UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Gallium-68 Bioorthogonal Tetrazine Polymers for the Multistep Labeling of Cancer Biomarkers

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Chemistry

by

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Committee in charge:

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2013

The Thesis of Brandon Edward Nichols is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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2013

# DEDICATION

There are so many people in my life that have had a positive impact on me that I could not possibly acknowledge them all in a meaningful manner within the space provided. There is however one person in my life that I would like to, in a page, try to give thanks to though. I think they might have earned it.

I have never seen my father. He called me once when I was in seventh grade but he did not have much to say. I am truly blessed though because if it was not for him I would not have had the chance to live my life under the wing of such an amazing woman. Here is where I would like to thank the strongest, the most loving and, undeniably, the most understanding person I have ever met in my life. I would like to thank my mother for, well… everything. I want to thank her for giving up so much of her life so that I could live mine in any direction or way I wanted. You are the reason I work so hard, the reason I am the way I am, and even though we argue still sometimes I want you to know that I love you. Not a day goes by where I do not think about all the sacrifices you have made for me and you did it with a smile on your face. You never put your setbacks on me, never complained about my father leaving you. If I can give a quarter of the love you gave me, to my children, I know that I will have done a good job.

To the best teammate, friend, and mother a boy could have, thank you for everything. I dedicate this thesis to you.

I love you mom,

Your Son

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ACKNOWLEDGEMENTS

The author would like to acknowledge support provided by the American Cancer Society Institutional Research Grant 70-002 provided through the NCI ICMIC program (P50 CA11475) UCSD *In vivo* Cancer and Molecular Imaging Center in the Moores Cancer Center and the University of California, San Diego. Research reported in this publication was also supported in part by the National Institute of Biomedical Imaging and Bioengineering of the National Institutes of Health under Award Number K01EB010078. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors acknowledge the support of Jesse Vargas and the NIH P50 Center of Excellence Grant to the San Diego Center for Systems Biology (SDCSB).

The author would also like to acknowledge Zhengtao Qin for his collaborative effort on this research project, Jun Yang for synthesizing necessary reagents, Michael Hardy for his guidance and thoughtful insights, Jolita Seckute for her guidance, patience, and malty malts, Christian Cole for his help with fluorescent microscopy, cell work, and guidance, Haoxing Wu for his synthesis guidance, Gage Brummer for his help and comedy, Dr. Vera for his instruction and allowance of the utilization of his 68Ge/68Ga Generator and PET imaging instruments/tools, Dr. Joseph for being on my thesis committee, and Dr. Devaraj for his unwavering patience and guidance on the project.

All chapters are, in part, being prepared for submission for publication material. Zhengtao Qin worked equally on this project and is a primary investigator and co-author of this material. Without Zhengtao's help this project could not have been completed, he was an asset to this project.

The author would like to announce his role as one of the leads on this project. Manuscript preparation, data preparation, and other information gathering were performed in part or in whole by this author.

The following material in part or in whole is under review at the following: Nichols B, Qin Z, Yang J, Vera DR, Devaraj NK. 68Ga Chelating Bioorthogonal Tetrazine Polymers for the Multistep labeling of Cancer Biomarkers. *ChemComm* Manuscript Submitted.

ABSTRACT OF THE THESIS

Gallium-68 Bioorthogonal Tetrazine Polymers for the

Multistep Labeling of Cancer Biomarkers

by

Brandon Edward Nichols

Master of Science in Chemistry

University of California, San Diego, 2013

Professor Neal K. Devaraj, Chair

This thesis reports the development of a metal chelating bioorthogonal tetrazine diethylene triamine pentaacetic acid (DTPA) dextran probe that is highly reactive with transcyclooctene modified monoclonal antibodies. Using this probe, we performed multistep labeling of the A33 antigen on human colon cancer cells using transcyclooctene

modified anti-A33 monoclonal antibodies followed by tetrazine DTPA dextran probes. Confocal imaging demonstrates the expected surface modification of A33 by fluorescently labeled tetrazine DTPA dextran. The availability of the DTPA chelating group allows rapid metallation of the reactive polymers with the positron emitting radionuclide gallium-68 ($^{68}$Ga) and it was demonstrated that $^{68}$Ga tetrazine dextrans can be used to label the A33 antigen on cancer cells using the bioorthogonal multistep approach. We have characterized the biodistribution of the $^{68}$Ga tetrazine dextran and have imaged its uptake *in vivo* using positron emission tomography (PET) imaging. Tetrazine modified metal-chelating polymers may prove to be important probes for multistep imaging of antigens using short-lived and highly convenient PET radionuclides such as $^{68}$Ga.

# CHAPTER 1:

# INTRODUCTION

There is clinical interest in the use of readily available positron emitting isotopes such as fluorine-18 (18F) and gallium-68 (68Ga) to image affinity ligands *in vivo* via positron emission tomography (PET). Unfortunately, despite the various advantages of PET imaging, development of novel translatable PET agents is extremely challenging. Though 18F is readily available, the requirement of a cyclotron for production and harsh labeling chemistry has sparked interest in exploring alternative radionuclides.[1] One of the most promising is 68Ga, which can be conveniently produced on-site using a variety of commercially available generators.[2] Also, unlike 18F, which is covalently attached by harsh substitution chemistry, 68Ga can be readily incorporated into a number of standard chelating agents such as diethylene triamine pentaacetic acid (DTPA). In particular, 68Ga modified peptides such as somatostatin are widely used for imaging neuroendocrine tumors.[3] However, even after an affinity ligand is labeled with 68Ga, the short positron decay half-life (68 minutes) requires that the affinity ligand must show significant target accumulation and high background clearance in hours. This is simply not possible with the most commonly used affinity ligands, monoclonal antibodies, which clear the blood slowly over days. Long blood residence times often enable antibodies to accumulate over time in the region of interest.[4] Several strategies have been used to circumvent this issue. Some focus on the affinity ligand and with optimizing the clearance kinetics by creating antibody fragments or searching for small peptides with affinity for a target. However, although smaller protein fragments have more desirable clearance kinetics this, along with lowered affinity constants, hinders their ability to accumulate effectively at the target. Simultaneously, there has been significant effort in application of PET nuclides with much longer decay half-lives such as 64Cu, 124I and 89Zr.[5-7] Recent elegant work

has also demonstrated that utility of inverse-electron demand tetrazine/norbornene cycloadditions for introducing long-lived PET radioisotopes such as 64Cu and 89Zr to monoclonal antibodies.[8] However, these isotopes suffer from a range of issues including limited availability (and subsequently high expense), toxicity from systemic exposure, poor resolution, high required radiation doses, delayed imaging, and tradeoffs with signal sensitivity.[9]

The complications involved with direct labeling of affinity ligands with PET radionuclides has led to the exploration of multistep pretargeting strategies.[10-15] Several groups have recently explored pretargeted imaging using bioogrthogonal cycloadditions that react with rapid kinetics and can be performed under biologically relevant conditions and in the presence of cellular functional groups.[16-22] Among the many reactions reported, the inverse electron demand Diels-Alder cycloaddition between 1,2,4,5 tetrazines and strained dienophiles such as norbornene, cyclopropene, and trans-cyclooctene has emerged as a valuable *in vivo* bioorthogonal coupling tool (Scheme 1a). These reactions can be extremely fast (up to $3x10^4$ $M^{-1}sec^{-1}$), do not require a catalyst, are high yielding/chemoselective, and operate best in aqueous solutions and serum. The reaction is irreversible and the coupling product stable. This next generation bioorthogonal chemistry has been utilized for a myriad of biomedical applications such as the labeling of antigens on live cells with tetrazine fluorophores, intracellular imaging of drug-like molecules using fluorogenic probes, and amplified multistep assembly of nanomaterials for point-of-care diagnostics.[23-25]

