Electronic Supplementary Information

Fabrication of folate bioconjugated near-infrared fluorescent silver nanoclusters for targeted in vitro and in vivo bioimaging

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EXPERIMENTAL SECTION

Materials and Chemicals. All the chemicals used are at least of analytical grade. Ultrapure water (Hangzhou Wahaha Group Co. Ltd., Hangzhou, China) was used throughout. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS), lipoic acid (LA), polyethyleneimine (PEI, MW, 1800) and sodium borohydride ($NaBH_4$) were obtained from Aladdin (Shanghai, China). Hoechst 33342 and Lysotracker Green DND-26 were obtained from Invitrogen. Folate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Silver nitrate ($AgNO_3$), $Na_3PO_4 \cdot 12H_2O$ and chloral hydrate were obtained from Guangfu Fine Chemical Research Institute (Tianjin, China). 10 mM phosphate buffer solution (PBS) (pH 7.4) was purchased from Shanghai Hongbei Reagent Co. (Shanghai, China). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2- H-tetrazolium bromide (MTT) and Diamidino-2-phenylindole (DAPI) were purchased from KenGen Biotech Co. Ltd. (Nanjing, China).

Instrumentation and Characterization. The transmission electron microscopy (TEM) image of AgNCs was obtained on a JEM-100CXII transmission electron microscope (Jeol, Tokyo, Japan) with an accelerating voltage of 100 kV. The hydrodynamic size of AgNCs were measured on Zetasizer Nano ZS90 (Malvern Instruments, Manchester, UK). X-ray photoelectron spectroscopy (XPS) measurements were carried out on a Axis Ultra DLD spectrometer fitted with a monochromated Al Kα X-ray source (hν = 1486.6 eV), hybrid (magnetic/electrostatic) optics, and a multichannel plate and delay line detector (Kratos Analytical, Manchester, UK). Fourier transform infrared (FTIR) spectra (4000−400 cm^{-1}) in KBr were recorded on a Magna-560 spectrometer (Nicolet, Madison, WI, USA). The

concentration of AgNCs was determined on an X series inductively coupled plasma mass spectrometer (ICPMS) (Thermo Elemental, UK).

Absorption spectra were recorded on a UV-3600 UV–vis–NIR spectrophotometer (Shimadzu, Tokyo, Japan). Fluorescence decay curves were measured on a FELIX32 system (Photon Technology International, NJ, USA). The fluorescence quantum yield of SH-PEI-AgNCs was determined on an FLS920 spectrometer with an integration sphere attachment under excitation of 380 nm (Edinburgh, UK). The photoluminescence measurements were performed on a Hitachi F-4500 spectrofluorometer (Tokyo, Japan). In vitro cytotoxicity of the probe was assessed using the MTT assay. The cells imaging were observed using PerkinElmer UltraView VoX laser scanning confocal microscope (Waltham, MA). The fluorescence images of the mice were obtained with a Bethold NightOWL LB 983 in vivo Imaging System (Bad Wildbad, Germany). The excitation filter was set as 530 nm, the emission filter was set as 700 nm. Fluorescence images were recorded by the CCD camera with constant exposure time.

Synthesis of SH-PEI. Typically, 38.4 mg of EDC (0.4 mmol) and 43.4 mg of Sulfo-NHS (0.4 mmol) were dissolved in 10 mL of DMSO, mixed with 41.3 mg of LA (0.2 mmol), and incubated at room temperature for 30 min. Then, 90 mg of PEI (0.05 mmol) was added to the resulting solution, and incubated for 12 h. The final conjugates were purified by dialyzing (MW cutoff = 600 Da) and lyophilized. The conjugates are hereafter referred to as SH-PEI.

