Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information for

Dendrimer-folate-copper conjugates as bioprobes for synchrotron x-ray fluorescence imaging

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Materials and methods

1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl (EDC, 98%), polyamidoamine (PAMAM) dendrimer generation 5 (G5), triethylamine，Folic acid (FA) were purchased from Aldrich Co., Ltd; Acetic anhydride, dimethyl sulfoxide (DMSO, 99%), dimethylformamide (DMF, 99%), Dialysis membrane (MWCO=3500) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai); KB cells and A549 cell were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. ^{1}H-NMR spectrum was performed on Bruker AVANCE DRX 500Hz spectrometer in D2O solution.

Preparation of dendrimer PAMAM generation 5 - folic acid conjugates (G5-FA)

4.90 mg of Folic Acid (MW = 441.4 g/mol) reacted with 29.69 mg of EDC·HCl (MW= 191.71 g/mol) in a mixture of 6 mL dry DMF and 2 mL dry DMSO for 1 h. The reaction mixture was added dropwise to the DI water solution (15 mL) of 1.39 μmol of G5 PAMAM and vigorously stirred for 3 days. After dialysis (using cellulose membrane with 3500 MWCO, PBS buffer and DI water 3 times with 1 L each time) and lyophilization, the yield was 95.4%. Further purification was carried out by membrane filtration with DI water. The obtained sample G5-FA was lyophilized and stored in a dry place before further modification. Yield: 92.1 %.

Preparation of acetylated dendrimer PAMAM generation 5 - folic acid conjugate

(Ac-G5-FA)

Acetylated PAMAM dendrimer generation 5(Ac-G5-FA) was prepared according to the reported procedure. Briefly acetic anhydride (150% of primary amine on the surface of PAMAM dendrimer G5) was slowly added to the dendrimer G5-FA solution (1.73 μmol dendrimer G5-Ac dissolved in 6 mL

methanol) in the presence of triethylamine (1.25 equivalent of acetic anhydride). The mixture was stirred under N2 atmosphere at room temperature. After 18 h methanol was evaporated on rotary evaporator. The residue was dissolved in water and dialyzed (using cellulose membrane with 3500 MWCO) against PBS buffer and double distilled (DI) water for 3 days. The obtained sample Ac-G5-FA was lyophilized and stored in a dry place before further modification and characterization. Yield: 94.7%.

Measurements of metal ion binding to PAMAM dendrimers in aqueous solutions

Cu (II) was selected as a model cation to probe the binding of metal ions with affinity toward the amine groups inside the PAMAM dendrimers. Reagent grade $CuSO_4 \cdot 5H_2O$ was used as a source of Cu (II). Briefly, a $CuSO_4$ solution (2mM, 5 mL) was added into a G5-Ac-FA (or G5-Ac) dendrimer aqueous solution (0.1mM, 5mL) under vigorous stirring. The reaction mixture turned a deep-blue from wather blue within a few seconds.

Cell culture and cytotoxicity assay

KB cells (folate-receptor positive, FR+) and A549 cells (folate-receptor negative, FR-) were procured from the Stem Cell Bank of the Chinese Academy of Sciences. The KB cell line is a human oral epidermoid carcinoma that overexpresses FR, especially when grown in a low folic acid medium. The KB cells were grown continuously as a monolayer at 37□ and 5% CO2 in folic-acid-deficient RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), yielding a final folate concentration roughly that of normal human serum[1-2]. A549 Cell line is a human lung adenocarcinoma cells without folate-receptor, was grown continuously as a monolayer at 37 ^{o}C, and 5% CO_2 in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS).

We first tested the cytotoxicity of G5-Ac-FA-Cu^{2+} conjugates to KB cells and A549 cell . In brief, KB cells were respectively seeded in 96-well plates at a density of 5×10^3cells per well and incubated for 24h. The medium in each well was replaced with 100μl of culture medium containing different concentration of treatments(0.1μM, 0.2μM, 0.4μM, 0.8μM,1.6μM,3.2μM) and cultured for another 24h. 10 μL WST-1 solution was added to wells, absorbance values were taken using a 96-well Opsys Microplate Reader at 450 nm.[3] Cell viability is expressed as a percentage of control. The cytotoxicity of G5-Ac-FA-Cu^{2+} conjugates to A549 cell have been performed according to the same procedure.

