Supplemental Data

The lipids 1,2-Dinonadecanoyl-*sn*-glycero-3-phosphocholine (DNPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (Ammonium Salt) (DSPE-PEG), 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[PDP(Polyethylene Glycol)2000] (Ammonium Salt) (PDP-PEG-lipid), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (ammonium salt) (Carboxy-PEG-lipid) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl) (Ammonium Salt) (DPPE-Rhodamine) were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification (all lipids at purity > 99 %). *p*-Isothiocyanatobenzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) was purchased from Macrocyclics (Dallas, TX). Cholesterol (Chol), sucrose, Diethylenetriaminepentaacetic acid (DTPA), calcium ionophore A23187, phosphate buffered saline (PBS), dithiothreitol (DTT), Sephadex G-50, Sepharose 4B, *N*-hydroxysuccinimide (NHS), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), sodium chloride (NaCl), glycine, citric acid monohydrate, Albumin from Bovine serum (BSA), 4-Morpholinrrthanesulfonic acid (MES), Endothelial cell growth supplement (ECGS), heparin sodium salt, sodium carbonate, sodium bicarbonate, and tetramethylammonium acetate (TMAA) were purchased from Sigma-Aldrich Chemical (Atlanta, GA). Ethylenediamine Tetraacetic Acid, Disodium Salt Dihydrate (EDTA), sodium citrate dihydrate and N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic Acid (HEPES) were purchased from Fisher Scientific (Pittsburgh, PA). Desalting PD10 columns and 10DG columns were purchased from BioRad (Hercules, CA). Actinium-225 (^{225}Ac, actinium chloride) was provided by The Institute on Transuranium Elements in Germany. The RNA A10 PSMA aptamer (A10 aptamer) with the sequence 5"-NH_2-spacerGGGAGGACGAUGCGGAUCAGCCAUGUUUACGUCACUCCUUGUCAAUCCU CAUCGGCiT-3" containing 2"-fluoro pyrimidines, a 3"-inverted T cap, and a 5"-amino

group attached by a hexaethyleneglycol spacer was purchased from Integrated DNA Technologies (Skokie, IL). The anti-PSMA antibody J591 was purchased from BZL Biologics, LLC (New York, NY). Hybri-Care medium, Eagle's Minimum Essential Medium (EMEM), RPMI 1640 and F12K medium were purchased from ATCC (Manassas, VA). Fetal bovine serum (FBS) was purchased from Omega Scientific (Tarzana, CA). BCA™ protein assay kit, dimethylformamide (DMF) and succinimidyl 4-[pmaleimidophenyl]-butyrate (SMPB) were purchased from Pierce (Rockford, IL). Quanti-iT™ RNA Assay Kit was purchased from Life Technologies (Grand Island, NY). CellTiter 96® Non-Radioactive cell proliferation assay (MTT) was purchased from Promega Corporation (Madison, WI). Growth Factor Reduced Matrigel was purchased from (BD Biosciences, San Jose, CA). Water which was used for the preparation of the citrate and HEPES buffers was previously passed thrice through Chelex® (Bio RAD, Hercules, CA) treatment followed by sterile filtration.

Supporting Information / Methods

Liposome preparation

In a 25 mL round bottom flask, all lipids and cholesterol, dissolved in chloroform, were combined at a final concentration of 10 µmoles and mole ratio of 6.61:2.83:0.47:0.09 for DNPC:cholesterol:DSPE-PEG:DPPE-Rhodamine. Chloroform was evaporated using a Buchi rotavapor R-200 (Buchi, Flawil, Switzerland) at 55 ^{o}C for ten minutes. The thin dried lipid film was further exposed to a stream of N_2 for five minutes. The dried lipid film was then hydrated in 1 mL of citrate buffer (0.75 mL of 160 mM citrate buffer prepared by mixing 15.72 mgr of citric acid monohydrate and 13.26 mgr of sodium citrate dihydrate in a total volume of 0.75 mL and 0.25 mL of 20 mg/mL DOTA, pH 4.0) for 2 hours at 55 ^{o}C. Ascorbic acid (0.012 M) was co-entrapped to minimize lipid oxidation due to radiation (1). To control the size distribution and lamellarity, the lipid suspension was extruded 21 times through two stacked polycarbonate filters of 100 nm pore diameter (Avestin Inc., Ottawa, Canada). Extrusion was carried out in a water bath at 80 ^{o}C. After extrusion, to remove the unentrapped contents and to exchange the suspension buffer, liposome suspensions were eluted at room temperature through a size exclusion chromatography (SEC) column eluted with an isosmolar HEPES buffer (20 mM HEPES, 200 mM sucrose at pH = 7.4). Size distribution of liposome suspensions was measured using a Zetasizer NanoSeries (Malvern Instruments Ltd, Worcestershire, UK).

