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Supporting Information

Indocyanine Green Encapsulated Nanogels for Hyaluronidase Activatable and Selective Near Infrared Imaging of Tumors and Lymph Nodes

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Experimental details

1. Materials and Methods

1.1. Materials

Hyaluronic acid (MW 20k) was purchased from Lifecore Biomedical (Chaska, MN, USA). The 2-[7-[1,3-dihydro-1,1-dimethyl-3-(4-sulfobutyl)-2H-benzo[e]indol-2-ylidene]-1,3,5-heptatrienyl]-1,1-dimethyl-3-[5-(3-sulfosuccinimidyl)oxycarbonylpentyl]-1H-benzo[e]indolium (ICG-Osu) was supplied by Dojindo Molecular Technologies, Inc. (Tokyo, Japan). Ethylene diamine, dimethyl sulfoxide (DMSO), potassium dioxide (KO_2), Toluidine blue solution, and bovine testicular hyaluronidase (type I-S) were purchased from Sigma (St. Louis, MO, USA). A desalting column (MW cut-off: 5000) was purchased from Pierce (Rockford, IL, USA). OVCAR-3 cells and human epithelial carcinoma cells (HeLa) were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute medium (RPMI), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA, USA).

1.2. Synthesis and characterization of HA-conjugated ICG (HA-ICG)

To activate the carboxyl group, 200 mg of HA (10 μmol of HA, 527 μmol of carboxyl group) was dissolved in 5 mL of phosphate buffer (pH 8.0) and reacted with 100 mg of EDC (527 μmol) and 60 mg of NHS (527 μmol) for 1 hr. Subsequently, the solution was mixed with 158.3 mg of ethylenediamine in DMSO and reacted for 24 hr at room temperature. The reactants were dialyzed in distilled water for 2 days to remove excess ethylenediamine and then freeze-dried. The degree of amine substitution in hyaluronic acid was determined by ^1H NMR (400 MHz, D_2O).

The amine groups (321 nmol) in HA were dissolved in 0.3 ml of phosphate buffer (pH 8.0) and reacted with ICG-Osu (321 nmol) in 0.3 ml of DMSO overnight at room temperature. The reactant was passed through a desalting column to remove free ICG-Osu. Each fraction was analyzed by UV spectroscopy at a wavelength of 800 nm to determine the amount of ICG present after being diluted in a 1% Triton X-100 (Tx-100) solution in PBS. The amount of HA was quantitatively analyzed with the Toluidine blue assay after gel electrophoresis (Fig. S2). Various amounts of amine-modified HA (2, 4, 6, 8, and 10 μg) and HA-ICG nanogels were loaded onto 0.5% agarose gel and then stained with Toluidine blue solution (0.2% in mixture solution,

ethanol: distilled water: acetic acid=50:49:1), as performed in a previous study [1]. The band intensity was quantitatively analyzed using Image J image-processing software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/).

The absorption spectra of free ICG and HA-ICG were analyzed by UV spectroscopy from 550 nm to 900 nm. The morphology of HA-ICG hydrogels was observed by transmission electron microscopy (TEM, 200kV, Phillips) and scanning electron microscopy (SEM, FEI company, Magellan 400) after loading the conjugate onto a carbon-coated copper grid and drying in air. The size of the HA nanogels in PBS was determined by dynamic light scattering (DLS, Otsuka Electronics, Japan). To disassemble HA-ICG nanogels, a KO_2 solution (0.5 mM), HAdase (500 unit/ml), and Tx-100 solution (1%) were added to HA nanogels in DW and incubated for 2 hr. The size of each sample was measured by DLS.

1.3. Physicochemical stability of HA-ICG

ICG, ICG-Osu, and HA-ICG nanogels were diluted with distilled water at a final concentration of 1 μg/ml and were placed into each well of a 12-well plate. Near-IR (NIR) light with an excitation wavelength of 780 nm was irradiated onto the samples for a predetermined time of 10, 30, 60, or 120 min at room temperature. After incubation, a 10% Tx-100 solution in DW was added to each sample to recover quenched ICG. The remaining fluorescence property of each sample in a final 1% Tx-100 solution was measured by a spectrofluorometer with an excitation and emission wavelength of 780 nm and 815 nm, respectively.