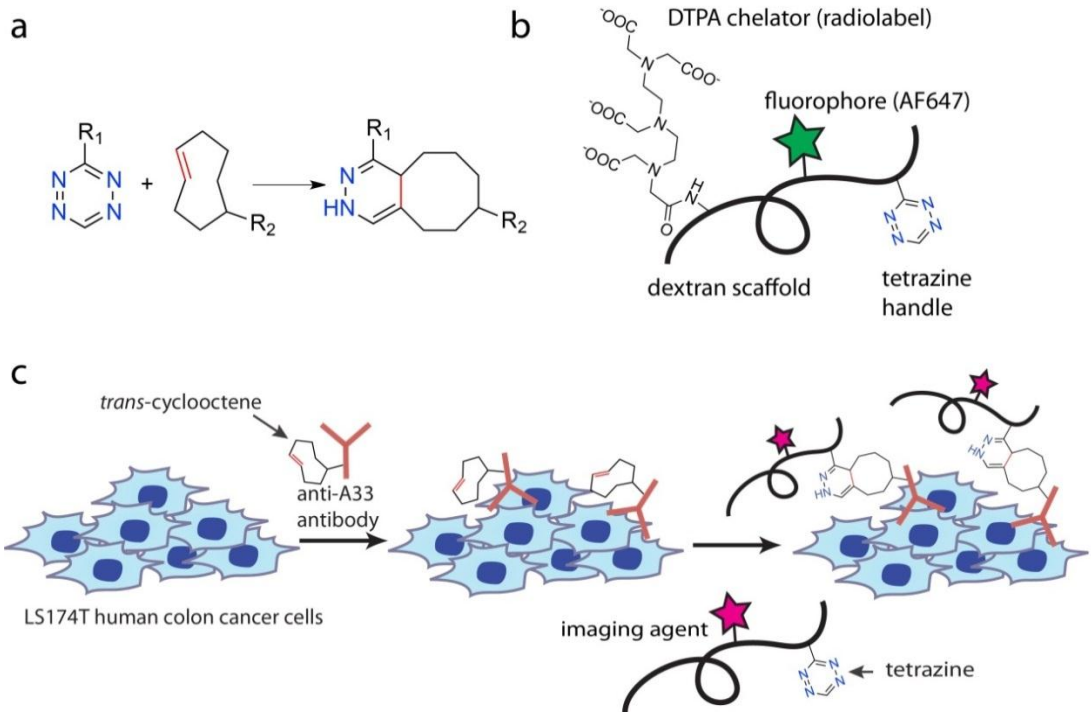
Recent work has demonstrated that inverse Diels-Alder tetrazine cycloadditions are capable of *in vivo* multistep imaging of tumor xenografts, using either single-photon

emission computed tomography (SPECT) or PET radionuclide probes.[19, 22] In these studies, a trans-cyclooctene modified monoclonal antibody is allowed to accumulate and clear over a lengthy time period. After clearance, a secondary tetrazine imaging agent is administered. In the case of short-lived PET agents, this approach is essential given the long circulating half-life of monoclonal antibodies. Previous work has demonstrated the need to optimize the pharmacokinetics of the secondary tetrazine agent to achieve high *in vivo* reaction yields. For example, the use of commercially available polymers modified with tetrazines and 18F PET probes enabled successful optimization of pharmacokinetics and high *in vivo* reaction yields.[22]

Here we report a novel metal chelating bioorthogonal tetrazine DTPA dextran probe that is capable of being radiolabelled using the positron emitting isotope 68Ga (Scheme 1b). These probes are highly reactive with transcyclooctene modified monoclonal antibodies. The colon cancer biomarker A33 is readily modified by multistep labeling using a trans-cyclooctene modified anti-A33 monoclonal antibody followed by tetrazine DTPA dextran (Scheme 1c). Using confocal microscopy, we verify surface localization of the dextran probes and minimal background uptake. Exposure of the metal chelating tetrazine-dextran to the radionuclide 68Ga results in convenient metallation and the formation of a PET tracer. These radionuclide diagnostic agents can also be utilized for multistep labeling of colon cancer cell lines and show well behaved pharmacokinetics in mice. Bioorthogonal labeling using metal-chelating tetrazines could enable the use of the short-lived and convenient 68Ga radionuclide for the multistep labeling and imaging of monoclonal antibodies bound to cancer related biomarkers.

This material in part or in whole is under review at the following:

Nichols B, Qin Z, Yang J, Vera DR, Devaraj NK. 68Ga Chelating Bioorthogonal Tetrazine Polymers for the Multistep labeling of Cancer Biomarkers. *ChemComm* Manuscript Submitted




Scheme 1.1: a) Bioorthogonal inverse Diels-Alder reaction between tetrazine and *trans*-cyclooctene proceeds extremely rapidly without the need for catalyst. These reactions can be used to couple imaging agents and affinity ligands together. B) Cartoon schematic of the dextran scaffold which has been aminated to allow attachment of tetrazine reactive groups as well as imaging agents such as near-infrared fluorophores and chelators that can be metalated with radioisotopes. C) Multistep labeling of cellular bound *trans*-cyclooctene anti-A33 with a reactive tetrazine DTPA dextran containing an imaging agent such as a near-infrared fluorophore (AF647) or a radionuclide ($^{68}$Ga).

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# CHAPTER 2:

# MATERIALS AND METHODS

All chemical reagents were purchased from Sigma-Aldrich and used as received unless otherwise noted. Tert-butyl 4-(6-H-1,2,4,5-tetrazin-3-yl)benzylcarbamate and (E)-cyclooct-4-enol precursors were synthesized as previously described. [1, 2] Trypsin (0.05% T / 0.53mM EDTA) and L-Glutamine were purchased from Mediatech – VWR (San Diego, CA) and 10x PBS was purchased from Biomiga, Inc. (San Diego, CA). Penicillin/ streptomycin, Alexa Fluor 647 Carboxylic Acid Succinimidyl Ester, and Texas Red-X, Succinimidyl Ester, single isomer were all purchased from Life Technologies. Bisbenzimide H (Hoescht Stain) was purchased from Sigma Aldrich. Amino-terminated DTPA dextran was synthesized as previously described.[3]

## 2.0 Tissue culture/cell growth conditions

LS174T cells were grown in cDMEM media supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin. Cells were incubated in 5.0% carbon dioxide, 95% humidity at 37°C. Generally, cells were grown in T-75 tissue culture flasks, seeded at densities between 500,000 and 750,000 cells per flask (cells were quantified with the Life Technologies Countess automated cell counter). The cells were trypsinized with TrypLE Express and resuspended in cDMEM. Cells were allowed to incubate for two days before incubating with probes as described below.

2.1 Synthesis of tetrazine NHS (2,5-dioxopyrrolidin-1-yl 5-((4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)-5-oxopentanoate)

$CF_3COOH$ (0.25mL) was added to a stirred solution of tert-butyl 4-(6-H-1,2,4,5-tetrazin-3-yl)benzylcarbamate (10.0 mg, 0.033 mmol) in $CH_2Cl_2$ (1.0 mL) at room temperature. The resulting solution was stirred 2.0 hours at room temperature and then evaporated to afford (4-(6-H-1,2,4,5-tetrazin-3-yl)phenyl)methanamine TFA salt. The resulting salt was dissolved in $CH_2Cl_2$, after which $Et_3N$ (10.0 mg, 0.10 mmol) was added, followed by glutaric anhydride (4.0 mg, 0.033 mmol). This resulting solution was stirred for 1 hour at room temperature after which N,N'-disuccinimidyl carbonate (13.0 mg, 0.05 mmol) was added. The reaction solution was stirred at room temperature for 1 hour and then evaporated. The residue was purified by preparative TLC (Hexanes:EtOAc=3:1) to afford 9.0 mg product as a pink solid, in 66% yield. 1H NMR (400 MHz, $CDCl_3$) δ 2.15-2.18 (2H, m), 2.41 (2H, t, J = 8 Hz), 2.70 (2H, t, J = 8 Hz), 2.84 (4H, bs), 4.57 (2H, d, J = 8 Hz), 6.48 (1H, bs), 7.52 (2H, d, J = 8 Hz), 8.58 (2H, d, J = 8 Hz), 10.21 (1H, s); 13C (100 MHz, $CDCl_3$) δ 21.15, 25.81, 30.10, 34.52, 43.49, 128.81, 128.83, 144.15, 158.01, 166.49, 168,55, 169.55. HRMS [M+Na]+ m/z calcd. for $[C_{18}H_{18}N_6O_5Na]+$ 421.1231, found 421.1229.

