ORIGINAL ARTICLE

Gold nanorods for target selective SPECT/CT imaging and photothermal therapy in vivo

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ABSTRACTThe development of theranostic agents with high detection sensitivity and antitumor efficacy at low concentration is a
challenging task for target selective imaging and therapy of cancers. In this study, folate-conjugated and radioactive-iodine-
labeled gold nanorods (GNRs) were designed and synthesized for target selective SPECT/CT imaging and subsequent
thermal ablation of folate-receptor-overexpressing cancers. Both (*ortho*-pyridyl) disulfide-poly(ethylene glycol)-folate and
a short peptide, H₂N-Tyr-Asn-Asn-Leu-Ala-Cys-OH, were conjugated on the surface of the GNRs through thiol chemistry.
The tyrosine in the peptide sequence was introduced for radioactive-iodine labeling through an iodine-125-labeled GNRs
in human serum showed that 91% of the iodine-125 remained intact on the GNRs after incubation for 24 h. *In vitro* and
in vivo results in this study confirmed the potential utility of folate-conjugated and iodine-125-labeled GNRs as smart
theranostic agents. This type of platform may also be useful for the targeted SPECT/CT imaging and photothermal therapy
of inflammatory diseases such as atherosclerosis and arthritis, in which folate-receptor-overexpressing macrophages play
pivotal roles.**KEY WORDS**Target selective imaging; gold nanorods; SPECT/CT; photothermal therapy

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Introduction

Theranostic agents for simultaneous cancer diagnosis and therapy have received a great deal of attention. In particular, a variety of nanomedicines have been designed as theranostics due to their advantageous properties, which include a relatively large surface area for tagging targeting ligands and contrast agents, enhanced permeation and retention (EPR) in the circulatory system, and size- and shape-dependant optical and magnetic

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Receptor molecules overexpressed on the surface of cancer cells have been used as important diagnostic and therapeutic targets for the detection and therapy of human cancers. However, there are a limited number of target receptors on the cancer cell surface for specific binding with targeting ligands. Therefore, development of theranostic agents with high detection sensitivity and antitumor efficacy at low concentration is desirable for the target selective imaging and therapy of cancers. Intriguingly, the detection sensitivity of single-photon emission computed tomography (SPECT) imaging is relatively high (i.e., in the picomolar range), which enables the visualization of whole-body biological processes in vivo without inducing pharmacological effects (7-9). Thus this technique has been widely used for the molecular imaging of various diseases after labeling probes with radioisotopes (for example, radioactive iodine isotopes and technetium-99m). In addition to SPECT imaging, which provides molecular information with no depth limitation, additional anatomic information using x-ray computed tomography (CT) is very helpful for accurate interpretation of the images obtained. Therefore, SPECT/CT fusion imaging is generally used in clinical applications to obtain an improved



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Scheme 1. Synthetic procedure for folate-conjugated and radioactive-iodine-labeled GNRs, FA-¹²⁵I-GNR.

imaging contrast and anatomic information (10,11).

Recently, photothermal therapy using biocompatible plasmonic nanomaterials has received considerable attention as a new candidate technique for cancer treatment. Among several types of plasmonic metal nanoparticles, including nanoprisms, nanoshells, and spherical particles, gold nanorods (GNRs) have proved to be the most suitable for in vivo photothermal therapy because of their high light absorption coefficient in the near-infrared (NIR) region (600-900 nm) (4,12), excellent photothermal stability during laser illumination (13,14), and a better heat generation rate than other nanoparticles (15). It would be ideal if GNRs can be simultaneously used for enhanced CT imaging and the subsequent photothermal therapy of the cancers. However, our recent study showed that the optimum concentration of GNR for effective and homogeneous thermal ablation is less than 0.5 nM which is considerably smaller than that required for CT enhancement (13). On the basis of this observation, we speculate that labeling GNRs with radioisotopes and targeting ligands will prove to be a successful application of GNRs as a theranostic tool for target selective imaging and therapy.

Folate receptor (FR) is one of the representative targets for cancer imaging or therapy because this receptor is overexpressed in various epithelial cancers, e.g., ovarian, brain, breast, and kidney (16). In particular, FR overexpression is observed in approximately 90% of ovarian carcinomas (17,18). Several FA-conjugated drugs for FR targeting are currently under assessment in human clinical trials (16,19). Here, we describe the application of folate-conjugated (FA-conjugated) and radioactive iodine-labeled GNRs for *in vivo* SPECT/CT imaging and subsequent photothermal therapy of folate receptor-overexpressing cancers (Scheme 1).

