Supplementary Information

Precise targeting of cancer metastasis using multi-ligand nanoparticles incorporating four different ligands

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SUPPLEMENTARY FIGURES

Figure S1. Bioluminescence (BLI) images show the metastatic burden in the lungs of various animals based on different types of breast cancer. Fig. 7 shows the PET images of these animals after injection with the $^{18}$F-labeled multi-ligand nanoparticles.

Figure S2. Coronal PET whole-body image shows a mouse 60 min after injection of the $^{18}$F-labeled multi-ligand nanoparticles. A mouse with D2.OR metastasis in the lungs was intravenously injected with a dose of 200 $\mu$Ci (0.2 mL) of the radiolabeled multi-ligand nanoparticle, and immediately followed by a PET acquisition. PET/CT imaging was performed using a Siemens Inveon microPET/CT scanner.
Figure S3. Quantification of the time-course of signal intensity in the heart is shown after injection of 200 µCi (0.2 mL) of the radiolabeled multi-ligand nanoparticle in healthy animals.

Fig. S4. Representative PET image of lungs ex vivo. A mouse with D2.A1 metastasis in the lungs was intravenously injected with a dose of 200 µCi (0.2 mL) of the radiolabeled multi-ligand nanoparticle, and immediately followed by a PET acquisition. To verify the findings of the in vivo imaging, mice were perfused and lungs were excised and imaged ex vivo using the Siemens Inveon microPET/CT scanner.
SUPPLEMENTARY METHODS

Nanoparticle fabrication

We prepared 100-nm liposomes using established methods. A lipid composition of DPPC, cholesterol and DSPE-PEG(2000)-ligand in the molar ratio of 60-X:40:X was used. Depending on the nanoparticle variant, X was 2, 4.2 or 5.6 mol% for the single-, dual- or quadruple-ligand nanoparticle, respectively. The dual- and quadruple-ligand particles used an equal ratio of the two or four ligand conjugates. The lipids were dissolved in ethanol and hydrated with PBS at 60 °C followed by sequential extrusion in a Lipex Biomembranes Extruder (Northern Lipids, Vancouver, Canada), to size the liposomes to 100 nm. The liposomes were then dialyzed against PBS for 1 day using a 100 kDa MWCO dialysis tubing (Spectrum Laboratories, CA). The size of the nanoparticles was characterized using dynamic light scattering (DLS, Brookhaven Instruments). The exact number of peptides on each liposome formulation was confirmed using direct protein assays (Bio-Rad Protein Assay using Coomassie Blue G-250 dyes).

For the fluorescence imaging studies, each liposome variant was labeled with an Alexa 647 fluorophore, which contained an NHS functional group. The fluorophore was conjugated directly onto the lipid 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) in chloroform at 55 °C in the presence of triethylamine. To reassure complete conjugation of the entire amount of DSPE with fluorophore, we used a 2-fold molar excess of fluorophore over DSPE. The progression of the reaction was monitored using thin layer chromatography (TLC). The unreacted fluorophore was eventually removed after the liposomes were formed using dialysis. Following evaporation of the solvent, DSPE-Alexa-647 was used as part of the lipid matrix at 2.5 mol%. The final levels of each fluorescent label on each liposome were directly measured using the Spectrum In Vivo Imaging System (IVIS, Perkin Elmer).

Functionalization of nanoparticles with targeting ligands

The αvβ3 integrin-targeting peptide c(RGDfC), P-selectin-targeting peptide CDAEWVDVS, fibronectin-targeting peptide CREKA, and the EGFR-targeting peptide CYHWYGYTPQNVI were used. Each peptide was conjugated on the distal end of DSPE-PEG-NH2 using standard conjugation chemistry. Briefly, the thiol of the cysteine residue on the peptides was conjugated to the amine of DSPE-PEG-NH2 via the sulfo-SMCC crosslinker. Sulfo-SMCC contains an amine-reactive N-hydroxysuccinimide (NHS ester) and a sulfhydryl-reactive maleimide group to form stable amide and thioether bonds. To guarantee complete conjugation of DSPE-PEG-NH2, the peptide was at a 2-fold molar excess over PEG. The completion of the reaction was confirmed using thin layer chromatography (TLC). TLC was carried out on silica gel coated fiber sheets using a mixture of CHCl3/MeOH as the mobile phase. These conjugates were then conveniently and accurately added to the recipe for each liposome variant.

