Supporting Information

In vivo Stealthified Molecularly Imprinted Polymer Nanogels Incorporated with Gold Nanoparticles for Radiation Therapy

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1. Experimental Sections

1-1. Materials

Sodium carbonate (Na_2CO_3), citric acid, sodium chloride (NaCl), magnesium sulfate ($MgSO_4$), diethyl ether, methanol (MeOH), sodium hydroxide (NaOH), tris(hydroxymethyl)aminomethane, sodium boron hydride ($NaBH_4$), hydrochlodic acid (HCl), sulfuric acid (H_2SO_4), bromophenol blue, glycerol, thiourea, 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonate (CHAPS), D-MEM (high glucose), D-PBS, Accutase, 0.25%-trypsin, 0.5% trypan blue stain solution, *N*-isopropylacrylamide (NIPAm), and *N,N'*-methylenebisacrylamide (MBAA) were purchased from Nacalai Tesque Co. (Kyoto, Japan). Sodium dihydrogen phosphate (Na_2HPO_4), dichloromethane (DCM), gold standard solution (1000 ppm), nitric aid (HNO_3)*N*-Boc-3-hydroxypyrrolidine, ethyl acetate (EtOAc), hexane (Hex), acetone, ethanol (EtOH), 2-aminoethanol, human serum albumin (HSA),sodium dodecyl sulfate (SDS), penicillin-streptomycin solution, glycine, urea, isoflurane, 10% formalin solution, 2,2'-azobis (2-methylpropionamidine)dihydrochloride (V-50), and cell counting kit-8 (CCK-8) were purchased from Wako Co. Ltd. (Osaka, Japan). Cystamine dihydrochloride, 11-mercaptoundecanoic acid, *N*-hydroxy succinimide (NHS), gold chloride trihydrate, fetal bovin serum (FBS), immunoglobulin G (IgG) were purchased from SIGMA-ALDRICH (USA). Disodium hydrogen phosphate (NaH_2PO_4) was purchased from Katayama Chemical Industries Co. Ltd. (Osaka, Japan). *N,N*-Diisopropylethylamine, acryloyl chloride, tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Tokyo Chemical Industries (Tokyo, Japan). 4*N* Hydrogen chloride (HCl) was purchased from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). 2-Methacryloyloxyethyl phosphorylcholine (MPC) was purchased from NOF Corporation (Tokyo, Japan). Pyrrolidyl acrylate (PyA) was prepared as previously reported.[1] Methacryloyloxyethyl thiocarbamoyl rhodamine B (MTRB) was purchased from Polysciences Inc. (PA, USA). Human serum was purchased from Cosmo Bio (Tokyo, Japan). BIO-Safe Coomassie G-250 stain was purchased from BIO-RAD (Tokyo, Japan). Corning matrigel was purchased from Corning (NY, USA). In-gel tryptic digestion kit was purchased from Thermos Scientific (MA, USA). Deionized water was obtained from a Millipore Milli-Q purification system. Fibrinogen from human plasma was purchased from Merck Millipore Co. (Darmstadt, Germany). Surface Plasmon resonance (SRP) sensor chips (unmodified Au) were purchased from GE Healthcare Japan (Tokyo, Japan).

1-2. Characterization

UV-Vis spectral measurements were performed using a V-560 spectrophotometer (JASCO Ltd., Tokyo, Japan). The particle size distribution and zeta potential were obtained using a DLS system (Zetasizer Nano ZS; Malvern Instruments Ltd., Malvern, UK). Transmission electron microscopy (TEM) images of the purified MIP-NGs were obtained using a JEM-1230 (JEOL, Tokyo, Japan). Fluorescent spectra were measured by using an F-2500 fluorescence spectrophotometer (Hitachi High-Technologies, Tokyo, Japan). SDS-PAGE was performed using PowerPac basic power supply (BIO-

RAD) and electrophoresis chamber (AE6500, ATTO) with e-PAGEL (crosslinking ratio 7.5%, ATTO). The cells were cultured in a CO_2 incubator (CO_2 Water Jacketed Incubator, Thermo Fisher Scientific Inc, Massachusetts, US) and observed under an inverted microscope (CKX31, Olympus, Tokyo, Japan). Cells were collected by centrifugation (KUBOTA2800, Kubota, Osaka, Japan). Ultracentrifugation was performed by OptimaTM MAX-XP (Bechman Coulter, Tokyo, Japan)

1-3. Synthesis of *N,N'*-cystamine bisacrylamide (CBA)

Cystamine dihydrochloride (5.8 g, 25 mmol) and NaOH (4.0 g, 100 mmol) were dissolved in pure water (60 mL). Acryloyl chloride (4.2 mL, 50 mmol) was added dropwise to the aqueous solution under stirring for 30 min in an ice bath. After 3 h reaction under room temperature, the precipitate was corrected by the filtration and washed by pure water. The corrected product was dried in vacuo.

