Development and Evaluation of Molecular Imaging Agents for Inflammation and Cancer

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Abstract

Molecular imaging is a technique to target a specific tissue or cell type using an imaging agent to provide non-invasive and direct visualization and characterization of *in vivo* biological processes at cellular and molecular level. The intrinsic tissue/cell characteristics act as *in vivo* target and against which the target-specific molecular imaging agent provides signal source that could be detected by various imaging modalities, such as optical imaging (fluorescence and bioluminescence), positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), and ultrasound, etc. Various imaging techniques are often more complementary than competitive. The choice for an imaging modality or combination of techniques is mainly determined by the specific biological question to be addressed. Moreover, many other factors are to be considered, such as efficiency of detection in a timely manner, cost ease of benefits, and availability of tools and instruments.

In this dissertation, studies involving development and evaluation of a series of peptide and antibody based imaging agents for non-invasive detection of inflammation are described. These imaging agents are designed and synthesized to target physiologically important receptors (e.g. formyl peptide receptor) on surfaces of leukocytes (e.g. neutrophil) for various imaging modalities, such as near-infrared fluorescence (NIRF) imaging, SPECT and PET. *In vitro* and *in vivo* evaluation studies of these imaging agents under inflammatory and non-inflammatory conditions are successfully performed, including partition coefficient, target-specificity, blood kinetics, biodistribution, imaging feasibility in various animal models and immunohistochemical

analysis. Highly promising results obtained from these studies suggest the potential of these imaging agents to advance into further pre-clinical and clinical research.

In addition to developing molecular imaging agents for inflammation, a tumor-specific heptamethine cyanine-based dual-modality PET/NIRF imaging agent is being investigated for cancer imaging. Data obtained from this proof-of-concept study, such as spectroscopic evaluation, tumor-specific cellular-uptake, biodistribution, blood kinetics, NIRF and nuclear imaging are highly encouraging. Initial results from these studies set a solid foundation for developing a new generation of tumor-specific agents to enable early detection, targeted therapy and simultaneous monitoring of treatment efficacy of cancers.

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Abbreviations

BCA	bicinchoninic acid
BSP	bromosulfophthalein
CAM	cellular adhesion molecules
CCD	charged coupled device
cFLFLF	cinnamoyl-phe-D-leu-phe-D-leu-phe
CT	computed tomography
Cy 5.5	cyanine 5.5
Cy 7	cyanine 7
DCC	N,N'-dicyclohexylcarbodiimide
DIEA	N,N-diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DOTA	2,2',2'',2'''-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid
EDC	1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide
<i>E.Coli</i>	<i>Escherichia coli</i> , a Gram-negative, rod-shaped bacterium
FDG	fluorodeoxyglucose- ¹⁸ F
FBS	fetal bovine serum
FPR	formyl peptide receptor
Gd-DTPA	gadolinium-diethylenetriaminepentaacetic acid
HA	hepatic arterial buffer
HBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
H/E	hemotoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
HSA	human serum albumin
HTS	high-throughput screening

HYNIC	hydrazinonicotinic acid
IgG	immunoglobulin G
i.v. (iv)	intravenous injection
i.p.(ip)	intraperitoneal injection
I/R	ischemia/reperfusion
ICAM-1	intracellular cell adhesion molecule-1
IHC	immunohistochemistry
LPS	lipopolysaccharides
LTB4	leukotriene B4
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy
MCF-7	a breast cancer cell line
MCF-10A	a non-tumorigenic breast epithelial cell line
MI	myocardial infarction
MPO	myeloperoxidase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MRI	magnetic resonance imaging
NHS	N-hydroxysuccinimide
NIR	near-infrared
NIRF	near-infrared fluorescence
NMR	nuclear magnetic resonance
OATP	organic anion transport polypeptide
OSEM	ordered-subset expectation maximization
p.i. (pi)	post injection
PBS	phosphate buffered saline
PDT	photodynamic therapy
PEG	polyethylene glycol
PET	positron emission tomography
PECAM-1/CD31	platelet endothelial cell adhesion molecule
PK	pharmacokinetic

PD	pharmacodynamic
PMA	phorbol-12-myristate-13-acetate
PMN	polymorphonuclear leukocytes
PS	photosensitizer
PSC	photosensitizer-carrier conjugate
RBC	red blood cell
ROI	region of interest
ROS	reactive oxygen species
RP-HPLC	reverse phase high performance liquid chromatography
SAR	structure-activity-relationship
SD	standard deviation
SUV	standardized uptake value
SPECT	single photon emission computed tomography
Sulfo-NHS	N-hydroxysulfosuccinimide
TFA	trifluoroacetic acid
TNF- α	human tumor necrosis factor- α
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	a nonionic surfactant
US	ultrasound
UV-Vis	ultraviolet–visible spectroscopy
VCAM-1	vascular cell adhesion molecule-1
VOI	volume of interest

Symbols

ε	molar extinction coefficient
$\lambda_{\max}$	maximum wavelength
E_x	excitation wavelength
E_m	emission wavelength
$\log P$	partition coefficient
$T_{1/2}$	half-life
%ID/g	percentage injected dose per gram tissue
%ID	percentage injected dose
Radioactivity	1 μCi =37 kBq

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Chapter 1: Introduction

1.1 Molecular Imaging

Molecular imaging has undergone explosive development over last few decades, because of its clinical utilities for detection, diagnosis and treatment of variety of pathophysiological conditions. Literally speaking, molecular imaging can be interpreted as imaging of molecules or imaging using molecules. It aims to target a specific tissue or cell type using a specific imaging agent to provide direct visualization and characterization of *in vivo* biological processes at the molecular or cellular level. The inherent tissue/cell characteristics act as *in vivo* target and the target-specific molecular imaging agents provide the source of signal that could be detected by corresponding imaging modalities, such as optical imaging (fluorescence and bioluminescence), positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), and ultrasound, etc.

Molecular imaging can be applied for early detection, characterization, and real-time monitoring of disease progression as well as evaluation of drug and treatment efficacy. For example, annexin V is a calcium-dependent protein that has a high affinity for the phosphatidylserine, a plasma membrane phospholipid. One of the earliest features of apoptosis is the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, thereby exposing phosphatidylserine to the external environment. Annexin V binds to the cell surface and identifies cells at an early stage of apoptosis (1, 2). Relying on this mechanism, various radio- and fluorescent-labeled annexin V based imaging agents have been developed (3, 4) (Figure 1-1).

In theory, principles for the design and development of molecular imaging agents are similar to those of drug discovery (5, 6) and follow the same footsteps in the development although a much smaller quantity of imaging agent is often required for detection purpose. As illustrated in Figure 1-2, early-phase development of imaging agents could be interpreted as an interacting relationship between “Structure” and “Activity”. Once a biological target, such as protein, is chosen, a series of chemical structures are synthesized as precursors for target-specific binding. These precursors could be either small molecules (7-9) or large molecules (peptides, proteins, polymers, etc) (10-14), and usually labeled with radioisotopes or fluorescence dyes for different imaging modalities. Evaluation of the biological activities of synthesized imaging agents are sequentially performed for a variety of parameters. These evaluations include, but are not limited to, *in vitro* cellular assays (e.g. to determine affinity and specificity), partition coefficient measurement (hydrophilicity/hydrophobicity), serum stability (how long the imaging agent is stable in blood serum), blood kinetics (how fast the imaging agent could be cleared through blood circulation), biodistribution (how the imaging agent distributes in various tissues and organs), *in vivo* animal imaging (to evaluate imaging feasibility and related parameters), and *ex vivo* examination (to observe the imaging agent at the cellular and molecular level along with histological analysis). Although experimental protocols may vary according to diverse requirements of imaging agents and diagnostic purposes, the structure-activity-relationship (SAR) would be a guide to follow for developing optimized imaging agents.

The major advantage of molecular imaging techniques over more conventional methods is that they can provide sufficient spatial and temporal information of *in vivo*

biological processes without performing invasive procedures, such as biopsy and surgery. Moreover, molecular imaging allows longitudinal studies of the same living subject to access all kinds of time-dependent profiles, such as pharmacokinetic/pharmacodynamic (PK/PD) and biodistribution of the imaging agent, treatment effects of therapeutics, and progress of disease in the drug discovery and development process, thus reducing required number of animals and cost (5).

Molecular imaging also effectively fulfills an essential criterion for a bench-to-bedside translational approach. Currently, most of the diagnostic imaging techniques that are routine in clinical radiology departments have a counterpart in the research setting, such as positron-emission tomography (PET), single-photon emission tomography (SPECT), magnetic resonance imaging (MRI) and ultrasound. Preclinical studies in animals, especially small animals (e.g. rodents), could be performed not only to predict the clinical imaging observations and facilitate the optimization of imaging protocols, but also provide a broad research platform for better understanding of biological responses. Take the workflow of small animal PET imaging for an example, the study could be divided into four sections (Figure 1-3): 1) synthesis and radiolabeling of the imaging agent which has been already optimized and used for the said pathophysiological conditions; 2) administration of the imaging agent into a particular animal model of interest; 3) PET imaging acquisition, and 4) data processing, reconstruction and analysis. The entire process of small animal imaging requires interdisciplinary collaboration among researchers from various fields, such as chemist/radiochemist, biologist, imaging scientist and clinicians with deep understanding of knowledge and skills in each area.

1.2 Imaging Modalities

The incessant technical advancement in imaging modalities has provided solid foundation for obtaining high-quality small animal images, as well as accurate performance of preclinical studies. In other words, the imaging technique must have adequate spatial resolution and sensitivity to detect biochemical events that occur at molecular level, are difficult to detect by routine method or visual examination, and are biologically significant to understand its outcome over time in small animals. The choice for an imaging modality or combination of techniques is determined by the specific biological question to be addressed, since each imaging modality has advantages and limitations and different imaging techniques are more complementary than competitive (Figure 1-4) (5, 15-17).

1.2.1 Optical (fluorescence and bioluminescence) Imaging

In vivo optical imaging can be broadly categorized into fluorescence (18, 19) and bioluminescence imaging (20, 21). Both imaging modalities are easily accessible and usually built as one modality owning both functions, such as the IVIS Spectrum manufactured by Caliper Life Science (Figure 1-5). Imaging in fluorescence and bioluminescence is straightforward: animals are anesthetized, injected intravenously or intraperitoneally with the respective imaging agent or substrate, and placed in a dark chamber with a thermoelectrically cooled Charged Coupled Device (CCD) camera. (5).

Fluorescence imaging is based on the absorption of energy from an external excitation light of one wavelength by a fluorophore such as green/red fluorescence protein, and near-infrared fluorescent (NIRF) imaging agents. Light is then emitted by the fluorophore at a longer wavelength of lower energy, and captured by the camera. NIRF

imaging agents are the most common choice for *in vivo* animal imaging because of better photon signal penetration and minimum tissue absorbance and autofluorescence in the NIR region (700–900 nm) (22-24). In bioluminescence imaging, light is generated by a chemiluminescent reaction between an enzyme and its substrate. The externally detected light is an indicator of the biological/molecular process (25). Moreover, a variety of genetically engineered recombinant reporter proteins allows investigation of specific protein expression in different disease states and the study of interactions between drugs and proteins *in vivo*.

On one side, optical imaging has several advantages, such as high sensitivity (up to $\sim 10^{-15}$ M), operational simplicity, fast and easy image acquisition and analysis, and relatively low instrumental cost. These features are particularly suitable for high-throughput screening to confirm target-specificity and access *in vivo* performance of compounds. On the other side, clinical application of optical imaging is somehow impeded due to the poor light penetration (a few millimeters) and tissue scattering in the biological system. However, optical imaging still has potential and will gain more importance in future clinics, especially for anatomical locations which are not limited by light penetration (e.g. breast), accessible via a catheter or endoscope or intra-operative surgery (26).

1.2.2 Positron Emission Tomography (PET)

In PET imaging, a compound is labeled with a positron emitting radioisotope and administered in trace quantities. The positron emitted from the radionuclide annihilates with a surrounding electron and transformed into two 511-keV gamma rays, which are emitted in two $\sim 180^\circ$ opposite directions. This coincidence event is detected by a

detector ring surrounding the living subject, and the gamma rays are converted into visible light and finally into electrons to allow localization and quantification of the radiolabeled compound (Figure 1-6).

The sensitivity of PET is very high ($\sim 10^{-11}$ – 10^{-12} M) and is practically independent of the depth due to accurate methods of attenuation correction for gamma rays. The spatial resolution of PET depends on the size of the single detector component, and measures ~ 4 – 8 mm³ in clinical and ~ 1 – 2 mm³ in small animal imaging systems.

Almost all PET radioisotopes are produced by a cyclotron and are introduced into the compound of interest by chemical synthesis or complexation using manual procedures or an automated synthesizer. Frequently used positron-emitting isotopes include ¹¹C (half-life time $t_{1/2}$ = 20 min), ¹³N ($t_{1/2}$ = 10 min), ¹⁵O ($t_{1/2}$ = 2 min), ⁶⁴Cu ($t_{1/2}$ = 12 hours), ¹²⁴I ($t_{1/2}$ = 4 days), and ¹⁸F ($t_{1/2}$ = 110 min). It is of note that two concurrently administered PET imaging agents could not be differentiated in the same living subject since the PET scanner only detects the gamma rays at 511keV energy level.

1.2.3 Single Photon Emission Computed Tomography (SPECT)

In SPECT imaging, a compound is labeled with a radioisotope that emits one or more gamma rays of characteristic energies, and thus allowing two or more compounds labeled with different radioisotopes to be distinguished within the same study, which is different from PET imaging. As illustrated in Figure 1-7, a gamma camera rotates around the living subject to acquire signals from various angles. These two dimensional images are then accumulated and applied to a tomographic reconstruction algorithm to build the three-dimensional image about the distribution of the radio-labeled compound. Small

animal SPECT instruments have a spatial resolution of $\sim 1 \text{ mm}^3$ and clinical systems have resolutions that are $\sim 12\text{--}15 \text{ mm}^3$.

Most commonly used SPECT isotopes are heavier isotopes such as $^{99\text{m}}\text{Tc}$ (140 keV; $t_{1/2} = 6 \text{ h}$), ^{111}In (171 keV and 245 keV; $t_{1/2} = 2.8 \text{ d}$), and ^{123}I (27 keV and 159 keV; $t_{1/2} = 8 \text{ d}$). Advantages of SPECT over PET include lower costs, potential for imaging with different isotopes simultaneously, and the more molecular probes available for clinical use. Disadvantages of SPECT compared to PET include the lower sensitivity of about one to two orders of magnitude and the more complex approximate attenuation correction.

The major drawbacks of nuclear imaging including PET and SPECT include, but is not limited to: 1) reliance on radiation, in which cases the harmful radiation is always “on”; 2) Since the radioisotopes have short half-lives, experiment has to be performed within limited time (usually 1-2 days), which largely increases the cost and difficulty of research; 3) Radiolabeling often requires a radiation-specific wet lab and specially trained staff. All these factors make the regular production and novel development of nuclear imaging agents expensive and restricted to locations with the necessary facilities.

1.2.4 Computed Tomography (CT)

For CT, X-rays are emitted from a focused X-ray source rotating around a living subject placed in the center of the CT scanner (also Figure 1-7). When crossing through the body of the subject, the X-rays get absorbed in inverse proportion to the density of the tissue of the body. The amount of absorption is detected by a detector on the other side of the X-ray source. Based on the X-ray absorption, high-resolution tomographic anatomical

images are reconstructed through a set of back calculations with a spatial resolution of up to $\sim 6\text{ }\mu\text{m}$ in small animal scanners.

CT is limited by the lack of soft tissue contrast. Due to the low detection sensitivity (gram amounts of contrast agents are intravenously administered) and due to the difficulty in labeling molecular probes with CT-compatible contrast agents (for example, iodine), CT is not currently used as a molecular imaging technique. However, CT provides a high quality anatomical framework for molecular imaging, especially, in combination with other imaging techniques such as PET and SPECT (27, 28).

1.2.5 Magnetic Resonance Imaging (MRI)

The physical principles of magnetic resonance imaging are an extension of the theory of nuclear magnetic resonance (NMR). MRI, unlike other techniques of optical and nuclear imaging, is relatively insensitive (10^{-3} – 10^{-4} M). Only certain nuclei, such as hydrogen or phosphorous, have magnetic properties and possess angular momentum or “spin” and are detectable by NMR. When these nuclei are exposed to a high static magnetic field they can have one of two orientations with respect to the applied field. These two orientations have slightly different energy levels proportional to the applied field and produce a net magnetization that represents a small fraction (determined by Boltzmann distribution) of the total number of nuclei. This accounts for the low signal intensities of the MRI experiment.

Signal of MRI experiment is derived from the hydrogen nuclei (protons) of water molecules due to abundance of water in the biological tissue. Therefore, image intensity is modulated by the local density of water. Since ^1H protons are sensitive to its local physicochemical microenvironment and the MRI experiment can be customized to

accommodate various pathophysiological conditions. Moreover, the specificity of MRI can be further increased by exogenous contrast agents such as gadolinium chelates (29).

1.3 Inflammation, Neutrophil Activation and Formyl Peptide Receptor

Tissue damage caused by trauma, infection, or other stimuli triggers a complex sequence of events collectively known as the inflammatory response. To overcome the inflammatory response and repair injured tissue, the mammalian body contains a variety of defense mechanisms. One foremost innate mechanism is an increase in blood circulation and deployment of leukocytes in injured areas. The mechanism of neutrophil activation and migration to the injured site through microvasculature has been studied (Figure 1-8) (30). Firstly, leukocytes adhere to the vascular endothelium due to locally enhanced expression of adhesion molecules. Subsequently, they pass through the endothelium and the basal membrane (extravasation) and migrate into the tissue and airways (31).

In the current study, we chose neutrophils as a target to identify and characterize the inflamed/injured tissue because of following reasons: i) neutrophils are generated in bone marrow, circulated in blood, under non-inflammatory conditions they are mostly in an inactive state, ii) during inflammation they become activated due to chemotaxis and are the first line of defense, iii) they have a very fast acute response due to their speediest mobility among the mammalian cell types (20 micrometer/minute), following the chemo-attractant gradient, iv) they are large in number (~50-70% of total white blood cells) with good characteristics for high sensitivity of detection, and v) they rapidly accumulate at inflammatory sites due to increased blood circulation and high permeability through microvasculature.

One of the most significant features of neutrophils in response to inflammation is that they undergo a variety of changes in morphology and surface composition of various proteins in an activated state (32). Expression of one such protein called formyl peptide receptor (FPR) is a cornerstone of neutrophil activation, and this process is conserved in mammalian species through evolution (33). It has been decisively established that FPRs are highly expressed on neutrophils to a major extent and to some degree in monocytes and macrophages (34), in response to biological metabolic by-products of protein oligopeptides such as fMLF (in literature it has been referred to as fMLP, a misnomer - formyl-methionyl-leucyl-phenylalanine) under infectious conditions. fMLF is a highly potent effector of neutrophil function, which induces neutrophil activation as demonstrated by chemotaxis, degranulation, oxygen radical generation, and arachidonate metabolite release (35-37), leading to transient changes in surface membrane depolarization, as well as subsequent fluctuations in intracellular calcium and cAMP levels (38),(39). It has been also demonstrated that other factors such as lipopolysaccharides (LPS), phorbol 12-myristate 13-acetate (PMA) may induce expression of FPRs on neutrophils (25).

1.4 Neutrophil-specific Imaging Agents for Inflammation

Timely detection and localization of inflammatory lesions is critical for appropriate treatment to patients, in which molecular imaging appears to be the most promising strategy. Current nuclear imaging agents for detecting inflammatory lesions can be classified as leukocyte labeling agents (with variable selectivity for specific leukocytes) and non-leukocyte-specific agents such as antibodies that bind non-leukocyte cells (e.g. protein labeling agents or carbohydrates. Leukocytes can be labeled either *in*

vivo or *ex vivo*, but neutrophils are notorious towards activation during isolation and hence are practically difficult to label *in vitro*. *Ex vivo* autologous leukocytes labeled with radioactive isotopes (^{111}In or $^{99\text{m}}\text{Tc}$) (40), although considered as a gold standard in the current clinical practice, involves laborious isolation and labeling of white blood cells which takes a trained technician approximately 3 hours to complete. In addition, handling of potentially contaminated blood poses severe biological hazard for transmission of blood-borne pathogens such as hepatitis virus and human immunodeficiency virus to both technicians and patients (41, 42). In comparison, *in vivo* labeling agents may evade the problems associated with *ex vivo* labeling with non-leukocyte-specific agents such as: 1) FDG (fluorodeoxyglucose- ^{18}F); 2) antibodies and peptides that bind to endothelial receptors; 3) microorganism-targeting molecules; and 4) radiopharmaceuticals that are accumulate in inflammatory sites based on vasculature permeability. However, imaging by non-leukocyte-specific agents suffers from low inflammation specificity and poor correlation between site-specific uptake and inflammatory activity. Autologous leukocytes-specific radiolabeled monoclonal antibody against surface antigen has advantages of *in vivo* labeling, but their high molecular weight, long plasma half-life, high liver uptake and possible allergic reaction hindered their wide application (43).

Leukocyte-targeting chemokines have emerged as a promising class of agents with attractive characteristics for scintigraphic detection of infection and inflammation (44). The issues associated with chemokines are cost and occasional undesired bioactivities. During the past twenty years, the development of new radiopharmaceuticals has gradually shifted from large proteins to receptor-specific small molecules and peptides (45). The peptide mediators and small molecules have several advantages such

as easy synthesis, ease of structural modification, handling, rapid clearance from blood and other non-target tissues thus improving selectivity and sensitivity of detection. Some leukocyte-binding peptides that have been investigated for scintigraphic imaging of infection and inflammation are listed in Table 1-1. The FPR specific ^{99m}Tc -labeled agonist peptides, fMLF and fNleLFNleYK (46) were reported to be used for imaging inflammation by neutrophil binding and appeared to be superior to ^{111}In -labeled leukocytes (47-49). These agonist-based imaging probes nevertheless have undesired bioactivities, such as transient mild granulocytopenia in Rhesus monkeys (50). Antagonist peptide probes, such as iBoc-MLF and cinnamoyl-F(D)LF(D)LF (cFLFLF) were developed for imaging of infectious sites to circumvent the undesirable biological effect of the radiolabeled agonist peptide (51). Further development of these high non-specific binding was not observed presumably due to the high lipophilicity of the probe (51). Besides FPR ligands, leukotriene B₄ (LTB₄) receptor antagonist ^{99m}Tc -RP517 has been investigated to visualize *E. coli* infections (52) and myocardial inflammation (53), however, its hepatobiliary clearance, causing high uptake in the organs of the digestive tract relatively early after the injection, which limits its applicability as an inflammation imaging agent (52). A new hydrophilic LTB₄-antagonist with comparable receptor binding affinity, DPC11870-11, was thus developed and radiolabeled with ^{111}In . Tuftsin antagonist peptide, TKPPR, is another neutrophil specific but non-FPR binder, which has been studied as potential *in vivo* imaging agent (54).

All information described above sets a solid foundation to target FPR on activated neutrophils (55) for monitoring inflammation. In perspective, FPR antagonists with high binding affinity as well as preferable PK/PD properties would be ideal for development

of molecular imaging agent. In the current study, we chose the FPR antagonist peptide cinnamoyl-F(D)LF(D)LF (cFLFLF) (cinnamoyl-phe-D-leu-phe-D-leu-phe) for further modification to optimize the physiochemical parameters as shown in Figure 1-9. In our study, the modified PET imaging agent cFLFLF-PEG-⁶⁴Cu (56) demonstrated improved PK/PD property and desirable imaging feasibility in detecting bacterial-induced lung inflammation in mouse (28). In Chapter 2, a series of near-infrared fluorescence (NIRF) versions of the imaging agent have been developed to confirm the neutrophil-specificity of this modified peptide at tissue and cellular level (57). In Chapter 3, detailed *in vivo* evaluation and performance of the optimized neutrophil-specific NIRF imaging agent has been demonstrated with a mouse model of acute ear inflammation (58). In Chapter 4, heterobivalent neutrophil-targeting peptides have been developed and evaluated towards the SPECT imaging feasibility (27). Platelet endothelial cell adhesion molecule (PECAM-1/CD31) is also known as important biological marker during acute inflammation, especially in assisting neutrophil extravasation. Chapter 5 describes the synthesis of a PECAM-1-specific PET imaging agent, as a complementary study to our project of inflammatory detection.