1.4. Enzyme treatment for HA-ICG

HA-ICG nanogels (110 μg) in PBS were incubated with different amounts of HAdase (0, 31, 62, 125, 250, and 500 units/ml) for 1 hr at 37℃. As a negative control, the same protein concentration of glucose oxidase (6.2 mg/ml) to HAdase (500 unit/ml) was incubated with HA-ICG nanogels under the same conditions. After incubation, each solution was analyzed by a spectrofluorometer (Perkin-Elmer) at the excitation and emission wavelengths of 750 nm and 800 nm, respectively, to measure the fluorescence intensities of each sample. To visualize each solution with HA-ICG nanogels and different amounts of HAdase, reactant was loaded onto a 96-well plate and analyzed with an IVIS® Lumina imaging system (Xenogen, Alameda, CA). For the quantitative analysis of each sample, regions of interest (ROIs) were drawn, and the total flux (photons/sec) was measured.

1.5. Cell culture and transfection

HeLa (human cervical cancer cells), MDA-MB-231 (human breast cancer cells), PC3 (human prostate cancer cells), and HEK 293 cells were cultivated in 10% serum containing DMEM medium supplemented with antibiotics (penicillin (100 units/mL) and streptomycin (100 μg/mL)) in a humidified atmosphere with 5% CO2 at 37°C. OVCAR-3 cells (human ovarian carcinoma cells), MCF-7 (human breast cancer cells), and HCT-116 (human colon cancer cells) cells were cultured in 10% serum containing RPMI medium with antibiotics at the same atmospheric conditions. For the ICG images, different types of cells were seeded in 96-well plates at a density of 2×10^4 cells/well for 24 hr prior to treatment with HA-ICG. The cells were treated with 4.58 nmol (110 μg) of HA-ICG in serum-deficient medium for 3 days. Then, the ICG fluorescence image was visualized with the IVIS® Lumina imaging system (Xenogen, Alameda, CA).

1.6. Animal studies

All animal care and experimental procedures were approved by the Animal Care Committees of the Korea Research Institute of Bioscience and Biotechnology (KRIBB). To examine accumulation time of HA-ICG nanogels, both free ICG and HA-ICG nanogels were injected to female BALB/c (SLC, Inc., Japan) (female, 6-7 weeks of age) via transdermal injection. The total amount of ICG in each sample was 5 μg per injection. After predetermined time, the fluorescence intensity at injected site was quantitatively analyzed by IVIS® Lumina imaging system. A xenograft tumor model was generated by injection of MDA-MB-231 cells (4×10^6 cells/tumor) subcutaneously into the flank region of nude mice [2]. When the size of the tumors exceeded 300 mm^3, 20 μg of ICG in HA nanogels was injected intravenously. After a predetermined amount of time, in vivo optical ICG imaging with the IVIS® Lumina imaging system (Xenogen, Alameda, CA) was used to visualize the tumors. For optical imaging of sentinel lymph nodes in vivo, ICG and HA-ICG nanogels were administered to the forepaw pad of anesthetized female BALB/c mice via intradermal injection. To mimic HAdase-overexpressed environment, HA-ICG nanogels were mixed with HAdase and then injected in the same way. The total amount of ICG in each sample was 5 μg per injection. After incubation for a predetermined amount of time, the ICG fluorescence of each forepaw was visualized using the IVIS® Lumina imaging system

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with an ICG filter set. The plot of free ICG-Osu signal was added as a control (Fig. S8). Different amounts of ICG-Osu (0, 100, 200, 500 ng/well) in distilled water were analyzed by IVIS® Lumina imaging system.

