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The effect of multistage nanovector targeting of VEGFR2 positive tumor endothelia on cell adhesion and local payload accumulation

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Abstract

Nanovectors are a viable solution to the formulation of poorly soluble anticancer drugs. Their bioaccumulation in the tumor parenchyma is mainly achieved exploiting the enhanced permeability and retention (EPR) effect of the leaky neovasculature. In this paper we demonstrate that multistage nanovectors (MSV) exhibit rapid tumorotropic homing independent of EPR, relying on particle geometry and surface adhesion. By studying endothelial cells overexpressing vascular endothelial growth factor receptor-2 (VEGFR2), we developed MSV able to preferentially target VEGFR2 expressing tumor-associated vessels. Static and dynamic targeting revealed that MSV conjugated with anti-VEGFR2 antibodies displayed greater than a 4-fold increase in targeting efficiency towards VEGFR2 expressing cells while exhibiting minimal adherence to control cells. Additionally, VEGFR2 conjugation bestowed MSV with a significant increase in breast tumor targeting and in the delivery of a model payload while decreasing their accumulation in the liver. Surface functionalization with an anti-VEGFR2 antibody provided enhanced affinity towards the tumor vascular endothelium, which promoted enhanced adhesion and tumorotropic accumulation of a reporter molecule released by the MSV.

Keywords

Drug Delivery; Tumor vascular targeting; Vascular endothelial growth factor receptor; Multi-stage Nanovectors; Nanoparticles

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1. Introduction

Chemotherapy is the most widely used form of therapy in the treatment of cancer. Traditional chemotherapeutic drugs indiscriminately target and destroy both malignant and healthy cells, resulting in severe side effects [1]. Therapeutics, such as monoclonal antibodies and small interfering RNA (siRNA), are well suited to target tumor cells or specific genes but are associated with an increased risk of adverse immune reactions [2, 3] and ineffective delivery to cells [4]. New treatments based on the targeted delivery of therapeutic agents to the tumor microenvironment [5] rely on nanoparticles (NP) able to encapsulate poorly soluble drugs [6], to provide protection from degradation [7], to enhance cellular internalization [8], or to trigger the release of payloads based on environmental cues [9]. NP can persist in blood circulation and exploit the enhanced permeability and retention (EPR) effect exhibited by tumor-associated blood vessels [10, 11].

In the late 1800s, Rudolf Virchow observed that tumors were highly vascularized but it was not until the early 1970s that controlling angiogenesis became a therapeutic option for cancer [12]. Today, the delivery of therapeutic agents to the tumor microvasculature is a clinical reality. Both endothelial and cancerous cells undergoing angiogenesis express high levels of vascular endothelial growth factor receptor 2 (VEGFR2), a primary mediator of cell proliferation and tumor growth [13–16]. The inhibition of VEGF-mediated angiogenesis with monoclonal antibodies against VEGF (the natural ligand and activator of VEGFR2) culminated in the development of bevacizumab (Avastin), a humanized monoclonal antibody that obtained U.S. Food and Drug Administration approval in 2004 [17, 18]. Based on the success of this approach, to selectively deliver a payload to the cancer site, we developed nanovectors capable of recognizing vascular endothelial cells expressing VEGFR2.

To circumvent the biological barriers encountered by NP during systemic administration, we developed multistage nanovectors (MSV) [19]. MSV were rationally designed to exhibit superior margination (i.e., tendency to drift towards the vessel wall) due to their disk-like shape and size [20–22] and demonstrated efficient adhesion towards endothelial cells [23, 24]. During systemic administration MSV demonstrated rapid (< 1 hour) tumorotropic accumulation independent of EPR [25, 26] and enhanced delivery to the bone marrow upon targeting with E-selectin [27]. The nanosized pores and increased surface area of MSV can accommodate a variety of payloads (e.g., chemotherapeutics [28, 29], drugs [30], NP [31], contrast agents [32], biologics [33]) and their release could be tuned by adjusting the pore size [34] or their surface coating [35–37]. Furthermore the loading of payloads into the nanopores, resulting in nanoconfinement, enabled the emergence of features at the nanoscale bestowing MSV with enhanced gene silencing, hyperthermia, and diagnostic potential [31, 38].