1.5 Heptamethine Cyanine Dye Based Imaging Agents for Cancer

Cancer is one of the leading causes of death and debilitation. It has been estimated that over 1.5 million Americans will be diagnosed with this dreaded disease and over half million people will die due to all types of cancer in 2011 (59-63). Early detection and accurate diagnosis of cancer greatly increases the chances for successful treatment. In most of cases, the detection and diagnosis happens very late in cancer progression (when cancer is larger and has metastasized to other organs), which ultimately leads to the need

for very aggressive treatment. A few screening tools are now available for breast, prostate and colon cancers (64-74), however, analysis and interpretation of the data sometime can be misleading due to lack of high cancer specificity and poor sensitivity. False negative screens cause unnecessary anxiety and undesirable follow-up procedures to the patients. Thus there is an ongoing need for imaging agents that provide a highly specific and sensitive readout of cancer, particularly in the very early stages of the disease.

Recently, heptamethine cyanines, MHI-148 and IR-783 (Figure 1-10) have been reported as tumor-specific fluorescence imaging probes for a broad spectrum of primary and metastasized cancers (75). Cyanine dyes are fully conjugated small organic molecules comprising two aromatic nitrogen-containing heterocycles linked by a polymethine bridge. Heptamethine cyanines show absorption at near-infrared region (NIR) beyond 700 nm with high molar absorption coefficient and decent fluorescence quantum yield and thus can be used for *in vivo* optical imaging for better photon signal penetration through animal tissues with low minimum tissue absorbance in NIR region (700–900 nm) (76-81). More excitingly, these multifunctional dyes have shown unique tumor-specific uptake compared with normal cells and are preferentially accumulated in various cancers, for example, prostate (ARCaP_M cells and orthotopic prostate tumor, p.o.), bladder (T24, s.c.), pancreatic (MIA PaCa-2, s.c.) yielding an excellent signal to background ratio (75). These new findings opened up an opportunity to advance a novel class of tumor-specific probes for cancer imaging.

The use of heptamethine cyanines as tumor-targeting agents has several advantages: 1) a versatile flexible structural design amenable to multitude functional group modifications (e.g., cyanine-base and ligand for metal chelation can be modified at

ease and conveniently accessible for conjugation); 2) the optimization of desired pharmacological properties (e.g., aqueous solubility and good blood clearance can be achieved through relatively easy structural modifications); 3) the small molecular weight allows operational and handling simplicity, chemical/biological stability and high permeability across physiological barriers; 4) this class of molecules has demonstrated low toxicity and appears to be biologically silent; 5) the high tumor uptake specificity of cyanine-base can be utilized to enhance the sensitivity for detection offered by PET labeling; 6) there is a possibility through chemical modifications to enhance the residence time of the probe once inside cancer cells which can be exploited to evaluate treatment regimes, and; 7) these agents can be made inexpensively.

In Chapter 6, the synthesis and evaluation of a dual-mode heptamethine cyanine-based tumor-specific PET/NIRF imaging agent is described. Data obtained from this proof-of-concept study, such as spectroscopic evaluation, tumor-specific cellular-uptake, organ distribution, blood kinetics, and optical/nuclear imaging feasibility is highly promising. The information gathered from these studies set a solid foundation for developing a new generation of tumor-specific agents to enable early detection, simultaneous monitoring of treatment and targeted therapy of cancers with minimal toxicity.

1.6 Figures and Table

Figure 1-1. Example of Cy5.5 labeled Annexin V as an optical imaging agent to detect apoptosis.

Figure 1-2. Relationship between chemical structure and biological activity in early-phase development of molecular imaging agents.

Figure 1-3. Workflow of MicroPET imaging.

	Optical Imaging	PET	SPECT	MRI	Ultrasound	CT
Advantages	<ul style="list-style-type: none">• High-throughput screening for target confirmation and probe evaluation• High sensitivity• Low cost• Simple operation	<ul style="list-style-type: none">• Clinical translation• High sensitivity with unlimited depth penetration	<ul style="list-style-type: none">• Clinical translation• Unlimited depth penetration	<ul style="list-style-type: none">• Clinical translation• High resolution and soft-tissue contrast	<ul style="list-style-type: none">• Clinical translation• High spatial and temporal resolution• Low cost	<ul style="list-style-type: none">• High spatial resolution• Clinical translation
Disadvantages	<ul style="list-style-type: none">• Limited clinical translation• Low depth penetration	<ul style="list-style-type: none">• Limited half-life of radioisotope• Radiation• Cost	<ul style="list-style-type: none">• Limited half-life of radioisotope• Radiation• Limited spatial resolution	<ul style="list-style-type: none">• Costs• Imaging time	<ul style="list-style-type: none">• Operator dependency• Targeted imaging limited to vascular compartment	<ul style="list-style-type: none">• No target-specificity• Radiation• Poor soft-tissue contrast
Molecular Imaging						
Functional Imaging						
				Anatomic Imaging		

Figure 1-4. Summary of modalities used for molecular imaging. Each modality has particular characteristics, advantages and limitations, which are highlighted here.

Figure 1-5. Small animal optical imaging scanner (IVIS spectrum, Caliper Life Science). Locations of CCD camera, dark imaging chamber and anesthesia system are indicated by red squares, respectively. Note: scanner shown above belongs to Molecular Imaging Core at University of Virginia.

Figure 1-6. MicroPET scanner (left) (Focus 120, Siemens) and detection mechanism (right). Note: scanner shown above belongs to Molecular Imaging Core at University of Virginia.

Figure 1-7. Customer-built SPECT-CT scanner. A. Overview of the entire scanner. B. SPECT and CT modules are indicated by red and yellow squares, respectively. Note: scanner shown above was constructed by Dr. Mark Williams Lab in Molecular Imaging Core at University of Virginia.

Figure 1-8. Mechanism of neutrophil activation and migration to inflamed tissue (30).

Table 1-1. Leukocyte-specific small molecules and peptides in previous studies.

Agent	Physiologic function	Tracer	Uptake in infection/inflammation	Accumulation in non-target tissues	Toxicity
α MLF	FPR agonist	^{99m}Tc	Early, specific	Spleen, liver, kidneys, bladder and bowel	Neutropenia
<i>i</i> Boc-MLF	FPR antagonist	^{99m}Tc	Early, low absolute uptake	bladder	No
α NleLFNleY	FPR agonist	^{99m}Tc	Early, moderate absolute uptake	Spleen, liver, and bowel	Little
cF(D)LF(D)L	FPR antagonist	^{99m}Tc	Early, moderate absolute uptake	Liver, spleen, marrow and bone	No
TKPPR	Tuftsins antagonist	^{99m}Tc	Early, low absolute uptake	Liver, kidneys, bladder and synovia	No
RP517	LTB ₄ antagonist	^{99m}Tc	Early, specific	Liver, spleen, and bowel	N/A
DPC11870	LTB ₄ antagonist	^{111}In	Early, specific	Spleen, marrow, liver, kidneys	No

Figure 1-9. Schematic design of novel peptide based molecular imaging agents for inflammation.

Figure 1-10. Structures of tumor-specific heptamethine cyanine dyes (IR 783 and MHI-148) (75).

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Chapter 2: Synthesis of the Cyanine 7 Labeled Neutrophil-Specific Agents for Noninvasive Near Infrared Fluorescence Imaging

2.1 Abstract

A neutrophil-binding peptide, cinnamoyl-F(D)LF(D)LF (cFLFLF), was modified with a various lengths of polyethylene glycol (PEG) moieties and labeled with the near-infrared (NIR) fluorophore, Cyanine 7 (Cy7). Solubility and bioavailability to circulating neutrophils of these imaging agents were evaluated. A preliminary noninvasive fluorescence imaging of the optimized imaging agent is also presented with mouse model of PMA-induced acute ear inflammation.

2.2 Introduction

Neutrophils are the most abundant type of white blood cells in mammals and form an essential part of the innate immune system. In a number of inflammatory disorders, neutrophils recruited to the site of injury and their subsequent release of reactive oxygen species (ROS) and proteolytic enzymes are responsible for the tissue damage associated with such disease conditions. Therefore, imaging technologies that could enable specific visualization of neutrophil activation and infiltration non-invasively are highly desirable. Non-invasive imaging of acute inflammation has been achieved by using nuclear imaging modalities with either *ex vivo* labeled leukocytes (1, 2) or *in vivo* imaging probes directly targeting formyl peptide receptor (FPR) of neutrophils (3-5). The strength of nuclear imaging is low tissue attenuation and quantitability of its nuclear signal. However, its wide application in research setting is impeded by the cost of the relevant instruments and the limited shelf life of the nuclear probes.

During last decade, the real time *in vivo* fluorescence imaging of small animals has gone through tremendous technological progress because of its merits of operational simplicity and low cost. As a result, considerable effort has been directed toward the development of new NIR fluorescent probes targeting *in vivo* molecular events and biochemical processes. However, to date, there has been no report on neutrophil-specific NIR fluorescence imaging agent.

In the current study, we synthesized and evaluated a series of fluorescent neutrophil-binding ligands: cFLFLF-PEG_n-Cy7 (n=0, 12, and 76). The hexapeptide, cFLFLF, exhibits high affinity to FPR (6), but its high hydrophobicity impedes the application in live animals. We addressed the issue by modifying the hydrophobic peptide with PEG moieties to enhance its bioavailability. The spectrum ($E_{\text{excitation}}/E_{\text{emission}}$: 745 nm/800 nm) of Cy7 provides better photon signal penetration through animal tissues because of the minimum tissue absorbance in the NIR region (700–900 nm) (7).

2.3 Materials, Methods and Results

All chemicals purchased commercially were of analytical grade and were used without further purification. Fmoc-PAL-PEG-PS resin (0.18mmol/g) was acquired from Applied Biosystems (Foster city, CA). *N*-Fmoc-amino-dPEGTM₁₂ acid was purchased from Quanta Biodesign Ltd, (Powell, OH). *t*-Boc-PEG-NHS-3.4 kD was obtained from Laysan Bio Inc (Arab, AL). CyTM7 Mono NHS ester was purchased from GE healthcare (Piscataway, NJ). High-performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific. All other chemicals were obtained from Sigma-Aldrich.

Synthesis of fluorescence imaging probes

As shown in Scheme 2-1, peptides cFLFLFK-CONH₂ **2-4** and cFLFLF-PEG₁₂-K-CONH₂ **2-5** were synthesized on solid phase using conventional F-moc chemistry starting from Fmoc-PAL-PEG-PS resin. The procedure followed our previously published paper (4), in which PEG₁₂ moiety (*N*-Fmoc-amino-dPEGTM₁₂ acid) was inserted into the peptide sequence as an amino acid residue, while PEG₇₆ moiety (*t*-Boc-PEG-NHS-3.4 kD) was conjugated to the ω-NH₂ of the lysine residue of **2-4** in aqueous solution to form peptide cFLFLFK-PEG₇₆-NH₂ **2-6**. The yield of the crude peptides was in the range of 80-85%. CyTM7 Mono NHS ester was conjugated to the ω-NH₂ of the lysine residue of **2-4** and **2-5** or the terminal -NH₂ of the PEGylated **2-6** in a 1:1 mixture of acetonitrile and sodium borate buffer (0.1N, pH 8.5) at 4 °C for 2 hrs, followed by incubation at room temperature for another 1 hour in dark. The yield of Cy7 conjugations was in the range of 59-97%. All of the intermediates and the final products were purified by semi-preparative reverse phase high performance liquid chromatography (RP-HPLC). HPLC was performed with a Varian system with ABI Spectroflow 783 UV detector using an Apollo C18 reversed-phase column (5μm, 250×10 mm). The mobile phase changed from 60% solvent A (0.1% trifluoroacetic acid in water) and 40% solvent B (0.1% trifluoroacetic acid in 80% aqueous acetonitrile) to 100% solvent B during 30min at a flow rate 3 mL/min. UV detector wavelengths were 215 nm for all the experiments.

The HPLC retention times of cFLFLF-Cy7 **2-7**, cFLFLF-PEG₁₂-Cy7 **2-8** and cFLFLF-PEG₇₆-Cy7 **2-9** were 19.2 min, 16.9 min, and 16.7 min, respectively. The chemical purities of Cy7 conjugates were >95% and these pure samples were used for further experiments. Final Cy7 conjugated products were characterized by MALDI-TOF

mass spectroscopy (W.M. Keck Biomedical mass spectrometry laboratory at University of Virginia) as followed (see Appendix section for detailed spectra): (1) cFLFLF-Cy7 **2-7**: calculated $[M]^+$: 1607.8; found $[M+H]^+$: 1608.2; (2) cFLFLF-PEG₁₂-Cy7 **2-8**: calculated $[M]^+$: 2207.1; found $[M+H]^+$: 2208.2; (3) cFLFLF-PEG₇₆-Cy7 **2-9**: average calculated $[M]^+$: 5007; found $[M+H]^+$: major peaks distributed from 4929 to 5150 with center peak at 5000.

Fluorescence spectra

Fluorescence spectra were recorded on a Fluorolog-3 spectrofluorometer (JOBIN YVON/HORIBA, Edison, NJ), with slit width of 5 nm and integration time of 0.2 s. Stock solutions (20 μ M) of Cy7 and its peptide conjugates **2-7**, **2-8**, and **2-9** were prepared by dissolving solid sample in 97% aqueous solution with 3% DMSO. Fluorescence spectra were measured by adding 3 mL aliquots of the stock solutions into a quartz cuvette. The fluorescence intensities were normalized and plotted versus emission wavelengths. It turned out that the conjugation of peptides (with or without PEG) and Cy7 did not significantly alter the fluorescence emission spectra of Cy7, as exhibited in Figure 2-1. The excitation wavelengths used in all experiment were 745 nm. The fluorescence emission maximum of Cy7 was measured to be 771 nm (vs 775 nm in PBS buffer, pH 7.45). The peptide conjugates exhibited 2–3 nm red shift in their fluorescence emission maximums versus that of Cy7.

Partition coefficients

The hydrophilicity of compounds **2-7**, **2-8**, and **2-9** was determined by measuring partition coefficient constant in *n*-octanol and water. In brief, the purified compound (5 nmol) was dissolved in an equal volume (0.6 mL/0.6 mL) mixture of *n*-octanol and

deionized water, resulting in a final concentration of 4.15 μM that is in the range of linear relationship between fluorescence probe concentration and fluorescence intensity. After vigorous vibration for 10 min, the mixture was centrifuged at 10,000 rpm for 5 min. Aliquots (in triplets) of 100 μL were aspirated out carefully from each layer and transferred into separate wells of a fluorescence 96-well plate. The plate was placed under the CCD camera of Xenogen IVIS Spectrum (Caliper life science, Hopkinton, MA) and the fluorescence was measured with a filter set of $E_x/E_m=745/800$ nm (auto exposure, medium binning, F stop=2). Fluorescence intensity of each well was measured by drawing ROI on the well boundaries and recorded as average radiance ($\text{p/s/cm}^2/\text{sr}$) using software LiveImage 3.1. Partition coefficient parameter $\log P$ was calculated using the equation: $\log ([\text{fluorescence intensity in octanol}]/[\text{fluorescence intensity in water}])$. Fluorescence intensities to calculate $\log P$ were corrected by a factor of solvent effect. The factor was calculated based on different fluorescence intensities of the same concentration of probes in *n*-octanol and water. The average and standard deviation of $\log P$ values were calculated. As exhibited in Figure 2-2, It turned out that compound **2-7** showed most positive $\log P$ (1.35) and compound **2-8** demonstrated moderate improvement toward water solubility ($\log P$, 0.49). Compound **2-9** exhibited the best hydrophilicity as $\log P$ of -1.13 which was comparable to our previously reported PET imaging probe cFLFLF-PEG₇₆-Cu-64 ($\log P$, -1.21) (5). Although Cy7 itself is quite water soluble with $\log P$ of -1.62, incorporation of this dye is not enough to compensate the hydrophobicity of the cFLFLF moiety. PEGylation with a larger PEG₇₆ moiety indeed improves the bioavailability of the FRP-targeting peptide sequence. As a result, compound **2-9** was further evaluated as an in vivo imaging probe with a mouse model of ear inflammation.

Animal model of acute ear inflammation

The mouse model of acute inflammation was induced by topical application of phorbol myristate acetate (PMA) [5 µg in 20 µl of DMSO and acetone (1/9,v/v)] onto the left earlobes of the FVB mice, male, 3 months old, purchased from National Cancer Institute at Frederick (Frederick, MD). It prompted acute ear dermatitis, manifested by local swelling, erythema and infiltration of neutrophils (8-10). The right ears of the same mice served as control and received only the vehicle [20 µl of DMSO and acetone (1/9, v/v)]. *In vivo* fluorescence imaging was performed at 24 hrs after PMA application. To test the *in vivo* FPR-specificity of probe **2-9**, a group of PMA treated mice was designed for blocking experiment. For the blocking mice, excess of the non-fluorescent peptide **2-6** was administered 1 hr before the probe **2-9** injection.

In vivo fluorescence imaging feasibility

All *in vivo* studies were carried out in compliance with University of Virginia Animal Study Committee's requirements for the care and use of laboratory animals in research. *In vivo* fluorescence imaging was performed on Xenogen IVIS Spectrum with excitation filter of 745 nm and emission filter of 800 nm. Images were acquired and analyzed on Live Image 3.1 software (Caliper life science, Hopkinton, MA). Mice were anesthetized with isoflurane (2%) during probe injection and imaging. After 24 hrs of PMA application, mice ($n=5$) were injected with 2 nmol of probe **2-9** via tail vein (i.v.). For the blocking group, mice were injected with 100 nmol (50 eq.) of peptide **2-6** at 1 hour before probe injection. *In vivo* fluorescence imaging was performed at various time points post injection (pi). All *in vivo* NIR fluorescence images were acquired using auto exposure (F stop=2). Measurement ROIs were obtained from both PMA-challenged ears

(left) and control ears (right). Background ROIs were recorded as the fluorescence emission from surrounding area of mice. Fluorescence intensities were normalized by subtracting the background ROIs from the measurement ROIs. A representative image is presented in Figure 2-3. The fluorescence intensity of PMA-challenged ear was significantly higher than control ear, as well as the PMA-challenged ear of blocking group. The ratio of the fluorescence intensity of the infected ear relative to the control ear (L/R) reached the peak value of 3.4 at 3 h pi while in blocking group it demonstrated a significant lower ratio of 1.9 ($P < 0.005$, compared to normal group). It proved the high FPR specificity of probe **2-9** *in vivo*.

Statistics

For statistical classification, a Student t test (2-tailed, unpaired) was performed using Prism software (GraphPad). Any P value less than 0.05 was considered statistically significant.

2.4 Conclusion

In conclusion, we have successfully synthesized cFLFLF-PEG₇₆-Cy7 **2-9** as a potential NIR fluorescence imaging probe for *in vivo* detection of neutrophilic activation and accumulation. The specificity of the probe was approved by an *in vivo* blocking experiment. In our mouse model of the PMA induced ear inflammation, probe cFLFLF-PEG₇₆-Cy7 **2-9** showed significantly higher accumulation in the infected ears than in the control ones. Further *in vivo* evaluation of this fluorescence imaging agent as well as correlation between the macroscopic live animal images and microscopic details at tissue and cellular level will be demonstrated in the following chapter.

Figure 2-1. Normalized fluorescence spectra of Cy7 conjugated peptides. Excitation wavelengths were 745 nm for all experiments.

Figure 2-2. Comparison of partition coefficient (log *P*) values among various fluorescence compounds 2-7, 2-8, 2-9 and Cy7. Positive values suggested the hydrophobic properties of peptide 2-7 and 2-8, while negative data indicated the hydrophilic properties of Cy7 and imaging agent 2-9 (after conjugation with polymeric PEG moiety).

Figure 2-3. Representative fluorescence image at 3 hrs post injection of cFLFLF-PEG₇₆-Cy7 **2-9** (2 nmol per 20g mouse): A, inflamed group; B, blocking group pre-injected with 100 nmol (50 eq.) of non-fluorescence peptide **2-6** at 1 hour before injection of probe **2-9**.

2.6 Appendix

Mass spectrum of compound **2-7** (cFLFLF-Cy7)

Mass spectrum of compound **2-5** [cFLFLF-PEG₁₂-K(NH₂)-CONH₂] Mass spectrum of compound **2-5** [cFLFLF-PEG₁₂-K(NH₂)-CONH₂] (expanded)

Mass spectrum of compound **2-8** (cFLFLF-PEG₁₂-Cy7)

Mass spectrum of compound 2-6

Mass spectrum of compound 2-6 (expanded)

Mass spectrum of imaging agent **2-9** (cFLFLF-PEG₇₆-Cy7) Mass spectrum of imaging agent **2-9** (cFLFLF-PEG₇₆-Cy7) (expanded)

2.7 Acknowledgements

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The neutrophil-specific near-infrared fluorescence imaging agent cFLFLF-PEG₇₆-Cy7 (compound 2-**9**) has been commercialized by Kerafast LLC (Winston- Salem, NC) since Sep 2011.

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Chapter 3: A Novel Near-infrared Fluorescence Imaging Probe for *in vivo*

Neutrophil Tracking

3.1 Abstract

In this chapter, the development and validation of a multiscope near-infrared fluorescence (NIRF) probe, cinnamoyl-F-(D)L-F-(D)L-F-PEG-Cyanine 7 (cFLFLF-PEG-Cy7), that target formyl peptide receptor (FPR) on neutrophils using mice ear inflammation model was described. Acute inflammation was induced in mice by topical application of phorbol-12-myristate-13-acetate (PMA) on left ears 24 h before probe administration. Non-invasive NIRF imaging was longitudinally performed up to 24 h post probe injection (p.i.). *In vivo* neutrophil-targeting specificity of the probe was characterized by a blocking study with pre-administration of excess of non-fluorescent peptide cFLFLF-PEG, and by an imaging study with a scrambled peptide probe cLFFFL-PEG-Cy7. NIRF imaging of mice injected with cFLFLF-PEG-Cy7 revealed fluorescence intensity for inflamed left ears was approximately 4-fold higher than that of control right ears at 24 h p.i. In comparison, the ratios acquired with the scrambled probe as well as from the blocking study were 1.5- and 2-fold at 24 h p.i., respectively. Moreover, microscopic immunohistological study confirmed that NIRF signal of cFLFLF-PEG-Cy7 associated with activated neutrophils in the inflammatory tissue. With this probe, *in vivo* neutrophil chemotaxis could be correlatively imaged macroscopically in live animals and microscopically at tissue and cellular levels.

3.2 Introduction

Neutrophils are the most abundant type of white blood cells in mammals and are an essential part of the innate immune system responding to a variety of stress signals. In a number of inflammatory disorders, such as bacterial infection, neutrophils are the first respondents activated and recruited to the site of injury with subsequent release of reactive oxygen species (ROS) and proteolytic enzymes from them to combat the infection. However, if the response from neutrophil is more than desired the tissue damage associated with such disease conditions ensues (1, 2). An understanding of how neutrophils interact with stressed tissue environment and to manage their response is crucial for the development of effective intervention and therapies to attenuate inflammation. Non-invasive imaging technologies that enable visualization of neutrophil chemotaxis and activation in live animals are thus highly desirable.

Study of gross neutrophil recruitment and chemotaxis in patients and animals has been achieved by using nuclear imaging modalities with either *ex vivo* labeled leukocytes (3, 4) or intravenously injected imaging probes that directly target formyl peptide receptor (FPR) expressed on neutrophils (5-7). Nuclear imaging has the advantage of high sensitivity resulting from high tissue penetration of gamma photon, but its application in small animal research setting is somehow impeded by the cost of the relevant instruments, laborious synthesis and the limited shelf life of the nuclear probes. Moreover, the inherent low resolution of autoradiography prevents the study of neutrophil trafficking from minute details at tissue and cellular levels.

