Folic acid-modified diatrizoic acid-linked dendrimer-entrapped gold nanoparticles as nanoprobes for targeted CT imaging of human cervical cancer

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ABSTRACT

Objective(s): The development of multifunctional nanoprobes for specific targeting and imaging of tumors presents a great challenge. Herein, we report the synthesis and characterization of folic acid (FA) gold nanoparticles (AuNPs) through diatrozic acid (DTA) linking for in vitro and in vivo targeted imaging of HeLa cells by computed tomography (CT).

Materials and Methods: In this study, G5 dendrimers were used as templates to synthesize AuNPs within the dendrimer interiors. The cytotoxicity and hemocompatibility of the particles were evaluated by morphological observation of cells after hematoxylin and eosin staining, cell viability assay, flow cytometric analysis of cell cycle and apoptosis, and hemolytic assay. The cellular uptake of the particles was confirmed through inductively coupled plasma atomic emission spectroscopy determination of silver and transmission electron microscopy. Finally, in vitro and in vivo targeted CT imaging performances were evaluated using HeLa cells and a xenografted HeLa tumor model, respectively.

Results: We showed that Au DENPs-FA-DTA did not significantly affect cell morphology, viability, or the cell cycle and apoptosis, thereby indicating their good biocompatibility and stability in the given concentration range. Micro-CT images showed that HeLa cells could be detected by X-ray after incubation with Au DENPs-FA-DTA in vitro and that the xenograft tumor model could be imaged after intravenous and intratumoral administration of the particles. The FA-modified AuNPs enabled targeted CT imaging of HeLa cells overexpressing FA receptors in vitro and the corresponding xenografted tumor model in vivo.

Conclusion: Our results demonstrated that the designed AuNPs show great potential as probes for targeted CT imaging of human cervical cancer.

Keywords: Cervical cancer, Computed tomography, Dendrimers, Folic acid, Gold nanoparticles

INTRODUCTION

Cervical cancer is one of the most common female reproductive tract diseases and the leading cause of female deaths worldwide [1]. When cervical cancer is detected at the early or preinvasive stages, it can be treated by resection with standard concurrent chemotherapy and radiotherapy. Unfortunately, a substantial proportion of patients diagnosed with invasive cervical cancer suffer relapses or progress in cancer stage after their initial treatment. Thus, the development of novel safe and effective approaches to treat the disease remains a priority [2]. Current cervical cancer therapy, including chemotherapy and radiation
therapy, often involves non-specific eradication of normal cells, which limits their therapeutic potential. Recent important findings in nanomedicine and nanotechnology research can potentially revolutionize numerous aspects of cervical cancer diagnosis and treatment [3-5].

Considerable research effort has been exerted to develop various nanoparticle (NP) systems that can selectively target, detect, and eliminate cancer cells at the early stages. Among the many different nanocarrier systems available, gold nanoparticles (AuNPs) have been shown to be a promising platform for molecular diagnostics, imaging, targeting, and treatment of cancer cells for a number of reasons [6-10]. First, because of its higher atomic number and electron density, Au has a higher X-ray attenuation intensity than iodine and can thus promote enhanced contrast in CT images. Second, AuNPs are nontoxic within a certain concentration range [11]. Third, they can be easily functionalized with moieties such as targeting agents, imaging dyes, or specific biomarkers [12]. Finally, appropriate surface treatment of AuNPs can help increase their blood circulation time by decreasing clearance via the reticuloendothelial system (RES) [13]. AuNPs can penetrate the leaky endothelium of the tumor vasculature and preferentially accumulate into tumor cells through the enhanced permeation and retention (EPR) effects of the tumor tissue, thereby enhancing imaging efficacy [14].

Dendrimers are a class of nanostructured macromolecules characterized by a three-dimensional highly regular branched structure with a large number of reactive end groups [15-17]. The exceptional physicochemical properties of dendrimers enable their facile use as templates to synthesize AuNPs [18-20]. Dendrimers can be constructed to feature varying molecular weights and chemical compositions besides having a polyfunctional surface that facilitates the attachment of drugs and pharmacokinetic modifiers or targeting moieties [21]. Moreover, the remaining amine groups of dendrimers can be easily acetylated to shield their positive potential, thereby avoiding nonspecific binding and toxicity [18]. Our previous study has shown that Au dendrimer-encapsulated NPs (DENPs) or Au dendrimer-stabilized NPs (DSNPs) can be covalently bound to targeting molecules for specific targeting of cancer cells [22-23]. These studies suggest that the “active” targeted CT imaging of cancer cells may be realized using acetylated Au DENPs after the surface is modified with targeting molecules.