2.2 Synthesis of trans-cyclooctene NHS ((E)-cyclooct-4-en-1-yl (2,5-dioxopyrrolidin-1-yl) glutarate)

DMAP (6.1 mg, 0.05 mmol) was added to a stirred solution of (E)-cyclooct-4-enol (5.0 mg, 0.040 mmol) in toluene (1.0 mL), followed by glutaric anhydride (6.0 mg, 0.05 mmol). The resulting reaction solution was heated to 100$^{o}$C and stirred at this temperature for 18 hours. After TLC indicated that the reaction had finished the solvent was evaporated and the residue was dissolved in $CH_2Cl_2$, followed by addition of N,N'-disuccinimidyl carbonate (13.0 mg, 0.05 mmol). After stirring at room temperature for 30 minutes, the reaction solution was evaporated and the residue was purified by preparative TLC (Hexanes:EtOAc=2:1) to afford 7.0 mg product as a colorless liquid, in 51 % yield. 1H NMR (500 MHz, $CDCl_3$) δ 1.59-1.71 (2H, m), 1.89-2.05 (6H, m), 2.30-2.40 (6H, m), 2.68 (t, J = 10 Hz, 2H), 2.83 (4H, bs), 4.42-4.45 (1H, t, m), 5.46-5.60 (2H, m); 13C (100 MHz, $CDCl_3$) δ 20.05, 25.80, 30.28, 31.18, 32.72, 33.30, 34.46, 38.81, 41.10, 80.64, 133.27, 135.13, 168.32, 169.27, 171.95; HRMS [M+Na]+ m/z calcd. For $[C_{17}H_{23}NO_6Na]$ + 360.1418, found 360.1419.

2.3 Synthesis of Tetrazine DTPA Dextran used for 68Ga labeling and *in vivo* experiments

The required aminated Tetrazine DTPA Dextran precursor was prepared from Amino-terminated Dextran DTPA (synthesized as previously described from dextran T10, final molecular weight approximately 16 kDa determined by dynamic light scattering)[3] and amine reactive tetrazine-NHS. One equivalent of DTPA Dextran was

reacted with 10 equivalents of tetrazine-NHS for two hours at 21°C in 0.1M $NaHCO_3$ buffer (pH 8.5). After reacting for 2 hours, the dextran was washed with Milli-Q deionized water 3 times using 3 kDa Amicon centrifuge filters. Tetrazine loading was quantified using the characteristic visible absorption band of tetrazine at approximately 530 nm. The tetrazine DTPA Dextran was subsequently reacted with an excess of acetic anhydride (1000 equivalents) in deionized water for 1 hour to ensure capping of any free amines to prevent nonspecific *in vivo* uptake.[4] The resulting solution was washed with 500 μL of Milli-Q water 3 times using 3 kDa Amicon centrifuge filters. After the final wash, the solvent was evaporated and the dried dextran stored at -20°C until needed. The polydispersity index 0.38 was measured by Dynamic Light Scattering with a Malvern Zetasizer Nano ZS.

## 2.4 Synthesis of Alexa Fluor 647 (AF 647) Tetrazine DTPA Dextran

Amino-terminated DTPA Dextran was reacted at room temperature for 1 hour with 1 equivalent of Alexa Fluor 647 (AF 647) in 0.1M $NaHCO_3$ buffer (pH 8.5). The product was rinsed with Milli-Q water 3 times using a 3 kDa Amicon centrifuge filter. The AF 647 DTPA Dextran was then reacted with tetrazine NHS as described above.

## 2.5 Synthesis of anti-A33 trans-cyclooctene (TCO)

Antibody trans-cyclooctene conjugates were synthesized as previously described.[5] 100 μg of Anti-A33 monoclonal antibody (R&D Systems) was dissolved in 100 μL of a

90:10 mixture of 0.1 M $NaHCO_3$ (pH 8.5) and anhydrous DMF. 50 equivalents of trans-cyclooctene NHS was added and the reaction mixture was gently shaken for two hours. The resulting solution was centrifuge filtered three times, using a 30 kDa Amicon filter and 90:10 $NaHCO_3$/ anhydrous DMF mixture as a wash. The antibody conjugate was resuspended in 0.1M $NaHCO_3$ at 1 mg/mL concentration (determined by monitoring the absorbance at 280 nm) and stored at 4°C.

## 2.6 Fluorescence Microscopy

LS174T human colon cancer cells were incubated for two days on a Lab-Tek chamber slide maintained in cDMEM medium. Cells were treated with 200 nM anti-A33 trans-cyclooctene in cell growth media and incubated for 30 minutes at 37°C. The media solution was aspirated and the cells were washed 3 times with cDMEM. After reincubation in cDMEM, the cells were treated with 10 μM of AF 647 tetrazine DTPA dextran and incubated for 30 minutes at 37°C. The media solution was aspirated, the cells were washed 3 times with cDMEM and reincubated in 200 μL cDMEM. To these cells, 200 μL of 2 μM Hoescht stain was added and incubated for 20 minutes at 37°C before imaging. All photos were collected with a 100x objective with a numerical aperature of 1.46 on a Zeiss AxioObserver Z1 inverted fluorescence microscope fitted with a ORCA-Flash 4.0 CMOS camera from Hamamatsu. The light source was a mercury arc-lamp from Sutter and images were processed using ImageJ 1.45j software package.

## 2.7 Radiochemistry

0.79 mg tetrazine DTPA dextran was dissolved in 300 μL 2M Sodium Acetate buffer solution (pH = 8.5). The dextran concentration for this stock solution was approximately $1.6 \times 10^{-4}$ M. Caution: 68Ga is radioactive and all procedures should be performed behind lead shields and by trained personnel equipped with radiation dosimetry monitoring badges. Radioactivity was measured on a Capintec CRC-15W dose calibrator. A 68Ga generator (Eckert & Ziegler Isotope Products IGG100) was eluted with 5 mL 0.1 M HCl. A 1.5 – 3 mL portion (~800 μCi) of the eluate was collected into an 8 mL multipurpose polypropylene tube. 100 μL of tetrazine DTPA dextran stock solution was added to the 68Ga containing vial. The mixture was shaken briefly and then incubated at room temperature for 15 minutes. The radiochemical purity (>99%) was confirmed by instant thin layer chromatography. This value was calculated as RCY = RCP * Product Activity / Activity added, where RCP is the Radiochemical Purity. The RCP was calculated through standard Instant Thin Layer Chromatography technique. This RCY was corrected for decay.

## 2.8 Multistep labeling of LS174T colon cancer cells with 68Ga tetrazine dextran

LS174T cells were checked for confluency, and then detached using trypsin/EDTA. The cells were split into 1 mL 130,000 cell aliquots in 1.7 mL sterile eppendorf tubes. Cell aliquots were incubated with either 200 nM of transcyclooctene anti-A33 (n=3) or a control antibody consisting of 200 nM unmodified anti-A33 (n=3) for

1.5 hours at 37°C. After incubation the cells were pelleted and washed with 500 μL of PBS 3 times. The cells were dispersed in 100 μL of PBS and were treated with 40 μCi of 68Ga tetrazine dextran for 1 hour. The cells were pelleted and washed three times with 500 μL of PBS containing 2% FBS. The radioactive supernatant was removed via pipette and put into plastic scintillation vials behind lead shielding for decay. The radioactivity bound to the cells was determined on a Beckman Gamma 9000 well counter.

## 2.9 Animals

Four female Swiss Webster mice (27-33 g, 8 weeks old) and five female nude mice (19-21 g, 6 weeks old) were purchased from Charles River Labs. The mice were maintained at the animal facilities of John and Rebecca Moores Cancer Center upon arrival. All *in vivo* procedures used in this study were approved by the University of California, San Diego, Institutional Animal Care and Use Committee review board. Mice were anesthetized with isoflurane prior to probe injection and imaging.

## 2.10 *In vivo* Tumor Imaging

Inject 0.2 nmol (~30 μg) trans-cyclooctene modified fluorescent antibody (anti-A33 coupled with Alexa Fluor 750: Ex: 749 nm; Em: 775 nm) through tail vein. Post injection (24 hours), image the mice *in vivo*. Scan the whole body on a Fluobeam 800 (Ex: 780 nm, Em: >800 nm).

## 2.11 Human Plasma Stability

Human whole blood was centrifuged at 3200 rpm (2000 g) for 15min. The top layer of plasma was then immediately transferred to a conical vial. 100 μL DTPA-Tetrazine-T10 (0.14 nmol) was incubated with 1000 μl (~1 mCi 68Ga) at room temperature for 15 min. The product was centrifuged and washed with a 3K Amicon filter at 3000 rpm for 10 min. The retentate was brought to 1.1 mL with PBS and the the 68Ga- DTPA-Tetrazine- T10 (200 μL) was incubated with 2.0 mL human blood plasma at 37°C. At 30 min, 1h, 2h, 3h post incubation, 0.1 mL of the mixture was removed for ITLC. (Solid phase: Whatman 31 ET strips; mobile phase: Acetone).