During the last decade, a novel modality, NIRF imaging system of small animals has gone through tremendous technological advances because of the merits of operational

simplicity and low cost (8-10). As a result, considerable efforts have been directed toward the development of new NIRF probes targeting *in vivo* molecular events and biochemical processes (2, 11). It has been widely used to monitor cell trafficking (12, 13) macroscopically in live animals by labeling the cells with a variety of NIRF probes. In principle, the NIRF probe labeled cells along with their associated *in vivo* pathophysiological process could be studied with either fluorescence microscopy or an intravital fluorescence imaging device at tissue or cellular levels. Imaging study at cellular level can be performed before and after *in vivo* settings to delineate function of the cells in various physiological and pathophysiological conditions. Correlation of whole body image with high resolution microscopic image should eventually provide better understanding of biochemical processes. Multi-resolution data sets acquired from the exact same probe would make the correlation simpler and more straightforward.

To study neutrophil trafficking and function *in vivo* by NIRF modalities, labeling the cells with a NIRF fluorophore is required. *In vivo* labeling procedure is preferred because *ex vivo* separation and labeling method might result in undesired consequences, such as contamination and activation of neutrophils, etc. We recently reported the synthesis of cFLFLF-PEG-Cy7 (cinnamoyl-F-(D)L-F-(D)L-F-PEG-Cyanine 7) for non-invasive NIRF imaging of neutrophils in small animals (14). In this chapter, validation of this probe *in vitro* and *in vivo* is presented and fluorescence images of live animals and microscopic tissue slices are correlated. The probe cFLFLF-PEG-Cy7 consists of three components: a FPR-binding peptide sequence – cFLFLF (15), a hydrophilic modifier moiety – PEG, and a fluorophore – Cy7 to attain desired pharmacological and optical properties. The fluorescence spectrum ($E_{\text{excitation}}$ / E_{emission} : 745 nm/800 nm) of Cy7

provides better photon signal penetration through animal tissues because of the minimum tissue absorbance and autofluorescence in the NIR region (700–900 nm) (10). The feasibility of *in vivo* neutrophil imaging was validated with a mouse model of acute ear inflammation. *In vivo* neutrophil-specificity of the probe was demonstrated by a blocking study and an imaging study with a scrambled peptide probe. Neutrophil-binding property of cFLFLF-PEG-Cy7 was also verified by fluorescence microscopy.

3.3 Materials and Methods

Chemicals used for synthesis were commercially available and of analytical grade and used without further purification. Fmoc-PAL-PS resin and Fmoc-amino acids were purchased from Applied Biosystems (Foster City, CA). *t*-Boc-PEG-NHS-3.4 kD was purchased from Laysan Bio Inc (Arab, AL). CyTM7 Mono NHS ester was purchased from GE healthcare (Piscataway, NJ). Fetal Bovine Serum (FBS) was purchased from Invitrogen Corporation (Carlsbad, CA). High-performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific (Pittsburgh, PA). All other chemical reagents and solvents were purchased from Sigma-Aldrich (St Louis, MO).

Semipreparative reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Varian ProStar system (models: pumps, 210; column valve module, 500; fraction collector, 701) (Varian Instruments, Walnut Creek, CA) equipped with ABI Spectroflow 783 UV detector and an Apollo C18 reversed-phase column (5 μ m, 250×10 mm) (Grace Davison Discovery Sciences, Deerfield, IL). For all chromatographic purification, the mobile phase gradient from 60% Solvent A, 0.1% trifluoroacetic acid (TFA) in water, and 40% Solvent B, 0.1% TFA in 80% aqueous acetonitrile (CH₃CN) to 100% Solvent B during 30 min at a flow rate 3 mL/min. was

used. In all purification experiments, the UV detector wavelength was set to 215 nm. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was performed on all peptide products at W.M. Keck Biomedical mass spectrometry laboratory at University of Virginia and the data was obtained on a Bruker Daltonics system (Billerica, MA) (14).

Analytical HPLC was performed on the same system mentioned above but equipped with a Varian reversed-phase analytical column (Microsorb MV 100-5 C18, 250×4.6 mm, Agilent Technologies, Santa Clara, CA). For all chromatographic analysis, Solvent A was 0.1% trifluoroacetic acid (TFA) in water, and Solvent B was 0.1% TFA in 80% aqueous acetonitrile (CH₃CN) and the flow rate at 1 mL/min was used. The UV detector wavelength was set to 215 nm.

Mice

Male FVB mice, 3 months old (about 20 g/mouse) were purchased from National Cancer Institute (Frederick, MD). All mice were housed in a controlled environment (12 h light/12 h dark photoperiod, 22±1°C, 60±10% relative humidity) and were provided with free access to autoclaved pellet food (Harlan, type 7912, Madison, WI) and tap water. All *in vivo* studies were performed in compliance with University of Virginia Animal Study Committee's requirements for the care and use of laboratory animals in research. Mice were anesthetized with isoflurane (2%) during probe injection and imaging.

Synthesis of cFLFLF-PEG-Cy7 3-6 and cLFFFL-PEG-Cy7 3-7

Peptide cFLFLF-PEG **3-4** and scrambled peptide cLFFFL-PEG **3-5** were synthesized using standard Fmoc chemistry, following previously reported procedure (6,

14). We scrambled the sequence of cFLFLF-PEG **3-4** and replaced (D)-Leu with (L)-Leu to form a scrambled peptide cLFFFL-PEG **3-5**. Cy7 conjugation was carried out following a previous reported procedure (14) (Scheme 3-1). All intermediates and final products were purified by RP-HPLC to homogeneity, and purity of the probes were >95%. Structural characterization of peptides **3-4**, **3-5** and Cy7 conjugates **3-6**, **3-7** was performed by MALDI-TOF mass spectroscopy.

Fluorescence spectra

Solutions of Cy7 conjugates (**3-6** and **3-7**, 20 μ M each) were freshly prepared by dissolving samples in a mixture solvent ($H_2O/DMSO$, 97/3, v/v). Fluorescence emission spectra were recorded on a Fluorolog-3 spectrofluorometer (JOBIN YVON/HORIBA, Edison, NJ). The slit width was 5 nm and integration time was 0.2 s for all measurements. The excitation wavelength used was 745 nm. The recorded fluorescence intensity was normalized and plotted versus emission wavelength (14).

Partition coefficient measurement

The measurement of partition coefficient ($\log P$) was performed following reported method (14). In brief, equal amount (5 nmol) of Cy7 conjugates **3-6** and **3-7** were dissolved in an equal volume (600 μ L) mixture of *n*-octanol and deionized water (pre-equilibrated overnight). The fluorescence intensities of 100 μ L aliquots (in triplicate) from both layers were measured by Xenogen IVIS Spectrum (Caliper life science, Hopkinton, MA). $\log P$ was calculated using the equation: $\log ([\text{fluorescence intensity in octanol}] / [\text{fluorescence intensity in water}])$, in which fluorescence intensity was corrected by a factor of solvent effect. The factor was calculated based on different fluorescence intensities of the same concentration of probes in *n*-octanol and water.

PMA-induced ear inflammation

Topical application of phorbol-12-myristate-13-acetate (PMA) [5 µg in 20 µL of vehicle (DMSO and acetone, 1/9, v/v)] onto the left earlobe of mouse could induce acute dermatitis, manifested by local swelling, erythema and infiltration of neutrophils (16, 17). The right earlobe served as control and received only the vehicle. We used this model for neutrophil-specific fluorescence imaging at 24 h after PMA application.

Measurement of ear thickness

The ear thickness was measured before and after PMA challenge on both ears. Measurements were made using an engineer's micrometer (Mitutoyo Corp, Japan), with care being taken to measure only the outer two-thirds of the ear, avoiding skin folds located at the base. Measurements were taken in quadruplicate for each mouse ($n=6$) (17).

PMA dosage test and neutrophil quantification

Various concentrations of PMA were applied on left ears of mice ($n=3$ per PMA dose) to observe the effect of dose on intensity of inflammation. The tested doses included 0.5, 1.5, 5 and 50 µg per 20 µL of vehicle. Twenty-four hours after PMA application, mice were sacrificed and both ears were excised and fixed with formalin before paraffin embedding. The embedded ear tissues were sliced to 5 µm thick histological sections that were specifically stained for neutrophils with a monoclonal rat-anti-mouse neutrophil IgG (MCA771G; Serotec, Raleigh, NC) as well as hemotoxylin. Stained tissue slices were analyzed under a light microscope (Zeiss Primo Star, Carl Zeiss MicroImaging, LLC, Thornwood, NY) equipped with a camera (AxioCam ICc1, Carl Zeiss) and pictures were taken by AxioVision LE software (Carl Zeiss). Five random segments were selected on each slices, three slices were examined for each ears.

Neutrophil and total cell population of PMA-challenged ears (left) were counted by Image J software (developed by National Institutes of Health, free downloadable at <http://rsbweb.nih.gov/ij/>) following the procedure described in Appendix 3-1. Neutrophils showed dark brown color due to staining with rat-anti-mouse neutrophil IgG, while all other cells exhibited blue color derived from hemotoxylin staining. Total cell count is calculated by summing the number of brown and blue cells. The percentage of neutrophil to total cell number was thus calculated. The statistical analysis is representation of whole ear. In addition, percentage increase in left ear thickness was also calculated against various PMA doses.

Luminol-bioluminescence imaging of myeloperoxidase

Myeloperoxidase (MPO) is the most abundant protein in azurophilic granules of neutrophils (18, 19). MPO activity has been assayed as an indicator of neutrophil migration and activation. MPO specific luminal-bioluminescence study was carried out to confirm the accumulation and activation of neutrophils in PMA-challenged ear (18). The mice were injected with 200 μ L of luminol (5 mg/100 mL DMF) intraperitoneally (i.p.) 24 h after PMA (5 μ g in 20 μ L of vehicle) application. Immediately after luminol injection, a 40-min kinetic bioluminescence scan was performed with a Xenogen IVIS Spectrum processed on Live Image 3.1 software (Caliper life science) with open filter and exposure time of 60 s.

Effective dose of imaging probe 3-6

Various doses of the probe cFLFLF-PEG-Cy7 **3-6** were tested (1 nmol, 2 nmol, 5 nmol and 10 nmol per 20 g mice) with PMA-challenged mice. It turned out that 1 nmol was not sufficient enough to provide fluorescence signal and 2, 5, 10 nmol gave the same

ratio of fluorescence signal of inflamed ear to control ear which meant 2 nmol was the minimal dose to observe reasonable signal contrast. As a result, 2 nmol was selected for all of the studies reported here.

In vivo NIRF imaging

In order to demonstrate the imaging feasibility and specificity of probe cFLFLF-PEG-Cy7 **3-6**, four groups of experiment ($n=5-6$ per group) were performed, among which group A, B and C were applied with PMA (5 μg per 20 μL of vehicle) on left earlobes: (A) Inflamed group: 2 nmol of probe **3-6** was injected intravenously (i.v.); (B) Blocked group: an excess of non-fluorescence peptide cFLFLF-PEG **3-4** (100 nmol, 50 equivalents) was i.v. injected 1 h prior to probe **3-6** (2 nmol) injection; (C) Scramble group: 2 nmol of scrambled peptide probe **3-7** was i.v. administered; (D) Negative control group: mice without any PMA treatment were i.v. injected with probe **3-6** (2 nmol).

In vivo NIRF imaging was performed on Xenogen IVIS Spectrum at various time points (30 min, 1 h, 2 h, 3 h, 6 h and 24 h p.i.) with a filter set of $E_x/E_m=745/800$ nm. Identical illumination settings (auto exposure, medium binning, F/Stop=2) were used for acquiring all images. The fluorescence emission was normalized to photons per second per centimeter squared per steradian ($\text{p/s/cm}^2/\text{sr}$). Images were acquired and analyzed on Live Image 3.1, and displayed in the same scale of fluorescence intensity. Measurement ROI (region of interest) was obtained from both PMA-challenged ear (left) and control ear (right). Background ROI was recorded as the fluorescence emission from dark area of the image. Corrected fluorescence intensity was determined by subtracting background ROI from measurement ROI (Figure 3-4A).

Organ distribution

At the completion of *in vivo* imaging (24 h p.i.), PMA-challenged mice that were injected with probe **3-6** (inflamed group) were euthanized ($n=4$). Organs and tissues (left and right earlobes, lungs, heart, liver, kidney, spleen, stomach, small intestine, bone, and muscle) were excised, rinsed with saline, lined up on a black paper and placed under the CCD camera of Xenogen IVIS spectrum. Fluorescence images were taken using the identical illumination settings as *in vivo* imaging. The normalized fluorescence intensities of each organ and tissue were measured and expressed as the average radiance (p/s/cm²/sr).

Immunohistochemical staining and fluorescence microscopy

Immunohistochemistry (IHC) analysis was performed on harvested ear tissues 27 h after PMA application on both inflamed and control earlobes (corresponding to the time point of 3 h p.i.). After excised, ears were immediately transferred into 1 mL conical tubes and frozen into liquid nitrogen for 1–2 min and then stored at -80°C freezer overnight. The frozen tissues were kept frozen and dissected to adjacent histological sections (3 µm thick) that were specifically stained for neutrophils with a monoclonal rat-anti-mouse neutrophil IgG (MCA771G; Serotec, Raleigh, NC) following the protocol described in Appendix 3-2 (7). Stained ear tissue slices were observed under a light microscope (Alpha & OMEGA microscope service, Gaithersburg, MD) with a camera (DP71, Olympus Corporation, Japan) and pictures were taken and processed by MicroSuite Pathology software. Furthermore, the fluorescence images were examined for the same batch of slices on a confocal fluorescence microscope (Carl Zeiss LSM 510-UV,

Germany) with laser excitation filter of 633 nm and long pass emission filter of 650 nm, and pictures were taken and processed by LSM Image Browser software.

Fluorescence stability in serum

Five nmol of each probes **3-6** and **3-7** (in 0.5 mL PBS buffer) were incubated separately with equal volume of FBS at 37°C (9). The purpose of the experiment was to test whether serum will affect the fluorescence emission property of this probe. Since mice serum was not available FBS was used instead. Aliquots of 50 µL were taken out at various time points (up to 24 h) and transferred into a black, round bottom 96-well plate (non-treated, Corning Incorporated, NY)). The plate was placed under the CCD camera of IVIS spectrum and fluorescence intensity was measured using the identical illumination settings as *in vivo* imaging.

Serum stability of probe 3-6 monitored by HPLC

Chemical stability of probe **3-6** in serum was monitored by analytical HPLC from 0 to 24 h after incubation with serum. The probe was essentially tested under two different solvent conditions on HPLC to ascertain the peak identity and purity prior to stability studies (Condition 1: 95% Solvent A and 5% Solvent B to 100% Solvent B in 15 min, retention time was around 10 min; Condition 2: 60% Solvent A and 40% Solvent B to 100% Solvent B in 15min, retention time was around 14 min, and Condition 2 was used for all following studies). The peak was analyzed by MS analysis to make sure the observed signal was from the probe. Once peak has been confirmed, UV detector (215 nm) was used to measure the stability of probe in serum as follows: The probe cFLFLF-PEG-Cy7 **3-6** (50 nmol) was dissolved in PBS buffer (100 µL) and incubated with equal volume (100 µL) of FBS at 37°C on a Thermomixer (Eppendorf, Hamburg, Germany).

Aliquots (15 μ L) were withdrawn at various time points (0 h, 1.5 h, 3 h, 4 h and 24 h) post incubation, and equal volume (15 μ L) of acetonitrile was added. The mixture was subjected to vortex for 1 min to ensure protein precipitation and followed by centrifugation for 1.5 min at 13,000 rpm (mini Spin, Eppendorf, Hamburg, Germany). A fixed volume (10 μ L) of supernatant solutions was withdrawn by syringe and injected into HPLC system. The retention time for FBS treated probe samples was monitored over time.

Statistics

Quantitative data are expressed as means $\pm$ standard deviation (SD). Means were compared using Student's *t* test. *P* values <0.05 were considered statistically significant.

3.4 Results

Synthesis of peptides

The synthesis of peptides was straightforward on the solid resin, following coupling sequences of c-F-L-F-L-F-K and c-L-F-F-F-L-K. PEG_{3.4kDa} was conjugated to ω -NH₂ of lysine (K) in aqueous solution. Cy 7 conjugation was accomplished with moderate yields of 60 $\pm$ 1% (*n*>3) (Scheme 3-1). The HPLC retention time of cFLFLF-PEG **3-4**, cFLFLF-PEG-Cy7 **3-6**, cLFFFL-PEG **3-5**, and cLFFFL-PEG-Cy7 **3-7** was 18.0 min, 16.7 min, 17.9 min and 16.6 min, respectively. The non-conjugated peptides could be readily separated from Cy7 conjugated products by semi-preparative HPLC. The chemical purities of Cy7 conjugates **3-6** and **3-7** were >95% based on analytical HPLC. The molecular weights of peptides and Cy7 conjugates were characterized as followed: (1) cFLFLF-PEG **3-4** was previously reported (7); (2) cLFFFL-PEG **3-5**, the average calculated mass was 4343 and major *m/z* peaks were observed at 4300–4477 with center

peak at 4345; (3) cFLFLF-PEG-Cy7 **3-6** (14), average calculated mass was 5007 and major m/z peaks were distributed at 4929–5150 with center peak at 5000; (4) cLFFFL-PEG-Cy7 **3-7**, the average calculated mass was 5007 and major m/z peaks were observed at 4864–5040 with center peak at 5000. Detailed mass spectra of various compounds are shown in Appendix 3-3.

Hydrophilicity of probes

The log *P* values of probe **3-6** and **3-7** were calculated to be -1.13 ± 0.01 and -1.02 ± 0.10 , indicating their similar hydrophilicity and blood solubility which enabled the direct comparison of *in vivo* imaging between the two probes. Moreover, the log *P* value of fluorescence probe **3-6** was also comparable to that of our previous reported PET probe (cFLFLF-PEG- ^{64}Cu , log *P*, -1.21) (7), suggesting the major contribution of PEG moiety in adjusting the hydrophilicity of the whole molecule.

Fluorescence spectra

Cy7 conjugates exhibited their maximum fluorescence emission wavelengths (λ_{max}) at 773–774 nm (Figure 3-1), while λ_{max} of Cy7 was at 771 nm in this solvent system (14). The conjugation of peptides and Cy7 did not significantly alter the fluorescence emission spectra of Cy7 dye. The peptide conjugates showed only 2-3 nm red shifts in their λ_{max} . Moreover, although λ_{max} of both Cy7 and Cy7 conjugates (**3-6** and **3-7**) were at around 770–780 nm, a slightly different emission wavelength (800 nm) was selected for fluorescence imaging on Xenogen IVIS Spectrum to take advantage of the existing filter pair of 745/800 nm ($E_{\text{x}}/E_{\text{m}}$) in the instrument.

In vivo model for monitoring inflammation

Ear redness could be visualized immediately after PMA (Figure 3-2A) application. Ear thickness was measured as a function of time until 96 h after PMA application (Figure 3-2C). Inflamed ear thickness increased significantly from 0.23 ± 0.02 mm to 0.45 ± 0.03 mm within 6 h and remained relatively at that level until 48 h and began to drop down slowly. Therefore, the time frame of 24–48 h after PMA application was chosen to inject fluorescence probes for monitoring neutrophil infiltration at inflamed areas and perform longitudinal NIRF imaging.

After 24 h of PMA treatment, bioluminescence was locally emitted from PMA-treated ears (left ears), suggesting local generation and accumulation of MPO as a measurement of neutrophil accumulation and activation at the inflammation site (Figure 3-2B).

Evaluation of the intensity of ear inflammation was sought by varying PMA doses applied on left ears. Microscopic images of both left and right ear sections were examined. All of the four doses chosen indeed invoked inflammation as suggested by large amount of neutrophil infiltration and accumulation (dark brown cells) in the left PMA-challenged ear (Figure 3-2D) while only a few neutrophils were observed in the right control ear (primarily in blood vessels) (Figure 3-2E). This observation also confirmed that inflammatory process was highly tissue selective (in this case only left ear) and triggered by PMA challenge at site-specific manner in the whole body of mouse. Quantification of PMA-induced inflammation was performed based on two factors: percentage of neutrophil accumulation to total cell amount and percentage of increase in left ear thickness after PMA application. Except the lowest PMA dose of $0.5 \mu\text{g}$ per $20 \mu\text{L}$ vehicle, all other doses did not show much of difference in both factors (the intensity

of inflammation in terms of neutrophil recruitment appears to remain the same), and therefore, the dose of 5 μ g per 20 μ L vehicle was selected for further studies (Figure 3-2F).

In vivo NIRF imaging

Representative NIRF images of all four groups (inflamed, blocking, scrambled and negative control) of mice were exhibited longitudinally (Figure 3-3). Fluorescence signal could be clearly visualized in the PMA-challenged ear as early as 30 min p.i. (image not shown). The inflamed group injected with probe cFLFLF-PEG-Cy7 **3-6** exhibited the strongest fluorescence signal in left ears among all groups at all time points. The absence of *in vivo* fluorescence signal from other organs was attributed to low injection dose (2 nmol) of probes and skin scattering.

The fluorescence intensities of PMA-challenged ears (left) for groups A, B and C (inflamed, blocked, scrambled) and those of non-PMA challenged group D (negative control) were plotted against various time points post probe injection as depicted in Figure 3-4B. For the inflamed group, fluorescence intensity in left ear reached the maximal level at 3 h p.i., and showed a two-phase decay: in the first phase, fluorescence intensity decreased relatively quickly by 6 h p.i.; in the second phase, a much slower decreasing trend was observed until 24 h p.i. The scrambled group demonstrated a similar trend of signal change: maximal signal intensity was also observed at 3 h p.i. but in an “attenuated” pattern compared with that of inflamed group. On the other hand, the blocking group did not show similar trend: it exhibited a consistent drop in intensity from the beginning and never attained any maximal value. It is interesting to note that fluorescence intensity of inflamed ears in the inflamed group were 2.5 and 3-fold greater

at 3 h p.i. and 3.2 and 4-fold greater at 24 h p.i. compared with that of scrambled and blocking groups, respectively. In addition, fluorescence intensity of negative control mice as expected exhibited low signal strength with pattern very similar to either blocked or scrambled group and signals from left and right ears were overlapping with each other at all time points. It was also interesting to note that fluorescence intensity of right ears of both scrambled and blocked group were lower than that of left ears and this is presumably due to redistribution of blood since the amount of blood flow in inflamed areas is more than that in non-inflamed ones.

Organ uptake of probe 3-6

Ex vivo evaluation of probe **3-6** on dissected organs at 24 h p.i. revealed the fluorescence signal mainly existed in inflamed ear, liver and kidney (Figure 3-5). The strong fluorescence intensity of PMA-challenged ear indicated the neutrophil binding of cFLFLF-PEG-Cy7 **3-6** led to a very slow clearance of the probe from the inflammation site. The high uptake in liver suggested that this probe might be subjected to hepatobiliary clearance pathway. This biodistribution pattern was comparable to our previous study on PET tracer cFLFLF-PEG-⁶⁴Cu that also demonstrated the highest uptake in liver and kidney 18 h p.i. (7). It is of note that liver or kidney was not visible by the whole animal imaging.

Immunohistochemical staining and fluorescence microscopy

IHC analysis was carried out to assess the relative amount and distribution of neutrophils in tissue slices of PMA-challenged ear versus control ear at 3 h p.i. (27 h after PMA application) due to the maximal fluorescence intensity of PMA-challenged ear at this time point. As can be seen in Figure 3-6A, PMA-challenged ear showed large

amount of neutrophil accumulation (A1, rat-anti-mouse neutrophil antibody stained neutrophils in dark brown; A2, black-and-white version of A1 under confocal microscope) and the fluorescence signal of probe **3-6** (A3, red color) correlated well with neutrophils (A4, fused image of A2 and A3). On the contrary, as indicated in Figure 3-6B, control ear revealed very few neutrophils (B1) and little fluorescence signal was observed (B3 and B4). This microscopic result further confirmed *in vivo* neutrophil-specificity of probe **3-6**.

Serum stability

Figure 3-7 demonstrated that the fluorescence signal of probe **3-6** and **3-7** remained stable up to 24 h in FBS, indicating that the fluorescence signal loss over time of *in vivo* imaging was attributed to the clearance and not due to decomposition of probes.