Synthesis of NIR Fluorescent AgNCs. All glassware was washed with aqua regia ($HCl:HNO_3$ volume ratio = 3:1), and rinsed with ethanol and ultrapure water sequentially *(Caution: Aqua regia is a very corrosive oxidizing agent, which should be handled with great care.)*. In a typical experiment, aqueous $AgNO_3$ solution (5 mL,

1 mM) was added to the SH-PEI solution (5 mL, 1.5 mM) under vigorous stirring, then $NaBH_4$ solution (0.1 mL, 0.5 M) was introduced 2 min later. The reaction was allowed to proceed under vigorous stirring for 2 h. The color of the solution changed from reddish brown to light yellow. The resulting SH-PEI-AgNCs were purified via centrifugal filtration at 6000 g for 30 min with Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane (Millipore, Billerica, MA, USA). For comparison, dihydrolipoic acid-coated AgNCs (DHLA-AgNCs) were also synthesized according to Adhikari and Banerjee.[23]

Bioconjugation of AgNCs. The carboxylic acid group of FA was conjugated with the free amine group on the surface of SH-PEI-AgNCs through a carbodiimide coupling strategy.[24] Typically, 15 mg of EDC and 16 mg of Sulfo-NHS were dissolved in 1 mL of PBS, mixed with 24.8 mg of FA, and incubated for 30 min. Then, the resulting solution was mixed with 4 mL of SH-PEI-AgNCs (10 mg mL^{-1}) and incubated for 12 h. The final conjugates were purified by a centrifugal filter device (Amicon Ultra-15; 10 KD), lyophilized and resuspended in 2 mL of PBS. The products were characterized by FTIR. The conjugates are hereafter referred to AgNCs-FA.

Cytotoxicity Assay. Human breast carcinoma cell lines (MCF-7, FR-α positive) and mouse embryo fibroblast cell lines (Balb/3T3, control) obtained from China Center for Type Culture Collection (CCTCC) (Wuhan, China) were cultured in Dulbecco's modified Eagle's high glucose medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C, with the medium changed every other day. MCF-7 cell lines and 3T3 cell lines were seeded at 1×10^4 mL^{-1} (96-well plate) for 24 h. Cell activity was evaluated in triplicate using AgNCs in a concentration range from 0 to 200 μM. The

cultured cells were then treated with SH-PEI-AgNCs, washed twice with PBS buffer before the addition of DMEM containing MTT (5 mg mL^{-1}) and further incubated at 5% CO_2 at 37 °C for another 4 h. Then, the medium containing MTT was replaced by 100 μL of DMSO to solubilize the formazan crystals precipitate. The plate was shaken for 15 min at 37 °C before the measurement of optical absorbance at 492 nm on a model 680 microplate reader (Bio-Rad).

Biodistribution and Toxicity Analysis. Athymic nude mice (female, age 6-8 weeks) were intravenously injected with 500 μL of PBS buffer or SH-PEI-AgNCs (0.2 μmol as Ag). The mice were euthanized humanely via carbon dioxide asphyxiation 24 h after injection. Necropsies were performed to collect the major organs and tissues (heart, lung, liver, kidney, spleen). The organs were weighed and dissolved in aqua regia for two weeks. The organs were then diluted 1000× in water and submitted for ICPMS determination. The %ID/gram values of SH-PEI-AgNCs in all analyzed organs were determined by comparing the Ag concentration to the injected solution as reported by ICPMS, giving a quantitative measure of SH-PEI-AgNCs biodistribution.

Intracellular Imaging with AgNCs After 24-h culture of the cells, the AgNCs was added to each plate to make its concentration of 80 μM. The cells were incubated with AgNCs or FA-AgNCs for 4 h. To gain the specific binding affinity of the fluorescent probe FA-AgNCs to FR, we performed receptor blocking studies with FA. The FR was blocked by pretreating MCF-7 cells with FA for 2 h, followed by 4 h incubation with FA-AgNCs. As a negative control, FA-AgNCs was added into the normal NIH 3T3 cell. The resulting cells were washed with PBS (10 mM, pH 7.40) three times. Then, Hochest 33342 (10 μL) and Lysotracker Green DND-26 (20 μL) were used to stain nucleus and endosomes/lysosomes for 10 min, respectively. After

the medium was washed, the cells were observed using laser scanning confocal microscope.