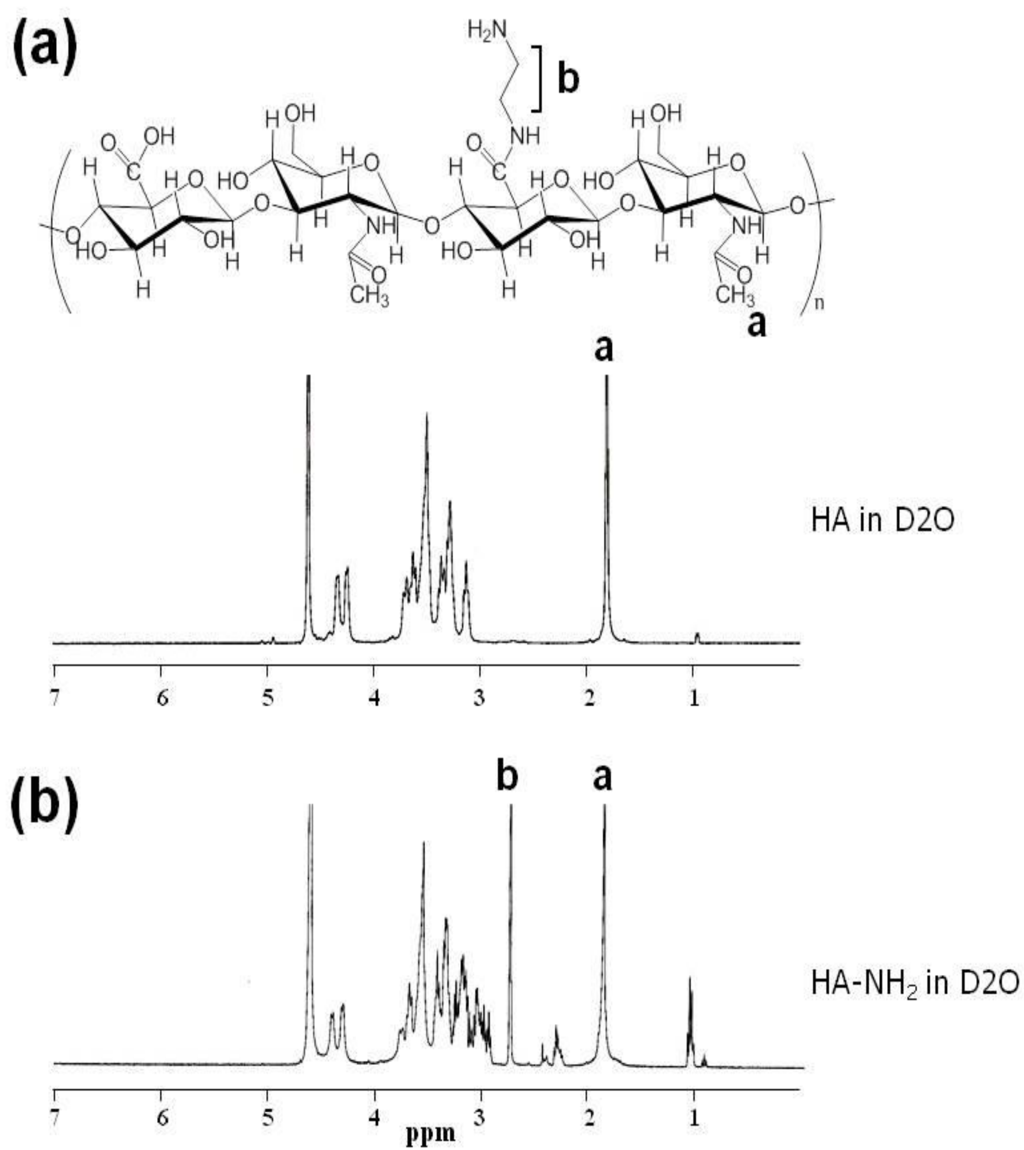

Fig. S1 NMR analysis of amine immobilized Hyaluronic acid (HA)

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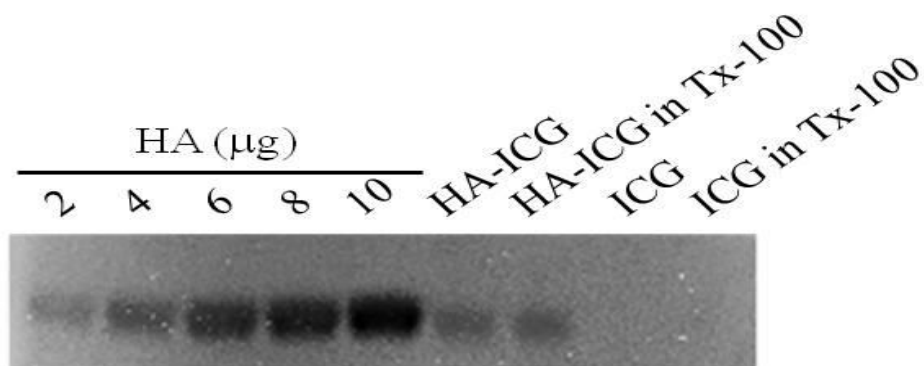

