Published in final edited form as:

Biomater Sci. 2018 May 29; 6(6): 1517–1525. doi:10.1039/c8bm00225h.

Bevacizumab and Near Infrared Probe Conjugated Iron Oxide Nanoparticles for Vascular Endothelial Growth Factor Targeted MR and Optical Imaging

Run Lin^{1,2}, Jing Huang², Liya Wang^{2,3}, Yuancheng Li², Malgorzata Lipowska², Hui Wu², Jianyong Yang¹, and Hui Mao^{2,*}

¹Department of Radiology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510080, China

²Department of Radiology and Imaging Sciences, Emory University School of Medicine, Atlanta, GA 30322, USA

³Department of Radiology, The People's Hospital of Longhua, Shenzhen, Guangdong 518109, China

Abstract

Vascular endothelial growth factor (VEGF) plays a pivotal role in the cascade of development and progression of cancers. Targeting this cancer hallmark is a logical strategy for imaging based cancer detection and monitoring the anti-angiogenesis treatment. Using Bevacizumab (Avastin®), which is a recombinant humanized monoclonal antibody directly against VEGF and angiogenesis inhibitor, as a targeting ligand, a multimodal VEGF targeted molecular imaging probe was developed by conjugating near infrared dye (NIR830) labeled bevacizumab to magnetic iron oxide nanoparticles (IONP) for optical and magnetic resonance (MR) imaging of cancers over-expressing VEGF. The targeting effect of NIR830-bevacizumab-IONPs to VEGF over-expressing cells was investigated by the receptor mediated cell uptake experiments and a blocking assay using VEGF over expressing 4T1 breast cancer cells. Systemic administration of VEGF-targeted NIR830-bevacizumab-IONPs into mice bearing 4T1 breast tumors resulted in higher accumulation of targeting IONPs in tumors compared to non-targeted IONPs. Quantitative analysis of T₂-weighted MRI at 48 h post injection revealed that the averaged percentage of signal intensity change in tumors treated with NIR830-bevacizumab-IONPs was $52.4 \pm 11.0\%$ comparing to $26.9 \pm 12.4\%$ in controls treated with non-targeted IONPs. The results demonstrated the feasibility and efficacy of NIR830-bevacizumab-IONPs as a VEGF targeting dual-modality molecular imaging probe that can be potentially used for imaging of cancers with VEGF over-expression and delivery of bevacizumab for imaging guided anti-cancer treatment.

Graphical Abstract

*Corresponding should be addressed to: Hui Mao, PhD, Department of Radiology and Imaging Sciences, Emory University School of Medicine, Atlanta, Georgia 30322, The United States of America, Telephone: (404) 712-0357, hmao@emory.edu.

Conflicts of interest

There are no conflicts of interest to declare

Keywords

magnetic resonance imaging; optical imaging; molecular imaging; magnetic iron oxide nanoparticles; vascular endothelial growth factor; cancer

Introduction

Vascular endothelial growth factor (VEGF) plays key roles in normal vascular functions and a broad spectrum of pathological conditions, which promotes great interests of using VEGF as a biomarker and target for molecular diagnosis and individualized treatment.^{1, 2} As over expression of VEGF is the hallmark of most solid tumors, the VEGF-targeting strategy has been broadly adopted as a therapy or as a part of therapeutic regimens in the clinical oncology.^{3, 4} Bevacizumab (Avastin®), a humanized monoclonal immunoglobulin G antibody targeting VEGF, was first approved by the US Food and Drug Administration (FDA) for the treatment of colorectal cancer in 2004 and have now largely extended to other cancers, including brain tumor, renal cell carcinoma, lung cancer, and cervical cancer.^{5–8} Further, a number of clinical trials are undergoing to evaluate the efficacy for other therapeutic applications.^{9–13} However, for the optimal efficacy of a targeted treatment, there is a need of non-invasive molecular imaging approaches for accurately and timely determining VEGF over expression level, in order to stratify the patients who can be benefited from the targeted treatment and then targeted delivery of therapeutic agents. VEGF targeted molecular imaging probe and image-guided delivery of anti-VEGF agents thus may enable the precision medicine and improve survival of cancer patients.^{14–16} Several earlier attempts have explored the feasibility of using VEGF as a biomarker for molecular imaging using ultrasound,¹⁷ X-ray computerized tomograph (CT),¹⁸ nuclear imaging,¹⁹ optical imaging²⁰ as well as magnetic resonance imaging (MRI),²¹ but the integration of multiple modalities for VEGF-targeted imaging, which can improve the accuracy of targeted imaging and the range of image-guided intervention, such as pre- and intra-operative imaging, has only received limited investigation.^{22–24}

The rapid advancements in nanotechnology provide versatile platforms for molecular imaging probe development and image-guided drug delivery, offering favorable pharmacokinetics and high sensitivity for receptor targeted imaging and theranostics.^{25–27} In particular, nanomaterials can be used to integrate multiple imaging modalities that not only provide high resolution anatomic and morphological information but also complementary with functional and biological information.^{28–30} On the other hand, imaging tools with

integration of different imaging modalities, such as positron emission tomography (PET) with computed tomography (PET-CT), single photon emission computed tomography with CT (SPECT/CT) and PET with magnetic resonance imaging (PET-MRI), have become clinically available.^{31–33} More recently, combining optical imaging and MRI enables both preclinical and clinical evaluations of targeted imaging probes and image-guided drug delivery and therapies.^{34–39}

Here we report a VEGF targeted MRI and optical dual-modality imaging probe prepared with bevacizumab conjugated magnetic iron oxide nanoparticles (IONP) and a near infrared (NIR) molecule (NIR830) for imaging of cancers with VEGF over-expression. MRI and optical contrast effects of the probe (NIR830-bevacizumab-IONP) was concentration dependent with the transverse relaxation rate R_2 or $1/T_2$ of $99.2 \text{ s}^{-1} \text{ mM}^{-1}$ at the magnetic field strength of 3 Tesla. Cell uptake experiments and blocking assay demonstrated the targeting effect of NIR830-bevacizumab-IONPs to VEGF over expressed 4T1 breast cancer cells, which led to VEGF targeted MRI and NIR imaging of 4T1 tumors in mice using NIR830-bevacizumab-IONPs.

