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

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Abstract

Metastasis displays a highly heterogeneous cellular population with cancer cells continuously evolving. As a result, a single-ligand nanoparticle cannot account for the continuously changing expression of targetable biomarkers over time and space. To effectively direct nanoparticles to metastasis, we developed a multi-ligand nanoparticle by using four different types of ligands on the same nanoparticle that target biomarkers on the endothelium associated with metastatic disease. These vascular targets included $\alpha_v\beta_3$ integrin, P-selectin, EGFR and fibronectin. Using terminal and *in vivo* imaging studies, the targeting performance of the multi-ligand nanoparticles was compared to the single-ligand nanoparticle variants. All four single-ligand nanoparticle variants achieved significant targeting of lung metastasis in the 4T1 mouse model of breast cancer metastasis with about 2.5% of the injected dose being deposited into metastasis. A dual-ligand nanoparticle resulted in a nearly 2-fold higher deposition into lung metastases than its single-ligand counterparts. The multi-ligand nanoparticle significantly outperformed its targeting nanoparticle counterparts achieving a deposition of ~7% of its injected nanoparticles into lung metastases. Using the high sensitivity of radionuclide imaging, PET imaging showed that a multi-ligand nanoparticle labeled with [¹⁸F]fluoride was able to precisely target metastatic disease at its very early stage of development in three different animal models of metastatic breast cancer.

Graphical Abstract

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1. Introduction

Various targeting ligands schemes have been employed to direct nanoparticles to tumors. However, traditional strategies using a single-ligand system often consider cancer as a monolithic disease and fail to account for tumor heterogeneity. This is particularly evident in the case of metastatic disease. Metastatic cancer cells continuously evolve and change the expression of targetable cell-surface biomarkers over time and space,^{1–7} which results in different metastatic lesions being targeted by different ligands. To capture the dynamic nature of metastasis, we designed multi-ligand nanoparticles that employed four different ligands on the same nanoparticle to effectively target variations in receptor presentation on the endothelium of metastatic lesions (Fig. 1). Our targeting concept is based on three key components that synergistically facilitate effective nanoparticle targeting of the heterogeneity of metastatic disease and its dissemination: 1) Vascular targeting of nanoparticles (rather than traditional deep-tissue targeting); 2) a selection of vascular targets that are overexpressed in metastatic microenvironments; and 3) a four-ligand targeting system that considers the dynamic microenvironment of metastasis by accounting for potential spatiotemporal alterations in the expression patterns of four targetable biomarkers, including $\alpha_v\beta_3$ integrin, P-selectin, EGFR, and fibronectin.

Targeting of upregulated receptors on cancer cells in the tumor interstitium (deep-tissue targeting) requires the nanoparticle has to successfully extravasate across the endothelium, navigate through the extracellular space and finally have meaningful interactions with the cell-surface receptors on the target cells. Unfortunately, the microenvironment of early metastasis does not favor deep-tissue targeting of nanoparticles.^{1, 8–10} The early spread of metastatic cancer cells to distant organs results in small clusters of cancer cells that lack leaky endothelium.¹¹ On the other hand, the endothelium is the closest point-of-contact for circulating nanoparticles. By having direct access to the vascular bed, circulating nanoparticles can continuously scavenge for vascular cancer biomarkers. Most importantly, the size and the multivalent avidity, due to formation of multiple receptor-ligand bonds, makes nanoparticles ideal for targeting vascular-associated pathologies. In previous studies, we showed that vascular targeting is more effective than deep-tissue targeting in mouse models of breast cancer metastasis.^{12–15}

Further, the endothelium associated with metastasis offers a diverse set of targetable biomarkers, which differ from that of healthy vascular beds. Metastatic cancer cells develop their own distinct microenvironment and upregulate specific biomarkers in the lumen of colonized blood vessels in distant organs.^{1, 8–10, 13} In this work, we selected mouse models of triple-negative breast cancer (TNBC), which are highly metastatic and exhibit a high recurrence rate and mortality.¹⁶ To target metastatic TNBC, we selected four different peptides that target 1) receptors on metastatic TNBC cells (*i.e.*, EGFR, $\alpha_v\beta_3$ integrin); 2) the adhesion molecules of the leukocyte adhesion cascade that circulating tumor cells (CTCs) use to attach to the endothelium of distant metastasis (*i.e.*, E- and P-selectin, $\alpha_v\beta_3$ integrin); and 3) overexpressed glycoproteins of the extracellular matrix in the near-perivascular regions of metastasis (*i.e.*, fibronectin). These vascular biomarkers often exhibit spatiotemporal variability representing different microenvironments.¹³