Fig. S2 Toluidine blue assay for quantification of HA in the HA-ICG conjugate.

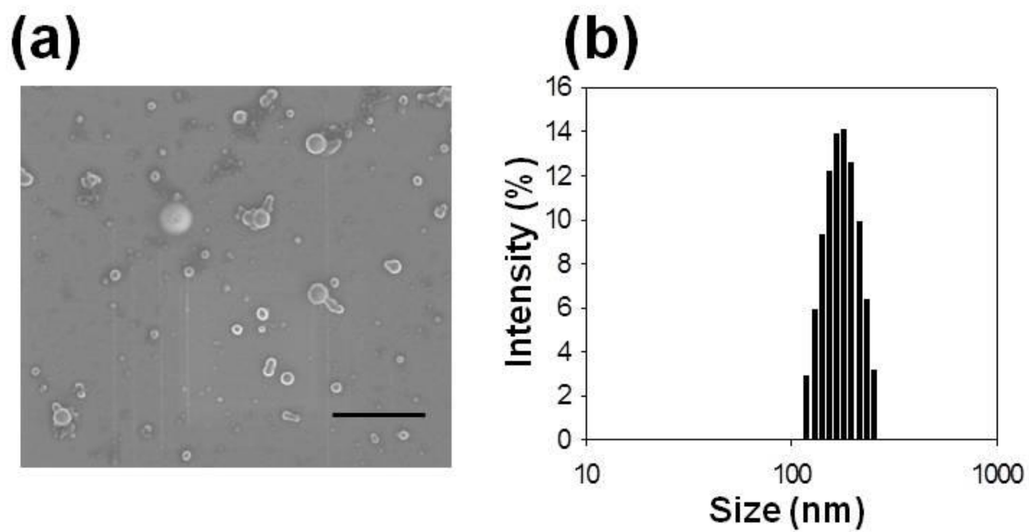

Fig. S3 **(a)** SEM images of as-prepared HA-ICG nanogels. The scale bar is 1 μm. (b) A histogram of the size distribution of HA-ICG nanogels determined by dynamic light scattering (DLS).

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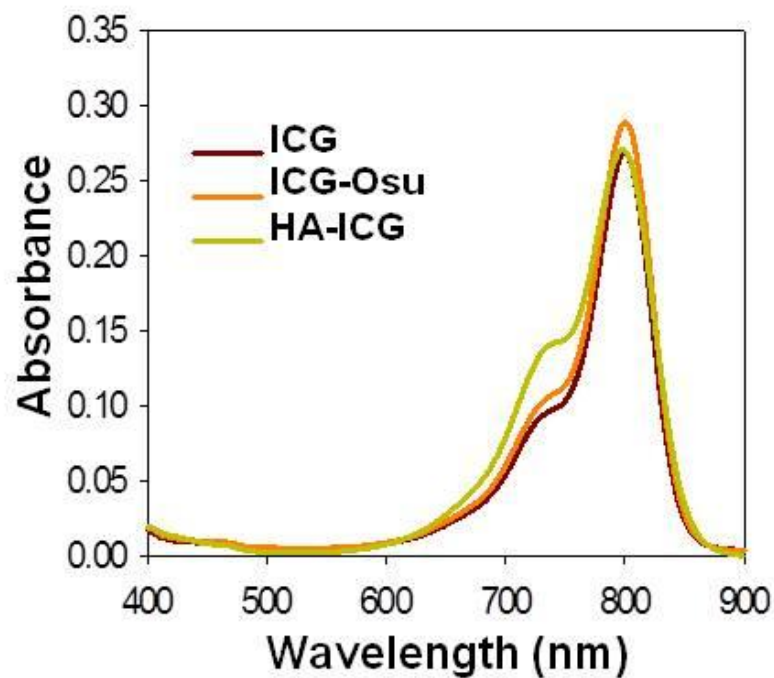

Fig. S4 Absorption spectra at a concentration of 1 μg/mL in 1% Tx-100 solution.