Material and Methods

1. Materials

Bevacizumab (Avastin[®]) was obtained from Roche (Genentech, South San Francisco, CA). Fluorescein isothiocyanate (FITC, 90%), dimethyl sulfoxide (DMSO, 99.9%), ethylenediaminetetraacetic acid (EDTA, 98.5%), bovine serum albumin (BSA, 98%), 0.1% nuclear fast red in 5% aluminum sulfate, and potassium hexacyanoferrate (II) trihydrate (98.5%) were purchased from Sigma-Aldrich (Saint Louis, MO). ProLong[®] Gold antifade mountant with 4',6-diamidino-2-phenylindole (DAPI) was purchased from Life Technologies (Gaithersburg, MD). Roswell Park Memorial Institute 1640 (RPMI 1640) medium and phosphate-buffered saline (PBS) were purchased from Corning (Manassas, VA). Polyacrylamide desalting column (MWCO: 6K), N-hydroxysulfosuccinimide (sulfo-NHS), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), borate buffer, BCA protein assay kit, streptomycin and penicillin (10,000 $\mu\text{g/mL}$ streptomycin and 10,000 U/mL penicillin in 0.85% saline), fetal bovine serum (FBS), acetone (99.5%), and hydrochloric acid solution (HCl, 36.5% - 38.0%) were purchased from Thermo Fisher Scientific (Waltham, MA). Water-soluble iron oxide nanoparticles with 10 nm core size and surface carboxyl terminals, activation buffer (pH 5.5), coupling buffer (pH 8.5) and EasySep Magnet were obtained from Ocean NanoTech LLC (San Diego, CA). PD10 desalting columns and Nanosep centrifugal devices (MWCO: 300 K) were purchased from GE Healthcare Life Sciences (Piscataway, NJ) and Pall Corporation (East Hills, NY), respectively. Tissue-Tek[®] Cryomold[®] standard disposable vinyl specimen molds and optimum cutting temperature compound (OCT) were purchased from Sakura Finetek USA, Inc (Torrance, CA).

2.1 Synthesis of NIR830-NHS

The procedure for synthesizing NIR830-NHS was prepared from the protocol reported previously.^{40, 41} The UV-vis absorption spectra and fluorescence spectra of NIR830-NHS

were measured using a Cary 100 Bio UV-Visible Spectrophotometer (Varian, Walnut Creek, CA) and a ISA FluoroMax-2 spectrofluorometer (Jobin Yvon-Spex, Edison, NJ), respectively.

2.2 Preparation and characterization of ligand-coupled IONPs

Bevacizumab was desalted using a polyacrylamide desalting column. To obtain purified fluorescent dye FITC labeled bevacizumab, FITC was dissolved in DMSO and mixed with bevacizumab at a molar ratio of 15:1 in 0.05 M borate buffer, pH 8.5. The solution was slowly rotated at 4°C for 24 h in dark, followed by purification with a PD10 column which was equilibrated with 0.05 M borate buffer. To label bevacizumab with the NIR dye, NIR830-NHS was dissolved and mixed with bevacizumab at a molar ratio of 15:1 in 0.05 M sodium carbonate aqueous solution with a pH of 8.7. The solution was gently rotated at room temperature for 6 h in dark to obtain the NIR830 labeled bevacizumab. Unconjugated dye was removed by a PD10 column, which was equilibrated with 0.05 M sodium carbonate aqueous solution. Protein concentrations were determined using the BCA protein assay kit. The UV-vis absorption spectra of bevacizumab and NIR830-bevacizumab were measured on a Cary 100 Bio UV-Visible Spectrophotometer. The average number of NIR830 dye labeled onto each bevacizumab (X_1) was estimated via the equation 1:

$$X_1 = C_{NIR830}/C_{bevacizumab} \quad (\text{eq. 1})$$

where C_{NIR830} and $C_{bevacizumab}$ were the concentrations (μM) of NIR830 dye and bevacizumab in the NIR830-bevacizumab sample, respectively (Supplementary Information).

