Supplementary Information

pH-Triggered Au- fluorescent Mesoporous Silica Nanoparticles
for $^{19}$F MR/Fluorescent Multimodal Cancer Cellular Imaging

Shizhen Chen, Yuqi Yang, Haidong Li, Xin Zhou*, Maili Liu

Key Laboratory of Magnetic Resonance in Biological Systems, State Key Laboratory for Magnetic Resonance and Atomic and Molecular Physics, Wuhan Center for Magnetic Resonance, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, Wuhan 430071, China

* Corresponding author. Tel.: +86-27-87198802, Fax: +86-27-87199291
E-mail address: xinzhou@wipm.ac.cn
**Experimental Section**

**Materials**

Tetraethylorthosilicate, 3-aminopropyltrimethoxysilane, 3-[N-(2-aminoethyl)amino-propyltrimethoxysilane, fluorescein isothiocyanate isomer I (FITC), folic acid, 1-H-pyrazole-carboxamidine hydrochloride, diisopropyl ethylamine, N-ethyl-N-(3-dimethylaminopropyl)carbodiimide, a 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), hydrochloride 4',6-diamidino-2-phenylindole (DAPI), and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) were purchased from Sigma-Aldrich. All chemicals were used as received.

**Synthesis of –NHNH₂-functionalized gold nanoparticles**

The first step involved preparation of the acid-functionalized gold nanoparticles (Acid-AuNPs), Acid-AuNPs were synthesized using a modification of the procedure published by Brust et al. In a typical experiment, hydrogen tetrachloroaurate (III) hydrate (300 mg, 7.63 x 10⁻⁴ mol) was dissolved in 15 mL of methanol, followed by the quick addition of 11-mercaptopoundecanoic acid (393 mg, 1.8 x 10⁻³ mol) and glacial acetic acid (3.0 mL). Under vigorous stirring, an aqueous solution of sodium borohydride (30 mL, 0.4 M) was added in small portions. Washing with methanol, ethyl ether, and water yielded the desired 5-10 nm Acid-AuNPs.

In the second step, the surface of the AuNPs was modified with bifunctional methyl-3-mercaptopropionate (HSCH₂CH₂COOCH₃), which was chemically bonded to the surface of the AuNPs via Au–S covalent bonds. To conjugate with carboxylic acid-functionalized FMSNs, the –OCH₃ group was then converted to the –NHNH₂ functional group by a hydrazinolysis reaction because the –NHNH₂ functional group facilitates the subsequent conjugation of the carboxylic acid-functionalized FMSNs. The hydrazinolysis reaction is required because hydrazide end-groups (–NHNH₂) that provide hydrazone linkage with the FMSNs are acidlabile linkers with the ability to cleaved in the low acidic environment (pH 5–6) present in the endosome of the lung cancer cells.
To accomplish the above, 100 mg of carboxylic acid-functionalized AuNPs were dispersed in 20 ml of diphenyl ether to form a colloid solution by sonication. In the next step, 33 μl of methyl-3-mercaptopropionate was added to the colloid solution and then refluxed at 260 ºC for 1 h. Subsequently, the solution was cooled to 100 ºC and 145μl of hydrazine monohydrate (N₂H₄·H₂O) was added dropwise to the solution, with the solution continuously stirred for 2 h. The resulting nanoparticles were separated by centrifuging at 15,000 rpm for 10 min, washed thrice with methanol and dried at 50 ºC for 24 h. As a consequence of the above procedure, the carboxylic acid-functionalized AuNPs were functionalized with an –NHNH₂ group on the surface.

