

Supporting Information

“Development of Target-Specific Multimodality Imaging Agent by Using Hollow Manganese Oxide Nanoparticle as a Platform”

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Synthesis and characterization

General consideration. Any reagent including $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (Aldrich), Sodium Oleate (TCI), Oleic acid (Aldrich), Phthalate buffer (pH 4.6, Samchun Chem.), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG-2000 PE, Avanti Polar Lipids, Inc.), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG(2000) biotin, Avanti Polar Lipids, Inc.), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG(2000) Amine, Avanti Polar Lipids, Inc.), Bolton-Hunter reagent (Thermo Scientific), and $\text{Na}[^{125}\text{I}]\text{I}$ (PerkinElmer) were used as purchased without any purification. Analyses of transmission electron microscopy (TEM) were conducted with JEOL JEM-2010. The negative staining for showing the organic shell around the **HMON** in TEM images was carried out by using 5% phosphotungstic acid at pH 7. Inductive coupling plasma atomic emission spectroscopy (ICP AES) was carried out with Direct Reading Echelle ICP (LEEMAN). ^1H NMR spectra were obtained on a Varian ^{Unity}Inova 500NB (500 MHz) spectrometer (Palo Alto, CA, USA). Fast atom bombardment (FAB) mass spectra were obtained on a JMS-600 (JEOL Ltd, Tokyo, Japan). The purification of radioactively labeled ligands (^{125}I -biotin) was carried out by using HPLC, Spectra System (Thermo Electron Corp., Fremont, CA, USA), equipped with an analytical column (YMC-Pack C18, 5 μ , 4.6 x 250 mm). During the HPLC work, the eluent was monitored simultaneously by using UV (254 nm) and $\text{NaI}(\text{Tl})$ radioactivity detectors. Thin layer chromatography (TLC) was performed on Merck F₂₅₄ silica plates and analyzed on a Bioscan radio-TLC scanner (Washington, D.C., USA). Radioactivity (gamma radiation) was measured in a dose calibrator (Biodex Medical Systems, Shirley, NY, USA) and tissue radioactivity in a Wizard² automatic gamma counter (PerkinElmer, Waltham, MA, USA).

Synthesis of biotin-HMON. The aqueous suspension containing 20 nm-sized hollow manganese oxide nanoparticles (**HMON**) were prepared by the previously reported method

including the etching of water-dispersible manganese oxide nanoparticles in an acidic solution.^[1]

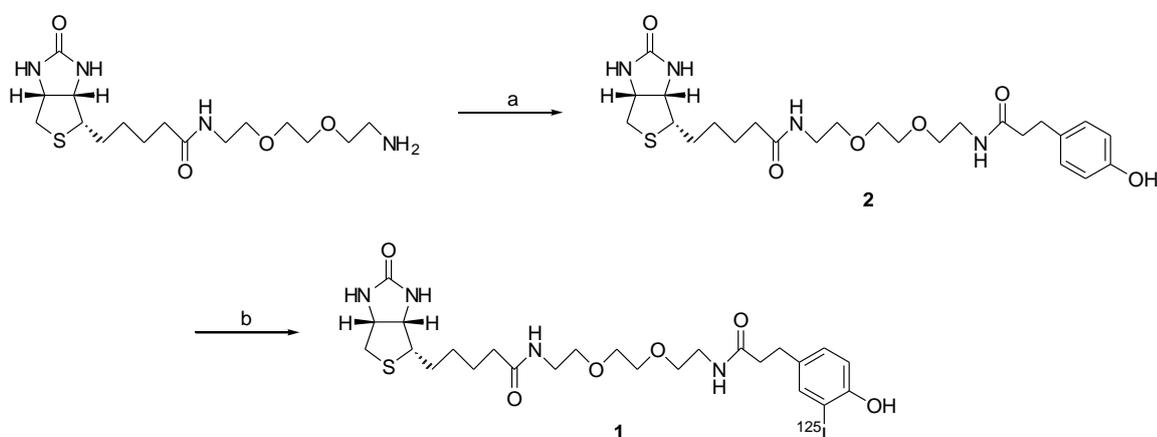
The **HMONs** were isolated as a solid from the aqueous suspension by the ultracentrifugation and dried *under vacuo*. The **HMON** solids were encapsulated with a phospholipid mixture composed of PEG-phospholipid and biotin-PEG-phospholipid to endow them with biocompatibility and affinity to streptavidine (SA). Typically, 4 ml of the **HMONs** in CHCl₃ (2.5 mg/ml) was mixed with 1 ml of CHCl₃ containing 30 mg of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-phospholipid) and 3 mg of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (biotin-PEG-phospholipid). After evaporating solvent, it was incubated at room temperature *in vacuo* for 5 min. The addition of 5 ml water resulted in a clear and dark-brown suspension. After filtration, excess phospholipids were removed by ultracentrifugation, providing biotinylated **HMONs** (biotin-**HMONs**).

