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TITLE: VEGF-Iron Oxide Conjugate for Dual MR and PET Imaging of Breast Cancer Angiogenesis

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**Abstract:**

The overall hypothesis of this concept award proposal is that a single probe that allows simultaneous acquisition of both positron emission tomography (PET) and molecular magnetic resonance imaging (mMRI) data will maximize the topographical, anatomical, functional, and molecular information of breast cancer. In this proposal, we choose to image breast cancer tumor vasculature by visualization and quantification of Flt-1 and Flk-1/KDR. Objectives: Aim 1: To develop iron oxide (IO) nanoparticles functionalized with both VEGF121 and PET isotope 64Cu (t1/2 = 12.7 h) and test the dual probe in vitro. Aim 2: To test the PET and mMRI efficacy of the dual-functional nanoprobe in breast cancer in vivo. Major Findings: We have developed VEGF121 mutant that is VEGFR-2 specific. We have developed water soluble iron oxide nanoparticles conjugated with macrocyclic chelating agent DOTA for 64Cu-labeling and cyclic RGD peptide for integrin alpha(v)beta(3) recognition. We have also developed IO-VEGF conjugate and tested the nanoconstruct.
Table of Contents

Introduction .................................................................................................................. 4

Body ............................................................................................................................. 5

Key Research Accomplishments .............................................................................. 14

Reportable Outcomes .............................................................................................. 15

Conclusions ............................................................................................................... 16

References .................................................................................................................. 17

Appendices ................................................................................................................ 18
INTRODUCTION

Breast cancer is the most common form of cancer in women. Despite vigorous efforts to diagnose, treat and prevent this disease, it remains a leading cause of death among women. Vascular endothelial growth factor (VEGF) and its receptors are essential for breast cancer angiogenesis and metastasis. The angiogenic actions of VEGF are mediated via two closely related endothelium-specific receptor tyrosine kinases, Flk-1/KDR and Flt-1. Both are largely restricted to vascular endothelial cells. It has been reported that both Flk-1/KDR and Flt-1 receptors are overexpressed on the endothelium of breast cancer vasculature, whereas they are almost undetectable in the vascular endothelium of adjacent normal tissues. The ability to non-invasively visualize and quantify tumor VEGF receptor expression level will provide new opportunities to document breast cancer angiogenesis status, more appropriately select patients considered for anti-angiogenesis treatment, and monitor anti-angiogenic treatment efficacy. We have recently demonstrated that recombinant human VEGF_{121} can be labeled with positron emitting radionuclide ^{64}\text{Cu} without loss of receptor affinity and functional activity of the protein. ^{64}\text{Cu}-\text{VEGF} is also able to delineate small tumors that are highly angiogenic. Although highly sensitive, positron emission tomography (PET) has relatively poor spatial resolution. Magnetic resonance imaging (MRI), on the other hand, provides exquisite high-resolution anatomical information, as well as access to volume specific chemical and physical information. Functional MRI is thus an excellent tool for assessing tumor angiogenesis and blood flow. Furthermore, signal amplification techniques have enabled both T_2 and T_1 mediated molecular MRI (mMRI), thus extending MRI further beyond the anatomical and physiological level.

We hypothesize that a single probe that allows simultaneous acquisition of both PET and mMRI data will maximizes the topographical, anatomical, functional, and molecular information of breast cancer. In this proposal, we choose to image breast cancer tumor vasculature by visualization and quantification of Flt-1 and Flk-1/KDR.

Objectives: Aim 1: To develop iron oxide (IO) nanoparticles functionalized with both VEGF_{121} and PET isotope ^{64}\text{Cu} (t_{1/2} = 12.7 h) and test the dual probe in vitro. Aim 2: To test the PET and mMRI efficacy of the dual-functional nanoprobe in breast cancer in vivo.