As exhibited in Figure 3-8, the retention times for FBS treated probe samples were observed at around 14 min for all time points. After 24 h incubation with FBS serum, peak area remained more than 95%, which suggested that the probe was stable at 24 h.

3.5 Discussion

In order to develop a NIRF imaging probe for *in vivo* imaging of inflammation and infection in live research animal, we have designed and synthesized a series of Cy7 conjugated agents with varied lengths of PEG moieties – cFLFLF-PEG_n-Cy7 (n = 0, 12, 76) (14). The suitable water solubility of the PEG_{3.4kDa} modified cFLFLF-PEG-Cy7 **3-6** along with other favorable pharmacokinetic parameters renders its further investigation *in vivo*.

One of the major concerns in the development of imaging agent for neutrophil to monitor inflammation has been the specificity of the probe and most of the current

research advancements are focused on this aspect (20). In this report, we demonstrated the neutrophil-specificity of fluorescence probe **3-6** by comparing *in vivo* NIRF imaging of the inflamed group with the blocked and scrambled groups as well as correlating IHC staining of neutrophils at cellular level with fluorescence microscopic study. In addition, evidence is provided by luminol-bioluminescence imaging of mice (24 h after PMA application) by comparing left inflamed ear with right control ear indicating presence of functionally activated neutrophils in the inflamed area.

The neutrophil-specificity of probe **3-6** was clearly demonstrated in the macroscopic *in vivo* imaging study (Figure 3-3). The imaging was highly distinct and clearly showed the left inflamed ear being more fluorescent intense than the right ear. The inflamed ear not only retained fluorescence over time but also showed improved signal to noise ratio when compared with the control ear along with mice without inflammation (negative control group). Although tissue distribution of the fluorescence probe after dissecting the organs indicated presence of significantly large amounts of probe in liver and kidney, this characteristic however, did not interfere with live imaging. We attribute this loss of fluorescence signal to the scattering of deep tissue. Advantage of this technique is to acquire good sensitivity for the fluorescence signal in inflamed areas compared with background but the disadvantage is that only surface organ or tissue can be monitored for inflammation and additional tools may be needed for the detection of inflammatory process in deeply located organs.

When probe **3-6** injected in live mice with inflammation on left ear, maximal fluorescence signal was observed within 3 h p.i. (climbing phase) and subsequent decrease in the signal intensity between 3 to 6 h p.i. (fast clearance phase) followed by

much slower loss of signal from 6 to 24 h p.i. (slow clearance phase) of the left ear, while the right ear has very less fluorescence signal (~ 3.4 fold less) and showed continuous loss of intensity indicating the amount of circulating neutrophils in the blood, its distribution volume and accumulation in inflamed areas and clearance from non-inflamed areas thus improving the signal to noise ratio (resolution) of the probe. The observed pharmacokinetics of probe **3-6** in mice can be attributed to the combination of three factors: (1) the trafficking and recruitment of activated neutrophils bound to fluorescence probe **3-6** in inflamed areas from circulation along with free flowing probe **3-6** in the blood reaching to inflamed site and targeting already recruited neutrophils; (2) the clearance of free probe **3-6** in blood from circulation; and (3) onsite leftover probe from dead neutrophils and its clearance. The climbing phase was assumed to be caused by factor (1) since inflammation manifests more blood flow. After maximal trafficking and trapping of probe **3-6** was reached in the inflammatory site, blood clearance of the free fluorescence probe **3-6** dominated the fast clearance phase between 3 to 6 h p.i. The nuclear version of this probe, cFLFLF-PEG- ^{64}Cu , was reported with blood half-life of 55 ± 8 min (7). The slow clearance phase of fluorescence signal (after 6 h p.i.) was indicative of retention of neutrophil-bound probe in inflamed areas, which was presumably attributed to poor neutrophil clearance and probe degradation from dead neutrophils. This observation was further confirmed by the blocking group, in which the fluorescence signal was consistently decreasing because neutrophils were pre-occupied with non-fluorescence peptide **3-4** and the blood clearance of floating fluorescent probe **3-6** dominated pharmacokinetics. For the scrambled group, similar pharmacokinetics was observed as the inflamed group for accumulation of signal in 3 h p.i., however in an

“attenuated” pattern due to absence of neutrophil-specificity of scrambled probe **3-7**. The maximal peak at 3 h p.i. was assumed to be caused by enhanced permeation (increased blood flow) to inflammatory site (Figure 3-4B).

The immunohistochemical and fluorescence microscopic data provided evidence that the fluorescence probe **3-6** was indeed associated with neutrophils at the inflammation site while few neutrophils and little fluorescence were observed in control tissue. It was also confirmed that microscopic study of neutrophil activation and trafficking with the fluorescence probe **3-6** at tissue and cellular levels is feasible. The results from the studies demonstrated here suggest that the high potential for *in vivo* imaging of inflammation by recruiting activated neutrophils to inflamed site is possible. However, to quantify the inflammation and to monitor it for therapeutic intervention will be the next highly challenging task.

3.6 Conclusion

We have successfully performed longitudinal NIRF imaging study of PMA-induced ear inflammation in live mice using fluorescence probe **3-6**. With this imaging probe, macroscopic and microscopic visualization of neutrophilic activation and infiltration is enabled and it will not only deepen our understanding on the inflammatory process but also facilitate the pre-clinical screening of anti-inflammatory agents.

3.7 Figures and Scheme

Scheme 3-1. Synthesis of fluorescence imaging probes. Reagents and conditions: (i) (ii) standard solid phase Fmoc chemistry; (iii) 1. *t*-Boc-NH-PEG_{3,4kD}-NHS, 2. pre-chilled trifluoroacetic acid (95%); (iv) Cyanine7 Mono NHS ester, CH₃CN/sodium borate buffer, pH 8.5, 4°C to room temperature.

Figure 3-1. Representative fluorescence emission spectra of cFLFLF-PEG-Cy7 **3-6** and cLFFFL-PEG-Cy7 **3-7** in DMSO/H₂O (v/v, 1/9) with the excitation wavelength at 745 nm and max emission observed at 773–774 nm.

Figure 3-2. *In vivo* model for inflammation monitoring. (A) Chemical structure of PMA. (B) Representative luminol-bioluminescence imaging of mice at 24 h after PMA application on left ears to locate the inflammation *in vivo*. (C) Inflamed ear (left) thickness of mice ($n=4$) at various time points before and after PMA application at the constant dosage of 5 μ g per 20 μ L vehicle (DMSO:acetone=1:9). (D) and (E) Immunohistochemical staining of neutrophils (with rat-anti-mouse neutrophil IgG) and hematoxylin staining of both left inflamed ear (D) and right control ear (E) of PMA-challenged mice (magnification, $\times 100$), (F) Correlation of increase in ear thickness and accumulation of neutrophils in the inflamed left ears as plotted against various PMA dosages [0.5, 1.5, 5 and 50 μ g per 20 μ L vehicle (DMSO:acetone=1:9)].

Figure 3-3. Representative *in vivo* NIRF imaging of a single mice at 1 h, 2 h, 3 h, 6 h and 24 h post probe injection. (A) Inflamed group: PMA-challenged mice injected with probe cFLFLF-PEG-Cy7 **3-6** (2 nmol). (B) Blocked group: PMA-challenged mice pre-injected with excess of non-fluorescence peptide cFLFLF-PEG **3-4** (100 nmol) 1 h before the injection of probe cFLFLF-PEG-Cy7 **3-6** (2 nmol). (C) Scrambled group: PMA-challenged mice injected with scrambled peptide probe cLFFFL-PEG-Cy7 **3-7** (2 nmol). (D) Negative Control group: mice without PMA challenge injected with probe cFLFLF-PEG-Cy7 **3-6** (2 nmol).

Figure 3-4. Analysis of epi-fluorescence images. (A) Three regions of interest (ROI) were depicted in a representative fluorescence image of inflamed group. ROI 1 and ROI 2 were drawn around both earlobes while ROI 3 was drawn in a dark area of the image as control. Fluorescence intensities of left and right ears were obtained by subtracting control ROI 3 from ROI 1 and ROI 2, respectively. (B) Fluorescence intensity of left and right ears as a function of post probe injection time. Eight lines represent four experimental groups (inflamed, blocked, scrambled and negative control) and two lines (left and right ears) for each group. For all groups, average fluorescence intensities ($n=5-6$) were plotted. Only for the inflamed group, standard deviation (STDEV) was shown due to the significant difference ($P < 0.0005$) between signal intensities from left and right ears.

Figure 3-5. Organ biodistribution of probe cFLFLF-PEG-Cy7 3-6 at 24 h pi. Fluorescence intensity measured from various organs and tissues represented in average radiance (p/s/cm²/sr) in the column. Upper right inset, representative fluorescence image of dissected organs and tissues.

Figure 3-6. Neutrophil-specificity of probe cFLFLF-PEG-Cy7 **3-6** at tissue and cellular level. Immunohistochemical staining of neutrophils (with rat-anti-mouse neutrophil IgG) and the corresponding microscopic fluorescence image of left ear (A1-A4) and right ear (B1-B4) of inflamed group at 3 h pi. Immunostained neutrophils appear dark brown (A1) while few neutrophil was shown in control ear (B1). Black-and-white images of the slices (A2, B2) could be fused with fluorescence images (A3, B3) to generate fused ones (A4, B4) that reveal the neutrophil-specific binding of the probe (magnification, $\times 400$).

Figure 3-7. Fluorescence stability probe cFLFLF-PEG-Cy7 and cLFFFL-PEG-Cy7 in serum. The fluorescence intensity of both probes was plotted against incubation time (5 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h and 24 h) with FBS at 37 °C (error bars were within the dots).

Figure 3-8. Representative HPLC chromatograms for serum stability study of probe cFLFLF-PEG-Cy7 **3-6**. HPLC chromatograph of intact probe 6 was demonstrated by red color with a retention time of 14.2 min; and after incubation of probe 6 with serum for 24 hours, little change (retention time and integration area “A”) could be observed on the new chromatograph (indicated by black color).

3.8 Appendix

Appendix 3-1. Quantification of cells by Image J

The number of brown and blue cells in pictures with 400 magnifications was counted using “Cell Counter” function of Image J software [ImageJ for microscopy. Tony J. Collins. BioTechniques. 2007; 43:S25-S30.]. The cells could be counted according to the procedure as followed: 1. Open the image 2. Select “Plugins”, go to “Analyze”, and select “Cell Counter”. A new window (Cell Counter) will be opened. 3. Click “initialize” in the “Cell Counter” window. 4. To begin counting, select a “counter type” and then click directly on a cell of interest. A representative image of the counting window of Image J is shown below.

In the image above, neutrophils (brown cells) were labeled by dark blue dots and all other cells (blue cells) were labeled by light blue dots. Both cell numbers could be clearly monitored in the “Cell Counter” window (type 1 and type 2). Total cell count is calculated by summing the number of brown and blue cells. The percentage of neutrophil to total cell number was calculated accordingly.

Appendix 3-2. Neutrophil immunohistochemical (IHC) staining protocol for mouse ear

1. Frozen ear tissue was cut into slices with 5 μ m thickness.
2. Allow dryness in air for 1-2 h.
3. Dip the slices into Aceton/EtOH (1:1) (pre-chilled to -80 °C for 15 min) for 10 min at -30 °C.
4. Wash slices with Dako Cytomation wash buffer (1 x) for 3 times, with 5 min each time.
5. Leave the rack of slices in Dako Cytomation wash buffer (1x)
6. Prepare reagents at the following dilution factors and
7. Load the slices on Autostaining, set up protocol following the table described as below. (The entire process took about 3 hours to complete.)

Protein blocking	Protein blocking	End Enzyme blocking	Pre-treatment	Primary Ab	Secondary reagent	Tertiary reagent	Substrate	Auxiliary
Avidine 100 μ L	Biotin 100 μ L	DEEB 100 μ L	No AR (manual step) 100 μ L	Neu or buffer 1:200 100 μ L	RAR-b 1:200 100 μ L	ST/AV 1:200 100 μ L	DAB+	Hematoxylin
15 min	15 min	10 min	30 min	30 min	30 min	30 min	10 min	5 min

- a. wash slices with DI water between each step.
- b. Buffer used in this protocol should be Dako Cytomation wash buffer
- c. Avi & Biotin used to block internal protein, no need to dilute
- d. Manuel step denotes that slices were treated prior to loading on the autostaining
- e. DEEB: Dual endogeneous enzyme block (mainly to block two enzymes, alkaline phosphatase and peroxidase)
- f. Neu Ab: Rat anti mouse neutrophils (Allotypic marker), MCA771G, AbD serotec
- RAR-b: Rabbit anti rat antibody
8. Take slice out , dip into 95%EtOH for three times, 100%EtOH for three times and finally xylene twice.
9. Add xylene based glue dropwise on slices and cover the cover slip carefully (try to avoid any air bubbles).
10. Allow dryness in air (attention: tissue slices with fluorescence probes should be shielded for light).

Appendix 3-3. MALDI-TOF mass spectra of various compounds

Mass spectrum of compound **3-3**

Mass spectrum of compound **3-5** Mass spectrum of compound **3-5** (expanded)

Mass spectrum of the scrambled imaging agent **3-7**

Mass spectrum of the scrambled imaging agent **3-7** (expanded)

Appendix 3-4. Microscopic images of ear tissue slices (inflamed and control) with hemotoxylin and eosin (H/E) staining at various magnifications.

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Chapter 4: Evaluation of A Neutrophil Targeting Heterobivalent SPECT Imaging

Probe: cFLFLF-PEG-TKPPR-^{99m}Tc

4.1 Abstract

A new heterobivalent peptide ligand specifically targeting polymorphonuclear leukocytes (PMNs) with favorable pharmacological parameters to monitor sites of inflammation for imaging is designed. The detailed synthesis, characterization and pharmacological evaluation of the ligands are reported here. Two separate peptide binding ligands for formyl peptide and tuftsin receptors were chosen to link together based on the high expression levels of the two receptors on activated PMNs. The heterobivalency and pegylated links were incorporated in the structural design to improve the sensitivity of the detection and to improve the bioavailability along with blood clearance profile, respectively. Two chemical constructs: cFLFLF-(PEG)_n-TKPPR-^{99m}Tc (n=4 and 12) were evaluated *in vitro* with human PMNs for binding affinity and bioavailability. As a result, cFLFLF-(PEG)₁₂-TKPPR-^{99m}Tc was found to have more favorable pharmacological properties and was therefore used for further *in vivo* studies. Preliminary *in vivo* assessment of the agent was performed using Single Gamma Emission Computed Tomography (SPECT) imaging of a mouse model of ear inflammation. The results of these studies indicate cFLFLF-(PEG)₁₂-TKPPR-^{99m}Tc may be a desirable imaging agent for binding to PMNs to identify sites of inflammation by SPECT.

4.2 Introduction

High-quality nuclear imaging can be a useful diagnostic and prognostic tool for detecting sites of inflammation. This should aid in designing therapies to control pathological conditions. However, the ability to detect and characterize inflammation has been so far elusive (1). One of the hallmarks of inflammation is migration and activation of leukocytes. Current leukocyte imaging techniques include *ex vivo* white blood cells labeled with ^{67}Ga -citrate, ^{111}In or $^{99\text{m}}\text{Tc}$ containing complexes and re-injection of the labeled cells back into patients. Although the utility of this method is proven, the process is laborious and involves handling of blood products, thereby increasing the risk of contamination. Tracking neutrophils by intravenous administration of highly specific radiolabeled binding probes would circumvent these limitations. As a result, numerous chemotactic peptides, such as fMLF, and its analogs have been investigated as potential neutrophil imaging probes (2, 3). However, exhibition of undesired biological side effects and/or poor pharmacokinetic parameters have limited their clinical utility.

We have recently reported a novel PET imaging agent, cFLFLF-PEG- ^{64}Cu (PEG = 3.4 kDa), which binds to the formyl peptide receptor (FPR) without prompting neutrophil activation and, in addition, has favorable pharmacokinetic characteristics such as increased hydrophilicity to minimize non-specific liver uptake (4, 5). However, the high cost of PET imaging and the limited supply of the cyclotron-produced ^{64}Cu isotope may restrict its widespread application in research and clinics. A $^{99\text{m}}\text{Tc}$ version of the probe for Single Gamma Emission Computed Tomography (SPECT) imaging of neutrophils provides an attractive alternative because of $^{99\text{m}}\text{Tc}$'s wider availability and lower cost. In addition, to further enhance the detection sensitivity, binding affinity and

specificity of the ligand towards neutrophils, we designed a heterobivalent peptide, cFLFLF-PEG-TKPPR-^{99m}Tc, that simultaneously targets the FPR and tuftsin receptor. Heterobivalent ligands have demonstrated higher affinity than monovalent and symmetrical bivalent binders (homodimer) (6, 7).

Because of inclusion of the polar TKPPR sequence in the present heterobivalent ligand design, the size of PEG linker was reduced compared with larger PEG linker reported earlier in cFLFLF-PEG-⁶⁴Cu (PEG = 3.4 kDa) in order to maintain roughly the same water solubility. Thus, the two binding peptide sequences were linked either through a bifunctional PEG₄ or PEG₁₂ moiety. An additional lysine residue was incorporated in the construct as a handle for HYNIC group and its coordination to radio-metal (Scheme 4-1). We wish to report here the detailed synthetic method, the observed pharmacokinetic parameters for these new probes and a preliminary demonstration of its application to SPECT imaging of inflammation *in vivo*.

4.3 Materials and Methods.

All chemicals obtained commercially were of analytical grade and used without further purification. Na^{99m}TcO₄ was obtained from Cardinal Health INC (Charlottesville, VA). 6-Boc-hydrazinonicotinic acid (6-Boc-HYNIC acid) was obtained from SoluLink. (San Diego, CA). Fmoc-Arg-PS resin was purchased from Applied Biosystems (Foster City, CA). Fmoc-amino acids were purchase from Anaspec Inc. (Fermont, CA). N-Fmoc-amino-dPEGTM₄ acid and N-Fmoc-amino-dPEGTM₁₂ acid were purchased from Quanta Biodesign, Ltd. (Powell, OH). N-hydroxysulfosuccinimide (Sulfo-NHS) and 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC) were purchased from PIERCE (Rockford, IL). All other chemical reagents and solvents were

obtained from Sigma-Aldrich. For purification of peptides precursors and ^{99m}Tc labeled products, semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC) was performed with an Apollo C18 reversed-phase column (5 μm , 250 $\times$ 10mm) on a Varian system with ABI Spectroflow 783 UV detector and Bioscan NaI solid scintillation Flow Count Radio-HPLC detector. The mobile phase was changed from 60% Solvent A (0.1% TFA in water) and 40% Solvent B (0.1% TFA in 80% aqueous acetonitrile) to 100% Solvent B at 30min at a flow rate 3mL/min. MALDI-TOF mass spectroscopy analysis was performed on samples of peptide products at the W.M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia (UVa) and the data was obtained on a Bruker Daltonics system (Billerica, MA). Peptides were synthesized manually by standard solid-phase method, following a conventional Fmoc strategy using 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as the coupling agent. Lysine was used as handle to incorporate hydrazinonicotinic acid (HYNIC) to the peptide. High performance liquid chromatographic (HPLC) analysis of the non-radiolabeled compounds was performed on a Varian Prostar system equipped with a ProStar 335 HPLC Diode Array Detector. HPLC solvents were purchased from Fisher Scientific (Pittsburgh, PA) and used without further purification. Typical yields of the crude peptides were 80-85%.

Human tumor necrosis factor- α (TNF- α) was procured from Perpotech, and fMLF was purchased from Sigma. Aliquots of both samples were taken (TNF- α , 10 U/mL, and fMLF, 10 mM) and stored at -20 °C. For every assay the solutions were thawed to ambient temperature and freshly diluted with hepatic arterial (HA) buffer before use. Multiscreen high-throughput screening (HTS) with glass fiber filter (FC) 96-well plates,

type C, with 1.2-mm glass filters were purchased from Millipore. Filtration from 96- well plates was performed under vacuum on a Brandel filtration device. The membranes from each well were collected by punching with the Millipore Multiscreen punching instrument. The radioactivity from ^{99m}Tc -bound ligand was measured with either Minaxi (Packard), Autogamma 5000 series (Packard), or Wallac 1420Wizard (Perkin-Elmer) γ -counters. Radioactivity was measured for 1 min per sample and was not corrected for decay.

Human neutrophils were prepared from normal heparinized (10 U/mL) venous blood by a 1-step Ficoll-Hypaque separation procedure (8, 9), yielding approximately 98% neutrophils; greater than 95% viable as determined with trypan blue containing less than 50 pg/mL of endotoxin. After separation, neutrophils were washed with Hank's balanced salt solution with heparin (10 U/mL) 3 times. After the third wash, neutrophils were resuspended in HA buffer, which was Hank's balanced salt solution supplemented with 0.1% human albumin (Bayer Healthcare). Neutrophil experiments were completed in HA buffer.

Female FVB mice, 5 months old, were purchased from the National Cancer Institute (Frederick, MD). Mice were housed in a controlled environment (12 h light/12 h dark photoperiod, $22\pm 1^\circ\text{C}$, $60\pm 10\%$ relative humidity) and were provided free access to autoclaved pellet food and tap water. All procedures were in accordance with current National Institutes of Health (NIH) guidelines and were approved by the University of Virginia Animal Care and Use Committee (ACUC).

SPECT/CT imaging was performed with a microSPECT/CT scanner designed and built at UVa under NIH funding (10). The scanner uses an open-barrel type gantry

consisting of two 38" steel wheels connected by aluminum profile pieces. CT and SPECT scanning are performed sequentially and the animal is translated axially from one subsystem to another between two fixed locations along a 1" diameter carbon fiber half cylinder that is located at the gantry axis of rotation. Reproducibility of animal table location permits consistent and simple fusion of CT and SPECT images using stored offset parameters. The scanner can be operated with two different types of x-ray detectors: CCD-based for high spatial resolution (modulation transfer function in the reconstructed CT image of 0.1 at a spatial frequency of 14.7 mm^{-1} when used with an 8 micron x-ray source) or CMOS-based for fast readout (2 frames per second). Microfocus x-ray sources with focal spot sizes ranging between 8 and 50 microns are available. SPECT scanning can be performed using up to four gamma cameras (10 cm x 10 cm fields of view) simultaneously. Respiratory and cardiac gating are available for both CT and SPECT scanning. CT projection images are preprocessed for detector sensitivity uniformity correction and dark count subtraction using a custom written IDL program and then are reconstructed with a Feldkamp 3-dimensional filtered backprojection algorithm (COBRA, Exxim, Inc., Pleasanton, CA). SPECT data are acquired using a custom written interface using Kmax (Sparrow Corp., Port Orange, FL) software and reconstructed using a custom written maximum-likelihood expectation-maximization algorithm (11).

