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Targeting and detecting cancer cells using spontaneously formed multifunctional dendrimer-stabilized gold nanoparticles[†]

Xiangyang Shi^a, Su He Wang^b, Mary E. Van Antwerp^b, Xisui Chen^b, and James R. Baker Jr^b

Xiangyang Shi: xshi@dhu.edu.cn; Su He Wang: shidasui@umich.edu; James R. Baker: jrbakerjr@umich.edu

^a College of Chemistry, Chemical Engineering, and Biotechnology, Donghua University, Shanghai 210620, P. R. China; Fax: +86-21-67792306-804

^b Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan, Ann Arbor, MI 48109, USA

Abstract

We develop a facile approach to fabricating multifunctional dendrimer-stabilized gold nanoparticles (Au DSNPs) for cancer cell targeting and imaging. In this work, amine-terminated generation 5 (G5) poly(amidoamine) (PAMAM) dendrimers pre-functionalized with folic acid (FA) and fluorescein isothiocyanate (FI) are complexed with Au(III) ions, followed by acetylation of the amine groups on the dendrimer surfaces. This one-step process leads to the spontaneous formation of 6 nm-sized Au nanoparticles stabilized by multifunctional dendrimers bearing both targeting and imaging functionalities. The multifunctional Au DSNPs are characterized by UV-Vis spectrometry, ¹H NMR, and transmission electron microscopy (TEM). The formed Au DSNPs are water-soluble, stable, and biocompatible. Combined flow cytometry, confocal microscopy, silver staining, and inductively coupled plasma-mass spectrometry (ICP-MS) analyses show that the FA- and FI-functionalized Au DSNPs can specifically target to cancer cells expressing high-affinity FA receptors *in vitro*. This approach to functionalizing Au DSNPs may be extended to other targeting molecules, providing a unique nanoplatform for targeting and imaging of a variety of biological systems.

Introduction

Current cancer therapy often involves non-specific killing of healthy cells, thus limiting the therapeutic efficiency. Recent advances in nanoscience and nanotechnology have the potential to revolutionize cancer diagnosis and therapy.¹ Much effort has been made in the development of various nanoparticle (NP) systems that can specifically target, detect, and kill cancer cells at early stages. Among many different nanocarrier systems, gold (Au) NPs have shown to be a promising platform for cancer cell targeting, imaging, and treatment because of their very good biocompatibility.^{2–10} However, optimizing an approach to preparing multifunctional Au NPs for cancer diagnosis and therapy still remains a great challenge.

Dendrimers are a novel class of highly branched, mono-dispersed, synthetic macromolecules with well-defined composition and architecture.^{11,12} Using dendrimers, especially

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Correspondence to: Xiangyang Shi, xshi@dhu.edu.cn; Su He Wang, shidasui@umich.edu; James R. Baker, Jr, jrbakerjr@umich.edu.

poly(amidoamine) (PAMAM) dendrimers, as templates or stabilizers one can readily synthesize dendrimer-entrapped Au NPs (Au DENPs)^{2,13–16} or dendrimer-stabilized Au NPs (Au DSNPs).^{17–19} In general, Au DENPs are synthesized using fast reduction and nucleation chemistry and have a size smaller than 5 nm; Au DSNPs are synthesized under slow reduction and nucleation chemistry and have a size larger than 5 nm.²⁰ Our previous work shows that Au DENPs can be modified and bio-functionalized effectively with dye tags and targeting ligands for cancer cell targeting and imaging.² Although metal DSNPs have been used for biological sensing applications,^{21,22} utilization of Au DSNPs as effective platforms for specific cancer cell targeting and imaging has not been reported. This is largely due to the difficulties in manipulating the particle surface chemistry and decreasing the toxicity of the formed particles. In comparison with Au DENP nanostructures composed of one or more metal NPs within one dendrimer molecule,²³ Au DSNPs are nano-structures composed of one metal NP surrounded with multiple dendrimer molecules. Therefore, it is possible to modify Au DSNPs with many more targeting ligands than those of Au DENPs, which significantly increases their targeting sensitivity and polyvalency effect.²⁴

Recently, we have shown that acetamide-functionalized Au DSNPs can be formed by acetylation of amine-terminated PAMAM dendrimers of generation 5 (G5.NH₂) complexed with HAuCl₄ and that dye-functionalized Au DSNPs can be formed by acetylation of dye-conjugated, amine-terminated G5 dendrimers complexed with HAuCl₄.²⁵ The formed, functionalized Au DSNPs have a neutral surface charge and are stable in aqueous solution. The formation occurred spontaneously, and the reaction took place at room temperature. This process provides a basis to further develop multifunctional Au DSNPs as a platform for cancer cell targeting and imaging through a simple spontaneous approach.