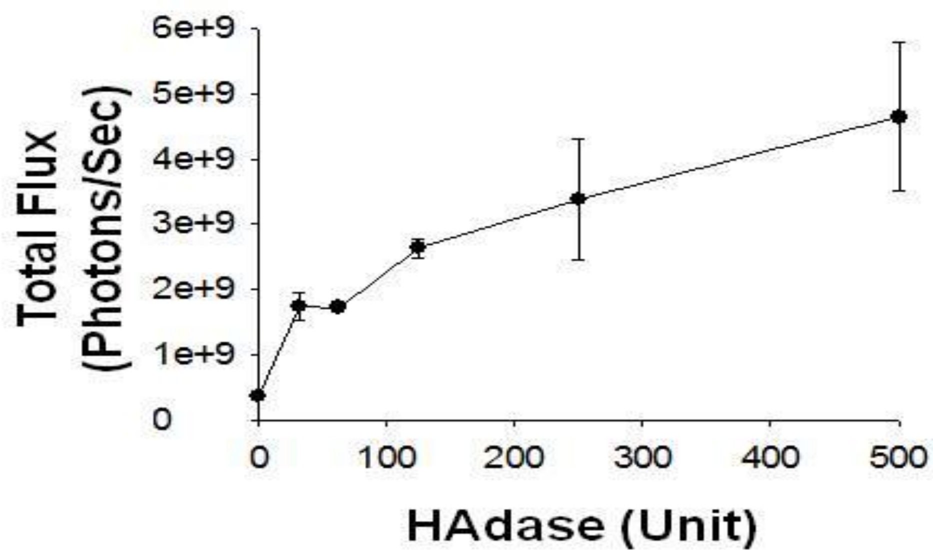

Fig. S5 The quantitative analysis of the ICG fluorescence intensity in HA-ICG nanogels at different HAdase concentrations.

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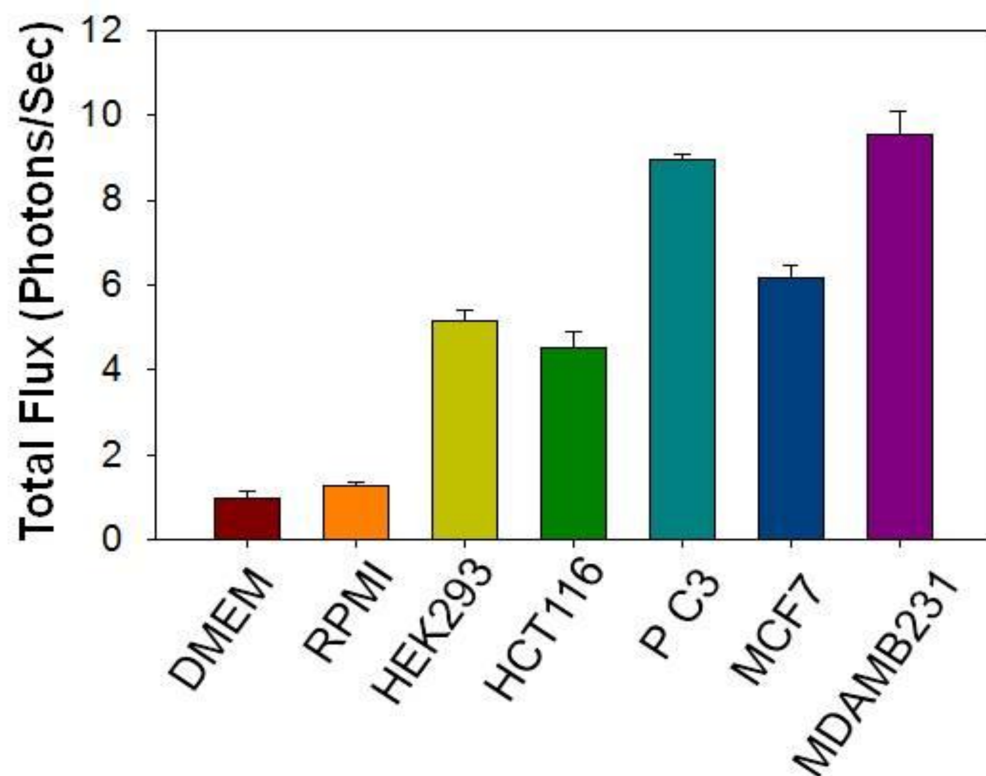