A 5 mL carboxylic acid-functionalized AuNPs solution was deaerated by pure nitrogen (>99%). Then, 15 μl of 0.1 mol L⁻¹ 3-MPA solution was added into the above solution. The resulting solution was stirred for 3 h for reaction, and then was dialyzed for another 3 h to remove the extra free small molecules. A 10 μl of EDC solution (0.05 mol L⁻¹ in dimethyl sulfoxide (DMSO)) and the same amount of NHS solution (0.05 mol L⁻¹ in DMSO) were added into the above dialyzed solution, the mixture of which was stirred for 30 min for reaction and then dialyzed again. After dialysis, FA was added into this solution, which was stirred overnight and then also dialyzed. The FA functionalized AuNPs were thus obtained in this final solution.

**Synthesis of fluorescein isothiocyanate-labeled MSNs (FMSNs)**

FMSNs were prepared by reacting 500 mg (1.11 mmol) of fluorescein isothiocyanate with 0.2 mL (1.15 mmol) of (3-aminopropyl) trimethoxysilane (APTMS) for 2 h. The resulting product was introduced to a co-condensation reaction of 10.0 mL (43.9 mmol) of tetraethylorthosilicate (TEOS), 2.04 g (5.32 mmol) of cetyltrimethylammonium bromide (CTAB), 960 mL of water, and 7.0 mL of sodium hydroxide (2 M). The reaction mixture was heated at 80ºC for 2 h, under vigorous stirring. The resulting orange-colored solid was filtered, washed thoroughly with methanol, and dried under vacuum for 20 h.

**Loading, capping, and release experiments**
To cap the C₆F₆-loaded FMSNs (C₆F₆@FMSNs), –NHNH₂ -functionalized AuNPs were first dispersed by sonication in 20 ml of anhydrous methanol containing three drops of acetic acid (catalyzer) to form a colloid solution. Then 60 mg FMSNs and 1 ml C₆F₆ were added to the colloid solution with continuous stirring, the reaction was carried out at room temperature for 48 h. Subsequently, the resulting colloidal solution was centrifuged at 15,000 rpm for 10 min and the precipitate obtained was redispersed in methanol by sonication, and again centrifuged. The process was continued until the solution became colorless and particles settled at the bottom of the test tube. The centrifuged solution was collected and diluted to 100 ml with methanol in a capacitance flask.

All of the supernatants from the washings (including those before capping was performed) were collected, and the loading of C₆F₆ (0.075 mmol g⁻¹) was calculated as the difference between the concentrations of the initial solution of C₆F₆, and that of the reaction medium combined with the subsequent washings. Fluorescein-loaded MSNs (FITC@MSNs) was loaded and capped following the same procedure.

The gating protocol was investigated by studying the release profiles of C₆F₆ from the Au-FMSNs, at 37°C in two different media, (a) phosphate buffer, pH 6.0, and (b) phosphate buffer, pH 7.4, using a dialysis bag diffusion technique. Briefly, 10 mg of the C₆F₆@FMSNs sample was dispersed in 5 mL of different buffer solutions, and sealed in a dialysis bag (molecular weight cutoff = 8000). The dialysis bag was submerged in 20 mL of the respective buffer solutions, and stirred at 37°C for 12 h. The released C₆F₆ in the buffer was collected at predetermined time intervals and analyzed using UV-Vis absorption spectrum at a wavelength of 231 nm.

**Characterization**

The chemical composition of the Au-MSNs was determined using an ESCALAB 220i-XL X-ray photoelectron spectrometer (XPS) (VG Scientific Ltd.). The microstructure of the MSNs and Au-FMSNs was observed using transmission electron microscopy (TEM JEOL JSM-3010). The static water contact angle of the MSNs and Au-FMSNs were measured at 25°C, using an OCA 20 contact angle system (Dataphysics, Germany). N₂ absorption isotherms were measured at 77 K
using a Micromeritics ASAP 2000 gas adsorption analyzer, after the samples were
degassed at 293 K and 10 µ torr for 5 hours. The surface area and total pore volume
were evaluated using the Brunauer- Emmett-Teller (BET) model, and the pore size was
evaluated using the Barett-Joyner-Halenda (BJH) model. UV-vis absorption spectra
were recorded in an Evolution 220 spectrophotometer (Thermo fisher scientific).
Fluorescence spectra were recorded at room temperature on a Hitachi 7000
fluorescence spectrophotometer.