It is known that folic acid (FA)- and fluorescein isothiocyanate (FI)-modified G5 dendrimers with neutral surface charge can specifically target to cancer cells overexpressing folic acid receptors (FARs).²⁶ We anticipate that using FA- and FI-modified G5 dendrimers to synthesize Au DSNPs may provide a means to create an Au NP-based multifunctional nanoplatform for cancer cell targeting and imaging. In this present work, we complexed FA- and FI-modified amine-terminated G5 dendrimers (G5.NH₂-FI-FA) with HAuCl₄, followed by acetylation of the dendrimer terminal amines to create multifunctional Au DSNPs using our one-step reaction at room temperature (Scheme 1). The formed FA- and FI-functionalized Au DSNPs are stable, water-soluble, and biocompatible. Flow cytometry, confocal microscopic imaging, silver staining, and inductively coupled plasma-mass spectrometry (ICP-MS) analyses show that the multifunctional Au DSNPs can specifically bind to cancer cells overexpressing high-affinity FAR *in vitro*. To our knowledge, this is the first report regarding the synthesis of a multi-functional DSNP-based nanoplatform for cancer cell targeting and imaging. The tunable dendrimer surface chemistry (*e.g.* surface functionalization and bioconjugation) in conjunction with the facile approach developed to form Au DSNPs open a new avenue to developing various biofunctionalized Au DSNPs for a range of interesting biomedical applications.

Experimental

Materials

Ethylenediamine core amine-terminated PAMAM dendrimers of generation 5 (G5.NH₂) with a polydispersity index less than 1.08 were purchased from Dendritech (Midland, MI). The FA, FI, acetic anhydride, triethylamine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and all the other chemicals and solvents were obtained from Aldrich and were used as received. Water used in all of the experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with resistivity higher than 18 MΩ.cm. Regenerated cellulose dialysis

membranes (MWCO = 10 000) were acquired from Fisher. The FI- and FA-functionalized generation 5 (G5.NH₂-FI-FA) PAMAM dendrimers were synthesized and characterized according to our previous work.²⁷ The FI-functionalized G5 dendrimers (G5.NH₂-FI) without FA conjugation were used as a control. The average numbers of FI and FA moieties conjugated onto each G5 dendrimer were estimated to be 4.5 and 4.8, respectively. The molecular weights of the G5.NH₂-FI and G5.NH₂-FI-FA conjugates were determined to be 29 564 and 33 484, respectively. The KB cells were from American Type Tissue Collection (ATCC, Rockville, Maryland). The penicillin, streptomycin, and fetal bovine calf serum (FBS) were purchased from Sigma (St. Louis, MO). The trypsin-EDTA, Dulbecco's PBS, RPMI 1640 medium (with or without FA), and bovine serum albumin were obtained from GIBCO-BRL (Gaithersburg, MD). The silver enhancement kit was from Sigma.

Synthesis of FI- and FA-functionalized Au DSNPs (Scheme 1a)

G5.NH₂-FI-FA/Au(III) complexes were first prepared by the addition of a methanol solution of HAuCl₄ (108 μ L, 39.6 mM) into a methanol/water (v/v = 1: 1) mixture solution of the G5.NH₂-FI-FA dendrimer (6 mL, 0.102 mM) with a molar ratio of G5.NH₂-FI-FA/Au atoms equivalent to 1: 7. The formed complex was denoted as {(Au³⁺)₇-G5.NH₂-FI-FA}. The primary amine groups of the {(Au³⁺)₇-G5.NH₂-FI-FA} complexes were acetylated using a procedure described in the literature.²⁸ Briefly, 55 μ L of triethylamine was added to the above formed {(Au³⁺)₇-G5.NH₂-FI-FA} solution. A methanolic solution (0.5 mL) of acetic anhydride (40.05 mg, more than a 500% molar excess of the total primary amines of {(Au³⁺)₇-G5.NH₂-FI-FA}) was added dropwise into the {(Au³⁺)₇-G5.NH₂-FI-FA}/triethylamine mixture solution while it was being stirred vigorously, and the mixture was allowed to react for 24 h. The mixture solution spontaneously changed to red after 6 h, indicating the formation of Au NPs. The methanol solution of the reaction mixture was extensively dialyzed against PBS buffer (3 times, 4 liters) and water (3 times, 4 liters) for 3 days to remove the excess of reactants and by-products, followed by lyophilization to obtain the {(Au⁰)₇-G5.NHAc-FI-FA} DSNPs. The control {(Au⁰)₇-G5.NHAc-FI} DSNPs without FA modification were prepared according to the procedure similar to that of {(Au⁰)₇-G5.NHAc-FI-FA} DSNPs (Scheme 1b).