Yield: 2.18 g (34%)

^{1}H-NMR (500 MHz, $CDCl_3$): δ= 6.33 (d, 2H, vinyl), 6.25 (m, 2H, vinyl), 5.69 (d, 2H, vinyl), 3.69 (q, 4H, ethyl), 2.90 (t, 4H, ethyl)

1-4. Preparation of MIP-NGs and NIP-NGs containing disulfide linkage

HSA (13.2 mg), PyA (42 mg, 0.30 mmol), NIPAm (387 mg, 3.42 mmol), MBAA (30.8 mg, 0.20 mmol), CBA (46.8 mg, 0.18 mmol), MPC (59 mg, 0.20 mmol), MTRB (6.66 mg, 0.01 mmol), and V-50 (217 mg) were dissolved in 10 mM PBS (pH 7.4). After N_2/vacuum cycles, emulsifier-free precipitation polymerization was carried out at 70°C for 12 h for preparation of MIP-NGs containing disulfide linkage. NIP-NGs were also prepared in the absence of HSA.

Size-exclusion chromatography using Sephadex G-100 (GE Healthcare Japan) and ion-exchange chromatography with DAEA Sephadex (SIGMA-ALDRICH) were carried out to purify the MIP-NGs and NIP-NGs to remove the remained monomer/initiator/oligomer species and template HSA. For size-exclusion chromatography. Sephadex G-100 was packed into a column (33 cm × 1.2 cm I.D.) and 10 mM PBS (pH 7.4) was used as the eluent. The nanogels concentrated with ultrafiltration (Amicon: 10 kDa) were dispersed in 10 mM PBS (pH: 7.4) (2 mL) and injected into the Sephadex G-100 packed column. For ion-exchange chromatography, DEAE-Sephadex was packed into a column (6.0 cm × 1.7 cm I.D.) and 10 mM PBS (pH 7.4) was used as the eluent. For these chromatography steps, each fraction (1.5 mL) was collected and fluorescent spectroscopy was measured to confirm the removal of template HSA. NIP-NGs were purified using the same procedure.

1-5. Preparation of Au MIP-NGs and Au NIP-NGs

The solvent of purified MIP-NGs was exchanged to pure water by using ultrafiltration (Amicon 10 kDa). TCEP (257 mg, 0.2 mmol) was added to the concentrated MIP-NGs (10 mL) and reacted for 2 h at room temperature to cleave the disulfide linkage to thiols. After washing with pure water by using ultrafiltration (Amicon 10 kDa), the thiolated MIP-NGs were then incubated with gold chloride

trihydrate (4.93 mg, 12.5 μmol) for 2 h at room temperature, and $NaBH_4$ aqueous solution (125 μmol, 0.25 mL) was slowly added under vigorous stirring. After 5 min reaction at room temperature, the Au MIP-NGs were washed with 10 mM PBS (pH 7.4) thrice. Au NIP-NGs were also prepared by same procedure from NIP-NGs.

1-5. DTNB assay

DTNB assay can estimate the number of free thiol groups in the solution. MIP-NGs reacted with TCEP were washed with 10 mM phosphate buffer (pH 7.4) by ultrafiltration. After the addition of DTNB (1 mM, 60 μL) and 10 mM phosphate buffer (pH 7.4, 1.94 mL) to the MIP-NGs dispersion (1 mL), the dispersion of MIP-NGs (final concentration: 1.31 mg/mL) was incubated at 25 °C for 1 h. The UV-Vis spectra were measured for MIP-NGs before and after TCEP treatments, and the number of thiol groups were estimated from the absorbance values at 412 nm of these solutions and absorptivity of the generated 5-mercapto-2-nitrobenzoic acid ($\varepsilon=1.55 \times 10^4\ M^{-1}cm^{-1}$).

1-6. Au contents in Au MIP-NGs and Au NIP-NGs

Au MIP-NGs and Au NIP-NGs (10 mg/mL, 50 μL) dispersed in 10 mM PBS (pH 7.4) were dried, and the HNO_3/H_2SO_4 (1:1 v/v) mixture (300 μL) was added and heated at 230°C. The treatment was repeated trice. The aqua regia (HCl/HNO_3 = 3/1 v/v, 1.0 mL) was added to the residue and reacted at room temperature for 1 h to ionize Au NPs. The aqueous solution was diluted with pure water (4.0 mL). Au contents were determined by ICP-AES (ULTIMA 2000, Jobin Yvon Horiba), where the calibration curve was prepared by using commercial gold standard solution (1000 ppm).