In this paper, we propose to bestow MSV with anti (α)-VEGFR2 antibodies to assess the specific targeting of MSV to tumor-associated blood vessels. The *in vitro* targeting efficiency was studied in static conditions and under physiological dynamic flow on endothelial monolayers expressing human VEGFR2. Breast tumor targeting and

biodistribution was assessed *in vivo* using near infrared fluorescent imaging and compared to untargeted MSV.

2. Materials and Methods

2.1 MSV Conjugation

MSV were modified with 3-aminopropyltriethoxysilanes (APTES, Sigma-Aldrich) as previously described [19]. Briefly, 1×10^8 MSV were suspended in isopropyl alcohol with 2% APTES and incubated at 35°C for two hours with mixing. MSV were washed three times and stored in a desiccator overnight. APTES-modified MSV were then functionalized with succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC, Thermo Scientific) in a 1 mg/mL solution and incubated for two hours with mixing. SMCC-modified MSV were washed three times and stored in a desiccator overnight. Rat anti-mouse VEGFR2 antibody (R&D Systems) was separately prepared and dye-labeled in the following manner. The VEGFR2 antibody was incubated at 4°C for one hour while mixing in phosphate buffer (PB, 10 mM, 7.5 pH), AlexaFluor 555 (Invitrogen), and Traut's reagent (2-iminothiolane hydrochloride; 2 mg/mL, Thermo Scientific) solution. The solution was then filtered through a desalting column to remove free dye. SMCC-modified MSV were suspended in PB with dye-labeled VEGFR2 antibody and incubated at 4°C for two hours with mixing. MSV were washed thrice and stored at 4°C. Labeling of anti-VEGFR2 conjugated MSV (α -VEGFR2) or SMCC-modified MSV (untargeted) was achieved by suspending MSV in PB solution containing AlexaFluor 647 (Invitrogen) and incubated for one hour with mixing. MSV were washed with PB and stored at 4°C.

2.2 PAEC Clones

Porcine aortic endothelial cells (PAEC) and hVEGFR2 plasmid were a gift from the laboratory of Dr. Mauro Giacca at the International Centre for Genetic Engineering and Biotechnology (ICGEB in Trieste, Italy). PAEC were maintained using F-12K media (Hyclone, Thermo Scientific) and supplemented with 10% fetal bovine serum. The sequence coding for hVEGFR2 protein was fused with yellow fluorescent protein (YFP). The overall sequence was cloned into a vector containing the geneticin (G418) resistance gene. The plasmid was transfected with into PAEC using lipofectamine 2000. PAEC "clones" were achieved using cloning by limiting dilution and arranged based on YFP fluorescence. PAEC clones were analyzed using flow cytometry to distinguish different levels of VEGFR2 expression (VEGFR-LOW, -MED, -HIGH). Stable transfection of PAEC clones (i.e., PAEC transfected with hVEGFR2) was achieved by supplementing complete media with 3 mg/mL of G418. Flow cytometry analysis of YFP status on PAEC was performed using a BD FACSFortessa housed within HMRI Flow Cytometry Core and equipped with four laser (405, 488, 561, 630 nm) excitation sources. The 488 nm source with a 525/50 bandpass filter was used to acquire YFP signal from control (i.e., wild-type (WT)) and VEGFR2-clones.

2.3 Targeting of MSV

The static (i.e., without flow) targeting potential of targeted (α -VEGFR2) and untargeted MSV was tested using 8-chamber CultureSlides (BDFalcon) placed on a flat rotator shaker (Thermo Scientific) within the incubator at 37 °C and 5% CO₂. PAEC were seeded at a

density of 17,500 cells/chamber for WT and 12,250 cells/chamber for HIGH PAEC, 24 hours prior to treatment. PAEC were treated with MSV at a ratio of 1:10 (PAEC:MSV) and analyzed at pre-determined time points. PAEC were then washed, fixed, and stained with Prolong gold and 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Slides were visualized and imaged using a Nikon Eclipse 80i fluorescence microscope, and data was analyzed using Nikon Elements.

