A Cationic Probe to Detect Microstructure in Fenestrated Organs

by

Scott Beeman

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved November 2012 by the
Graduate Supervisory Committee:

Kevin Bennett, Chair
Vikram Kodibagkar
Zahi Fayad
Vincent Pizziconi
James Pipe

ARIZONA STATE UNIVERSITY

December 2012
ABSTRACT

The goal of the works presented in this volume is to develop a magnetic resonance imaging (MRI) probe for non-invasive detection of extracellular matrix (ECM) underlying fenestrated endothelia. The ECM is the scaffold that supports tissue structure in all organs. In fenestrated structures such as the kidney glomerulus and the hepatic sinusoid the ECM serves a unique role in blood filtration and is directly exposed to blood plasma. An assessment of the ECM in fenestrated organs such as the kidney and liver reports on the organ’s ability to filter blood - a process critical to maintaining homeostasis. Unfortunately, clinical assessment of the ECM in most organs requires biopsy, which is focal and invasive.

This work will focus on visualizing the ECM underlying fenestrated endothelia with natural nanoparticles and MRI. The superparamagnetic ferritin protein has been proposed as a useful naturally-derived, MRI-detectable nanoparticle due to its biocompatibility, ease of functionalization, and modifiable metallic core. We will show that cationized ferritin (CF) specifically binds to the anionic proteoglycans of the ECM underlying fenestrated endothelia and that its accumulation is MRI-detectable. We will then demonstrate the use of CF and MRI in identifying and measuring all glomeruli in the kidney. We will also explore the toxicity of intravenously injected CF and consider other avenues for its application, including detection of microstructural changes in the liver due to chronic liver disease. This work will show that CF is useful in detected fenestrated microstructures in small animals and humans alike, indicating that CF may find broad application in detecting and monitoring disease in both preclinical and clinical settings.
ACKNOWLEDGMENTS

I would like to gratefully acknowledge my advisor, Kevin Bennett. His guidance (while sometimes unorthodox), has changed me from student into a self-driven scientist. Kevin has become one of my closest friends and is my strongest advocate. I look forward to many more years of friendship, collaboration, and beer.

I would also like to thank my family. Their unyielding support is the foundation of any of my successes in life. To them I owe my confidence and passion for life.

And of course I must thank Perri, who’s limitless love and patience are the pinnacle of my already charmed life.
### TABLE OF CONTENTS

| LIST OF FIGURES | ........................................................................................................... viii |
| LIST OF TABLES | ............................................................................................................... x |
| CHAPTER | |
| 1 INTRODUCTION | ........................................................................................................... 1 |
| Magnetic resonance imaging | .......................................................... 1 |
| Magnetism, relaxation, and NMR/MRI | ........................................... 2 |
| MRI | .......................................................... 6 |
| The gradient echo pulse sequence | ........................................... 6 |
| MRI contrast agents | .......................................................... 7 |
| Paramagnetism and superparamagnetism | ........................................... 7 |
| Nanoparticles as MRI contrast agents | ........................................... 9 |
| Ferritin | .......................................................... 10 |
| The kidney glomerulus | .......................................................... 11 |
| Measuring kidney glomeruli | .......................................................... 12 |
| Clearance of CF from the nephron | .......................................................... 13 |
| Detecting microstructural changes to the hepatic sinusoid | .......................................................... 14 |
| The chapters to come | .......................................................... 16 |

| 2 MEASURING GLOMERULAR NUMBER AND SIZE IN PERFUSED RAT KIDNEYS USING MRI | ........................................................................................................... 19 |
| Introduction | .......................................................... 19 |
| Methods | .......................................................... 20 |
| In vitro preparation and imaging | .......................................................... 20 |
| Post-processing | .......................................................... 20 |
| Histology | .......................................................... 21 |

iii
<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statistics</td>
<td>22</td>
</tr>
<tr>
<td>Results</td>
<td>22</td>
</tr>
<tr>
<td>Discussion</td>
<td>26</td>
</tr>
<tr>
<td>Conclusions</td>
<td>30</td>
</tr>
<tr>
<td>3 DETECTING, COUNTING, AND MEASURING THE SIZE OF GLOMERULI IN HUMAN TRANSPLANT KIDNEYS - A PROOF OF CONCEPT</td>
<td>31</td>
</tr>
<tr>
<td>Introduction</td>
<td>31</td>
</tr>
<tr>
<td>Methods</td>
<td>32</td>
</tr>
<tr>
<td>Sample preparation</td>
<td>32</td>
</tr>
<tr>
<td>MRI</td>
<td>32</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>33</td>
</tr>
<tr>
<td>Image processing</td>
<td>33</td>
</tr>
<tr>
<td>Results</td>
<td>34</td>
</tr>
<tr>
<td>Discussion</td>
<td>37</td>
</tr>
<tr>
<td>Conclusions</td>
<td>38</td>
</tr>
<tr>
<td>4 A HIGH T1-RELAXIVITY NANOPARTICLE FOR IN VIVO MRI-DETECTION OF GLOMERULI</td>
<td>39</td>
</tr>
<tr>
<td>Introduction</td>
<td>39</td>
</tr>
<tr>
<td>Methods</td>
<td>41</td>
</tr>
<tr>
<td>Loading the apoferritin core</td>
<td>41</td>
</tr>
<tr>
<td>Cationization of WFe ferritin</td>
<td>41</td>
</tr>
<tr>
<td>Animal preparation and imaging</td>
<td>42</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>42</td>
</tr>
<tr>
<td>Data analysis</td>
<td>43</td>
</tr>
<tr>
<td>CHAPTER</td>
<td>Page</td>
</tr>
<tr>
<td>-----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Results</td>
<td>43</td>
</tr>
<tr>
<td>In vivo detection of glomeruli</td>
<td>43</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>46</td>
</tr>
<tr>
<td>Discussion</td>
<td>46</td>
</tr>
<tr>
<td>Conclusions</td>
<td>47</td>
</tr>
</tbody>
</table>

5 TOXICITY, BIODISTRIBUTION, AND EX VIVO MRI

DETECTION OF INTRAVENOUSLY INJECTED CATIONIZED FERRITIN

Introduction                                                                 49
Methods                                                                      51
Synthesis of cationized ferritin                                            51
Animal preparation and blood collection                                     52
Toxicity and ex vivo MRI detection of CF                                    52
Immune response                                                            53
Biodegradation                                                              53
Imaging                                                                     54
Tissue Preparation                                                          54
Immunohistochemistry                                                       54
Ex vivo MRI-detection of CF                                                55
Ex vivo MRI to assess biodegradation                                       55
Data analysis                                                               55
Results                                                                     56
Toxicity                                                                    56
Biodegradation                                                              60
Tissue distribution studies                                                60
<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunofluorescence</td>
<td>60</td>
</tr>
<tr>
<td>MRI</td>
<td>63</td>
</tr>
<tr>
<td>Discussion</td>
<td>67</td>
</tr>
<tr>
<td>Conclusions</td>
<td>71</td>
</tr>
<tr>
<td><strong>6</strong></td>
<td><strong>CATIONIZED FERRITIN AS A MRI PROBE TO DETECT MICROSTRUCTURAL CHANGES IN A RAT MODEL OF NON-ALCOHOLIC STEATOHEPATITIS</strong></td>
</tr>
<tr>
<td>Introduction</td>
<td>72</td>
</tr>
<tr>
<td>Methods</td>
<td>75</td>
</tr>
<tr>
<td>Synthesis of Cationic Ferritin</td>
<td>75</td>
</tr>
<tr>
<td>Animal Preparation</td>
<td>75</td>
</tr>
<tr>
<td>In vivo MRI</td>
<td>77</td>
</tr>
<tr>
<td>Ex vivo MRI</td>
<td>78</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>78</td>
</tr>
<tr>
<td>Transmission Electron Microscopy</td>
<td>79</td>
</tr>
<tr>
<td>Inductively Coupled Plasma Optical Emission Spectroscopy</td>
<td>79</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>79</td>
</tr>
<tr>
<td>Results</td>
<td>80</td>
</tr>
<tr>
<td>Intravenous CF labels the healthy perisinusoidal ECM and is detected with MRI</td>
<td>80</td>
</tr>
<tr>
<td>Perisinusoidal ECM labeling by CF decreases with steatohepatitis</td>
<td>86</td>
</tr>
<tr>
<td>Discussion</td>
<td>95</td>
</tr>
<tr>
<td>Conclusions</td>
<td>97</td>
</tr>
</tbody>
</table>
8 SUMMARY AND FUTURE DIRECTIONS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>98</td>
</tr>
<tr>
<td>Summary</td>
<td>98</td>
</tr>
<tr>
<td>Detecting kidney glomeruli in vivo and ex vivo</td>
<td>98</td>
</tr>
<tr>
<td>Nanoparticle toxicity and biodistribution</td>
<td>100</td>
</tr>
<tr>
<td>Detection of microstructural changes in chronic liver disease</td>
<td>101</td>
</tr>
<tr>
<td>Future directions</td>
<td>102</td>
</tr>
<tr>
<td>$T_2^*$ susceptibility volume versus the actual volume of glomerulus</td>
<td>102</td>
</tr>
<tr>
<td>Glomerular populations</td>
<td>103</td>
</tr>
<tr>
<td>Assessing glomerular health by dynamically measuring CF-uptake</td>
<td>105</td>
</tr>
<tr>
<td>Tubules</td>
<td>106</td>
</tr>
<tr>
<td>translation to humans</td>
<td>110</td>
</tr>
<tr>
<td>Conclusions</td>
<td>111</td>
</tr>
</tbody>
</table>