Cell Culture

Human lung adenocarcinoma cell line A549 and normal human lung fibroblast
cell line MRC5 were obtained from the Cell Bank of Shanghai Institute of Cell
Biology, China. The A549 and MRC5 cells were maintained in OptiMEM medium at
pH 7.2, supplemented with 10% inactivated fetal bovine serum and 1%
penicillin/streptomycin 10000 U/ml, and were grown in an incubator at 37°C,
supplied with 5% CO2.

Confocal laser scanning microscopy

To measure the cellular uptake, A549 cells were cultured in incubation medium
(DMEM) for 24 h, in a 12-well chamber slide with one piece of cover glass at the
bottom of each chamber. FITC-labeled MSNs were added to the incubation medium
at a concentration of 100 µg mL⁻¹, under 5% CO₂ and at 37°C, for different
incubation times. To achieve fixation, the glass that the cells were adhered on was
immersed in 4% paraformaldehyde in PBS for 10 min, at room temperature.
Following fixation, the glass was washed with PBS, and mounted on a slide, where
nucleic staining was performed using DAPI (blue), and cell membrane staining was
achieved using DiI (red); the slides were washed three times with PBS (pH 7.4) and
visualized under a laser scanning confocal microscope Nikon A1 (Nikon, Tokyo,
Japan).

Cellular ¹⁹F MRI

All ¹⁹F MRI experiments were performed on a 9.4 T microimaging system
(Bruker Biospec, Germany) with a 30mm inner diameter ¹⁹F birdcage coil (376.4
MHz) for both radiofrequency transmission and reception. NMR spectroscopy was
used to determine the T₁ relaxation with an inversion recovery pulse sequence, in order to choose the parameters for the best contrast MR images. The FLASH (fast low angle shot) pulse sequence was employed for all MRI acquisitions. For Figure 3, a FOV (field of view) = 33x33 mm², TR=3s and TE=200 ms were used. For Figure S5, a FOV=20x20 mm², TR=200ms and TE=6 ms were used. All raw data were processed using the Matlab (Mathworks, Natick, MA).

Fig. S1. The surface hydrophilicity of (a) FMSNs (b) Au-FMSNs and (c) Au-FMSNs after incubated in pH 6.0 PBS for 60 min.

To gain a better understanding of uncapping process, the surface hydrophilicity of the FMSNs and Au-FMSNs was characterized by measuring their contact angles. The FMSNs exhibited the largest contact angle (Fig. S1a), indicating their hydrophobic nature. When AuNPs were used to cap the FMSNs, the resulted Au-FMSNs presented a significantly decreased contact angle (Fig. S1b), revealing a remarkable improvement in the surface hydrophilicity; this improvement was due to the unique chemical and physical properties of AuNPs. Such hydrophilic surface provided an excellent microenvironment for the cell adhesion. After being incubated in pH=6.0 PBS for 60 min, the Au-FMSNs showed an increase in the contact angle (Fig. S1c) because the AuNPs were uncapped from the Au-FMSNs.
X-ray photoelectron spectroscopy (XPS) was performed to analyze the synthetic FMSNs (curve a) and Au-FMSNs (curve b), as shown in Fig. S2. The peaks at 533 eV, 104 eV, and 88 eV were associated with significant O1s, Si2p, and Au4f signals, respectively. The FMSNs (Fig. S2a) showed a major peak component at a binding energy (BE) of 533 eV, which was attributed to O1s; the minor peak component at a BE of 285.6 eV was assigned to C1s, and the peak at a BE of 104.0 eV corresponded to Si2p. Curve b indicated the presence of Au. Two new peaks appeared at 84.2 and 87.7 eV (Fig. S2b), and were related to the Au 4f doublet of 4f7/2 and 4f5/2, respectively. These results confirmed the successful AuNPs capping of the FMSNs.
**Fig. S3** BET nitrogen adsorption/desorption isotherms of FMSNs and Au-FMSNs. Insert: BJH pore size distribution of FMSNs and Au-FMSNs.