## 2.12 Positron Emission Tomography (PET) Imaging

Nuclear imaging was performed on a GE healthcare eXplore VISTA dual-ring small-animal PET Scanner. Doses of 68Ga tetrazine DTPA dextran (2.2 nmol in 150 μL, approximately 30-70 μCi) were injected into mice through the tail vein. PET acquisitions were conducted in two beds static emission mode with a 400-700 keV energy window. All mice were scanned three times at 10, 30 and 60 minutes after injection. Immediately after their third scan, mice were euthanized with $CO_2$. Blood, liver, spleen, kidneys and intestine were harvested and weighed after which their radioactivity was determined on a Beckman Gamma 9000 well counter. For A33/TCO *in vivo* conjugation experiments, PET acquisitions were performed approximately 1 hour after injection.

The author would also like to acknowledge Zhengtao Qin for his collaborative effort on this research project, Jun Yang for synthesizing necessary reagents, Dr. Vera for his instruction and allowance of the utilization of his 68Ge/68Ga Generator and PET imaging instruments/tools, and Dr. Devaraj for his unwavering patience and guidance on the project.

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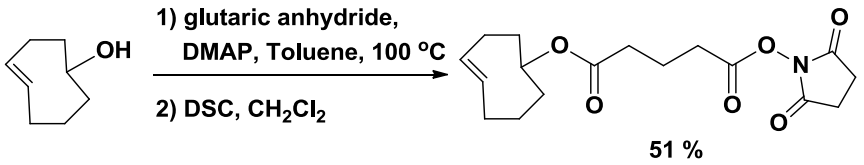
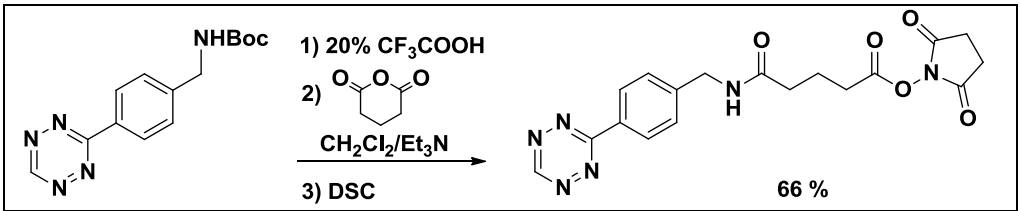
Nichols B, Qin Z, Yang J, Vera DR, Devaraj NK. 68Ga Chelating Bioorthogonal Tetrazine Polymers for the Multistep labeling of Cancer Biomarkers. *ChemComm* Manuscript Submitted.




Scheme 2.1: Synthesis of trans-cyclooctene NHS ((E)-cyclooct-4-en-1-yl (2,5-dioxopyrrolidin-1-yl) glutarate)




Scheme 2.2: Synthesis of tetrazine NHS (2,5-dioxopyrrolidin-1-yl 5-((4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)-5-oxopentanoate)