This final report will summarize the major achievements based on the originally proposed studies and further discoveries that derived from this DOD BCRP Concept funding mechanism.
Part I: Development of VEGFR-2 Specific Mutant

We recently labeled wild-type VEGF$_{121}$, the shortest VEGF-A isoform that exists in nature, with $^{64}$Cu ($t_{1/2} = 12.7$ h) for PET imaging of VEGFR expression (1). MicroPET imaging revealed rapid, specific, and prominent uptake of $^{64}$Cu-DOTA-VEGF$_{121}$ (~15 %ID/g) in highly vascularized small U87MG tumors with high VEGFR-2 expression, but significantly lower and sporadic uptake (~3%ID/g) in large U87MG tumors with low VEGFR-2 expression. For this tracer, the highest uptake was in the kidneys. Because the wild-type VEGF$_{121}$ binds to both VEGFR-1 and VEGFR-2 (2). The kidney is usually the dose-limiting organ because it has high VEGFR-1 expression, which can take up VEGF-A based tracers (1, 3, 4). In our previous study, we found that the uptake of $^{64}$Cu-DOTA-VEGF$_{121}$ in the kidneys was mainly due to VEGFR-1 binding (and some renal clearance), while the tumor uptake was mainly related to VEGFR-2 expression (1). The high renal uptake may limit its future clinical applications.

Based on alanine scanning mutagenesis, we identified a D63AE64AE67A mutant of VEGF$_{121}$ (VEGF$_{DEE}$) (Fig. 1), which binds specifically to VEGFR-2. Cell binding assay reveals that the IC$_{50}$ values of VEGF$_{121}$ and VEGF$_{DEE}$ for VEGFR-1 were 4.2 nM and 78.1 nM, respectively, the IC$_{50}$ values of VEGF$_{121}$ and VEGF$_{DEE}$ for VEGFR-2 were 2.9 nM and 11.7 nM, respectively. Taken together, VEGF$_{DEE}$ has about 20 fold lower VEGFR-1 binding and only 4-fold lower VEGFR-2 binding affinity when compared to VEGF$_{121}$. VEGF$_{121}$ and VEGF$_{DEE}$ were then conjugated with macrocyclic chelating agent 1,4,7,10-tetraazacyclododecane N,N′,N″,N‴-tetraacetic acid (DOTA) and labeled with positron emitting radionuclide $^{64}$Cu ($t_{1/2} = 12.7$ h) for positron emission tomography (PET) imaging in an orthotopic 4T1 murine breast cancer model. Quantitative microPET imaging studies indicated that both $^{64}$Cu-DOTA-VEGF$_{121}$ and $^{64}$Cu-DOTA-VEGF$_{DEE}$ had rapid and prominent activity accumulation in VEGFR-2 expressing 4T1 tumors. The renal uptake of $^{64}$Cu-DOTA-VEGF$_{DEE}$ was significantly lower than
that of $^{64}$Cu-DOTA-VEGF$_{121}$ as rodent kidneys expressed high levels of VEGFR-1 based on immunofluorescence staining. Blocking experiments and biodistribution studies confirmed the VEGFR specificity of $^{64}$Cu-DOTA-VEGF$_{DEE}$ (Fig. 2).

**Fig. 2** MicroPET imaging studies of 4T1 tumour-bearing mice. **a** Serial microPET scans of 4T1 tumour-bearing mice injected intravenously with 5-8 MBq of $^{64}$Cu-DOTA-VEGF$_{121}$ or $^{64}$Cu-DOTA-VEGF$_{DEE}$. Mice co-injected with 200 µg of VEGF$_{121}$ are also shown (denoted as “+ Block”). Coronal slices containing the tumours (arrowheads) are shown. **b** Coronal, sagittal and axial slices containing the kidney (arrowheads) at 4 h post-injection of $^{64}$Cu-DOTA-VEGF$_{121}$ or $^{64}$Cu-DOTA-VEGF$_{DEE}$. Note the different scale in **a** and **b**.
Part II: Development of Iron Oxide Nanoparticles

We have developed two types of novel superparamagnetic iron oxide nanoparticles (USPIO), namely, PVP-IO and PASP-IO.

Polyvinylpyrrolidone (PVP)-coated iron oxide (PVP-IO) nanoparticles were synthesized by a one-step thermal decomposition method (Fig. 3). The overall size of the particles is dependent on the PVP/Fe(CO)₅ reaction ratio and the core size of the nanoparticles is dependent on the reaction time. In order to prepare stable PVP-IO nanoparticles with large core size and thin coating for macrophage uptake, we found the optimal molar ratio of PVP/Fe(CO)₅ to be 0.16 and the reaction time to be 5 h. Moreover large amount of uniform PVP-IO nanoparticles were obtained after acetone purification and dialysis. Transmission electron microscope (TEM) image shows monodisperse PVP-IO with core size of 8-10 nm. Dynamic light scattering (DLS) measurement indicates that the colloidal particles in aqueous solution have hydrodynamic diameter of about 40-50 nm. X-ray diffractometer (XRD) pattern is well indexed to the crystal plane of spinel ferrite. The magnetization curve finds the saturation magnetization of PVP-coated iron oxide nanoparticles to be around 110 emu/g Fe, which is much higher than that of Feridex (ca. 70 emu/g Fe).