WORK CITED

APPENDIX

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>119</td>
</tr>
<tr>
<td>Permissions</td>
<td>119</td>
</tr>
<tr>
<td>Permissions from scientific journals</td>
<td>120</td>
</tr>
<tr>
<td>Permissions from co-authors</td>
<td>122</td>
</tr>
<tr>
<td>B</td>
<td>123</td>
</tr>
<tr>
<td>INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE FORMS</td>
<td>123</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.</td>
<td>Whole-kidney identification of individual glomeruli</td>
<td>22</td>
</tr>
<tr>
<td>2.2.</td>
<td>Histogram of apparent glomerular volumes</td>
<td>26</td>
</tr>
<tr>
<td>3.1.</td>
<td>MRI of CF-labeled human kidney</td>
<td>34</td>
</tr>
<tr>
<td>3.2.</td>
<td>Histogram of apparent glomerular volume distribution</td>
<td>35</td>
</tr>
<tr>
<td>4.1.</td>
<td>In vivo paraCF detection of glomeruli</td>
<td>43</td>
</tr>
<tr>
<td>4.2.</td>
<td>Confocal microscopy of paraCF-labeled kidneys</td>
<td>44</td>
</tr>
<tr>
<td>5.1.</td>
<td>Blood serum measurements of kidney biomarkers</td>
<td>55</td>
</tr>
<tr>
<td>5.2.</td>
<td>Blood serum measurements of liver biomarkers</td>
<td>56</td>
</tr>
<tr>
<td>5.3.</td>
<td>Total leukocyte counts</td>
<td>58</td>
</tr>
<tr>
<td>5.4.</td>
<td>Confocal imaging of kidneys, livers, lungs, and spleens</td>
<td>61</td>
</tr>
<tr>
<td>5.5.</td>
<td>MRI of kidneys, livers, lungs, and spleens</td>
<td>65</td>
</tr>
<tr>
<td>5.6.</td>
<td>Normalized MRI signal magnitudes of kidneys, livers, lungs, and spleens</td>
<td>66</td>
</tr>
<tr>
<td>6.1.</td>
<td>CF-labeling of the perisinusoidal ECM is detectable with MRI</td>
<td>82</td>
</tr>
<tr>
<td>6.2.</td>
<td>CF-labeling of the perisinusoidal ECM is confirmed with confocal microscopy</td>
<td>82</td>
</tr>
<tr>
<td>6.3.</td>
<td>Transient labeling of the perisinusoidal ECM</td>
<td>85</td>
</tr>
<tr>
<td>6.4.</td>
<td>The MCD diet</td>
<td>87</td>
</tr>
<tr>
<td>6.5.</td>
<td>Reduced CF-labeling of the perisinusoidal ECM of the liver is detectable in vivo in the rat model of NASH</td>
<td>88</td>
</tr>
<tr>
<td>6.6.</td>
<td>$T_2^*$ maps of control and CF-inoculated livers</td>
<td>90</td>
</tr>
<tr>
<td>6.7.</td>
<td>Immunofluorescence microscopy of NASH livers</td>
<td>92</td>
</tr>
<tr>
<td>6.8.</td>
<td>Transmission electron microscopy of NASH livers</td>
<td>93</td>
</tr>
<tr>
<td>6.9.</td>
<td>Liver iron concentrations</td>
<td>94</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>7.1.</td>
<td>A three-dimensional histogram of relative glomerular labeling intensity versus the normal distance of a glomerulus from the surface of the kidney.</td>
<td>104</td>
</tr>
<tr>
<td>7.2.</td>
<td>CF-labeling of the proximal tubule.</td>
<td>107</td>
</tr>
<tr>
<td>7.3.</td>
<td>Identification of proximal tubules.</td>
<td>109</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>24</td>
</tr>
</tbody>
</table>

### Table 2.1
The number of labeled glomeruli and apparent glomerular volumes ($V_{\text{glom}}$) measured with the MRI-based technique, compared to the values obtained with acid maceration and stereology.

24
CHAPTER 1

INTRODUCTION

The goal of the works presented in this dissertation is to develop a cationic, MRI-detectable probe for non-invasive detection of kidney glomeruli and the perisinusoidal space of the liver. Targeted MRI contrast agents have been developed to detect molecules and cells throughout the body (Cormode, Jarzyna, Mulder, & Fayad, 2010; Geninatti Crich et al., 2005; S. Zhang, Merritt, Woessner, Lenkinski, & Sherry, 2003). Superparamagnetic iron oxide nanoparticles (SPIOs) of approximately 10-50 nm are one such type of contrast agent that create a detectable change in the MRI signal. Ferritin, a 13 nm protein-based SPIO, has been proposed as a useful natural nanoparticle contrast agent due to its uniform size, biocompatibility, and ease of functionalization (Bennett, Shapiro, Sotak, & Koretsky, 2008; Bulte et al., 1995; Uchida et al., 2008). Because the ferritin nanoparticle has a superparamagnetic iron oxide core, its accumulation creates a darkening in the MR image that can be detected using $T_2^*$-weighted MRI.

We will describe the development and application of this extracellular matrix-specific, cationized ferritin probe in three parts: First, in chapters 2-4 we will discuss the use of CF in detecting, identify, and measuring kidney glomeruli; second, in chapter 5 we will explore the toxicity of intravenously injected CF and other avenues for its application; and finally, in chapter 6, we will show that CF may be used to detect microstructural changes to the liver in chronic liver disease. In this chapter we will provide the necessary background for the studies presented in Chapters 2-6.

MAGNETIC RESONANCE IMAGING

Magnetic resonance imaging (MRI) is a vital tool in clinical diagnostics and preclinical research. MRI has found wide application in clinical and preclinical
settings due to its versatility in inducing image contrast from tissue and its ability to produce very high resolution images. MRI is derived from the older chemical characterizing tool called nuclear magnetic resonance (NMR) which, as the name suggests, relies on atomic nuclei to precess at a resonant frequency when exposed to a magnetic field. It is the manipulation of these precessing nuclei with an assortment of circuitry that yields a spatially encoded signal that can be used to create an MRI image. NMR and MRI are separated only by MRI's use of magnetic gradients to spatially encode nuclei to produce an image.