In the present study, FA- and FI-modified G5 were complexed with HAuCl₄, after which the dendrimer terminal amines were acetylated to create multifunctional Au DENPs using a one-step reaction at room temperature (Scheme 1).

The PEGylated dendrimers were then used as templates to entrap AuNPs within the dendrimer interiors; this step was followed by acetylation to neutralize the remaining dendrimer terminal amines (Scheme 1). The synthesized DENPs were characterized using ultraviolet–visible light (UV–Vis) spectrometry, proton nuclear magnetic resonance (¹H NMR) spectroscopy, transmission electron microscopy (TEM), and X-ray attenuation measurements. The cytotoxicity and hemocompatibility of the particles were evaluated by morphological observation of cells after hematoxylin and eosin (HE) staining, cell viability assay, flow cytometric analysis of the cell cycle and apoptosis, and hemolytic assay.

The cellular uptake of the particles was confirmed through silver staining of cells and TEM. Finally, in vitro and in vivo targeted CT imaging performances were evaluated using HeLa cells and a xenografted HeLa tumor model, respectively. To the best of our knowledge, this work is the first to report the development of Au DENPs for in vitro and in vivo CT imaging of cervical cancer cells. Through unique dendrimer nanotechnology and PEGylation conjugation chemistry, FA-modified multifunctional Au DENPs can be synthesized for targeted CT imaging of cervical cancer via an active folic acid receptor (FAR)-mediated targeting strategy.

Scheme 1. Schematic illustration of the preparation of Au DENPs-FI-DTA and Au DENPs-FI-FA-DTA
MATERIALS AND METHODS

Experimental

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). FA, FI, DTA, acetic acid, and all other chemicals and solvents employed in this work were obtained from Sigma-Aldrich and used as received. The water used in all of the experiments was purified using a Milli-Q Plus 185 water purification system with a resistivity greater than 18 MΩ.cm. Regenerated cellulose dialysis membranes were acquired from Fisher. The FI, FA, and DTA functionalized G5 were synthesized and characterized according to our previous work [26]. FI- and DTA-functionalized G5 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 4.1, respectively. Penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Sigma. Trypsin–ethylenediaminetetraacetate (EDTA), Dulbecco’s phosphate-buffered saline (PBS), Dulbecco’s modified Eagle medium (DMEM), and bovine serum albumin were obtained from GIBCO-BRL. A silver enhancement kit was obtained from Sigma.

Synthesis of FI-, FA-, and DTA-functionalized Au DENPs

Au DENPs-FI-FA-DTA complexes were first prepared by adding a methanol solution of HAuCl₄ into a methanol/water mixture solution of G5 at a molar ratio of G5 atoms equivalent to 1:50. The formed complex was further modified by FI, FA, and DTA, followed by acetylation of the remaining dendrimer terminal amines, as described in our previous reports [10, 17, 23, 27-28].

Characterization techniques

UV–Vis spectra were collected using a Perkin-Elmer Lambda 20 UV–Vis spectrometer.

The ¹H NMR spectra of the Au DENPs were recorded on a Bruker DRX 400 NMR spectrometer. Samples were dissolved in D₂O before the NMR measurements. TEM measurements were performed at 200 kV using a JEOL 2010F analytical electron microscope with an energy dispersive spectroscopy system attached. A 5 µL aqueous solution of Au DENPs (3 mg/mL) was dropped onto a carbon-coated copper grid and air-dried before the measurements.

Cell cultures and biological evaluation

HeLa cells were purchased from Shanghai Cell Bank and continuously grown in two 10 cm culture dishes, one in FA-free media and the other in regular DMEM cell culture medium supplemented with penicillin, streptomycin, 10% heat-inactivated FBS, and 2.5 µM FA. The cells grown in FA-free media expressed high-level FAR (HFAR), whereas the cells grown in FA-containing media expressed low-level FAR (LFAR).

