Supplementary Information

A Novel Affibody Bioconjugate for Dual-modality Imaging of Ovarian Cancer

Yihong Wang,[a,b] Zheng Miao,[b] Gang Ren,[b] Yingding Xu,[b] and Zhen Cheng[b]*

[a]*Department of Chemistry and Chemical Engineering, Southeast University, Nanjing, Jiangsu, 211189, China*
[b]*Molecular Imaging Program at Stanford (MIPS) and BioX Program, Canary Center at Stanford for Cancer Early Detection, Department of Radiology, Stanford University, California, 94305*

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Materials and General methods:

Materials: PAMAM G0 was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Cy5.5-NHS was purchased from Amersham Biosciences (Piscataway, NJ). 2,2',2''-(10-(2-(2,5-dioxopyrrolidin-1-yloxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (DOTA NHS) was purchased from Macrocyclics Inc. (Dallas, TX). Sulfo-SMCC (Sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate) and Pierce® Pre-Coated Iodination Tubes were purchased from Thermo Scientific (Rockford, IL). The Ac-Cys-$Z_{HER2:342}$ (Ac-CVDNKFNKEMRNAYWEIALLPNLNNQQKRAFIRSLY DDPSQSANLLAEAKKLNDAQAPK-NH_2) was synthesized (purity > 95%) as we previously reported and used directly for the chemical modification.[1] All other standard synthesis reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and used without further purification. ^{64}Cu was provided by the Department of Medical Physics, University of Wisconsin at Madison (Madison, WI), and $Na^{125}I$ was purchased from PERKIN Elmer (Waltham, MA). Human ovarian

cancer SKOV3 cell line was obtained from the American Type Tissue Culture Collection (Manassas, VA). Female athymic nude mice (*nu/nu*) were purchased from Charles River Laboratories (Boston, MA).

Instruments: Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) was performed by Stanford Protein and Nucleic Acid Biotechnology Facility, Stanford University. A Dionex Summit high-performance liquid chromatography (HPLC) system (Dionex Corporation, Sunnyvale, CA) equipped with a 170U 4-Channel UV-Vis absorbance detector was used for purification and analysis of Cy5.5 labeled compound. UV detection wavelengths were 218 nm, 280 nm and 590 nm for all the experiments. Both semipreparative (Zorbax SB-C18, 9.4 mm × 250 mm) and analytical (Dionex Acclaim 120 C18, 4.6 mm × 250 mm) reverse phase HPLC columns were used. The mobile phase was solvent A, 0.1% trifluoroacetic acid (TFA) in water and solvent B, 0.1%TFA in acetonitrile (CH_3CN). A CRC-15R PET dose calibrator (Capintec Inc., Ramsey, NJ) was used for all radioactivity measurements. A Dionex Summit HPLC system equipped with a 170U 4-Channel UV-Vis absorbance detector and radioactivity detector (Carroll & Ramsey Associates, model 105S, Berkeley, CA) was used for purification of radiolabeled compound. Analytical RP-HPLC columns [(Vydac, Hesperia, CA. 218TP510-C18, 10 mm × 250 mm) was used for analysis of labeled proteins. The mobile phase was solvent A, 0.1% trifluoroacetic acid (TFA)/H_2O, and solvent B, 0.1%TFA/acetonitrile.

Synthesis of DPCZ: First, PAMAM G0 (4.08 mg) was conjugated with Cy5.5-NHS ester (4 mg) in 2000 μL of 0.15M $NaHCO_3$(1800 μL) and 900 μL saturated $NaHCO_3$ solution (pH 8.5) in a molar ratio of 2: 1 for 3 h at 4 °C. The resulting PAMAM G0(Cy5.5) conjugate was then purified using HPLC. The mobile phase was solvent A, 0.05%TFA/H_2O (v/v), and solvent B, 0.05% TFA/acetonitrile (v/v) and gradients (12-26%) were used for purification of the conjugate. The flow rate was typically 1.0