Antibody conjugation to liposomes

PDP-labeled liposomes were reacted with DTT for introducing terminal thiol groups which were then reacted with maleimide-activated J591 antibodies using standard published protocols (2). Briefly, out of the 4.8 mole % PEGylated lipids, 2.4 mole % consisted of PEG-chains with PDP end groups. Liposomes (2.5 µmoles in 1 mL of PBS buffer, pH 5.5, 300 mOSM) containing a total of 0.06 µmoles of lipids with PDP groups, were activated by reacting with 25 µmoles of DTT in 0.05 mL of PBS (pH 5.5) (500 mM

DTT) on a shaker for 30 minutes at room temperature. Antibodies were activated independently by reacting with SMPB dissolved in DMF (1:20 mole ratio of antibody:SMPB in 0.038 mL total volume) on a shaker for 60 min at room temperature. Purified activated antibodies, following a PD10 column eluted with PBS buffer, pH 7.2, 300 mOSM, were incubated with the activated liposomes overnight on a shaker at room temperature. Non conjugated antibodies were separated using a Sepharose 4B SEC column (11 cm length) eluted with PBS buffer (pH 7.4, 300 mOSM). Lipid concentration was determined by the fluorescence intensity of Rhodamine-labeled lipids contained in liposomes (excitation: 550 nm; emission: 590 nm). Antibody concentration was determined using a BCA^{TM} protein assay kit as per manufacturer's instructions. The number of antibodies conjugated per liposome was calculated using the mean size of liposomes for an average lipid headgroup surface area of 48 $Å^2$ (assuming gel lipid phase) (3). Immunoreactivity of J591 after conjugation to liposomes was evaluated on the stably PSMA positive LnCaP cell line in monolayers. Briefly, LnCaP cells (2×10^7) were washed with ice cold PBS and were resuspended in PBS containing 2% BSA. J591-labeled liposomes (or the radiolabeled J591) were incubated with cells at a liposome (or antibody)-to-receptor ratio of 1-to-100 on ice for 60 minutes. At the end of incubation, cells were washed twice with ice cold PBS and the cell associated activity was measured after secular equilibrium was reached using a Beckman Coulter LS6500 scintillation detector (Brea, CA). Selectivity of anti-PSMA binding was evaluated by blocking of PSMA receptors on cells by preincubation with excess J591 antibody (4μgr /10^6 cells) in PBS containing 1% BSA at 4 ^oC for 30 minutes followed by introduction of J591-targeted liposomes and measurement of their cell association.

A10 aptamer conjugation to liposomes

Liposomes containing Carboxy-PEG-lipids were activated with EDC/NHS to convert the terminal carboxylic acid groups to amine reactive esters, which reacted with the NH_2

group on the A10 PSMA aptamer (4). Out of the 4.8 mole % PEGylated lipids, 2.4 mole % consisted of Carboxy-PEG-lipids. Liposomes (1.25 mM total lipid in 1 mL MES buffer (10mM MES, 230 mM Sucrose prepared in DNAse-RNAse free water, pH 5.9, 300 mOSM) were reacted with 100 µL of 800 mM EDC (prepared in DNAse-RNAse free water) and 100 µL of 200 mM NHS (prepared in DNAse-RNAse free water) for 20 minutes on a shaker at room temperature. The unreacted EDC and NHS were separated by passing the liposome suspension through a Sephadex-G50 column (eluted in MES buffer (10 mM MES, 230 mM Sucrose prepared in DNAse-RNAse free water, pH 5.9, 300 mOSM)). Five nanomoles of the A10 aptamer suspended in 30 µL of DNAse-RNAse free water was added to 2.5 µmoles of liposome suspension (1.6 mL total volume) and the reaction was carried out for 4 hours at room temperature on a shaker. Unbound aptamer was separated using a Sepharose 4B column (eluted with PBS, pH 7.4, 300 mOSM). Concentration of the bound aptamer was quantified using a Quanti-iT^{TM} RNA Assay Kit as per manufacturer's instructions. Lipid concentration was determined as above by the fluorescence intensity of Rhodamine-labeled lipids. The number of aptamers bound per liposome was calculated using the same rationale as above. Immunoreactivity of A10 aptamer after conjugation to liposomes was evaluated following the exact protocol described in the previous paragraph for J591-labeled liposomes. Selectivity of anti-PSMA binding was evaluated by blocking of PSMA receptors on cells by preincubation with excess aptamer (0.03 nmoles/ 10^6 cells) in PBS containing 1% BSA at 4 ^oC for 30 minutes followed by introduction of aptamer-targeted liposomes and measurement of their cell association.