MTT quantitation of cell viability

Cytotoxicity of acetylated Au DENPs

Cell viability was measured using the 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, approximately 5 × 10⁵ HeLa cells per well were seeded into a 96-well plate. After overnight culture, functionalized Au at concentrations ranging from 0 µM to 2 µM in PBS were added to each well. After 24 h of incubation with Au, the medium in each well was replaced by medium without serum containing MTT solution; the plate was then incubated for 4 h at 37 °C. The medium was then carefully removed, the cells were washed with PBS buffer, and dimethyl sulfoxide was added to the wells. The absorbance values of each well were measured at a wavelength of 490 nm using a microplate reader.

Cell morphology after treatment with acetylated Au DENPs was observed after HE staining. Approximately 3 × 10⁵ HeLa cells were plated in each well of 6-well cell culture plates, and each well was covered with a poly-L-lysine -coated cover slip. The cells were cultured for 24 h to grow to 80% confluence. Acetylated Au DENPs were then incubated with the cells for 4 h, after which the cells were washed with PBS three times and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 2 h. The cell samples on the cover slips were washed twice for 5 min with PBS, dipped into a Coplin jar containing hematoxylin for 30 s, rinsed with water for 1 min, and stained with 1% eosin Y solution for 30 s. Finally, the cover slips were dehydrated and mounted onto glass slides. The morphology of the cells was observed using an optical microscope.

Flow cytometry analysis

HeLa cells were seeded in 12-well plates at a density of 5 × 10⁴ cells per well in four replicates and grown to confluence after overnight culture at 37 °C and 5% CO₂. Then, the medium was removed and replaced with fresh medium containing different Au DENPs-FA concen-
trations; the cells were subsequently incubated at 37 °C and 5% CO<sub>2</sub> for 4 h, trypsinized, and suspended in PBS containing 0.1% bovine serum albumin. The cells were analyzed using a Becton Dickinson FACScan analyzer, the FL1 fluorescence of 1 × 10<sup>5</sup> cells was measured, and the mean fluorescence of the gated viable cells was quantified.

**Cellular uptake of acetylated Au DENPs**

The cellular uptake of acetylated Au DENPs was confirmed by inductively coupled plasma atomic emission spectroscopy (ICP-AES), TEM, and silver staining. HeLa-HFAR cells were plated in a 12-well plate at a density of 5 × 10<sup>5</sup> cells/well 1 d prior to the experiment. The cells were incubated with 50 nM or 200 nM of functionalized Au in FA-free media or FA-DMEM at 37 °C and 5% CO<sub>2</sub> for 24 h. After incubation, the cells were washed once with Versene, twice with PBS, and three times with Hank’s buffered salt solution. The cells were then lifted with trypsin–EDTA, resuspended in 200 µL of PBS, and dispersed into 1.8 mL of 10% FBS in PBS. The cell suspension was counted. The remaining cells were centrifuged to form pellets and lysed using an aqua regia solution. The Au NPs were digested after aqua regia treatment, and Au uptake was measured with a Finnigan instrument.

Approximately 1.25 × 10<sup>4</sup> cells were seeded into 3 wells of a 24-well plate 1 d prior to the experiments. An hour before initiating the experiment, the cells were rinsed four times with serum-free and FA-deficient DMEM. Functionalized Au DSNPs were added at a final concentration of 50 nM. After 24 h of incubation with the functionalized Au DSNPs at 37 °C, the HeLa-HFAR and HeLa-LFAR cells were rinsed with PBS and treated with 0.1% trypsin. The cells were then washed with PBS, divided into four equal portions, and spun onto slides using a Shandon Cytospin 3 cyt centrifuge. The slides were rinsed, treated with Lugol’s solution, and stained with a silver enhancement kit according to the manufacturer’s instructions. The silver staining time was optimized to differentiate the cell images under different conditions better. Silver staining for 5 min yielded the best contrast between the control cells and cells treated with Au DSNPs. The cell morphology after silver staining was also observed using a microscope.