![Fig. 3. Schematic structure of PVP-IO.](image)

Transverse T₂ weighted spin echo images were acquired using a 3 Tesla Siemens Tim Trio MR Scanner. A gradient echo acquisition was used with repetition time of 2000 ms, echo time of 1.8 ms, slice thickness of 12 mm, and a flip angle of 20 degrees. The normal first order shim process was applied, and the phantoms were imaged at room temperature. The measured $r_2$ and $r_2^*$ were 174.8 mM⁻¹s⁻¹ and 294.3 mM⁻¹s⁻¹. It is remarkable that PVP-IO show the highest $r_2$ among those reported in literature (e.g., $r_2 = 151.9$ mM⁻¹s⁻¹, $r_2^* = 275.0$ mM⁻¹s⁻¹ for Feridex). The higher relaxivity of PVP coated

![Fig. 4. Mouse macrophage cells were incubated with Feridex or PVP-IO for 48 h at different iron concentrations and then stained by Prussian blue and then counterstained with nuclear fast red. Higher macrophage uptake of large core PVP-IO than Feridex is observed at all concentrations examined.](image)
magnetic nanoparticle is likely attributed to the high magnetic moment of PVP-IO (110 emu/g Fe) and effective magnetic relaxations to the proton spins around PVP-IO.

It is highly desirable that PVP-IO nanoparticles possess high and persistent uptake by macrophages for detection of inflammatory disease by MRI. To investigate this property, in vitro cell uptake experiments were carried out using a mouse macrophage cell line RAW 264.7 (American Type Culture Collection, Manassas, VA). The uptake of PVP-IO by macrophages was compared to that of Feridex that is currently used clinically for MRI. To detect the presence of iron oxide nanoparticles in cells, Prussian blue staining was carried out after 48 h and 72 h of incubation of Feridex and large core PVP-IO nanoparticles with macrophages at 20, 50, and 100 µg/mL. As shown in Fig. 4, macrophage uptake of large core PVP-IO is higher than Feridex at all the concentrations examined even though the overall size of PVP-IO is smaller than Feridex.

To confirm the ability of macrophage to take up the newly synthesized large core PVP-IO, the cells were incubated with different concentrations of PVP-IO or Feridex and T2*-weighted MR images were obtained. As shown in Fig. 5, PVP-IO exhibit significantly more negative contrast enhancement as compared to Feridex at relatively low concentrations of Fe. Such difference is diminished when large excess of Fe is used (e.g. 100 mg Fe/mL).

After successful demonstration of the
uptake of large core PVP-IO by macrophages in culture, we tested the same particles in vivo for liver imaging. $T_2^*$-weighted MR images were obtained before and after the administration of large core PVP-IO or Feridex (6 mg Fe/kg) through the marginal vein of rabbits at 3, 7 and 20 min, respectively. Both Feridex and large core PVP-IO are able to lower signal intensity in the rabbit liver parenchyma but the $T_2^*$ effect of PVP-IO is more obvious than Feridex (Fig. 6).

Poly(aspartic acid) (PASP)-coated IO nanoparticles (PASP-IO) were synthesized using a co-precipitation method and functionalized as shown in Fig. 7. TEM revealed that the average size of iron oxide nanoparticles was ~5 nm. A selected area diffraction pattern showed that the iron oxide nanoparticles were Magnetite ($\text{Fe}_3\text{O}_4$). In solution, the colloidal particles had a diameter of ~40 nm as determined from DLS data. This data showed that the colloidal particles consisted of iron oxide crystals covered with PASP layer. The hysteresis loop of PASP-coated iron oxide had no coercive force showing superparamagnetic behavior and the saturation magnetization of PASP-coated iron oxide nanoparticles was 116.9 emu/g Fe.

![Fig. 7](image-url)

**Fig. 7.** (A) The synthesis of the PET/MRI dual functional probe DOTA-IO-RGD. DOTA-IO was prepared similarly except that no RGD peptide was used. (B) Illustration of PET/MRI probe based on iron oxide nanoparticle.