To thoroughly evaluate targeting variants of nanoparticles, we used various mouse models of breast cancer metastasis that represent a broad spectrum of the disease. In particular, we focused on the imaging and diagnostic application of targeted nanoparticles. While current clinical modalities can detect large metastases (>1 cm) with accuracy,^{17–19} today's imaging rarely detects disseminated disease (*i.e.*, micrometastasis), which prohibits early and effective interventions.²⁰ By the time metastatic disease becomes clinically evident, long-term patient outcome is not favorable. In previous work, we have also demonstrated effective drug delivery to hard-to-reach tumors using vascular targeting and single-ligand schemes.^{14, 21, 22} To illustrate the broad application of multi-ligand targeting schemes, we selected two types of nanoparticles, which are all-purpose, versatile nanoparticles for numerous types of therapeutic and imaging agents. Specifically, we used 100-nm liposomes and silica nanoparticles.^{23–26} Importantly, these nanoparticles can conveniently accommodate multi-ligand strategies. Presentation of different types of ligands can be easily controlled on the surface of the nanoparticles in terms of ligand density and ratio among different ligands types. Using fluorescence and radionuclide imaging, we show that multi-ligand nanoparticles are capable of precise and effective targeting and recognition of metastatic disease at its early stages of development.

2. Experimental section

2.1 Nanoparticle fabrication

We prepared 100-nm liposomes using established methods.^{4, 13, 23, 24, 27, 28} The DPPC and DSPE-PEG(2000)-ligand were used as the lipid matrix for the liposomes. 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. After extrusion, the nanoparticles were dialyzed against PBS for 1 day using a 100 kDa MWCO dialysis tubing (Spectrum Laboratories, CA). 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. For the fluorescence imaging studies, each liposome variant was labeled with an Alexa 647 fluorophore. The fluorophore was conjugated directly onto the lipid DSPE. DSPE-Alexa-647 was used as part of the lipid matrix at 2.5 mol%. More details on the synthesis and characterization of the nanoparticles are provided in the Supplementary Information.

2.2 Functionalization of nanoparticles with targeting ligands

Functionalization was performed using previously established methods.^{13, 29, 30} The $\alpha_v\beta_3$ integrin-targeting peptide c(RGDfC),^{12, 13, 31} P-selectin-targeting peptide CDAEWVDVS,^{13, 32, 33} fibronectin-targeting peptide CREKA,³⁴ and the EGFR-targeting peptide CYHWYGYTPQNVI^{30, 35} were used. Each peptide was conjugated on the distal end of DSPE-PEG-NH₂ using standard conjugation chemistry. 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. More details are provided in the Supplementary Information.

2.3 Animal models

All animal studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University. The well-being of the animals took priority over precise measurements in decisions regarding euthanasia or other interventions. All animals received standard care, including *ad libitum* access to food and water, a 12/12 light/dark cycle, appropriate temperature and humidity. Teklab Global #2018 was fed to mice that were imaged with fluorescent imaging. This is because the standard rodent diet has chlorophyll, which adds artifacts to fluorescent images.

We developed three different models of breast cancer metastasis based on the 4T1, D2.A1, and D2.OR cancer cells. All cell lines were engineered to stably express firefly luciferase and green fluorescent protein (GFP). In the case of the 4T1 model, we inoculated 0.5×10^6 4T1-luc-GFP cells orthotopically in a no. 9 mammary fat pad of female BALB/c mice that was surgically exposed while mice were anesthetized. We have previously established that disseminated metastases are developed within 14 days of 4T1 inoculation.^{12–15} At that point, the primary tumor was resected. The other three models of metastasis were based on direct injection of cancer cells into blood circulation in nude or wild-type female mice. Tail vein injection of cancer cells resulted in colonization in the lung. Mice were injected *via* the tail vein with 5×10^5 metastatic D2.A1 or D2.OR cells. The animals were monitored on a daily basis to ensure that they did not suffer from any adverse effects resulting from tumor inoculation.

2.4 Bioluminescence imaging

Using the IVIS Spectrum system, bioluminescence imaging (BLI) was performed 10 min after intraperitoneal administration of 200 μ l of D-luciferin (10 mg/ml). BLI was performed every 3–7 days until the terminal point of the study. At the terminal point, organs were extracted for *ex vivo* organ imaging or histological analysis.