Note that the number '7' in the nomenclatures is the number of Au atoms per dendrimer molecule, according to the reaction stoichiometry. The denotation of {(Au⁰)₇-G5.NHAc-FI-FA} and {(Au⁰)₇-G5.NHAc-FI} does not necessarily represent any single particle.

Characterization techniques

UV-Vis spectra were collected using a Perkin Elmer Lambda 20 UV-Vis Spectrometer. All Au DSNP and dendrimer-Au(III) complex samples were dissolved in water at the concentration of 3 mg/mL. ¹H NMR spectra of Au DSNPs were recorded on a Bruker DRX 500 nuclear magnetic resonance spectrometer. Samples were dissolved in D₂O before NMR measurements. Zeta potential measurements were performed using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, UK) equipped with a standard 633 nm laser. TEM measurements were performed at 200 kV using a JEOL 2010F analytical electron microscope with an energy dispersive spectroscopy (EDS) system attached. A 5 μ L aqueous solution of Au DSNPs (3 mg/mL) was dropped onto a carbon-coated copper grid and air-dried before the measurements. The size distribution histograms of the Au DSNPs were measured using ImageJ software (<http://rsb.info.nih.gov/ij/download.html>). For each sample, 200 NPs were randomly selected to analyze the size distribution.

Cell cultures and biological evaluation

The KB cells were continuously grown in two 10 cm culture dishes, one in FA-free media and the other in regular RPMI 1640 cell culture medium supplemented with penicillin (100

units/mL), streptomycin (100 µg/mL), 10% heat-inactivated FBS, and 2.5 µM FA. The cells grown in FA-free media express high-level FAR (denoted as KB-HFAR), while the cells grown in FA-containing media express low-level FAR (denoted as KB-LFAR).

MTT quantitation of cell viability

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to quantify the viability of the cells. Briefly, approximately 5×10^4 KB cells per well were seeded into a 96-well plate. After overnight culture, functionalized Au DSNPs at concentrations ranging from 0 to 2 µM in PBS were added to each well. After 24 h incubation with Au DSNPs, the metabolically active cells were then detected by adding MTT to a final concentration of 0.5 mg/mL for 2–4 h at 37 °C. Isopropanol with 0.04 N HCl was added, and the plates were read at 570 nm with a 630 nm reference. The mean and standard deviation for the triplicate wells were reported.

Flow cytometry analysis

Approximately 5×10^5 cells per well were seeded in 12-well plates the day before the experiments. An hour before initiating an experiment, the cells were rinsed four times with serum-free and FA-deficient RPMI 1640 medium. Functionalized Au DSNPs were added in the final concentrations of 0–300 nM. After 1 h of incubation with the functionalized Au DSNPs, the KB-HFAR and KB-LFAR cells were trypsinized and suspended in PBS containing 0.1% bovine serum albumin and analyzed using a Becton Dickinson FACScan analyzer. The FL1-fluorescence of 10 000 cells was measured, and the mean fluorescence of the gated viable cells was quantified.

Confocal microscopy

Confocal microscopic analysis was performed using an Olympus FluoView 500 laser scanning confocal microscope (Melville, NY). The cells were plated on a plastic cover-slip before measurements. The FI fluorescence was excited with a 488 nm argon blue laser, and the emission was measured through a 505–525 barrier filter. The optical section thickness was set at 5 µm. The cells were incubated with functionalized Au DSNPs (25 nM) for 2 h, followed by rinsing with PBS buffer. The nuclei were counterstained with 1 µg/mL of Hoechst33342, using a standard procedure. Samples were scanned using a 60× water-immersion objective lens and magnified with FluoView software. All samples were imaged under similar instrumental conditions.