Synthesis of cFLFLF-PEG_n-K(HYNIC)TKPPR 4-6 and 4-7

As shown in Scheme 4-1, Fmoc-Arg-PS resin **4-1** (150 mg, 0.21 mmol/g) was loaded to a peptide synthesis vessel, suspended in DMF (2mL) and shaken at 600 rpm for 5 min and drained. Fmoc was removed by adding 2 mL of 20% piperidine-DMF solution to the synthesis vessel. The vessel was shaken on a vibrator (Labnet Shaker 20,

Woodbridge, NJ) for 5 min and the solution was drained. The same deprotection step was repeated once again by shaking for 20 min. The deprotected resin was washed six times each with 2mL of DMF. The resin was then coupled to the Fmoc-protected amino acid by successively adding 4 molar excess of the Fmoc-protected amino acid (0.84 mmol) in DMF (0.1 M), 4 equivalents of HBTU in DMF (0.1 M), and 8-fold excess of DIEA (1.68 mmol). The reaction vessel was shaken for 90 min. The reaction solution was drained off and the resin was washed with 3 x 2mL of DMF. The peptide resin was subsequently capped by adding 10 fold molar excess of acetic anhydride (0.5mL) and DIEA and shaking at 600 rpm for 30 min. After draining off the solution the resin was washed with 6 x 2mL of DMF and was then ready for the next cycle of coupling. To obtain the resin-bound peptide sequence **4-4**, the amino acids and special residues were coupled in following order: Fmoc-Pro, Fmoc-Pro, Fmoc-Lys(*t*-Boc), Fmoc-Thr(*t*-Bu), Fmoc-Lys(ivDDE), N-Fmoc-amino-dPEGTM₄ acid or N-Fmoc-amino-dPEGTM₁₂ acid, Fmoc-Phe, Fmoc-(D)Leu, Fmoc-Phe, Fmoc-(D)Leu, Fmoc-Phe, and Trans-cinnamic acid. The ivDDE protecting group of **4-4** was removed to free the ω -NH₂ of the lysine by treatment with 2% hydrazine in DMF for 60 min. After draining, the peptide resin was repeatedly washed with DMF (2 mL) for six times. To conjugate the chelating HYNIC moiety at the ω -NH₂ of the lysine a solution of 4 molar excess of 6-Boc-HYNIC acid, 4 equivalents of HBTU, and 8 equivalents of DIEA in 1.6 mL of DMF was added into the reaction vessel. The vessel was shaken for 90 min, the solution was drained off, and the resin was washed with DMF (2 mL) for three times. The peptide cFLFLF-PEG_n-K(HYNIC)TKPPR **4-6** or **4-7** was cleaved from the resin support using a 95% TFA. The crude products were

purified with HPLC conditions described above. The molecular weights of the purified precursors were verified by MALDI-TOF Mass Spectroscopy.

*Synthesis of cFLFLF-PEG_n-TKPPR-^{99m}Tc **4-8** or **4-9***

To a 1.5 mL vial was consecutively added 100 μ g of cFLFLF-PEG_n-K(HYNIC)TKPPR **4-6** or **4-7** in 200 μ L of citrate buffer (10 mM, pH 5.2), 200 μ L of tricine solution (30 mg/mL in 10 mM citrate buffer, pH 5.2), 100 μ L of nicotinic acid solution (10 mg/mL in 10 mM citrate buffer, pH 5.2), 37 MBq of ^{99m}TcO₄⁻ solution (370 MBq/mL in saline), and 25 μ L of SnCl₂ solution (1.0 mg/mL in 0.1 N HCl). The reaction mixture was stirred by shaking and heated at 60°C for 15 min. After being cooled to room temperature for 10 min, the reaction mixture was purified by same HPLC conditions mentioned above. The observed retention times of cFLFLF-PEG₄-TKPPR-^{99m}Tc **4-8** and cFLFLF-PEG₁₂-THPPK-^{99m}Tc **4-9** were 16.8 and 17.8 min, respectively.

Partition coefficient

Freshly HPLC purified cFLFLF-PEG_n-KTKPPR-^{99m}Tc **4-8** or **4-9** (~350 kBq) in 500 μ L of water was mixed with 500 μ L of octanol in an Eppendorf microcentrifuge tube. The tube was sonicated for 10 min and then centrifuged at 4,000 rpm for 5 min (Fisher Scientific Marathon Micro-A). Aliquots (100 μ L) of octanol and aqueous layers were carefully separated and radioactivities of samples were counted. Log *P* is derived as the log of the ratio of radioactivity in octanol to radioactivity in water. The measurement was repeated in triplicate and data presented is the average of three measurements.

Neutrophil binding

Freshly isolated human neutrophils (4x10⁶ cells/mL) were treated with TNF- α (10 U/mL, Peprotech) twenty minutes prior to binding studies and transferred to a 96 well

plate (Multiscreen® HTS FC by Millipore, Billerica, MA. 1.2 μm glass filter type C, 50.0 μL , $\sim 2.0 \times 10^5$ cells/well). Saturation assays were carried out using eight different concentrations of cFLFLF-PEG₁₂-TKPPR-^{99m}Tc ranging from 1.0 μM to 0.01 nM incubated at 25 °C for 90 min. Neutrophils were incubated with the excess radioligand at 25 °C for 90 minutes to obtain total binding. Following incubation, the plates were filtered rapidly under vacuum using Brandel filtration device (Brandel Inc. Gaithersburg, MD), washed three times with cold Tris-Mg buffer (-5 °C, 10mM, 150 μL each time/well) to remove the unbound radioligand, and dried under vacuum. The plates were then wiped with Kimwipes EX-L at room temperature and membranes from each well were collected by Millipore multiscreen punching instrument (Billerica, MA). The bounded radioactivity remaining on the membranes was measured in a gamma counter. Specific binding was calculated as the difference between total binding and nonspecific binding at the highest concentration. Non-radioactive peptide (cFLFLF-PEG₁₂-TKPPR-^{99m}Tc) was used in excess (100 μM) to determine non-specific binding. Saturation experiment data (K_d and B_{max} values) were obtained by computer analysis using PRISM 4.0 (GraphPad).

Neutrophil cell viability

Mice neutrophils were separated following previous established method (Appendix 4-2) and re-constituted in PBS with a cell density of 2.2×10^6 cells/mL. The cell suspension was divided into two groups. Both groups included three conical tubes with 200 μL /tube. Group 1 was added with 100 μL of radio-labeled peptide (370 kBq, 10 μCi) and group 2 was added with 100 μL of PBS at room temperature. After adding the radio-labeled compound for 10 min and 30 min, 10 μL of cell suspensions were taken out

from each tube and mixed with equal volume of trypan blue. The cell viability was determined by counting the non-stained and stained cell amounts on a hemocytometer.

Blood clearance

Blood kinetics of cFLFLF-PEG₁₂-TKPPR-^{99m}Tc was studied in 3 control (non-inflamed) mice. Approximately 50 µL of blood from the contralateral tail vein were collected in capillary tubes at 15, 30, 45, 60, 120, and 180, 360 min after tracer injection (0.37–0.74 MBq). The capillary tubes were placed in a vial that was weighed beforehand and afterward. Activity in each blood sample was measured in a radioisotope calibrated well counter (CRC-15W, Capintec, Inc, NJ), decay corrected back to the time of tracer injection, normalized for injected dose and animal body weight, and expressed as %ID/g of blood.

Serum stability of cFLFLF-PEG₁₂-TKPPR-^{99m}Tc 4-9

Fifty microcuries of the cFLFLF-PEG₁₂-TKPPR-^{99m}Tc was added in to 100 µL of fetal bovine serum (Invitrogen, Grand Island, NY). After incubation at 37°C for 1, 3, and 6 hrs, aliquots of the mixture was filtered through a 0.2 µM micro spin filter. The filtrates were analyzed by reverse-phase HPLC with a Bioscan Flow Count Radio-HPLC detector. All of newly formed γ-peaks besides the original cFLFLF-PEG₁₂-TKPPR-^{99m}Tc peak would be considered as degraded products.

Biodistribution

Body distribution of radioactivity was determined in control ($n=3$) mice 3 h and 18 h after injection of tracer. After a single blood sample had been taken from the tail vein, mice were euthanized by deep halothane anesthesia. The organs and tissues (ear, heart, lungs, muscle, bone, liver, kidney, spleen, small intestine, and stomach) were

collected, rinsed with PBS, wiped with filter paper and weighed in a pre-weighed vial. The radioactivity of each sample was measured in a γ -well counter and decay-corrected back to the time of tracer injection. Biodistribution values are expressed as a percentage of the injected dose (%ID) and normalized by body and organ/tissue mass.

Mouse ear inflammation model

A female FVB mouse, 5 month old, with left ear inflammatory lesion was used to preliminarily assess the ability of the cFLFLF-PEG₁₂-TKPPR-^{99m}Tc to detect inflammation as a result of neutrophils migration and accumulation. The animal model is similar to that described by Gross et al (12). Topical application of PMA (2.5 μ g in 20 μ l DMSO) onto the left earlobe induces acute dermatitis, manifested by local swelling, erythema and infiltration of neutrophils (13, 14). The right ear served as control and received only DMSO (vehicle). The SPECT/CT imaging was correlated with myeloperoxidase (MPO) activity determined by non-invasive luminol-bioluminescence imaging (12).

Luminal-bioluminescence imaging of myeloperoxidase

Since MPO is the most abundant protein in azurophilic granules of neutrophils, MPO specific luminal-bioluminescence study was carried out in support of the accumulation and activation of neutrophils in PMA-treated ear. The mice were injected with 200 μ l of luminol (5mg/0.1L DMF) intraperitoneally 22 h after PMA application. Immediately after luminal injection, a 40 min kinetic bioluminescence scan was performed on Xenogen IVIS Spectrum (Caliper life science, CA) and processed on Live Image software (Caliper life science) with open filter and exposure time of 60 s.

SPECT/CT imaging

Twenty-four hours after PMA challenge, 3.7 MBq (1 mCi) of cFLFLF-PEG₁₂-TKPPR-^{99m}Tc in 200 µl of saline was administered via tail vein. Three hours later, CT/SPECT imaging was performed using the microSPECT/CT scanner described above. Anesthesia (1%-2% isoflurane in oxygen) was delivered throughout the imaging procedure. CT projection data acquisition used the CMOS X-ray detector and obtained 200 evenly spaced projections spanning 240 degrees over approximately 5 minutes. For SPECT scanning sixty evenly spaced views were obtained over 180 degrees using two opposing gamma cameras simultaneously, resulting in a total of 120 projections over 360 degrees at an angular increment of 3 degrees. The acquisition time was approximately 45 minutes. The two cameras were fitted with 0.5 mm diameter tungsten pinholes. The reconstructed CT voxel size was 0.164×0.164×0.164 mm³ on a 320×320×384 image matrix. The reconstructed SPECT voxel size was 0.4×0.4×0.4 mm³ on a 60×60×60 image matrix. All SPECT images were corrected for radioactivity decay but not for gamma ray attenuation.

Image Analysis

Co-registration of SPECT and CT images was performed using ASIPRO software (Siemens Medical Solutions USA, Inc., Knoxville, TN) and a transformation matrix previously obtained with a dual modality phantom. To characterize the accumulation of the tracer in the ears, volume-of-interest (VOI) analysis was performed to identify the number of detected counts originating in the ears. CT images were used to identify voxels lying within the ear and the corresponding SPECT voxels were included in each VOI. Inflamed VOI was drawn tightly around the ear inflammation area on the SPECT

transaxial image slice, and copied to the control ear. Six slices were taken in both ears. The upper 60% signal (in counts/mL) was taken into calculation in each slice. The known gamma camera detection efficiency was used to compute the activity contained in the ears. This activity was expressed as percentage of the injected dose per mouse body mass (%ID/g).

4.4 Results and Discussion

Probe synthesis and radiolabeling

The precursors, cFLFLF-PEG_n-K(HYNIC)TKPPR **4-6** and **4-7**, were synthesized using standard Fmoc peptide chemistry on resin. Briefly, starting from arginine residue loaded on resin at carboxyl group, the TK(*t*-Boc)PPR peptide was constructed first, an additional lysine residue [Lys(ivDDE)] was added to form intermediate **4-2** prior to addition of varied pegylated linker. Then on the other side of the PEG the second cFLFLF peptide was built to produce the desired peptide sequence **4-4**. Finally the removal of ivDDE protecting group of lysine linker using basic hydrazine conditions released the side chain ω -NH₂ derivative **4-5** for conjugation with HYNIC. The resin bound HYNIC conjugated bivalent peptides **4-6** and **4-7** were cleaved with standard TFA chemistry. The two precursor peptides were purified by reverse phase HPLC with total yield in the range of 20 ~25%. The purified peptide constructs were characterized by mass spectroscopy for its composition. The calculated and observed MALDI-TOF-MS [M+H]⁺ were 1905 and 1906 for cFLFLF-PEG₄-K(HYNIC)TKPPR **4-6**, 2258 and 2259 for cFLFLF-PEG₁₂-K(HYNIC)TKPPR **4-7** (Appendix 4-1). The two radiolabeled cFLFLF-PEG_n-K(HYNIC)-TKPPR-^{99m}Tc **4-8** (*n*=4) and **4-9** (*n*=12) were obtained by conjugating **4-6** and **4-7** with stannous chloride reduced [^{99m}Tc]pertechnetate with

nicotinic acid and tricine as co-ligands, respectively. The radiolabeled peptides were purified from unlabeled precursors and other reagents by reverse phase semi-preparative HPLC with radiochemical yield >75% and radiochemical purity of 98%. The detailed synthetic method is described in Scheme 4-1.

In vitro evaluation

The *in vitro* binding studies to isolated human neutrophils exhibited K_d values 47.8 and 15.1 nM for ligand **4-8** and **4-9** respectively. The radiolabeled ligand **4-8** was roughly 3 fold less potent than compound **4-9**, possibly due to the longer PEG₁₂ linker allows the simultaneous binding to both the formyl peptide and the tuftsin receptors on activated neutrophils. The purified radioligands **4-8** and **4-9** were first evaluated for their log *P* values to estimate approximate lipophilicity of the individual compounds. The observed log *P* values for **4-8** and **4-9** are in agreement with the expected trend as PEG₁₂ linked found to be more hydrophilic (-0.89) compared to PEG₄ derivative (-0.77). Based on the better solubility in water and higher binding affinity the radioligand **4-9** was chosen for further *in vivo* studies.

The cFLFLF-PEG₁₂-TKPPR-^{99m}Tc is stable for at least 6 hours in serum. HPLC analysis of serum samples mixed with ligand displayed no appreciable fragments of ^{99m}Tc labeled peptide at each time point (1, 3, and 6 hours).

For neutrophil cell viability studies, no significant change in the population of viable cells was observed between group 1 (radioligand treated) and group 2 (PBS treated). Cell viabilities were >95% at both time points (10 and 30 min) for both groups.

In vivo evaluation

In order to further analyze the pharmacokinetic parameters of the ligand **4-9**, blood clearance studies were conducted *in vivo*. The data revealed a mono-exponential clearance of the radiotracer as shown in Figure 4-1. The elimination half-life for blood clearance ($T_{1/2}$) was calculated to be about 84 min.

Biodistribution of cFLFLF-PEG₁₂-TKPPR-^{99m}Tc in liver increases from 2.3 to 5.3 %ID at 3 and 18 h post injection which indicated the radiotracer excretes mainly through hepatobiliary pathway (Figure 4-2). The longer blood retaining of cFLFLF-PEG₁₂-TKPPR-^{99m}Tc, 2.1 and 1.2 %ID at 3 and 18 h pi vs. 0.2 %ID of cFLFLF-PEG-⁶⁴Cu (*15*) at 18 h pi correlates the longer blood half-life time ($T_{1/2}$) of 84 min (Figure 4-1) vs. 55 min. This indicates that a longer PEG moiety might be needed for fast blood clearance of cFLFLF-PEG_n-TKPPR-^{99m}Tc and, therefore benefiting the earlier imaging quality by lowering background signals from blood.

At 22 hours post PMA treatment, bioluminescence as observed by MPO assay was locally emitted from PMA-treated earlobes of FVB (female) mice after luminol administration, reaching levels of 7 fold over vehicle-treated ears. The image (Figure 4-3A) of 21 min after luminol injection was chosen as a representative due to the highest ratio of photons emitted from infected ear (left) to control ear (right). At 27 hours after PMA challenge and 3 hours post injection of cFLFLF-PEG₁₂-TKPPR-^{99m}Tc, CT/SPECT imaging shows that the accumulation of the tracer measured in the infected ear (left) is about 3.15 folds higher than in the control ear (Figure 4-3B).

These data suggest that *in vivo* detection of inflammation through neutrophil activation and migration in live animal is feasible with gamma or SPECT modalities. In

real inflammatory situation the intensity of radiotracer signal will be dependent on the intensity of inflammation and amount of PMNs accumulation at the inflammatory site.

4.5 Conclusion

In conclusion, two newly synthesized neutrophil-targeting heterobivalent SPECT imaging probes: cFLFLF-PEG₄-TKPPR-^{99m}Tc and cFLFLF-PEG₁₂-TKPPR-^{99m}Tc were evaluated *in vitro* and *in vivo*. The peptide ligands bound to neutrophils with high affinity and demonstrated stability in serum and sufficient hydrophilicity. The cFLFLF-PEG₁₂-TKPPR-^{99m}Tc is a feasible probe for *in vivo* imaging of acute neutrophilic inflammation. Further biological evaluation of this novel imaging agent is ongoing, with the goal of refining the biological properties of the agent to facilitate studies that assess the efficacy of novel anti-inflammatory therapeutic drug candidates.

4.6 Figures and Scheme

Scheme 4-1. Synthetic scheme of $^{99\text{m}}\text{Tc}$ radiolabeled peptides. Reagent and conditions: i) standard solid phase Fmoc chemistry; ii) (1) piperidine, (2) Fmoc-PEG-COOH, HBTU, DIEA; iii) standard solid phase Fmoc chemistry; iv) (1) 2% hydrazine, (2) 6-Boc-HYNIC acid, HBTU, DIEA; v) TFA(95%); vi) NaTcO₄, SnCl₂, Nicotinic acid, pH 5.2.

Figure 4-1. Monoexponential blood clearance curve of cFLFLF-PEG₁₂-TKPPR-^{99m}Tc in control mice ($n=3$): the normalized blood radioactivity was plotted against various time points after tail vein injection of the tracer.

Figure 4-2. Tissue and organ accumulation of cFLFLF-PEG₁₂-TKPPR-^{99m}Tc at 3 and 18 h post injection of normal mice ($n=3$) expressed as the percent injected dose per gram of tissue (decay corrected).

Figure 4-3. *In vivo* bioluminescence imaging of MPO and SPECT imaging of cFLFLF-PEG₁₂-TKPPR-^{99m}Tc. A. Representative MPO-BLN image of mouse subjected to topical application of PMA on left ear for 24 hours. B. Representative transaxial images of MicroCT (left), MicroSPECT (right) and fused micro-CT and micro-SPECT (middle) of mice subjected to PMA application on left ears. Both SPECT and CT images were obtained 3 hr after tail vein injection of cFLFLF-PEG₁₂-TKPPR-^{99m}Tc. SPECT scans revealed PMA infected ear had visually more tracer uptake compared to control ear, which was quantified by ROI analysis.

4.7 Appendix

Appendix 4-1. MALDI-TOF mass spectra of compounds **4-6** and **4-7**

Appendix 4-2. Mouse PMN purification protocol

1. Collect blood via eye bleed or heart (prevent clotting with heparin), fill the needle part of the syringe with heparin (from CCM, heparin sodium, injection, usp, 1000 usp U/mL)
2. Transfer all blood into a culture test tube (fisher, 17x100mm, cat. 14-956-6B)
3. Let lympholyte-Mammal (accurate chemical and scientific corp.) warm up to rt and shake it and wait until bubbles disappear. Add lympholyte-mammal (L-M) into another test tube (same volume as blood) and carefully transfer the blood on the top of L-M layer.
4. Centrifuge at 800 rcf, 20min, rt
5. Remove top layers carefully, only leave the red pellet (neutrophil and red blood cells) in the tube
6. Add sterile 3% dextran (sigma, from leuconostoc spp, Lot# 0001375672) with 0.9% NaCl (same volume as blood) and mix it until homogeneous. Sit it at rt for 20min until separation completes.
7. Take the top layer (clear, pink solution) into a new tube and add twice volume of PBS (1X), centrifuge it at 400rcf, 10min, rt.
8. Remove the supernatant and remain the RBC+neutrophil pellet, lyse RBC with 4.5mL water within 30sec and equalized with 10X PBS buffer, mix and centrifuge it at 400 rcf, 10min, at rt.
9. White pellet on the bottom are neutrophils, resuspend it to 1X PBS with desired volumn.

Notes: this method could achieve a neutrophil recovery yield of 90%.

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Chapter 5: Synthesis of PECAM-1-specific ^{64}Cu PET Imaging Agent: Evaluation of Myocardial Infarction Caused by Ischemia-reperfusion Injury in Mouse

5.1 Abstract

As part of our project in developing molecular imaging agents for inflammation, the synthesis and *in vivo* evaluation of a PECAM-1 specific PET imaging agent, PECAM-1-Ab-DOTA- ^{64}Cu , are described in the current chapter. The PET imaging agent was synthesized by conjugating the anti-mouse PECAM-1 antibody with 2,2',2'',2'''-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid (DOTA) and subsequent labeling with ^{64}Cu . Positron Emission Tomography (PET) was successfully performed in a mouse model of myocardial infarction (MI) induced by an ischemia/reperfusion (I/R) injury, indicating the elevated expression of PECAM-1. Preliminary organ distribution data of this imaging agent was also obtained in control mice.

5.2 Introduction

Ischemia-reperfusion (I/R) injury is associated with the pathogenesis and management of numerous inflammatory conditions, for example myocardial infarction and stroke (1-4). It has been established that reperfusion of an ischemic tissue results in significant leukocyte infiltration and tissue damage (2, 5-8). A variety of cellular adhesion molecules (CAM) are expressed on the cell surfaces during such abnormal events. These adhesion molecules play an important role in regulating leukocyte-endothelial cell specific interactions (5, 9, 10) which ultimately result in leukocyte trafficking and extravasation in mammalian species (11, 12). These CAM molecules include intracellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule (PECAM-1/CD31)

(11, 13). PECAM-1 a 130 kDa protein has been known as a member of the immunoglobulin superfamily of CAM (14, 15), and is constitutively expressed on all cells within vasculature, such as circulating platelets, monocytes, neutrophils, endothelial cells, and certain T-cell subsets. Most importantly it has been demonstrated to express abundantly at the interjunction of endothelial cell lining of the capillaries where the neutrophil extravasation occurs (14, 16, 17). PECAM-1 plays a significant role in vascular biology, for example in angiogenesis, platelet function, leukocyte adhesion, transendothelial migration, and transmitting signals (18-20).

PECAM-1 antibody has been labeled with fluorescence dyes and radioiodine (^{125}I or ^{131}I) for immunostaining in a variety of inflammatory conditions and used for *ex-vivo* analysis (10, 21-23). However, to the best of our knowledge the labeled PECAM-1 antibody prepared under mild conditions to acquire high labeling specificity has not been used non-invasively in live animal imaging. Thus, there is a great need to assess *in vivo* PECAM-1 function using non-invasive detection method. Herein we report the synthesis of PECAM-1-Ab-DOTA- ^{64}Cu , a PECAM-1 specific PET imaging agent for non-invasive studies and its biological evaluation in mouse myocardial infarction model (Figure 5-1).

5.3 Materials, Methods and Results

The synthesis of the antibody conjugate and its ^{64}Cu radio-labeling was achieved by using an optimized protocol according to the literature.(24, 25) As depicted in Scheme 5-1, DOTA (12.1mg, 24 μmol) (Macrocyclics, Inc., Dallas, TX) in 500 μL deionized H_2O (resistivity 18.2 $\text{M}\Omega\text{ cm}$, Milli-Q Direct Ultrapure Water System from Millipore) was mixed with 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC) (2.8 mg, 12 μmol) (PIERCE, Rockford, IL) in 130 μL H_2O . NaOH (0.1 M, $\sim 300\text{ }\mu\text{L}$) was added to

adjust pH to 5. N-hydroxysuccinimide (2.1 mg, 9.6 μ mol) (Sulfo-NHS) (PIERCE, Rockford, IL) was then added to the stirring mixture on ice-bath and followed by addition of $\sim$ 70 μ L NaOH solution (0.1 M) until pH reached 5.5. The reaction mixture was stirred at 4°C for 30 min, resulting in the formation of activated DOTA-NHS **5-1**. The concentration of **5-1** was considered to be $\sim$ 9.6 mM. Meanwhile, 100 μ g anti-mouse PECAM-1 (CD31) antibody (PECAM-1-Ab) (eBioscience Inc, San Diego, CA) was prepared for conjugation as follows: The antibody was dissolved in 1 mL of 0.1 M PBS buffer (pH 7.4) and was dialyzed against 1 L of 0.1 M PBS buffer (pH 7.4) containing 2 g Chelex 100 Resin (Analytical Grade, Bio-Rad Laboratories, Inc., Hercules, CA) at 4 °C for 48 h, with one buffer change at 24 h post incubation. The neutralized antibody in 200 μ L PBS buffer (0.1 M, pH 7.4) was then treated with 65 μ L solution DOTA-NHS **5-1** ($\sim$ 600 nmol) at an approximate ratio of 1:1000, and allowed to incubate at 4 °C for overnight. The conjugate PECAM-1-Ab-DOTA **5-2** was then transferred to 30 kDa cut-off filter (Micron YM-30 Centrifugal Filter, Millipore Inc, Billerica, MA), centrifuged and repeatedly washed with ammonium acetate buffer (400 μ L, 0.1 M, pH 5.5) by centrifugation (Marathon Micro-A, Fisher Scientific, Pittsburgh, PA) (4000 rpm, 10 min/each time x 6 times). The conjugate **5-2** was reconstituted in 50 μ L ammonium acetate buffer was recovered by inverted centrifugation at 2000 rpm for 2 min. The radio-labeling was performed by treating 50 μ L of conjugate **5-2** with $\sim$ 800 μ Ci of $^{64}\text{CuCl}_2$ (Washington University, School of Medicine, St. Louis, WA) in 200 μ L of 0.1M ammonium acetate buffer (pH 5.5) at 37°C for 30 min incubation. The reaction was quenched by addition of 5 mL of EDTA solution (0.01 M) and concentrated using a 30

kDa cut-off filter. The concentrated PECAM-1-Ab-DOTA-⁶⁴Cu **5-3** was washed with 400 μ L of PBS buffer (0.1 M, pH 7.4) by repeated centrifugation (4000 rpm, 10 min/each time) for three times. The efficiency of radio-labeling was $\sim$ 70—90%. Radio-labeling efficiency was calculated as follows: (radioactivity of radio-labeled PECAM-1 antibody)/(total radioactivity of ⁶⁴Cu added) $\times$ 100%. All vials, containers, buffers and related consumables used in this protocol were sterilized prior to use.