To conjugate bevacizumab with IONPs that have carboxylic acid ($-\text{COOH}$) groups on the surface, EDC (0.25 mg/mL) and sulfo-NHS (0.125 mg/mL) were added to IONPs (1.0 mg/mL) suspended in the activation buffer. After incubating for 5 min at room temperature, free EDC and sulfo-NHS were removed by centrifugation with centrifugal devices (MWCO: 300 K) at 2500 rpm for 10 min. Then 1.0 mg of FITC-bevacizumab or NIR830-bevacizumab were mixed with activated IONPs in 1.0 mL coupling buffer and stirred slowly for 2 h. NIR830 or FITC-labeled bevacizumab conjugated IONPs (FITC-bevacizumab-IONPs or NIR830-bevacizumab-IONPs) were purified by the magnetic separator three times at 4 °C. The average number of NIR830-bevacizumab conjugated onto each IONPs (X_2) was estimated according to the equation 2:

$$X_2 = C_{NIR830 - bevacizumab}/C_{IONPs} \quad (\text{eq. 2})$$

where $C_{NIR830-bevacizumab}$ and C_{IONPs} were the concentrations (μM) of NIR830-bevacizumab and IONPs in the NIR830-bevacizumab-IONPs samples, respectively (Supplementary Information). BSA was used to make the non-targeted IONPs (NIR830-BSA-IONPs) as the control using the similar preparation of NIR830-bevacizumab-IONPs. The core sizes and hydrodynamic sizes of the nanoparticles were measured using

transmission electron microscope (TEM, H-7500, Hitachi, Japan), and dynamic light scattering (DLS, Zetasizer Nano S90, Malvern, UK), respectively. The Fe concentration was determined by spectrophotometry.⁴² The UV–vis absorption spectra of IONPs and NIR830-bevacizumab-IONPs were measured on a Cary 100 Bio UV-Visible Spectrophotometer.

To evaluate the MRI contrast effect, we measured the relaxivity of NIR830-bevacizumab-IONPs on a 3 Tesla MRI scanner (Tim Trio, Siemens, Erlangen, Germany) using a volumetric coil. Aqueous solutions of NIR830-bevacizumab-IONPs with Fe concentrations ranged from 0.03 to 0.50 mM were prepared and evaluated by a series of imaging sequences, including a T₂-weighted fast spin echo (SE) imaging sequence with time of echo (TE) of 65 or 80 ms, time of repetition (TR) of 2400 or 3200 ms, field of view (FOV) of 90 mm × 90 mm, flip angle of 150°, and slice thickness of 1.0 mm. To measure the transverse relaxation rate R_2 or $1/T_2$, a multi-echo SE sequence was used with a TR of 2400 ms and 15 different TEs, starting at 11.6 ms with increments of 11.6 ms, flip angle of 180°. The T₂ values at different concentrations were calculated using a method previously reported.⁴³ The optical contrast effect of NIR830-bevacizumab-IONPs was confirmed with optical imaging scan using a Kodak 4000MM Image Station (Eastman Kodak, Rochester, NY).

2.3 Cell specific uptake of bevacizumab-labeled nanoparticles

Human breast cancer 4T1 cells with high VEGF over expression¹⁹ were grown in RPMI 1640 medium containing 1% penicillin-streptomycin and 10% fetal bovine serum at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. 1.5×10^4 4T1 cells were seeded into each well in an 8-well chamber slide and grown for 24 h before experiments were performed. To test and verify the cell uptake of the nanoparticles, cells were cultured in nanoparticle-containing medium (NIR830-bevacizumab-IONPs or FITC-bevacizumab-IONPs, 0.2 mg/mL Fe) for 3 h at 37 °C followed by Prussian blue staining for iron, or DAPI staining for cell nuclei. The presence of IONPs in the cells can be visualized and analyzed based on FITC signals. For Prussian blue staining, each well of the chamber slide was filled with 0.25 mL of 5% potassium ferrocyanide (II) trihydrate in 10% HCl solution, incubated for 15 min and washed three times with PBS. Afterward, cells were counterstained with nuclear fast red solution for 20 min and dehydrated consecutively with 70% and 100% ethanol. Targeted specific uptake was further validated by the blocking assay, in which 200-fold molar excess of bevacizumab was incubated with 4T1 cells for 30 min to block VEGF prior to adding NIR830-bevacizumab-IONPs or FITC-bevacizumab-IONPs (0.2 mg/mL Fe). After incubating VEGF blocked cells with the probe for 3 h, the cells were washed with PBS, then fixed and stained with the Prussian blue staining agents or DAPI for analysis under microscope (BX41, Olympus, Melville, NY).

2.4 MRI and NIR fluorescence optical imaging of tumor bearing animals,

All animal experiments were conducted in compliance with the state and federal Animal Welfare Acts, the standards and policies of U.S. Public Health Service. Experimental procedures performed on mice, including anesthesia, tumor cell implantation and euthanasia, were following a protocol approved by Institutional Animal Care and Use Committee (IACUC) at Emory University, which is fully and continuously accredited by AAALAC International. Female Balb/c mice (6 to 8 weeks of age, Harlan Laboratories, Indianapolis,

IN) were subcutaneously implanted with 1.0×10^6 4T1 breast cancer cells suspended in 100 μ L of PBS into mammary fat pads bilaterally. Tumors were allowed to grow for approximately 10 days until they reached the size of 7 mm (in either dimension) before imaging experiments.

NIR830-bevacizumab-IONPs or NIR830-BSA-IONPs were injected intravenously (i.v.) into tumor bearing mice ($n = 3/\text{group}$) via the tail vein at a dosage of 20 mg Fe/kg body weight, respectively. MRI and NIR optical imaging were performed before or 48 and 96 h after administration of VEGF targeted NIR830-bevacizumab-IONPs or NIR830-BSA-IONPs as the non-targeted control. MRI scans were performed using a volumetric coil with a T_2 -weighted fast SE imaging sequence with TE of 86 ms, TR of 3600 ms, FOV of $120 \text{ mm} \times 56 \text{ mm}$, flip angle of 150° , and slice thickness of 1.0 mm.