mL/min. The desired fractions were collected, frozen immediately and lyophilized. The identity of the target product was confirmed by MALDI-TOF-MS. $[M+H]^+$ Expected: 1419.71, measured: 1419.68. Second, PAMAM G0(Cy5.5) (3.10 mg) was conjugated with DOTA-NHS ester (2.79 mg) in a molar ratio of 1: 2.55 in DMSO and 135 μL of DIPEA for 2 h at 4 °C. The resulting DOTA-PAMAM G0(Cy5.5) conjugate was also purified by HPLC. $[M+H]^+$ Expected: 1804.11, measured: 1803.57. Third, the purified DOTA-PAMAM G0(Cy5.5) 3.37mg then reacted with 11.5 mg of Sulfo-SMCC in in 200 μL DMSO at molar ratio of 1:14.12 and in a total volume of 2.8 mL PBS (pH 7.4) for 2 h at 4 °C. After purified by HPLC, 1 mg Ac-Cys-$Z_{HER2:342}$ was site specifically conjugated to the DOTA-PAMAM G0(Cy5.5)-SMCC via the cysteine residue. The reaction was performed in a molar ratio of 1:3 using 173.9 μg of DOTA-PAMAM G0(Cy5.5)-SMCC and 1.77 mg of Ac-Cys-$Z_{HER2:342}$ in a total volume of 250 μL in PBS for 2 h at room temperature. The final resulting bioconjugate, DOTA-PAMAM G0(Cy5.5)- $Z_{HER2:342}$ (DPCZ,78.42 μg), was then purified using HPLC and characterized by MALDI-TOF-MS. $[M+H]^+$ Expected: 8969.29, measured: 8969.31.

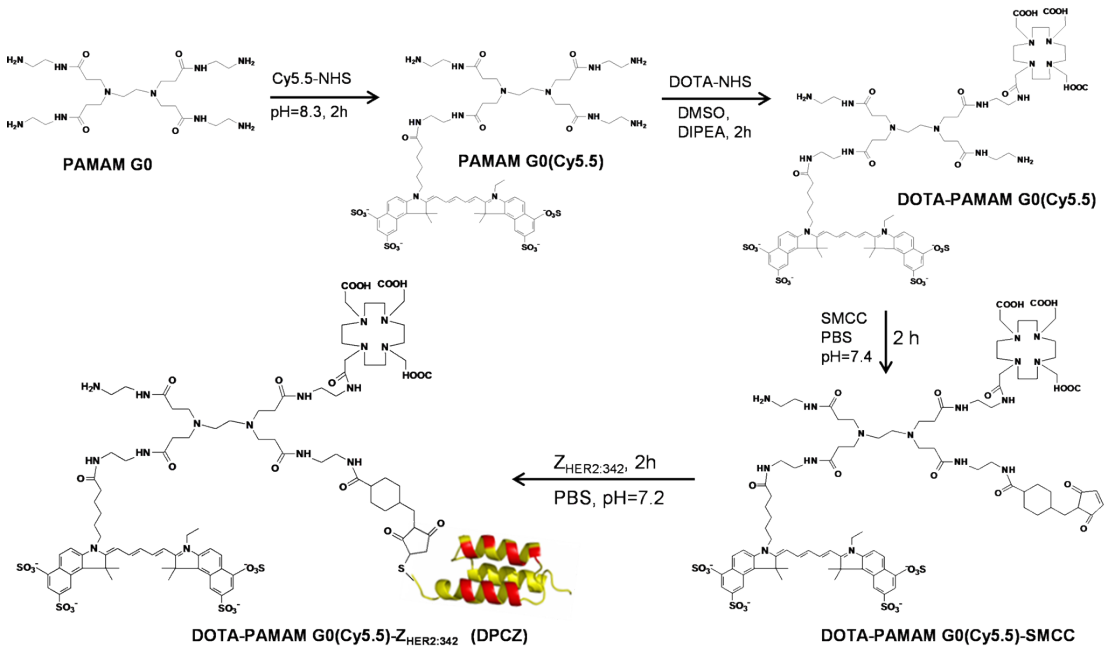

Figure S1. Synthetic schemes for DPCZ.