For further TEM imaging of the distribution of the acetylated Au DENPs within the cells, HeLa cells were plated in 6-well cell culture plates at a density of 3 × 10<sup>5</sup> cells per well in DMEM medium with 10% FBS in a humidified incubator for 24 h to grow to 80% confluence. Then, acetylated Au DENPs were added to each well at a final concentration of 2,000 nM, and cells incubated for 12 h at 37 °C. The culture medium was discarded and the cells were washed with PBS, trypsinized, centrifuged, washed three times with PBS, and finally fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer for 12 h at 4 °C and post-fixed with 1% OsO<sub>4</sub> in 0.2 M phosphate buffer for 2 h at 4 °C. After additional washing in PBS, the cells were dehydrated in 30%, 50%, 70%, 95%, and 100% ethanol solutions. The cell samples were then embedded with Epon 812 and polymerized. Then, the embedded cells were sectioned using a Reichart instrument. Sections with a thickness of 75 nm were mounted onto 200-mesh copper grids and counterstained with uranyl acetate and lead citrate for 5 min before TEM measurements. The grids were visualized using an H600 transmission electron microscope with an operating voltage of 60 kV.

Confocal microscopic analysis was performed using an Olympus Fluoview 500 laser scanning confocal microscope. The cells were plated on a plastic cover slip before measurement. FI fluorescence was excited with a 488 nm argon blue laser, and emissions were measured through a 505–525 barrier filter. The optical section thickness was set to 5 µm, and cells were incubated with functionalized Au DSNPs for 24 h, followed by rinsing with PBS. The nuclei were counterstained with DAPI for 10 s. Samples were scanned using a 60× water-immersion objective lens and magnified with Fluoview software. All samples were imaged under similar instrumental conditions. The same method was also applied to analyze the xenograft tumor tissues.

**In vitro micro-CT imaging**

HeLa cells were incubated with Au DENPs-FI-FA-DTA and Au DENPs-FI-DTA with concentrations of 0, 100, or 300 nM for 24 h at 37 °C. After washing with PBS three times, cells were trypsinized, centrifuged, and resuspended with 100 µL of PBS in a 0.5 mL Eppendorf tube containing approximately 1.5 × 10<sup>6</sup> cells in each tube. The cell suspension in each tube was placed in a self-designed scanning holder and then scanned using a micro-CT imaging system. Images were reconstructed on a micro-CT imaging workstation.
In vivo micro-CT imaging

Animal experiments and animal care were carried out according to protocols approved by the Institutional Committee for Animal Care and the policy of the National Ministry of Health. Male 4-to 6-week-old BALB/c nude mice were subcutaneously injected in the right side of their backs with $1 \times 10^6$ HeLa cells/mouse. When the tumor nodules had reached a volume of $1.0 \pm 0.15 \text{ cm}^3$ after approximately 3 weeks post-injection, the tumor was confirmed by gross specimen and HE staining to show the HeLa cell features. The mice were placed in a scanning holder and then scanned using a micro-CT imaging system using parameters similar to those applied for in vitro experiments. CT scanning was performed both before and after intratumoral or intraperitoneal injection of Au DENPs at time points of 0, 2, 4, and 6 h post-injection [29]. Images were reconstructed on a micro-CT imaging workstation. CT values were acquired on the same workstation using the software supplied by the manufacturer.

In vivo biodistribution of Au

After CT imaging of the tumor model 6 h after injection of the DENPs via intraperitoneal and intravenous injection, the mice were euthanized by CO$_2$ inhalation, their heart, lung, stomach, spleen, liver, intestines, kidney, testicle, blood, tumor, and brain were extracted and weighed. The organs were cut into 1–2 mm$^2$ pieces and incubated in aqua regia solution for 4 h. Au content was determined by using an ICP-AES. After CT imaging, animals were anesthetized by intraperitoneal injection of chloral hydrate and subjected to endovascular perfusion with 4% paraformaldehyde via an open-chest, left cardiac ventricle puncture approach. The heart, lung, spleen, liver, intestines, and kidney were subjected to rapid frozen sectioning, cut into sections (8 µm) with a cryostat, and collected on poly-D-lysine coated slides for HE staining and silver staining. Mice were sacrificed at 3, 6, 9, and 12 h. Blood and organs were collected and kept in liquid nitrogen.

Statistical analysis

All data were expressed as means ± S.D. Comparisons between two groups were analyzed by Student’s $t$-test, and those between multiple groups were evaluated by one-way analysis of variance (ANOVA) followed by LSD’s tests using SPSS 15.0 software. The p values $<0.05$ were considered significant.

