Generation of NODAGA-PSMA-Mb and NODAGA-PSMA-IgG

The anti-PSMA antibody huJ591 (PSMA-IgG) and the corresponding minibody variant (PSMA-Mb) were buffer exchanged into 0.1 M Sodium Borate pH 8.5 containing 5 mM Ethylenediaminetetraacetic acid (EDTA). Buffer exchanged PSMA-IgG and PSMA-Mb were each incubated with an N-hydroxysuccimide (NHS) -derivatized version of 1,4,7-triazacyclononane,1-glutaric acid-4,7-acetic acid (NHS-NODAGA) to non-selectively conjugate the chelator to lysine side chains. NODAGA-conjugated PSMA-IgG and PSMA-Mb were purified by gravity filtration over PD10 columns (GE Healthcare) to remove non-reacted NHS-NODAGA and to adjust buffer conditions to 0.25M Ammonium Acetate (pH 7). Conservation of protein purity and lack of aggregation attributable to conjugation were confirmed by Sodium dodecyl sulfate-Polyacrylamide Gel Electrophoresis and size exclusion chromatography. The PSMA-IgG and NODAGA-PSMA-IgG conjugate were analysed for high molecular weight aggregation by size exclusion high pressure liquid chromatography (HPLC) at 25°C at a flow rate of 0.25mL/min over a Tosoh TSK SuperSW3000 column using 25mM sodium phosphate, 250mM sodium sulfate, 5% isopropyl alcohol, pH 6.8 as running buffer. The PSMA-Mb and NODAGA-PSMA-Mb conjugates were likewise analyzed over a Phenomenex Yarra SEC-2000 column at a flow rate of 0.5mL/min at 30°C using 0.1M sodium phosphate (pH 6.8) as the mobile phase. All steps were carried out under metal-free conditions. Reverse phase-HPLC was used to quantify conjugation ratios and FACS analysis with the PSMA-expressing xenograft-derived human PCa cell line C4-2 XCL (a gift from ImaginAb) was used to measure the retention of binding activity.

^{64}Cu radiolabeling

The radionuclide, ^{64}Cu, was produced in high radiochemical and radionuclidic purity by the Washington University School of Medicine (St. Louis, MO) and supplied as $^{64}CuCl_2$ in 0.1N HCl. In separate reactions, 181.3MBq/mg (4.9mCi/mg) and 309.32MBq/mg (8.36mCi/mg) of $^{64}CuCl_2$ (1.73 x 10^4 mCi/μmol, 0.1N HCl) was added to NODAGA-PSMA-IgG and NODAGA-PSMA-Mb in Chelex®-treated 0.25M Ammonium Acetate buffer (pH 7), respectively. The mixtures were incubated at room temperature on a shaker for 15 minutes and quenched by adding 25μL of 100mM EDTA (pH 7). The reaction mixtures were then passed through a PD10 column followed by elution in Chelex®-treated Phosphate Buffered Saline (PBS; pH 7). Labeling efficiency and radiochemical purity of the radiotracer was tested by instant Thin Layer Chromatography.

Immunoreactivity fraction determination of ^{64}Cu-NODAGA-PSMA-Mb and ^{64}Cu-NODAGA-PSMA-IgG

Human PCa cell lines, PC-3 (PSMA-negative, #CRL-1435) and CWR22RV1 (PSMA-positive, #CRL-2505) were obtained from the American Tissue Type Collection (ATCC). Cells were incubated for 30 minutes with various protein concentrations (20, 50, 100 and 200 ng) of ^{64}Cu-NODAGA-PSMA-Mb or ^{64}Cu-NODAGA-PSMA-IgG in PBS (pH 7) with 1% bovine serum albumin (BSA). A 10-fold volume of PBS, 1% BSA was added to the tubes and cells were pelleted by centrifugation at 800 x *g* for 10 minutes. Supernatants were transferred to separate tubes and the percent active fraction determined as previously described (*1*).

Imaging-based characterization of radiotracer performance

All animal experiments were performed in accordance with guidelines set by the Fox Chase Cancer Center IACUC. For tumor implantation, PC-3 and CWR22Rv1 cells, at concentration of 4 x 10^7 cells/mL, were mixed 1:1 (v/v) with Matrigel (Corning Life Sciences) and injected subcutaneously on the left (PC-3) and right shoulders (CWR22Rv1) of male athymic nude (NCr nu/nu, Taconic) mice (injection volume = 0.2 mL). The CWR22Rv1 cells were injected 1 week prior to PC-3 cells to account for their more rapid growth rate. Tumor growth rate was monitored by caliper measurements and imaging studies were initiated once the tumors reached approximately 150-300 mm^3 using the formula tumor volume = $(\text{longer measure})^2 \times \text{shorter measure}/2$.

The imaging parameters for CLI using the IVIS Spectrum optical imaging system (PerkinElmer Inc, Hopkinton, MA) were: exposure time = 300 seconds, medium binning and 19.6 cm field-of-view. The average radiance ($p/s/cm^2/sr$) from three regions – one covering each tumor, and a third covering the posterior dorsal region, but remote from the tumors to serve as a measure of the non-specific background probe retention from each image, were used for region-of-interest (ROI) analysis. ROIs covered the same regions of the mice across all time points.