Labeling liposomes and J591-antibody with Actinium-225

One mL of the liposome suspension (5 mM total lipid) in HEPES buffer was incubated at 65-67 ^oC (in a dry heating bath) with a total volume of 0.08 mL of a mixture containing the radionuclide and the ionophore (0.03 mL of actinium chloride in 3 mM HCl, and 0.05

mL equimolar mixture of ethanol and water containing A23187 ionophore (at 0.37 mg/mL)) (1). After 60 minutes of incubation, 0.05 mL of 10 mM DTPA was added to the liposome suspension to complex any unentrapped ^{225}Ac or ^{225}Ac adsorbed on the liposome surface that was then removed using SEC through a 10 cm Sephadex G-50 column, eluted with phosphate buffer (PBS, pH = 7.4, 300 mOSM) (1). Loading efficiency of ^{225}Ac was determined by measuring the radioactivity of liposome suspensions before and after SEC by counting the *γ*-emissions of Bismuth-213 decay (^{213}Bi, 360-480 keV) using a Cobra *γ*-counter (Packard Instrument Co., Inc.) upon reaching secular equilibrium (^{213}Bi $t_{half\text{-}life}$= 45.59 minutes). Equilibrium values were also confirmed by comparing to counts of ^{221}Fr emissions (^{221}Fr $t_{half\text{-}life}$= 4.9 min, 185-250 keV).

Antibody radiolabeling was performed following a published protocol described elsewhere (5). Briefly, for the chelation reaction, a solution of 1.56 μCi of ^{225}Ac dissolved in 200 mM HCL, 0.025 mL of DOTA-NCS (10 mg/mL), 0.02 mL of ascorbic acid (150 g/L) and 0.025 mL of TMAA was prepared at final pH of 5.5, and was heated at 60^{o}C for 60 minutes. For the antibody labeling, the resultant solution was then combined with a second solution containing the antibody (0.45 mg), 0.02 mL of ascorbic acid and 0.025 mL of 1 M carbonate buffer. The combined mixture was then heated at 37^{o}C for a period of 60 minutes. Upon completion of incubation, the reaction mixture was treated with 0.02 mL of 10 mM DTPA and was purified through a 10 DG size exclusion chromatography column eluted using saline. The protocols reported by McDevitt et al. were followed in order to evaluate the radiochemical purity of the final eluted sample using ITLC and the radiolabeling efficiency.

Induction of PSMA expression on HUVEC cells

Monolayers of HUVEC on Matrigel^{TM} were exposed to MDA-MB-231 Cell Conditioned Media (CCM) and were induced to express PSMA following a published method (6). In

detail, the surface of each well of a 24-well plate was coated with 150 µL of Matrigel™ (thawed overnight at 4 °C) and was incubated at 37 °C for 30 minutes. HUVEC cells suspended in Cell Conditioned Media (CCM) were then plated on Matrigel™ (50,000 cells per well). After 18 hours of incubation in a humidified incubator at 37 °C and 5% CO_2, tube formation was observed and images were acquired using bright field transmission microscopy on an Olympus IX 70 inverted microscope (Olympus America Inc, PA) with a 10x, 0.25 NA dry objective. CCM was generated from the MDA-MB-231 breast cancer cell line (6). MDA-MB-231 breast cancer cells were cultured using EMEM. When a 225 cm^2 cell culture flask was 100% confluent, the cell culture growth medium was removed, centrifuged, sterile filtered and stored at -23 °C upon further use.

Detection of induced PSMA expression on HUVEC by immunofluorescence

PSMA expression induced on HUVEC was determined using J591-based immunofluorescence (7). After tube formation, HUVEC cells were washed and fixed for 20 minutes using 4% paraformaldehyde. The antibody J591 (5 µg/mL, suspended in PBS containing 1% BSA) was added to cells following a 60 minute incubation at room temperature. Cells were then washed thrice using PBS containing 1% BSA followed by incubation with a FITC-labeled goat anti-mouse secondary antibody (25 µg/mL, suspended in PBS containing 1% BSA), obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) for 60 minutes in the dark at room temperature. After the incubation period, cells were then thoroughly washed with PBS containing 1% BSA and the detection of PSMA expression was evaluated by acquiring fluorescence images using an Olympus IX 70 inverted microscope with an exciter bandpass filter (480 ± 40 nm) and an emitter bandpass filter (535 ± 50 nm) (Chroma Technology Corp., Bellows Falls, VT).

In parallel experiments, binding of Rhodamine-labeled J591-conjugated liposomes to PSMA expressing HUVEC cells was confirmed by incubating liposomes with cells

(plated as above) for 6 hours. After completion of incubation, cells were washed thrice with sterile PBS and imaged as above using an exciter bandpass filter (540 ± 25 nm) and an emitter bandpass filter (605± 55 nm) (Chroma Technology Corp., Bellows Falls, VT).

Detection of relative PSMA expression on different cell lines

LnCaP, Mat-Lu and HUVEC cells were plated on a 6 well plate at a density of 250,000 cells per well. For HUVEC cell line, the plates were pre coated with 0.5 mL Matrigel® and the cells cultured in presence of Cell Conditioned media (CCM) obtained from MDA-MB-231 cells. PSMA expression was determined using J591-based immunofluorescence. Briefly, the cells were washed using sterile PBS, scrapped using a cell scrapper and re-suspended in sterile PBS containing 1% BSA (in order to block the non specific receptors) and incubated on ice at 4^{o}C for 30 minutes, at a density of 250,000 cells per mL. Cells were then washed using PBS and incubated with J591 antibody suspended in PBS containing 1% BSA at a concentration of 5 µg/mL for 60 minutes on ice. At the end of the incubation, cells were again washed and then incubated with goat anti-mouse FITC conjugated secondary antibody suspended in PBS containing 1% BSA at a concentration of 25 µg/mL for 60 minutes in dark and on ice at 4^{o}C. After the incubation period cells were washed and re-suspended in sterile PBS at a density of 250,000 per mL. The relative PSMA expression on the cells was then determined by fluorescence counting of cell suspensions (10,000 events) using a Becton Dickinson FACS Calibur flow cytometer (San Jose, California) with a 488 nm Argon laser and analyzed using the Cell Quest software (San Jose, California).