Experimental

Materials

Cetyltrimethylammonium bromide (CTAB, 99%), Gold (III) chloride trihydrate (HAuCl₄, 99.9%), sodium borohydride (NaBH₄, 99%), silver nitrate (AgNO₃, 99%), folic acid (97%), dimethyl sulfoxide (DMSO, HPLC grade) and acetonitrile (ACN, HPLC grade) were obtained from Sigma-Aldrich (St. Louis, USA). RPMI 1640, heat-inactivated fetal bovine serum and Antibiotic & Antimycotic were purchased from Gibco[®] (Calsbad, USA). (ortho-pyridyl) disulfide-poly(ethylene glycol)-succinimidyl ester (OPSS-PEG-NHS, MW 5 kDa) was purchased from Creative PEGworks (Winston Salem, USA). Monomethoxy poly(ethylene glycol)-thiol (mPEG-SH, MW 5 kDa) was purchased from SunBio (Anyang, Republic of Korea). Peptide (YNNLAC, MW 696.29 Da) was purchased from Peptron Co., Ltd (Daejon, Republic of Korea). Iodogen (Pierce pre-coated iodination tube) and BCA assay kit were purchased Pierce Biotechnology (Rockford, USA). APC-conjugated antihuman FOLR1 (folate receptor 1) antibody was purchased from R&D Systems, Inc. (Minneapolis, USA). [I-131]NaI was purchased from Korea Atomic Energy Research Institute (KAERI) (Daejeon, South Korea). [I-125]NaI was purchased from PerkinElmer (Santa Clara, USA). Inrared camera (Thermovision A40) was obtained from FLIR Systems, Inc. (Wilsonville, USA). Ultrapure distilled water was used in all experiments.

Preparation of CTAB-coated GNRs

CTAB-coated GNRs were prepared by a seed-mediated method as described by Hongwei *et al.* (20). Briefly, CTAB solution (7.5 mL,



100 mM CTAB in deionized water) was mixed with 250 μ L of 10 mM HAuCl₄ aqueous solution. While the mixture was stirring at 400 rpm, 600 μ L of 10 mM NaBH₄ were added, stirred at 900 rpm for 2 min, and then maintained at 25 °C for 2 h, thereby preparing a seed solution. Meanwhile, 1.7 mL of a 10 mM HAuCl₄ aqueous solution were added to 40 mL of a 100 mM CTAB aqueous solution, followed by sequential addition of 250 μ L of a 10 mM AgNO₃ aqueous solution and 270 μ L of a 100 mM ascorbic acid aqueous solution. Then, 420 μ L of the seed solution were added and reacted for 12 h. The product solution was centrifuged at 12,000 rpm at 25 °C for 15 min, thereby obtaining the CTAB-coated GNRs.

Preparation of folate-conjugated GNRs, FA-GNR

Amine-functionalized folate, FA-miniPEG-NH₂, was synthesized as described by Dhar *et al.* (see Figure S1) (21).

An aqueous solution containing CTAB-coated GNRs was centrifuged at 12,000 rpm for 15 min, decanted, and resuspended in deionized water to remove excess CTAB. The particle concentration of CTAB-coated GNRs was 50 nM after resuspension. FA-miniPEG-NH₂ (1.25 μ mol) was reacted with OPSS-PEG-NHS (357 nmol) in PBS (6.7 mM, pH 7.4, NaCl 154 mM) for 2.5 h at 25 °C to prepare FA-PEG-OPSS. Deionized water (500 µL) containing both FA-PEG-OPSS (75 nmol) and mPEG-SH (175 nmol) were added to 500 µL of CTAB-coated GNRs at 25 °C for 1 h to couple both PEGs on the GNR surface, thereby obtaining FA-coupled GNRs. After 1 h, the mixture was centrifuged at 12,000 rpm for 15 min, decanted, and resuspended in 500 µL of PBS to remove unconjugated FA-PEG-OPSS and mPEG-SH. Then, a tyrosine-containing short peptide, YNNLAC, was further conjugated on the surface of the FA-coupled GNRs. The YNNLAC peptide was dissolved in PBS to prepare a 2.5 mM YNNLAC peptide solution. Then, the YNNLAC solution (100 μL) was added to 500 μL of FA-coupled GNR solution and stirred at 25 °C for 12 h. To remove unconjugated peptides, the mixture was centrifuged at 12,000 rpm for 15 min, decanted, and resuspended in 500 µL of PBS, thereby finally obtaining FA-GNR in which both the FA-PEG and YNNLAC peptides were conjugated on the surface of the GNRs.