2.5 Fluorescence imaging *ex vivo*

Lungs were imaged *ex vivo* using the IVIS Spectrum system. Briefly, mice were injected with a dose containing $\sim 5.3 \times 10^{11}$ nanoparticles of each formulation. Each nanoparticle formulation was labeled with Alexa 647. Using phantoms of each nanoparticle variant, the IVIS Spectrum system was calibrated to take quantitative deposition measurements of regions with lung metastasis. After 3 h from nanoparticle injection, the animals were anesthetized with an IP injection of ketamine/xylazine and transcardially perfused with PBS. After the organs were explanted, the lungs were precisely sliced in 500 μ m sections using a mouse brain slicer. We confirmed that this low tissue thickness nearly eliminated losses of fluorescence signal. Organs from animals injected with saline were also imaged to determine background fluorescence at all excitation wavelengths. This enabled quantitative *ex vivo* imaging of the nanoparticle concentration in the lungs.

2.6 Histological evaluation

Details on the histological analysis are provided in the Supplementary Information.

2.7 Radiolabeling of nanoparticle

Silica nanoparticles were prepared by using a base-catalyzed sol–gel process. 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 1.5× molar excess of each desired peptide was dissolved in each container and allowed to react with the silane-PEG-maleimide for 2 hours. 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.

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 stream 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-triethylammoniumbenzoate (1, 5mg in 0.5 mL of anhydrous acetonitrile) was added to the above dried complex, and the mixture was heated at 100°C for 10 min. Afterwards, tetratylammonium hydroxide solution (TBAH, 20μl in 0.5 mL acetonitrile) was added and heated at 100 °C for 10 min. The solvent was then removed and N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (TSTU, 10mg in 1 ml MeCN) was added and 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, t_R=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).

2.7 PET imaging

MicroPET/CT imaging was performed using a Siemens Inveon microPET/CT scanner. For better anatomic localization, CT co-registration was applied. Before microPET imaging, CT scout views were taken to ensure the whole body of the mouse was placed in the co-scan field of view (FOV) where the highest image resolution and sensitivity are achieved. Under anesthesia, radiotracer (200 μCi, 0.2 ml) was administered *via* tail vein injection, and immediately followed by a PET acquisition up to 120 min. After the microPET acquisition was done, the mouse was moved into the CT field and a two-bed CT scan was performed. A two-dimensional ordered subset expectation maximization (OSEM) algorithm was used for image reconstruction using CT as attenuation correction. For quantitative analysis, the

resultant PET images were registered to the CT images, which enabled us to accurately define the ROI and quantify the radioactivity concentrations.

2.8 Statistical analysis

Means were determined for each variable in this study and the resulting values from each experiment were subjected to one-way analysis of variance with *post hoc* Bonferroni test (SPSS 15, Chicago, IL). A P value of less than 0.05 was used to confirm significant differences. Normality of each data set was confirmed using the Anderson-Darling test.

3. Results and discussion

3.1 Histological evaluation of vascular biomarkers

In this work, we explored a multi-ligand targeting strategy to target a nanoparticle to four different vascular markers related to metastasis. The selected targets involve surface receptors on the remodeled endothelium as well as foci resident on the endothelium of metastasis. Metastatic TNBC displays a highly heterogeneous cellular population, including rapidly dividing cancer cells, cancer stem cells and dormant disseminated cancer cells.^{36, 37} To consider the dynamic and complex nature of metastatic TNBC, we sought to target upregulated biomarkers expressed by metastatic TNBC cells (*e.g.*, EGFR, $\alpha_v\beta_3$ integrin), the remodeled endothelium associated with metastasis (*e.g.*, P-selectin, $\alpha_v\beta_3$ integrin), and the extracellular matrix in near-perivascular regions of metastasis (*e.g.*, fibronectin). In addition to the adhesion-specific biomarkers on the remodeled endothelium of metastasis,^{1, 38–47} TNBC cells carry a continuously evolving phenotype including varying overexpression of cell surface receptors. Specifically, EGFR contributes to tumor formation, invasiveness and metastasis and is overexpressed in ~70% of TNBC patients.^{48–56}

To assess the expression of vascular biomarkers on the endothelium associated with metastasis, we performed histological analysis in two mouse models of metastatic TNBC. Fig. 2 shows representative images in the 4T1 and D2.A1 mouse models. The main findings are summarized here: 1) After identifying clusters of cancer cells dispersed in the lungs, cancer cells were found on vascular beds. 2) These vascular beds were exactly the locations with highly targetable $\alpha_v\beta_3$ integrin, P-selectin, fibronectin and EGFR. Abundance and selective perivascular expression of all four biomarkers could be seen in regions with cancer cells. 3) These biomarkers were not observed on the endothelium of healthy lung tissues, which indicates that they are highly selective vascular targets associated with metastasis. The histological analysis shown in Fig. 2 illustrates the presence of these four biomarkers on the vascular beds of locations with metastatic TNBC in different animal models, indicating their availability for vascular targeting of nanoparticles.