In order to characterize the radio-labeled conjugate, the “cold” counterpart PECAM-1-Ab-DOTA-Cu was synthesized with CuCl₂ following the same procedure described above. Both radioactive and non-radioactive counterparts were analyzed by size-exclusion HPLC (radio-HPLC) on a RAININ chromatography system equipped with a Macrosphere GPC column (300mm $\times$ 7.5mm; silica based particle size 300Å; Alltech, Nicholasville, KY), an ABI Spectroflow 783 UV detector (Kratos Analytical Instrument, Kyoto, Japan) and a NaI solid scintillation Flow Count Radio-HPLC detector (Bioscan, Inc., Washington, DC). The column was eluted with PBS buffer at a flow rate of 1 mL/min. The HPLC retention time of Radio-labeled antibody was assigned by co-injection with the “cold” counterpart, as presented in Figure 5-2.

Optimized condition of saturation loading of PECAM-1 antibody was studied by varying the molar ratio of activated DOTA-NHS **5-1** while keeping the rest of parameters same and subsequent complexing with ⁶⁴Cu. Complexation of ⁶⁴Cu to DOTA was assumed to be >90% since excess of ⁶⁴CuCl₂ was used (for 100 μ g antibody; $\sim$ 800 μ Ci ⁶⁴CuCl₂). As shown in Table 5-1, four conditions were explored (10:1, 100:1, 1000:1 and 5000:1). The optimal ratio of 1000:1 was considered for radio-labeling since the higher

ratio (5000:1) of NHS-DOTA resulted in similar loading efficiency. Therefore, calculated specific activity of the probe used for following experiment was 5.2 ± 0.6 mCi/mg.

All animal use and imaging protocols were approved by the University of Virginia Animal Care and Use Committee. The newly synthesized imaging agent PECAM-1-Ab-DOTA- ^{64}Cu **5-3** was evaluated in a mouse model of acute myocardial infarction (MI) induced by ischemia-reperfusion (IR) injury. The acute MI was initiated in male C57Bl/6 mice ($n=4$) by coronary occlusion via ligation of left anterior descending coronary artery for 45 min, followed by reperfusion (26, 27). Immediately after reperfusion, approximately 80-100 μCi of PECAM-1-Ab-DOTA- ^{64}Cu **5-3** was injected into mice through the tail vein. For contrast-enhanced MR imaging, a 0.3-0.6 $\mu\text{mol/g}$ bolus of gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA) was injected through tail vein prior to image acquisition (28). Contrast-enhanced MR imaging (29) (7T ClinScan Bruker/Siemens MR scanner, acquisition time: 30—45 min) and PET imaging (Siemens Focus 120 MicroPET scanner, acquisition time: 10—20 min) were sequentially performed at 3 h and 24 h post injection (pi). After the last PET scan (24 h pi), the mice were euthanized by deep halothane anesthesia and hearts were harvested immediately. *Ex vivo* MR/PET scans of harvested hearts were performed to further delineate the infarction area resulting from IR injury.

Both *in vivo* and *ex vivo* imaging data were reconstructed (30). MicroPET and MR images were fused with AMIDE software (31). It was observed that the mid-ventricular myocardial infarct tissues of IR injured mice was delineated clearly by Gd-enhanced MRI, PET and fuse PET/MRI images from both *in vivo* and *ex vivo* data sets, as shown in Figure 5-3. The *ex vivo* heart image (Figure 5-3 lower row) confirmed that the

imaging agent accumulated specifically in left ventricle myocardium wherein MI was induced.

Standardized Uptake Values (SUV) of PECAM-1-Ab-⁶⁴Cu **5-3** in the infarct and healthy myocardial tissues were measured based on boundaries defined by the Gd-enhanced MR images. SUVs were calculated as the ratio of the average region of interest (ROI) activity concentration normalized by the injected dose and the weight of animal (30). As described in Figure 5-4, the mean SUV at the infarct zone is about a factor of 2 times higher than the healthy zone ($p < 0.01$). Previous biological studies demonstrated that elevated PECAM-1 expression was observed in various ischemia reperfusion animal models (32-34), which is in agreement with our results.

At 24 h pi, both IR-injured and control mice were anesthetized by 2% isoflurane (with oxygen flow). Blood samples (~50 μ L) were taken from the tail veins and mice were euthanized by cervical delocalization under deep anesthesia. Organs and tissues (heart, lungs, muscle, bone, liver, kidney, spleen, small intestine, and stomach) were harvested, rinsed with PBS buffer, wiped with filter paper and weighed in a pre-weighed tube. The radioactivity of each sample was measured using Wallac 1420 Wizard gamma counter (Perkin-Elmer), decay corrected to the injection time, normalized for injected dose and organ/tissue weight, expressed as percentage injected dose per gram tissue (%ID/g) as shown Figure 5-5.

5.4 Conclusion

In conclusion, we have successfully synthesized a radio-labeled PECAM-1 specific PET imaging probe PECAM-1-Ab-DOTA-⁶⁴Cu. PET/MR imaging was performed to evaluate the ability of newly developed probe for PECAM-1 expression in

the infarction region of mouse myocardium by ischemia-reperfusion injury. The elevated expression of intravascular PECAM-1 was indeed observed by *in vivo* and *ex vivo* imaging and confirmed by SUV image analysis in the injured tissue.

5.5 Figures, Table, and Scheme

Table 5-1. Optimization of reaction conditions.

Data shown here was the average $\pm$ standard deviation of three parallel experiments.

DOTA-NHS 5-1 : PECAM-1-Ab	10:1	100:1	1000:1	5000:1
Specific Activity (mCi/mg)	0.4 ± 0.15	2.2 ± 0.6	5.2 ± 0.6	5.4 ± 0.9

Scheme 5-1. Synthesis of PECAM-1 specific PET imaging probe. Conditions and reagents: (i) EDC, pH 5–5.5, 4 °C (ii) PECAM-1-Ab, 0.1 M PBS buffer, pH 7.4, 4°C (iii) $^{64}\text{CuCl}_2$, 0.1 M ammonium acetate buffer, pH 5.5, 37°C, 30 min.

Figure 5-1. Schematic of PECAM-1 specific labeling and PET imaging.

Figure 5-2. Characterization of PECAM-1 specific PET imaging agent by HPLC. (A) Radio-HPLC chromatogram of PECAM-1-Ab-DOTA-⁶⁴Cu detected by gamma detector (B) HPLC chromatogram of the “cold” counterpart PECAM-1-Ab-DOTA-Cu detected by UV detector at 280 nm. Y-axis for both chromatograms was removed for comparison of retention times.

Figure 5-3. Dual modality images of a representative mouse heart at 24 h pi. *In vivo* live animal transaxial images (upper row) and *ex vivo* isolated heart images (lower row) of Gd-enhanced MR image (left column), fused MRI and PET images (middle column) and PET image of PECAM-1-Ab- ^{64}Cu (right column).

Figure 5-4. SUV analysis of infarct and healthy myocardial regions of I/R injured mice ($n=4$) ($P<0.01$, infarct zone vs. healthy zone). The statistical analysis was performed with one tailed, unpaired Student t-test by GraphPad Prism software.

Figure 5-5. Organ distribution profile of IR-injured and control mice at 24 h post injection of probe PECAM-1-Ab-DOTA-⁶⁴Cu 5-3.

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Chapter 6: ^{64}Cu -DOTA-Heptamethine Cyanine Based PET/Fluorescence Dual-mode Molecular Imaging Probe for Tumor: Synthesis and *in vivo* Evaluation

6.1 Abstract

Non invasive detection and diagnosis of cancer can be improved by dual-mode analysis in real time and space. Herein synthesis, characterization, *in vitro* and *in vivo* evaluation of PC-1001 (**6-4b**), a novel tumor-specific dual-modality PET/fluorescence imaging probe, are described. The utility of probe PC-1001 is demonstrated both *in vitro* human breast cancer cells and *in vivo* human breast tumor xenograft studies.

Methods: Tumor-specific heptamethine-cyanine dye was conjugated with DOTA and radiolabeled with ^{64}Cu for dual-modality imaging. Detailed spectroscopic properties of the PC-1001 probe were obtained. *In vitro* cellular uptake studies were performed in the breast cancer MCF-7 cell line and normal breast epithelial MCF-10A cells as controls to establish tumor uptake specificity. Inhibition studies were performed to uncover the mechanism of tumor-specific uptake. *In vivo* time-dependent fluorescence and PET imaging of breast tumor xenografts in mice were performed. Blood clearance (half-life in blood), biodistribution, and tumor-specific uptake and plasma binding of PC-1001 were quantified. *Ex vivo* histology (H/E staining) and confocal fluorescence imaging of selected tissue specimens were examined.

Results: The conjugation of a DOTA-Cu moiety to a heptamethine cyanine dye exhibited similar fluorescence properties ($\epsilon=82,880\text{ cm}^{-1}\text{M}^{-1}$, $E_x/E_m=750/820\text{ nm}$) to the parental dye, indicating no significant effect of PET part attachment to the optical properties. Time-dependent cellular accumulation studies suggest significantly higher probe uptake (>2-fold, 30 min) in MCF-7 breast cancer cells than control non-

tumorigenic breast MCF-10A epithelial cells. The probe uptake in tumor cells is mediated by an organic anion transport polypeptide (OATP) system. *In vivo* studies reveal that the dual-modality PET/fluorescence imaging probe has desirable accumulation profiles in tumor tissues, with tumor versus muscle uptake of 5.8 ± 0.2 fold at 48 h post probe injection. Blood clearance half-life of the probe was calculated to be 4.3 ± 0.2 h. Microscopic fluorescence imaging of *ex vivo* harvested tissue indicated that the probe was mainly associated with viable tumor but not with necrotic tissues.

Conclusion: A highly effective dual-modality (PET/fluorescence) imaging probe is characterized and shown to be an effective tumor-specific imaging agent. The promising results of the study indicate that the dual-modality imaging probe PC-1001 is well suited for pre-clinical evaluation.

6.2 Introduction

Cancer diagnosis and treatment are the most demanding challenges facing clinicians and scientists because of the lack of appropriate agents for early cancer detection and follow-up of cancer progression, and clinical response to therapeutic intervention. Molecular imaging approaches could offer effective tools for cancer detection and prognosis allowing visualization of tumors in real time and space (1). Because of a lack of cancer specific targets, new highly sensitive molecular probes are needed for accurate identification of cancer (2, 3). The present investigation focuses on the further development of a dual PET/fluorescence imaging probe for the visualization of human breast cancers from deep tissues, based on our previous discovery of a class of carbocyanine dye, heptamethine cyanine, which successfully detected human and mouse tumors with high degree of sensitivity and specificity (3-5).

The importance of early cancer detection is evident. In a study of 5-year survival results, over 90% of patients survived when the disease was detected early (6). A variety of diagnostic imaging techniques, such as magnetic resonance imaging (MRI), nuclear imaging [positron emission tomography (PET), single photon emission computed tomography (SPECT)], ultrasound (US) and optical imaging are being developed and utilized mostly as single modality (2, 5, 7). For example, PET is a very sensitive, non-invasive, biological function dependent technology widely utilized in pre-clinical and clinical imaging of cancer (8, 9). PET imaging, however, has been limited by the short half-life of radioactive probes. In contrast, near infrared fluorescence (NIRF) optical imaging has the capability to provide detailed information at the molecular and cellular level over a long period, but has limited ability to present 3D images due to low spatial resolution and poor light penetration through deep tissues (10-12). Thus combining both nuclear and optical techniques to develop multi-modal imaging probes would be an ideal way to obtain high sensitivity and specificity for cancer detection and overcome the limitations of complementary singular techniques. Currently, a few dual-mode imaging probes are being developed and investigated (13-16).

Recently Yang *et al* (17) and others (12) reported on a class of heptamethine cyanine dye that yields tumor-specific NIRF signals for the detection of a broad-spectrum of human and mouse tumor cell lines, tumor xenografts and mouse tumors from transgenic animals. To further improve the sensitivity and efficacy of this class of cyanine dye for deep-tissue detection of tumors, we synthesized a dual modality PET/fluorescence imaging agent and tested this new probe in cultured human breast cancer cells and breast tumor xenografts in mice. The dye was chosen because of its

tumor-specific targeting properties and low tissue interference from NIRF signals. These features allowed us to monitor tumor progression, including metastases to deep tissues such as bone and visceral organs in host mice. In this study we synthesized a heptamethine cyanine dye, MHI-148, covalently conjugated with a DOTA ligand to form a complex with a variety of radionuclides (e.g. ^{64}Cu) for dual PET/fluorescence imaging. The PET part of the dual-mode probe provides high sensitivity and deep tissue spatial resolution to detect tumors and their metastatic lesions. Both *in vitro* and *in vivo* evaluations of probe PC-1001 for cancer imaging in the breast tumor model is described.

6.3 Materials and Methods

All chemicals purchased commercially were of analytical grade and were used without further purification. 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) was obtained from Macrocyclics, Inc. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Invitrogen. All other chemicals were purchased from Sigma-Aldrich Chemical Co. $^{64}\text{Cu}-\text{CuCl}_2$ (half-life, 12.7 h; β^+ , 17.8%, $E_{\beta^+\text{max}}$, 656 keV; β^- , 38.4%, $E_{\beta^-\text{max}}$, 573 keV) was purchased from Washington University. [^{18}F]FDG (Fluorodeoxyglucose- ^{18}F), was purchased from PETNET Solutions. Ultrapure water (resistivity, 18.2 M Ω cm) used for making solutions was obtained from Milli-Q Direct Ultrapure Water System from Millipore. High-performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific. Chemistry and radiochemistry products were analyzed and purified by preparative and semi-preparative reversed-phase HPLC. HPLC was performed on a Varian ProStar system (models: pumps, 210; column valve module, 500; fraction collector, 701) (Varian Instruments) equipped with either a DENALITM 238 DE C18 SPRING preparative

column (120 Å, 250×25 mm) and an Alltech Apollo C18 semi-preparative column (5 µm, 250×10 mm) (Grace Davison Discovery Sciences) and ABI Spetroflow 783 UV detector as well as a NaI solid scintillation Flow Count Radio-HPLC detector (Bioscan). Two mobile phases were used for HPLC: Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in 80% aqueous acetonitrile). The mobile phase gradient varied according to characteristics of compounds for 30 min at a flow rate 12 mL/min for the preparative column and 3 mL/min for the semi-preparative column monitored at 254 nm. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) analysis was performed on compounds at the W.M. Keck Biomedical Mass Spectrometry Laboratory using a Bruker Daltonics system at the University of Virginia. ¹H and ¹³C nuclear magnetic resonance (NMR) spectra were recorded on Varian Innova spectrometer (300 MHz).

MCF-7 cells (kindly provided by Dr. R. Bruggemeier, Ohio State University) were grown in IMEM containing 5% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (GIBCO) (18). MCF-10A cells are an immortalized, non-transformed epithelial cell line derived from human fibrocystic mammary tissue and was purchased from American Type Culture Collection (ATCC). MCF-10A cells were cultured in DMEM/F12 medium supplemented with epidermal growth factor 40 ng/ml (BioVision), insulin 10 µg/ml (Sigma), hydrocortisone 500 ng/ml (Sigma), cholera toxin 100 ng/ml (Calbiochem), 5% horse serum (Invitrogen), 1% penicillin and streptomycin. Unless otherwise mentioned, all cell lines were cultured in the incubator at 37°C in a 5% CO₂ humidified atmosphere (CO₂ water Jacketed incubator, series II, Forma Scientific). Phosphate buffered saline (PBS) (0.1 M, pH 7.45) was purchased from Invitrogen.

Human serum albumin (HSA) was obtained from Sigma-Aldrich. Krebs-Henseleit (KH) buffer (118 mM NaCl, 23.8 mM NaHCO₃, 4.83 mM KCl, 0.96 mM KH₂PO₄, 1.20 mM MgSO₄, 12.5 mM HEPES, 5.0 mM glucose, and 1.53 mM CaCl₂, pH adjusted to 7.4) was prepared as reported (19, 20).

A breast tumor xenograft model was used for *in vivo* studies. This model used ovariectomized athymic mice (3-4 weeks old) obtained from Charles River Laboratory and handled according to procedures approved by the University of Virginia Animal Care and Use Committee in accordance with current guidelines of National Institutes of Health Model Procedure of Animal Care and Use. MCF-7 breast tumor cells (ten million cells in 0.1 ml) suspended in Matrigel (BD Biosciences) were inoculated into the mammary gland fat pad of athymic mice (four inoculants per mouse; $n_t=4$). Tumor growth was supported by a subcutaneous Silastic capsule implant containing a mixture of 17- β -estradiol and cholesterol at a ratio of 1:3, which provided ~80 pg/ml of plasma 17- β -estradiol (18). Tumor sizes were monitored by measuring tumor diameters, r_1 and r_2 , with calipers (both length and width) once a week and tumor volumes were calculated using the formula $3/4\pi r_1^2 r_2$ where $r_1 < r_2$ (21). Tumors were allowed to grow for 6-8 weeks before initiation of the studies. Mice with tumors of 150 mm³ or above were used for *in vivo* experiments.

The probe solutions for injection were prepared in sterile PBS buffer (0.1 M, pH=7.45). Mice were administered with imaging probes (PBS volume 100-150 μ L) by two different methods for blood collection, probe biodistribution, and fluorescence and PET imaging: 1) intravenous (iv) injection via mouse tail vein using a syringe (1/2 cc U-100 Insulin Syringe, 28G1/2, Fisher Scientific) under anesthesia (2% isoflurane in

oxygen); 2) intraperitoneal (ip) injection via mouse lower abdominal area using the same syringe as described. Radioactive doses of probe PC-1001 (**6-4b**) for animal injection were prepared and measured with a well counter (CAPINTEC Radioisotope Calibrator CRC-7, code 115). Radioactive samples for partition coefficient analysis, *in vitro* cell studies, blood clearance and organ distribution were measured for radiation using an automatic gamma counter (2480 Wizard, PerkinElmer). Whole animal fluorescence imaging was performed by IVIS Spectrum (Caliper Life Science) and the PET scans were acquired using a microPET Focus 120 (Siemens Medical Solutions).

Synthesis and radiolabeling of 6-4b

As described in Figure 6-1, the heptamethine cyanine dye MHI-148 (2-[2-[2-chloro-3-[2-[1,3-dihydro-3,3-dimethyl-1-(5-carboxypentyl)-2H-indol-2-ylidene]-ethylidene]-1-cyclohexen-1-yl]-ethenyl]-3,3-dimethyl-1-(5-carboxypentyl)-3H-indolium bromide) (compound **6-1**, see Appendix 6-1 for detailed synthesis) and DOTA-SulfoNHS were synthesized according to a process described previously (17, 22, 23). One of the carboxylic group of MHI-148 was conjugated with N- α -Boc-Lys-OH (50 mg, 0.2 mmol) through NHS-activation followed by deprotection of Boc using TFA to afford compound **6-2** that was purified by HPLC (70-100% Solvent B in 30 min) with a retention time of 12.3 min. The amine group of compound **6-2** (84.8 mg, 0.1 mmol) was conjugated with DOTA-NHS (116.3 mg, 0.2 mmol) to yield compound **6-3** (55.2 mg, yield 44.7%) which was purified by semi-preparative HPLC (50-100% Solvent B in 30 min) with a retention time of 17.7 min. Metal chelation of DOTA by copper yielded the final imaging probe PC1001 (**6-4a** and **6-4b**) which could be purified by HPLC (50-100% Solvent B in 30 min) with a retention time of 18.6 min and metal chelation yield >90%. All intermediates

and final products were purified by RP-HPLC to homogeneity, and purity of probe **6-4a** and **6-4b** was > 95%. Structural characterization of compound **6-2** and **6-3** was performed by ^1H and ^{13}C NMR and compound **6-3** and **6-4a** were analyzed by MALDI-TOF-MS (see Appendix 6-2).

Spectroscopic evaluation

UV-Vis

Solutions (10 μM) of MHI-148 **6-1**, MHI-DOTA **6-3** and MHI-DOTA-Cu **6-4a** were prepared separately in 0.1 M PBS buffer (pH 7.45) with 0.25% (v/v) DMSO. Measurements of absorption spectra were performed on a UV-Vis spectrometer with a path length of 1 cm (Biomate 5, Thermo Spectronic, Rochester, NY), scanned from 300-900 nm and analyzed by VISIONlite software. Molar extinction coefficients (ϵ) were then calculated.

Fluorescence

Fluorescence emission spectra were performed with a Horiba FluoroMax 4 spectrofluorometer (JOBIN YVON/HORIBA, Edison, NJ) with a variety of excitation wavelengths, with slit width of 5 nm and integration time of 0.2 s for all measurements. Spectra data were recorded, processed and plotted with Origin software. The solution of probe **6-4a** (10 μM , 3 mL) was prepared in 0.1 N PBS buffer (pH 7.45) with 0.25% (v/v) DMSO and was excited with a series of excitation wavelengths (650, 675, 700, 725, 750 and 775 nm) to optimize the fluorescence emission spectrum. All spectra were normalized to the maximum emission intensity and plotted against emission wavelength.

Human albumin binding

Stock solution of probe **6-4a** (100 μ M, 100 mL) was prepared in 0.1 M PBS buffer (pH 7.45) and 1% (v/v) DMSO and diluted to 10 μ M with PBS. Various final concentrations (0%, 0.1%, 0.5%, 1%, 3%, and 5%, v/v) of human serum albumin (HSA) were mixed with 10 μ M solutions of **6-4a**. The fluorescence spectra of each sample was measured with an excitation wavelength of 750 nm (other conditions were kept constant as mentioned above). The recorded fluorescence intensity was normalized by the maximum fluorescence intensity and plotted against emission wavelength.

Concentration-dependent fluorescence emission

Stock solutions (100 μ M, 10 mL) of MHI-148 **6-1** and probe **6-4a** were freshly prepared in 0.1 M PBS buffer (pH 7.45) with 0.25% (v/v) DMSO and a series of dilutions were made with PBS. Fluorescence emission spectra of both compounds were measured over the concentration range of 0-100 μ M with the excitation wavelength of 750 nm while keeping the rest of conditions the same as above. Maximum fluorescence intensities were plotted against each concentration.

pH effect

Stock solution of probe **6-4a** (100 μ M, 100 mL) was prepared in 0.1 M PBS buffer (pH 7.45) and 1% (v/v) DMSO and diluted to 10 μ M with various buffers to form a series of resultant pH conditions (pH=4.0, 5.0, 5.8, 6.2, 6.8, 7.0 and 7.4). For pH 4.0-5.8, stock buffer solution (acetic acid/sodium acetate) was used. For pH 6.2-7.0, stock buffer solution (dipotassium phosphate/monopotassium phosphate) was used. The commercial PBS buffer (0.1 M) was used for dilution at pH 7.4. All pH values were measured by Orion pH meter (Model 410A, Thermal Scientific). Fluorescence spectra of all solutions were measured and recorded as described above.

