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# Supporting information

## Biological Reduction of Nitroimidazole-Functionalized Gold Nanorods for Photoacoustic Imaging of Tumor Hypoxia

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## Experimental Section

**General methods**. All reagents were purchased from standard suppliers and used without further purification. Absorption spectra were recorded using a HITACHI U-3010 UV/Vis spectrophotometer. Zeta potential was measured by Otsuka electronics ELS-800SA. Transemission electron microscopy (TEM) images were taken at 200 kV using HITACHI H-800 and cellular TEM images were taken at 80 kV HITACHI H-7650 and EDX mapping images were taken at 80 kV JEOL JEM-1400. Photoacoustic imaging (PAI) was performed on ENDRA Nexus 128. Confocal microscopy was performed on ZEISS LSM 710. NI-$CO_2H$ were synthesized as described previously (Son, A.; Kawasaki, A.; Hara, D.; Ito, T.; Tanabe, K. *Chem. Eur. J.* **2015**, *21*, 2527–2536.). Human lung carcinoma cell line, A549, was purchased from American Type Culture Collection (Manassas, VA) and colon 26 was given from Prof. Michiyuki Matsuda and Dr. Yuji Kamioka (Department of Medicine, Kyoto University). These cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen Corp., Carlsbad, CA) containing 10% of fetal bovine serum in a humidified incubator with 5% $CO_2$, 95% air at 37 ˚C. Hypoxic cell culture condition was made using Ruskinn Invivo 400. Female BALB/c mice, 6-9 weeks of age were purchased from SLC (Shizuoka, Japan). The mice were housed at the Institute of Laboratory Animals at Kyoto University Graduate School of Medicine.

**Preparation of gold nanorods (GNRs).** To a 3mL reaction mixture containing 2 mM $HAuCl_4$-$3H_2O$ and 80 mM CTAB, 10 μL of cyclohexanone and 20 μL of acetone ware added. Then, 25-30 μL of 10 mM $AgNO_3$ solution and 200 μL of 42 mM ascorbic acid solution were added to the reaction solution. The solution was mixed and the color changed from dark orange to colorless. The resulting solution was transferred to a quartz test tube, and was irradiated by UV light from a Xe lamp (Hamamatsu) through a band pass filter (Sigma, UTVAF-50S-33U) for 30 min. The resulting dark purple solution was centrifuged at 16,100 rcf for 20 min, and supernatant was discarded. The precipitate was redispersed in deionized water, and centrifuged again at same conditions. The supernatant was discarded and the precipitate was redispersed in 200 μL of deionized water (GNRs).

**Preparation of G-PEGs, G-NIs and G-NI-Fs.** To GNR dispersion, 200 μL of 20 mM HS-PEG-$NH_2$ solution was added and stand still for 12-18 h at room temperature. The resulting solution was centrifuged at 16100 rcf for 20 min twice and the precipitate was collected to re-disperse in 200 μL of deionized water to give G-PEGs. To G-PEGs

dispersion, 200 μL of 50 mM EDC, 5 mM DMAP, 50 mM HOBt, and 40 mM NI-$CO_2H$ in DMF (200 μL) solution was added and stand still for 18 h at room temperature. The resulting solution was centrifuged at 16100 rcf for 20 min twice, and the precipitate was collected to re-disperse in 200 μL of deionized water to give G-NIs. In the same way, we prepared fluorescein-labeled nanoparticles, G-NI-Fs. To G-PEGs dispersion, 200 μL of DMF solution containing 50 mM EDC, 5 mM DMAP, 50 mM HOBt, 40 mM NI-$CO_2H$, and 3 mM Atto 647N NHS ester was added and stand still for 12-18 h at room temperature. The resulting solution was centrifuged at 16100 rcf for 20 min and washed with deionized water twice. Then the precipitate was collected to re-disperse in 200 μL of deionized water to give G-NI-Fs.

**Characterization of GNR and its Derivatives.** UV-vis-NIR spectra were acquired on a HITACHI U-3010 spectrophotometer. Transmission electron microscopy (TEM) images were taken at 120 kV JEOL JEM-1400. Zeta potential was measured by Otsuka electronics ELS-800SA. IR spectra were acquired on a JASCO FT/IR-4100 fourier transform infrared spectrometer. Freeze dried GNRs, G-PEGs and G-NIs samples were measured by KBr method.

**Cell Culture.** A549 human lung cancer cells and colon 26 mouse rectal cancer cells were cultured at 37 ˚C and 5% $CO_2$ in DMEM supplemented with 10% fetal bovine serum (FBS), 500 units Penicillin and 500 μg Streptomycin.

**Cytotoxicity Assay (WST Assay).** WST assays were performed to assess the mitochondrial activity of cells treated as the following. The cytotoxicity assays were performed in 96-well plates with a seeding density of $1 \times 10^4$ cells per well. The cells were pre-incubated overnight at 37 ˚C in 5% $CO_2$ before adding test substances. Then, they were incubated for 24 h with medium containing 0 - 2.5 nM G-NIs. After incubation, 10 μL of WST-8 solution was added to each well and the plates were incubated for 2 h at 37 ˚C. After the incubation, absorbance at 450 nm was measured with micro-plate spectrophotometer (BIORAD, xMark).