3.2 Nanoparticle targeting variants for fluorescence imaging

In the first set of studies, we used a liposomal nanoparticle with a composition and size identical to previously published work.¹³ We prepared 100-nm liposomes using established methods.^{4, 13, 23, 24, 27, 28} To be detectable in the fluorescence imaging studies, the nanoparticle variants were labeled with the Alexa 647 fluorophore that contained an NHS functional group. The fluorophore was conjugated directly onto the lipid DSPE. To ensure

for complete conjugation of the entire amount of DSPE with the Alexa fluorophore, a 2-fold molar excess of fluorophore was used over DSPE. Completion of the reaction was confirmed by thin layer chromatography (TLC). The final levels of the fluorescent label on the nanoparticles were directly measured using the Spectrum In Vivo Imaging System (IVIS, Perkin Elmer). Further, there was no change in the fluorescence signal of the nanoparticle formulations during a 24 h dialysis, indicating stable fluorescence labeling.

The average diameter of the nanoparticles and their targeting variants was verified by dynamic light scattering and determined to be ~103 nm (with a polydispersity index of 0.027).

The nanoparticle variants included an $\alpha_v\beta_3$ integrin-targeting nanoparticle (RGD-NP), a P-selectin-targeting nanoparticle (PSN-NP), an EGFR-targeting nanoparticle (EGFR-NP), a fibronectin-targeting nanoparticle (CREKA-NP), an EGFR and $\alpha_v\beta_3$ integrin-targeting nanoparticle (dual-NP), and a quadruple-targeting nanoparticle (multi-NP). As ligands, we selected peptides that bind specifically and with high affinity to $\alpha_v\beta_3$ integrin (RGD), P-selectin (PSN), fibronectin (CREKA), and EGF receptor (EGFR). More specifically, the $\alpha_v\beta_3$ integrin-targeting peptide c(RGDfC),^{12, 13, 31} P-selectin-targeting peptide CDAEWVDVS,^{13, 32, 33} fibronectin-targeting peptide CREKA,³⁴ and the EGFR-targeting peptide CYHWYGYTPQNVI^{30, 35} were used. Before preparing liposomes, the DSPE-PEG-ligand was synthesized separately for each peptide. Briefly, peptides were conjugated on the distal end of DSPE-PEG-NH₂ using standard conjugation chemistry as reported previously.¹³ In previous studies, we have confirmed the structure of similarly synthesized conjugates using MALDI-MS and NMR.^{29, 57} The thiol of the cysteine residue on the peptide was conjugated to the amine of DSPE-PEG-NH₂ *via* the heterobifunctional crosslinker sulfo-SMCC, which is reactive towards amine and sulfhydryl groups. To guarantee complete conjugation of the DSPE-PEG-NH₂ with the peptide, we used a two-fold molar excess of the peptide over DSPE-PEG-NH₂. The progression of the reaction was monitored using thin layer chromatography (TLC), which confirmed complete conjugation of the entire amount of DSPE-PEG-NH₂ with the peptide. TLC was carried out on silica gel coated fiber sheets using a mixture of chloroform/methanol as the mobile phase. DSPE-PEG-NH₂ traveled a short distance (Rf=0.2), whereas the free peptides migrated to closer to the solvent front (Rf=0.75–0.9 depending on the peptide). In addition iodine vapor staining, spots were also ninhydrin positive. The products appeared in a new spot (Rf=0.5–0.6). At the end of the reaction, there was only the spots of DSPE-PEG-peptide and the remaining excess of peptide. The absence of a spot at Rf=0.2 indicated the complete depletion of DSPE-PEG-NH₂. The excess peptide was completely removed using a 1-day dialysis against PBS.

These conjugates were then conveniently and accurately added to the recipe for each nanoparticle variant. Using Bio-Rad Protein Assay and Coomassie Blue G-250 dyes, the number of peptides on the nanoparticle variants was determined. The single-ligand nanoparticles contained ~2,000 peptides per particle, whereas the dual-ligand variant had a total ~4,200 peptides per particle. The quadruple-ligand NP contained a total number of ~5,800 peptides per particle with the four peptides having an equal share.