Preparation of samples for Synchrotron X-ray Fluorescence analysis

KB cells (folate-receptor positive, FR+) and A549 cells (folate-receptor negative, FR-) were procured from the Stem Cell Bank of the Chinese Academy of Sciences. These cells were grown continuously as a monolayer at 37 ^{o}C, and 5% CO_2 in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS). After trypsinization, cells were collected in a tube, centrifuged and resuspended in fresh RPMI 1640 medium to a final concentration of 1×10^5 cells/ml and seeded in 24 well plates with Mylar film. Copper uptake studies were performed in the same medium but supplemented with 0.2μM G5-Ac-FA-Cu^{2+} at 37°C for 4h, then washed with PBS for three times, fixed with paraformaldehyde in isotonic phosphate-buffered saline, and washed twice with ammonium acetate or deionized water to avoid formation of residual salt crystals that might increase background X-ray fluorescence. Cell samples were stored at 4°C before SXRF measurements.

Synchrotron radiation experiment

The experiment was performed at the Shanghai Synchrotron Radiation Facility in China. Scanning

X-ray fluorescence analysis was performed at XRF microprobe station. The X-ray light source comes from the BL15U1 beam line of Shanghai Synchrotron Radiation Facility (SSRF, Shanghai, China). The sample were placed onto a kinematic specimen holder suitable for both optical and X-ray Xuorescence microscopy. The holder was then mounted on a light microscope (Leica DMXRE) and target cells imaged previously by standard fluorescence microscopy were located on the grid relative to a reference point using a high spatial resolution motorized x/y stage (Ludl Electronic Products, Hawthorne, NY). Coordinates were determined and used to precisely locate the target cell once the grid was transferred to the microprobe. A Fresnel zone plate was used to focus the monochromatic X-ray beam from an undulator source to a spot size of $2\times2\mu m^2$ on the specimen. An incident photon energy of 7.9-8.2keV was chosen to ensure excitation of the K-line of Cu, and the sample was raster scanned through the beam at 298K under a helium atmosphere. The pixel step size was set to 1μm and the entire X-ray spectrum was recorded at each pixel using an energy dispersive germanium detector. This beam line can provide multi-chromatic X-rays (white light), who's energy range from 3.5 – 22.5keV. The electron energy in the storage ring is 1-20 KeV, the spot size of the X-ray beam was $2.5\times2.6\mu m^2$.

1. Myc, A.; Majoros, I. J.; Thomas, T. P.; Baker, J. R., Jr. *Biomacromolecules* **2007**, 8, 13–18.
2. Majoros, I. J.; Myc, A.; Thomas, T.; Mehta, C. B.; Baker, J. R., Jr. *Biomacromolecules* **2006**, 7, 572–579.
3. Worle-Knirsch, J. M.; Pulskamp, K.; Krug, H. F. *Nano Lett.* **2006**, 6, 1261.