**Magnetism, relaxation, and NMR/MRI.** The foundation of NMR and MRI is not in macroscopic magnetism, but instead lies in the intrinsic spin and magnetic moment of fundamental particles. The physical basis of magnetic resonance may be described in either a classical or quantum framework. The background presented here will be in the classical framework with a few necessary quantum principles. Furthermore, this work will focus exclusively on acquisition of MRI signal from the hydrogen protons (though we will also discuss the effects of electron magnetism on hydrogen protons). The hydrogen nucleus is constructed from a single proton and has a spin \( I = 1/2 \). We will hereby simply refer to hydrogen nuclei as ‘protons’.

Magnetism stems from three different sources. The first source is the circulation of electrons about a nucleus. Elementary classical physics informs us that a loop of electric current induces a magnetic field. The orbit of electrons about a nucleus is a parallel situation in which orbiting electrons are a circulating current that induce a small magnetic field. The second and third sources of magnetism come from protons and electrons. Protons and electrons each have a magnetic moment, \( \mu \), that is linearly proportional to the spin angular momentum, \( S \), offset only by a particle’s gyromagnetic ratio, \( \gamma \). The spin angular momentum
of protons and electrons, and therefore their magnetic moments, are intrinsic properties of the particle - they simply exist. Indeed, the spin and the magnetic moment of a particle are so strongly linked that they always point either parallel or anti-parallel to one another. The net moment is the sum of the spin moments and the orbital moment, where the nuclear contribution is orders of magnitude lower than the electronic contribution. In the classical model of NMR/MRI it turns out that, when placed into a magnetic field, the spin-axis of a proton or electron will precess about the field with a constant angle between the spin-axis and the applied magnetic field vector, $B_0$, and at a constant frequency called the “Larmor” frequency, defined by $\omega_0 = \gamma B_0$. While the remainder of this section will focus on the proton (it is, in fact, the hydrogen protons of water molecules that ultimately yield the bulk of the signal in the type of MRI discussed in this work), it is of special importance to note that the magnetism of an unpaired electron is orders of magnitude stronger than that of a proton. In most materials all of the electrons are paired (according to the Pauli Principle) and their magnetism cancelled, though there exist elements such as the lanthanides which have many unpaired electrons in their outer orbital. These elements are strongly paramagnetic and find heavy use as MRI contrast agents - a topic that we will return to soon.

Of course a sample will never contain only a single proton. Let us instead consider a sample of water. Our sample now contains many moles of hydrogen protons with an isotropic distribution of nuclear magnetic moments. Immediately upon application of an external magnetic field, $B_0$, there exists no net magnetization in our water sample but as time goes on a bulk sample magnetization grows in the direction of $B_0$ due to the alignment of a very small number of nuclear magnetic moments with the external field. This effect stems from the thermodynamic nature of our system which dictates that the protons in
our system slightly favor a low energy parallel orientation of their nuclear magnetic moments with $\mathbf{B}_0$ over a high energy anti-parallel orientation. The growth of sample magnetization in the direction of $\mathbf{B}_0$ is referred to as the ‘longitudinal relaxation’ and results a macroscopic magnetism in the sample that points parallel to $\mathbf{B}_0$ at thermodynamic equilibrium. Longitudinal relaxation is modeled by the equation $M_z(t) = M_0(1-e^{-t/T_1})$, where $M_z$ is the longitudinal magnetization of the sample, $t$ is the amount of time after the application of a field (or, in future discussion, the amount of time after an RF pulse), and $T_1$ is the fitted time constant for the regrowth of longitudinal magnetization. It should be noted that, though the bulk sample magnetization along $\mathbf{B}_0$ is stable, any given spin in the system is free to point in any direction. The presence of $\mathbf{B}_0$ merely makes it slightly more probable that a nuclear moment will orient itself along $\mathbf{B}_0$ (or, in the quantum framework, the presence of $\mathbf{B}_0$ merely makes it slightly more probable that a spin will occupy the parallel versus anti-parallel spin state).

Detecting a net magnetism that is pointing in the direction of an external magnetic field is difficult because the number of nuclear magnetic moments that align with field is very small (approximately 1 in 1-10 million) and they do not precess in a coherent manner. NMR/MRI overcomes this by creating a coherent alignment of spins in the plane transverse to $\mathbf{B}_0$. It turns out that the polarization of spins along the direction of $\mathbf{B}_0$ may be rotated into the transverse plane using radio frequency (RF) coils mounted orthogonally to $\mathbf{B}_0$ that are tuned to the Larmor frequency. These coils are used to ‘tilt’ the sample magnetization away from $\mathbf{B}_0$, which effectively sums all of the longitudinally aligned nuclear magnetic moments into a single coherent sample magnetization in the transverse plane. When the RF pulse is shut off, the spins resume their precession around $\mathbf{B}_0$, except now all of the spins precess about $\mathbf{B}_0$ in phase with one another yielding a
net magnetization in the transverse plane called ‘transverse magnetization’. The transverse magnetization precesses about $B_0$ at the Larmor frequency because it is simply a coherent ensemble of spins with their moments aligned in the transverse plane. The transverse magnetization is detectable because the spins rotate in a coherent (summed) manner, at a well defined frequency. Faraday’s law and Maxwell’s equations inform us that this rotating net magnetization may be used to induce an oscillating current in an orthogonally mounted receive coil.

Transverse magnetization also relaxes, this time modeled as an exponential decay. Coherent spins ultimately become incoherent due to the fact that each spin in the system samples a unique set of microenvironments and local magnetic fields. The different magnetic environments that each spin experiences cause the Larmor frequency of each proton to fluctuate with time and ultimately yields a ‘dephasing’ of all spins in the transverse plane. This dephasing in the transverse plane is called ‘transverse relaxation’. In its most simple form, transverse relaxation can be modeled by the equation $M_\perp(t) = M_0 e^{-t/T_2^*}$, where $M_\perp$ is the transverse magnetization of the sample, $t$ is the amount of time after the RF pulse, and $T_2^*$ is the fitted time constant describing the decay of transverse magnetization. The rate at which the transverse magnetization decays is strongly affected by the protons’ environment and this model can be expanded to a multi-exponential in more complex systems to account for multiple source of transverse relaxation. In fact, $T_2^*$ can further be dissected by defining it as $T_2^* = T_2' + T_2$, where $T_2'$ is the time constant fitted to reversible transverse relaxation caused by static field inhomogeneities and $T_2$ is the time constant fitted to irreversible transverse relaxation that is intrinsic to the system. In tissue, $T_2$ can be a strong indicator of disease so it is important to be able to isolate $T_2$. This may be accomplished by applying a second RF pulse that flips the transverse
magnetization 180° to reverse the static dephasing. This causes a regrowth of transverse magnetization that results in a ‘echo’ at the time point where static dephasing has been completely reversed.

**MRI.** So far we have described the classical physical foundation of NMR - a system that represent all protons in the sample equally, regardless of their spatial location. The distinction between NMR and MRI lies in MRI’s use of electromagnetic gradients to spatially encode protons to produce an image. The application of electromagnetic gradients in the system described above produces a spatially varying Larmor frequency, defined by $\omega(x)=-\gamma B(x)$, where $x$ is the spatial location along the field gradient. Therefore, electromagnetic gradients may control the Larmor frequencies of protons as a function of their spatial location in the sample. The periodicity of spin precession in a magnetic field and the ability to manipulate the Larmor frequencies of protons as a function of their spatial location with gradients provides the opportunity spatially encode protons in a sample. The data collected are in the spatial frequency domain and can be reconstructed into an image via a Fourier transform. Multiple gradient coils can be applied dynamically to manipulate the magnetic field in three dimensions, allowing for imaging of all three spatial dimensions at a one time.

**The gradient echo pulse sequence and MRI contrast.** The gradient echo (GRE) is the MRI pulse sequence that is used in all of the work presented in this volume. Briefly, a one dimensional GRE would include an initial RF pulse that creates a coherent bulk magnetization in the transverse plane followed by an application of a negative magnetic gradient, causing a spatially-dependent dephasing effect across the sample. A positive gradient is then applied for twice the duration of the negative gradient. Data collection begins at the onset of the positive gradient and is centered around the ‘echo time’ (TE) - the time at which
the dephasing caused by the negative gradient is cancelled by the rephasing from the positive gradient. This experiment may be generalized to two and three dimensions. The experiment is repeated to apply different gradient combinations such that all of 2D or 3D k-space (the spatial frequency domain in which MRI data is collected) may be filled. The time duration at which the experiment is repeated is defined by the ‘repetition time’ (TR). Both TE and TR are user defined parameters of the experiment and can be adjusted to manipulate image contrast between tissue types.