Histological evaluation

Immunohistochemistry was performed to evaluate the microdistribution of P-selectin, αvβ3 integrin, fibronectin and EGFR in metastatic sites in the lungs. Mice were anesthetized with an IP injection of ketamine/xylazine and transcardially perfused with heparinized PBS followed by 4% paraformaldehyde in PBS. Organs were explanted and post-fixed overnight in 4% paraformaldehyde in PBS. The tissues were soaked in 30% sucrose (w/v) in PBS at 4 °C for cryosectioning. Serial tissue sections of 12 μm in thickness were stained with the nuclear stain DAPI and the specific antibody for P-selectin, αvβ3 integrin, fibronectin or EGFR. The tissue sections were imaged at 5, 10 or 20x on a Zeiss Axio Observer
Z1 motorized FL inverted microscope. To obtain an image of the entire large tissue section (i.e., lung lobe), a montage of each section was made using the automated tiling function of the microscope.

**Radiolabeling of nanoparticle**

Silica nanoparticles were prepared by using a base-catalyzed sol–gel process. Specifically, tetraethylorthosilicate (TEOS, 200 μL) was added in a solution of 10 mL ethanol and 42 mL water under sonication. After 20 min, 200 μL of 2M NaOH was added as a catalyst to promote the condensation reaction. Sonication was continued for 60 min to get a white turbid suspension. All reactions were conducted at room temperature. Finally, aggregates were removed by repeated centrifugation. Functionalization of the nanoparticles with the four targeting ligands was performed using the four peptides described above via standard conjugation chemistry. Briefly, 2 mg of silane-PEG-maleimide was dissolved in 2 mL of deionized water and separated into 4 equal volumes. A 1.5X molar excess of each desired peptide was dissolved in each container and allowed to react with the silane-PEG-maleimide for 2 hours. The completion of the reaction was confirmed using thin layer chromatography (TLC). Finally, the peptide-PEG-silane conjugates and 1 mg of silane-PEG-NH₂ were added to the silica particles and allowed to react for 24 hours. The size of the nanoparticles was characterized using dynamic light scattering (DLS, Brookhaven Instruments). The exact number of peptides on each nanoparticle formulation was confirmed using direct protein assays (Bio-Rad Protein Assay using Coomassie Blue G-250 dyes).

No carrier-added (n.c.a) [¹⁸F] fluoride was produced by a cyclotron via the nuclear reaction ¹⁸O (p,n) ¹⁸F. At the end of bombardment, the activity of aqueous [¹⁸F]fluoride (50-100 mCi) was transferred to the GE Tracerlab FXn synthesizer by high helium pressure. After delivery, the radioactive solution was passed through a Sep-Pak light QMA cartridge and was eluted by K₂CO₃ solution (6 mg in 0.6 ml water) followed by K₂₂₂ solution (12 mg in 1 ml acetonitrile). The solvent was evaporated under a steam of helium at 85 °C for 5 min and the residue was vacuumed at 55 °C for another 3 min to get the anhydrous K₂₂₂/ [¹⁸F] complex. A solution of the triflate salt of tert-butyl 4-N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (TSTU, 10mg in 1 mL acetonitrile) was added to the above dried complex, and the mixture was heated at 100°C for 10 min. After cooling down, 8 mL of 5% acetic acid was added and the resulting mixture was loaded onto a Sep-Pak plus C18 cartridge, which was then washed with 10 ml water and 10 ml 10% MeCN. The crude [¹⁸F]SFB was eluted with 1 mL of acetonitrile which was further purified by semi-preparative HPLC (Phenomenex C-18, 10 mm × 250 mm, acetonitrile : water (0.01%TFA) = 1:1, flow rate of 3 mL/min, tᵣ=11.5 min). The radioactive fraction containing the desired products was collected, diluted with water, loaded onto a Sep-Pak C-18 cartridge and eluted with 1 ml acetonitrile. After evaporation, the residue was reconstituted in 0.1 mL DMSO, mixed with 0.1 mg of MSN nanoparticles and 20 μL DIPEA. The mixture was reacted at 40 °C for 10 min, the radiolabeling yield were 80~91% (n=6) as determined by thin layer chromatography (TLC).

**References**