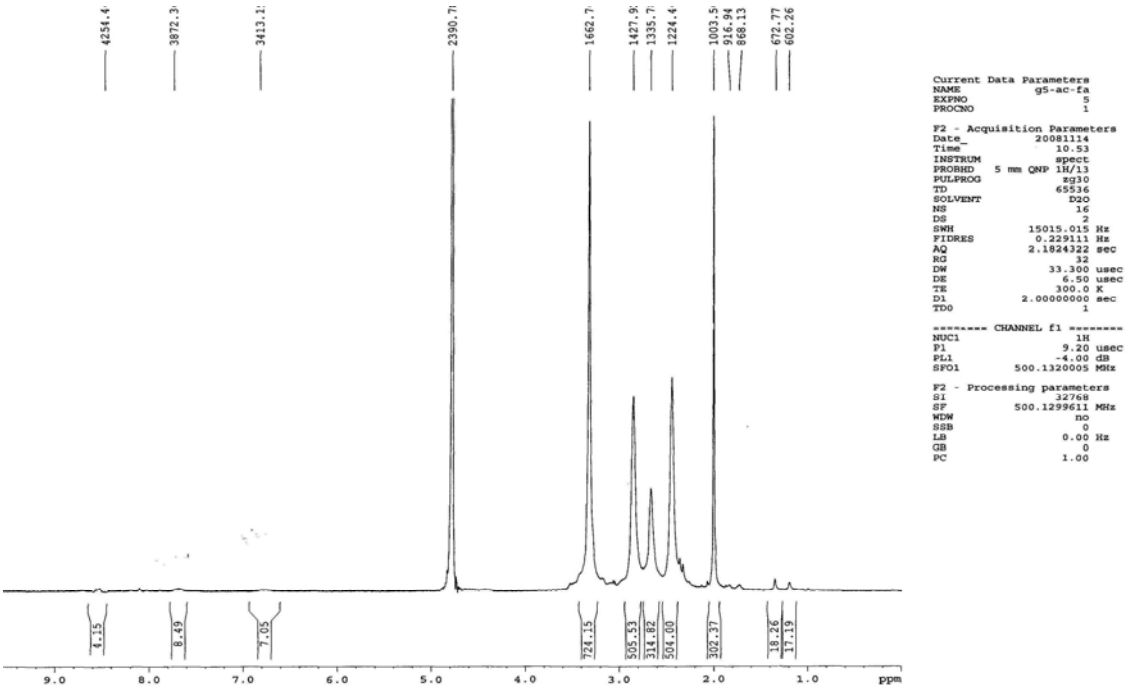

Fig. S1 The ^{1}H-NMR spectrum of G5-FA-Ac

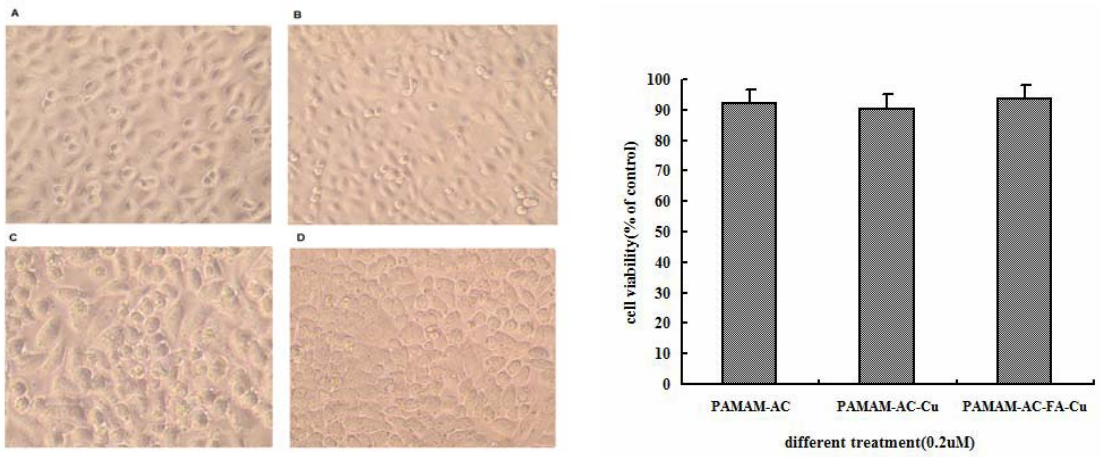

Fig. S2 The cytotoxicity assessments G5-Ac-FA-Cu^{2+} conjugates in concentration of 0.2μM. A) KB cell, B) KB cell cultured with G5-Ac, C) KB cell cultured with G5-Ac-Cu, D) KB cell cultured with G5-Ac-FA-Cu