RESULTS AND DISCUSSION

Synthesis and characterization of Au DENPs-FI-FA-DTA

Similar to our previous work on the formation of acetamide-functionalized Au DENPs and dye-functionalized Au DENPs with acetamide surface groups [30-33], we found that FI-, FA-, and DTA-functionalized Au with close-to-neutral surface charges could be spontaneously formed by acetylation of the remaining dendrimer terminal amines complex (Scheme 1). The UV–Vis spectra of the formed Au DENPs are shown in Fig 1. Both the Au DENPs-FI-DTA and Au DENPs-FI-FA-DTA show absorbance peaks at 510 nm, which corresponds to the characteristic absorption of FI and AuNPs because the surface plasmon resonance peak of AuNPs at 510 nm overlaps the absorption of FI moieties. By contrast, only the featured FA absorbance peak at 280 nm was observed in the Au DENPs. The $^1$H NMR spectra of the Au DENPs are shown in Fig. 2. The peaks located at 2.30–3.40 ppm and 1.95 ppm belong to the –NH protons of G5 and –COCH$_3$ protons of DTA. The appearance of –COCH$_3$ protons located at 1.87 ppm in both Au DENPs-FI-DTA and Au DENPs-FI-FA-DTA confirms further acetylation of the remaining amines of the dendrimers. The peak of protons of FI and FA was observed between 6.00–8.00 ppm. A slight difference could be found in Fig. 2 at approximately 7.5 ppm, which belongs to FA of Au DENPs-FI-FA-DTA. These results are consistent with the obtained UV-Vis spectra.

![UV-vis spectra of the formed Au DENPs-FI-DTA (a) and Au DENPs-FI-FA-DTA (b)](image)

The formed Au DENPs were also characterized by TEM. TEM images of the Au DENPs are shown in Fig. 3. The size of both AuNPs clearly falls within a relatively narrow range of 2–6 nm, and nearly no difference between their morphologies was observed. The formed Au DENPs are water-soluble and stable for at least 6
months in both PBS and cell culture medium. No significant difference in DTA was observed between the PBS buffer (Fig 4b) and cell culture (Fig.4c).

**Cytotoxicity of acetylated Au DENPs**

To evaluate the morphological changes of HeLa cells after incubation with the acetylated Au DENPs, the treated cells were stained with HE (Fig 5). Results showed that even if the concentration of DENPs was increased to 2,000 nM, the cells did not show significant morphological changes when compared with untreated control cells. These results indicate that acetylated Au is non-cytotoxic and does not affect the morphology of HeLa cells within a certain concentration window. Compared with the control group, no changes were observable in the HeLa cell morphologies. FA by itself did not influence cell viability compared with the control in our experiment. In addition, the MTT assay results did not indicate any cellular toxicity after incubation of HeLa cells with the NPs for 24 h (Fig 6). Even at ten-fold doses of FA, cell viability was not significantly influenced by the NPs, likely because the NPs possess both an essentially inert, nontoxic Au core and biocompatible surface-confined FA [34-35]. Cell cycle damage is one of the most important features of cytotoxicity. Cell phase distributions are usually analyzed by determining DNA content, and the fraction of DNA content in the sub-G1 phase is an indicator of cell apoptosis [37-38]. To investigate the influence of acetylated Au DENPs on cell apoptosis, the treated cells were analyzed by flow cytometry [8]. The sub-G1 fractions of HeLa cells incubated with acetylated Au DENPs at concentrations of 1,000 nM and 2,000 nM were 4.87% ± 0.87% and 4.98% ± 0.91%, respectively, in the quadruplicated experiment (Fig 7). No statistically significant differences were observed with that of the untreated negative control cells. As shown in Table 1, acetylated Au DENPs had no effect on the cell cycle of HeLa cells. These results further confirm that acetylated Au DENPs are non-cytotoxic at the given concentration range.

![Representative microscopic images of HE-stained HeLa cells: negative control cells without treatment (a), and cells incubated with ([Au0]300-G5.NHAc-(PEGFA)-mPEG) DENPs at a Au concentration of (b) 100 mM and (c) 300 mM for 24 h](image)
Table 1. Apoptosis and cell cycle analysis of HeLa cells after incubation with the \((\text{Au}0)_{50}-\text{G5.NHAc-FA5}\) DENPs for 4 h (mean \(\pm\)S.D., \(n = 4\)).