Binding and internalization of liposomes by cell monolayers

LnCaP, Mat-Lu and BT474 cells were plated on 6 well plates at a density of 10^6 cells per well. Rhodamine-labeled liposomes were incubated with cells at a liposome-to-receptor

ratio of 1-to-10 and 1-to-1. HUVEC cells were plated on 6 well plates at a density of 250,000 cells per well. Prior to plating cells, wells were coated with 0.5 mL Matrigel™. Rhodamine-labeled liposomes were incubated with cells at a liposome-to-receptor ratio of 1-to-1. After 6 and 24 hours of incubation periods, cells were washed thrice using ice cold PBS, then scrapped and suspended in 2 mL PBS. The fraction of liposomes bound to cells was quantified by measuring the fluorescence intensity of Rhodamine (excitation: 550 nm, emission: 590 nm) using an SLM AMINCO 8000 spectrofluorometer. The fluorescent signal was corrected for potential light scattered by cells; the measured intensity of suspensions with identical density of non-treated cells was subtracted from the fluorescent intensity. The cell suspension was then incubated with 1 mL of a stripping buffer (50 mM glycine, 100 mM NaCl, pH 2.8) for 5 minutes at room temperature to remove the surface bound liposomes. At the end of incubation, samples were again washed twice with ice cold PBS and resuspended in a volume of 2 mL PBS for measurement. These studies were repeated for the radiolabeled J591 antibody on all cell lines at the same antibody-to-receptor ratios.

Cytotoxicity studies on cell monolayers

LnCaP, Mat-Lu and BT474 cells were plated on 96 well plates at a density of 40,000 cells per well. HUVEC cells were plated on 96 well plates at a density of 20,000 cells per well. Radioactivities ranging from 3 to 9 μCi/mL were added to cell monolayers in a total final volume of 0.2 mL per well. The cells were then incubated for 6 and 24 hours. At the end of incubation, cells were gently washed thrice with sterile PBS, and were further incubated with sterile fresh complete medium for a period of one doubling time (for the 24 hours of treatment), and for 18 hours plus a period of one doubling time (for the 6 hours of treatment) at 37 ^{o}C and 5% CO_2 to ensure evaluation of cell viability at the same time point relative to the beginning of all incubations. Measured doubling times

were 34 hours for LnCaP, 30 hours for Mat-Lu, and 48 hours for BT474. Cell viability was evaluated using an MTT assay.

Uptake of delivered Actinium-225 by cells in monolayers

LnCaP, Mat-Lu and BT474 cells were plated on 96 well plates at a density of 40,000 cells per well as in the parallel cytotoxicity studies. ^{225}Ac loaded J591-labeled liposomes, A10 aptamer-labeled liposomes, non-targeted liposomes and non-liposomal (free) chelated radionuclide were incubated with cells at identical lipid-to-cell ratios as in cytotoxicity studies but with lower radioactivities for 6 and 24 hours. At the end of incubation, cells were washed, trypsinized and lysed using acidified isopropanol, and internalized radioactivity per cell was measured using a Cobra *γ*-counter or a scintillation counter depending upon the radioactivity levels. HUVEC cells were plated on 96 well plates precoated with 40 µL of Matrigel™ at a density of 20,000 cells per well as in cytotoxicity measurements. Intracellular uptake was then measured as described above.

Supplemental Table 1. Binding and internalization of non-targeted liposomes by cells in monolayers.