Partition coefficient (log P)

Freshly purified probe **6-4b** (185 kBq) by HPLC was dried over blowing N₂ flow to remove TFA and acetonitrile, solublized in 500 µL DI water and mixed with 500 µL of octanol in an Eppendorf tube (1.5 mL). The tube was sonicated for 10 min and centrifuged at 4000 rpm for 5 min (Marathon Micro-A, Fisher Scientific). Aliquots (100 µL) from the octanol and water layers were carefully removed. Radioactivities from both layers were measured with a gamma counter. Partition Coefficient (log *P*) was calculated by the following equation: $\log P = \log_{10} \left[\frac{\text{(radioactivity in octanol)}}{\text{(radioactivity in water)}} \right]$. The measurement was recorded in triplicate and presented as an average.

In vitro cellular studies

Time-dependent cellular uptake of radioactive probe 6-4b

MCF-7 and MCF-10A cells were pre-seeded in 48-well microplates with a density of $\sim 2 \times 10^5$ cells/mL (500 µL/well). After incubation at 37°C for 24 h, probe **6-4b** (74-111 kBq, 20 µL per well) was added into each well. Medium from selected wells was removed at various time points after probe addition (3, 5, 15, 20, 30 and up to 60 min), and those wells were then washed successively with KH buffer (19, 20) for three times. At the end of 60 min time point, lysis buffer (20 mM Tris and 0.4% Triton X-100, pH adjusted to 9.0) (500 µL) was added to all wells. The plate was gently shaken for 10 min (400 rpm, Labnet Shaker, National Labnet Laboratory). The lysate from each well was transferred to separate tubes and each well was washed with PBS buffer for two times and combined with the lysate from the same well. Radioactivity of each tube was measured by gamma counter, with decay corrected to the time point of probe addition, normalized to per mg protein, and plotted against incubation time using Prism 5.0

(GraphPad). The protein amounts (mg protein) of cell lysates were determined using a standard BCA (bicinchoninic acid) Protein Assay Kit (Pierce, Thermal fisher Scientific Inc., Rockford, IL) following the product manual. A 96-well microplate reader (SPECTRAMax 190; Molecular Devices) was used to measure the absorbance of assay solution at 562 nm. All measurements were conducted in triplicate and reported as an average $\pm$ SD.

Mechanism-based cellular uptake of radioactive probe 6-4b

MCF-7 and MCF-10A cells were pre-seeded on 48-well microplates with a density of $\sim 2 \times 10^5$ cells/mL (500 μ L/well) and incubated for 24 hours. Both cell types were divided into three groups as 1) control, 2) inhibitor addition and 3) for determination of the effects of temperature. Each group was first processed as follows: Group 1) addition of blank PBS buffer; Group 2) addition of a uptake inhibitor, bromosulphophthalein (BSP) (250 μ M as final concentration), which is known to block organic anion transport polypeptides (OATPs) (24-26); Group 3) addition of blank buffer and incubation at lower temperature (0 $^{\circ}$ C) with probe **6-4b** (74-111 kBq/well) for 30 min, respectively. At the end of incubation, supernatants were removed and each well was washed with KH buffer for three times. The cells were lysed and processed as mentioned in the previous experiment. Radioactivity of each tube was measured by gamma counter, decay corrected to the time point of probe addition, normalized to per mg of protein. The measurement was in triplicate and reported as an average $\pm$ SD. The control MCF-7 cell uptake was assigned as 100% and was used as standard.

Blood Clearance

PC-1001 (0.74-1.11 MBq/~20 g mouse) was injected via the iv tail vein route in a group of tumor-bearing mice ($n=4$). Approximately 30-50 μL of blood from the contralateral tail vein was collected in capillary tubes at 5, 15, 30, 60, 180, 360 and 1280 min post probe injection. The capillaries were placed in pre-weighed tubes to measure the weight and radioactivity of the blood withdrawn. Radioactivity from each sample was measured by a gamma counter with radioactivity decay corrected to the time of probe injection, normalized for injected dose and blood weight, and expressed as percentage injected dose per gram blood (%ID/g). The data obtained was processed with OriginPro 7.5 (OriginLab Corp.).

Biodistribution

Tumor and organ distribution of probe **6-4b** was determined in five groups of tumor-bearing mice (number of mice; $n=4$, number of tumors; $n_t=16$, each mouse was bearing 4 tumors). Mice were injected with probe 4b, either via the iv or ip (1.11-1.85 MBq) route and results were categorized based on post probe injection time (6, 24, and 48 h pi) and route of probe administration (iv or ip). At various time points post injection, a single blood sample was taken from the tail vein and mice were euthanized by deep halothane anesthesia immediately. Organs and tissues (tumors, heart, lungs, muscle, bone, liver, kidney, spleen, small intestine, and stomach) were harvested, rinsed with PBS buffer, wiped with filter paper and weighed in a pre-weighed tube for the gamma counter measurements. The radioactivity of each sample was measured, decay corrected to the injection time, normalized for injected dose and organ/tissue weight, and expressed as percentage injected dose per gram tissue (%ID/g).

In vivo imaging protocol

Fluorescence imaging

A group of tumor-bearing mice ($n=4$, $n_t=16$, each mouse bearing four tumors) were iv injected with 10 nmol of probe **6-4a** (in 100 μ L PBS) and *in vivo* NIRF imaging of mice was performed at various time points (1, 3, 6, 24, 48, 72 and 168 h pi) with a filter pair of $E_x/E_m=745/820$ nm. Identical illumination settings (auto exposure, medium binning, F/Stop=2) were used for acquiring all images. The fluorescence emission was normalized to photons per second per centimeter squared per steradian (p/s/cm²/sr). Images were acquired and analyzed by Live Image 4.0 (Caliper Life Science), and displayed in the same scale of fluorescence intensity. The fluorescence emission was measured from the region of interest (ROI) of all four tumor inoculants as well as from the muscle of the hind legs as control. For all the time points, fluorescence intensity of tumor was presented as an average of all tumors ($n=4$, $n_t=16$) in each group. Fluorescence intensity of muscle was shown as an average of four mice. Both fluorescence intensities for tumor and muscle were plotted in a time-dependent manner using Prism 5.0.

PET imaging

Tumor-bearing mice were administered with probe **6-4b** (8.88-9.62 MBq per ~20g mouse) via iv ($n=2$, $n_t=8$) and ip ($n=2$, $n_t=8$) injections. Small-animal PET scan was performed on each animal at various time points (3, 6, 24, and 48 h pi). Acquisition time for each scan was 10 min for 3h and 6h, 20 min for 24 h and 30 min for 48 h time points. After ⁶⁴Cu PET imaging was performed and waited to allow ⁶⁴Cu decay (7 days, >10 half-lives), those two IP administered mice were further injected with FDG-¹⁸F (30.97-31.38MBq per ~20g mouse) via tail vein. The PET scan with an acquisition time of 10

min was performed on each mouse at 1.5 h and 3 h pi for comparative studies. PET data for both probe **6-4b** and FDG were reconstructed with the OSEM3D/MAP algorithm (zoom factor, 2.164) using microPET Manager (version 2.4.1.1, Siemens). The reconstructed pixel size was $0.28 \times 0.28 \times 0.79$ mm on a $128 \times 128 \times 95$ image matrix. All PET images were corrected for decay but not for attenuation. Each image analysis was performed using ASIPRO software (Siemens). To characterize the accumulation of probes in tumors, region-of-interest (ROI) analysis was performed manually by visualizing the tumor sites as they appeared as bumps under the skin to identify the radioactivity originating in the tumor region. For both probes at all time points, 8-10 contiguous transaxial tumor ROIs were drawn to cover the entire tumor region, and standardized uptake values (SUVs) were computed using the following formula: $SUV = ([nCi/cc] \times [animal\ weight\ (g)] / injected\ dose\ (nCi))$ (27).

Distribution of probe in blood

Probe **6-4b** was injected at a dose of 9.2 MBq per 20 g mice via tail vein. At 6 h pi, blood (~200 μ L) was collected from each mouse ($n=3$) by cardiac puncture using syringes and Eppendorf tubes (1.5 mL) pre-rinsed with heparin (10 U/mL in PBS). Half of the blood (~100 μ L per mouse) was transferred to gamma counter tubes to measure the radioactivity of whole blood and the other half (~100 μ L per mouse) was centrifuged at 3000 rpm for 5 min (Eppendorf Centrifuge 5810R, Brinkmann Instruments, Inc). From the centrifuged sample, supernatant (majority of serum, >90 μ L) was collected from the settled red blood cells (RBC) and transferred to separate tubes. Both the supernatant and RBC were measured for radioactivity. Radioactivity of all samples for each mouse were

normalized by the value of whole blood (100%) and binding data of each blood component was averaged for three mice (%).

Histological analysis

The mice treated with probe **6-4a** (10 nmol per ~20g mice) were euthanized to harvest the tumors. The harvested tumors were cut into two sections and fluorescence imaging by IVIS Spectrum was performed as described above. The fluorescence imaged tumors were further used for histological analysis. Tumors were fixed by immersing into 10% neutral buffered formalin solution for 48 h before transferring to 70% ethanol. The fixed tissues were dissected to adjacent histological sections (5 μ m thick) that were stained with hemotoxylin and eosin (H/E staining) while keeping two consecutive slice blank (no staining) for direct microscopic visualization of fluorescence signal. H/E stained slices were observed under a light microscope (Zeiss PrimoStar) with a camera (Zeiss AxioCam Cc1) and pictures on ROI were recorded by AxioVision 4.8 (Zeiss). Meanwhile blank slices consecutive to the H/E stained ones were examined on a confocal microscope (Olympus IX81 microscope) equipped with a Hamamatsu digital camera ORCA-ER, with a laser excitation filter of 633 nm and long pass emission filter of 650 nm. The probe distribution and accumulation in tumor was acquired at a microscopic level.

Statistics

Quantitative data are expressed as means $\pm$ standard deviation (SD). For statistical classification, a Student t test (2-tailed, unpaired) was performed using Prism 5.0 software (GraphPad). Any P value less than 0.05 was considered statistically significant.

6.4 Results

Synthesis and Characterization of probe 6-4b

As shown in Figure 6-1, the dual-modality imaging probe consisted of three modules: tumor-targeting carrier (NIRF dye), linker and a radio-metal chelating moiety. PC-1001 was synthesized in straightforward three chemical steps. At each step, the intermediates were purified by HPLC and the compounds were characterized by NMR and MALDI-TOF mass analysis. ^1H and ^{13}C NMR of compounds **6-2** and **6-3** are presented in Appendix 6-2. MALDI-TOF analysis of compound **6-3** and **6-4a** exhibited similar mass spectra with a $[\text{M}^+]$ peak of 1197.648 (Appendix 6-2). The final radiolabeled probe **6-4b** exhibited a single peak by HPLC with a chemical and radio-purity more than 95%, and was compared with its non-radioactive counterpart **6-4a** for confirmation of the structure.

The log P value **6-4b** was calculated to be 1.25 ± 0.20 .

Spectrum properties

The molar extinction coefficient (ϵ) of **6-4a** was measured to be $82,880 \text{ cm}^{-1}\text{M}^{-1}$ at the wavelength of 750 nm (MHI-148 **6-1**, $\epsilon=55,320 \text{ cm}^{-1}\text{M}^{-1}$). As shown in Figure 6-2A, the optimal excitation wavelength of probe **6-4a** was 750 nm, which was used for all subsequent studies. Fluorescence spectra of **6-4a** demonstrated enhanced intensity after mixing with various concentrations of human serum albumin (HSA) and the fluorescence intensity ($E_m=820 \text{ nm}$) reached peak value when the HSA concentration was 0.5% (~2 fold increase) compared to that of no HSA in the system (Figure 6-2B). The fluorescence intensity of both probe **6-4a** and MHI-148 **6-1** (the carrier molecule) demonstrated a linear increasing correlation with the probe concentration within the range of 0-10 μM

(Figure 6-2C). However an inverted decreasing trend was exhibited when the probe concentration was beyond 10 μ M (data not shown). This information provided a window of probe dosing for *in vivo* fluorescence imaging. The fluorescence emission spectra of **6-4a** was also measured under a series of pH conditions (pH=4.0, 5.0, 5.8, 6.2, 6.6, 7.0 and 7.4) and observed to be independent of pH (Figure 6-2D).

In vitro cellular uptake

The comparison between tumor and non-tumor cellular uptake was measured and quantified by the more sensitive PET probe **6-4b**. Figure 6-3A depicts time-dependent uptake of probe **6-4b** in MCF-7 and MCF-10A cells. MCF-10A cells exhibited a slow but steady probe uptake over the entire incubation period (60 min). In contrast, probe uptake in MCF-7 cells showed fast accumulation in the initial 30 min and then a steady increasing trend (> 2 fold higher compared to normal MCF-10A cells) until 60 min post probe incubation. The preliminary mechanism-based cellular uptake study was performed as shown in Figure 6-3B. At 30 min post incubation, probe accumulation in control MCF-7 group (100%) was 3 fold higher than that of MCF-10A ($34 \pm 2\%$) ($P < 0.0001$). Probe uptake in the group of MCF-7 cells with an OATP inhibitor, BSP, revealed a decrease up to $55 \pm 6\%$ ($P < 0.0001$). In contrast, however, a similar experiment with MCF-10A cells failed to demonstrate the ability of BSP to inhibit probe uptake ($P > 0.05$), suggesting the probe uptake by normal breast epithelial cells could be OATP-independent. In addition, the effect of temperature on cellular uptake of **6-4b** was studied, and showed that in both cell lines the cellular uptake decreased with lowering temperatures (MCF-7 at 0°C , $64 \pm 13\%$, $P < 0.0001$ when compared to control MCF-7, 100%; MCF-10A at 0°C , $9 \pm 1\%$, $P < 0.001$ when compared to control MCF-10A, $34 \pm 2\%$).

In vivo dual-modality imaging

Time-dependent *in vivo* NIRF imaging was performed on tumor-bearing mice as described in Materials and Methods using probe **6-4a**. The corresponding analysis provided a preview of the pharmacokinetic profile for radioactive probe **6-4b**. As noted in Figure 6-4A and Figure 6-4B, the contrast for tumors started to be visibly distinct by fluorescence imaging as soon as 1 h pi, with fluorescent intensity exhibiting a sharp increase and reaching peak value at 48 h pi, and remained significantly higher but with a subtly decreased trend until 168 h (7 days) pi. In contrast, the fluorescence intensity of muscle exhibited much slower rate of uptake up to 24 h pi, reaching a plateau (~4 fold less than tumor fluorescence at 48 h pi) and then steadily decreasing trend from 72 h to 7 days pi.

PET imaging and analysis

Based on the data obtained from fluorescence imaging of tumor for probe **6-4b**, a window of opportunity for nuclear imaging was determined. PET was then performed in that time frame to achieve an optimum contrast. As described in the experimental section, *in vivo* PET imaging data was collected at various time points (3, 6, 24 and 48 h pi) and SUV were computed. Representative PET images at 24 and 48 h pi are shown in Figure 6-4C. SUV data [tumor:muscle uptake ratios were 0.63:0.33, 0.64:0.36, 0.94:0.22, and 1.07:0.19 for time points of 3, 6, 24 and 48 h pi, respectively] are presented in Figure 6-4D.

*Comparison of FDG with probe **6-4b** in tumor imaging*

Two of the four mice used for PET imaging after the decay of ^{64}Cu radio-nuclide at 7 days (>10 half lives) were further subjected to comparative studies with FDG uptake in tumor. Figure 6-5A and 6-5B demonstrate that mice administered with probe **6-4b** exhibited better tumor-specific uptake and contrast (tumor:muscle SUV values were 0.94:0.22, and 1.07:0.19 for 24 and 48 h pi, respectively) when compared with FDG injected mice (tumor: muscle SUV values were 0.19:0.26 and 0.11:0.16 respectively at 1.5 and 3 h pi). The specific uptake of FDG in this tumor model is reported to be relatively poor compared with other tumor models (28), which leads to poor imaging contrast as shown by tumor-to-muscle SUV ratios. Furthermore, complete analysis of whole mice FDG images revealed that there were huge amount of uptake in other organs when compared with probe **6-4b**, such as heart, spine, etc (Figure 6-6).

Blood clearance and biodistribution

The tumor to blood ratio of probe **6-4b** over a time period of 48 h pi increased from ~ 0.2 to ~ 0.9 . The data is presented in Figure 6-7.

The radioactivity of blood at various time points was measured and fitted into a mono-exponential equation, $y = y_0 + A_1 \times \exp[-(x-x_0)/t_1]$ with $R^2 = 0.9794$. The blood clearance data is presented as the inset of Figure 6-8A. The elimination half-life for blood clearance ($T_{1/2}$) was calculated to be 4.3 ± 0.2 h.

The tumor and organ uptake profile of probe **6-4b** at various post injection time points and via different injection routes is shown in Figure 6-8A. The majority of probe radioactivity was retained in the blood at 6 h pi (0.73 ± 0.04 %ID/g), which then dramatically decreased until 24 h pi (0.34 ± 0.04 %ID/g for iv and 0.38 ± 0.11 %ID/g for ip injection, $\sim 50\%$). Later on the radioactivity appeared to remain constant through 48 h pi,

irrespective of injection route. The probe **6-4b** demonstrated a much higher rate of tumor-specific uptake and accumulation over time (0.14 ± 0.02 %ID/g at 6 h pi for iv, 0.16 ± 0.03 %ID/g at 24 h pi for iv, 0.25 ± 0.05 %ID/g at 24 h pi for ip, 0.27 ± 0.08 %ID/g at 48 h pi for iv, and 0.30 ± 0.04 %ID/g at 48 h pi for ip). On the other hand, organs such as heart, liver, lungs and spleen exhibited poor rates of uptake, and in kidney, muscle and bone, the amount of probe accumulation remained constant over time.

The radioactivity of both serum (~90%) and RBC (<10%) components of the blood was measured and normalized to whole blood (100%). The majority of radioactivity was observed to be associated with serum as exhibited in Figure 6-8B.

Correlation of probe accumulation with tumor size

The amount of radioactivity for various tumors in each individual mouse ($n=20$, $n_t=80$) was measured and decay corrected to the probe injection time point. Mice were randomly selected from various batches and separate time points, independent of route of probe administration. Analysis of all four sizes of tumors from each of these mice was performed and all mice showed a similar correlation between probe accumulation and tumor weight. Data for a representative mouse is shown in Figure 6-8C, suggesting that probe accumulation (measured as whole tumor radioactivity, Bq) linearly correlated with individual tumor weights (g) ($R^2=0.9837$).

Histological analysis

As depicted in Figure 6-9A, viable (V) and necrotic (N) tumor regions were distinguished visually and macroscopically. Viable tumor regions tended to be more fresh and pink than necrotic areas that were usually off-white or light colored due to lack of blood supply. The fluorescence signals were mainly emitted from viable tumor regions

while the necrotic parts showed little or no fluorescence signal (Figure 6-9A and 6-9B). Microscopically, viable and necrotic tumor cells (darker nucleus) were identified by their characteristic H/E stained morphology as shown in Figure 6-9C (see more detailed histological images in Figure 6-10). The adjacent non-stained slices were observed under a fluorescence microscope to confirm the signal was mainly emitted from the viable tumor and not from the necrotic regions (Figure 6-9C and 6-9D).

6.5 Discussion

The molecular imaging of cancer by a single modality has intrinsic limitations in space and time. These limitations include the low sensitivity of some techniques (MRI, CT) and the poor spatial resolution and non-optimal imaging conditions due to insufficient half-life of probes such as PET radionuclide. In addition other pharmacodynamic and kinetic properties such as the stability of a probe, bioavailability and clearance upon detection and minimum interference in the biological system may restrict the usefulness of a single mode probe for continuous monitoring of disease progression. The cyanine based probes demonstrated high tumor-specific uptake and have prolonged half-life but poor deep tissue penetration and scattering of light that limits their utility for quantitative 3D imaging. However, the tumor-specific uptake properties of these molecules offer opportunities for chemical conjugation to carry other complementary agents into the cancer tissue. In particular, we sought to study a PET imaging probe conjugated to the cyanine as a tumor-specific dual-modality imaging agent.

Traditionally, near-IR fluorescence compounds such as Cy 7 and IR dye 800 are used as optical imaging probes and usually conjugated with other carrier systems for target site-specific delivery and detection of biological disturbances (10, 13, 14). A

variety of delivery systems have been developed in preclinical and clinical settings towards this goal using peptides, proteins/monoclonal antibodies, liposome and polymers (10, 29-32). However, the application of these delivery systems to carry imaging probes has met with severe difficulties due to physiological barriers (33) such as spatial and temporal blood flow, vascular permeability and heterogeneous tissue distribution. Moreover, large molecule carrier systems have sample preparation, chemical/biological stability and storage issues associated with their clinical development. Small molecule tumor-specific delivery agents on the other hand have several advantages: i) ease of chemical modification to achieve optimal pharmacological properties, ii) ease of storage and handling due to their chemical stability, iii) high permeability across cell membranes, and iv) cost effectiveness.

The dual mode probe **6-4b** was synthesized with the goal of realizing complementary properties of each method considering the intrinsic limitations of the NIRF agent. Probe **6-4a** as analyzed by UV/Vis spectroscopy showed an enhanced molar extinction coefficient compared with MHI-148, suggesting that conjugation with DOTA-Cu is not deleterious for probe design. Both the *in vitro* cellular uptake study and the *in vivo* imaging study suggested that modification of the dye structure with linker and Cu-DOTA moiety did not affect the tumor-targeting property of the dye carrier.

Probe **6-4a** exhibited a significant (> 2 fold) fluorescence enhancement after mixing with HSA as described in Figure 6-2B. Meanwhile, the distribution profile of probe **6-4b** in blood (Figure 6-8B) suggested that the majority of the probe was associated with serum not red blood cells. Concurrently, both results indicated the interaction between the probe and the protein (albumin), which might be a major cause of

the long half-life (4.3 ± 0.2 h) (top inset of Figure 6-8A) of **6-4b** in blood circulation and may be a factor to improve tumor-specific uptake over time (34).

In vitro cellular uptake studies observed by radioactivity measurement suggested the selectivity for tumor cells (MCF-7) uptake of probe **6-4b** compared with normal breast epithelial cells (MCF-10A) as exhibited in Figure 6-3A. Furthermore, the preliminary mechanistic studies (Figure 6-3B) demonstrated that OATP may be an important receptor responsible for the probe's active transport across cell membrane (17), although OATP-independent uptake was also identified (Figure 6-3A and 6-3B).

In order to understand the efficacy of the newly designed dual-mode probe using an *in vivo* mice model, we first investigated tumor progression with time and discovered that in a given single mouse. Even when four separate tumors were grafted at four mammary pads, tumor growth was irregular (35). Although the causes behind such uneven tumor growth in mice are not fully understood, we took this observation as an opportunity to define tumor-specific probe uptake, probe accumulation with respect to tumor size, redistribution in various tissues over time, clearance using one single dose of probe and monitoring the probe distribution by fluorescence imaging over a week.

First of all, fluorescence imaging data acquired over a long period of time (up to 5 day pi) provided an accurate preview of the pharmacokinetic profile for probe **6-4a**. Since PET imaging is mainly limited by the decay of the radioisotope, the long term evaluation of pharmacokinetic properties could not be performed by nuclear imaging. At 24 and 48 h pi, PET/NIRF imaging revealed a ~ 4.27 and 5.78 fold (for PET) and 2.71 and 3.54 fold (for NIRF) increase in intensity of signal in tumor compared with muscle tissue. Both fluorescence and nuclear imaging techniques indicated significant tumor

uptake of probe **6-4a** and **6-4b** over time, indicating a similar selective accumulation trend in tumor (Figure 6-4) and the validity of probe for cancer imaging. At the end of 5 days pi, the NIR fluorescence imaging indicated very little change compared to that of 2 days pi, suggesting good retention in tumor with significant contrast between tumor and host normal tissues of the probe over time. These results suggest that PET imaging has better sensitivity compared with fluorescence imaging and provides more accurate and quantitative analysis. On the other hand, NIRF imaging offers high throughput *in vivo* probe screening and therapy evaluation along with qualitative *ex vivo* tissue analysis at the microscopic level (Figure 6-9) (36). The development of a dual imaging probe is thus necessary for a comprehensive understanding of the disease state (13, 14).

