO}_3 \cdot 9\text{H}_2\text{O}, 98\%)\), sodium oleate \((\text{NaOA}, 97\%)\), hexane, ethanol, 1-octadecene \((90\%)\), chloroform, dimethylformamide \((\text{DMF}, 99\%)\), D-\((+)-\)glucose, dimethyl sulfoxide \((\text{DMSO}, 90\%)\), ammonium hydroxide \((\text{NH}_4\text{OH}, \text{ACS grade})\), sodium bicarbonate \((\text{NaHCO}_3)\) buffer \((0.1 \text{ M}, \text{pH}=8.5)\), fluorescein isothiocyanate \((\text{FITC})\), tetramethylrhodamine \((\text{TRITC})\), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide \((\text{EDC})\), n-hydroxysuccinimide \((\text{Sulfo-NHS})\), 4',6-diamidino-2-phenylindole \((\text{DAPI})\), paraformalin, potassium ferrocyanide \((\text{II})\) trihydrate \((\text{K}_4\text{Fe(CN)}_6)\), nuclear fast red solution, hematoxylin and eosin Y solution, and optimal cutting temperature compound \((\text{OCT})\) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oleic-acid coated iron oxide nanoparticles \((\text{IONPs})\) with an averaged core size of 30 nm, activation buffer \((\text{pH} = 5.5)\) and coupling buffer \((\text{pH} = 8.5)\) were purchased from Ocean Nanotech LLC (San Diego, CA, USA). Phosphate-buffered saline \((\text{PBS})\), fetal bovine serum \((\text{FBS})\), transferrin \((\text{Tf})\), RPMI-1640 medium, Dulbecco's Modified Eagle Medium \((\text{DMEM})\), trypsin-EDTA, penicillin-streptomycin solution, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide \((\text{MTT})\) kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amicon Ultra-4 centrifugal filters \((50 \text{ kDa})\) were purchased from Millipore (Burlington, MA, USA). Balb/c mice were ordered from Harlan Laboratories (Indianapolis, IN, USA).
**Figure S1.** Cell viability studies of 4T1 breast cancer cells treated with (a) FITC-Tf-uIONPs and (b) TRITC-uIONPs;
Figure S2. Confocal fluorescence images of a selected 4T1 tumor section co-stained with FITC labeled Tf. a: DAPI for nuclei, b: Tf; c: merged image with DAPI and Tf, (d) histological H&E staining from a tumor section adjacent to the section used for fluorescence imaging. The scale bar for all images is 100 µm.
Figure S3. Confocal fluorescence images of a selected tumor section co-stained with FITC-Tf-uIONPs and TRITC-uIONPs. a: DAPI for nuclei, b: FITC-Tf-uIONPs (green), c: TRITC-uIONPs (red), and d: merged image (DAPI + FITC-Tf-uIONPs + TRITC-uIONPs); (e) histological H&E staining from a tumor section adjacent to the section used for fluorescence imaging. The dashed red lines outline the tumor regions with high cellularity (mainly colocalized with active targeting FITC-Tf-uIONPs) from the tumor stromal regions (mainly colocalized with non-targeting TRITC-uIONPs); (f) Prussian blue staining for iron on the same tumor section used for fluorescence imaging. The scale bar for all images is 200 µm.
Figure S4. Merged confocal fluorescence images (DAPI + FITC-Tf-uIONPs + TRITC-uIONPs) of selected tumor slices collected from mice receiving co-injection of active-targeting FITC-Tf-uIONPs and non-targeting TRITC-uIONPs with the core size of 3 nm. Images were taken at different time points after co-injection of FITC-Tf-uIONPs and TRITC-uIONPs (a-e: 1 hour; g-k: 3 hours; m-q: 24 hours). The pixels of different fluorescent signals, i.e., FITC-Tf-uIONPs (green) and TRITC-uIONPs (red), were segmented from each image and counted using an in-house program. Pixel ratios, defined as the pixel intensity of FITC-Tf-uIONPs over pixel intensity of TRITC-uIONPs, are plotted to demonstrate the time-dependent change of the ratios between active-targeting FITC-Tf-uIONPs and non-targeting TRITC-uIONPs (f: 1 hour, l: 3 hours, and r: 24 hours). The scale bar for all images is 200 µm.
Figure S5. Characterizations of TRITC-IONPs and FITC-Tf-IONPs with the core size of 30 nm. TEM images of (a) TRITC-IONPs, and (b) FITC-Tf-IONPs; The size distribution (c) and zeta potential (d) of IONPs measured by DLS; Fluorescent emission spectra (e) and (f) of free dye, IONPs, and FITC-Tf or TRITC conjugated IONPs.
Figure S6. Merged fluorescent images of tumor sections collected from mice receiving co-injection of ligand conjugated active targeting FITC-Tf-IONPs and passive or non-targeting TRITC-IONPs with a 30 nm core size (a-e: 1 hour; g-k: 3 hours; m-q: 24 hours) and corresponding segmentation analysis (f: 1 hour, l: 3 hours, and r: 24 hours). The pixel ratio is defined as the pixel intensity of FITC-Tf-IONPs over pixel intensity of TRITC-IONPs. The scale bar for all images is 200 µm.
Figure S7. Images of a selected 4T1 tumor tissue slice stained for CD68 macrophage (a) and corresponding H&E staining (b) (scale bar: 200 µm); Fluorescent images of mouse macrophage RAW264.7 cells treated with FITC labeled IONPs of different sizes (c-f) (scale bar: 20 µm), table summarizing pixel reading of different IONPs from fluorescent images to show the level of macrophage uptake (g).