**Confocal Microscopy.** A549 cells ($1 \times 10^4$ cells) were plated into 35 mm glass bottom dish and cultured for 24 h at 37 ˚C in 5% $CO_2$, and then incubated under hypoxic (0.3% $O_2$) or aerobic conditions (20% $O_2$) for 24 h. The oxygen concentration was set up using chamber device (Ruskinn in vivo 400), which could control the oxygen concentration outside of the cells. G-NI-Fs was added to the medium at a final concentration of 1.25

nM and incubated for 3 h under hypoxic or aerobic conditions. After the cells were washed, they were further incubated in fresh medium for 1 h under aerobic conditions. Then the imaging by microscopy was conducted. Fluorescent images of G-NI-Fs in A549 cells at designated oxygen concentration were acquired using an argon laser (633 nm) for excitation and a 638 - 759 nm band-pass filter for emission. The specimens were viewed at high magnification using a 63 × oil objective (Carl Zeiss).

**Total gold uptake levels.** A549 cells ($5 \times 10^5$ cells) were exposed to gold nanoparticles under hypoxic or aerobic conditions for 24 h at 37 ˚C. After wash by PBS, the cells were collected and treated by aqua regia. The resulting samples were subjected to ICP MS (Thermo X-series).

**Transmission electron microscopy.** A549 cells ($1 \times 10^4$ cells/well) were plated into 8 well Permanox® slide dish and cultured for 24 h under aerobic conditions. Then the cells were pre-incubated at 37 ˚C in 5% $CO_2$, and then incubated under hypoxic (0.3% $O_2$) or aerobic conditions (20% $O_2$) for 24 h.
G-PEGs or G-NIs were added to the medium at a final concentration of 1.25 nM and incubated for 24 h under hypoxic or aerobic conditions. After the cells were washed, they were fixated by 4% glutaraldehyde and 4% paraformaldehyde solution. Then, the samples were stained by osmium tetroxide and embedded in Epon resin.

**Photoacoustic imaging (PAI).** 200 μl of 25 nM G-NIs or G-PEGs in saline were administered intravenously to mice. Before injection and at 5 min, 30 min, 1 h, 2 h and 24 h after injection, photoacoustic imaging was acquired with 680 nm laser light.

**Measurement of Anti-PEG IgM.** GNRs (5 pmol), G-NIs (5 pmol) or G-PEGs (5 pmol) in saline were administered intravenously to mice. A week after injection, their blood were collected and centrifuged, and then serum was collected. Anti-PEG IgM levels in serum were determined by ELISA using Mouse Anti-PEG IgM ELISA kit.