The number of conjugated YNNLAC per GNR was calculated by analyzing the amount of unconjugated peptides in the supernatant by using high-performance liquid chromatography (HPLC). HPLC analysis was performed on a Waters 2690 (Waters, USA) HPLC system equipped with XTerra RP18 (5 μ m, 4.6 mm ×250 mm) reverse-phase column. The mobile phase for HPLC analyses was acetonitrile (0.1% trifluoroacetic acid) with a flow rate of 1 mL/min at room temperature. The concentration of the GNRs was calculated

using the extinction coefficients $(1.3 \times 10^9 \text{ and } 4.6 \times 10^9 \text{ M}^{-1} \text{ cm}^{-1}$ at 510 and 785 nm, respectively) (12). The absorption spectrum of the GNR solution was measured using a UV/Vis scanning spectrophotometer (DU730, Beckman Coulter, Brea, CA, USA). TEM experiments were performed using a transmission electron microscope (TEM; JEM-1010, JEOL, Tokyo, Japan), operating at 40-100 kV.

Dispersion stability of FA-GNR

To check the dispersion stability of FA-GNR in the various aqueous solutions, CTAB-coated GNR and FA-GNR were dispersed in each of deionized water, PBS, and a cell culture medium (RPMI 1640 without phenol red) containing 10% fetal bovine serum (FBS). The final concentration of GNRs in the solutions was adjusted to 1 nM. The solutions were maintained at 25 °C and observed for 7 d. White-light images and UV/Vis spectra of the sample solutions were taken periodically.

In vitro cell studies

In vitro cell uptake and phototoxicity tests were performed to evaluate the ability of FA-GNR to selectively target FR-positive ovarian cancers. A human ovarian carcinoma (SKOV3) cell line and human lung adenocarcinoma epithelial cell line (A549) were obtained from the American Type Culture Collection (Rockville, MD, USA), and were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% Antibiotic & Antimycotic in a humidified 5% CO_2 incubator at 37 °C. In this study, both cell lines were adapted to grow in folate-deficient RPMI medium for 1 week.

Flow cytometric analysis of folate receptor (FR) expression

The flow cytometric analysis of folate-receptor expression in the cell lines was carried out using an APC-conjugated antihuman FOLR1 (folate receptor 1) antibody according to the manufacturer's instructions. SKOV3 and A549 cells were trypsinized and then centrifuged at 1000 rpm for 3 min. The cells were washed with PBS and resuspended in 1 mL of PBS at a concentration of 2×10^5 cells/mL. 10 µL of APC-FOLR1 were added and incubated in the dark for 15 min. The cells were washed with PBS to remove unbound antibodies. The cells were resuspended in PBS for the final flow cytometric analysis.

In vitro cell uptake test

Labeling of tyrosine (Y) with the radioisotope I-131 was performed



for quantitative analysis of FR-mediated cell uptake. FA-GNR (39 μ L, 43.8 nM particle concentration) and 103.5 MBq of [¹³¹I] NaI in 0.2 mL of saline were added to an Iodogen (Pierce precoated iodination tube) and then incubated at 25 °C for 10 min. Radio iodination was checked by ITLC (0.9% saline). Since the labeling efficiency was more than 99%, no further purification was performed to obtain the ¹³¹I-labeled FA-GNRs (i.e., FA-¹³¹I-GNR).

In brief, SKOV3 and A549 cells were both seeded in triplicate in each well of 24-well plates at a density of 3×10^4 cells/well and incubated for 24 h for cell attachment. The growth medium was removed from the cell cultures and replaced with 200 µL of FAfree culture medium containing 37 kBq/mL of FA-¹³¹I-GNR. In free-FA competition studies, the cells were pre-incubated with 1 mM free-FA-containing culture medium at 37 °C for 30 min and then co-incubated with FA-¹³¹I-GNR for an additional 4 h. The cells were then quickly washed twice with 500 μ L of icecold Dulbecco's phosphate-buffered saline (DPBS, Welgene) to remove the unbound GNRs. Cellular radioactivity was released with 200 μ L of lysis buffer (sterile water containing 0.1% SDS) and counted in a gamma-counter. The protein content of each well was determined using a BCA assay kit. In vitro cell uptake was quantitated by measuring the radioactivity of cell extracts. The amount of protein per well was used for normalization of CPM results. The numerical values $(CPM/\mu g)$ for the case and control group were represented by normalized fold ratios. Statistical significance was calculated by determining *p* values using Student's t-test.