Dynamic targeting (i.e., with flow) of MSV in flow conditions was tested using ibidi μ -slide I^{0.4} Luer pre-coated with fibronectin at 75 μ g/mL. 24 hours prior to MSV treatment, PAEC were seeded at a concentration of 1.25×10^6 cells/mL. Slides were flowed with 3×10^7 MSV (targeted and untargeted) at 100 μ L/minute for 30 minutes, as previously described [35]. Slides were continuously imaged using an inverted Nikon Eclipse Ti fluorescence microscope equipped with a Hamamatsu ORCA-Flash 2.8 digital camera and fitted with an induction chamber maintaining samples at 37°C and 5% CO₂. Data was analyzed using Nikon Elements.

2.4 Animal Care

Animal studies were performed in accordance with the guidelines of the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals based on approved protocols by The University of Texas M.D. Anderson Cancer Center's Institutional Animal Care and Use Committee. Female athymic nude mice (NCr-Fox1^{nu}; 4–6 week old) were purchased from Charles Rivers Laboratories and maintained as previously described [39]. Mouse breast cancer tumors were established by implanting 5×10^5 4T1 tumor cells suspended in PBS into the mammary fat pad of female nude mice for non-invasive imaging.

2.5 Non-invasive imaging and biodistribution

Nude mice containing 4T1 tumors were randomly divided into groups (n = 4) to compare α -VEGFR2 (i.e., targeted) at 2 and 4 hours versus IgG (i.e., non-targeted) at 2 hours. Mice were injected intravenously in the tail vein with 5×10^7 MSV labeled with DyLight 800, as previously described [40]. Mice were imaged using an IVIS Lumina equipped with the indocyanine green excitation and emission filters at pre-determined times. Mice were sacrificed and organs were harvested, washed in PBS, and imaged for fluorescence in each organ. Images of whole animal and organs were analyzed and exported using Living Image 4.0 software.

2.6 Statistical analysis

Statistics were calculated with Prism GraphPad software. Statistics for static targeting, dynamic targeting, and *in vivo* targeting experiments was analyzed using a Two-Way ANOVA followed by a Bonferroni post-test to compare replicate means by rows.

3. Results and Discussion

3.1 Characterization

3.1.1 MSV Characterization—Hemispherical MSV were fabricated as previously reported [41] yielding a homogenous and uniform distribution of 3.2 μ m MSV with 15 nm

pores. The multistep process required to produce α -VEGFR2 MSV is illustrated in Fig. 1a. FTIR (Fig. 1b, c) and zeta potential (Fig. 1d) measurements were collected after each modification to verify successful conjugation. Inspection of the FTIR spectrum between 1300 and 1900 cm^{-1} (Fig. 1c), revealed a broad peak at 1565 cm^{-1} after APTES modification which corresponds to N-H (i.e., amine groups) and confirmed successful conjugation [42]. SMCC conjugation prompted a peak at 1704 cm^{-1} , consistent with terminal maleimide groups present on the cross-linker [42]. Upon conjugation with VEGFR2 antibodies, the peak at 1704 cm^{-1} diminished suggesting a decrease in maleimide groups due to the reaction between the sulfhydryl groups on the antibody (enriched using Traut's reagent) and the available maleimide groups on the surface of MSV [42]. Zeta potential measurements corroborated the modifications to the surface resulting in dramatic changes in surface charge (Fig. 1d). For example, upon APTES modification the surface charge increased by +29 mV (consistent with previous publications [19, 34, 40]) followed by decreases of -13 and -22 mV for SMCC and α -VEGFR2, respectively. Fluorescence spectroscopy revealed that 1500 ng of anti-VEGFR2 antibody was successfully conjugated to 1×10^6 MSV, equating to approximately 1.5 pg of antibody per MSV (Supplementary Information, Fig. S1). In addition, the successful conjugation of VEGFR2 antibody to MSV was confirmed using fluorescent microscopy and did not hinder further incorporation of fluorescent molecules within the MSV (Fig. 1e). MSV modified with α -VEGFR2 antibodies conserved the ability to retain a therapeutic payload, melittin the primary active component in bee venom and has shown promise for cancer therapy [43] (Fig. 1f). Thus upon functionalization with antibodies, MSV conserved the ability to retain payloads for imaging and therapy.