Silver stains

Approximately 1.25×10^5 cells were seeded into 3 wells of a 24-well plate the day before the experiments. An hour before initiating an experiment, the cells were rinsed four times with serum-free and FA-deficient RPMI 1640 medium. Functionalized Au DSNPs were added at a final concentration of 50 nM. After 2 h of incubation with the functionalized Au DSNPs at 37 °C, the KB-HFAR cells were rinsed with PBS and treated with 0.1% trypsin. To enhance the silver staining, the cells were then washed with PBS (containing Mg^{2+} and Ca^{2+}) buffer (Hyclone, Logan, UT), divided into 4 equal portions, and spun onto slides using a Shandon Cytospin 3 cytocentrifuge. The slides were rinsed, treated with Lugol's solution (0.1% iodine/2% aqueous KI), and stained with a silver enhancement kit according to the manufacturer's instructions. The silver staining time was optimized in order to better differentiate the cell images under different conditions. It was found that 5 min of silver staining gave the best contrast between the control cells and the cells treated with Au DSNPs. The cell morphology after silver staining was observed by an Olympus IX70 fluorescent inverted microscope.

Inductively coupled plasma-mass spectrometry (ICP-MS)

To measure the uptake of functionalized Au DSNPs by the target cells, the KB-HFAR cells were plated in a 12-well plate at a density of 5×10^5 cells/well one day prior to the experiment. The cells were incubated with 50 nM or 200 nM of functionalized Au DSNPs in RPMI folate-free medium at 37 °C, 5% CO₂ for 2 h. After the incubation, the cells were washed once with Versene (Gibco), twice with PBS, and three times with Hanks' Balanced Salt Solution (HBSS). The cells were then lifted with trypsin/ethylenediaminetetraacetate, resuspended in 200 μ L of PBS, and dispersed into 1.8 mL of 10% FBS in PBS. The cell suspension (100 μ L) was counted. The remaining cells were centrifuged to form pellets and lysed using an aqua regia solution (0.1 mL). The Au nanoparticles were also able to be digested after the treatment with the aqua regia solution. The Au uptake was measured with a FinniganTM ELEMENT2 ICP-MS instrument (Thermo Electron Corporation, Waltham, MA 02454).

Results and discussion

Similar to our previous work on the formation of acetamide-functionalized Au DSNPs and dye-functionalized Au DSNPs with acetamide surface groups,²⁵ in this present work we found that FI- and FA-functionalized Au DSNPs with close to neutral surface charges could be spontaneously formed by acetylation of the $\{(Au^{3+})_7-G5.NH_2-FI-FA\}$ complex (Scheme 1). In this process, no additional reducing agents were added and the reactions were performed at room temperature.

Fig. 1 shows the UV-Vis spectra of the dendrimer conjugates G5.NH₂-FI (Curve 1) and G5.NH₂-FI-FA (Curve 2), and the formed DSNPs $\{(Au^0)_7-G5.NHAc-FI\}$ (Curve 3) and $\{(Au^0)_7-G5.NHAc-FI-FA\}$ (Curve 4), respectively. Both the G5.NH₂-FI-FA dendrimers and the $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSNPs show absorbance peaks at 280 nm and 500 nm, which correspond to the characteristic absorption of FI and FA, respectively. In contrast, only the featured FI absorbance peak at 500 nm was observed for both the G5.NH₂-FI dendrimers and the $\{(Au^0)_7-G5.NHAc-FI\}$ DSNPs. Because the surface plasmon resonance peak of Au NPs at 520 nm overlapped the absorption of FI moieties, it is very hard to confirm the existence of Au NPs through UV-Vis spectroscopy. However, one can see a broad shoulder of absorbance at 550 nm for both the $\{(Au^0)_7-G5.NHAc-FI\}$ and the $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSNPs (as indicated by the arrow in Fig. 1a), providing an indication of the existence of the NPs. Neither the G5.NH₂-FI nor the G5.NH₂-FI-FA shows this specific absorbance feature, indicating that the NP-resulted light scattering effect does not exist in dendrimers in the absence of Au NPs. We note that under the preparation conditions, the reductive transformation of Au(III) ions to Au(0) is complete. Further addition of the strong reducing agent NaBH₄ (3–5 molar excess of Au atoms) did not induce any changes in the UV-Vis spectrum and the color of the solution. The formed Au DSNPs are water-soluble and stable for at least 6 months in water, PBS buffer, or serum (a photograph of Au DSNPs *versus* dendrimer conjugates in PBS buffer (pH = 7.4) is shown in Fig. 1b). The stability of the Au DSNPs in both water and PBS buffer was also occasionally confirmed using UV-Vis spectrometry within 6 months. We do not see any appreciable changes in the absorption features of these Au DSNPs.

