A Dendrimer-Based Dual Radiodense Element-Containing Nanoplatform for Targeted Enhanced Tumor Computed Tomography Imaging

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Part of experimental section

Materials. Amine-terminated generation 5 poly(amicodeamine) (PAMAM) dendrimers (G5.NH₂) with a polydispersity index less than 1.08 were purchased from Dendritech (Midland, MI). Chloroauric acid (HAuCl₄), acetic anhydride (Ac₂O), triethylamine (TEA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), sodium borohydride (NaBH₄) and N-hydroxysuccinimide (NHS) were obtained from Aldrich (St. Louis, MO). Diatrizoic acid (DTA) and folic acid (FA) were from J & K Chemical (Shanghai, China). Polyethylene glycol (PEG) with one end of monomethyl ether group and the other end of carboxyl group (mPEG-COOH) and with one end of amine group and the other end of carboxyl group (NH₂-PEG-COOH) were from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). Water used in all the experiments was purified by a Milli-Q plus 185 water purification system (Millipore, Bedford, MA) with a resistivity higher than 18 MΩ.cm. Regenerated cellulose dialysis membranes (molecular weight cut-off, MWCO = 10 000 or 1 000) were acquired from Fisher (Pittsburg, PA). KB cells (a human epithelial carcinoma cell line) were from Institute of Biochemistry and Cell Biology, the Chinese Academy of Science (Shanghai, China). RPMI-1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

Characterization Techniques. ¹H NMR spectra of samples dissolved in D₂O were acquired by a Bruker DRX 400 NMR spectrometer. UV-vis spectra were collected using Lambda 25 UV-vis spectrophotometer (Perkin-Elmer, Boston, MA). Fourier transform infrared (FTIR) spectra were recorded using a Nicolet 5700 spectrometer (Thermo Nicolet Corp., Waltham, MA) in a wavenumber range of 4000–500 cm⁻¹. Powder samples were mixed with KBr crystals, grinded fully and tableted before measurements. Transmission electron microscopy (TEM) images were recorded by a JEOL 2010F analytical electron microscope (Tokyo, Japan) under a voltage of 200 kV. A typical TEM sample was prepared by depositing an aqueous solution of the particles (1 mg/mL, 5 µL) onto carbon-coated
copper grid and air-dried before measurements. The particle size distribution was measured using Image J 1.40 G software (http://rsb.info.nih.gov/ij/download.html). For each sample, at least 300 particles from different images were randomly selected and analyzed.

**Stability Assay of [(Au$^{0}_{120}$-G5.NHAc-DTA-(PEG-FA)-mPEG] DSNs.** The stability of the formed Au DSNs-DTA-FA under different pH and temperature conditions was evaluated by UV-vis spectrometry. The particles were dissolved in water at a concentration of 0.5 mg/mL. The pH values of Au DSNs-DTA-FA solution were adjusted to be in a range of 5-8 using HCl and NaOH (0.1 M). The samples were respectively maintained for 30 min under different pHs (5-8) and temperatures (4, 25, 37 and 50 °C, respectively) before measurements. In addition, the sample was also dispersed in phosphate buffered saline (PBS) and cell culture medium, respectively for one month in order to check their colloidal stability.

**X-Ray Attenuation Measurements.** [(Au$^{0}_{120}$-G5.NHAc-DTA-(PEG-FA)-mPEG] DSNs, [(Au$^{0}_{120}$-G5.NHAc-mPEG] DENs and Omnipaque (300 mg I/mL, GE Healthcare) were dispersed in water with different Au/I concentrations ranging from 0 to 0.1 M. The samples were prepared in 1.5-mL Eppendorf tubes and placed in a self-designed scanning holder. [(Au$^{0}_{120}$-G5.NHAc-mPEG] DENs and Omnipaque were used as controls. CT scanning were carried out in a CT imaging system (GE Medical Systems, Milwaukee, WI) with 100 kV, 200 mA, and a slice thickness of 0.625 mm. X-ray attenuation intensity was evaluated in Hounsfield units (HU) by selecting a uniform round region on the digital CT images loaded in a standard display program.