Cell viability test

SKOV3 cells were seeded in each well of 96-well plates at a density of 0.7×10⁴ cells/well and incubated for 24 h for cell attachment. FA-GNR in aqueous solution was diluted with a cell culture medium containing 10% FBS to obtain various concentrations of FA-GNR (0.25, 0.5, 1, and 2 nM, based on GNR). The existing culture medium was replaced with 200 µL of fresh medium containing FA-GNR, and the cells were incubated for 4 h. For the untreated control group, the same volume of fresh culture medium, without GNRs, was added to the plate. After the cells were washed twice, a fresh cell culture medium was added, and further incubated for an additional 24 h. The cell viability of SKOV3 was analyzed using a CCK-8 solution. The absorbance was measured at 450 nm (reference =650 nm) using a microplate reader (Tecan Safire 2, Switzerland). Untreated control cells served as 100% viable cells, and the medium served as the background. Data are expressed as the mean (SD) from four data samples. The Student's t-test was used for statistical analyses.

Selective photothermal cancer therapy using FA-GNR

SKOV3 cells were seeded in each well of 24-well plates at a density of 3×10^4 cells/well and incubated for 24 h for cell attachment. The existing culture medium was replaced with 200 µL of fresh medium containing FA-GNR (0, 0.25, 0.5, 1, and 2 nM, based on GNR), and the cells were incubated for 4 h. In free-FA competition studies, 1 mM FA was pre-incubated at 37 °C for 30 min and then coincubated with FA-GNR for further 4 h. After the cells were washed twice, a fresh cell culture medium was added. For the photothermal therapy studies, light was applied to the central part of the cell layer using an 810-nm CW laser $(2 \text{ W/cm}^2 \text{ for 4 min, light-spot})$ diameter: 3.5 mm). A calcein AM staining kit (Molecular Probes) was used as an indicator in live/dead viability and cytotoxicity tests. Following illumination, the cells were immediately stained with calcein AM for fluorescence microscopy. Live cells were stained with calcein (green 494/517 nm) and the nuclei of dead cells were stained with ethidium bromide dimer (red 528/617 nm). Fluorescence images were taken from more than three different areas in each case. All the data were acquired using identical settings on the microscope in order to ensure reproducibility.

In vivo animal studies

All animal studies were approved by the Institutional Animal Care and Use Committee. Female athymic nude mice (Balb/c-nu, ca. 19-21 g) were used for the *in vivo* experiments. SKOV3 cells $(1 \times 10^7 \text{ cells}/50 \ \mu\text{L}$ of RPMI) were implanted subcutaneously into the left hind flank of each mouse, and the tumor size was measured daily (22). The animals were chosen for *in vivo* studies when their tumor sizes reached about 60 mm³.

Prepareation of ¹²⁵I-labeled FA-GNR, FA-¹²⁵I-GNR

Labeling of tyrosine (Y) with the radioisotope I-125 was performed for an *in vivo* SPECT imaging. FA-GNR (39 μ L, 32.1 nM particle concentration) and 74 MBq of [¹²⁵I]NaI in 0.1 mL of saline were added to an Iodogen (Pierce pre-coated iodination tube) and then incubated at 25 °C for 15 min. Radioiodination was checked by ITLC (0.9% saline). Since the labeling efficiency was more than 99%, no further purification was performed to obtain the ¹²⁵I-labeled FA-GNRs (i.e., FA-¹²⁵I-GNR). Radiochemical stability tests were done by ITLC (saline) in human serum at 37 °C.

SPECT/CT imaging of ovarian cancer xenografts

For in vivo SPECT/CT fusion imaging, three mice received FA-



¹²⁵I-GNR at a dose of 40.7 MBq/mouse (equal to 0.841 pmol FA-GNR/mouse), and three other mice received FA-¹²⁵I-GNR at a dose of 18.5 MBq/mouse (equal to 0.374 pmol FA-GNR/mouse). SPECT images were obtained using animal SPECT (NanoSPECT, Bioscan) at 4 h and 24 h post-injection. CT images were obtained using animal PET/CT (Explore Vista-CT, GE) at 24 h post-injection. The obtained SPECT and CT scans were fused using InVivoScope[™] software (Bioscan). For comparison, SPECT images were also obtained from two mice without tumors, after intravenous injection of FA-¹²⁵I-GNR at a dose of 18.5 MBq/mouse.