Serial PET/CT images were acquired on a Siemens Biograph™ Truepoint™ PET/CT scanner with a 16-slice CT configuration. CT acquisition parameters were: 130kVp, 21mAs, pitch=1, 16 x 0.6mm collimation, and 0.6s rotation. PET acquisition was performed in 3-D mode for 10 minutes. PET images were reconstructed using the TrueX reconstruction algorithm with 2 iterations (I) and 21 subsets (S), using 35cm field-of-view (FOV), matrix size of 336, 2mm slice thickness, with corrections applied for

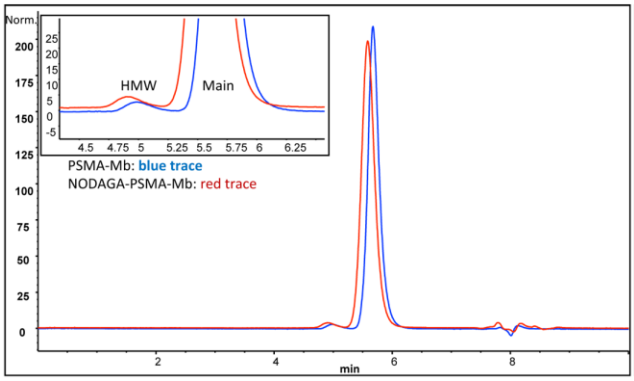
scatter and attenuation, and with a 2mm full width at half maximum Gaussian post-processing filter.

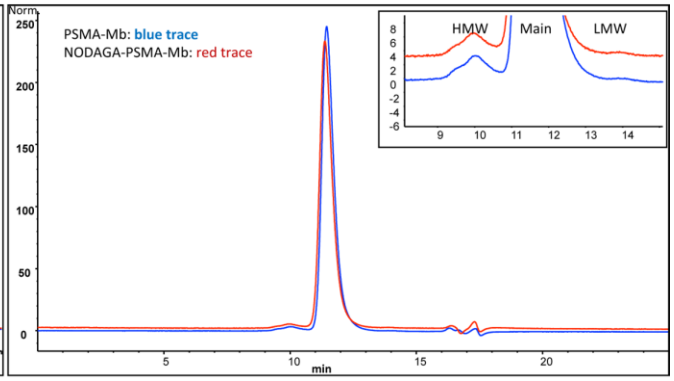
1. Robinson MK, Doss M, Shaller C, et al. Quantitative immuno-positron emission tomography imaging of HER2-positive tumor xenografts with an iodine-124 labeled anti-HER2 diabody. *Cancer Res.* 2005;65:1471-1478.

Supplemental Table 1. Radiotracer Characteristics

	^{64}Cu-NODAGA-IgG	^{64}Cu-NODAGA-Mb
Radiochemical purity (%)	99.7	98.8
Radiochemical yield (%)	81	67
Specific activity (mCi/mg)	3.96	5.60
Immunoreactivity (%)	58 ± 10.9	56.7 ± 13.6
Relative immunoreactivity (fold)	15.7	24.2

A

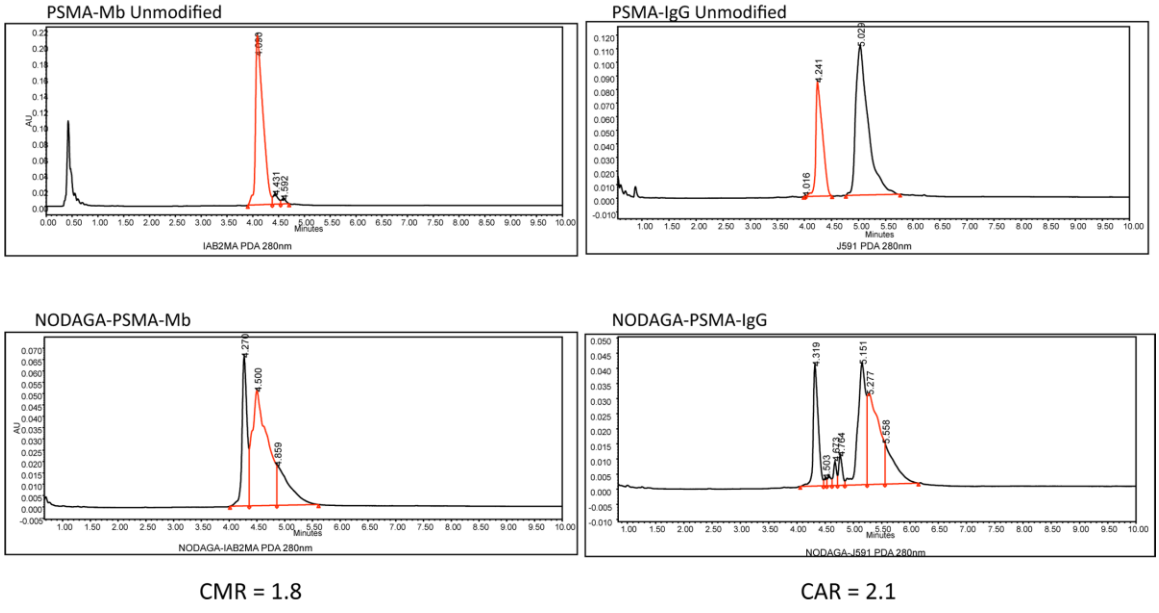




Sample	Main Peak Retention Time / %Area	HMW Peak Retention Time / %Area
PSMA-Mb	4.98 / 1.6%	5.67 / 98.4%
NODAGA-PSMA-Mb	4.90 / 1.7%	5.58 / 98.3%

Sample	HMW Retention Time / %Area	Main Peak Retention Time / %Area	LMW Retention Time / %Area
PSMA-IgG	10.00 / 2.2%	11.44 / 97.6%	13.88 / 0.2%
NODAGA-PSMA-IgG	9.97 / 2.0%	11.37 / 97.7%	13.91 / 0.3%

B



CMR = 1.8

CAR = 2.1