**Cytotoxicity Assay.** KB cells were regularly cultured and passaged in RPMI 1640 cell culture medium supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin under 37 °C and 5% CO$_2$. The cytotoxicity of Au DSNs-DTA-FA was tested by MTT viability assay of KB cells according to the literature protocols.$^1$ Briefly, KB cells were seeded into a 96-well plate with a density of 1×10$^4$ cells per well and incubated overnight to bring the cells to about 80% confluence. The next day, medium of each well was replaced with fresh medium containing Au DSNs with different concentrations (0-2000 nM), and the cells were continuously cultured for 24 h. Thereafter, MTT (10 µL,
5 mg/mL) in PBS was added to each well and the cells were incubated for another 4 h. The medium was then carefully removed, and DMSO (200 μL) was added. The absorbance at a wavelength of 570 nm for each well was measured using a Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA). Mean and standard deviation for the triplicate wells for each sample were reported. After treatment with the Au DSNs-DTA-FA for 24 h, the cell morphology was also observed using a Leica DM IL LED inverted phase contrast microscope (Wetzlar, Germany) with a magnification of 200× for each sample.

**Hemolytic Assay.** Fresh human blood stabilized with EDTA was provided by Shanghai General Hospital (Shanghai, China) after approval by Ethical Committee of Shanghai General Hospital. Human red blood cells (HRBCs) were acquired according to the procedure reported in the literature.² The obtained HRBCs were diluted 4 times with PBS. After that, 100 μL of the diluted HRBC suspension was separately added to Eppendorf tubes (1.5 mL) containing 0.9 mL of water (positive control), 0.9 mL of PBS (negative control), or 0.9 mL of PBS containing the Au DSNs-DTA-FA with different final concentrations (50-2000 nM), respectively. After a gentle shaking, the mixtures were kept still for 2 h at 37 °C. Then, the samples were centrifuged (10,000 rpm, 1 min), the photos of the samples were taken and the absorbance of the supernatants (hemoglobin) was recorded by a Perkin Elmer Lambda 25 UV-vis spectrophotometer. A group of Au DSNs-DTA-FA solutions with the same concentrations excluding HRBCs were used as the background contrast to be subtracted. The hemolysis percentages of different samples were calculated by dividing the difference in absorbance at 541 nm between the samples and the negative control by the difference in absorbance at 541 nm between the positive and negative controls.

**Cellular Uptake Assay.** The cellular uptake of Au DSNs-DTA-FA by target cells was evaluated by Leeman Prodigy inductively coupled plasma-optical emission spectroscopy (ICP-OES, Hudson, NH) according to protocols reported in the literature.³ In brief, KB cells were seeded into 12-well plates at a density of 8 × 10⁵ cells per well for 24 h. The medium of each well was replaced with 2 mL of fresh medium containing Au DSNs-DTA-FA or Au DSNs-DTA ([Au] = 50, 500 or 1000 nM, respectively)
and the cells were then incubated at 37 °C and 5% CO₂ for 4 h. After that, the cells were washed 3 times with PBS, lifted with trypsinization, and resuspended in 2 mL of RPMI-1640 medium containing 10% FBS. The concentration of cells was determined by hemacytometry. Then, cells in each well were collected by centrifugation at 1000 rpm for 5 min, lysed by an *aqua regia* solution (0.2 mL, nitric acid/hydrochloric acid, v/v = 1:3), and diluted with water before ICP-OES analysis of Au concentration. Three parallel wells for each sample were measured.

**In Vitro CT Imaging of KB Cells.** In *vitro* CT imaging of KB cells was carried out prior to tumor CT imaging *in vivo*. KB cells were incubated with the [(Au⁰)₁₂₀-G5.NHAc-DTA-mPEG] DSNs or [(Au⁰)₁₂₀-G5.NHAc-DTA-(PEG-FA)-mPEG] DSNs at different dendrimer concentrations (0, 500, 1000, 1500 and 2000 nM, respectively) for 4 h. The cells were washed 3 times with PBS and then trypsinized, centrifuged and resuspended into PBS (100 μL, in a 1.5 mL Eppendorf tube for each sample). The samples were placed in a self-designed scanning holder and CT scanning was performed using a clinical CT imaging system (GE Medical Systems) under the following parameters: tube voltage, 100 kV; tube current, 200 mA; and slice thickness, 0.625 mm. CT values in Hounsfield units (HU) was analyzed by selecting a uniform round region on the digital CT images loaded using a standard display program.