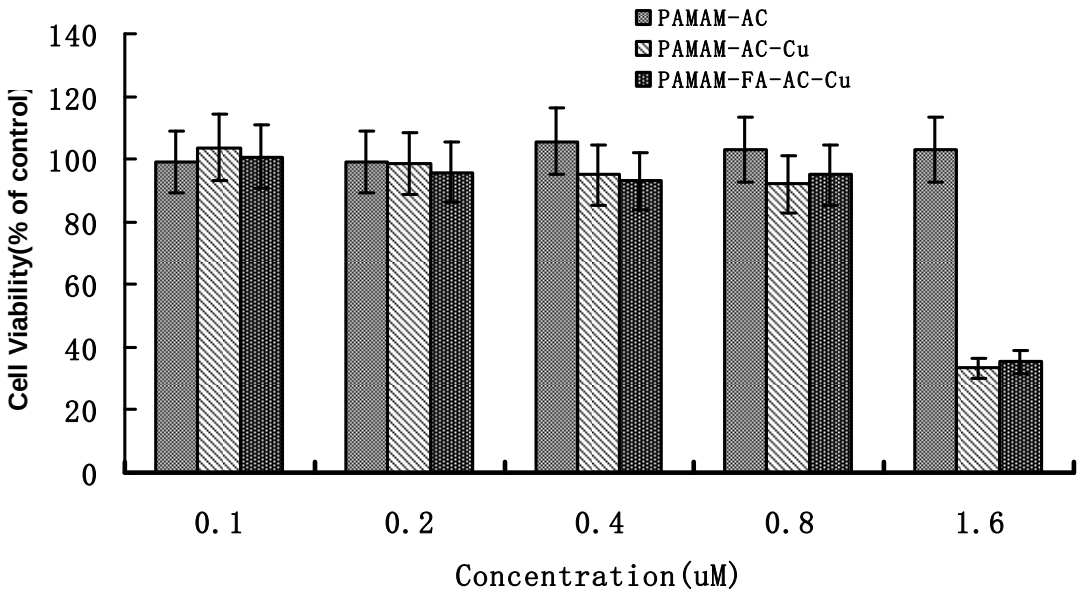

Fig. S3 The cytotoxicity assessments G5-Ac-FA-Cu^{2+} conjugates to A549 cells in different concentrations.

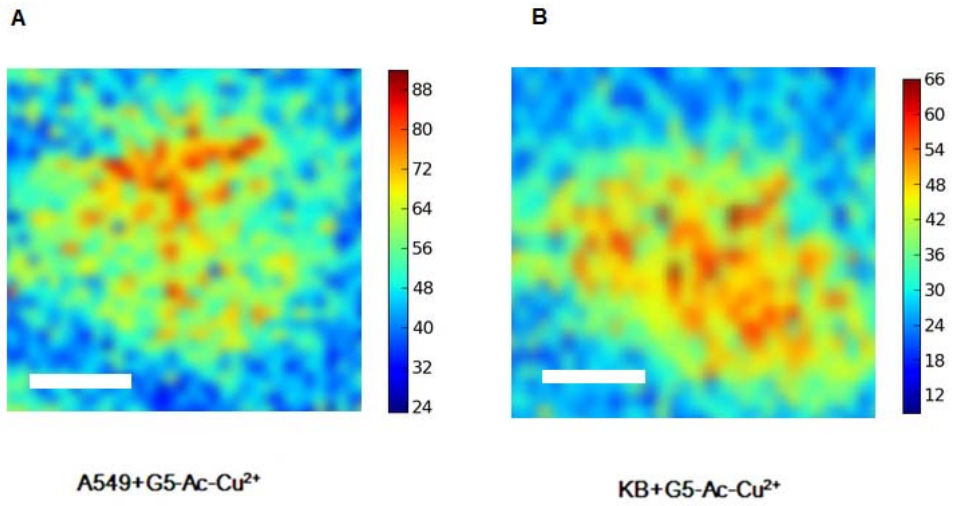

Fig. S4 The Synchrotron X-ray fluorescence analysis of KB cells and A549 cells cultured with G5-Ac-Cu2+ conjugates.

Scare bar：5μm.

A, A549 cell cultured with G5-Ac-Cu2+ conjugates B, KB cells cultured with G5-Ac-Cu2+ conjugates