The three main mechanisms for MRI contrast when using a GRE pulse sequence are longitudinal relaxation ($T_1$), transverse relaxation ($T_2^*$), and spin density ($\rho$). Each of these mechanisms has an effect on the the MRI signal and the way in which the MRI signal is acquired can drastically change the proportions by which each mechanism imparts contrast on the image. Typically, the user defined TE will modulate the $T_2^*$-weighting of an image and the TR will modulate the $T_1$-weighting. A longer TE will increase the $T_2^*$-weighting while a shorter TR will increase the $T_1$-weighting. One would want to minimize both $T_2^*$-weighting (very short TE) and $T_1$-weighting (very long TR) to obtain an image primary defined by spin density.

While this description of MRI is a hugely simplified version of a very complex phenomenon, it lays a sufficient foundation to discuss MRI contrast agents - the focus of this work.

**MRI CONTRAST AGENTS**

**Paramagnetism and superparamagnetism.**

Most MRI contrast agents rely on the effects that the electron magnetism of a metal-based agent has on hydrogen protons of the surrounding water. The magnetization of the agents used in this work is of either paramagnetic or
superparamagnetic form and depends on the strength of the applied magnetic field $B_0$ according to the equation $M = X B_0$, where $M$ is the magnetism of the nanoparticle, $X$ is the magnetic susceptibility constant for the magnetic material of the particle, and $B_0$ is the applied magnetic field. A material is paramagnetic or superparamagnetic if the measured susceptibility of a material is greater than zero. Typically a superparamagnetic material has a susceptibility much greater than zero and its magnetization will saturate and therefore fall out of the linear $M = X B_0$ regime at the $B_0$ magnetic field strengths employed in MRI. A material that has only paired electrons and a susceptibility of less than zero is called diamagnetic, though diamagnetism will be neglected for the remainder of the conversation about contrast agents.

Paramagnetism is usually associated with single metal atoms with unpaired electrons such as gadolinium and manganese. Unpaired electrons have a large permanent magnetic moment (orders of magnitude greater than a proton’s magnetic moment) and are what define an atom as paramagnetic. The magnetic moments of the unpaired electrons in paramagnets are free to rotate and tend to distribute themselves isotropically at thermal equilibrium in the absence of an external magnetic field. Once a magnetic field is ‘turned on’, the electron magnetic moment will couple with the external field. This interaction between the paramagnet and the applied field is similar to the interaction between a proton and a magnetic field. The electronic magnetic moment of a paramagnet is so strong that, when in a solution of water, it can exchange energy with the surrounding hydrogen protons. This dipolar interaction can shorten the longitudinal relaxation time of the hydrogen protons and ultimately yield a brightening of MRI signal in $T_1$-weighted MRI.
Now consider a material in which bound atoms in a crystal work together to form domains; each domain with its own large magnetic moment. A large material (greater than 30 nm in diameter) can have many such domains that, when magnetized, can couple to one another and perpetuate a large magnetic field in the absence of an external field. This is called a ferromagnet (an example of a ferromagnet would be a refrigerator magnet). Now imagine a ferromagnet that has been cut up until it contains only a single magnetic domain with only a single large magnetic moment. Such particles with a single magnetic domain are called ‘superparamagnets’. In the absence of an external magnetic field, the magnetic moments of these superparamagnets will oscillate along the principle axis of the crystal thereby appear to have zero magnetization. However, once exposed to a magnetic field these superparamagnetic particles will couple so strongly with the applied field that its magnetization will saturate (reach its highest possible magnetization, regardless of the field it is in) and produce a strong magnetic field that reaches far beyond the physical boundaries of the particle. The strong magnetic field of a superparamagnet coupled with an external field causes dephasing of nearby coherent protons and a shortened transverse relaxation time, ultimately yielding a darkening of MRI signal in T2-weighted MRI.

**Nanoparticles as MRI contrast agents.** Paramagnetic gadolinium chelates are by far the most common MRI contrast agents in use in the clinic, though they have a very low relaxivity (the measurement of an agents ‘strength’). Alternatively, nanoparticles may be synthesized with paramagnetic or superparamagnetic cores for use as highly detectable contrast agents that shorten longitudinal relaxation or transverse relaxation, respectively. Typically these synthetic nanoparticles are coated with a saccharide such as dextran to protect the body from the toxic metal core of the particle and can be as small as
a few nanometers in diameter. The strength of a nanoparticle contrast agent is reported in terms of relaxivity ($r_1$ for agents that shorten longitudinal relaxation or $r_2$ for agents that shorten transverse relaxation), and indicates the agent’s ability to brighten (an $r_1$ agent) or darken (an $r_2$ agent) the MR image. The relaxivity of a nanoparticle is strongly linked to its size, aggregation state, saturation magnetization, and the composition of its metal core (Bennett, Shapiro, Sotak, & Koretsky, 2008; Yablonskiy & Haacke, 1994).

**Ferritin.** Natural macromolecules have been employed as biocompatible nanoparticle contrast agents that carry paramagnetic or superparamagnetic payloads. The ferritin protein is one such natural nanoparticle that comes pre-packaged with a superparamagnetic iron oxide core. Ferritin is an endogenous 24-mer protein of approximately 475 kDa that is used by the body to store iron in a non-toxic state. The iron is stored in the hollow center of the spherical protein shell, which is about 1 nm thick, until it is required for hemoglobin or other iron-rich cellular components. At 12 nm in diameter, the superparamagnetic ferritin protein serves as an excellent naturally derived, biocompatible magnetic nanoparticle for $T_2^*$-weighted MRI.

Apolferritin, the empty (iron-free) version of ferritin, provides even greater versatility. Without the iron oxide core in place, the apoferritin protein is free to be modified in a number of useful ways. The apoferritin protein shell may be loaded with large amounts of iron, rendering a naturally derived nanoparticle called ‘magnetoferritin’, which has a $r_2$ relaxivity of approximately 100 times that of native ferritin (Bulte et al., 1995; Clavijo Jordan, Caplan, & Bennett, 2010). More recently we have modified this synthesis process with a tungsten dopant. The exchange coupling within the crystal is prevented by strategically doping the iron
oxide core of apoferritin with tungsten ions, resulting in a large paramagnetic nanoparticle with a very high $r_1$ and low $r_2$.

The surface of the ferritin protein is also readily modified. Aime, et al. have shown that the surface of the ferritin protein may be targeted to tumor blood vessels by incubating the protein in streptavidin and biotinylated C3d peptides - peptides that are specific to receptors expressed by vascular cells (Geninatti Crich et al., 2007). The remainder of this work though will focus on the use of cationized ferritin (CF) as a targeted molecular imaging agent. CF has long been used as an electron dense probe in transmission electron microscopy to label charged surfaces in tissue sections (Brody, Vaccaro, Hill, & Rounds, 1984; Danon, Goldstein, Marikovsky, & Skutelsky, 1972; Schmidley & Wissig, 1986; Voelz, Kondziella, Berens Von Rautenfeld, Brinker, & Lüdemann, 2007) and in previous work we have shown the iron oxide core of CF to be MRI-detectable using $T_2^*$-weighted MRI (Beeman, Georges, & Bennett, 2012; Beeman et al., 2011; Bennett, Shapiro, Sotak, & Koretsky, 200811; Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008). The work presented in this document will focus on the use of CF as a charge-specific MRI contrast agent to detect anionic extracellular matrix underlying fenestrated endothelium.