**In Vivo Micro-CT Imaging of KB Xenograft Tumor Model.** All animal experiments were carried out and approved by ethical committee for animal care of Shanghai Ninth People’s Hospital and also in accordance with the policy of the National Ministry of Health. Male BALB/c nude mice of 4- to 6-week old (Shanghai Slac Laboratory Animal Center, Shanghai, China) were subcutaneously injected with 1 × 10⁶ cells in the right side of their back for each mouse. The constructed xenografted tumor model with a volume of 0.7-1.5 cm³ at approximately 3 weeks post-injection of KB cells were utilized for micro-CT imaging. The mice were divided into three groups (n = 3 for each group): Group 1, [(Au⁰)₁₂₀-G5.NHAc-mPEG] DENs (single-elemental group); Group 2, [(Au⁰)₁₂₀-G5.NHAc-DTA-mPEG] DSNs (non-targeted dual-elemental group); and Group 3, [(Au⁰)₁₂₀-G5.NHAc-DTA-(PEG-FA)-mPEG] DSNs (targeted dual-elemental group), respectively. The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg). For each group, the particles (dispersed 0.1 mL PBS, [Au]
= 0.1 M) were intravenously injected to each tumor-bearing mouse through tail vein. CT scanning was performed both before and at 2, 4 and 6 h post injection using a micro-CT imaging system (eXplore Locus, GE Healthcare). Images were reconstructed on a micro-CT imaging workstation (GEHC microView, GE Healthcare) based on voxels of 45 μm × 45 μm × 45 μm. The parameters of CT scanning and measurements were similar to those described in our previous report.4-5

**In Vivo Biodistribution.** The above tumor-bearing BALB/c nude mice after CT scanning at 6 h post-injection were euthanized and different organs including heart, liver, spleen, lung, kidney, stomach, intestines, testicle, blood, tumor and brain were extracted and weighed. The organs were then cut into 1-2 mm² pieces and digested by *aqua regia* solution for 24 h. Then, Au content in different organ pieces was determined by ICP-OES.

**Statistical Analysis.** The experimental results between groups were analyzed through the one-way analysis of variance (ANOVA) statistical method. A p value of 0.05 was set as the significance level, and the data were marked with (*) p < 0.05, (**) p < 0.01, and (***) p < 0.001, respectively.
Figure S1. $^1$H NMR spectra of G5.NH$_2$ dendrimer (a) and Au DSNs-DTA-FA (b).

Figure S2. FTIR spectra of G5.NH$_2$ dendrimer (a), [(Au$^0$)$_{120}$-G5.NHAc-DTA-(PEG-FA)-mPEG] DSNs (b) and free DTA (c).
Figure S3. TEM image and size distribution histogram of [(Au\(^0\))\(_{120}\)-G5.NH\(_2\)] DENs (a) and [(Au\(^0\))\(_{120}\)-G5.NH\(_2\)-DTA-(PEG-FA)-mPEG] DSNs (b).

Figure S4. UV-vis spectra of [(Au\(^0\))\(_{120}\)-G5.NHAc-DTA-(PEG-FA)-mPEG] DSNs under (a) different pHs (5-8) at 25 °C and (b) different temperatures (4-50 °C) at pH 7.
Figure S5. Photographs of blank cell culture medium (1), [(Au\(^0\))\(_{120}\)-G5.NHAc-DTA-(PEG-FA)-mPEG] DSNs dispersed in cell culture medium (2) and PBS (3) after one month’s storage at room temperature.

Figure S6. Microphotographs of KB cells treated with the [(Au\(^0\))\(_{120}\)-G5.NHAc-DTA-(PEG-FA)-mPEG] DSNs at different concentrations (0 (a), 100 (b), 300 (c), 500 (d), 1000 (e) and 2000 nM (f), respectively) for 24 h.

The morphology of KB cells after incubation with the particles at different concentrations for 24 h was observed by phase contrast microscopy. It can be seen that most of KB cells maintain a normal fusiform morphology, indicative of the fact that the Au DSNs do not show obvious cytotoxicity to cells in the concentration range of 50-2000 nM.
Figure S7. TEM image and size distribution histogram of (a) $[(\text{Au}^0)_{120}-\text{G5.NHAc-mPEG}]$ DSNs and (b) $[(\text{Au}^0)_{120}-\text{G5.NHAc-DTA-(PEG-FA)-mPEG}]$ DSNs.

References