1-7. Surface plasmon resonance measurements

After washing with water and EtOH, the SPR sensor chip was immersed in EtOH solution of 1 mM 11-mercaptoundecanoic acid at 25°C for 24 h. After washing with EtOH, the SPR sensor chip was docked into the Biacore 3000. Aqueous solution of EDC (final concentration: 0.2 M) and NHS (final concentration: 0.05 M) was injected for 7 min, where the flow rate was 20 μL/min. The proteins solution dissolved in 10 mM PBS (pH 7.4) were injected for 7 min (flow rate was 20 μL/min). Then, 1M ethanolamine aqueous solution (pH 8.5) was injected to treat the activated carboxylic acid groups for 7 min (flow rate was 20 μL/min). The MIP-NGs solution (0, 800, 1600, 3200, 6400, and 12800 ng/mL) were then injected for 7 min (flow rate was 20 μL/min). All procedures in Biacore 3000 were performed at 25°C. Data were obtained at 13 min after each injection.

1-8. Protein corona analysis

Human serum was centrifuged (45000 rpm) at 37°C for 5 h to remove coagulated proteins. Au MIP-NGs or Au NIP-NGs were incubated in 55% human serum for 12 h at 37 °C (final concentration: 500 μg/mL). Ultracentrifugation with 45000 rpm was carried out at 37°C for 5 h to correct Au MIP-NGs or Au NIP-NGs as pellet, and the supernatant was carefully removed. The Au MIP-NGs and Au NIP-

NGs were then redispersed and incubated in the urea-thiourea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 800 μL) for 2 h at room temperature to desorb the proteins from the nanogels. The Au MIP-NGs and Au NIP-NGs were removed by ultrafiltration (300 kDa), and the solvent of the protein solution was changed to 10 mM PBS (pH 7.4) using ultrafiltration (10 kDa) fifth. The protein solution (1600 μL) was obtained.

The protein solution (20 μL) was mixed with SDS-PAGE migration solution (20 μL) and treated for 5 min at 90 °C. The mixture (20 μL) was loaded into acrylamide gel (crosslinking ratio: 7.5 %) and electrophoresis was performed at 20 mA for 80 min. The gels were stained with BIO-Safe Coomassie G-250 stain solution.

1-9. Cell culture

Freeze human pancreatic cancer cell line MIAPaCa-2, obtained from American Type Culture Collection (Rockville, MD, USA), was incubated at 37 °C for 1.5 min, and the cells were washed with DMEM (10 mL) once by centrifugation with 1100 rom for 5 min. The cells (10,000 cells/dish) were redispered in DMEM (11 mL) and poured in dish (diameter: 100 mm). The cell culture was performed in CO_2 incubator at 37 °C. The DMEM medium was changed to fresh one in each 3 day. When the cells reached subconfluent, the cells were detached from the dish by incubating in Accutase (3 mL) in CO_2 incubator at 37 °C for 20 min, and corrected by centrifugation with 1100 rom for 5 min. After redispersion of the cells in DMEM (1 mL), the number of cells was calculated by using Burker-Turk calculator. The cells were then cultured in each dish as 10,000 cells/dish using DMEM medium.

1-10. Pancreatic cancer model mice

BALB/c nude mice (4 weeks, male) purchased from (Charles River Laboratories Japan Inc. Yokohama, Japan) were maintained under isothermal conditions with regulated photoperiods in specific pathogen-free animal care facilities. Subcutaneous injections of 3×10^6 MIAPaCa-2 cells suspended in Matrigel (100 μL) were performed for each mouse.

1-11. Biodistribution

Biodistribution of Au MIP-NGs was investigated using the pancreatic cancer mice. Intravenous administration of Au MIP-NGs or Au NIP-NGs (10 mg/mL, 100 μL) was demonstrated to each pancreatic cancer mouse. After 6 h, the mice were anesthetized by isoflurane and sacrificed, then organs of mice were preserved in 10% formalin solution. The organs were heated at 1000°C for 3 h, and the residues were treated in HNO_3/H_2SO_4 (1:1 v/v) mixture (300 μL) at 230°C thrice. The aqua regia (HCl/HNO_3 = 3/1 v/v, 1.0 mL) was added to the residue and reacted at room temperature for 1 h to ionize Au NPs. The aqueous solution was diluted with pure water (4.0 mL). Au contents were determined by ICP-AES (ULTIMA 2000, Jobin Yvon Horiba), where the calibration curve was prepared by using commercial gold standard solution (1000 ppm).