The signal contrast enhancement effect of $T_2^*$- and $T_1$-weighted MR images of PASP-IO and Feridex in a same concentration gradient in distilled water ranging from $4 \times 10^{-4}$ to $1.25 \times 10^{-5}$ M iron concentration is shown in Fig. 8. $T_2$ signal intensity of PASP-IO was reduced faster than Feridex at the lower iron concentration and $T_1$ signal intensity was increased faster than Feridex at the lower concentration, showing that PASP-IO could be used as both a $T_2$ and $T_1$ contrast agent. Transverse $T_2$ weighted spin echo images were acquired using a 3 Tesla Siemens Tim Trio MR Scanner. The measured $r_2$ and $r_2^*$ were 105.5 mM$^{-1}$s$^{-1}$ and 165.5 mM$^{-1}$s$^{-1}$, respectively, which were somewhat lower than Feridex ($r_2 = 151.9$ mM$^{-1}$s$^{-1}$, $r_2^* = 275.0$ mM$^{-1}$s$^{-1}$).
Part III: Development of IO conjugates for tumor angiogenesis imaging

Polyaspartic acid (PASP) has two kinds of functional groups: carboxylates (-COOH) and amines (-NH₂). Therefore, iron oxide nanoparticles were coated with PASAP through the carboxyl group and the remaining amine group were used for DOTA or hetero-linker conjugation with NHS-PEG-MAL. The maleimide residues on the IO surface were then coupled with RGD-SH through Michael addition reaction. The DOTA-IO-RGD conjugate showed integrin specificity according to cell based receptor binding assay (Fig. 9).

Fig. 10 shows coronal microPET images of a female mouse at different time points after injection of 3.7 mBq radiotracer. All microPET images were decay corrected and the tumor was clearly visualized with high contrast relative to the contralateral background from 1 to 21 h after injection of ⁶⁴Cu-DOTA-IO-RGD in U87MG tumor mice. Compared with the RGD conjugated nanoparticle, the non-targeted particle ⁶⁴Cu-DOTA-IO showed significantly lower tumor uptake which might be caused by passive targeting. Tumor accumulation 1 h after injection of ⁶⁴Cu-DOTA-IO-RGD was 7.9 ± 0.8 percentage injected dose per gram (%ID/g) and steadily increased and peaked at
about 4 h after injection (10.1 ± 2.1 %ID/g). At 21 h after injection, tumor uptake slightly decreased to 9.8 ± 3.2 %ID/g. A blocking experiment with 200 µg of RGD injected before ⁶⁴Cu-DOTA-IO-RGD revealed a significant reduction in ⁶⁴Cu-DOTA-IO-RGD uptake.

![Image](image_url)

**Fig. 10.** (A) Decay-corrected whole-body coronal microPET images of nude mouse bearing human U87MG tumor at 1, 4, and 21 h after injection of 3.7 MBq of ⁶⁴Cu-DOTA-IO, ⁶⁴Cu-DOTA-IO-RGD or ⁶⁴Cu-DOTA-IO-RGD with blocking dose of cold c(RGDyK) peptide. (B) Time-activity curves of U87MG tumor after injection of 3.7 MBq of ⁶⁴Cu-DOTA-IO, ⁶⁴Cu-DOTA-IO-RGD or ⁶⁴Cu-DOTA-IO-RGD with blocking dose of cold c(RGDyK) peptide.

To investigate the integrin αᵣβ₃ targeting ability and MRI visibility of DOTA-IO-RGD conjugates, *in vivo* T2-weighted fast spin-echo MR imaging was performed with mice bearing U87MG tumors (**Fig. 11**). MR signal intensity decreased significantly after injection of DOTA-IO-RGD as compared with IO injection and DOTA-IO-RGD co-administration with integrin αᵣβ₃ blocking agent. Signal intensity decreased after injection with IO and had a different distribution pattern compared with DOTA-IO-RGD with particle accumulation predominantly at the lateral tumor borders. MR signal intensity also decreased slightly after injection of free ligand to block integrin αᵣβ₃ receptors followed by administration of DOTA-IO-RGD.

Uptake of PASP-IO, DOTA-IO-RGD, and DOTA-IO-RGD plus free RGD was assessed histologically using Prussian blue staining (**Fig. 12**). After 20 min, blue spots on the surface of U87MG cells were observed, indicating a strong uptake of DOTA-IO-RGD, whereas there was no significant uptake for plain particles. Blocking integrin αᵣβ₃ receptors with free RGD effectively reduced the number of blue spots on the surface of U87MG cells, showing that the accumulation of PASP-IO-RGD was specifically mediated by its integrin αᵣβ₃ binding. Prussian Blue staining was also performed with different tissue slides such as tumor, liver, spleen, muscle, and kidney after injection of...
DOTA-IO-RGD or DOTA-IO. In the liver and spleen, both DOTA-IO-RGD and DOTA-IO had significant uptake with no visible difference between the two particles.