<table>
<thead>
<tr>
<th>Group</th>
<th>Apoptosis (%)</th>
<th>Cell cycle (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>G0-G1</td>
</tr>
<tr>
<td>Control</td>
<td>5.17(\pm)0.93</td>
<td>59.45(\pm)6.67</td>
</tr>
<tr>
<td>100 mM (Au)</td>
<td>4.87(\pm)0.87</td>
<td>65.42(\pm)6.21</td>
</tr>
<tr>
<td>300 mM (Au)</td>
<td>4.98(\pm)0.91</td>
<td>66.68(\pm)6.93</td>
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**In vitro cellular uptake of Au DENPs-FI-FA-DTA**

In the presence of the targeting ligand FA, cervical cancer cells overexpressing high-affinity FAR should be able to take up the developed DENPs. We explored the targeted cellular uptake of the AuNPs. After incubation with the DENPs for 4 h, both HeLa-HFAR and HeLa-LFAR cells were silver stained for optical microscopic observation (Fig 8), the cells incubated with Au DENPs-FI-FA-DTA (at the Au concentration of 100 mM (Fig.8b and e) and 300 mM (Fig.8c and and f) turned to black, and the color became darker at the higher Au concentration (Fig. 8c and f).

This finding suggests that the linked FA moiety enables the specific uptake of the particles by cervical cancer cells with high-level FAR expression via a receptor-mediated pathway [23].

The non-specific uptake of the particles by HeLa cells could be due to two possible mechanisms: phagocytosis and diffusion via cell walls [36-39].

To confirm the distribution of Au in cancer cells, TEM images were obtained. Our study showed that the electron-staining particles in cells treated by FA were significantly increased (Fig 9b) in the cytoplasm of the control HeLa cells compared to those no electron-staining particles without treatment (Fig 9a).

The TEM results confirmed that the Au DENPs-FI-FA-DTA were internalized by the cells instead of adhering to the surface. Based on previous studies, the uptake of Au likely occurs through two distinct mechanisms: phagocytosis and diffusion via cell walls [39]. Taking these results together, we can conclude that greater amounts of Au can be taken up by HeLa cells than Au without FA, primarily because of the linked FA ligands that induce receptor-mediated endocytosis [40]. The specific uptake of FA enables the use of the particles as imaging probes for targeted CT imaging of HeLa cells in vitro.

**Fig. 6.** MTT assay of the viability of HeLa cells treated with \([\text{Au0}]_{300}-\text{G5.NHAc-(PEG-FA)-mPEG}\) DENPs at different Au concentrations for 24 h (\(n = 5\)).

**Fig. 7.** Flow cytometry analysis of HeLa cells treated without (a) or with\(([\text{Au0}]_{50}-\text{G5.NHAc-FA5}\) DENPs at the concentration of 1000 nM (b) and 2000 nM (c), respectively for 4 h (\(n = 3\)).
**Targeted micro-CT imaging of HeLa cells in vitro**

With the established targeting specificity of Au DENPs-FI-FA-DTA to FAR-overexpressing cancer cells and the suitable X-ray attenuation property of the particles, we next explored the potential to use them as nanoprobes for targeted CT imaging of HeLa cells in vitro. After incubation with Au DENPs-FI-DTA and Au DENPs-FI-FA-DTA for 4 h, HeLa-HFAR cells were imaged by CT. Considering the difficulty associated with differentiating the brightness of the CT images of the cells treated with Au with different Au concentrations by the naked eye, the CT signal intensity must be quantitatively evaluated via the manufacturer’s standard display program. The CT values of the HeLa-HFAR cells showed that all of the HeLa-HFAR cells treated with either Au DENPs-FI-DTA or Au DENPs-FI-FA-DTA had higher CT values than control cells without treatment (Fig 11). Moreover, the HeLa-HFAR cells incubated with DENPs showed significantly higher X-ray attenuation than those treated with Au DENPs-FI-DTA at the same Au concentrations. These results verify the effects played by the linked FA moieties, which enable specific CT imaging of FAR-overexpressing cancer cells through a FA-mediated targeting pathway.