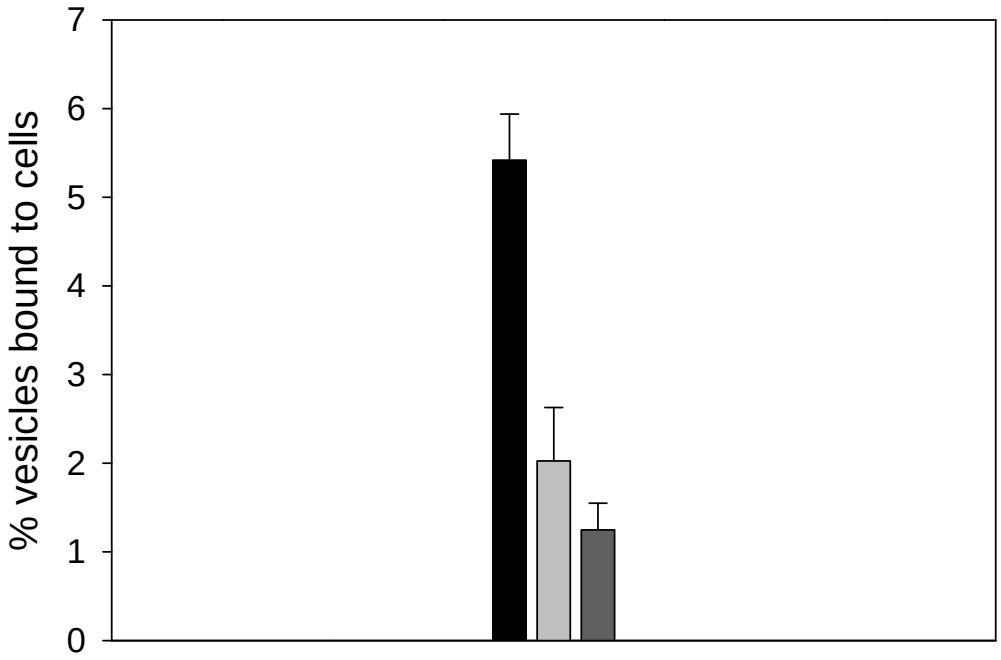
Incubation time (hours)	Cell line	Liposome : Receptor Ratio (Liposome:Cell Ratio)	Liposomes (L_P-PDP)	Liposomes (L_P-COOH)

			% bound and internalized liposomes	% bound and internalized liposomes
6	LnCaP	1:1 ($18x10^4$:1)	0.1 ± 0.1	0.0 ± 0.0
		1:10 ($18x10^3$:1)	0.0 ± 0.5	0.1 ± 0.7
	Mat-Lu	1:1 ($9.6x10^4$:1)	0.0 ± 0.1	0.3 ± 0.0
		1:10 ($9.6x10^3$:1)	0.3 ± 1.0	0.6 ± 1.3
	HUVEC (CCM + Matrigel™)	1:1 ($4.7x10^4$:1)	0.4 ± 0.9	0.2 ± 1.0
	BT474	- ($13.8x10^4$:1)	0.2 ± 0.1	0.1 ± 0.0
		- ($13.8x10^3$:1)	0.3 ± 0.8	0.5 ± 1.1
	HUVEC (Matrigel™)	- ($4.7x10^4$:1)	0.2 ± 0.9	0.0 ± 0.8
	HUVEC	- $4.7x10^4$:1)	-0.2 ± 0.9	-0.1 ± 1.0
24	LnCaP	1:1 ($18x10^4$:1)	0.2 ± 0.1	0.2 ± 0.1
		1:10 ($18x10^3$:1)	0.1 ± 0.5	0.3 ± 0.5
	Mat-Lu	1:1 ($9.6x10^4$:1)	0.0 ± 0.1	0.2 ± 0.0
		1:10 ($9.6x10^3$:1)	-0.1 ± 1.0	0.3 ± 0.9
	HUVEC (CCM + Matrigel™)	1:1 ($4.7x10^4$:1)	-0.8 ± 0.8	-0.7 ± 0.9
	BT474	- ($13.8x10^4$:1)	0.2 ± 0.1	0.2 ± 0.1
		- ($13.8x10^3$:1)	0.5 ± 1.1	0.3 ± 1.0
	HUVEC (Matrigel™)	- ($4.7x10^4$:1)	-0.8 ± 0.8	-0.9 ± 0.8
	HUVEC	- ($4.7x10^4$:1)	-1.0 ± 0.8	-1.0 ± 0.8

Supplemental Table 2

Non-specific association to Matrigel™ of ^{225}Ac-DOTA and ^{225}Ac-DOTA encapsulated in PEGylated liposomes measured via the % radioactivity associated with Matrigel™ following 6 and 24 hours of incubation relative to total radioactivity introduced per well.