The changes in MRI signal intensity and T_2 value of the tumor were calculated from four tumor containing slices selected from images of each mouse using the region of interest (ROI) and ImageJ software (National Institutes of Health, Bethesda, MD) for data analysis. Average signal intensity of each ROI was measured for comparison. The levels of MRI signal intensity changes were calculated using the following equation (Eq. 3)

$$\Delta S (\%) = \left(\frac{S_{pre} - S_{post}}{S_{pre}} \right) \times 100\% \quad (\text{Eq. 3})$$

where ΔS was the percent of MRI signal intensity change, and S_{pre} and S_{post} were the MRI signal intensity before and after injection, respectively.

Following each MRI scan, NIR images were taken on a Kodak 4000MM Image Station using a 720/830 nm excitation/emission filter set with a exposure time of 180 seconds, f-stop 2.80, 2×2 binning, FOV of 98.5 mm, and a Gamma value of 0.2. Corresponding X-ray images were recorded to provide anatomic locations of the tumors.

2.5 Histological analysis

Mice were sacrificed by cervical dislocation after *in vivo* imaging examinations were completed. Tumors and organs were collected and placed in specimen molds for *ex vivo* NIR imaging scan using the same system and parameters as described in 2.4. Samples were then placed into OCT blocks, frozen in liquid nitrogen and were cut into 8- μ m-thick slices for Prussian blue staining for iron to detect the presence of iron oxide nanoparticles in the tissue using the procedure described in 2.3. Afterward, slides were counterstained with nuclear fast red for 20 min and observed on a light microscope.

2.6 Statistical analysis

The results were obtained by averaging data from multiple measurements and were presented as mean $\pm$ SD (standard deviations). For comparison studies, two-tailed unpaired t-test was typically used to analyze the statistical significance of the results. The level of significance was set at $p < 0.05$.

3. Results and discussions

3.1 Characterizations of IONPs conjugated with NIR830 labeled bevacizumab

NIR830 labeled bevacizumab was successfully conjugated onto IONPs with 10 nm core size (*i.e.*, NIR830-bevacizumab-IONPs). Obtained NIR830-bevacizumab-IONPs are highly dispersed and stable in the aqueous solution as observed in TEM images (Fig. 1A) and DLS data (Fig. 1B and Table 1). NIR830-bevacizumab-IONPs maintained NIR830 specific absorbance peak at 830 nm (Fig. 1C). The conjugations of ligands with IONPs were further verified by the observed increase in the hydrodynamic size from 16.78 ± 0.51 nm to 23.79 ± 3.96 nm and change in the surface charge from -34.37 ± 1.40 mV to -26.63 ± 1.30 mV measured by the zeta potential (Table 1) as negative charged carboxyl groups on the surface of IONPs were neutralized by the coupling with ligands. The average number of NIR830-bevacizumab labeled onto each IONP was estimated at 1.77 (Supplementary Information). The optical and MRI contrast effects of NIR830-bevacizumab-IONPs were concentration dependent and the R_2 relaxation rate of the probe was determined as $99.2 \text{ s}^{-1} \text{ mM}^{-1}$ at the magnetic field strength of 3 Tesla (Fig. 1D–F).

3.2 VEGF targeting and cell specific uptake of bevacizumab functionalized IONPs

The VEGF targeting effect of NIR830-bevacizumab-IONPs and FITC-bevacizumab-IONPs was demonstrated by cellular uptake experiments using 4T1 breast cancer cells with VEGF over expression and confirmed with the blocking assay. After 3 h of incubating 4T1 cells with NIR830-bevacizumab-IONPs or FITC-bevacizumab-IONPs, substantial cell uptake of the targeted probes was observed from the images of Prussian blue staining for iron and fluorescence imaging for FITC labeled probes and cells (Fig. 2A–D). However, when adding bevacizumab to block the VEGF, binding and cellular uptake of NIR830-bevacizumab-IONPs or FITC-bevacizumab-IONPs was significantly reduced (Fig. 2E–H), confirming the specific binding and targeting of the probes to the VEGF over expressed 4T1 cells.

3.3 VEGF targeted MRI and NIR optical imaging of tumor bearing mice

Nanoparticles can penetrate into and retain within the tumor mass through the mechanisms of passive or active targeting.^{44, 45} Passive targeting is mostly driven by the enhanced permeability and retention (EPR) effect,^{46, 47} in which tumor hyper vascularization and leaky vascular walls with enlarged junctions between endothelial cells allow nanoparticles extravasate from the blood vessels into the tumor tissue and retain there due to slow clearance as the result of impaired lymphatic drainage. In addition to the EPR driven delivery, active targeting is achieved through binding between affinity moieties on the nanoparticles specifically to the targeted biomarkers, which are highly expressed in tumor cells.^{48, 49} Active targeting requires imaging probes have antibody-like strong binding affinity and proper kinetics. Bevacizumab is a 150 kDa recombinant, humanized monoclonal antibody that binds all isoforms of VEGF-A, a member of the VEGF family that includes five related glycoproteins, VEGF-A, -B, -C, -D and placental growth factor (PLGF). The cell based binding study showed that bevacizumab has a K_D of 35.1 pM for VEGF-A,⁵⁰ therefore, it is an efficient ligand for VEGF targeted imaging.