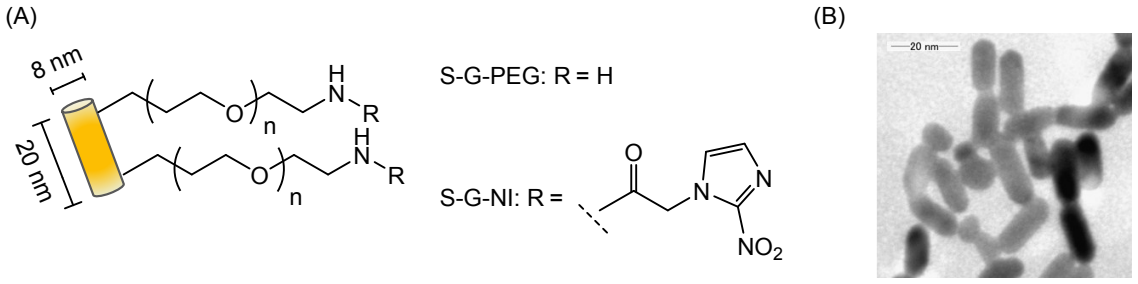


**Figure S1.** (A) Chemical structure of small gold nanorods (S-G-PEG and S-G-NI). (B) TEM image of S-G-NI

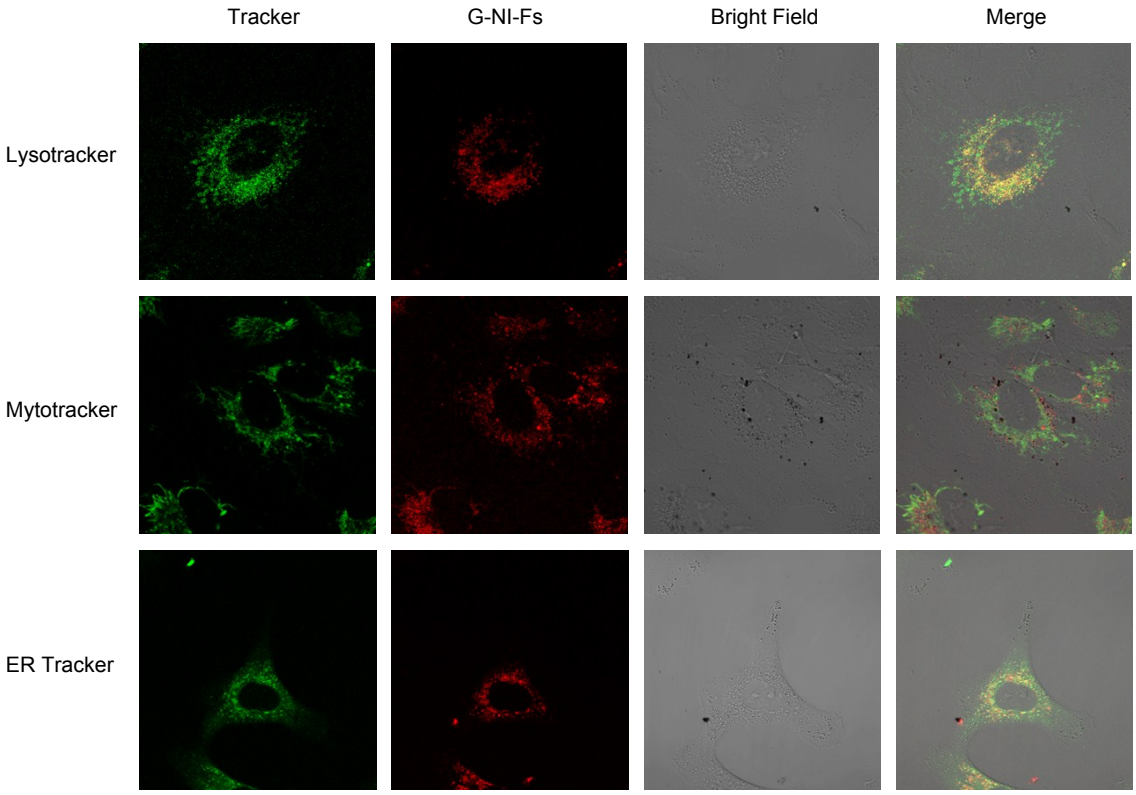


**Figure S2.** Localization of G-NI-Fs in A549 cells. Cells were incubated with G-NI-Fs and then lysosome, mitochondria and ER were stained by organelle markers, Lysotracker, Mytotracker and ER tracker, respectively.

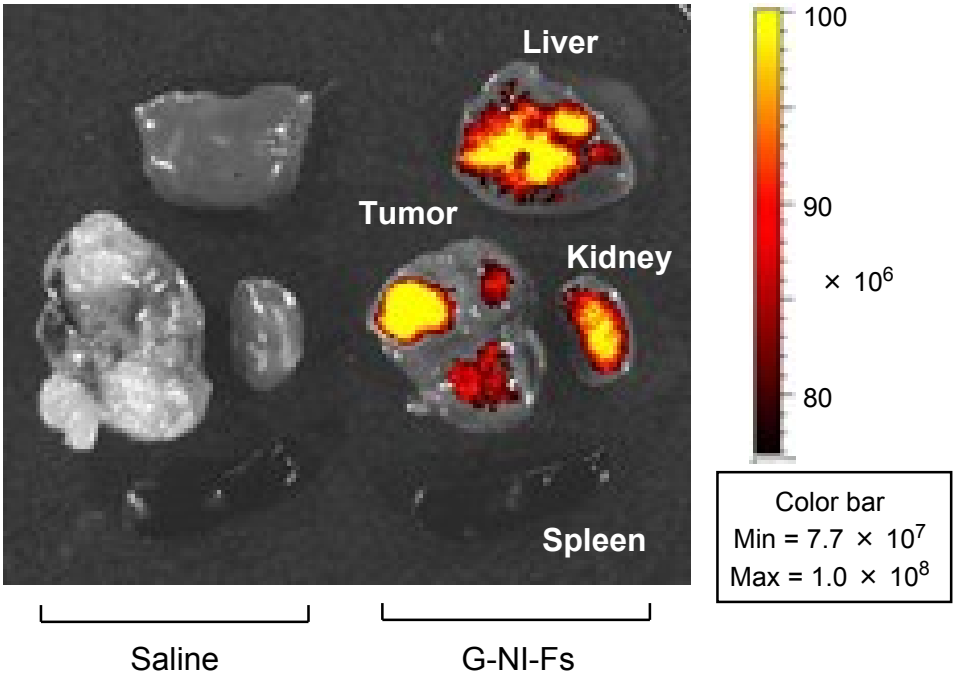


**Figure S3**. Ex vivo imaging of organs of tumor-bearing mice. Analysis of the mice was conducted after injection of saline or G-NI-Fs. The all images were monitored by in vivo imaging system (excitation at 570 nm, emission at 620 nm).