**Table S1.** Surface properties of FMSNs and Au-FMSNs

<table>
<thead>
<tr>
<th>Material</th>
<th>BET Surface Area (m²/g)</th>
<th>Average Pore Width (nm)</th>
<th>Pore Volume (cm³/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMSNs</td>
<td>790</td>
<td>3.1</td>
<td>0.735</td>
</tr>
<tr>
<td>Au-FMSNs</td>
<td>49</td>
<td>&lt;1.2</td>
<td>0.147</td>
</tr>
</tbody>
</table>

The average pore diameter was 3.1 nm, calculated using the Barrett-Joinder-Halenda (BJH) method, as shown in **Fig. S3**. The Brunauer-Emmett-Teller (BET) surface area and pore volume of the FMSNs were measured to be 790 m²/g and 0.735 cm³/g, respectively. In contrast with the surface area, pore volume and average pore diameter for the FMSNs, the values for the Au-FMSNs decreased dramatically, as outlined in **Table S1**. These results indicated that the FMSNs were highly porous and had a large surface area, and also demonstrated that AuNPs efficiently blocked the pores of the FMSNs, which enabled them to encapsulate and deliver large quantities of ¹⁹F contrast agents.

**Fig. S4** Release behavior of FITC loaded in Au-MSNs incubated in (a) pH 7.4 PBS, (b) MRC-5 cells, (c) A549 cells and (d) pH 6.0 PBS over 5 hours.
To investigate the pH-triggered release behavior of the Au-MSNs system, fluorescein isothiocyanate (FITC) dye was loaded as a guest by soaking the MSNs (FITC@MSNs) in a pH 7.4 PBS of FITC. To examine the capping efficiency, the FITC-loaded Au-MSNs were first dispersed in pH 7.4 PBS, and then a suspension of MRC-5 cells (4 × 10^5 cells mL^-1). The Au-MSN system exhibited less than 3.0% FITC release in pH 7.4 PBS (Fig. S4a), and less than 5.0% in the MRC-5 cell suspension (Fig. S4b), over a period of 5 h. This result indicated that no significant leakage of the entrapped FITC molecules occurred, suggesting the good capping and leaching-prevention efficiency of the AuNPs for the encapsulation of the FITC molecules.

The triggered release of FITC was investigated by incubating the FITC-loaded Au-MSNs in pH 6.0 PBS and a suspension of A549 cells (4 × 10^5 cells mL^-1). In the weak acid solution, the hydrazone linkage between the MSNs and the Au could be cleaved, and the AuNPs would be uncapped from the MSNs. As shown in Fig. S4, a considerable release of FITC from the Au-MSNs was indeed observed in the A549 cells (4 × 10^5 cells mL^-1) (Fig. S4c) and in pH 6.0 PBS (Fig. S4d). In pH 6.0 PBS, when the incubation time was longer than 120 min, the release of FITC tended toward a stable value. However, in the A549 cells, a maximum release was observed at 200 min, because a finite period of time was required for the MSNs to be efficiently endocytosed by the A549 cells. The rate of release of FITC slightly increased over time, until 85% of the total FITC was released. These results indicated that the amount of FITC released from the Au-MSNs was dependent on the incubation time.
and pH. The release of FITC molecules was monitored using a fluorescence spectrophotometer.

![Fig. S5](image)

**Fig. S5** $^{19}$F MRI of C$_6$F$_6$@Au-FMSNs in lung cancer cells (A549) at different incubation time: (a) 0 min, (b) 10 min, (c) 20 min, (d) 30 min, (e) 60 min, (f) 90 min, (g) 120 min, and (h) 180 min. FOV=20x20 mm$^2$