The transformation of the remaining terminal amine groups of G5.NH₂-FI and G5.NH₂-FI-FA dendrimers after being complexed with HAuCl₄ was confirmed by ¹H NMR spectroscopy (Fig. S1, ESI[†]). After the acetylation reaction, the appearance of the peak at

[†]Electronic supplementary information (ESI) available: ¹H NMR spectra of the DSNPs $\{(Au^0)_7-G5.NHAc-FI\}$ and $\{(Au^0)_7-G5.NHAc-FI-FA\}$. See DOI: 10.1039/b902199j

1.85 ppm related to the $-\text{CH}_3$ protons of the acetyl groups clearly indicates the formation of acetamide groups on the dendrimer surface of both Au DSNPs.²⁸ However, the aromatic proton peaks related to both FI and FA did not show any significant changes, compared to the ^1H NMR spectra of the $\text{G5.NH}_2\text{-FI}$ and $\text{G5.NH}_2\text{-FI-FA}$ dendrimers, respectively.²⁷ Zeta potential measurements show that after the acetylation reaction, the surface potentials of the $\{(\text{Au}^{3+})_7\text{-G5.NH}_2\text{-FI}\}$ (+35.6 mV) and the $\{(\text{Au}^{3+})_7\text{-G5.NH}_2\text{-FI-FA}\}$ (+30.8 mV) complexes significantly decreased when the $\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$ (+9.2 mV) and the $\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$ (+5.3 mV) DSNPs were formed, further confirming the successful transformation of the dendrimer terminal amines to acetamide groups. We note that the surface potentials of both Au DSNPs are not close to zero, which is opposed to our previous work related to Au DENPs after either FI- or both FI- and FA-functionalization.² This might be due to the fact that some of the amine groups of multiple dendrimers that stabilize Au NPs experience more difficulty to be acetylated as compared to the acetylation of the amine groups of single dendrimers with Au NPs entrapped.

The morphology of the formed Au DSNPs was observed through TEM imaging. Fig. 2 shows the bright-field TEM images and size distribution histograms of the $\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$ and the $\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$ DSNPs, respectively. The average diameters of both the $\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$ and the $\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$ DSNPs are around 6 nm, with approximately similar standard deviation (Fig. 2c, 2f). This implies that both NPs with or without FA conjugation display a similar morphology. Both the $\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$ and the $\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$ DSNPs are crystalline, as confirmed by the selected area electron diffraction (SAED) pattern. The indexed (111), (200), (220), and (311) rings are indicative of the face-centered cubic (*fcc*) crystal structure of the Au DSNPs (data not shown). The EDS spectra of both the $\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$ and the $\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$ DSNPs confirm the existence of the Au element. TEM micrographs (Fig. 2a, 2b, 2d, and 2e) also reveal that both the $\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$ and the $\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$ DSNPs do not aggregate even when dried for TEM sample preparation, suggesting that the dendrimers can effectively stabilize the Au NPs. It is worthwhile to note that under the TEM conditions, dendrimers on the surfaces of the Au NPs are invisible in the TEM images because of their low electron contrast, compared with metal Au. We also note that the size of the FI- and FA-modified Au DSNPs is smaller when compared with the acetamide-functionalized Au DSNPs and the Alexa Fluor 594 dye-functionalized Au DSNPs with acetamide terminal groups described in our previous work.²⁵ This may be attributed to the different dye molecules attached on the dendrimers and the different reaction solvents used.