1-12. Radiation therapy

Au MIP-NGs (10 mg/mL) dispersed in 10 mM PBS (pH 7.4) were injected from tail-vein of the BALB/c nude mice bearing pancreatic cancer tumors. 10 mM PBS (pH 7.4, 140 mM NaCl) was injected as a control group. After 2 days, the mice were anesthetized by intraperitoneal administration of somnopentyl (0.1 mg/g body weight), and the bodies of mice, excluding tumor tissues, were protected from irradiation damage using a lead plate. X-ray irradiation (0.5 Gy/min at the target; total dose: 0, 2, or 4 Gy) was carried out using an MBR-1505R2 (Hitachi Medical Co., Tokyo, Japan) at a current of 5 mA and a voltage of 150 kV with a 1-mm-thick aluminum filter. The data of mice body weights and therapeutic efficacy of radiation were expressed as mean ± standard deviation. The tumor volume was estimated by calculating by using the formula $L \times W^2 \times (\pi/6)$, where L and W are the longest and shortest diameters of the tumor, respectively.[2-4] The mouse tumors were 954 - 1174 mm^3 in volume on day zero. The statistical significance of differences in therapeutic efficacy between two groups possessing unequal variance was calculated using the Student's t-test with two-sided test. Differences were considered significant at the 95 % confidence level ($p < 0.05$).

2. Particle size distribution of MIP-NGs and NIP-NGs

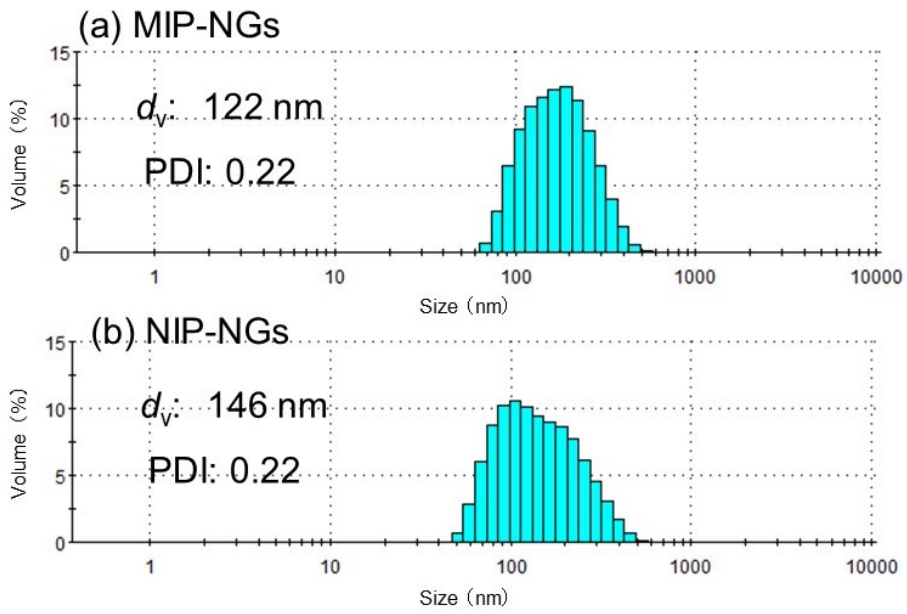

Figure S1. Particle size distribution measured by DLS of MIP-NGs and NIP-NGs. MIP-NGs: molecularly imprinted polymer nanogels. NIP-NGs: non-imprinted polymer nanogels.

3. Particle size distribution of Au NIP-NGs

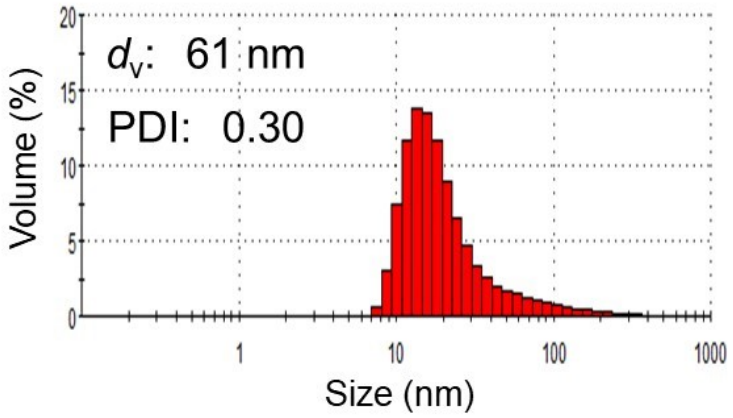

Figure S2. Particle size distribution measured by DLS of Au NIP-NGs. Au NIP-NGs: non-imprinted polymer nanogels incorporating gold nanoparticles.