To test the active targeting of VEGF over expressed tumors *in vivo*, mice bearing 4T1 breast tumors were imaged before and 48 or 96 hours after i.v. injection of either VEGF targeted NIR830-bevacizumab-IONPs, or NIR830-BSA-IONPs as a non-targeted control. With tumors typically showing hyperintense signals on the T₂ weighted MRI images, the signal loss observed in the tumor regions after injection of NIR830-bevacizumab-IONPs, or NIR830-BSA-IONPs (Fig. 3A–F) indicated that both targeted and non-targeted probes accumulated in the tumors. However, tumors treated with NIR830-bevacizumab-IONPs exhibited stronger T₂ “darkening” contrast at both 48 and 96 hours after injection compared to the controls. When using the ROI analysis to quantify the contrast changes in the tumor, it was found that the averaged MRI signal intensity in the group receiving VEGF targeted NIR830-bevacizumab-IONPs (405.4 ± 103.9) was significantly lower than that of the group receiving non-targeted control NIR830-BSA-IONPs (588.2 ± 125.8) at 48 h post injection ($n = 3$, $p < 0.05$). This T₂ contrast effect and the difference between targeted and control probes remained at 96 h post injection with the averaged signal intensity at 415.0 ± 132.7 for targeted NIR830-bevacizumab-IONPs comparing to 595.5 ± 142.3 from non-targeted NIR830-BSA-IONPs. When we further analyzed the percentages of MRI signal intensity decreases in tumors, the group receiving NIR830-bevacizumab-IONPs showed $52.4 \pm 11.0\%$ signal drops at 48 h post-injection comparing to the baseline at pre-injection (Fig. 3G). However, the percentage of signal change was found to be only $26.9 \pm 12.4\%$ in the group receiving NIR830-BSA-IONPs 48 h post-injection ($n = 3$, $p < 0.05$, Fig. 3G). Similarly, we observed $51.3 \pm 14.6\%$ signal changes in the group receiving NIR830-bevacizumab-IONPs comparing to $26.2 \pm 13.6\%$ in the group receiving NIR830-BSA-IONPs at 96 h post injection ($n = 3$, $p < 0.05$), respectively (Fig. 3G).

It is worth noting that the observed accumulation of non-targeted probe is likely due to intrinsic passive targeting driven by the EPR effect, as macromolecules (10 to 200 nm or 40 to 800 kDa), such as the nanoparticles used in this study (*i.e.*, core size of 10 nm and hydrodynamic of size 18–24 nm), can accumulate in tumor tissues more than in normal tissues.⁵¹ However, VEGF targeted NIR830-bevacizumab-IONPs can accumulate in the tumor first through the EPR effect, followed by receptor-mediated active targeting. While it is difficult to determine the relative contribution of the EPR effect and receptor-mediated targeting in the current system of the targeted probe and the tumor model, it is estimated from the probe induced change of MRI signal intensity that VEGF targeted NIR830-bevacizumab-IONPs had approximately 2-fold higher accumulation in the tumor comparing to non-targeted NIR830-BSA-IONPs 48 hours after injection of the probes. This observation is similar to that from an earlier study of using super-paramagnetic iron oxide (SPIO) nanoparticles functionalized with tripeptide arginine-glycine-aspartic acid (RGD) to targeted imaging of integrin $\alpha_v\beta_3$ in mice carrying subcutaneous tumors from CT26 colon carcinoma cells.⁵² The biodistribution study with electron spin resonance spectroscopy showed that mice injected with RGD-functionalized SPIO nanoparticles exhibited a 2.5-fold higher signal in tumor tissues compared to the mice treated with non-targeted SPIO nanoparticles 4 h after injection. Taking together, our results indicated that although the non-targeted probe could reach the tumor mass through the EPR effect and passive targeting, improved delivery could be achieved via active targeting as demonstrated by the observed stronger contrast effect in T₂ weighted MRI.

To correlate with MRI, NIR optical imaging was performed on each animal immediately after MRI scans at each time point. Similar to the observations from MRI experiments, no NIR signal could be detected in the tumors before administering agents. At 48 and 96 h post-injection, strong NIR signal appeared in the tumors received NIR830-bevacizumab-IONPs whereas those received NIR830-BSA-IONPs exhibited low NIR signal (Fig. 4A–F). Therefore, these results supported the preferential and enhanced accumulation of VEGF targeted NIR830-bevacizumab-IONPs over the non-targeted control as observed in both *in vivo* MRI and NIR imaging of mice bearing VEGF over expressed 4T1 tumors. Taking together, the results suggested that NIR830-bevacizumab-IONPs could be used as MRI-NIR dual modal imaging probe for actively targeting tumors with VEGF over-expression.

To validate the *in vivo* MRI and NIR imaging findings, *ex vivo* optical images and histological analysis were performed in the organs and tumor tissue samples collected from the animals after imaging experiments. The *ex vivo* optical images of tumor tissues and organs (Fig. 5) showed that there is little NIR signal from NIR830 labeled IONPs in brain, heart, lung, spleen and muscle (Fig. 5A–C, E–G, I–K, M–O), indicating minimal uptake of the probes in these organs. Because the relatively small core size at 10 nm for IONPs used in this study, there is a low level of both targeted and non-targeted IONPs in the kidney (Fig. 5F, N), likely resulting from the small percentage of renal clearance of the probes. On the other hand, NIR830-bevacizumab-IONPs appeared to be accumulated mostly in the liver (Fig. 5D, L) compared to other normal tissues and organs. The accumulation of NIR830-bevacizumab-IONPs comparing appeared to be more than 10 times higher than that of kidney. This is consistent with general biodistribution profile of nanoparticles, as most IONPs, even with mere 10 nm core size, are cleared from the blood circulation by the reticuloendothelial system (RES) and typically taken up by liver, which degrades and metabolizes IONPs eventually. Interestingly, NIR830-bevacizumab-IONPs showed almost three times higher uptake than non-targeted IONPs in the liver based on comparison of intensities of the NIR signal measured in each organ, possibly due to the expression of VEGF in normal liver tissues.^{53, 54} Importantly, tumors received targeting NIR830-bevacizumab-IONPs had more than 3 times higher averaged NIR signal (Fig. 5H) compare to those received non-targeted NIR830-BSA-IONPs (Fig. 5P), although this estimation did not taken into account of heterogeneous uptake of the probe by the different regions of the tumor and depth limitation in measuring NIR signals.