THE KIDNEY GLOMERULUS

The human kidney has ~200,000 - 2,000,000 nephrons responsible for removing waste from the body and maintaining proper blood ion, glucose, and pH levels. Each nephron is composed of a single glomerulus and tubule. The glomerulus plays a critical role in renal blood filtration, serving to retain vital macromolecules and cells in the blood stream while allowing smaller plasma components to flow into the nephron tubules for resorption or expulsion in the form of urine.
Measuring glomeruli. A low count of kidney glomeruli (Nglomeruli) may suggest a number of renal and systemic diseases including diabetes, obesity, and hypertension (Bertram, Soosaipillai, Ricardo, & Ryan, 1992; Brenner, Garcia, & Anderson, 1988; Hoy et al., 2008). Furthermore, changes in Nglomeruli and glomerular volume (Vglomeruli) have been linked to ~90% of all chronic renal diseases (Puelles et al., 2012). A recent study performed by the National Kidney Foundation revealed that ~88% of chronic kidney disease (CKD) cases go undiagnosed in patients with Type 2 diabetes - a patient population already known to be at risk for CKD. Furthermore, current techniques to measure renal function are known to report normal measurements when the kidney has actually lost much of its filtration capacity, rendering them insufficient to detect renal disease early on (Cullen-McEwen, 2003). The number of patients requiring dialysis and kidney transplant could be substantially reduced by improving the screening of CKD in the offices of primary care physicians and developing robust diagnostic tools to detect the early indicators of CKD.

Current techniques that estimate glomerular number and size have provided substantial insight into renal physiology and its roll in health and disease (Beeman et al., 2011; Brenner, Troy, Daugharty, Deen, & Robertson, 1972; Cullen-McEwen, 2003; Menini et al., 2004; Puelles et al., 2012) but are destructive and fail at characterizing individual glomeruli. At this point, measurements of Nglomeruli and Vglomeruli are not clinically available; histological techniques to measure Nglomeruli and Vglomeruli require resection and destruction of the kidney and only yield extrapolated estimates of Nglomeruli and Vglomeruli from small sample populations (Bertram et al., 1992). A non-invasive technique to measure the number and size of every glomerulus would prove invaluable to preclinical and clinical assessment of renal disease.
A potential strategy to non-invasively measure Nglom and Vglom is to develop a MRI contrast agent that specifically targets the glomerular basement membrane (GBM). We have previously demonstrated that intravenously injected CF can be used to detect individual glomeruli with MRI (Beeman et al., 2011; Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008). This technique is based on selective electrostatic binding of CF to the anionic proteoglycans of the GBM, the accumulation of which renders each perfused glomerulus visible with MRI. Based on this, we can locate and count each perfused glomerulus in the kidney and measure its volume. Furthermore, the three dimensional nature of MRI will allow us to study glomerular populations defined by their size and location in the kidney - providing a unique insight into intra-renal distributions of glomeruli.

**Clearance of CF from the nephron.** An understanding of the mechanism of CF clearance from the GBM is of vital importance to this project. The traditional model for macromolecular filtration in the glomerulus involves a capillary wall that is composed of three layers collectively referred to as the filtration barrier. The glomerular capillary wall has a hydrostatic pressure gradient across it that drives plasma filtration across the capillary wall and into the proximal tubule of the nephron. The first layer of the glomerular capillary wall is a fenestrated endothelial layer with 70 - 100 nm fenestrae that allow the passage of plasma and macromolecules across the filtration barrier but restricts cells to the capillary space. This endothelial layer can also phagocytize macromolecules that enter into the second layer - the glomerular basement membrane (GBM). The anionic GBM is thought to be the main player in plasma filtration, filtering particles by both size and charge. Finally, there exists an epithelial layer consisting of cells called podocytes that are thought to serve as a size-selective filter. Furthermore, there exist mesangial cells inside the glomerular capillary wall...
lumen that work to clear accumulated macromolecules that have been filtered by the GBM. It is very likely that the bulk of intravenously delivered CF that has accumulated in the GBM is cleared via phagocytosis by the intra-glomerular mesangial cells and the endothelium. Nevertheless, a small amount of protein can escape the glomerular filtration barrier and enter into the proximal tubule. The vast majority of the protein that enters into the proximal tubule of a healthy glomerulus will bind to the epithelial glycocalyx and be absorbed by the proximal tubule. This process of reabsorption of protein in the proximal tubule is saturable, therefore an unhealthy nephron in which large amounts of protein enter the proximal tubule will ultimately allow the protein to pass through the entire nephron and be excreted in the urine. This is quite damaging to the nephron.

**DETECTING MICROSTRUCTURAL CHANGES TO THE HEPATIC SINUSOID**

The liver is a multifaceted organ, serving to synthesize and store proteins, produce bile, and filter blood, among a variety of other functions. Chronic liver disease affects a huge number of americans per year and accounts for ~30,000 deaths per year in the United States alone (National Center for Health Statistics, 2011). There is a strong likelihood of chronic liver disease progressing to cirrhosis in the event that it goes untreated. In the case of cirrhosis, a number of serious complications arise, including hepatic encephalopathy, renal failure, and hepatocellular carcinoma. Excessive deposition of extracellular matrix (ECM) and a loss of endothelial fenestrations (Farrell, Teoh, & Mccuskey, 2008; Friedman, 2000; Iredale, 2007; Lough, Rosenthall, Arzoumanian, & Goresky, 1987; Martínez-Hernandez & Amenta, 1995) are characteristic of advanced chronic liver disease and lead to a reduction in plasma access to the liver parenchyma. This ultimately yields reduced hepatic function (Friedman, 2000; Iredale, 2007).
A non-invasive method for detecting such microstructural changes would prove vital in clinical and preclinical diagnostics.

Chapter 6 of this work will demonstrate a new molecular MRI technique to detect microstructural changes to the perisinusoidal space of the liver caused by the chronic liver disease non-alcoholic steatohepatitis (NASH). The perisinusoidal space is the site of exchange between the blood plasma and the hepatocytes and is situated between the hepatocytes and a fenestrated layer of endothelia. These fenestrated endothelia serve as a size-selective sieve, allowing plasma molecules to enter into the perisinusoidal space to interact with the hepatocytes while retaining blood cells in the capillary space (Wisse, 1970). Situated inside of the perisinusoidal space is a layer of ECM that regulates cell migration, proliferation, differentiation and gene expression (Bedossa & Paradis, 2003; Friedman, 2000; Iredale, 2007). The advancement of NASH causes thickening of the ECM and a loss of endothelial fenestrations (Farrell et al., 2008; Friedman, 2000; Iredale, 2007; Lough et al., 1987; Martinez-Hernandez & Amenta, 1995) which in turn reduces plasma access to the perisinusoidal space and hepatocytes. This is detrimental to hepatic function (Friedman, 2000; Iredale, 2007).

NASH is differentiated from the more benign fatty liver disease using tissue biopsy. Liver fibrosis can be diffuse and a loss of endothelial fenestrations is difficult to detect with using an electron microscope, therefore liver biopsy can be subject to sampling errors (Bedossa, Dargère, & Paradis, 2003; Siddique, El-Naga, Madda, Memon, & Hasan, 2003). Clever imaging techniques such as ultrasound elastography and magnetic resonance elastography have been developed to diagnose liver fibrosis via detection of viscoelastic changes caused by the stiffening of the tissue due to fibrosis (Sandrin et al., 2003; Yeh et al.,
Despite these excellent new, non-invasive tools that detect gross anatomical changes, there remains a strong need to detect changes to microstructure that follow the development of chronic liver disease.

In Chapter 6 we will investigate the use of CF to detect reduced macromolecular access to the perisinusoidal space in a rat model of NASH. We will show that intravenously injected CF binds transiently to the perisinusoidal ECM in healthy rats, allowing visualization of the perisinusoidal space with MRI. Furthermore, we will show that CF accumulation is reduced in animals with NASH, suggesting that access of CF to the perisinusoidal space is blocked by excess uncharged ECM and abutted endothelia. The reduced accumulation of CF is detectable using MRI. Reduced CF accumulation may be an early imaging biomarker for changes in macromolecular access to the hepatic parenchyma due to structural changes to the endothelium and perisinusoidal ECM.