Synthesis of HMON/SA. For the conjugation of SAs to biotin-**HMON**, a 13.5 ml aqueous solution of the biotin-**HMONs** (0.67 mg/ml) was mixed with a 4.5 ml of aqueous solution containing excess amount of SAs (1 mg/ml) and stirred at room temperature. After 30 min incubation, the reaction suspension was centrifuged and the supernatant containing leftover SAs were removed. The resulting **HMON/SA** was purified by repeating the re-dispersion of the precipitate in a distilled water and centrifugation.

Synthesis of biotinylated CET. Prior to the biotinylation reaction of Cetuximab (CET), the solvent in a CET solution (5 mg/ml, Merck, Erbitux) was exchanged with distilled water by using PD-10 desalting column (GE healthcare, SephadexTM G-25 M). For the biotinylation, an aqueous solution of NHS-biotin (0.5 ml, 4.4mg/ml, Thermo Scientific, EZ-LinkTM Sulfo-NHS-biotin) was added to a 3 ml of aqueous solution containing CET (5 mg/ml) and incubated with gentle shaking at 4 °C. After the reaction for 1 hr, the biotinylated CET was purified by eluting the resulting solution through PD-10 column.

Conjugation of CET to HMON/SA: synthesis of CET-HMON/SA. In order to conjugate CET to HMON/SA, a 2.8 ml of aqueous solution of biotinylated CET (14 mg) was added to a 4.2 ml of aqueous suspension of HMON/SA (1.67 mg/ml). After the incubation for 60 min, nanoparticles were isolated from the reaction suspension by the centrifugation. The resulting CET/HMON/SA conjugates were purified by repeating the re-dispersion in water and the centrifugation.

Synthesis of N-(8-(3-(4-Hydroxy-3-[¹²⁵I]iodophenyl)propanamido)-3,6-dioxaoctanyl)-biotinamide (¹²⁵I labeled biotin, ¹²⁵I-biotin). The synthesis of ¹²⁵I labeled biotin (¹²⁵I-biotin) was carried out through the protocol in Scheme 1. Bolton-Hunter reagent (19.3 mg, 0.073 mmol) was added to a 1:1 mixture of acetone and water solution (600 μL) containing 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (25 mg, 0.067 mmol) and sodium bicarbonate (16.8 mg, 0.2 mmol) and stirred at room temperature. After the reaction for 3 hrs, the reaction solution was concentrated by evaporating the solvent *under vacuo*. The resulting N-(8-(3-(4-hydroxyphenyl)propanamido)-3,6-dioxaoctanyl)-biotinamide (**2**) was obtained as a white solid by the extraction with distilled water and the lipophilization (43% yield). ¹H NMR (D₂O) of **2**: δ 7.05 (d, *J*=8.5 Hz, 2H), 6.75 (d, *J*=8.5 Hz,



Scheme S1. Reagents and conditions: (a) Bolton-Hunter reagent, sodium bicarbonate, water, acetone, rt, 3 hr; (b) Na[¹²⁵I]I, Iodogen tube, phosphate buffer, r. t., 30 min

2H), 4.5 (m, 1H), 4.3 (m, 1H), 3.71-3.18 (m, 13H), 2.92-2.60 (m, 4H), 2.45-2.40 (m, 2H), 2.19-2.14 (m, 2H), 1.64-1.43 (m, 4H), 1.32-1.23 (m, 2H). HRMS: mass calcd for C₂₅H₃₈N₄O₆S (M + H)⁺, 523.2512; found, 523.2590.