In Vivo Imaging with AgNCs. Nude mice harboring MCF-7 tumors (8 mm) were obtained from the Institute of Hematology & Hospital of Blood Diseases, Chinese Academy of Medical Sciences & Peking Union Medical College (Licence Number: SCXK-2004-001, Tianjin, China). All animal experiments were carried out according to the guidelines of the Animal Experimentation Ethics Committee of Nankai University. For in vivo imaging, FA-AgNCs (0.02 μmol, 200 μL) were intravenously injected into MCF-7 tumor-bearing mice. To further validate the imaging specificity, excess free FA (0.01 mmol, 100 μL) was first injected into MCF-7 tumor-bearing mice via the tail vein to block FR-α. After 12 h, AgNCs-FA (0.02 μmol, 200 μL) was administered into the mice by intravenous injection. As a negative control, FA-AgNCs (0.02 μmol, 200 μL) was intravenously injected into the normal nude mice. The mice were anesthetized with intraperitoneal administration of 4% chloral hydrate at a dosage of 400 mg kg^{-1}. At the special time points after injection, the fluorescence profiles in normal and MCF-7 tumor-bearing mice were imaged. The resulting images were processed by subtracting the background tissue autofluorescence from the fluorescence from SH-PEI-AgNCs with the software of the imaging system.

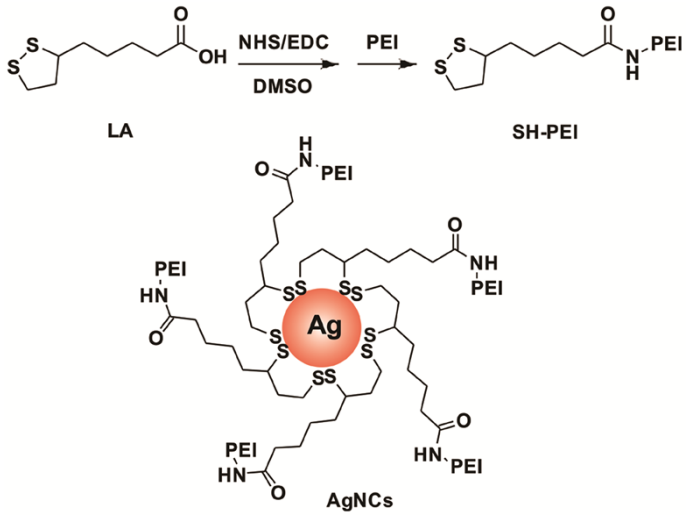

Fig. S1 Synthesis of SH-PEI and NIR fluorescent SH-PEI-AgNCs.

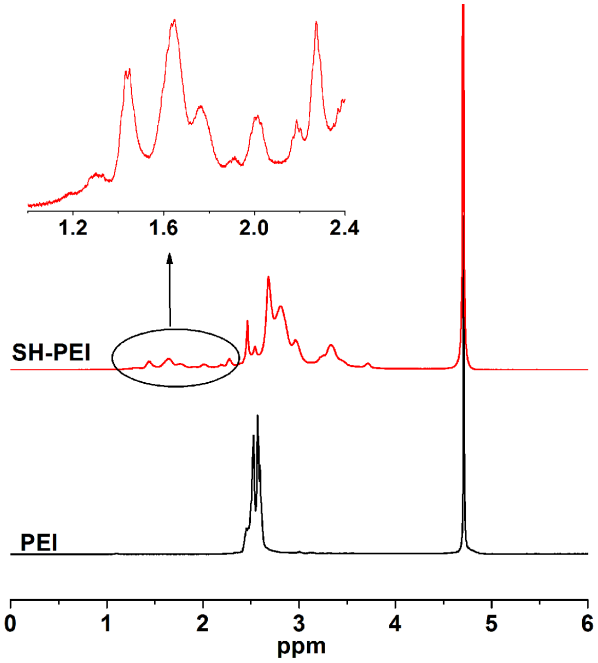

Fig. S2 1H NMR spectrum of SH-PEI and PEI.