We should note that the DSPE-PEG-ligands were dissolved and well-mixed with the rest of the lipids in ethanol before hydration and formation of liposomes. The Bio-Rad Protein Assay confirmed the amounts of DSPE-PEG-peptide, indicating negligible losses during extrusion. While we didn't measure the surface distribution of the different types of ligands relative to each other dual and multi-ligand nanoparticles, we assumed that there was uniform distribution of the peptides because the different types of peptides did not exhibit greatly different sizes and charges. Overall, in the case of the dual- and quadruple-ligand nanoparticles, we sought to ensure the ligand density for each type of peptide by using an equal ratio for the two or four ligand conjugates. Zeta potential measurements revealed a near neutral charge for these liposomes (about 2–4 mV), indicating that all the amines were conjugated with peptides.

3.3 Evaluation of targeting efficacy of different nanoparticle variants *in vivo*

For these studies, we used the 4T1 model, which is one of the standard models to study the development of metastatic TNBC in immunocompetent mice. After inoculation in a mammary fat pad, 4T1 tumors rapidly develop spontaneous metastases at organ sites reminiscent of those observed in human patients.^{12, 13, 15, 22} The primary tumor was then surgically removed, so that metastatic disease could be studied in an animal setting comparable to the adjuvant clinical scenarios where the primary tumor is removed, and metastatic foci remain intact. Further, the 4T1 cells stably expressed fluorescent and bioluminescent reporter genes, which were needed to longitudinally track the dissemination and growth of developing metastasis in mice. Fig. 3a shows representative bioluminescence (BLI) images of a mouse bearing a 4T1 tumor indicating the timeline of metastatic progression. Once BLI imaging indicated that metastasis was present in the lungs (day 16), a nanoparticle formulation labeled with Alexa 647 was systemically injected into the animal. To quantitatively image the fluorescence signals from nanoparticles deposited in metastases, we used the IVIS Spectrum system to image the lungs *ex vivo*. Our previous studies have showed that maximum deposition of targeted nanoparticles onto the endothelium of metastasis occurs within 3 h post-injection.^{13, 15} After 3 h from injection of the nanoparticles, lungs were perfused, excised and precisely sliced in 500- μ m sections using a mouse organ slicer. Phantom studies confirmed that the 500- μ m tissue thickness resulted in negligible attenuation of fluorescence signal, enabling accurate quantification of the concentration of each nanoparticle variant in metastatic sites in the lungs. Fig 3b shows representative images of the deposition of CREKA-NP and multi-NP in the lungs.

We quantitatively evaluated the different targeting variants of the nanoparticles in the 4T1 mouse model at a time point representing the early spread of metastatic disease (day 16 after tumor inoculation). Using calibrations from phantoms of the Alexa 647-labeled nanoparticles, quantitative measurements of the nanoparticle were taken from lung slices. Lungs from animals injected with saline were also imaged to determine background fluorescence at the selected excitation wavelengths. Furthermore, the accuracy of the quantitative measurements of the fluorescence imaging method was confirmed by directly measuring the concentration of deposited nanoparticles in the tissues in a separate study (n=4). Specifically, besides the surface Alexa 647 label, a red fluorophore was encapsulated into the nanoparticle. Using the same methodology, 3 h after injection, the lungs were

perfused, excised, sliced in 500- μ m sections and imaged with the IVIS Spectrum system. The lung sections were then homogenized and the secondary label was extracted following established methods.^{22, 58, 59}

Specifically, we compared the quadruple-ligand nanoparticle targeting all four biomarkers to its single-ligand nanoparticle variants targeting only one of the four biomarkers and a dual-ligand nanoparticle targeting EGFR and $\alpha_v\beta_3$ integrin. All formulations were injected at the same dose containing $\sim 5.3 \times 10^{11}$ nanoparticles. The summary of the measurements is shown in Fig. 4a (n=4–6 mice in each group). It should be noted that injection of the nanoparticles to healthy animals resulted in negligible signals (nearly baseline levels) from the lungs. Consistent with our previous studies,¹³ the intratumoral deposition of the non-targeted nanoparticles (NT-NP) resulted in low levels (1.26% of dose), indicating low EPR-driven deposition into early metastasis. On the other hand, all four single-ligand nanoparticle variants achieved higher levels of deposition in lung metastasis, which was about 2.5% of the injected dose of nanoparticles. Notably, the deposition among the four single-ligand variants was statistically insignificant. Among the four potential ligand combinations to generate dual-ligand nanoparticles, we selected EGFR and $\alpha_v\beta_3$ integrin, which represent two diverse biomarkers, including receptors on metastatic TNBC cells (*i.e.*, EGFR, $\alpha_v\beta_3$ integrin) and receptors on the remodeled endothelium metastasis (*i.e.*, $\alpha_v\beta_3$ integrin). The dual-ligand nanoparticle resulted in a nearly 2-fold higher deposition into lung metastases than its single-ligand counterparts. Finally, the multi-NP particle significantly outperformed all the formulations achieving a deposition of $\sim 7\%$ of the injected nanoparticles into lung metastases. It is important to mention that we sought to maintain a reliable assay system by selecting animals with very similar metastatic burden as shown in Fig. 4b.