4. SDS-PAGE analysis of HSA in human serum

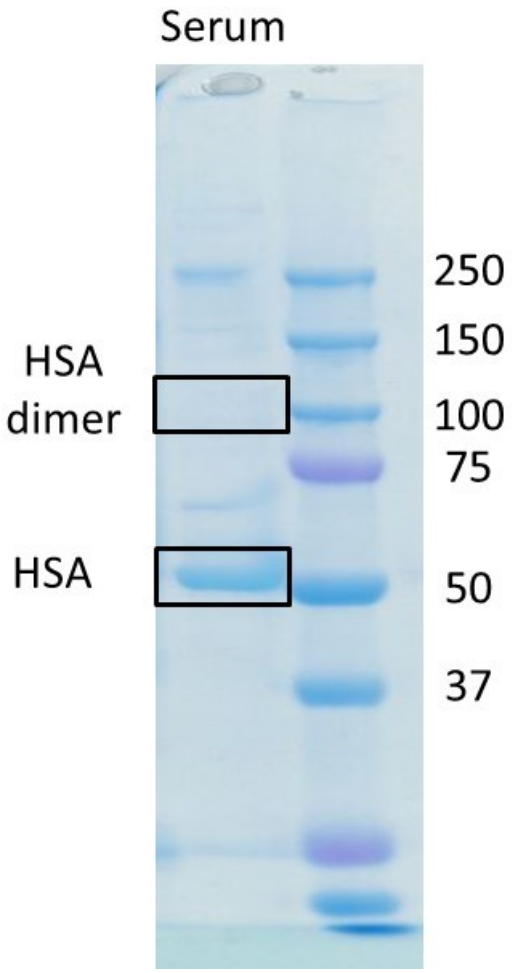

Figure S3. Protein bands derived from human serum containing proteins analyzed by SDS-PAGE

5. Cell cytotoxicity of Au MIP-NGs

Cell cytotoxicity of Au MIP-NG was examined by CCK-8 assay using a standard fibroblast cell line, NIH3T3. Cultured NIH-3T3 cells seeded into each well in the 96 well microplates (5,000 cells/well) were cultured in 100 μL DMEM medium (10% FBS, 1% penicillin streptomycin). After incubation in under a CO_2 atmosphere for 24 h, the Au MIP-NGs dispersion (10 μL, final concentration: 0, 1.2, 12, 120, 1200 μg/mL) were added into each well, and the 96 well plate was incubated for 24 h. CCK-8 solution (10 μL) was added into each well. After 4 h incubation, the absorbance in each well was measured by using microplate reader (λ= 450 nm). Cell viability was calculated from equation 1.

$$Cell\ viability\ (\%) = \frac{sample(A_{570} - A_{650}) - blankA(A_{570} - A_{650})}{control(A_{570} - A_{650}) - blankB(A_{570} - A_{650})} \times 100 \quad \text{(eq. 1)},$$

with *sample* containing cells and Au MIP-NGs, *control* containing cells only, *blank A* containing Au MIP-NGs only, and *blank B* containing no cells or Au MIP-NGs.

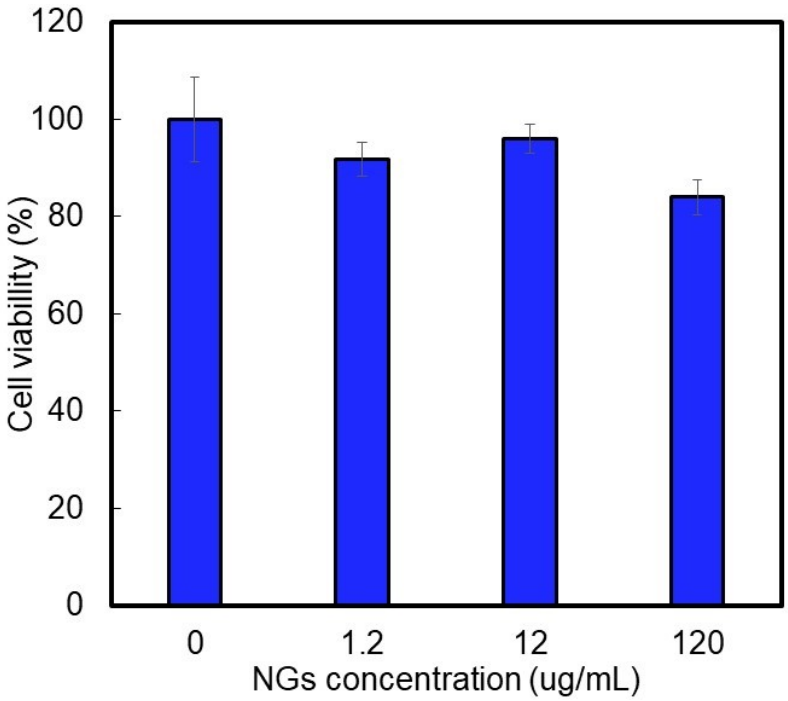

Figure S4. Cell cytotoxicity of Au MIP-NGs evaluated by CCK-8 assay with normal cells, NIH-3T3.