FDG is commonly used for cancer imaging in both research and clinical settings.(8, 37-39) However, tumor uptake of FDG is metabolism-dependent and varies in different forms and phases of tumors (40). As shown in Figure 6-5, the tumor-to-muscle uptake ratios (by SUV) for probe **6-4b** are 4.27 at 24 h pi and 5.78 at 48 h pi and for FDG are 0.73 at 1.5 h pi and 0.69 at 3 h pi, indicating the tumor specificity of probe **6-4b** in this mouse model. It has been reported that MCF-7 cells demonstrate a low glucose consumption rate compared to other more aggressive breast tumor cells such as MDA cell line (28). Unlike probe **6-4b**, FDG is not tumor-specific and hence cannot provide sufficient signal/background ratios for successful detection of tumors in the current breast cancer models employed.

Probe **6-4b** accumulated selectively in tumor with respect to post injection time and tumor weight, while it achieved steady state distribution quickly in organs such as heart, liver, lungs, and kidney (Figure 6-8A and 6-8C). Interestingly the dual-modality

molecular imaging probe **6-4b** was observed to be present mainly in growing and live tumor cells and not in necrotic and dead tumor cells (Figure 6-9). This observation suggests that the probe **6-4b** can be used to detect live tumor cells and potentially offers a new tool for distinguishing live from dead cancer cells that could have significant utility in preclinical trials of drugs using animal models.

6.6 Conclusion

The dual-modality molecular imaging probe PC-1001 described in the current study has demonstrated its applicability for tumor detection and quantitative image analysis in a mouse breast cancer model. The probe PC-1001 is accumulated specifically in cancerous tissue with good contrast to normal tissue as compared with FDG. This probe could be useful in assisting the evaluation of anti-cancer therapies, anti-cancer drug discovery, and cancer-related biological studies. Further biological and toxicological evaluation of this imaging agent is ongoing, with the aim of advancing into pre-clinical studies.

6.7 Figures

Figure 6-1. Design and synthesis of dual-modality imaging agent PC1001. Reagents and conditions: i, a. N-hydroxysuccinimide (NHS), N,N'-dicyclohexylcarbodiimide (DCC), CH_2Cl_2 , rt, 12 h; b. *t*Boc-lysine, 0.1 N sodium borate buffer, pH 8.5, 4°C , 12 h; c. pre-chilled 95% TFA, 4°C , 2 h. ii, DOTA-SulfoNHS (structure shown above), 0.1 N sodium borate buffer, pH 8.5, 12 h. iii, CuCl_2 or $^{64}\text{CuCl}_2$, DI H_2O , rt, 10 min.

Figure 6-2. Spectroscopic analysis. A. Optimization of emission wavelength of MHI-DOTA-Cu **6-4a**; B. Effect of protein concentration on fluorescence intensity of MHI-DOTA-Cu **6-4a**; C. Working plot of MHI-148 **6-1**, MHI-Lys **6-2**, and MHI-DOTA-Cu **6-4a**; Note: Fluorescence intensity (counts per second) was plotted against various concentrations of fluorescent dyes and formed linear relationships up to 10 μM . D. Fluorescence property of MHI-DOTA-Cu **6-4a** was independent of pH.

Figure 6-3. *In vitro* cellular uptake studies of **6-4b**. A. Time dependent cellular uptake in MCF-7B and MCF-10A cells. B. Mechanism-based cellular uptake in MCF-7B (shown as 7B) and MCF-10A (shown as 10A). BSP (OATP inhibitor) and 0°C were used to demonstrate the active transportation mechanism. Note: 37°C was used to incubate cells if not mentioned.

Figure 6-4. *In vivo* dual-modality fluorescence/PET imaging. A. Representative fluorescence imaging of mice at various time points (1, 3, 6, 24, 48, 72 and 168 h pi) post probe **6-4a** (10 nmol per 20 g mice) injection (iv). Four tumor sites are indicated with yellow arrows. B. Fluorescence intensities of tumor and muscle were plotted against post injection time (1 h up to 168 h pi) ($n=4$, $n_t=16$). C. Representative PET images of tumor-bearing mice ip administered with probe **6-4b**. Images are presented in three directions (transaxial, coronal and sagittal) at 24 and 48 h pi. Tumor regions are surrounded by white dotted circles. The tumor-of-interest in all images is indicated with bold yellow arrows. D. Mean SUV values of tumor and muscle at various time points (3, 6, 24 and 48 h pi).

Figure 6-5. Comparison of PC1001 and FDG uptake in tumor. Representative transaxial PET images of tumor-bearing mice administered with PC1001 (6-4b) (A) at 24 and 48 h pi and with FDG at 1.5 and 3 h pi (C) (white arrow indicates the position of spine area). SUV_{mean} analysis of tumor and muscle uptake of two tracers was shown in (B) and (D), respectively. Radioactivity used to calculate SUV_{mean} was decay-corrected to the probe injection time point.

Figure 6-6. Transaxial and coronal images of tumor-bearing mice administered with FDG at 1.5 h pi. Images are exhibited at various radioactivity thresholds. From left to right, threshold went from low to high. Horizontal white dotted lines (level 1 and level 2) in upper transaxial images indicated the position of corresponding coronal slices. White arrows indicated spine area and yellow arrows indicated the tumor.

Figure 6-7. Tumor-to-blood ratio at various time points post injection and via different routes of administration (iv and ip). Error bars represented the standard deviation of the ratio value in each group ($n=3-4$).

Figure 6-8. Pharmacokinetic/pharmacodynamic properties of probe PC1001. A. Organ and tissue distribution of PC1001 at various time points (6, 24, and 48 h pi) and via different administration routes (ip vs. iv) ($n=4$ per group). Top inset, blood clearance profile of PC1001. Equation $y = y_0 + A_1 \times \exp[-(x-x_0)/t_1]$ was used to fit the data points and the blood half-life was calculated to be 4.3 ± 0.2 h ($n=3$) ($R^2 = 0.9794$ by Origin). B. Blood distribution of PC1001 at 6 h pi suggested that about 90% of probe was binding to serum instead of red blood cells (RBC) ($n=3$). C. Representative linear correlation ($R^2=0.9837$ by Origin) of PC1001 uptake by tumor radioactivity with tumor weight within a single mouse after 48 h.

Figure 6-9. Probe distribution in tumor tissue at the microscopic level. (A) Representative picture of freshly harvested tumor sectioned in half. Pink area could be observed as viable region (V) while necrotic (N) area was recognized as white or off-white color due to absence of blood supply. (B) Fluorescence image (by IVIS spectrum) of the same tumor segment suggested that fluorescence signal (yellow) was mainly associated with viable regions. (C) and (D) demonstrated microscopic images of tumor area indicated by dotted line square ($\times 100$ fold magnification), which were also a zoomed-in version of (A) and (B). While hemotoxylin/eosin staining (C) clearly exhibited the boarder of the viable and necrotic regions that were recognized by cell morphology, fluorescence microscopic image (D) revealed that the majority of fluorescence signal was associated with viable regions.

Figure 6-10. Tumor histology by H/E staining at various magnifications. A, $\times 40$; B, $\times 100$ and C, $\times 400$. All images indicated that necrotic (N) and viable regions (V) that could be clearly visualized by cell morphology.

6.8 Appendix

Appendix 6-1. Synthesis of carrier molecule MHI-148 **6-1**

Reagents and conditions: i) 110°C, 8h; ii) **6-9**, reflux in ethanol.

[illegible]

^1H -NMR spectrum of compound **6-3** in d^6 -DMSO.

^{13}C -NMR spectrum of compound **6-3** in d^6 -DMSO.

MALDI-TOF-MS of compound **6-3**. MS of probe **6-4a** showed similar mass spectroscopic data as compound **6-3**.

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Dr. Yi Zhang performed the compound synthesis and radiolabeling. Dr. Xiuzhen Xie performed the cellular uptake experiment. Li Xiao conducted all spectroscopic measurements, biodistribution, blood kinetics, imaging-related experiments, histological studies and all data analysis of the entire project.

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Chapter 7: Conclusion and Future Directions

7.1 Conclusion

In this dissertation, Chapters 2, 3, 4 and 5 mainly describe the development and evaluation of a series of peptide and antibody based imaging agents for non-invasive detection of inflammation. These imaging agents are designed and synthesized to target physiologically important receptors, such as FPR and tuftsin receptors on neutrophils, and PECAM-1 molecules expressed on cells within vasculature. Various molecular imaging (NIRF, SPECT and PET) along with anatomic assistant from CT and MRI are successfully carried out in live animals. *In vitro* and *in vivo* evaluation studies of these imaging agents under inflammatory and non-inflammatory conditions are also performed, including partition coefficient, target-specificity, blood kinetics, biodistribution, imaging feasibility in various animal models and immunohistochemical analysis. Highly promising results obtained from these studies suggest the great potential of these imaging agents to advance into further pre-clinical and clinical research. Future study of these imaging agents will focus on the non-invasive detection of inflammatory conditions in various animal models with the aim to widen their clinical applications and facilitate anti-inflammatory drug discovery.

As introduced in earlier chapters, NIRF imaging agents hold great promise in high-throughput screening of a variety of pre-clinical studies in the research setting. In the following section 7.2, the neutrophil-specific NIRF imaging agent cFLFLFL-PEG₇₆-Cy7 (described in Chapter 2 and 3) is tentatively used to detect lung ischemia-reperfusion injury which closely associates with lung transplantation surgery. Preliminary results of

fluorescence imaging are promising and exhibit the potential of these peptide agents in detecting lung IR-injury.

In addition to developing molecular imaging agents for inflammation, Chapter 6 demonstrates development and evaluation of a tumor-specific heptamethine cyanine-based dual-modality PET/NIRF imaging agents for cancer. This is a proof-of-concept study: one side of the carboxylic acid of MHI-148 is modified to load the PET radioisotope (Figure 6-1). Data obtained such as spectroscopic evaluation, tumor-specific cellular-uptake, biodistribution, blood kinetics, NIRF and nuclear imaging are highly promising. These initial results suggest that this type of structural modification does not hurt the biological performance of the original dye. Therefore, several indications for the future direction of the NIRF dye can be generalized as follow: 1) the dye molecule could be used as a carrier for developing a new generation of tumor-specific agents to enable early detection, targeted therapy and simultaneous monitoring of treatment efficacy of cancers (a preliminary study of synthesis and evaluation of photosensitizer-carrier conjugate is described in section 7.3); 2) additional structure-activity-relationship (SAR) of the dye molecule could be investigated to address the following questions (e.g. Does chemical modification on other sites of the molecule affect the tumor-targeting property? Can the hydrophilicity/hydrophobicity and PK/PD properties be further adjusted?); 3) conjugation of the dye with other radioisotopes (e.g. ^{99m}Tc , ^{111}In) could be examined for more versatile design and application of the imaging agent; 4) detailed biological mechanism of the tumor-specific uptake could be explored.

7.2 Neutrophil-specific NIRF imaging Agent cFLFLFL-PEG-Cy7 for Lung Ischemia-reperfusion Injury

7.2.1 Lung Ischemia-Reperfusion Injury

The yearly number of transplants worldwide has increased 130-fold over the last twenty years (1). Despite advances in surgical management, critical care, and immunosuppression, long-term survival has not improved. In fact, outcomes for lung transplantation are the worst of any solid organ transplant. In most series, 30-day mortality rates are near 15%, and ~50% of lung transplant patients die within three years (1, 2). Lung ischemia-reperfusion (IR) injury following transplantation continues to be a common and significant cause of morbidity and mortality in the early postoperative period, with rates as high as 41% reported (2). The 30-day mortality of patients with IR injury is about 40% compared to 7% in patients without (3). Patients with IR injury demonstrate immediate impairment in lung function after transplant accompanied by pulmonary edema, increased pulmonary vascular resistance, and decreased airway compliance (4, 5). Patients with IR injury require prolonged mechanical ventilation, longer hospital stays, and are at an increased risk of multi-organ failure. Lung IR injury has long-term consequences as well, where IR injury is also a risk factor for late graft rejection (6). Treatment strategies for patients that develop IR injury consist primarily of maintaining oxygenation and lung function. Use of inhaled nitric oxide has offered only limited benefits. IR injury occasionally is so severe that extracorporeal membrane oxygenation is required to maintain oxygenation.

One of the critical steps mediating lung IR injury is the direct engagement of circulating neutrophils with vascular endothelium after reperfusion (7-13). This

interaction results in the release of destructive proteases, oxygen radicals and cytokines, which promote the tissue injury (8, 14, 15). Many studies have shown that neutrophils have a substantial role in post-transplant lung IR injury (8-12). Studies have shown that neutrophil-activating compounds cause lung injury (16-18); neutrophil depletion attenuates lung IR injury (19-21); and immunoneutralization of neutrophil adhesion prevents lung IR injury (22-24). Neutrophils are end-effectors of injury, and the activation and chemotaxis of neutrophils into damaged lung is orchestrated by a variety of chemokines produced by multiple cell types including macrophages, epithelial cells, and T cells. The resulting damage is then manifested by increased capillary permeability, altered vascular tone, and acute graft failure.

In that case, lung IR injury, if remained unresolved for a long time, may lead to irreversible tissue damage and physiological dysfunction of other organs due to loss of oxygenated blood supply. The blood supply to IR injured tissue is thus highly necessary for recovery and repair; however, the reperfusion is now considered a double-edged sword and may cause excessive damage because of sudden formation of reactive oxygen species in ischemic tissues. Recent evidence has now been established that the recruitment of neutrophils in response to infectious and ischemic injuries occur by distinctly different signaling mechanism (25), hence studying inflammation and recruitment of neutrophils in different injuries is highly critical to understand the nature of disease.

Expression of FPR and its involvement are demonstrated in a variety of IR tissue injury, thus having an FPR-specific nuclear or fluorescent imaging agent with appropriate

animal model will be useful for detection and treatment of such injury, as exhibited in Figure 7-1.

7.2.2 Preliminary Results of Fluorescence Imaging

In vivo left lung IR injury was performed using a hilar-clamp model as previously described Dr. Laubach's laboratory, Division of Thoracic & Cardiovascular Surgery, University of Virginia (26). In the preliminary studies the left lung was injured by 1 hour ischemia, and subsequent reperfusion was achieved by removing the clip and the tube/suture to initiate a 2-hour reperfusion period. Sham animals received only thoracotomy without hilar occlusion. Both IR injured and sham mice were administered with the neutrophil-specific fluorescence imaging agent (cFLFLF-PEG₇₆-Cy7, 10 nmol/20 g mice) through jugular vein before reperfusion. Three hours after probe administration, mice were sacrificed and *ex-vivo* NIRF imaging of both harvested right and left lungs from IR injured and sham mice was performed using IVIS Spectrum. As shown in Figure 7-2, the IR injured mice display notable left lung inflammation as indicated by high intensity of fluorescence. Figure 7-3 exhibits normalized fluorescence intensity (in average radiance) of lungs for both IR injured and sham mice. Column A presents that the fluorescence intensity of IR-injured left lung displays ~4-fold more intensity than the right lung (despite the fact that the right lung is larger than the left). When compared with sham mouse, fluorescence intensity ratio is further elevated to ~5-fold, indicating higher uptake of the fluorescent imaging agent in the injured lung (27). It must be noted that the injured mouse has a slightly higher number of overall PMNs than sham mouse as observed in other inflammation model (28).

7.3 Cancer-specific Heptamethine Cyanine Dye for Photodynamic Therapy

7.3.1 Photodynamic Therapy for Cancer

Cancer remains one of the most stressful diseases to treat, causing significant morbidity and mortality with over 10 million new cases per year worldwide. Photodynamic therapy (PDT) for cancer although is currently used in clinical setup and offers good advantages over other types of therapies, it still has limitations. As illustrated in Figure 7-4, the most severe limitation of PDT for cancer treatment is the overall phototoxicity due to lack of tumor-specific uptake of photosensitizer (PS). Improving tumor-specific PS uptake can be addressed by its chemical modification.

Carrier-mediated delivery of PS is demonstrated to increase accumulation in the tumor and thus broaden the clinical applications and minimize the amount of precision needed in light delivery. A variety of delivery systems have been developed in preclinical models towards this goal using large molecules such as proteins/monoclonal antibodies, liposomes and polymers (29-32). However, application of the large molecule delivery of PS has severe difficulties because of impediment of physiological barriers, such as spatially and temporally heterogeneous blood flow and vascular permeability. Moreover, large molecule carrier systems have sample preparation, chemical/biological stability and storage issues associated with development. In contrast, the use of small molecule tumor-specific as delivery agents has several advantages: (1) ease of chemical modification to achieve optimal pharmacological properties, (2) ease of storage and handling, (3) high permeability across cell membranes, and (4) cost effectiveness. Conjugation of small molecule carrier with PS should yield a photosensitizer-carrier conjugate (PSC) with superior tumor-specific delivery properties, resulting in high accumulation of drug in

tumor compared with normal tissue. If realized, this in turn will be a huge advancement in photodynamic therapy by lowering down PS dose and therapy related toxicity.

A versatile design of small molecule carrier-conjugated PSC theranostic agent in modular fashion (PS, linker and carrier) is proposed as exhibited in Figure 7-5. The design allows flexibility to optimize the diagnostic and therapeutic properties either separately or synergistically for given PSC. The design includes: a) the novel PSC combines dual functions of imaging and therapy for cancer in a single molecule entity, b) the small molecule carrier moiety; as demonstrated in Chapter 6, MHI-148 **6-1** is known to be able to specifically target a broad spectrum of cancer cell lines and tumor models, and c) optimization of pharmacological properties through structural modification to yield maximum therapeutic benefits with minimum possible side effects in a highly efficient fashion. Overcoming limitation of PS accumulation in tumor tissue by conjugating with tumor-specific and biologically silent carrier molecule is one of attractive strategies to reduce overall toxicity associated with PDT, which is addressed in this proposal.

7.3.2 Synthesis of Conceptual Photosensitizer-Carrier Conjugate (PSC)

The chemical synthesis of this conceptual carrier conjugated PSC (MHI-Ce6) is demonstrated in Scheme 7-1. The multimilligram scale synthesis of non-metalated compound MHI-Ce6 **7-3** was achieved in four straight forward chemical steps. MHI-Ce6 was analyzed for its purity by HPLC and characterized by mass spectroscopy for its molecular composition. The compound was then coordinated with radioactive ^{64}Cu to yield product **7-4** for PET imaging. The composition of this new PET imaging agent was

compared with its cold counterpart **7-4a** by HPLC. The cold Cu compound **7-4a** was characterized by mass analysis.

7.3.3 *In vivo* NIRF and PET Imaging of PSC

The newly conjugated PSC MHI-Ce6 was evaluated for its *in vivo* clinical utility of in mice breast cancer xenograft. *In vivo* mice xenograft model of breast cancer was developed according to previously published protocols using MCF-7B human breast cancer cell lines (36). After visual observation of good size tumor development MHI-Ce6 was i.p. injected and the whole animal fluorescence imaging was performed (IVIS Spectrum) at various time points post probe injection (Figure 7-6A). Fluorescence intensities of all organs (including tumors) were measured by Live Image 4.0 and normalized to that of muscle to indicate the tumor-specific uptake (Figure 7-6B and 7-6C). In this example the maximum tumor-to-muscle ratio was observed to be 23 (Figure 7-6C). The tumor specific-uptake of MHI-Ce6 was demonstrated to be correlated linearly with the tumor size as measured by its volume in the same animal as shown in one of the representative mice (Figure 7-6D).

The mice model mentioned in the section above was used for PET imaging by intraperitoneally injecting ^{64}Cu -labeled compounds (100-200 μCi). Transaxial PET images are presented in Figure 7-7A with mice model administered with MHI-Ce6- ^{64}Cu (PSC) along with controls Ce6- ^{64}Cu and MHI- ^{64}Cu at 24 h and 48 h post probe injection (pi). SUV analyses were performed on tumors and muscles defined by CT scans (data not shown) and tumor-to-muscle ratio was calculated for each group at both time points. Interestingly, tumor-to-muscle ratio of Ce6- ^{64}Cu remained unchanged, however, the ratio for carrier MHI- ^{64}Cu and for MHI-Ce6- ^{64}Cu (PSC) increased over time (Figure 7-7B).

Indeed, this result suggests Ce6 as is not accumulated in tumor over time which may not be efficient for PDT. Excitingly, this limitation of Ce6 was overcome by conjugating it with carrier for tumor-specific delivery. PSC not only accumulated in tumor with time but also improved tumor-to-muscle ratio with more than 6 fold at 48 h pi. This result validated the hypothesis that conjugation of carrier molecule with PS might improve the efficiency of PDT.

Although many other experiments need to be performed to evaluate theranostic properties of the newly synthesized PSC, preliminary NIRF/PET imaging results suggest great potential of this type of agent in realizing both detection and treatment functions of cancer. Success of this project will provide a novel class of theranostic agents with reduced toxicity associated with PDT, thus improving quality of life as well as survival rate. In addition, the success of this project will also provide molecular imaging scientist and clinicians a new technique to tackle cancer.

7.4 Figures and Scheme

Figure 7-1. General schematic of imaging strategy for lung IR injury.

Figure 7-2. Preliminary studies of fluorescence imaging of lung IR injury model. Panel A. IR injury caused to left lung of the mice, right lung was untied. Panel B. Sham mouse no IT injury to either lung.

Figure 7-3. Normalized fluorescence intensities for IR injured (A) and control (sham) (B) lungs of mice. ROIs were drawn on lungs using software auto-ROI function, as outlined by blue boundaries in Figure 7-2.

Figure 7-4. General schematic of photodynamic therapy for tumor.

Figure 7-5. Conceptual design of photosensitizer-carrier conjugates (PSC). Versatile multimodal design to deliver PSC specifically to tumor cell by independent modification of carrier, linker and PS to optimize the therapeutic utility and diagnostic tool for molecular imaging (fluorescence, PET and SPECT).

Scheme 1. Synthesis of MHI-Ce6

Scheme 7-1. Synthesis of MHI-Ce6. Reaction conditions: i. DCC, N-hydroxysuccinimide, CH₂Cl₂, rt; ii. N-a-Boc-Lysine, 0.1 M borate buffer-acetonitrile; iii. EDC, DMF, rt; iv. Chlorin e6 anhydride, DMF, rt; v. ⁶⁴CuCl₂, MeOH, rt.

Figure 7-6. Tumor-specificity of MHI-Ce6 manifested by fluorescence imaging. A. Longitudinal *in vivo* epi-fluorescence imaging of breast tumor bearing mouse with multiple sites of tumor inoculation administered MHI-Ce6 (10 nmol) intra-peritoneally. Fluorescence imaging was performed at various time points post probe injection (1h, 3h, 6h, 24h and 48h pi) with filter pair $E_x/E_m=745/820$ nm. B. Fluorescence image of harvested organs of euthanized mouse at 48 h post MHI-Ce6 injection: T1, T2 and T3, tumors; H, heart; Lu, lung; Sp, spleen, Li, liver; Ki, kidney; Sm In, small intestine; Mus, muscle; St, stomach; Bo, bone and skin. C. Organ uptake profile by normalizing with muscle as organ-to-muscle ratio. D. Correlation of MHI-Ce6 uptake by fluorescence intensity with tumor size by volume.

Figure 7-7. Comparison of PET imaging and SUV analysis. A. Transaxial PET images of tumor-bearing mice administered with Ce6-⁶⁴Cu (left), MHI-⁶⁴Cu (middle) and MHI-Ce6-⁶⁴Cu (right) at 24 h (upper row) and 48 h (bottom row) post injection. Tumor regions are highlighted by white dotted circles and positioning of mice are defined “D” for dorsal and “V” for ventral in all images. B. Time-dependent accumulation of imaging agents defined as tumor-to-muscle uptake ratio.

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7.6 References

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