3.1.2 PAEC Characterization—PAEC cells were transfected with the hVEGFR2-YFP plasmid, selected for successful integration using G418, sorted using FACS, and cloned using limiting dilution to generate cell populations expressing various amounts of VEGFR2 (Supplementary Information, Fig. S2). From these cells, three were selected to illustrate the low, medium, and high (LOW, MED, and HIGH) range of possible VEGFR2 expression within tumor vasculature [44, 45]. Prior to selection, each clone sub-type exhibited several cells absent in YFP expression (Fig. 2a). Clones were then sorted and selected using G418 to yield stable populations of clones (Fig. 2b–d and Supplementary Information, Fig. S3). Confocal imaging (Fig. 2b) validated the pattern of expression for VEGFR2/YFP, progressively increasing from the LOW to HIGH VEGFR2 PAEC consistent with flow cytometry analysis (Fig. 2c). High magnification of HIGH PAEC displayed uniform YFP fluorescence (i.e., VEGFR2) on the surface surrounding the entire cell (Supplementary Information, Fig. S4). Western blot analysis confirmed the increasing expression of VEGFR2 in the LOW, MED, and HIGH PAEC populations (Fig. 2d & Supplementary Information, Fig. S3). The 15-fold increase in VEGFR2 expression observed in HIGH PAEC was below results obtained in experimental animal models, which displayed greater than a 40-fold increase in VEGFR2 expression in tumor endothelial cells with negligible expression found in tumor cells [46] thus demonstrating the applicability of HIGH PAEC to model the tumor endothelia. Furthermore, HIGH PAEC clones exhibited sustained and uniform expression of VEGFR2 for several weeks (Supplementary Information, Fig. S3) and

were used for all the *in vitro* targeting experiments while non-transfected wild-type (WT) PAEC cells were used as controls.

3.2 In vitro Targeting of MSV with α -VEGFR2

3.2.1 Static Targeting of MSV—To evaluate the influence of α -VEGFR2 on MSV targeting, we performed an adhesion assay in static conditions. MSV targeted with α -VEGFR2 showed a preferential targeting and significant accumulation on endothelial cells expressing VEGFR2 (HIGH) versus control (WT) cells (Fig. 3). Quantitative data was assessed using low magnification images of the entire well (Supplementary Information, Fig. S5) and then the number of MSV that initially settled down within the first 15 minutes were normalized to 1.0 to compensate all conditions. In WT PAEC, gradual increases were observed for both untargeted and α -VEGFR2 MSV exhibiting a 2-fold increase over 60 minutes (Fig. 3a). At all time-points collected on WT cells, no significant difference was detected between untargeted versus targeted MSV. Untargeted MSV demonstrated similar adhesion dynamics on both WT cells and HIGH PAEC clones (Fig. 3c). These values served as the baseline for MSV adhesion to PAEC in order to distinguish unspecific adhesion from targeting. On the contrary, α -VEGFR2 MSV showed a superior targeting effect on HIGH PAEC, exhibiting a significant ($p < 0.001$) 3-, 4-, and 5-fold increase at 30, 45, and 60 minutes respectively (Fig. 3c). High magnification images of static targeting at 15 and 60 minutes supported the enhanced targeting of α -VEGFR2 MSV to HIGH PAEC and demonstrated a substantial increase in the number of MSV associated with the cells over time (Fig. 3b, d).

3.2.1 Dynamic Targeting of MSV—To simulate the shear forces experienced during systemic administration, the targeting of α -VEGFR2 MSV was tested under dynamic flow conditions (Fig. 4). A continuous monolayer of PAEC (HIGH or WT) was seeded into slides pre-coated with fibronectin and stimulated with tumor necrosis factor – alpha to recreate the tumor vasculature for dynamic testing. α -VEGFR2 MSV demonstrated enhanced targeting and firm adherence towards VEGFR2 expressing cells exhibiting a significant increase in MSV adhesion after 20 minutes. At all time-points, untargeted and α -VEGFR2 MSV showed similar accumulation on WT PAEC (Fig. 4a and Supplementary Information, Movie S1 & S2), and provided information on the unspecific basal binding of MSV to endothelia under flow conditions. When α -VEGFR2 MSV were flowed on HIGH PAEC (Fig. 4c and Supplementary Information, Movie S3 & S4) we reported a significant increase in targeting after 20 minutes ($p < 0.01$ at 20 & 22 min; $p < 0.001$ from 24–30 min). In HIGH PAEC, α -VEGFR2 MSV exhibited greater than a 4-fold increase in targeting efficiency from 20 to 30 minutes over untargeted MSV. The corresponding images at 10, 20, and 30 minutes of PAEC under flow confirmed the progressive increase of MSV accumulation over time (Fig. 4b, d).