¹²⁵I labeling was conducted by mixing *N*-(8-(3-(4-Hydroxyphenyl)propanamido)-3,6-dioxaoctanyl)-biotinamide (0.3 mg, 0.57 mmol) and Na[¹²⁵I]I (19.4 MBq) in a PBS buffer solution using a pre-coated iodination tube for 30 min at room temperature. The completion of the reaction was monitored by using radio-TLC with 9:1 dichloromethane-methanol of eluting solvent. The ¹²⁵I-labelled product (¹²⁵I-biotin) was purified from the reaction mixture by using reverse phase HPLC column (YMC C18, 4.6 x 250 mm, 5 μ) with eluting an 80:20 mixture solvent of 0.05% trifluoroacetic acid/H₂O-acetonitrile in an 1 mL/min rate. The overall radiochemical yield was 50.7±8.3%.

Conjugation of ¹²⁵I-biotin: Synthesis of ¹²⁵I-HMON/SA and ¹²⁵I-CET-HMON/SA.

The ¹²⁵I-HMON/SA and ¹²⁵I-CET-HMON/SA were synthesized through conjugating ¹²⁵I-biotin to **HMON/SA** and **CET-HMON/SA**, respectively. For the synthesis of ¹²⁵I-HMON/SA, the purified ¹²⁵I-biotin (45.21 MBq) was added to 0.2ml of aqueous suspension containing **HMON/SA** (1mg/ml) and stirred at room temperature. After the conjugation reaction for 30 min, the nanoparticles were isolated from the reaction suspension by the centrifugation. The resulting ¹²⁵I-HMON/SA was purified by repeating the dispersion in water and centrifugation. The synthesis of ¹²⁵I-CET-HMON/SA was carried out through the same procedure with that applied for ¹²⁵I-HMON/SA, except for the use of **CET-HMON/SA** instead of **HMON/SA**.

The radio activity of the nanoparticles was measured by counting gamma-radiation by using a dose calibrator (Biodex Medical Systems, Shirley, NY, USA). The radiations counted from ¹²⁵I-HMON/SA and ¹²⁵I-CET-HMON/SA were 22.829 MBq and 5.2 MBq, respectively. The number of ¹²⁵I-biotin molecules on the retrieved nanoparticles was derived based on the radiation count and the amount of manganese ions measured by ICP-AES. The estimated

numbers of ^{125}I -biotin on ^{125}I -HMON/SA and ^{125}I -CET-HMON/SA were 6.3 and 1.4, respectively, for each nanoparticle.

In-vitro experiments for evaluating the cancer cell binding specificity.

Cell culture. A431, human epidermoid carcinoma cells, and MCF7, human breast cancer cells, were purchased from Korean Cell Line Bank (KCLB, Seoul, South Korea). A431 cells were cultured in MEM medium and MCF7 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and plus 1% antibiotic-antimycotic, respectively. Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C and passaged twice a week at a split ratio of 1:3 routinely.

Investigation of cancer cell binding specificity with MRI. A431, MCF7 cells were plated at a density of 5×10^3 cells/ml in 96-well plates and incubated with the **CET-HMON/SA** or **HMON/SA** at 37°C . The concentration of the nanoparticle was 2 mg/ml (based on the manganese ion concentration determined by ICP-AES). After the incubation for 4 hrs, the cells were washed with PBS three times to remove the unbound nanoparticles and trypsinized to be detached from the plate. The cell suspension was concentrated by the centrifugation and the resulting cell pellets were mixed with 1% agarose solution (1 ml). The mixture was transferred into Eppendorf-tube for MRI analysis. T_1 - and T_2 -weighted MRI of the cells mixed with agarose solution were performed with a 3.0 T whole body MRI system (Philips, Achieva ver. 1.2, Philips Medical Systems, Best, The Netherlands) equipped with 80 mT/m gradient amplitude and 200 ms/m slew rate. (Figure 2, S1)