While the averaged values of entire groups provided useful information, examination of the lung deposition for each animal separately provided further insights (Fig. 4c). When we looked closely at the single-ligand formulations, the targeting efficacy was very inconsistent. For instance, EGFR-NP achieved a deposition of 0.97% of injected dose into lung metastases of one animal and 4.34% in another animal, which represents a nearly 450% difference between those two animals. Similar observations can be made for the other single-ligand formulations. In the case of the dual-ligand nanoparticle, the targeting efficacy was higher than the single-targeting variants with significant consistency among all animals. Most notably, the multi-ligand nanoparticle targeting all four biomarkers consistently achieved the highest deposition in lung metastases for all the animals ranging from 6.1–8.4% of the injected dose.

Comparing the non-targeted to the CREKA-targeted nanoparticle variant, the organ distribution was similar in mice bearing 4T1 metastasis (Fig. 4d). For both formulations, the majority of the particles ended up being cleared by the organs of the reticuloendothelial system (liver and spleen), while the nanoparticle levels in the heart and kidneys were very low. As expected, the presence of peptide on the CREKA-NP accelerated the particle's recognition and clearance as indicated by the higher levels in the liver compared to the non-targeted variant.

Rather than targeting cancer cells in the tumor interstitium, we sought to target biomarkers on the endothelium associated with metastatic disease. Generally, traditional targeting schemes seek to direct nanoparticles onto upregulated receptors on the cancer cells in the tumor interstitium. Unfortunately, the microenvironment of early metastasis lacks the leaky endothelium often found in solid tumors and therefore passive intratumoral deposition of nanoparticle is not favored.^{13, 15} As a consequence, ‘deep-tissue’ targeting of surface receptors on cancer cells is limited in early and transient metastasis. On the other hand, the remodeled endothelium associated with metastasis serves as the early and obvious targetable site of the disease. While our targeting studies in Fig. 4 show that the single-ligand nanoparticle variants exhibited significant deposition into metastatic sites, the targeting efficacy was not consistently high in all the animals. To further increase targeting efficacy, we demonstrated in a previous study¹³ that a dual-ligand nanoparticle can target two receptors overexpressed in metastasis facilitating effective targeting of metastatic disease that was otherwise missed by single-ligand strategies. In fact, the dual-ligand strategy was able to account for spatiotemporal alterations in the expression patterns of the targetable receptors in metastatic sites. Here, we further expanded from two to four different targeting ligands on the same nanoparticle. Compared to all the single-ligand and the dual-ligand nanoparticle variants, the four-ligand nanoparticle achieved consistently higher targeting of metastatic disease.

3.4 PET imaging

Finally, we sought to evaluate the ability of the multi-ligand nanoparticles to target and detect metastasis at its very early stages of development. While fluorescence labelling provided sufficient contrast sensitivity to be able to image the nanoparticles *ex vivo*, targeting of the very early spread of the disease requires even higher sensitivity. Thus, we employed radiolabeling and radionuclide-based imaging due to its extraordinary sensitivity (down to the picomolar level).⁶⁰ More specifically, positron emission tomography (PET) exhibits many advantages, including high sensitivity and deep tissue penetration.^{60, 61} To evaluate the performance of the multi-ligand targeting strategy, we used mice bearing different models of breast cancer metastasis, representing a broad range of the disease. In this study, we included mice with D2.A1, D2.OR and 4T1 metastasis in the lungs as well as healthy mice (n=6). In addition to the 4T1 model, the D2-Hyperplastic Alveolar Nodules (HAN) series consists of metastatic D2.A1 cells and their isogenic and dormant D2.OR counterparts. The D2.OR cells differ dramatically in their ability to initiate metastatic outgrowth in the lungs of syngeneic Balb/C mice.^{62, 63} Dormant breast cancer cells are “potential time bombs” that remain in quiescent/senescent states for long periods of time prior to initiating their “explosive” metastatic outgrowth. Representative BLI images show the vast difference of the progression of the disease in the D2.A1 and D2.OR models (Fig. 5).