The *in vitro* static and dynamic studies were instrumental to optimize MSV for efficient targeting. In the experiments for static conditions we removed the impact of flow and demonstrated the ability of MSV to preferentially dock to endothelial cells expressing increased amounts of VEGFR2 (Figure 6c). The experiments in dynamic conditions were instead devoted to investigate the ability of the targeting molecule to increase the firm

adhesion of MSV to the endothelial cells in the presence of a physiological flow, as depicted in Figure 6d. Combined, the *in vitro* static and dynamic results demonstrated that a 4-fold increase was observed upon targeting MSV with α -VEGFR2. Furthermore, no significant difference in docking (static) or firm adhesion (dynamic) was observed for MSV directed at control cells or for untargeted MSV directed towards VEGFR2 expressing cells. Selective targeting is critical for drug delivery platforms to avoid unnecessary accumulation in healthy tissues and to improve the delivery of payloads to the target site (i.e., increased therapeutic index).

Upon docking on the cell surface, MSV were internalized regardless of their surface modification. The cells retained normal cytoskeletal structure displaying parallel actin filaments and microtubules radiating throughout the cell (Supplementary Information, Fig. S6 & Movie S5). The static and dynamic images (Fig. 3 & 4) showed that MSV initially adhere to PAEC at 15 minutes concentrating distantly from the nucleus and eventually become internalized as they migrate to the perinuclear region of the cell, as previously demonstrated in other endothelial cell lines [47]. To further validate the biocompatibility of MSV upon internalization by endothelial cells, PAEC treated with up to 20 MSV/cell continued to proliferate and displayed similar metabolic activity as untreated cells (Supplementary Information, Fig. S7).

3.3 Tumor targeting and biodistribution of α -VEGFR2 MSV

The biodistribution of untargeted and α -VEGFR2 targeted MSV was followed in breast tumor bearing mice using whole body near infrared (NIR) fluorescent imaging, a technique that has shown excellent correlation to other quantitative techniques to assess MSV biodistribution such as inductively coupled plasma – atomic emission spectroscopy [25, 26, 39, 40, 48]. The invasive murine 4T1 cells were chose to establish the breast cancer model. 4T1 cells have shown substantial VEGFR2 expression in the tumor-associated endothelium [49] and demonstrated tumor reduction upon treatment with anti-VEGFR2 monoclonal antibodies [50] thus validating its use to assess the targeting of α -VEGFR2 MSV.

Untargeted and α -VEGFR2 MSV were conjugated with NIR fluorescent dyes after antibody conjugation as shown in Fig. 1e. The signal from targeted and untargeted NIR-labeled MSV (Fig. 5a) was equivalent to APTES modified MSV previously used in other studies [39, 40]. Quantification of the injected dose was measured using the NIR signal within the syringe prior to the injection and subtracting the residual signal after the injection (Fig. 5a). Mice were imaged at 0 (i.e., immediately after injection), 2, and 4 hours. All animals displayed a prominent NIR signal from the abdomen with a predominant left side accumulation near the anatomical location of the liver (Fig. 5b, d). NIR imaging of harvested organs confirmed a considerable accumulation of MSV in the liver, spleen and kidney (Fig. 5c, e and Supplementary Information, Fig. S8). The heart, lungs, and tails (Fig. 5a) of mice treated either with untargeted or targeted MSV showed minimal NIR signal. Higher accumulation in tumors treated with α -VEGFR2 MSV increasing from 2 to 4 hours was observed (Fig. 5e). Fluorescence quantification of the NIR signal of MSV from each organ confirmed the biodistribution of α -VEGFR2 MSV (Fig. 5f). At 2 hours, α -VEGFR2 MSV exhibited a significantly ($p < 0.05$) lower accumulation in the liver along with a highly ($p < 0.001$)

significant increase in tumor accumulation yielding a 3-fold increase in targeting (9.5% versus 30.5%). Other organs (e.g., spleen, kidney, lung, and heart) demonstrated no significant difference at 2 hours. At 4 hours, α -VEGFR2 MSV continued to accumulate in the tumor exhibiting a 4-fold increase in targeting. In addition, α -VEGFR2 MSV exhibited superior tumor-to-liver ratios of 0.982 and 0.997 for 2 and 4 hours, respectively, compared to 0.210 for untargeted equating to a cumulative 5-fold increase in tumor biodistribution.