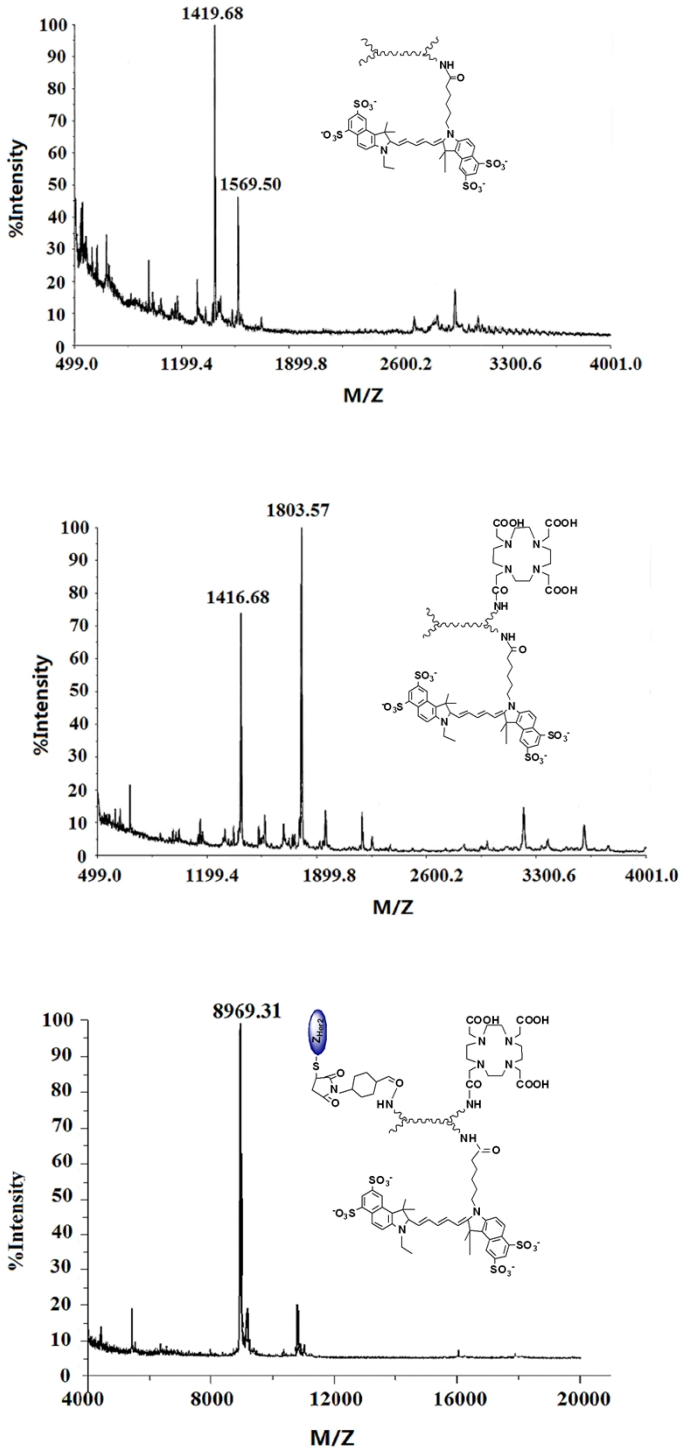

Figure S2. MALDI-TOF-MS results of compounds prepared.

Cell Culture: SKOV3 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C, with the medium changed every other day. The 80% confluent monolayer was trypsinized and dissociated into a single-cell suspension for further cell culturing.

Radioiodination and In Vitro Binding Assay: The compound, Ac-Cys-$Z_{HER2:342}$ was radiolabeled by the Iodogen method. Briefly, Ac-Cys-$Z_{HER2:342}$ (5 μg/μL in DMF) was added into a Pierce pre-coated iodination tube, and carrier free $Na^{125}I$ (500 μCi per 100 μg protein) was added to the tube and vortexed. After 10-15 min, the reaction mixture was removed from the reaction vessel and purified by a PD-10 column. The ^{125}I-Ac-Cys-$Z_{HER2:342}$ was used directly for the following in vitro cell binding assay. The *in vitro* cell binding assays were performed with the SKOV3 cells. Briefly, cells were seeded at a density of 0.2 million/well in 24 well tissue culture plates and allowed to attach overnight. Following a wash with the binding medium [Modified Eagle's Media with 25 mM HEPES and 0.2% bovine serum albumin (BSA)], the cells were incubated at 37 ^{o}C for 2 h with different concentrations of DPCZ (varying from 10^{-11}-10^{-6} M) and approximately 180,000 counts per minute (cpm) of ^{125}I-Ac-Cys-$Z_{HER2:342}$ in 0.5 mL of serum free binding media. The cells were rinsed twice with 0.01 M PBS (pH 7.4)/0.2% BSA and lysed in 0.5 mL of 1.0 M NaOH for 5 min, and the radioactivity of the cells was measured. The data was analyzed using the ORIGIN6.0 computer program (Northampton, MA), and the IC_{50} value, the concentration of competitor required to inhibit 50% of the radioligand binding, of the DPCZ was calculated.