6. Blood biomarker concentrations related to liver damage

BALB/c mice (male, 4 weeks) were used for evaluating secretion of blood biomarker after injection of Au MIP-NGs and PBS. Au MIP-NGs (10 mg/mL) dispersed in 10 mM PBS (pH 7.4) were injected from tail-vein of the BALB/c mice. As a control groups, 10 mM PBS (pH 7.4, 140 mM NaCl) was injected. After 24 h, the mice were anesthetized by isoflurane and sacrificed, then blood was corrected. The blood concentrations of various biomarker were measured by Oriental Yeast. Co. Ltd. (Tokyo, Japan).

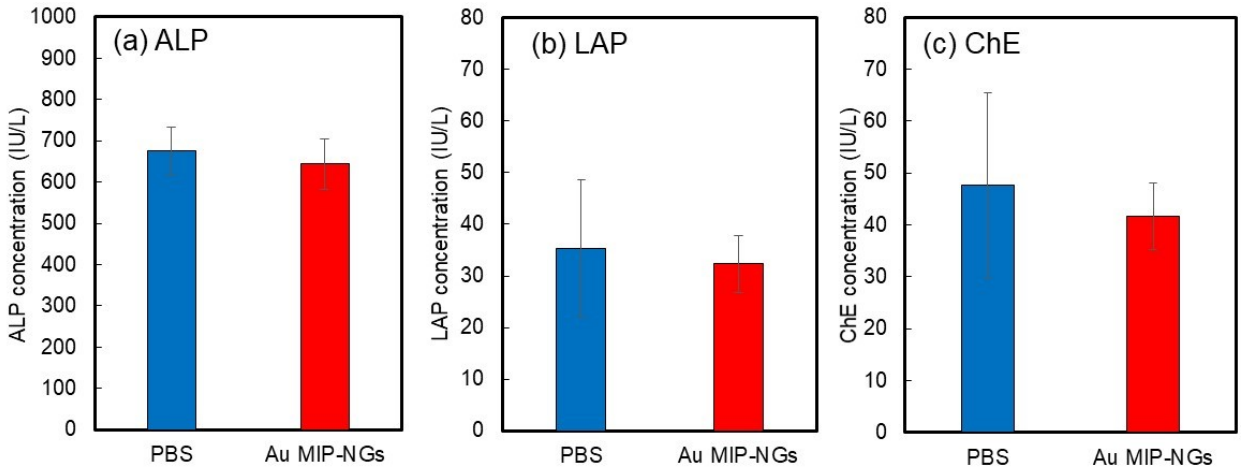

Figure S5. Blood biomarker concentrations of BalB/c mice after injection of Au MIP-NGs (red) and PBS (blue): (a) alkaline phosphatase; (b) aminopeptidase; (c) cholinesterase.

7. In vivo confocal laser scanning microscopy

The following animal experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals as stated by the University of Tokyo. The retention of MIP-NGs in the blood stream was evaluated by intravital confocal laser scanning microscopy (IVCLSM) according to a previously reported method.[5-7] BALB/c mice (6 weeks old, female, Oriental Yeast Co., Ltd., Tokyo, Japan) were anesthetized with 4 % isoflurane (Mylan Inc., Tokyo, Japan) using an anesthesia unit (Narcobit-E; Natsume Seisakusho Co., Ltd., Tokyo, Japan). A catheter was inserted into the lateral tail vein of a mouse, which comprised a 30-gauge needle (Dentronics Co., Ltd., Tokyo, Japan) connected to a non-toxic, medical-grade polyethylene tube (SP10; Natsume Seisakusho Co., Ltd., Tokyo, Japan). The mouse was placed on the microscope stage with temperature controller (Thermoplate; Tokai Hit Co., Ltd., Shizuoka, Japan), and the anesthesia was maintained with a decreased flow of 2 % isoflurane. The anesthesia depth of the mouse was monitored using a pulse oximeter (MouseSTAT; Kent Scientific, Torrington, USA).