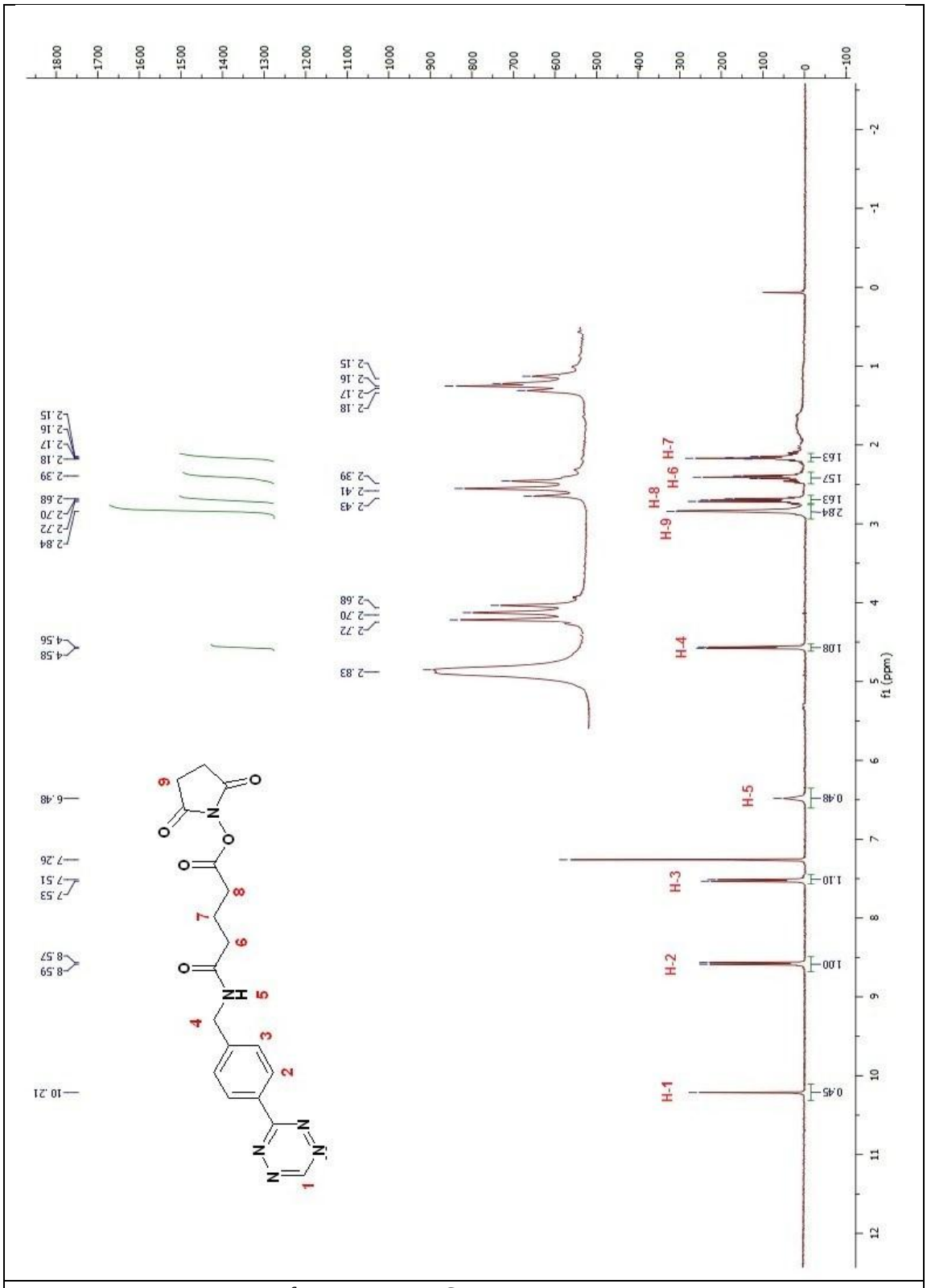



Figure 2.1: Proton NMR of Tetrazine NHS ester

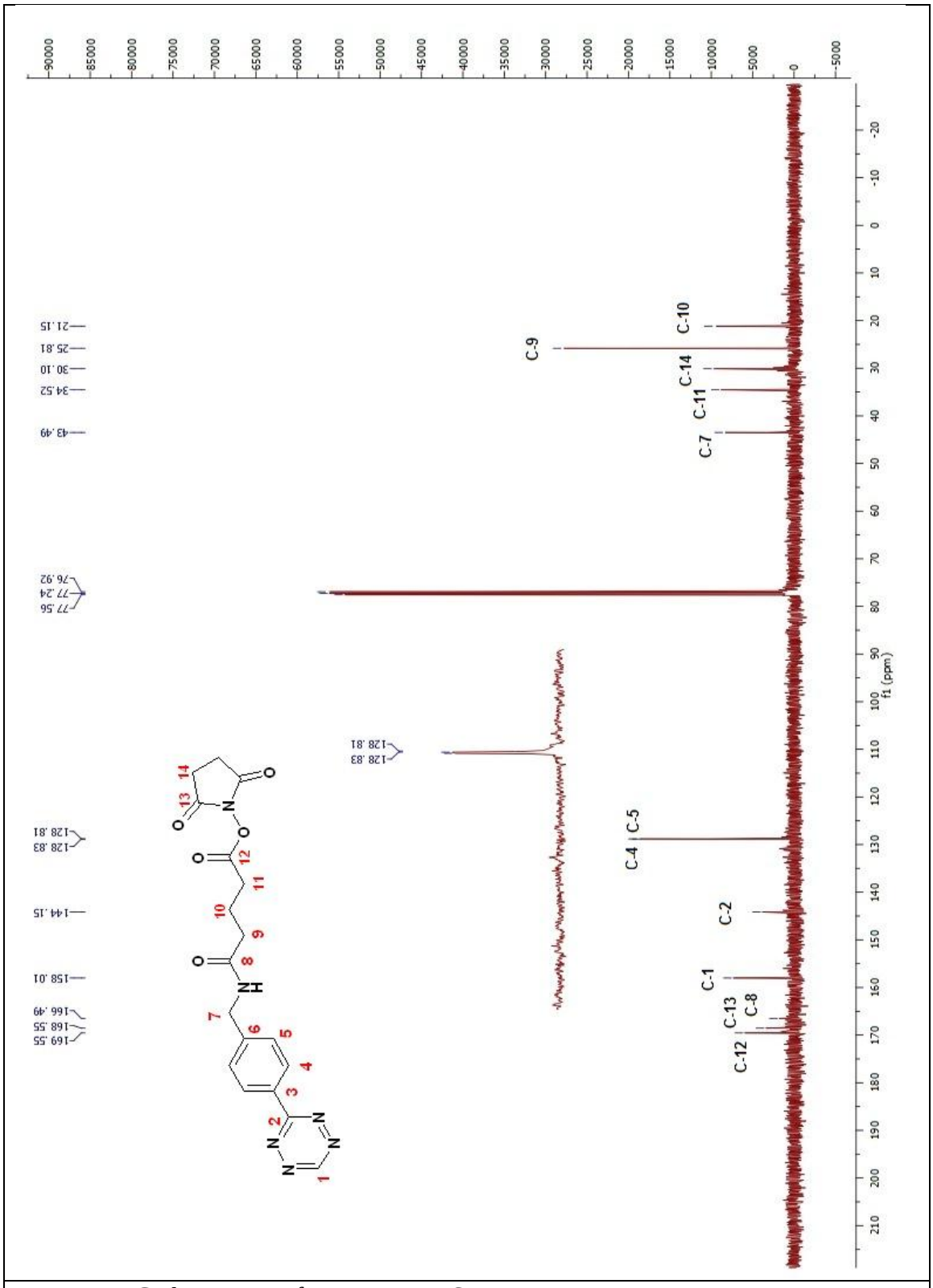



Figure 2.2: Carbon NMR of Tetrazine NHS ester

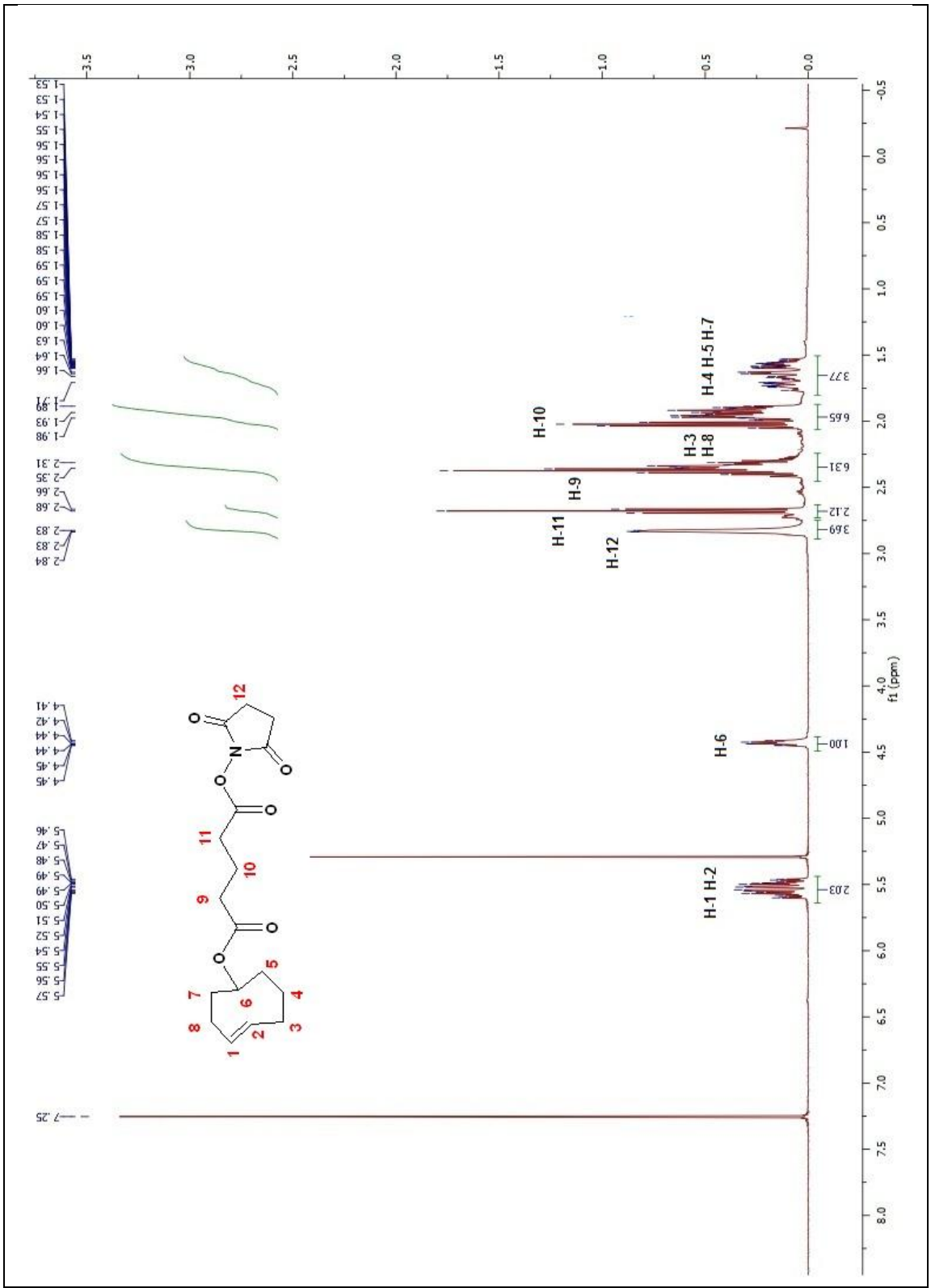



Figure 2.3: Proton NMR of Trans-Cyclooctene NHS Ester

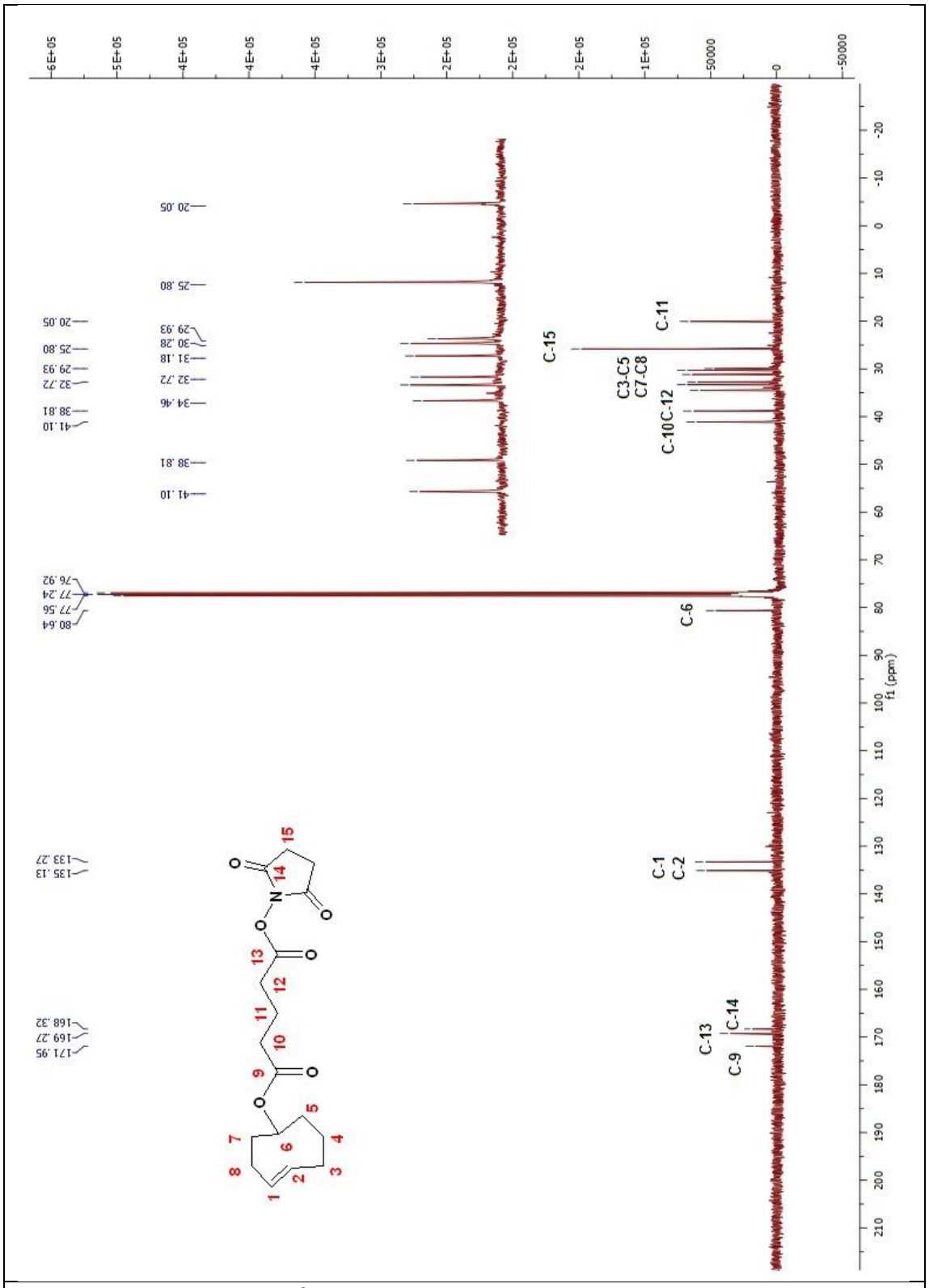



Figure 2.4: Carbon NMR of Trans-Cyclooctene NHS Ester

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# CHAPTER 3:

# RESULTS AND DISCUSSION

The goal of this work was to develop and characterize a bioorthogonal 68Ga tetrazine dextran imaging reagent suitable for multistep *in vivo* PET imaging. Previous results have determined that the pharmacokinetics of the secondary tetrazine agent can dramatically affect the efficiency of bioorthogonal chemical reactions *in vivo*.[1] The pharmacokinetics of the secondary imaging agent can be improved by adding tetrazines to amino-terminated dextran creating polymer modified tetrazine of different chain lengths but with identical reactivities to decouple and independently tune the clearance and reaction rates. These constructs were used to achieve *in vivo* multistep labeling using the PET radionuclide 18F. Here we extend this technology to 68Ga modification using a previously introduced biocompatible metal chelating DTPA dextran leading to the development of 68Ga labeled tetrazine DTPA dextrans.[2,3] We have verified that tetrazine DTPA dextran agents are highly reactive with surface bound transcyclooctene labeled monoclonal antibodies using fluorescence microscopy. LS174T human colon cancer cell lines pretargeted with trans-cyclootene modified anti-A33 monoclonal antibodies can be labeled *in vitro* using 68Ga tetrazine DTPA dextrans. We also tested the dextran imaging agents for multiple metrics, the clearance profile, and bioconjugation of 68Ga tetrazine DTPA dextran with A33 antibody labeled tumors and neutral biodistribution with minimal non-specific uptake.

In choosing a polymer scaffold, we decided to explore the use of dextrans due to their well-established clinical safety record, hydrophilicity, low expense, ready availability in numerous molecular weights, and our previous experience working with dextran imaging agents.[1,4,5] Dextrans with amine leashes and radionuclide agents have been extensively studied as radionuclide imaging agents for sentinel lymph node

detection.[2,6,7] Our motivation for working with 68Ga as a radiolabel was its convenient generator based production which is unique for a PET radiotracer.[8] The incorporation of 68Ga by chelation is also very straightforward and mild compared to covalent radiochemistry with labels such as 18F and 11C.[9] This is particularly relevant for tetrazine conjugates, which are unstable to the typical 18F labeling conditions. [10] In order to chelate the 68Ga, we decided to utilize DTPA chelation groups based on prior clinical work with DTPA albumins and dextrans and the known stability of DTPA chelates[11,12] which exhibits adequate *in vivo* stability for gallium during the moderately short biological half-life of the dextran conjugate.[13] However, although the DTPA chelate is suitable for the intended proof-of-principle studies, future clinical implementation of the proposed 68Ga imaging probes would likely utilize alternative chelators such as those based on 1,4,7-triazacyclononane-1,4,7- triacetic acid (NOTA) which possess more desirable *in vivo* properties.[14,15]

Although a multistep PET imaging approach would have application to numerous disease models, to initially test and optimize our method we chose to work with a human colon cancer model and target the A33 antigen.[16,17] A33 is a cell surface glycoprotein of the small intestine and colonic epithelium and its restricted tissue localization and high level of expression have led to its use as a target in colon cancer immunoimaging and therapy. Although the antigen is also present in normal intestine, radiolabeled antibodies against A33 are selectively retained by tumors in the gut as well as in metastatic lesions for as long as 6 weeks.[18] Recent studies of the trafficking and kinetic properties of the antigen have demonstrated that A33 is highly immobile and surface persistent.[19] These features make the antigen promising for use in multistep protocols as most of the anti-