Fluorescence Microscopy and Cell Uptake Studies of DPCZ: For fluorescence microscopy studies, SKOV3 cells (1×10^5) were cultured on 35 mm MatTek glass bottom culture dishes (Ashland, MA). After 24 h, the cells were washed with PBS and

then incubated at 37 ºC with DPCZ (1 nM) for 2 h. HER2 binding specificity of the conjugate in cell culture was verified by pre-incubation the SKOV3 cells with blocking dose of the non-fluorescent Ac-Cys-$Z_{HER2:342}$ (10 nM) for 30 min, then further incubation the cells with 1 nM of DPCZ for 2 h at 37 ºC. After the incubation period, cells were washed three times with ice-cold PBS. The fluorescence signal of the cells was recorded using an Axiovert 200M fluorescence microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with a Cy5.5 filter set (Exciter, HQ 650/20 nm; Emitter, HQ 675/35 nm). An AttoArc HBO 100W microscopic illuminator was used as a light source for fluorescence excitation. Images were taken using a thermoelectrically cooled charge-coupled device (CCD) (Micromax, model RTE/CCD-576, Princeton Instruments Inc., Trenton, NJ) and analyzed using MetaMorph Software version 6.2r4 (Molecular Devices Corporation, Downingtown, PA).

^{64}Cu Radiolabeling: Approximately 39.21 μg of DPCZ was radiolabeled with ^{64}Cu by addition of 185 MBq (5 mCi) of $^{64}CuCl_2$ [1 μg DPCZ per 7.4 MBq ^{64}Cu] in 0.1 N NaOAc (pH 5.5) buffer followed by a 1 hour incubation at 40°C. The radiolabeled complex was then purified by a PD-10 column (GE Healthcare, Piscataway, NJ). The product was washed out by PBS and passed through a 0.22 μm Millipore filter into a sterile vial for animal experiments. Radioanalytical HPLC was used to analyze the purified ^{64}Cu labeled DPCZ.

Tumor Xenografts: All animal studies were performed in compliance with federal and local institutional rules for animal experimentation. Protocols were approved by the Stanford Administrative Panel on Laboratory Animal Care. Female athymic nude mice (*nu/nu*) were obtained from Charles River Laboratories, Inc. (Cambridge, MA) at 4-6 weeks of age. Approximately 5×10^6 SKOV3 cells suspended in 50 μL of PBS were subcutaneously implanted in the right upper shoulders of the nude mice. Tumors

were allowed to grow to a size of 0.5-1.0 cm in diameter (3-4 weeks). The tumor bearing mice were subjected to *in vivo* optical and PET imaging studies.

Optical Imaging of Tumors in Mice: *In vivo* fluorescence imaging was performed with an IVIS 200 small animal imaging system (Perkin Elmer, Waltham, MA). A Cy5.5 filter set was used for acquiring ^{64}Cu-DPCZ fluorescence *in vivo*. Mice bearing SKOV3 (n = 3) were injected via tail vein with 0.5 nmol DPCZ and subjected to optical imaging at various time points postinjection (p.i.). Identical illumination settings (lamp voltage, filters, f/stop = 4, 1 second exposure time, field of views, binning) were used for acquiring all images, and fluorescence emission was normalized to photons per second per centimeter square per steradian (p/s/cm^2/sr) and displayed in the same scale of fluorescent intensity. Images were acquired and analyzed using Living Image 2.5 software (Xenogen, Alameda, CA).