To observe the blood stream by IVCLSM, the earlobe of the mouse was used. All image was obtained by a Nikon A1R confocal laser scanning microscope system connected to an upright ECLIPSE Ni-E equipped with a CFI Plan Apo Lambda 20× objective (Nikon, Tokyo, Japan). Fluorescence images were acquired with a 561-nm excitation laser and a bandpass emission filter (570 nm/620 nm). The pinhole diameter was set to result in a 10 μm optical slice. The earlobe was attached beneath a coverslip with a small drop of immersion oil. Data were acquired at a video rate of 15 frames/s for the first 1 min, followed by snap-shots at every 5 min. Au MIP-NGs (10 mg/mL in 10 mM PBS, pH 7.4) and Au NIP-NGs (10 mg/mL in 10 mM PBS, pH 7.4) were administered (100 μL) via the tail vein catheter 10 s after the imaging was initiated.

The acquired video data were analyzed by selecting regions of interest (ROIs) within the blood vessels and the extravascular skin tissues, where the average intensity of fluorescence per pixel for each time point was determined by Nikon NIS-Elements software (Nikon, Tokyo, Japan). Retention was defined as relative fluorescence intensity (%), which is a ratio of the observed intensity of fluorescence (F-F0) to the maximum intensity (Fmax-F0) observed, where the background fluorescence intensity (F0) was determined by the intensity of fluorescence before injecting the nanogels. The background values were subtracted from the observed fluorescence intensity (F and Fmax) measured after injecting the nanogels to obtain the background-corrected intensity for each time point.

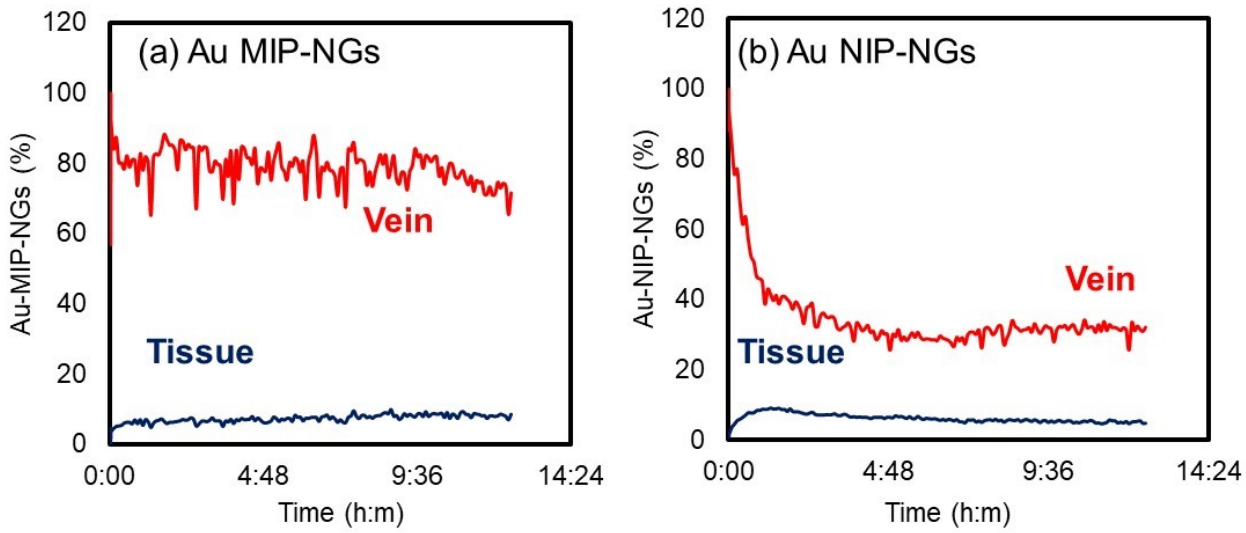

Figure S6. Retention profiles in the blood vessel (red lines) and tissue accumulation (blue lines) for Au MIP-NGs (a) and Au NIP-NGs (b)

8. Cancer treatment effect of Au MIP-NGs without X-ray irradiation

The following animal experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals as stated by the Kobe University. Au MIP-NGs (10 mg/mL) dispersed in 10 mM PBS (pH 7.4) were injected from tail-vein of the BALB/c nude mice bearing pancreatic cancer. 10 mM PBS (pH 7.4, 140 mM NaCl) was injected as a control group. After 11 days, the procedure of injection of Au MIP-NGs dispersion or PBS were repeated. The tumor volume was estimated by calculating by using the formula $L \times W^2 \times (\pi/6)$, where L and W are the longest and shortest diameters of the tumor, respectively. [2-4]