This biodistribution profile observed in *ex vivo* optical images of different tissues, including tumors, was further validated by the investigation of Prussian blue staining of iron presented in the specimens of different organs and the tumors (Fig. 6). Similarly, Prussian blue staining showed heavy iron stain in the liver (Fig. 6D) and tumor (Fig. 6H) of the animals received VEGF targeted NIR830-bevacizumab-IONPs. Blue staining was also observed in the liver and tumor in control animals treated with non-targeted NIR830-BSA-IONPs (Fig. 6L and P). There is no iron staining observed in the Prussian blue stained tissue samples from brain, lung, heart, kidney and muscle. However, it should be noted that the results from the tissue staining is sample specific and not quantitative comparing to those from optical imaging of the whole organ or large tissue block.

Conclusion

We have prepared a VEGF targeted MRI and NIR dual-modality imaging probe using well characterized magnetic IONPs. The *in vivo* imaging of mice bearing VEGF over expressed 4T1 tumors demonstrated that the targeted probe is capable of selectively targeting tumors over-expressing VEGF. Given the potent therapeutic effect of bevacizumab itself and the drug loading capacity of the IONP, the developed VEGF-specific imaging probe can be applied for imaging diagnosis and monitoring of diseases with VEGF over-expression as well as providing imaging guidance for the VEGF targeted treatment. Further investigation is warranted to explore theranostic applications of the NIR830-bevacizumab-IONPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work is supported in parts by Oversea Study Program of Guangzhou Elite Project (GEP, 11YB18) to RL and by the grants from NIH (R01CA154846-02 and Cancer Nanotechnology Platform Project U01CA151810-02) to HM.

References

1. Ferrara N, Endocr. Rev, 2004, 25, 581–611. [PubMed: 15294883]
2. Goel HL and Mercurio AM, Nat. Rev. Cancer, 2013, 13, 871–882. [PubMed: 24263190]
3. Ellis LM and Hicklin DJ, Nat. Rev. Cancer, 2008, 8, 579–591. [PubMed: 18596824]
4. Ferrara N and Adamis AP, Nat. Rev. Drug. Discov, 2016, 15, 385–403. [PubMed: 26775688]
5. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R and Kabbinavar F, N. Engl. J. Med, 2004, 350, 2335–2342. [PubMed: 15175435]
6. Escudier B, Pluzanska A, Koralewski P, Ravaud A, Bracarda S, Szczylik C, Chevreau C, Filipek M, Melichar B, Bajetta E, Gorbunova V, Bay JO, Bodrogi I, Jagiello-Gruszfeld A and Moore N, Lancet, 2007, 370, 2103–2111. [PubMed: 18156031]
7. Tewari KS, Sill MW, Long HJ, 3rd, Penson RT, Huang H, Ramondetta LM, Landrum LM, Oaknin A, Reid TJ, Leitao MM, Michael HE and Monk BJ, N. Engl. J. Med, 2014, 370, 734–743. [PubMed: 24552320]
8. Seto T, Kato T, Nishio M, Goto K, Atagi S, Hosomi Y, Yamamoto N, Hida T, Maemondo M, Nakagawa K, Nagase S, Okamoto I, Yamanaka T, Tajima K, Harada R, Fukuoka M and Yamamoto N, Lancet. Oncol, 2014, 15, 1236–1244. [PubMed: 25175099]
9. Scott AM, Wolchok JD and Old LJ, Nat. Rev. Cancer, 2012, 12, 278–287. [PubMed: 22437872]
10. Nagai T, Sato M, Kobayashi M, Yokoyama M, Tani Y and Mochida J, Arthritis. Res. Ther, 2014, 16, 427. [PubMed: 25230745]
11. Loupakis F, Cremolini C, Masi G, Lonardi S, Zagonel V, Salvatore L, Cortesi E, Tomasello G, Ronzoni M, Spadi R, Zaniboni A, Tonini G, Buonadonna A, Amoroso D, Chiara S, Carlomagno C, Boni C, Allegrini G, Boni L and Falcone A, N. Engl. J. Med, 2014, 371, 1609–1618. [PubMed: 25337750]
12. Pujade-Lauraine E, Hilpert F, Weber B, Reuss A, Poveda A, Kristensen G, Sorio R, Vergote I, Witteveen P, Bamias A, Pereira D, Wimberger P, Oaknin A, Mirza MR, Follana P, Bollag D and Ray-Coquard I, J. Clin. Oncol, 2014, 32, 1302–1308. [PubMed: 24637997]
13. Zalcman G, Mazieres J, Margery J, Greillier L, Audigier-Valette C, Moro-Sibilot D, Molinier O, Corre R, Monnet I, Gounant V, Riviere F, Janicot H, Gervais R, Locher C, Milleron B, Tran Q,