Tumor photothermal therapy

For *in vivo* tumor photothermal therapy, tumors in the FA-¹²⁵I-GNR-treated mice (3 mice, 0.841 pmol FA-GNR/mouse) were illuminated with an 810-nm CW laser (2 W/cm², 600 J/cm²) for photothermal therapy at 24 h post-injection. For comparison, additional mice received intravenous injections of sterilized PBS solution (4 mice, 90 μ L/mouse), followed by light illumination using an 810-nm CW laser (2 W/cm², 600 J/cm²) 24 h after injection. Temperature increases in the tumor tissues during light illumination were monitored in real time using an infrared (IR) camera to obtain thermal images of both the PBS-treated and FA-¹²⁵I-GNR-treated mice. The temperature increases were analyzed using ThermaCAMTM Researcher software. Thereafter, tumor volumes were measured periodically until day 30.

Results and Discussions

A total synthetic procedure for radioactive-iodine-labeled and FA-conjugated GNRs is illustrated in Scheme 1. Cetyltrimethylammonium bromide (CTAB)-coated GNR was synthesized by a seed-mediated method (20). Because of its cytotoxicity, excess CTAB was removed by centrifugation and redispersed in distilled water (DW). For the introduction of FAcoupled poly(ethylene glycol) (PEG) on the GNR surface, we synthesized an amine-functionalized folate, FA-miniPEG-NH₂, and then coupled the FA-miniPEG-NH₂ with (ortho-pyridyl) disulfide-poly(ethylene glycol)-succinimidyl ester (OPSS-PEG-NHS, MW 5 kDa), obtaining an FA-coupled PEG, FA-PEG-OPSS. Both FA-PEG-OPSS and monomethoxy-poly(ethylene glycol)-thiol (mPEG-SH, MW 5 kDa) were conjugated to CTAB-coated GNRs by a gold-thiol reaction. Then a short peptide, H2N-Tyr-Asn-Asn-Leu-Ala-Cys-OH (YNNLAC), was further conjugated on the surface of the GNRs through thiol chemistry, thereby finally obtaining FA-GNR in which both the FA-PEG and YNNLAC peptides were conjugated on the surface of the GNRs. The tyrosine (Y) in the peptide sequence is introduced for radioactive-iodine labeling through an iodinetyrosine interaction. Hydrophobic amino acids leucine (L) and alanine (A) were introduced to promote self-assembly of the peptides on the GNR surface. From the high-performance liquid chromatography (HPLC) analysis, the molar ratio of conjugated YNNLAC per GNR was calculated at 6382:1.

The prepared FA-GNR was characterized by transmission electron microscopy (TEM) and UV/Vis absorption spectroscopy (Figure 1A). The average length and diameter of the GNRs were 32.9 ± 3.5 nm and 9.58 ± 1.5 nm, respectively. The absorption band of FA-GNR is unchanged after surface modification of the GNRs.

To check the dispersion stability, FA-GNR was dispersed in each of deionized water, PBS solution, and cell culture medium containing 10% fetal bovine serum (FBS). The white-light images and UV/Vis spectra of all the test solutions were taken after 10 min, 4 h, 24 h, and 168 h of incubation at 25 °C (Figure S2). The color and UV/Vis spectra of all the test solutions did not change for 168 h, as shown in Figure 1B. On the other hand, CTAB-coated GNRs rapidly formed aggregates in cell culture medium containing 10% FBS (23). This result indicates that PEGs on the surface of the FA-GNR were enough to prevent interaction between serum proteins and the GNRs, which is particularly important for achieving lengthy blood-circulation of the GNRs *in vivo*.

We evaluated the ability of FA-GNR to selectively target FR-positive ovarian cancers. A human ovarian carcinoma (SKOV3) cell line and human lung adenocarcinoma epithelial cell line (A549) were used as FR-positive and FR-negative controls, respectively. The expression levels of FR on both cell lines were confirmed by fluorescence-activated cell sorting (FACS) analysis after staining the cells with APC-conjugated antihuman FOLR1 (folate receptor 1) antibodies (Figure S3). For quantitative analysis of FR-mediated cancer targeting, a radioisotope, iodine-131, was used to label tyrosine residues in FA-GNR through iodine-tyrosine interactions to obtain ¹³¹I-labeled FA-GNR, FA-¹³¹I-GNR. Both SKOV3 and A549 cells were incubated with FA-¹³¹I-GNR for 4 h in the absence or presence of free FA (1 mM) as a competitor. The level of uptake was then calculated by measuring the radioactivity of cell lysates. The cellular uptake of FA-¹³¹I-GNR in SKOV3 cells was about 2.7-fold higher than in A549 cells (Figure 2A). When free FA (1 mM) was treated in SKOV3 cells and A549 cells along with FA-¹³¹I-GNR, the cellular uptake of FA-¹³¹I-GNR decreased in both cell lines. In particular, an obvious decrease in the uptake of FA-¹³¹I-GNR was observed in SKOV3 cells.