Investigation of cancer cell binding specificity by using radioactivity counting method. A431, MCF7 cells were plated at a density of 5×10^3 cells/ml in 96-well plates and treated with the ^{125}I -CET-HMON/SA at 2 mg/ml (based on the manganese ion concentration determined by ICP-AES). The gamma radiation counted from the ^{125}I -CET-HMON/SA

containing 1 mg of manganese ions was 3.3 MBq. After incubation for 3 hrs, the cells were washed with PBS for three times, trypsinized to be detached from the plate, and concentrated by the centrifugation. Radioactivity of the cells was measured in tissue radioactivity using a Wizard² automatic gamma counter (PerkinElmer, Waltham, MA, USA). The radioactivity from A431 and MCF7 cells treated with the ¹²⁵I-CET-HMON/SA was counted to be 5.8×10^5 cpm, and 6.6×10^3 cpm, respectively. The radioactivity from the untreated A431 and MCF7 cells were both less than 50 CPM.

In vivo experiments for the tumor-specific imaging using a mouse model

Mouse xenograft tumor model. Male BALB/c nude mice (7 weeks old) were purchased from Orient Bio (Seoul, South Korea) and housed in specific pathogen-free conditions. They were cared for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and all studies with mice were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Samsung Biomedical Research Institute (SBRI). SBRI is an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility and abide by the Institute of Laboratory Animal Resources (ILAR) guide.

Ectopic xenograft tumors were established by subcutaneous inoculation of 2×10^6 A431 cells in a total volume of 0.1 mL of a serum-free medium containing 50% Matrigel (BD Bioscience, Erembodegem, Belgium) into the right thigh under isoflurane anesthesia. A431 cell lines (human epithelial carcinoma) established from an epidermoid carcinoma in the vulva, which express abnormally high levels of EGFR. The tumors became visible and palpable about 10 days after cell inoculation, and appreciable 3 weeks after cell inoculation. Tumor volumes were determined by digital caliper measurement of the largest diameter (length) and

perpendicular to the smallest diameter (width) and height. Data were calculated according to the formula (length \times width \times height) $\pi/6$.

In vivo MRI study. *In vivo* MRI was performed on 9 male ectopic tumor bearing mice. For 6 mice, **CET-HMON/SA** was injected intravenously through the tail of mouse, for 2 mice, **HMON/SA** was injected intravenously, and for 1 mouse, **IgG-HMON/SA** with was injected. MRI were obtained before and after injection of the agents 0.5 hr, 3 hr, 6 hr, 24 hr. MR images obtained when tumor volume reached around 200 mm³ (about 3 weeks after tumor cell injection) under 7T/20 animal MRI System (Bruker-Biospin, Fallanden, Switzerland) equipped with a 20 cm gradient set capable of supplying up to 400mT/m in 100 μ sec rise-time. A birdcage coil (72 mm i.d.) (Bruker-Biospin, Fallanden, Switzerland) was used for excitation, and an actively decoupled phased array coil was used for receiving the signal. **HMON** contrast enhanced multi-slice MR images were obtained using a fast spin-echo T₁-weighted MRI sequence (repetition time (TR) / echo time (TE) = 300/7.9 ms, number of experiment (NEX) = 4, echo train length = 2, 100 \times 100 μ m² in plane resolution with a slice thickness of 800 μ m and 10 slices), and a fast spine-echo T₂-weighted MRI sequence (TR/TE = 3000/60ms, NEX=4, echo train length = 4, 100 \times 100 μ m² in plane resolution with a slice thickness of 800 μ m and 10 slices). MR images were obtained before and ~1 hr (15/30/45 minutes), 3 hrs, 6 hrs and 24 hrs after contrast agent administration (Figure S2).