**Targeted micro-CT imaging of HeLa cells in vivo**

The suitable performance of the developed FA-targeted Au for specific targeting and CT imaging of cancer cells in vitro led us to investigate their potential for CT imaging of cervical cancer in vivo. A xenografted HeLa cervical cancer model was established in BALB/c nude mice (Fig 12a and Fig 12b). For intravenous injection, the tumor CT values treated with DENPs were much higher than those treated with the non-targeted DENPs at the same time points. Moreover, cancer mice pre-injected with free FA showed much less tumor CT values after injection of the targeted Au DENPs than those without free FA injection (Fig 12b). These results demonstrate that the tumor CT enhancement is mediated by FA targeting and that the tumor tissue pre-treated with free FA blocks the binding of the Au. Hence, at the same time points, the tumor CT values for Groups 3 and 5 did not show significant differences.

Similarly, for intraperitoneal injection, the tumor CT values in Group 2 were much higher than those in Groups 4 and 6 at the same time points, and FA blocking treatment did not result in notable differences in tumor

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**Expression of FR on both HeLa cells and xenografts**

Conjugation of the FI moiety onto Au DENPs enables confocal microscopic imaging of the uptake of the cells. Only HeLa-HFAR cells treated with Au DENPs-FI-FA-DTA showed fluorescence signals, which is related to the specific uptake of DENPs onto the membrane and into the cytoplasm of the cells (Fig. 9). By contrast, the same HeLa cells treated with DENPs without FA modification did not display any fluorescence signals (Fig. 10a). Likewise, Fig. 10b shows the expression of FR in the nude mice HeLa xenograft tumor. Fluorescence signals of the HeLa xenograft tumor tissue treated with DSNPs could be clearly detected. These findings confirm that both HeLa cells and HeLa xenograft tumor model express FR and that binding and intracellular uptake do not occur in the cells treated with Au.

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**Fig. 8.** Representative microscopic images of HeLa-HFAR (a–c) and HeLa-LFAR (d–f) cells with silver staining. (a) and (d) are negative control cells without treatment. The cells incubated with Au DENPs-FI-FA-DTA at the Au concentration of 100 mM (b and e) and 300 mM (c and f), respectively.

**Fig. 9.** TEM images of HeLa cell: (a) negative control cells without treatment, (b) cells incubated with Au DENPs-FI-FA-DTA at a concentration of 2000 nM for 12 h. The black arrow indicates particles in the lysosomes of the cells.
CT values. Intravenous injection of Au DENPs-FI-FA-DTA led to greater tumor CT enhancement than intraperitoneal injection of the same particles at the same time points. However, for Au DENPs-FI-DTA or Au DENPs-FI-FA-DTA injected into a free FA-blocked tumor model, the injection route did not noticeably affect tumor CT enhancement at the same time points. Thus, the optimized tumor CT imaging conditions are as follows: intravenous injection; targeted Au DENPs; and CT scanning 6 h post-injection. Our results clearly demonstrate that the developed DENPs have great potential as nanoprobes for targeted CT imaging of tumors overexpressing FAR. The biodistribution of the DENPs 6 h post-injection through the two administration routes employed in this work was studied using ICP-AES to analyze Au concentrations in the tumor as well as in several major organs, such as the heart, lung, stomach, spleen, liver, intestine, kidney, testicle, blood, and brain. For both intraperitoneal and intravenous injections, the spleen showed the most significant Au uptake at the two time points evaluated. Less uptake of Au in other major organs revealed that Au can escape from RES located in other major organs, thereby allowing for effective transport of the particles to the tumor tissues via passive enhanced EPR effects [10]. For Au DENPs-FI-FA-DTA, besides passive EPR effects, FA-mediated active targeting affords much higher Au uptake in the tumor tissue for both injection routes than that in the nontargeted and free FA-blocked groups; such a phenomenon allows specific targeted CT imaging of the tumors. The tumor Au uptake data are in accordance with the CT imaging results. Detailed pharmacokinetic studies of the Au DENPs over extended time periods are essential to understanding the biodistribution of the NPs fully.