A33 affinity ligands delivered will be on cell surfaces and readily accessible to the secondary tetrazine imaging agent.

Initially we were interested in determining whether the chelating tetrazine DTPA dextrans were capable of specifically targeting trans-cyclooctene modified monoclonal antibodies bound to the extracellular A33 marker. To verify extracellular localization, we decided to perform fluorescence microscopy studies using tetrazine DTPA dextran modified with a near-infrared emitting fluorescent probe, AlexaFluor 647 (AF647). DTPA dextran containing reactive amine "leashes" was synthesized as previously described and modified with approximately 1 equivalent of AF647 (verified by measurement of the absorbance at 647 nm).[2] The remaining amines were then modified with 5 equivalents of tetrazine NHS creating AF647 tetrazine DTPA dextran. A33 expressing LS174T human colon cancer cells were targeted with a trans-cyclooctene modified anti-A33 antibody, washed, and subsequently reacted with 10 μM of the fluorescent dextran for 30 minutes. After washing, the cells were imaged using fluorescence microscopy. As shown in Figure 3.1a, the cells surfaces were brightly stained (green), indicating that the fluorescent tetrazine DTPA dextran had modified the surface bound trans-cyclooctene antibodies. Staining is absent inside the cells, indicating that the dextran has not internalized, as expected for the non-internalizing A33 antigen.[19] Control cells that were not exposed to trans-cycloctene anti-A33 but were exposed to the AF647 tetrazine DTPA dextran showed minimal surface staining (Figure 3.1b), demonstrating that the observed fluorescence was not due to non-specific binding. We can therefore conclude that the chelating tetrazine DTPA dextrans are highly reactive with cellular bound dienophiles such as trans-cyclooctenes, similar to previously

introduced tetrazine imaging agents.[20] We also note that previous work has demonstrated that a fluorescent tag does not alter the receptor affinity or *in vivo* behavior of the receptor-specific dextran conjugate, Tc-99m-labeled Cy7-tilmanocept.[6]

Having demonstrated that tetrazine DTPA dextrans were capable of multistep labeling of strained dienophile modified surface biomarkers, we proceeded to explore metalation of tetrazine DTPA dextran with the positron emitting radionuclide 68Ga. In previous work, 18F was used as a PET imaging radionuclide for multistep imaging. However, 18F chemistry is harsh and typically requires heating under basic conditions, thus making it not compatible with reactive tetrazines, which degrade under these conditions.[10] Previous work created 18F tetrazine labeling agents using dextran scaffolds possessing excess tetrazine followed by reaction with a limiting quantity of a novel 18F transcyclooctene.[1,21] Though this process yields an 18F tetrazine PET agent, the synthetic protocol requires multiple steps, synthesis of a trans-cyclooctene 18F probe, necessitates consumption of reactive tetrazines, and is not applicable to the synthesis of PET probes containing a single tetrazine reactive group.

In contrast to 18F, 68Ga is an emerging generator produced PET radionuclide that, in addition to not requiring a cyclotron, is also appended to molecules via non-covalent and mild chelation chemistry.[22] Thus we expected that tetrazine reactive groups would be compatible with the conditions required for 68Ga chelation of pendant DTPA ligands. 68Ga was chelated to tetrazine modified DTPA dextran following previously published procedures in 99% radiochemical yield (RCY) (Graph 3.1).[3] Tetrazine reactive groups were compatible with the reaction conditions, and the presence of tetrazines on the dextran was verified by observing the tetrazine absorption signal at 530 nm.