Fig. S6 Quantification of ICG fluorescence intensities for different types of cancer cells after incubation with HA-ICG nanogels in vitro. HEK 293 cells (embryonic kidney cells) were used as non-cancer cells.

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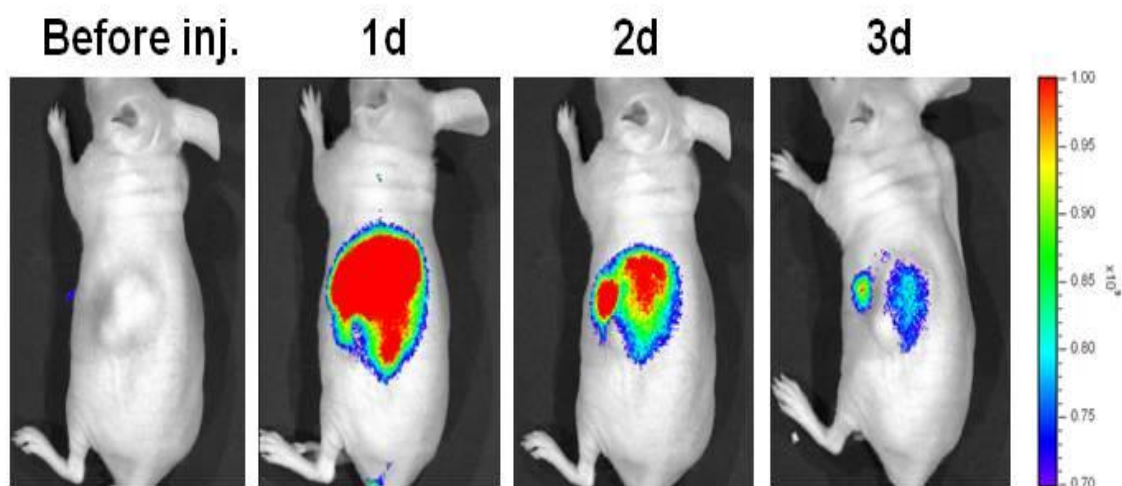

Fig. S7 Optical imaging of tumor tissues after HA-ICG nanogels were injected intravenously in MDA-MB-231–tumor xenograft mice.

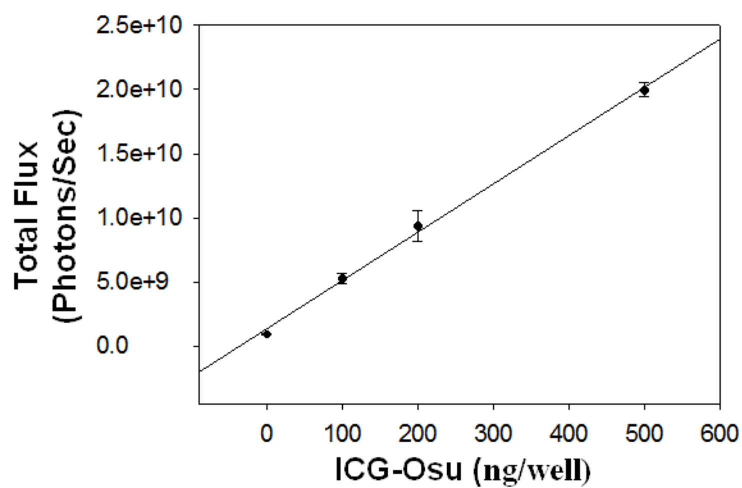

Fig. S8 Plot of ICG-Osu fluorescence signal using IVIS® Lumina imaging system.

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References

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