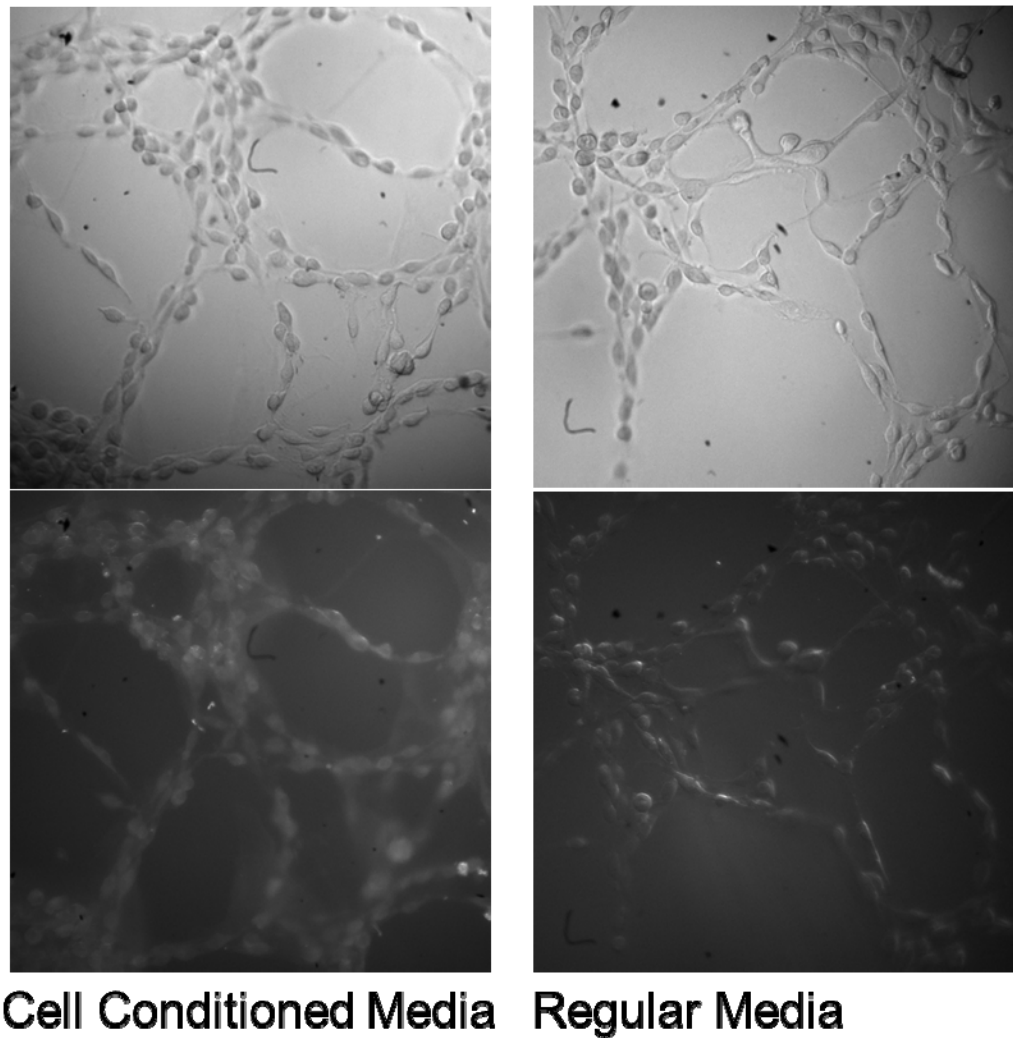
Incubation time (hours)	% Associated with Matrigel™	
	^{225}Ac-DOTA	^{225}Ac-L_{PDP}
6	13.89 ± 4.68	4.31 ± 2.17
24	25.10 ± 3.05	6.49 ± 1.56



Supplemental Figure 1

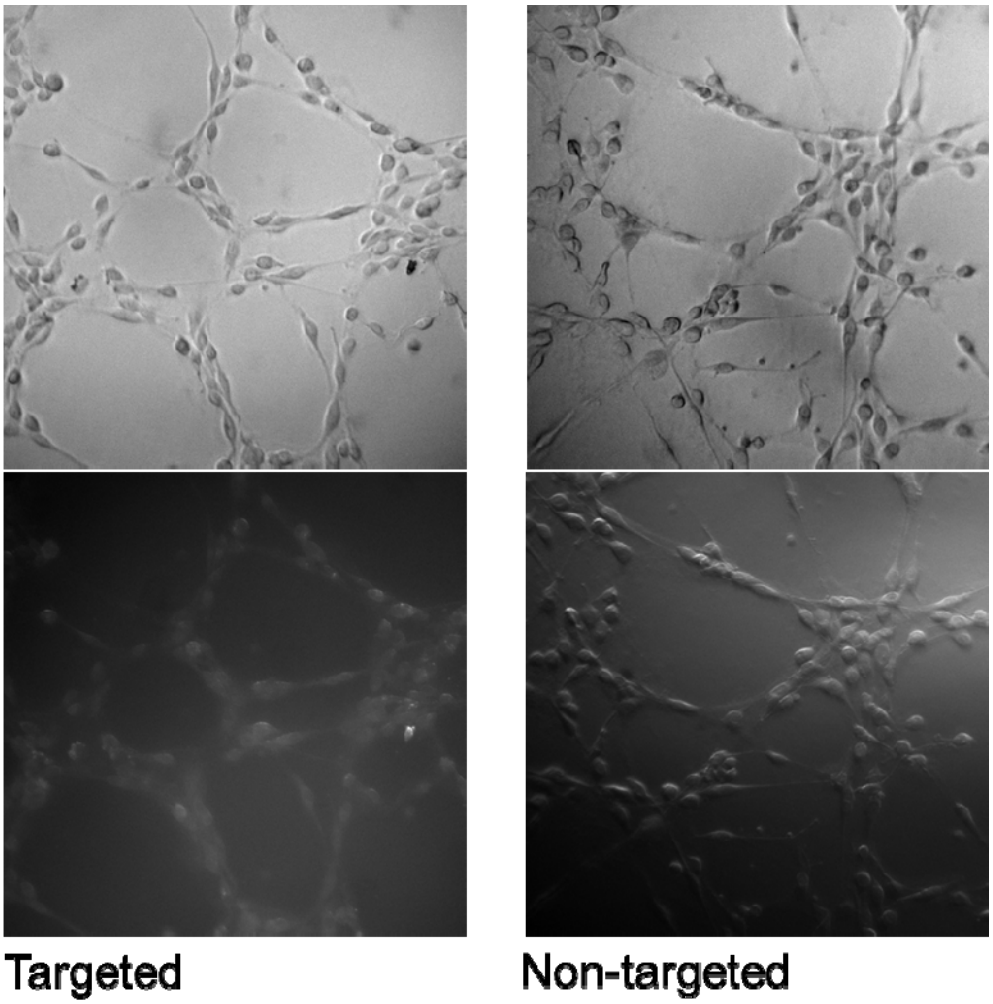
Optimization of A10 aptamer conjugation conditions by variation of the ratio of the liposome's carboxyl-terminus to aptamer during liposome conjugation and evaluation of binding of A10 aptamer-labeled liposomes to LNCaP cells in suspension following a 6 hour incubation.