We next determined if the resulting 68Ga tetrazine DTPA dextran was suitable for multistep cellular labeling similar to the fluorescent AF647 tetrazine DTPA dextran. LS174T cells were labeled with trans-cyclooctene anti-A33 monoclonal antibodies and subsequently exposed to 40 μCi 68Ga tetrazine DTPA dextran. Radiolabel uptake was quantified and compared to cells that received a control lacking dienophile (Graph 3.2). We also compared how trans-cyclooctene antibody loading affected the radiolabel uptake. Previous work has demonstrated that stoichiometric amplification of secondary imaging agents can be achieved by increasing the number of bioorthogonal reactive groups bound on the pretargeted monoclonal antibodies.[23] Indeed, decreasing the amount of trans-cyclooctenes that the antibodies were exposed to (30 equivalents versus 50) resulted in decreased 68Ga uptake, though this was still well above the nonspecific binding uptake observed in controls.

Although the pharmacokinetics of related DTPA dextrans have been previously characterized, we were unsure of the effect of the tetrazine on the biodistribution of the dextran imaging agent.[2] We therefore decided to monitor the *in vivo* pharmacokinetics and biodistribution of 68Ga tetrazine DTPA dextran with PET imaging followed by sacrifice and measurement of the percent injected dose of 68Ga probe in various tissues of interest. Figure 3.2 depicts a typical PET image of a mouse 60 minutes after receiving 50 μCi of 68Ga tetrazine dextran. Imaging for mice (n=3) indicated that the tetrazine probe showed moderate clearance and the expected uptake pattern for a DTPA dextran imaging agent in the blood pool. Mice were sacrificed after the 60 minute PET scan, and key organs and tissues were dissected, weighed, and the radioactivity counted to determine the percent injected dose (Graph 3.3). Time curves of radioactivity uptake for

the liver and heart were calculated from the PET experiment as well (Graph 3.4). Based on the percent injected dose in the blood pool, we estimate that the blood half-life of the 68Ga tetrazine dextran to be slightly less than one hour. Thus, this agent should be compatible with the 68 minute decay half-life of 68Ga. Blood stability tests were performed in human plasma with 68Ga DTPA Dextran tetrazine. It was found that, after a 3 hour incubation period, no free 68Ga was present in the plasma (Graph 3.5). Thus, the blood stability is compatible with the blood clearance times. The combined PET and biodistribution data demonstrate that tetrazine modification does not have a significant effect on 68Ga DTPA dextran distribution *in vivo* and indicates that the agent is appropriate for future *in vivo* multistep imaging studies.

The author would also like to acknowledge Zhengtao Qin for his collaborative effort on this research project, Jun Yang for synthesizing necessary reagents, Dr. Vera for his instruction and allowance of the utilization of his 68Ge/68Ga Generator and PET imaging instruments/tools, and Dr. Devaraj for his unwavering patience and guidance on the project.

The following material in part or in whole in under review at the following: Nichols B, Qin Z, Yang J, Vera DR, Devaraj NK. 68Ga Chelating Bioorthogonal Tetrazine Polymers for the Multistep labeling of Cancer Biomarkers. *ChemComm* Manuscript Submitted

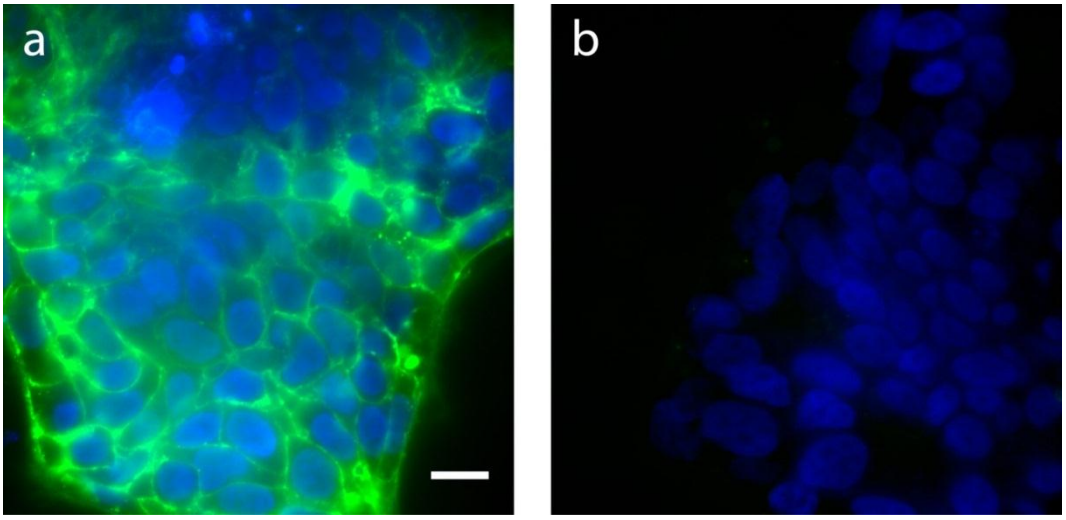
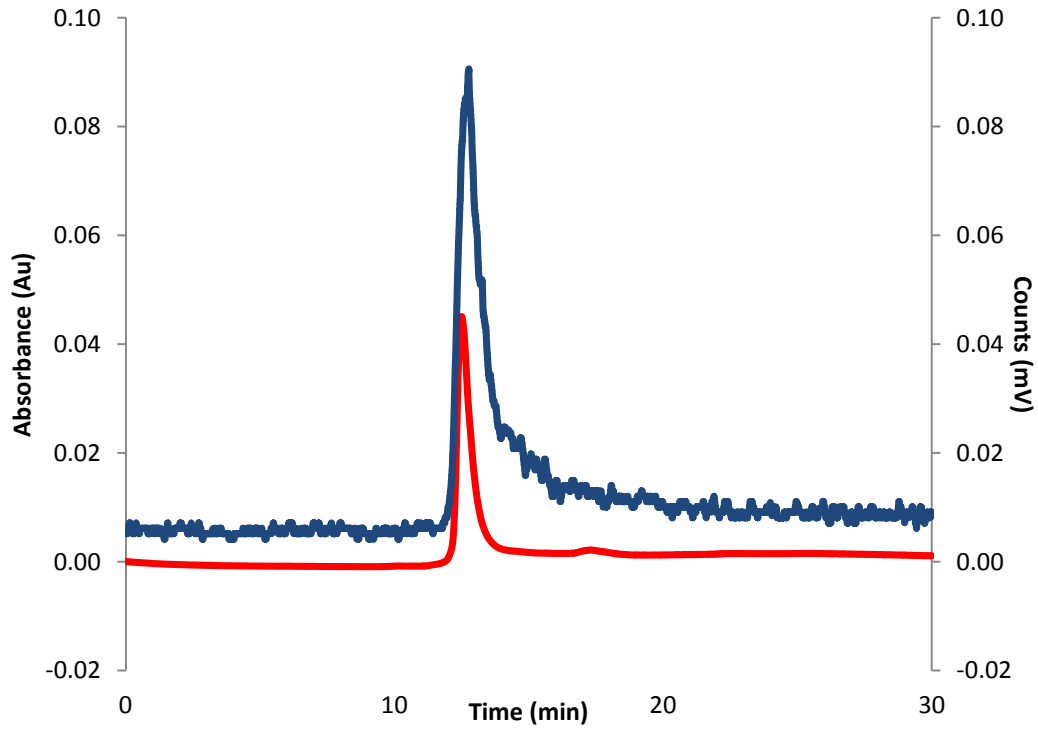
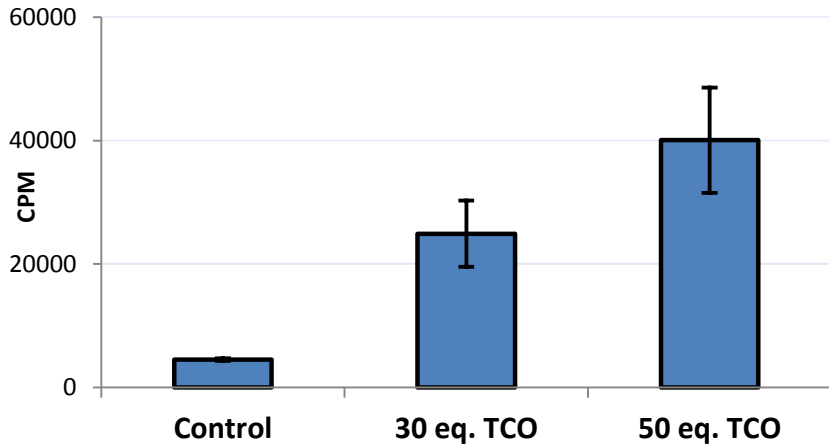