Immunohistochemical study

For the subsequent histological analysis the mice were sacrificed under anesthetic condition immediately after MRI data acquisition. The tumor tissues were removed carefully, fixed in 10% neutral buffered formalin for 24 hrs and then sliced in accordance with MRI data. Formalin-fixed, paraffin-embedded sections were sliced into 5 μ m sections for investigation of EGFR expression by an immunohistochemical staining method. After heat induced antigen

retrieval for 20 min in citrate buffer (pH 6.0), endogenous peroxidase was blocked with 3% hydrogen peroxide in phosphate buffered saline (PBS) for 10 min. Nonspecific epitopes were blocked with blocking solution (2.5% normal horse serum) for 20 min at room temperature followed incubation with anti-mouse EGFR (1:200; Abcam, Cambridge, UK) antibody diluted in PBS containing 1% bovine serum albumin at room temperature for 30 min. After washing with PBS, the tissue sections were incubated with biotin-conjugated IgG for 30 min and then washed again followed by incubation with horseradish peroxidase (HRP)-conjugated streptavidin for 30 min. The color reaction was developed using the chromogen 3,3-diaminobenzidine (DAB) for 5 min. After washing, the tissue sections were lightly counterstained with Mayer's hematoxylin before dehydration and mounting (Figure S3).

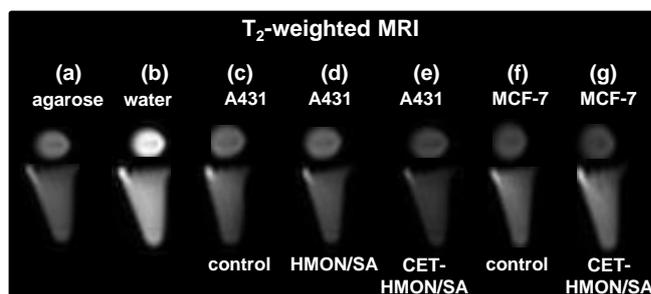


Figure S1. T₂-weighted MR images of (a) agarose, (b) water, (c) untreated A431 cells, (d) **HMON/SA** treated A431 cells, (e) **CET-HMON/SA** treated A431 cells, (f) untreated MCF7 cells, and (g) **CET-HMON/SA** treated MCF-7 cells, showing the specific image enhancement of A431 cells incubated with the **CET-HMON/SA** conjugate.