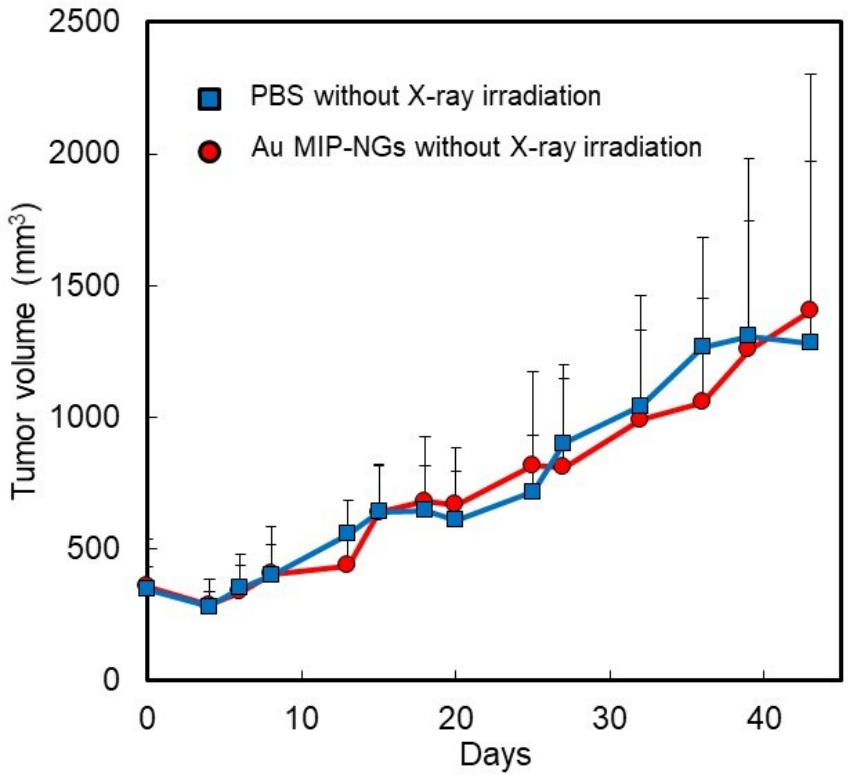

Figure S7. Tumor volume changes in pancreatic cancer model mice injected with (red) and without (blue) Au MIP-NGs without X-ray irradiation.

9. Body weight of pancreatic cancer mice

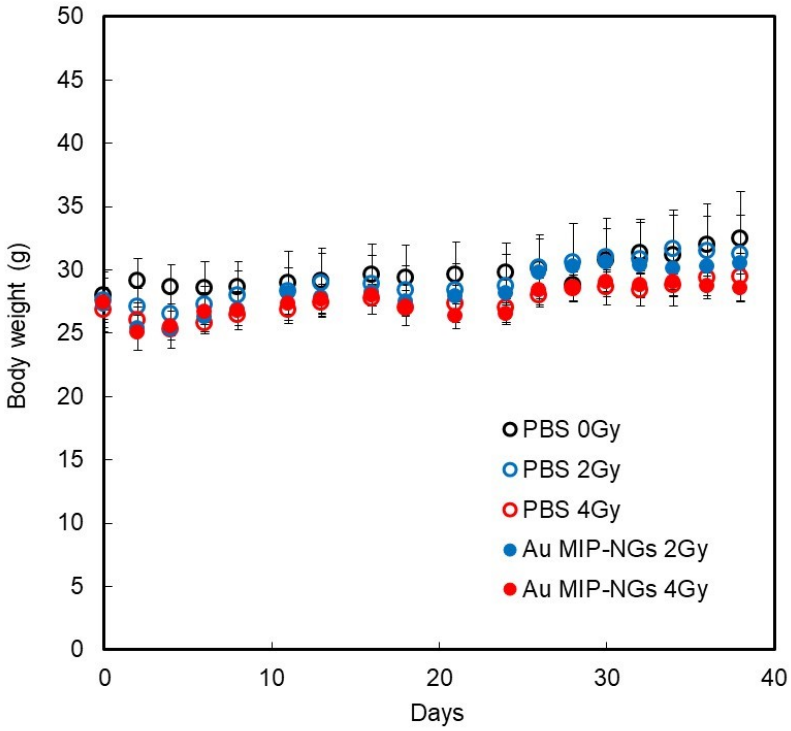

Figure S8. Body weight changes in X-ray irradiated pancreatic cancer model mice injected with Au MIP-NGs (closed circles) and PBS (open circles) and control mice without X-ray irradiation and Au MIP-NGs injection (black). X-ray irradiation dose: 2 Gy (blue); 4Gy (red). Au MIP-NGs: Au NPs incorporated molecularly imprinted polymer nanogels; Au NIP-NGs: Au NPs incorporated non-imprinted polymer nanogels

10. Reference

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