![Images of mice with T2-weighted MR images](image)

**Fig. 11.** T₂-weighted MR images of nude mice bearing U87MG tumor before injection of iron oxide nanoparticle (A, E) and 4 h after tail vein injection of DOTA-IO (B, F), DOTA-IO-RGD (C, G), and DOTA-IO-RGD with blocking dose of cold c(RGDyK) peptide (D, H).

![Images of Prussian blue stained tissues](image)

**Fig. 12.** Prussian blue stained U87MG tumor, liver and spleen after injection of ⁶⁴Cu-DOTA-IO-RGD, ⁶⁴Cu-DOTA-IO, and ⁶⁴Cu-DOTA-IO-RGD with blocking dose of cold c(RGDyK) peptide.

We have also developed DOTA-IO-VEGF₁₂₁ conjugate. To evaluate the binding affinity of DOTA-IO-VEGF₁₂₁ for VEGFR, Prussian blue staining on the porcine aorta endothelial (PAE) cells (VEGFR negative) and PAE/KDR cells (PAE cells stably transfected with VEGFR-2) were carried out in 6-well Chamber slides, respectively.
PAE or PAE/KDR cells were incubated with DOTA-IO-VEGF$_{121}$ or DOTA-IO for 30 min at the 30 µM Fe concentration and cells were then washed 3 times with PBS and dried at 37 °C for 4 h. The attached cells were then incubated with 10% potassium ferrocyanide in 20% hydrochloric acid for 20 min and washed in distilled water 3 times. The cells were incubated with 1% eosin solution for 10 min and washed in distilled water for counterstaining with nuclear fast red. For dehydration, the cells were incubated with absolute ethanol for 3-5 min. Mounting medium was then placed on the slides along with a coverslip and the iron particles in cells were observed as blue dots using an optical microscope with phase contrast. Only PAE/KDR cells showed prominent staining of DOTA-IO-VEGF, demonstrating VEGFR specificity of this iron oxide conjugate. In vivo test of this conjugate in breast cancer models is currently in progress.

Fig. 13. Prussian blue staining of PAE (A, B) and PAE/KDR (C, D) cells with DOTA-IO (A, C) and DOTA-IO-VEGF$_{121}$ (B, D).
KEY RESEARCH ACCOMPLISHMENTS

- We have successfully produced both wild-type VEGF_{121} protein and VEGFR-2 specific mutant VEGF_{DEE} by recombinant technique.
- We have developed two types of novel iron oxide nanoparticles PVP-IO and PASP-IO;
- Polyvinylpyrrolidone (PVP)-coated iron oxide (PVP-IO) nanoparticles outperforms Feridex for macrophage targeting;
- Poly(aspartic acid) (PASP)-coated iron oxide (PASP-IO) nanoparticles were simultaneously conjugated with tumor vasculature specific ligands VEGF_{121} and cyclic RGD peptide as well as macrocyclic chelating agent DOTA for MR and PET dual modality imaging.
REPORTABLE OUTCOMES

Publications:

Abstracts:
   In Vitro and In Vivo Test of PVP-coated Iron Oxide Nanoparticles as Liver-Specific MRI Contrast Agent
   Joint Molecular Imaging Conference, Providence, Rhode Island, September 2007
   Radiolabeled Iron Oxide Nanoparticle Conjugate for PET/MRI Dual Modality Imaging of Tumor Vasculature
   Joint Molecular Imaging Conference, Providence, Rhode Island, September 2007
   A VEGF2 Specific PET Tracer
   Joint Molecular Imaging Conference, Providence, Rhode Island, September 2007
CONCLUSIONS

We have designed, synthesized, and tested a novel bifunctional iron oxide-based nanoprobe for dual PET/MRI imaging of tumor angiogenesis markers integrin $\alpha_v\beta_3$ and VEGFR-2. The success of this imaging approach may allow for early clinical tumor detection with a high degree of sensitivity while also providing anatomic and molecular information specific to the tumor of interest.
REFERENCES


APPENDICES

None.