We next evaluated the in vitro cytotoxicity of FA-GNR in





Figure 1. A. Absorption spectrum and transmission electron microscopy (TEM) image of FA-GNR; and B. Dispersion stability of FA-GNR in deionized water (DW), phosphate-buffered saline (PBS) solution, and RPMI 1640 (without phenol red) containing 10% fetal bovine serum (FBS). The solutions were maintained at 25 °C and observed for 168 h. Photographs and absorption spectra shown above were taken after 168 h of incubation.



Figure 2. A. Intracellular uptake of FA-¹³¹I-GNR against SKOV3 cell line (n=3) and A549 cell line (n=3). FR-positive SKOV3 and FR-negative A549 cells were both incubated with FA-¹³¹I-GNR for 4 h in the absence (\blacksquare) or presence (\square) of free FA (1 mM) as a competitor. *Dotted line*: radioactivity measured from the cells treated with free I-131; **B. Cell viability of SKOV3 cells treated with FA-GNR at various concentrations (0, 0.25, 0.5, 1, and 2 nM, based on GNR, n=3).**

SKOV3 human ovarian carcinoma cells (Figure 2B). SKOV3 cells were incubated with FA-GNR solutions at various concentrations (0, 0.25, 0.5, 1, and 2 nM, based on GNR) and the viability of the cells was then checked. No cytotoxic effects of FA-GNR were observed at the tested concentrations.

The *in vitro* photothermal therapy of FA-GNR was then evaluated in SKOV3 cancer cells. SKOV3 cells were incubated with various concentration of FA-GNR (0, 0.25, 0.5, and 2 nM based on GNR). After 4 h, the cells were washed and illuminated with an 810-nm CW laser (2 W/cm^2 , 4 min) and then stained





Figure 3. *In vitro* **photothermal therapy using FA-GNR at various concentrations (0, 0.25, 0.5 and 2 nM, based on GNR) against SKOV3 cells.** SKOV3 cells were incubated with FA-GNR for 4 h in the absence or presence of free FA (1 mM) as a competitor, washed, and received light illumination with an 810-nm laser (2 W/cm², 4 min). Light was applied to the area inside the dotted white line. The cells were then stained with calcein AM. Live cells and the nuclei of dead cells are shown in green and red, respectively.



Figure 4. Radio TLC chromatogram of FA-¹²⁵**I-GNR.** Labeling efficiency was measured by instant thin layer chromatography (ITLC) with saline as the developing eluant, and was shown to be more than 99%.





Figure 5. Radiochemical stabilities of FA-¹²⁵**I-GNR in human serum.** ITLC of FA-¹²⁵I-GNR showed high radiochemical stability in human serum at 37 °C after incubation for 24 h (n=3). The radiostability was maintained at 90.9 \pm 0.3% for up to 24 h.

with calcein AM. The cells which were treated with 0.25, 0.5, and 2 nM FA-GNR were shown to be dead in the light-illuminated region (Figure 3, upper row). However, cells co-incubated with 1 mM free FA as a competitor were alive upon laser illumination (Figure 3, bottom row). These results support the idea that FA-GNR was selectively internalized into SKOV3 cells by FR-mediated endocytosis, and the subsequent photothermal effect of FA-GNR was adequate for cancer therapy *in vitro*.

Prior to *in vivo* experiments, we used iodine-125 to label FA-GNR to obtain FA-¹²⁵I-GNR for *in vivo* SPECT imaging. The labeling efficiency was more than 99% (Figure 4). Radiochemical stability of FA-¹²⁵I-GNR in human serum showed that 91% of the ¹²⁵I remained intact on the GNRs after 24 h of incubation at 37 °C (Figure 5).