The multi-ligand nanoparticle was radiolabeled with [¹⁸F]fluoride, which was produced by an on-site cyclotron. In addition to the four ligands (~500 ligands of each ligand type per particle), we left 100 PEG-amines on each particle available for conjugation with the [¹⁸F] label (Fig. 6a). In the first step of the radiochemistry (Fig. 6b), N-succinimidyl 4-[¹⁸F]fluorobenzoate ([¹⁸F]SFB) was synthesized, which was 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, t_R =11.5 min). Having an NHS ester, [^{18}F]SFB reacts effectively with the available amines on the nanoparticles. The radiolabeling yield was 80–91% (n=6 batches), as determined by thin layer chromatography (TLC).

To resemble the early-stage development of metastatic disease, we used mice bearing lung metastasis of 4T1 (Day 10), D2.A1 (Day 7) and D2.OR (Day 35) cancer cells. Fig. S1 (Supplementary Information) shows that the BLI photon count from the regions with metastasis was very low ($\sim 10^5$ p/sec/cm²/sr). The radiolabeled multi-ligand nanoparticle (200 μCi , 0.2 ml) was administered *via* tail vein injection, and immediately followed by a PET acquisition for 120 min. MicroPET/CT imaging was performed using a Siemens Inveon microPET/CT scanner. For anatomic localization, CT co-registration was applied. After the microPET acquisition was done, the mouse was moved into the CT field and a two-bed CT scan was performed. Fig. 7 (top row) shows representative coronal images of the mice 1 h after administration of the multi-ligand nanoparticles. PET imaging showed that vascular targeting of the nanoparticle resulted in “hot spots” in the lungs of all the mice with metastasis. As expected, the liver also displayed significant signal, since nanoparticles are cleared by Kuppfer cells in the liver (Fig. S2 in Supplementary Information). It should be emphasized that the thoracic region of healthy animals exhibited no signal, indicating the absence of non-specific accumulation of the multi-ligand nanoparticles in healthy lungs. Further, there was no signal from the heart 60 min after injection of the agent as shown in the pharmacokinetic profile of the agent in Fig. S3 in Supplementary Information. Most importantly, the findings of *in vivo* imaging were verified by *ex vivo* organ imaging. The lungs of the mice were perfused and excised after the last PET imaging session. Planar fluorescence imaging confirms the presence and topology of early metastasis (Fig. 7, bottom row). In addition, *ex vivo* PET imaging of lungs confirmed that the signal is from cancerous tissues in the lungs (Fig. S4 in Supplementary Information).

It is important to note that it is essential for a nanoparticle to be able to target ‘clinically silent’ micrometastatic disease. Numerous retrospective studies with long-term follow-up showed increased mortality in breast cancer patients with micrometastasis.^{64–68} Thus, it is essential to evaluate the risk of an individual patient of harboring clinically silent micrometastatic disease. An accurate diagnostic testing would be deployed for use in early-stage disease to optimally assess the risk of patients and impact treatment decisions for optimal local control. In more advanced disease scenarios, such imaging would be used for guiding treatment decisions around locoregional versus systemic treatment. In conclusion, early and accurate detection of metastasis can become one of the most consistent prognostic factors in therapy decision-making.

As a case study for the multi-ligand nanoparticle, triple-negative breast cancer (TNBC) was selected, which is an extremely metastatic subtype of breast cancer with frequent relapse and shorter metastasis-free-survival.^{69, 70} In this context, our animal modeling included an extremely metastatic subtype of breast cancer (*i.e.*, metastatic TNBC) as well as dormant disseminated disease (*i.e.*, latent breast cancer). Our studies showed that an ^{18}F -labeled nanoparticle coupled with a four-ligand vascular targeting facilitated precise detection of early disease, transient and dormant metastatic disease. Using the extraordinary sensitivity

of radionuclide imaging and a very low dose of the nanoparticles, PET imaging achieved remarkable diagnostic accuracy with high sensitivity (*i.e.*, correctly identified all the animals with micrometastasis) and high specificity (*i.e.*, correctly identified healthy animals as not having the disease).