**THE CHAPTERS TO COME**

The following chapters are presented unchanged from their forms required for publication.

Chapter 2 will present the first study to measure glomerular number and size with MRI. This will be presented verbatim from our 2011 publication in the American Journal of Physiology - Renal Physiology (Beeman et al., 2011). The goal of this work was to nondestructively and comprehensively measure Nglom and Vglom ex vivo in rat kidneys with MRI. In this study we exploit the negatively charged glomerular basement membrane as a target for intravenously delivered CF. We will discuss the labeling of the glomerulus with CF and how each glomerulus may be identified using $T_2^*$-weighted MRI. We will also present a method for counting and measuring the size of each labeled glomerulus from the MRI volumes.
Chapter 3 discusses the extension of the research presented in Chapter 2 to human transplant kidneys. This work is prepared for publication in the Journal of the American Society of Nephrology. The goal of this work is to measure the number and size of all glomeruli in human kidneys. In this study fresh human transplant kidneys were acquired through a donation network (The International Institute for the Advancement of Medicine) within 24 hrs of patient death and perfused with CF via the renal artery. In this chapter we will discuss how CF and MRI may be used to detect, count, and measure glomeruli in human transplant kidneys. This work is intended as a proof of concept that human glomeruli may indeed be identified with CF and MRI.

The work presented in Chapter 4 will discuss the development of a new cationized MRI probe for improved detection of kidney glomeruli in vivo. Normal CF (like that used in the studies of chapters 2 and 3) is superparamagnetic, which creates a dark contrast that is hard to detect above the background of blood in vivo. This study sets out to develop a ferritin based paramagnetic probe that may be cationized and injected to detect glomeruli in vivo. The goal of this work is to render glomeruli as bright spots when labeled with the probe and imaged with T1-weighted MRI.

Chapter 5 will be presented as it appears in our 2012 article published in Magnetic Resonance in Medicine (Beeman et al., 2012). The goal of this work was to establish the toxicity and biodistribution of intravenously delivered CF. The three chapters preceding this one make strong assertions about the prospects of using cationized ferritin-based agents for detection of kidney glomeruli both ex vivo and in vivo. With the ultimate goal of these chapters being translation of this technology to the clinic for assessment of renal health, it is vital to establish an understanding of how intravenous CF affects the body. The work presented in
this chapter is an investigation of the nephro-, hepato-, and immunotoxicity of intravenous CF as well as its clearance rate. We also study a number of organs for CF-labeling using MRI and immunofluorescence microscopy.

Chapter 6 presents a CF-based MRI technique to detect changes in plasma access to the perisinusoidal space of the liver in chronic liver diseases such as non-alcoholic steatohepatitis (NASH). This work is in review with the journal Magnetic Resonance in Medicine. The perisinusoidal space of the liver is the site of exchange between the blood plasma and the hepatic parenchyma. Chronic liver disease causes excessive deposition of extracellular matrix and loss of endothelial fenestrations in the hepatic sinusoid, thereby reducing blood access to the perisinusoidal space. In this work we will discuss the application of CF as a probe to detect microstructural changes to the hepatic sinusoid in the chronic liver disease non-alcoholic steatohepatitis.

Finally, Chapter 7 will summarize the work presented in this volume and discuss the future direction of this work.
CHAPTER 2
MEASURING GLOMERULAR NUMBER AND SIZE IN PERFUSED KIDNEYS USING MRI

INTRODUCTION

Changes in the number and size of glomeruli have been linked to a number of renal and systemic diseases (Brenner et al., 1988; Hoy et al., 2008). However, current techniques for counting and measuring glomeruli, such as acid maceration (Bonvalet, Champion, Wanstok, & Berjal, 1972) and the disector/fractionator stereology technique (Bertram et al., 1992), require the destruction of the entire kidney. Furthermore, conventional histological techniques extrapolate the total number and size of glomeruli from a selected number of histological sections or isolated glomeruli. Current techniques are thus estimates rather than direct measurements, and do not allow for localization of identified functioning glomeruli to specific parts of the kidney. A method for directly and non-destructively measuring number and size of all glomeruli in the kidney would serve as a useful tool in animal studies and potentially in the clinic.

Recently we demonstrated that intravenous injections of the iron binding protein ferritin, functionalized with cationic amine groups (Danon et al., 1972), can be used to detect individual glomeruli both in vivo and ex vivo with magnetic resonance imaging (MRI) (Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008). This method is based on the electrostatic binding of cationic ferritin (CF) to the anionic macromolecules of the glomerular basement membrane (GBM) and subsequent perturbation of the magnetic field around the labeled GBM by the ferritin, resulting in a decrease in the MRI signal at the location of the glomerulus. Here we develop and validate the technique of systemic injection of CF to count individual glomeruli and measure individual glomerular size with 3D MRI. We
demonstrate that glomerular counts obtained from 3D MRI volumes are consistent with counts obtained with established histological methods, with the advantage of retaining the intact kidney.

**METHODS**

**In vitro preparation and imaging.** Cationic ferritin (CF) was synthesized by conjugating horse spleen ferritin (Sigma Aldrich, St Louis, MO) to N,N-Dimethyl-1,3-propanediamine (DMPA) using 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), according to Danon et al. (Danon et al., 1972). Male Sprague-Dawley rats weighing between 215 g and 245 g were given 3 intravenous bolus doses (11mg/ml in phosphate buffered saline at pH 7.2) of 5.75 mg/100 g of CF (n=6) or native ferritin (NF) (n=6) with 1.5 hours between injections. Kidney were perfused and fixed via transcardial perfusion of PBS followed by 10% neutral buffered formalin, then resected and stored in glutaraldehyde. All experiments involving animals were carried out according to a protocol approved by the Arizona State University Institutional Animal Care and Use Committee. The perfused left kidneys were imaged in glutaraldehyde on a Varian 19T 89 mm bore NMR (Varian, Inc. Palo Alto, CA), equipped with a DOTY 3-axis imaging probe and a gradient with a maximum strength of 300 G/cm (DOTY Scientific, Inc. Columbia, SC). Scans were acquired with a 3D gradient echo (GRE) sequence with TE/TR = 7/40 ms and a resolution of 62x62x78 µm. Total scan time was 6 hours per kidney.

**Post-processing.** Labeled glomeruli in the 3D MRI dataset were counted using custom software written in Matlab (The Mathworks, Inc.). A bicubic interpolation method was used to resize the 3D MRI datasets and change the spatial resolution to 31x31x62 µm. Spatial signal magnitude gradients were then calculated to extract any dramatic spatial changes in signal magnitude.
throughout the volume. Voxels exceeding a signal magnitude difference of 0.11, on a signal magnitude scale of 0 to 1, defined regions to be included in following operations. Regional minima were located in these areas using an upper signal magnitude threshold of 0.4, based on a signal magnitude scale of 0 to 1. Regions considered to be glomeruli were then defined based on morphological thresholds, assuming that a glomerulus is approximately spherical. A watershed transform was computed on these regions to distinguish individual glomeruli where signal overlap of multiple glomeruli might occur (Bernardis & Yu, 2010; Meyer, 1994; Roerdink & Meijster, 2000).

MRI-based apparent glomerular volume measurements were made based on the number of voxels per glomerulus isolated by the counting algorithm multiplied by the post-interpolation voxel dimensions (31x31x62 µm). A superior threshold of 64 voxels per glomerulus (38x10^{-4} mm^3 or a glomerular diameter of 194 µm) was set to eliminate possible ‘false’ glomeruli. Glomeruli containing less than 64 voxels were included in the results. We have defined glomerular volumes measured using the MRI-based technique as “apparent glomerular volume” because the relationship between amount of CF accumulated in the GBM and the volume of susceptibility is yet unresolved in this system.