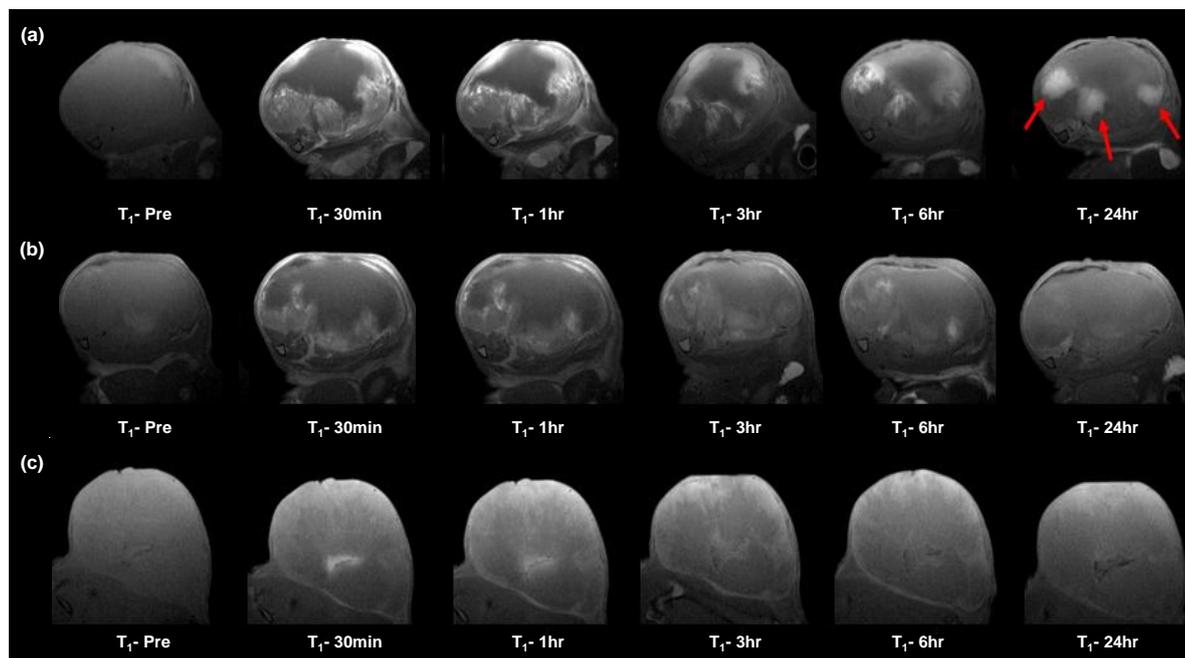


Figure S2. T_1 weighted MR images of the thigh of the mouse model inoculated with A431 cells. a) **CET-HMON/SA** injected intravenously, b) **HMON/SA** injected, and c) as a control, **HMON/SA** was conjugated with control antibody, immunoglobulin-G, **IgG-HMON/SA**. Only the **CET-HMON/SA** conjugate remained and showed as bright signal enhanced areas (arrows) until 24 hours, which may be attributed to the specific targeting of CET with EGFR over expressed cells on the surface.

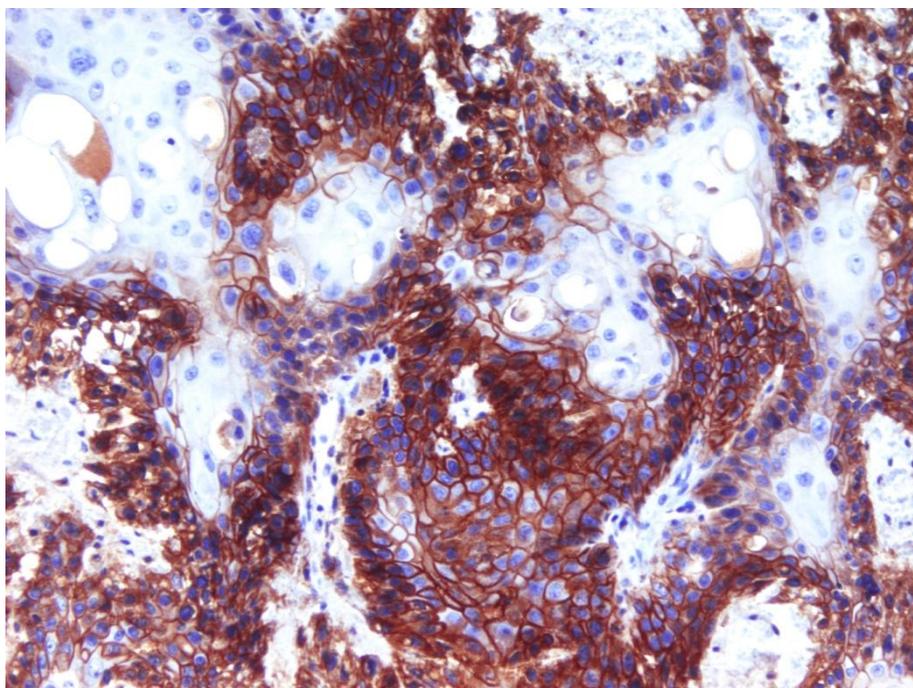


Figure S3. Representative expression of EGFR in A431 human epidermoid carcinoma tissue. EGFR proteins were measured by immunohistochemical staining method. Formalin-fixed and paraffin-embedded sections were stained with EGFR antibody. EGFR were stained a brown color in the section. Note the strongly increased staining for EGFR in A1431 cell membrane. Magnification, $\times 200$.

References for the Supporting Information

- [1] J. Shin, R. M. Anisur, M. K. Ko, G. H. Im, J. H. Lee, I. S. Lee, *Angew. Chem. Int. Ed.* **2009**, 48, 321.