The data suggest that the fluorescent signal in the tumors could originate from the accumulation of the NIR dye released from the surface of MSV (20% and 60% release at 2 and 4 hours)[40]. These results validate the working mechanism of MSV as illustrated in Figure 6. The shape of MSV was designed to flow and drift towards the vessel wall [21, 23, 25] while the surface allows for the attachment of α -VEGFR2 antibodies (Fig. 6b) and fluorescent molecules (Fig. 6b). α -VEGFR2 MSV are bestowed with enhanced recognition and docking features (Fig. 6c) and displayed firm adhesion on endothelial cells expressing VEGFR2 even in the presence of physiological flow (Fig. 6d). After targeting the tumor vasculature, MSV begin to shed the fluorescent molecules from within the pores of the MSV (Fig. 6e), which locally accumulate in the tumor. As previously demonstrated, upon release, the MSV undergo degradation and are either cleared by liver macrophages or are internalized by endothelial cells (Fig. 6f, Supplementary Information, Fig. S6) [51, 52].

Supporting the results of the *in vitro* static and dynamic analyses, the systemic administration of α -VEGFR2 MSV in breast tumor bearing mice confirmed the ability of MSV to target the tumor vasculature and showed a significant increase in the delivery of reporter molecules released from the pores and surface of MSV. The increased accumulation of a therapeutic payload at the tumor site and the avoidance of healthy tissues is a key feature of effective treatments with high therapeutic index. The liver-to-tumor ratios suggest that α -VEGFR2 successfully redirected MSV to the tumor vasculature while simultaneously reducing liver accumulation. The rapid accumulation of the model payload to tumors bestowed by MSV at 2 and 4 hours was comparatively greater than the previously reported accumulation of bevacizumab in tumors at 24 hours acquired using positron emission tomography imaging [53].

Targeting the tumor vasculature represents a promising strategy for the delivery of cancer chemotherapeutics. Due to their geometry and increased margination towards the vessel wall, MSV are endowed with the ability to drift during systemic circulation with an increased probability of preferentially docking to the tumor vasculature. The functionalization of the surface of the MSV with a α -VEGFR2 antibody increased the specificity towards VEGFR2 expressing cells *in vitro* and promoted the preferential targeting of the tumor-associated vessels. The increased affinity, docking, and sustained adhesion to VEGFR2 expressing cells favored the accumulation of the model payload effectively demonstrating the mechanism of action of the multistage delivery strategy.

4. Conclusion

The preferential delivery of therapeutic agents to a target location while minimizing the distribution in healthy tissues is the Holy Grail of drug delivery. MSV have been exploited

to locally deliver chemotherapeutics, anti-angiogenic agents, biologics, contrast agents, and nanoparticles for therapeutic and diagnostic applications [32, 54–57]. We demonstrate that MSV functionalized with α -VEGFR2 could recognize, dock, and firmly adhere to endothelial cells and tumor vessels overexpressing VEGFR2 resulting in a greater than 5-fold enhancement. Targeted MSV accomplished this task independently from the EPR effect, and rapidly homed to the tumor site exhibiting a significant increase in tumor accumulation. The delivery of a model payload to the tumor mass was demonstrated following the release of fluorescent molecules from the MSV pores, thus recapitulating the working mechanism that governs MSV [19]. Due to their ideal physical and chemical properties, their ability to enhance therapeutic agents [38], and their tunable nature that allows the controlled delivery of payloads [34], MSV can enable the spatial and temporal control of drug release at specific cellular targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. MSV characterization after conjugation with α -VEGFR2