Three mice bearing subcutaneous SKOV3 tumors received intravenous injections of FA-¹²⁵I-GNR when their tumor size reached about 60 mm³. The injection dose of FA-¹²⁵I-GNR was 40.7 MBq/mouse, which equals 0.841 pmol FA-GNR/mouse. SPECT images were then obtained 4 h and 24 h after injection

(Figure 6). CT images were also obtained 24 h after injection and then fused with SPECT images using InVivoScopeTM (Bioscan, USA) software. As shown in Figure 6, the tumor sites were starting to be discriminated from the surrounding tissues, and were clearly visualized, with good signal-to-noise ratio, at 24 h post-injection. Signals from the heart at 24 h post-injection indicated that FA-GNR was still circulating in the bloodstream. Signals from the thyroid and bladder resulted from accumulation of released ¹²⁵I from the FA-¹²⁵I-GNR during circulation. It is known that hepatic deiodases are capable of deiodonating mono- and di-iodotyrosine, resulting in the production of free radioiodide and eventually leading to thyroid uptake (24).

The *in vivo* therapeutic efficacy was further evaluated by measuring tumor growth rates. When their tumor sizes had reached approximately 60 mm³, the mice received an intravenous injection of either sterilized PBS solution (n=4) or FA-¹²⁵I-GNR solution (n=3, 0.841 pmol/90 μ L/mouse) (day0). Tumors in the PBS-treated and FA-¹²⁵I-GNR-treated mice were illuminated with an 810-nm CW laser (2 W/cm², 5 min) for photothermal





Figure 6. A. Coronal and sagittal plane SPECT images of the mice at different time points. The mice received intravenous injections of FA-¹²⁵I-GNR at a dose of 40.7 MBq/mouse (equal to 0.841 pmol FA-GNR/mouse). White arrows indicate tumor sites. B. Representative SPECT/CT fusion image of SKOV-3 xenograft-bearing mouse at 24 h after injection of FA-¹²⁵I-GNR (Th: thyroid, B: bladder, T: tumor).

therapy at 24 h post-injection at the same light dose and dose rate (day1). The temperature increase at the tumor sites was monitored in real-time during laser illumination using an IR camera. The tumor temperature in the FA-¹²⁵I-GNR-treated group rose to 50 °C within 1 min, and thereafter gradually reached 59 °C at 5 min. The tumor temperature in the PBS-treated group did not increase above 40 °C (Figure 7A and B). It is well known that the conditions for thermal ablation therapy are dependent on the operating temperature and time. It took 60 min to induce irreversible cellular damage at 46 °C, but 4-6 min is sufficient at 50-52 °C (25).

In vivo therapeutic effects were observed in the FA-GNRtreated groups compared with PBS-treated group after day 2 (Figure 7C). As expected, tumors in the FA-¹²⁵I-GNR-treated group were shown to be mostly ablated the next day and disappeared after light illumination, whereas no antitumor effect was observed in the PBS-treated group. No apparent tumor mass was detected in any of the 3 mice in the FA-¹²⁵I-GNR-treated group until day 15. On day 23, regrowth of the tumor mass was observed in 1 of the 3 mice. However, no tumor regrowth was detected in the other 2 mice until day 30. The mean tumor sizes in the FA-¹²⁵I-GNR-treated group on day 30 was 15.9% (P=0.004) that in the PBS-treated group. We also tested the dose effect of FA-¹²⁵I-GNR on cancer imaging and photothermal therapy. Three additional mice with subcutaneous tumors received FA-¹²⁵I-GNR at a dose of 18.5 MBq/mouse (equal to 0.374 pmol FA-GNR/mouse), and then SPECT images were obtained 4 h and 24 h post-injection. Light illumination at the tumor sites was performed at 24 h postinjection under the same conditions as described above. The tumor sites were clearly visualized from the SPECT images at 24 h post-injection (Figure S4). However, the temperature in the tumor tissues reached a plateau after 2 min and reached only 46 °C, even after 3 min of laser illumination, which is not enough to induce irreversible damage of tumors (Figure S5).

Conclusion

In conclusion, we showed in this study that FA-conjugated and radioactive-iodine-labeled GNRs are promising for SPECT/CT imaging and subsequent thermal ablation of FR-overexpressing ovarian cancer. This type of platform may also be useful for the targeted SPECT/CT imaging and photothermal therapy of inflammatory diseases such as atherosclerosis and arthritis, in which FR-overexpressing macrophages play pivotal roles (26-30).