The cytotoxicity of the functionalized Au DSNPs was evaluated by an MTT colorimetric assay. The cell viability data (Fig. 3) show that the KB cells (a human epithelial carcinoma cell line) treated with both $\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$ and $\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$ DSNPs with or without FA conjugation display similar OD (570 nm) values at an Au DSNP concentration range of 0–2000 nM. This implies that the formed functionalized Au DSNPs are biocompatible at an Au DSNP concentration of even up to 2000 nM. Note that the maximum concentration of Au DSNPs (2000 nM) investigated in this study is similar to that used in our previous studies.^{2,16} We think that the concentration of 2000 nM is adequate for the effective cytotoxicity assay.

The vitamin FA and the dye FI conjugated onto the G5 dendrimer surface were used as a targeting ligand and an imaging molecule, respectively, providing the formed Au DSNPs with both targeting and imaging functionalities. The folic acid receptor (FAR) is well known to be overexpressed in several human carcinomas including breast, ovary, endometrium, kidney, lung, head and neck, brain, and myeloid cancers.^{29–31} In this study, both KB-HFAR (KB cells expressing high-level FAR) and KB-LFAR (KB cells expressing low-level FAR)

cells were selected for the intracellular uptake of functionalized Au DSNPs. Fig. 4 illustrates the dose-dependent binding of the $\{(Au^0)_7-G5.NHAc-FI\}$ and the $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSNPs with KB-HFAR and KB-LFAR cells, investigated by flow cytometry. The cellular uptake of the $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSNPs shows a dose-dependent trend, with saturation and 50% binding occurring at approximately 100 nM and 34 nM, respectively (Fig. 4a), which is much higher than the binding capacity of FA-modified Au DENPs.² For KB-LFAR cells, neither the $\{(Au^0)_7-G5.NHAc-FI-FA\}$ nor the $\{(Au^0)_7-G5.NHAc-FI\}$ DSNPs show any significant binding, even at concentrations up to 300 nM (Fig. 4b). These results indicate that the specificity of the $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSNPs' binding to KB cells is restricted to cells containing high levels of FAR, confirming that the high affinity of FAR mediates the specific uptake of the Au DSNPs. The higher cellular uptake of the $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSNPs as compared with the FA-functionalized Au DENPs described in our previous work² is ascribed to the significantly more FA targeting ligands on each particle than those on each Au DENP, significantly enhancing the polyvalent binding effect.

The conjugation of the FI moiety onto Au DSNPs also enables confocal microscopic imaging of the cellular uptake. Fig. 5a–c shows that only KB-HFAR cells treated with FA-modified $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSNPs display fluorescence signals, which is associated with the specific uptake of $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSNPs into the cytoplasm and also onto the membrane of the cells (Fig. 5c). In contrast, the same KB cells treated with $\{(Au^0)_7-G5.NHAc-FI\}$ DSNPs without FA modification do not show any fluorescence signals (Fig. 5b), which is similar to KB cells treated with PBS buffer (Fig. 5a). This result suggests that the binding and intracellular uptake do not occur in the cells treated with non-FA-modified Au DSNPs. In order to confirm the Au NP uptake, the Au DSPNP-treated cells were stained by a silver enhancement kit. Ag staining of Au has been generally used to image the tissue uptake of Au.³² In our study, it is clear that only the $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSPNP-treated KB-HFAR cells show the prominent brown color (Fig. 5f), which is related to the Au NPs stained by the silver enhancement kit. In contrast, the same KB-HFAR cells treated with PBS buffer and the $\{(Au^0)_7-G5.NHAc-FI\}$ DSNPs do not show any significant silver enhancement (Fig. 5d and 5e).

In order to gain more quantitative information regarding the cellular uptake of functionalized Au DSNPs, ICP-MS was used to analyze the Au concentration within the cells. The results of the intracellular uptake experiments are shown in Table 1. The $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSNPs exhibited a concentration-dependent uptake by the KB-HFAR cells. The same KB cells incubated with $\{(Au^0)_7-G5.NHAc-FI\}$ DSNPs at the concentrations of 50 and 200 nM do not show appreciable uptake of Au, which is quite similar to the KB cells treated with PBS buffer. The higher cellular uptake and internalization of Au indicates that the binding and uptake of the $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSNPs is solely ascribed to FAR-mediated endocytosis.