- Lebitasy MP, Morin F, Creveuil C, Parienti JJ and Scherpereel A, *Lancet*, 2016, 387, 1405–1414. [PubMed: 26719230]
14. Etzioni R, Urban N, Ramsey S, McIntosh M, Schwartz S, Reid B, Radich J, Anderson G and Hartwell L, *Nat. Rev. Cancer*, 2003, 3, 243–252. [PubMed: 12671663]
 15. Weissleder R, *Science*, 2006, 312, 1168–1171. [PubMed: 16728630]
 16. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A, *CA. Cancer. J. Clin.*, 2015, 65, 87–108. [PubMed: 25651787]
 17. Wang J, Qin B, Chen X, Wagner WR and Villanueva FS, *Mol. Pharm.*, 2017, 14, 781–790. [PubMed: 28165246]
 18. Backer MV and Backer JM, *Theranostics*, 2012, 2, 502–515. [PubMed: 22737188]
 19. Heskamp S, Boerman OC, Molkenboer-Kuennen JD, Sweep FC, Geurts-Moespot A, Engelhardt MS, van der Graaf WT, Oyen WJ and van Laarhoven HW, *Mol. Pharm.*, 2014, 11, 4249–4257. [PubMed: 25294389]
 20. Sun X, Ma T, Liu H, Yu X, Wu Y, Shi J, Jia B, Zhao H, Wang F and Liu Z, *Eur. J. Nucl. Med. Mol. Imaging*, 2014, 41, 1428–1439. [PubMed: 24562646]
 21. Zhao H, Li Z, Yang B, Wang J and Li Y, *J. Biomat. Sci. Polym. E*, 2015, 26, 1178–1189.
 22. Backer MV, Levashova Z, Patel V, Jehning BT, Claffey K, Blankenberg FG and Backer JM, *Nat. Med.*, 2007, 13, 504–509. [PubMed: 17351626]
 23. Nagengast WB, de Vries EG, Hospers GA, Mulder NH, de Jong JR, Hollema H, Brouwers AH, van Dongen GA, Perk LR and Lub-de Hooje MN, *J. Nucl. Med.*, 2007, 48, 1313–1319. [PubMed: 17631557]
 24. Hsieh WJ, Liang CJ, Chieh JJ, Wang SH, Lai IR, Chen JH, Chang FH, Tseng WK, Yang SY, Wu CC and Chen YL, *Int. J. Nanomed*, 2012, 7, 2833–2842.
 25. Thakor AS and Gambhir SS, *CA. Cancer. J. Clin.*, 2013, 63, 395–418. [PubMed: 24114523]
 26. Min Y, Caster JM, Eblan MJ and Wang AZ, *Chem. Rev.*, 2015, 115, 11147–11190. [PubMed: 26088284]
 27. Shi J, Kantoff PW, Wooster R and Farokhzad OC, *Nat. Rev. Cancer*, 2017, 17, 20–37. [PubMed: 27834398]
 28. Jennings LE and Long NJ, *Chem. Commun*, 2009, DOI: 10.1039/b821903f, 3511–3524.
 29. Lee SY, Jeon SI, Jung S, Chung IJ and Ahn CH, *Adv. Drug. Deliv. Rev.*, 2014, 76, 60–78. [PubMed: 25064554]
 30. Hussain T and Nguyen QT, *Adv. Drug. Deliv. Rev.*, 2014, 66, 90–100. [PubMed: 24064465]
 31. Cherry SR, *Semin. Nucl. Med.*, 2009, 39, 348–353. [PubMed: 19646559]
 32. Torigian DA, Zaidi H, Kwee TC, Saboury B, Udupa JK, Cho ZH and Alavi A, *Radiology*, 2013, 267, 26–44. [PubMed: 23525716]
 33. Yankeelov TE, Abramson RG and Quarles CC, *Nat. Rev. Clin. Oncol.*, 2014, 11, 670–680. [PubMed: 25113842]
 34. Zhu H, Tao J, Wang W, Zhou Y, Li P, Li Z, Yan K, Wu S, Yeung KW, Xu Z, Xu H and Chu PK, *Biomaterials*, 2013, 34, 2296–2306. [PubMed: 23274069]
 35. Debroye E and Parac-Vogt TN, *Chem. Soc. Rev.*, 2014, 43, 8178–8192. [PubMed: 25211043]
 36. Yan K, Li H, Li P, Zhu H, Shen J, Yi C, Wu S, Yeung KW, Xu Z, Xu H and Chu PK, *Biomaterials*, 2014, 35, 344–355. [PubMed: 24103655]
 37. Guo Q, Kuang L, Cao H, Li W and Wei J, *Colloids Surf., B*, 2015, 136, 687–693.
 38. Wang H and Zhou S, *Biomater. Sci.*, 2016, 4, 1062–1073. [PubMed: 27184106]
 39. Zhang L, Liang S, Liu R, Yuan T, Zhang S, Xu Z and Xu H, *Colloids Surf., B*, 2016, 144, 344–354.
 40. Strekowski L, Mason CJ, Lee H, Gupta R, Sowell J and Patonay G, *J. Heterocyclic. Chem.*, 2003, 40, 913–916.
 41. Zhou Z, Chen H, Lipowska M, Wang L, Yu Q, Yang X, Tiwari D, Yang L and Mao H, *J. Biomater. Appl.*, 2013, 28, 100–111. [PubMed: 23812946]
 42. Huang J, Wang L, Lin R, Wang AY, Yang L, Kuang M, Qian W and Mao H, *ACS. Appl. Mater. Interfaces*, 2013, 5, 4632–4639. [PubMed: 23633522]