Figure 7. A. Thermographic monitoring in the tumors of PBS-treated mice (n=4, 90 μ L/mouse) and FA-¹²⁵I-GNR-treated mice (n=3, dose: 40.7 MBq/mouse) during 810-nm light illumination (+L) for 5 min (2 W/cm²); B. Thermographic images captured after 5 min of 810-nm light illumination; C. Tumor size after the therapy session. The mice received intravenous injection of either PBS or FA-¹²⁵I-GNR on day 0, and the tumors were then illuminated with light on day 1. Points, mean; bars, standard deviation; PBS+L (n=4) and FA-¹²⁵I-GNR +L (n=3); n = the number of mice.

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Supplementary material

Synthesis of a mine-functionalized folate, FA-miniPEG-NH $_{\rm 2}$

For the synthesis of $H_2N(CH_2)_3O(CH_2)_2O(CH_2)_2O(CH_2)_3NHBoc$, 4,7,10-trioxa-1,13-tridecanediamine (7.5 g, 34.1 mmol) was dissolved in 1,4-dioxane (80 mL), deoxygenated by Ar (g) purging. Then, a solution of Boc₂O (3.7 g, 16.95 mmol) in 1,4-dioxane (20 mL) was added slowly. The reaction mixture was stirred at room temperature for 12 h. The solvent was removed, and the resulting yellow oil was purified by silica-gel chromatography (methanol/ dichoromethane/triethylamine = 8/91/1) to produce compound 1 in 30% (3.23g) yield. ¹H-NMR (CDCl₃, 400 MHz); δ 5.09 (s, 1H), 3.66-3.50 (m, 12H), 3.22 (m, 2H), 2.80 (t, J = 8.0 Hz, 2H), 1.80-1.70 (m, 4H), 1.43 (s, 9H).

For the synthesis of FA-miniPEG-NH₂, folate (3.8 g, 8.588 mmol)

was dissolved in DMSO (130 mL), deoxygenated by Ar (g) purging. Dicyclohexylcarbodiimide (4.44 g, 21.77 mmol) and pyridine (75 mL) were added and then a solution of $H_2N(CH_2)_3O(CH_2)_2O(CH_2)_2O(CH_2)_3NHB$ OC (3.03 g, 9.456 mmol) in DMSO (20 mL) was added. The reaction mixture was stirred at room temperature for 18 h in the dark. The solid product, dicyclohexylurea, was filtered off, and diethyl ether (2 L) was added slowly. The formed yellow solid was collected and washed with diethyl ether several times.

After evaporation, the resulting compound 2 was dissolved in trifluoroacetic acid (25 mL) and stirred for 2 h. Trifluoroacetic acid was removed under vacuum and diethyl ether was added. A red precipitate formed and was washed with diethyl ether. Finally, compound 3 was isolated in 36% (1.996 g) yield. FA-miniPEG-NH₂ was identified by HPLC, NMR and ESI-Mass.



Figure S1. Synthesis of FA-miniPEG-NH₂.





Figure S2. Dispersion stability of FA-GNR. FA-GNR (final particle concentration: 1 nM) was added to deionized water (DW), PBS, and RPMI 1640 (without phenol red) containing 10% fetal bovine serum (FBS). The solutions were maintained at 25 °C and observed for 168 h. Photographs and UV/Vis absorption spectra of the solutions were taken after 10 min, 4 h, 24 h, and 168 h of incubation at 25 °C.



Figure S3. FACS analysis of two cell line (SKOV3 and A549) for aFR expression levels. The percentages of expression are 52.21% (SKOV3) and 1.39% (A549) with reference to their isotype control. The mean fluorescence intensity histogram shows high levels of fluorescence in SKOV3.





Figure S4. Coronal and sagittal plane SPECT images of the mice at different time points. The mice received intravenous injections of FA-¹²⁵I-GNR at a dose of 18.5 MBq/mouse (equal to 0.374 pmol FA-GNR/mouse). White arrows indicate tumor sites.



Figure S5. Thermographic monitoring in the tumors of FA-¹²⁵I-GNRtreated mice during 810-nm laser illumination (2 W/cm²). The mice received intravenous injections of FA-¹²⁵I-GNR (18.5 MBq/mouse, equal to 0.374 pmol FA-GNR/mouse). Temperature of tumor tissues in the FA-GNR-treated mice increased for 1 min, but reached a plateau after 2 min and reached only 46.4 °C, even after 3 min of laser irradiation. This indicates that the concentration of FA-GNR in the tumor is not enough to induce irreversible thermal damage of the cancer cells.