It is interesting to note that for the purpose of cancer cell targeting and imaging using multifunctional Au NPs, one might use an alternative approach to modify preformed Au NPs with the FI- and FA-modified dendrimers through ligand exchange. However, this approach seems to be more complicated than our one-step spontaneous approach: (1) Au NPs have to be synthesized and purified before ligand exchange; (2) after dendrimer ligand exchange, the non-adsorbed excess dendrimers have to be removed; and (3) the surface charge of the dendrimer-modified Au NPs has to be neutralized to avoid any non-specific binding with cell membranes. Our approach to spontaneously formed multi-functional Au DSNPs with neutral surface charge is rather unique, simple, and adaptable for different biomedical applications.

Conclusions

In summary, we have developed a facile approach to synthesizing multifunctional Au DSNPs through a one-step reaction at room temperature. The formed FI- and FA-functionalized Au DSNPs are water-soluble, stable, and biocompatible. Combined flow cytometry, confocal microscopy, silver enhancement, and ICP-MS analyses show that the FA-modified Au DSNPs can specifically bind to cancer cells overexpressing high-affinity FAR. The developed multifunctional Au DSNPs may be applicable for photothermal therapy of tumors that specifically uptake the Au NPs through inductive heating. This work is currently underway. In addition, recent advances in dendrimer-based nanomedicine show that dendrimers can be covalently linked with various targeting moieties (*e.g.* RGD peptides,^{33,34} hormones,³⁵ growth factors,³⁶ antibodies,³⁷ *etc.*) and drug molecules (*e.g.* methotrexate³⁸ and taxol^{39,40}) for targeted cancer therapy. We envision that the present simple Au DSNP synthetic approach along with tunable dendrimer surface chemistry holds great promise to open new avenues for the fabrication of functionalized NPs for diverse biomedical applications, especially for targeted cancer imaging and therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. (a) UV-Vis spectra of the dendrimer conjugates G5.NH₂-FI (Curve 1) and G5.NH₂-FI-FA (Curve 2), and the formed DSNPs {(Au⁰)₇-G5.NHAc-FI} (Curve 3) and {(Au⁰)₇-G5.NHAc-FI-FA} (Curve 4). (b) Photographs of the corresponding dendrimer conjugates and Au DSNPs in PBS (pH 7.4) buffer. 1, 2, 3 and 4 represent G5.NH₂-FI, G5.NH₂-FI-FA, {(Au⁰)₇-G5.NHAc-FI}, and {(Au⁰)₇-G5.NHAc-FI-FA}, respectively.

Fig. 2. TEM images and size distribution histograms of the DSNPs $\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$ (a–c) and $\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$ (d–f). (b) and (e) show magnified TEM images of $\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$ and $\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$ DSNPs, respectively.

Fig. 3. MTT assay of KB cell viability after treatment with $\{(Au^0)_7-G5.NHAc-FI\}$ and $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSNPs for 24 h. The data are expressed as mean $\pm$ standard deviation.

Fig. 4. Dose-dependent binding of $\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$ and $\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$ DSNPs with KB cells expressing high (a) and low (b) levels of FAR, respectively.

Fig. 5. Confocal microscopy images (a–c) and silver-staining optical microscopy images (d–f) of KB-HFAR cells with different treatments for 2 h. (a) and (d) KB cells treated with PBS buffer; (b) and (e) KB cells treated with $\{(Au^0)_7-G5.NHAc-FI\}$ DSNPs; and (c) and (f) KB cells treated with $\{(Au^0)_7-G5.NHAc-FI-FA\}$ DSNPs. In confocal images, the nucleus of the cells was blue-stained with Hoechst33342, and the green color was originated from the FI dye conjugated on dendrimers.

Scheme 1.

Schematic representation of the approaches to synthesizing $\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$ (a) and $\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$ (b) DSNPs.

Table 1

Cellular uptake of Au DSNPs determined by ICP-MS

Materials used to treat cells	Conjugate concentration (nM)	Au concentration (nM)	Cellular uptake of Au (fg/cell)
Non-treated control cells	0	0	0.385 ± 0.15
$\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$	50	350	0.375 ± 0.55
$\{(\text{Au}^0)_7\text{-G5.NHAc-FI}\}$	200	1400	0.44 ± 0.15
$\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$	50	350	5.78 ± 0.20
$\{(\text{Au}^0)_7\text{-G5.NHAc-FI-FA}\}$	200	1400	12.97 ± 0.50