**Histology.** We performed two techniques to validate the MRI-based measurements of glomeruli: 1) acid maceration and counting and 2) stereology. Acid maceration was based on techniques established in the literature (Bonvalet et al., 1972). Briefly, kidneys were cut into 1 mm^3 pieces immediately after resection and incubated in 5 ml of 6 N HCl for 1.5 hours. Incubated tissue was crushed and strained until homogenous. The solution was then brought up to 30 ml with deionized water. Glomeruli in solution were counted in a counting chamber (1 mm^2 scored 35 mm culture dish, Nuncion delta). The total number of
nephrons for each kidney was calculated based on the average number of glomeruli per area.

Stereology was performed on two kidneys. Serial sections of the left (imaged) kidneys were sampled and individual glomeruli across serial sections were compared and measured using the physical disector/fractionator method as previously described (Bertram et al., 1992).

**Statistics.** All statistical analyses comparing the MRI and acid maceration or stereological measurements from each respective animal were run as paired two-tailed Student’s t-tests to test the null hypothesis that the mean difference between pairs is zero at the significance level $\alpha = 0.05$ (The Mathworks, Inc. Natick, MA).

**RESULTS**

Excised, perfused kidneys were imaged after systemic CF or NF injections. $T_2^*$-weighted 3D gradient echo MR images of the kidneys showed dark MRI signal at the location of glomeruli after CF injections, but not after NF injections. The specific binding of CF, but not NF, was previously confirmed with immunofluorescence and electron microscopy (Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008).
Figure 2.1. Whole-kidney identification of individual glomeruli. A representative axial kidney image from a 3D MRI dataset from a CF-injected animal is shown (A). The data were analyzed with a 3D counting algorithm to identify glomeruli. Regions defined as glomeruli by the computational 3D counting algorithm were assigned an arbitrary color exclusively for visualization purposes (B).
Figure 2.1 shows the results of an automated 3D segmentation algorithm to identify individual glomeruli. In the figure, identified glomeruli are arbitrarily colored in axial slices of the 3D images of perfused, excised kidneys of CF-injected rats (Fig. 2.1B). Counting of CF-labeled glomeruli from 3D MRI datasets yielded $33,786 \pm 3,753$ glomeruli (n=5). One kidney was removed from group analyses as an outlier due to erratic stereological and imaging measurements (Table 2.1). The same counting algorithm yielded $1,916 \pm 806$ nephrons from 3D MRI datasets of kidneys from NF-injected rats (n=6). Acid maceration counting of contralateral kidneys from CF-injected rats yielded $30,585 \pm 2,053$ glomeruli (n=6).

The disector/fractionator stereology yielded counts of 35,421 and 34,504 glomeruli, compared to MRI-based counts in the same kidneys of 32,789 and 35,203 glomeruli, respectively. Paired two-tailed Student's t-tests to test the null hypothesis that the mean difference between pairs is zero showed that there were no significant differences between glomerular counts from the MRI-based method and from the acid maceration or stereological methods (at the significance level $\alpha = 0.05$). Counting results of all CF-injected rats are reported in Table 2.1. False positives counted in the control kidneys indicate a counting error of approximately 2,000 extra glomeruli per kidney, or ~5.5% error.
Table 2.1

The number of labeled glomeruli and apparent glomerular volumes ($V_{\text{glom}}$) measured with the MRI-based technique, compared to the values obtained with acid maceration and stereology.

<table>
<thead>
<tr>
<th></th>
<th>MRI #</th>
<th>Maceration #</th>
<th>Stereology #</th>
<th>$V_{\text{glom}}$ mm$^3 \times 10^{-4}$</th>
<th>$\text{Av } IV_{\text{glom}}$ mm$^3 \times 10^{-4}$</th>
<th>$IV_{\text{glom}}$ SD mm$^3 \times 10^{-4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat A</td>
<td>32,789</td>
<td>27,504</td>
<td>34,504</td>
<td>5.01</td>
<td>5.2</td>
<td>4.87</td>
</tr>
<tr>
<td>Rat B</td>
<td>35,203</td>
<td>31,190</td>
<td>35,421</td>
<td>4.98</td>
<td>4.58</td>
<td>4.3</td>
</tr>
<tr>
<td>Rat C</td>
<td>31,772</td>
<td>31,075</td>
<td>-</td>
<td>4.07</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Rat D</td>
<td>39,482</td>
<td>33,321</td>
<td>-</td>
<td>5.7</td>
<td>4.92</td>
<td></td>
</tr>
<tr>
<td>Rat E</td>
<td>29,682</td>
<td>31,478</td>
<td>-</td>
<td>4.89</td>
<td>4.33</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>33,786</td>
<td>30,914</td>
<td>34,963</td>
<td>4.99</td>
<td>4.89</td>
<td>4.36</td>
</tr>
<tr>
<td>SD</td>
<td>3,753</td>
<td>2,112</td>
<td>648</td>
<td>0.02</td>
<td>0.62</td>
<td>0.61</td>
</tr>
<tr>
<td>Outlier</td>
<td>37,625</td>
<td>28,944</td>
<td>24,156</td>
<td>8.37</td>
<td>9</td>
<td>7.28</td>
</tr>
</tbody>
</table>

Table 2.1. Measurements of intra-renal standard deviation in apparent glomerular volumes are unique to the MRI-based glomerular counting technique. One kidney was removed from group analyses (labeled ‘outlier’) due to erratic stereological and imaging measurements. $V_{\text{glom}}$, mean glomerular volume; $\text{Av } IV_{\text{glom}}$, the average apparent glomerular volume of all individual glomeruli in a kidney; $IV_{\text{glom}}$ SD, the standard deviation of apparent glomerular volumes of all glomeruli in a kidney; SD, standard deviation.
Individual apparent glomerular volumes were calculated based on the number of voxels per individual glomerulus and the voxel dimensions. Apparent volume measurements of labeled glomeruli using the MRI-based technique yielded $4.89 \times 10^{-4}$ mm$^3$ (n=5), compared to stereological volume measurements of $4.99 \times 10^{-4}$ mm$^3$ (n=2). Using the MRI-based technique, we were also able to calculate the intra-renal standard deviation of apparent glomerular volumes. This measurement is not obtained by stereology because the average glomerular volume measured using stereology is based on the extrapolated total volume of glomeruli divided by the extrapolated total number of glomeruli per kidney. Glomerular volumes are reported in Table 2.1 and volume distributions are shown as a histogram in Figure 2.2.

DISCUSSION

In this work we have developed a nondestructive method for measuring nephron number based on the accumulation of CF in the GBM. There were insignificant systematic differences between MRI-based measurements and measurements obtained with acid maceration and stereology. MRI-based counts yielded slightly more glomeruli than acid maceration and slightly fewer than stereology. Over counting is due to the identification of non-glomerular regions that are of similar signal magnitude and shape of glomeruli. This error is observed in the measurement of ~2,000 glomeruli per kidney in unlabeled kidneys.
Figure 2.2. Histogram of apparent glomerular volumes in the kidneys of 5 rats from 3D MRI data. Apparent glomerular volumes were measured from 3D magnetic resonance images based on the number of image voxels in a 3D cluster considered glomerular, multiplied by the imaging voxel dimensions. Average measured apparent glomerular volume was $4.89 \times 10^{-4}$ mm$^3 \pm 0.617 \times 10^4$. Error bars show mean ± SD between 5 rats.
We note that both of the established techniques for counting glomeruli, and thereby nephrons, are based on extrapolations of a limited number of counts to the entire kidney, while the MRI-based technique counts all glomeruli in the entire kidney. The MRI-based technique is advantageous for measuring glomerular volume because the volume of each labeled glomerulus in the entire kidney is measured and recorded. The intra-renal distribution of apparent glomerular volumes is a potentially new parameter for investigation.

While our average apparent glomerular volume measurements compare well to the volumes measured using stereology, care should be taken when interpreting glomerular volumes measured using MRI and superparamagnetic agents such as CF, as the volume of magnetic susceptibility may be dependent upon the amount and the manner in which the agent accumulates in the GBM. Thus, we have defined the MRI-based glomerular volume measurements as “apparent glomerular volume” to address the uncertainty of the origin of this measurement. The strong correlation between MRI-based measurements of apparent glomerular volume and those from stereology suggests that the fringe field of the accumulated CF does not extend beyond the glomerulus. It is likely that larger superparamagnetic particles of higher relaxivity would not be suitable for such measurements due to a larger fringe field.