4. Conclusions

In conclusion, we explored a new paradigm on how to design multi-ligand nanoparticles to consider the complex, dynamic and heterogeneous microenvironment of metastatic disease. Compared to the non-targeted nanoparticle, all four variants of single-ligand nanoparticles achieved superior deposition into early metastasis. The dual-ligand nanoparticle achieved a nearly 2-fold higher deposition into metastasis than any of the single-ligand variants. Most notably, the four-ligand nanoparticle exhibited further and significant improvements, resulting in a nearly 3-fold higher deposition than the single-ligand nanoparticles (~7% of the injected dose). Finally, we demonstrated the diagnostic accuracy of the multi-ligand nanoparticle to detect 'clinically silent' micrometastatic disease using PET imaging, which exhibits high sensitivity. Overall, precise targeting of a nanoparticle imaging agent early-stage metastasis can be a companion diagnostic and provide prognostic and diagnostic value in therapy decision-making.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Illustration of the multi-ligand concept shows a nanoparticle capable of targeting the dynamic microenvironment of metastatic disease.

Figure 2.

Histological evaluation of the microdistribution of vascular biomarkers in two mouse models of metastatic TNBC. Representative fluorescence images of serial histological sections of lungs show the spread of metastatic cancer cells in mice bearing 4T1 metastasis (top) and D2.A1 metastasis (bottom). Location of metastatic cancer cells is shown with respect to endothelial cells and expression of $\alpha_v\beta_3$ integrin, P-selectin, fibronectin and EGFR (10 $\times$ magnification; scale bar= 50 μ m; blue: nuclear stain; green: cancer cells; red: CD31 endothelial marker or $\alpha_v\beta_3$ integrin or P-selectin or fibronectin or EGFR).

Figure 3.

Representative *in vivo* bioluminescence (BLI) images and *ex vivo* fluorescence images of mice with 4T1 metastasis. (a) BLI images of the same animal show the progression of 4T1 metastasis in the lungs of mice. (b) The nanoparticle targeting variants were labeled with Alexa 647. The particles were intravenously injected into animals with 4T1 metastasis. After 3h from injection, lungs were perfused, excised, sectioned into thin slices of equal thickness and imaged *ex vivo* using an IVIS Spectrum system.

Figure 4.

Quantitative evaluation of the ability of the nanoparticle targeting variants to target metastasis in the 4T1 mouse model. (a) Each formulation was systemically administered via a tail vein injection at the same dose (NT-NP indicates the non-targeted nanoparticle). The fluorescence signal of the fluorescence images of the lung sections was quantified for each formulation at 3 h after injection ($n = 5$ mice). On the basis of phantom measurements of each formulation using the IVIS imaging system, the fluorescence signal was converted to nanoparticle concentration (mean $\pm$ SD; * indicates $p < 0.05$ by Student's t test; $n=4-6$ mice per group). (b) The bioluminescence signal from the thoracic region of each animal was quantified and summarized for each group, representing the metastatic burden of the different groups used in the targeting studies. Data are presented as mean $\pm$ SD. (c) The nanoparticle deposition of the nanoparticle targeting variants in lung metastasis is presented separately for each animal. (d) Organ distribution of NT-NP and CREKA-NP was evaluated in the 4T1 mouse model ($n=5$).

Figure 5. Representative BLI images shows the timeline of the progression of aggressive metastasis (top; D2.A1 model) and latent metastasis (bottom; D2.OR model) in the lungs of mice after tail vein injection of cancer cells.

Figure 6.

Radiolabeling of the multi-ligand nanoparticle with [^{18}F]fluoride. (a) Illustration of the nanoparticles shows that incorporation of the functionalization with the 4 targeting peptides and the availability of a small number of amine groups on the nanoparticle's surface. (b) Reaction scheme of the radiochemistry shows the synthesis of [^{18}F]SFB and its conjugation onto the nanoparticles.

Figure 7.

Imaging of micrometastasis using an ^{18}F -labeled multi-ligand nanoparticle and PET. Healthy mice and mice with 4T1 or D2A1 or D2.OR metastasis in the lungs were intravenously injected with a dose of 200 μCi (0.2 mL) of the radiolabeled multi-ligand nanoparticle, and immediately followed by a PET acquisition up to 120 min. PET/CT imaging was performed using a Siemens Inveon microPET/CT scanner. Coronal images show the thoracic region of the mice 60 min after injection of the nanoparticles (top row). At the end of the PET imaging session, the lungs of the animals were perfused, excised and imaged *ex vivo* using a CRi Maestro fluorescence imaging system, indicating the locations with metastasis in the lungs (bottom row).