A10 aptamer-labeled liposomes prepared using 10:3 (■), and 10:1 (▭) mole ratios of the carboxyl PEG-to-free A10 aptamer resulting in 11 ± 2 and 5 ± 1 aptamers per liposome, respectively. Non-targeted liposomes (▭).



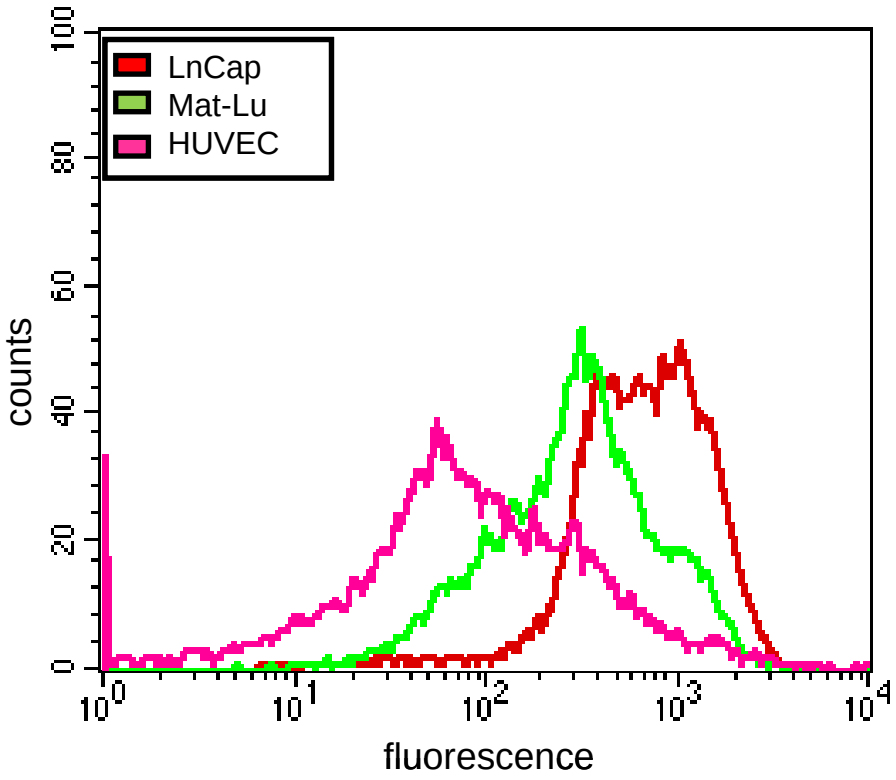
Supplemental Figure 2

Expression levels of PSMA on HUVEC monolayers cultured on Matrigel™ under different conditions. Cells were incubated with mouse anti-human PSMA J591 antibody and stained with FITC-labeled goat anti-mouse secondary antibody. HUVEC exposed to MDA-MB-231 derived conditioned medium (left) and regular (F12K) cell culture medium (right). Top panel: bright field images. Bottom panel: fluorescence images.