43. Duan H, Kuang M, Wang X, Wang YA, Mao H and Nie S, J. Phys. Chem. C, 2008, 112, 8127–8131.
44. Danhier F, Feron O and Preat V, J. Control. Release, 2010, 148, 135–146. [PubMed: 20797419]
45. Doane TL and Burda C, Chem. Soc. Rev, 2012, 41, 2885–2911. [PubMed: 22286540]
46. Fang J, Nakamura H and Maeda H, Adv. Drug. Deliv. Rev, 2011, 63, 136–151. [PubMed: 20441782]
47. Maeda H, Nakamura H and Fang J, Adv. Drug. Deliv. Rev, 2013, 65, 71–79. [PubMed: 23088862]
48. Bertrand N, Wu J, Xu X, Kamaly N and Farokhzad OC, Adv. Drug. Deliv. Rev, 2014, 66, 2–25. [PubMed: 24270007]
49. Mahon E, Salvati A, Baldelli Bombelli F, Lynch I and Dawson KA, J. Control. Release, 2012, 161, 164–174. [PubMed: 22516097]
50. Papadopoulos N, Martin J, Ruan Q, Rafique A, Rosconi MP, Shi E, Pyles EA, Yancopoulos GD, Stahl N and Wiegand SJ, Angiogenesis, 2012, 15, 171–185. [PubMed: 22302382]
51. Maeda H, J Control Release, 2012, 164, 138–144. [PubMed: 22595146]
52. Schleich N, Po C, Jacobs D, Ucakar B, Gallez B, Danhier F and Preat V, J Control Release, 2014, 194, 82–91. [PubMed: 25178270]
53. DeLeve LD, Hepatology, 2015, 61, 1740–1746. [PubMed: 25131509]
54. Poisson J, Lemoine S, Boulanger C, Durand F, Moreau R, Valla D and Rautou PE, J. Hepatol, 2017, 66, 212–227. [PubMed: 27423426]

Highlights:

A VEGF targeted optical and MRI dual-modality imaging probe was prepared for imaging of cancers with VEGF over-expression *in vitro* and *in vivo*.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1. (A) A TEM image of NIR830-bevacizumab-IONPs; (B) hydrodynamic sizes (in diameter) of NIR830-bevacizumab-IONPs determined by DLS; (C) UV-vis absorption spectra of NIR830-bevacizumab-IONPs and IONPs without NIR830 labeled ligands; (D) T_2 weighted MRI images showing the contrast effect of NIR830-bevacizumab-IONPs at different Fe concentrations (mM); (E) R_2 relaxation rates of NIR830-bevacizumab-IONPs at different Fe concentrations (mM); (F) NIR signal intensities of NIR830-bevacizumab-IONPs at different Fe concentrations (mM). All nanoparticles have the same core size of 10 nm in diameter.

Fig. 2.

Prussian blue, DAPI and FITC staining images of 4T1 cells treated with NIR830-bevacizumab-IONPs (A) or FITC-bevacizumab-IONPs (B, C); 4T1 cells with VEGF blocked by bevacizumab before being treated with NIR830-bevacizumab-IONPs (E) or FITC-bevacizumab-IONPs (F, G); and 4T1 cells only (I, J, K). DAPI and FITC merged fluorescent images of 4T1 cells in different treatments are shown in D, H and L. Scale bars indicate 20 μm .

Fig. 3. T₂ weighted MR images of a mouse bearing a 4T1 breast tumor (A) before, (B) 48 h post, and (C) 96 h post injection of VEGF targeted NIR830-bevacizumab-IONPs; and a mouse (D) before, (E) 48 h post and (F) 96 h post injection of non-targeted NIR830-BSA-IONPs. The red dashed lines delineate the area of the tumors in the respective slices. (G) Comparison of IONP induced MR signal decrease measured in the tumors (%) at the different time points after injection of the different probes, * $p < 0.05$.

Fig. 4. NIR images of a mouse bearing two 4T1 breast tumors in the flank (A) before, (B) 48 h post and (C) 96 h post injection with NIR830-bevacizumab-IONPs; and a tumor bearing mouse (D) before, (E) 48 h post, and (F) 96 h post injection with NIR830-BSA-IONPs. The blue dashed lines indicate the location and area of the tumors.

Fig. 5. Organ samples and corresponding optical images of (A) brain, (B) heart, (C) lung, (D) liver, (E) spleen, (F) kidney, (G) muscle, and (H) tumors treated with NIR830-bevacizumab-IONPs, and (I) brain, (J) heart, (K) lung, (L) liver, (M) spleen, (N) kidney, (O) muscle, and (P) tumors treated with NIR830-BSA-IONPs.

Fig. 6. Prussian blue staining images of (A) brain, (B) heart, (C) lung, (D) liver, (E) spleen, (F) kidney, (G) muscle, and (H) tumors treated with NIR830-bevacizumab-IONPs, and (I) brain, (J) heart, (K) lung, (L) liver, (M) spleen, (N) kidney, (O) muscle, and (P) tumors treated with NIR830-BSA-IONPs. Scale bars indicate 20 μm.

Table 1

Hydrodynamic diameters and zeta potentials of nanoparticles coupled with different ligands^a.

	IONPs	NIR830-bevacizumab-IONPs	NIR830-BSA-IONPs
D _H (nm)	16.78 ± 0.51	23.79 ± 3.96	17.67 ± 0.46
ζ potential (mV)	-34.37 ± 1.40	-26.63 ± 1.30	-25.17 ± 2.50

^a All nanoparticles have the same core size of 10 nm in diameter.