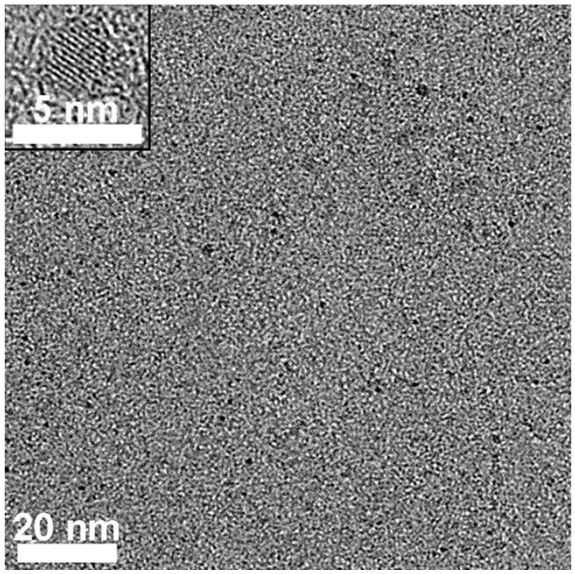

Fig. S3 HRTEM image of the as-prepared SH-PEI-AgNCs (scale 20 nm). Inset: HRTEM image of single AgNCs (scale 5 nm).

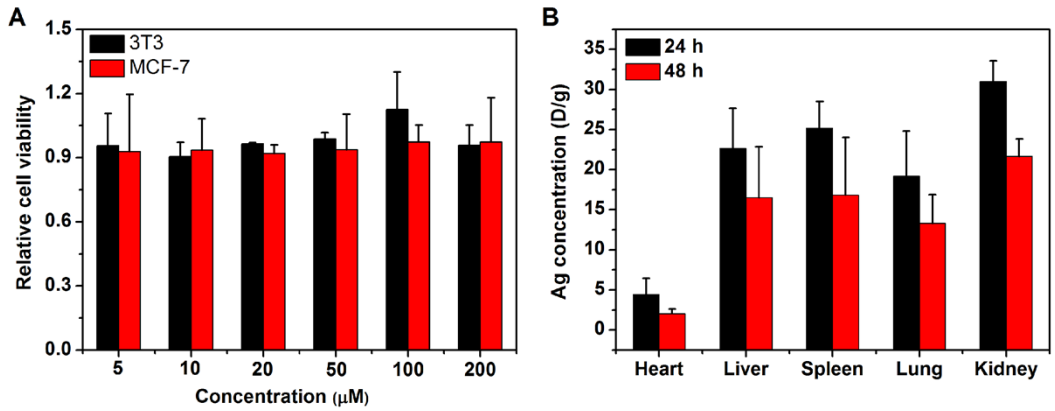

Fig. S4 (A) Histogram plots for the viability of 3T3 and MCF-7 cells in the presence of SH-PEI-AgNCs with different concentrations. Error bars represent one standard deviation (n = 3). (B) In vivo biodistribution and toxicity analysis of SH-PEI-AgNCs over a period of 48 h in athymic nude mouse. The Ag concentration in the organs was determined at different time points after tail-vein injection of the SH-PEI-AgNCs (0.2 μmol) by ICPMS (n = 3). Here %ID/g indicates the Ag element concentration in terms of the percentage of the injected dose (ID) per gram of tissue.

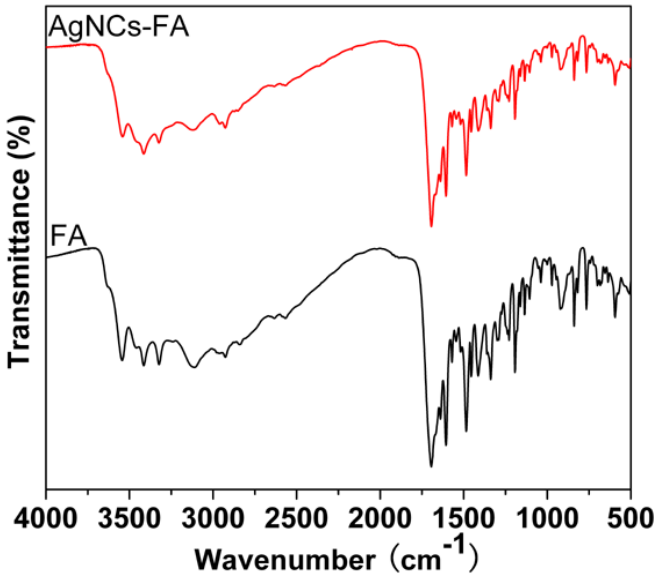

Fig. S5 FTIR spectra of FA and FA-AgNCs.