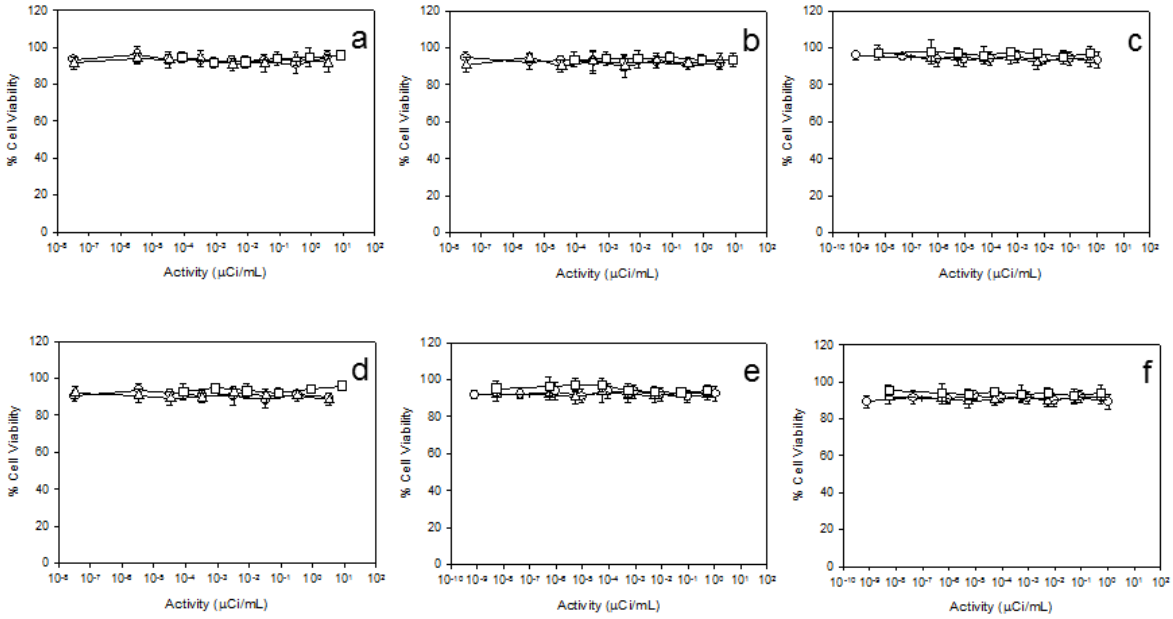
Supplemental Figure 3

Extent of association of fluorescent J591-labeled liposomes (left) and non-targeted liposomes (right) with PSMA-expressing HUVEC. Top panel: bright field images. Bottom panel: fluorescence images.



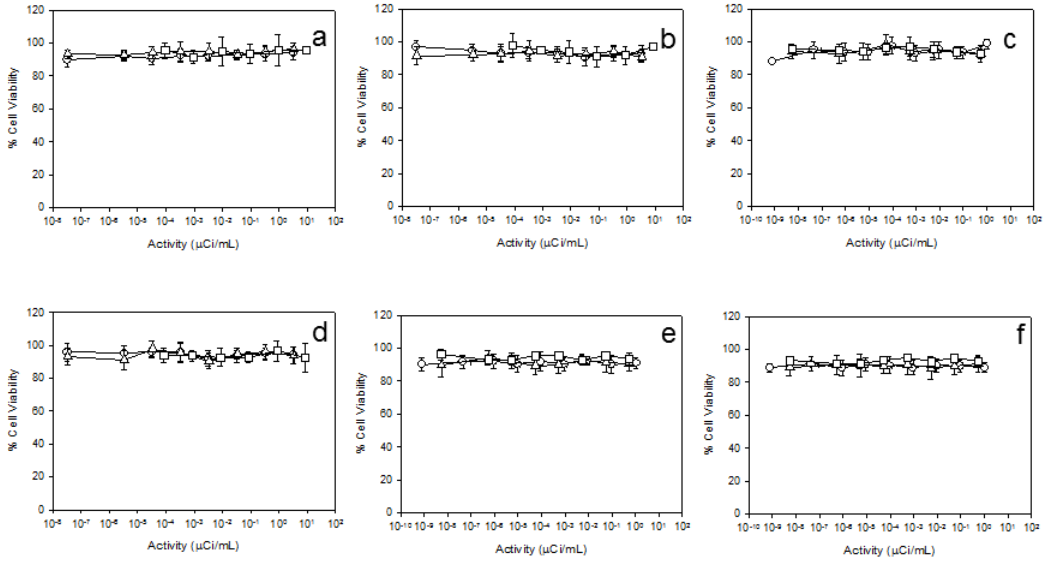
Supplemental Figure 4

The relative PSMA expression on LnCaP, Mat-Lu, and induced HUVEC cells determined by fluorescence counting of cell suspensions incubated with J591 antibody and stained with a FITC-labeled goat anti-mouse secondary antibody.



Supplemental Figure 5

Toxicity of liposomes, not containing radioactivity, following a 6 hour incubation on cell monolayers of (a) LNCaP, (b) Mat-Lu, (c) PSMA-expressing HUVEC, (d) PSMA-negative BT474, (e) PSMA-negative HUVEC grown on Matrigel™, and (f) PSMA-negative HUVEC grown in the absence of Matrigel™. J591-targeted liposomes (circles), A10 aptamer-labeled liposomes (squares), and non-targeted liposomes (triangles).



Supplemental Figure 6

Toxicity of liposomes, not containing radioactivity, following a 24 hour incubation on cell monolayers of (a) LNCaP, (b) Mat-Lu, (c) PSMA-expressing HUVEC, (d) PSMA-negative BT474, (e) PSMA-negative HUVEC grown on Matrigel™, and (f) PSMA-negative HUVEC grown in the absence of Matrigel™. J591-targeted liposomes (circles), A10 aptamer-labeled liposomes (squares), and non-targeted liposomes (triangles).

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