Small Animal PET Imaging: PET imaging of tumor-bearing mice was performed on a microPET R4 rodent model scanner (Siemens Medical Solutions USA, Inc., Knoxville, TN). The mice bearing SKOV3 were injected with ^{64}Cu-DPCZ (55.7μCi/ 6.7 nmol per mouse) via the tail vein. At different time p.i. (30 min, 1 h and 2 h), the mice were anesthetized with 2% isoflurane, and placed in the prone position and near the center of the field of view of microPET. The 5-min static scans were obtained and the images were reconstructed by a two-dimensional ordered subsets expectation maximum (OSEM) algorithm. No background correction was performed. For each small-animal PET scan, 2-dimensional ROIs (Regions of interest; 5 coronal and transaxial slices) were drawn over the tumor, liver, kidney and muscle on decay-corrected whole-body coronal images. The average radioactivity concentration was obtained from the mean counts values (per pixel per minter) within the ROI volume, which were converted to counts per milliliter per minute by use of a predetermined conversion factor. By assuming a tissue density of 1 g/mL, the ROIs were converted

to counts/g/min. An image ROI-derived %ID/g of tissue was then determined by dividing counts per gram per minute with injected dose (ID).

Biodistribution analysis: After PET imaging study, biodistribution studies were conducted at 20 h post injection of ^{64}Cu-DCDZ. The tumor and major tissues and organs were dissected, wet weighted, and placed on black papers. The fluorescence images were acquired. The radioactivity of major organs was also measured by the γ-counter. The radioactivity uptake was expressed as %ID/g.

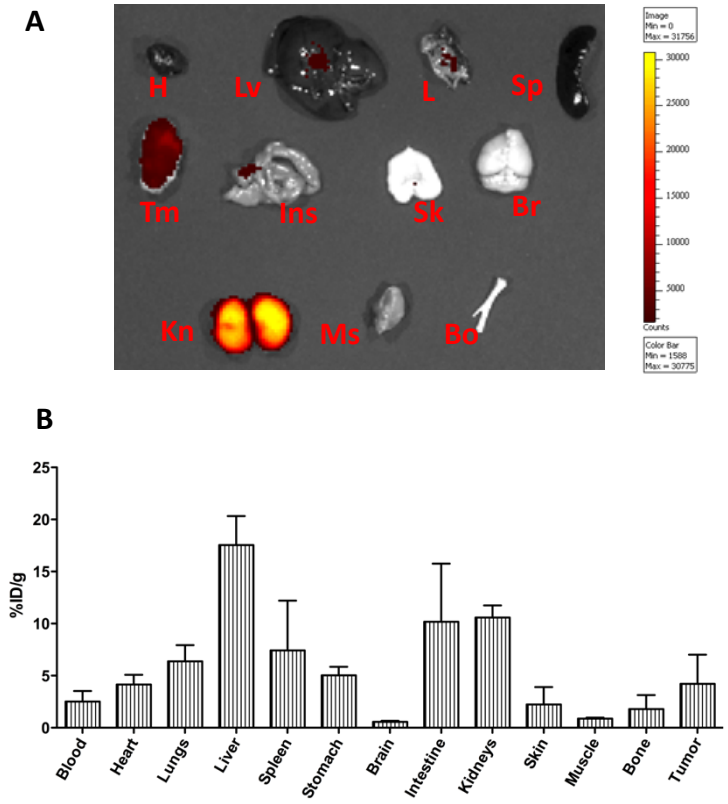

Figure S3. (**A**) Representative *ex vivo* NIRF image of tumor and normal tissues of SKOV3 tumor-bearing nude mice (n=3) injected with ^{64}Cu-DCDZ and sacrificed at 20 h p.i. H: heart; Lv: liver; L: lung; Sp: spleen; Tm: tumor; Ins: intestine; Sk: Skin; Br: brain; Kn: kidney; Ms: muscle; Bo: bone. (**B**) Biodistribution of ^{64}Cu-DPCZ in SKOV3 tumor mice models (n=3) at 20h p.i. as determined by radioactivity counting.

Statistical Methods: Statistical analysis was performed using the Student's *t*-test for unpaired data. A 95% confidence level was chosen to determine the significance between groups, with $P < 0.05$ being significantly different.

References:

1. Z. Cheng, O. P. De Jesus, D. J. Kramer, A. De, J. M. Webster, O. Gheysens, J. Levi, M. Namavari, S. Wang, J. M. Park, R. Zhang, H. Liu, B. Lee, F. A. Syud, S. S. Gambhir, *Mol. Imaging Biol.,* 2010, **12**, 316.