Figure 3.1: Confocal images of cells treated with fluorescent AlexaFluor 647 (AF647) tetrazine DTPA dextran. A) Cells pretargeted with *trans*-cyclooctene anti-A33 (sample). Scale bar (lower right) indicates 15 microns. B) Cells lacking A33/TCO (control)

Graph 3.1: Radiolabeling of tetrazine DTPA dextran with generator produced $^{68}$Ga



Graph 3.2: *In vitro* radiolabeling labeling of *trans*-cyclooctene modified LS174T cells with $^{68}$Ga tetrazine DTPA dextran



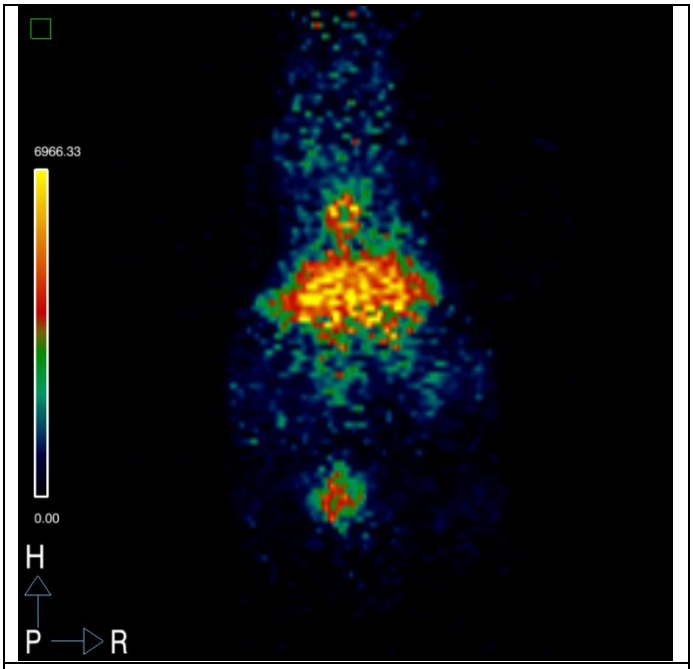
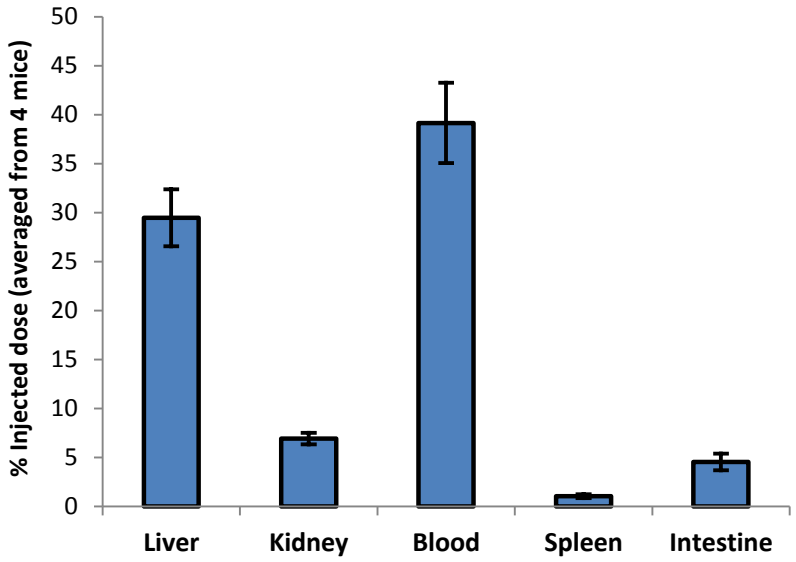
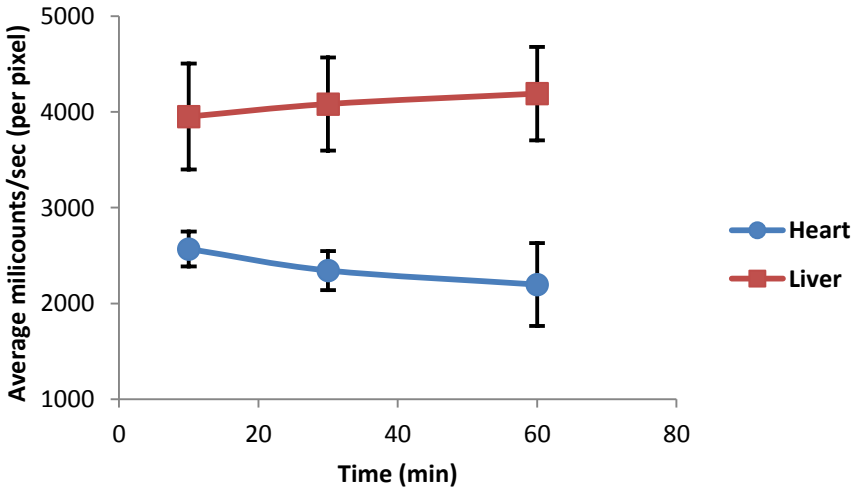




Figure 3.2: PET reconstruction (coronal slice) 1 hour after injection of 50µCi of $^{68}$Ga dextran tetrazine

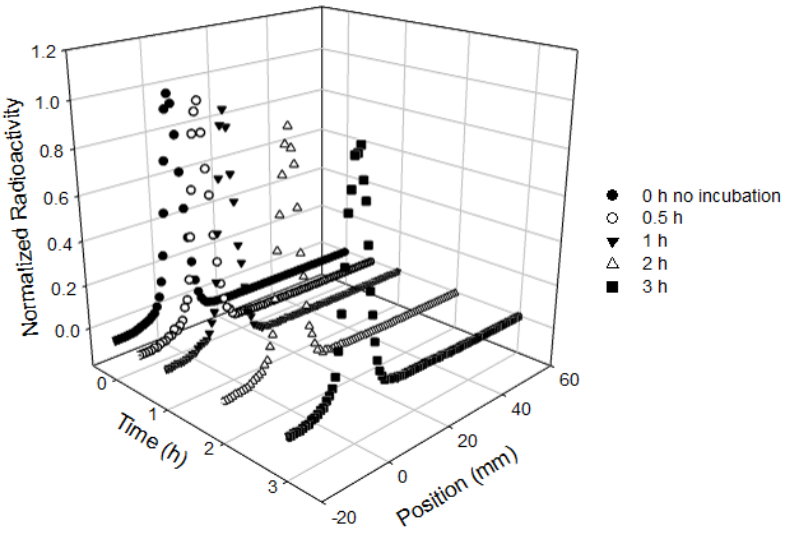
Graph 3.3: Biodistribution of tetrazine dextran after sacrifice




Graph 3.4: Time-activity curves of liver and heart



Graph 3.5: ITLC radioactivity after 0-3 h incubation with human plasma



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# Chapter 4

# Conclusion

We have developed a multistep bioorthogonal approach for labeling surface accessible cancer biomarkers with the generator produced PET radionuclide 68Ga. This approach relies on the use of a novel tetrazine DTPA dextran that is capable of chelating 68Ga under the mild conditions required for preserving tetrazine stability. This facilitates direct radiolabeling of tetrazine dextran probes with a PET radioisotope, which has been previously difficult to achieve with 18F. Tetrazine modified DTPA dextrans are highly reactive with surface bound transcyclooctene mod ified monoclonal antibodies, as demonstrated using a nearinfrared fluorescent AF647 tetrazine DTPA dextran and fluorescence imaging. 68Ga tetrazine DTPA dextrans are also reactive with colon cancer cells modified by trans-cyclooctene anti-A33 antibodies. The radiolabeled dextran construct shows well behaved pharmacokinetics and biodistribution *in vivo*, indicating that the tetrazine reactive groups do not negatively affect the distribution of the specific dextran scaffold, which has previously been successfully used for *in vivo* imaging. Our multistep approach is highly modular, and it is conceivable that alternative tetrazines, chelators, polymers, and dienophiles may be utilized. Indeed, although DTPA chelates are adequate for these initial proof-of-principle studies, clinical implementation would likely make us of more stable gallium chelators such as NOTA.[1,2] Future studies will further test the ability of 68Ga tetrazine probes to target biomarkers in *in vivo* models of human cancers. We believe that bioorthogonal tetrazine dextrans may eventually enable the multistep labeling of a broad array of surface biomarkers using the convenient short-lived PET radioisotope 68Ga.

Once again the author would like to thank Zhengtao Qin, Jun Yang, Dr. Vera, and Dr. Devaraj for their contributions and making this project possible.

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