(a)

GFP

DAPI

Merge

HeLa-LFAR

HeLa-HFAR

(b)

Fig. 10. Confocal microscopy images of HeLa-LFAR cells and HeLa-HFAR cells with Au DENPs-FI-FA-DTA for 2 h (a) and HeLa tumor xenografts (b). The nucleus of the cells were stained blue with DAPI, and the green coloration originated from the FI dye conjugated to the dendrimers.
AuNPs for targeting cervical cancer

Histological studies

To verify uptake of Au DENPs in the targeted cervical cancer cells, tumor xenografts were sliced and treated with a silver enhancement kit (Fig 13). In contrast to the control sample without treatment, which did not exhibit brown spots, all tumor slices treated with either targeted DENPs or non-targeted DENPs by different routes 6 h post-injection exhibited numerous dark brown spots in the cytoplasm of the cells; these spots are related to the AuNPs. Moreover, the Au density in the staining appeared to be in the same order as that determined by ICP-AES, further demonstrating the targeted cervical cancer cells Au uptake of the Au DENPs through different injection sites. Moreover, HE staining of tumor sections in different groups indicates that the morphology of tumor cells is not influenced after the injection of the Au through different routes. To explore the effect and metabolism of DENPs in healthy nude mice in response to intraperitoneal or intravenous injection for one month, HE and silver staining of major organs were performed on tumor tissue sections and examined using an optical microscope.

Compared with the control group with no treatment, the morphologies of major organs do not appear to have marked changes, implying that the injected DENPs through either intraperitoneal or intravenous injection do not display apparent in vivo toxicity to the organs. In the meantime, the results of silver staining demonstrate that after a month of metabolism, nearly no Au was observed in all the organs for both intraperitoneal and intravenous injection, suggesting that the injected PEGylated Au DENPs are excreted from the body over time, which is of great importance for their biomedical imaging applications.
Fig. 13. HE and silver staining of the HeLa tumor section in nude mice before (g) and after injection with [(Au0)300-G5.NHAc-FA]-mPEG DENPs (a–d) and [(Au0)300-G5.NHAc-FA]-mPEG DENPs (e and f) by different injection routes for 6 h.

Biodistribution of Au DENPs-FA

Determining the biodistribution of the Au DENPs-FI-FA-DTA is critical for their application as nanoprobes for in vivo CT imaging. The biodistribution of the Au DENPs-FI-FA-DTA in several major organs, such as heart, liver, lung, stomach, spleen, kidney, intestines, testicle, brain, and tumor, 6 h after intraperitoneal or intravenous injection of the DENPs was analyzed using ICP-AES (Fig 14).

Besides the kidneys and tumor, large amounts of elemental Au were detected in the lung, spleen, and liver, which are known as the RES organs [41]. This finding demonstrates that besides tumor accumulation, Au can be excreted mainly through the RES and renal-urinary routes.

Further efforts to reduce capture by RES, as well as detailed pharmacokinetic studies of the nanoprobes, should be carried out to achieve better understanding of the mechanism involved in this process.

In general, our biodistribution studies illustrated that the developed Au can escape from the RES and be taken up by tumor cells through different administration methods, allowing effective CT imaging of tumors.

Taken together, Au DENPs were synthesized and evaluated for targeted CT imaging of a HeLa in vitro and in vivo. Our results suggest that HeLa cells could be detected through X-ray attenuation after
incubation with Au in vitro, and that xenograft tumor tissues can be effectively imaged after intravenous, intraperitoneal, and intratumoral administration of the NPs. Morphological observation of cells after HE staining, MTT assay of cell viability, flow cytometry analysis of the cell cycle and apoptosis, and ICP-MS analysis showed that the FA-modified Au DSNPs can specifically bind to cancer cells overexpressing high-affinity FAR and that Au DSNPs are non-cytotoxic, and have no remarkable effects on cell morphology, viability, and the cell cycle and apoptosis. These findings indicate suitable biocompatibility at the studied concentration range.

TEM data further indicated that Au is taken up mainly in the lysosomes of the cells.

The in vivo results from this study demonstrated that the developed Au DSNPs are promising nanoprobes for targeted CT imaging of human cervical cancer. Considering the facile nature of dendrimer nanotechnology and versatility of surface conjugation chemistry, various targeted AuNPs may be synthesized in the future for targeted CT imaging of different types of cancer.

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