a, Schematic illustrating the steps and chemical reactions performed to conjugate MSV with α -VEGFR2 antibodies. b, Complete FTIR spectra after each conjugation step: oxidation (i), APTES (ii), SMCC (iii), and α -VEGFR2 (iv); and c, inset from b, displaying FTIR spectra of samples between 1300 to 1900 cm^{-1} to emphasize the emerging peaks within this range. d, Zeta potential values of MSV after each conjugation displaying changes in surface chemistry with each line representing a separate run. e, Confocal images of untargeted and α -VEGFR2 MSV showing conjugation of MSV with fluorescent dye (purple) and anti-VEGFR2 antibodies (green). f, Confocal images demonstrating melittin (red) loading into MSV with or without anti-VEGFR2 antibodies (green). (e, f: scale bar, 1 μm).

Figure 2. Characterization of hVEGFR2 transfection in PAEC

a, Fluorescent images demonstrating expression of VEGFR2 (YFP, yellow) on WT (no VEGFR2) and PAEC clones expressing varying amounts of VEGFR2 (LOW, MED, and HIGH) before selection and (b) after selection with G418 to remove unsuccessfully transfected cells. Nuclei of PAEC in blue. (scale bar, 50 μ m) c, Flow cytometry and (d) western blot showing fluorescence and protein expression, respectively, of VEGFR2/YFP in PAEC WT, LOW, MED, and HIGH after selection.

Figure 3. Interaction of MSV with VEGFR2 expressing cells in static conditions
 a, Quantitative analysis of the targeting index of MSV and α -VEGFR2 MSV on WT PAEC (i.e., control), obtained at 15 minutes normalizing the number of MSV per cell in each field of view. b, Fluorescent images at 15 and 60 minutes of WT PAEC imaged with DAPI (blue) and MSV (red). c, Quantitative analysis of targeting in HIGH PAEC (i.e., VEGFR2 expressing cells); d, Fluorescent images at 15 and 60 minutes show α -VEGFR2 MSV targeting to HIGH PAEC. The data are plotted as mean with SEM. *** = $p < 0.001$; (scale bar, 20 μ m).

Figure 4. Dynamic targeting of MSV to VEGFR2 expressing cells under physiologic flow conditions

a, Quantitation on time-lapse microscopy images on WT PAEC comparing the number of untargeted and α -VEGFR2 MSV bound to cells. b, Representative images at 10, 20, and 30 minutes from time-lapse microscopy merging transmitted light and fluorescence (MSV, red). c, Quantitative analysis of dynamic targeting in HIGH PAEC. d, Representative images showing targeting to HIGH PAEC at 10, 20, 30 minutes. The data are plotted as mean curve $\pm$ SEM. ** = $p < 0.01$; *** = $p < 0.001$; (scale bar, 50 μ m).

Figure 5. Targeting and biodistribution of α -VEGFR2 MSV to breast tumors

a, MSV (untargeted and α -VEGFR2) were labeled with NIR fluorescent dyes and imaged in the syringe before and after injection, minimal signal was found in the tails after tail vein injection. b, Whole body longitudinal non-invasive NIR optical imaging of mice treated with untargeted MSV at 0 and 2 hours. c, Harvested organs from mice treated with untargeted MSV at 2 hours. d, Imaging of mice injected with α -VEGFR2 MSV at 0, 2, and 4 hours. e, Organs from mice with α -VEGFR2 MSV at 2 (top) and 4 (bottom) hours. f, Quantification of the biodistribution of MSV comparing untargeted and α -VEGFR2 in the collected organs based on NIR signal. The data are plotted as mean values with SEM. * = $p < 0.05$; *** = $p < 0.001$.

Figure 6. Schematic representation of the multistage delivery strategy with α -VEGFR2 MSV
 a, MSV are conjugated with α -VEGFR2 antibodies (b) followed by conjugation with fluorescent molecules in the pores of MSV to serve as a model reporter payload. c, MSV functionalized with α -VEGFR2 demonstrated preferential docking and (d) exhibited targeting and firm adhesion to VEGFR2-expressing endothelia under physiological flow. e, After adhesion, the payload is released from the MSV and (f) diffuses into the tumor microenvironment. MSV then detach and are either internalized by endothelial/tumor cells or are degraded and cleared by the body.