The magnetic field strength of the MRI scanner should also be taken into consideration when making these measurements. While we have seen adequate labeling of glomeruli with a 5.75 mg/100g dose of CF at 7T (data not shown), it should be noted that the effects of CF accumulation on signal decrease with field strength. This means that greater doses of CF or particles of higher relaxivity may be required for visualization of glomeruli at fields less than 7T. Magnets of lower field strength may achieve the same spatial resolution, though this may
require improved RF probes built specifically for kidney imaging. Conversely, magnets of higher field strength have their own unique challenges, including $B_1$ inhomogeneities and substantial RF deposition into tissue. While unnecessary in the present study due to the small size of the kidneys imaged, corrections to the $B_1$ field and RF coils may be required to image larger kidneys at high fields.

One potential complication of MRI-based counting is the possibility that CF labels only functioning glomeruli, or that labeling of glomeruli may be affected by disease. Indeed, our recent work demonstrated that CF labeling of the GBM is affected by early- and late-stage puromycin-induced glomerulosclerosis (Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008). These effects may be focal or global, depending on the nature of the disease. Care must be taken to establish the effectiveness of MRI-based counts in any animal disease model. In addition, while MRI is faster and less labor intensive than stereological techniques, it requires both clean anatomical perfusion to avoid MRI artifacts from blood in the excised kidney as well as significant expertise in scanner operation. There is room for significant improvements in MRI acquisition strategies to reduce sensitivity to artifacts and shorten scan times via improvements in pulse sequence and acquisition design.

While this work has focused on labeling and measuring glomeruli ex vivo, this is a first step in establishing whole-kidney nephron measurements in vivo. We have shown in previous work (Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008) that labeled glomeruli may be detected in vivo using the same labeling and MRI techniques. Future work will be focused on establishing the optimum dose of CF required to detect individual glomeruli in vivo, and overcoming partial volume effects due to decreased resolution to achieve reasonable in vivo scan times. Furthermore, thorough in vivo toxicity screens of CF will be required if this
technique is to be used as an *in vivo* tool for assessment of renal health. Initial toxicology suggests that intravenously delivered CF is not acutely toxic, though these studies are ongoing. Nonetheless, *in vivo* measurements of nephron number and single glomerular function could be important for longitudinal studies of focal and segmental renal diseases and to monitor the progress of therapy.

**Conclusions.** We conclude that the MRI-based technique is capable of measuring the number and apparent size of individual glomeruli in 3D and yields comparable results to established methods. This result, along with previous *in vivo* detection of glomeruli with MRI, suggests the possibility of measuring glomeruli in living animals and in the clinic. To the best of our knowledge, this is the first report of a technique to nondestructively measure every glomerulus in the whole kidney.
CHAPTER 3
DETECTING, COUNTING, AND MEASURING THE SIZE OF GLOMERULI IN HUMAN TRANSPLANT KIDNEYS - A PROOF OF CONCEPT

INTRODUCTION

The goal of this work is to count the number (Nglom) and measure the volume (Vglom) of every glomerulus in human kidneys with magnetic resonance imaging (MRI). Changes in the number and size of glomeruli have been linked to ~ 90% of all chronic renal diseases as well as systemic diseases including diabetes and hypertension (Brenner et al., 1988; Puelles et al., 2012). Unfortunately, current histological methods to assess Nglom and Vglom require resection and destruction of the entire kidney and merely extrapolate measurements from small sample populations (Bertram et al., 1992). These techniques are neither clinically viable due to their destructive nature nor comprehensive in their assessment of Nglom and Vglom in preclinical research. A method to assess Nglom and Vglom in humans that is 1) non-invasive and 2) comprehensive (measures every glomerulus) would prove invaluable to clinicians and preclinical scientists alike.

Recently it has been shown that Nglom and Vglom may be measured ex vivo by simply injecting the extracellular matrix (ECM) specific cationic ferritin (CF) probe and imaging with MRI (Beeman et al., 2011; Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008; Heilmann et al., 2012). This method shows promise for application in humans as it does not require destruction of the kidney and identifies and measures every CF-labeled glomerulus in the kidney. CF is the cationized version of the iron-binding ferritin protein which, due to its endogenous origins, exhibits minimal toxic effects at MRI-detectable doses (Beeman et al., 2012). These previous studies have exploited the anionic proteoglycans of the
glomerular basement membrane (GBM) to bind CF. The superparamagnetic iron oxide core of the protein to induce local proton de-phasing (MRI-signal darkening) in regions where CF has accumulated. Since CF specifically accumulates in the GBM, all perfused glomeruli in the kidney are rendered MRI-visible after intravenous injection of CF. Here we propose to adapt this same technique to count every glomerulus in human transplant kidneys.

This work is presented as a proof of concept that human glomeruli may be targeted by a molecular imaging probe and subsequently detected with 3D MRI. Furthermore, we will show that, once glomeruli have been imaged, a number of glomerulus-specific measurements may be made including glomerular number and size.

METHODS

Sample preparation. Cationized ferritin (CF) was synthesized according to Danon, et al. (Danon et al., 1972). Four human kidneys were acquired though a donation network (The International Institute for the Advancement of Medicine). Kidneys were received in University of Wisconsin preservation solution within 24 hours of donor death. Kidneys were viable organs throughout the procedure, up until formalin fixation. The renal artery of three of the kidneys (one from a male, two from females) was catheterized and the kidney was perfused with phosphate buffered saline (PBS), then a 44 mg of CF in PBS, then PBS, then formalin. The fourth kidney was prepared in the same way but remained naive to CF (control). ~ 1 mm³ tissue biopsies were taken from the renal cortex from each kidney and prepared for immunofluorescence microscopy (detailed below). All kidneys were stored in 10% neutral buffered formalin.

MRI. Prior to imaging, kidneys were removed from formalin storage and washed three times in 500 ml PBS. The kidneys were then imaged using a
Bruker 7T/35 MRI scanner and a 72-mm quadrature transmit/receive radio frequency coil (Bruker, Billerica, MA). A T$_2^*$-weighted (TE/TR = 20/39 ms) three-dimensional gradient echo fast low angle shot (FLASH) sequence was used to image the entire kidney. MRI volumes were acquired with a 117x117x117 µm resolution (field of view = 6x6x10.5 cm, matrix size = 512x512x896, 5 averages, total scan time = 10 hr 39 min).

**Immunofluorescence.** ~ 1 mm$^3$ tissue biopsies were taken during sample preparation (described above). The biopsies were placed in 10% neutral buffered formalin for four hours. Then washed in PBS overnight. Samples were then cryoprotected in sucrose, rapidly frozen, and sectioned at 35 µm. Sections were washed in PBS, permeabliced, incubated in rabbit anti-horse spleen ferritin, an Alexa594 goat anti-rabbit secondary antibody and 4,6-diamidino-2-phenylindole (Invitrogen), and imaged on a Zeiss 710 laser scanning confocal microscope.

**Image processing.** Nglom and Vglom were measured from the MRI volumes using custom software written in MATLAB (The Mathworks, Natick, MA). Briefly, we derived the Hessian matrix for each voxel in the volume. This matrix describes the ‘curvature’ of each voxel based on its magnitude value relative to its neighbors’. This algorithm robustly detects the centroid voxels of glomeruli (assumed to be the darkest voxel of a single glomerulus) and their surrounding ‘glomerular’ voxels. Voxels that constitute a glomerulus are the clustered using a gaussian mixture model. Minimum and maximum size thresholds were then placed at 1 voxel and 16 voxels, respectively, as it was assumed that human glomeruli would be no less than 1.60x10$^6$ µm$^3$ and no more than 25.6x10$^6$ µm$^3$. 
RESULTS

CF-labeled, fixed human kidneys were imaged on a 7T MRI scanner. MRI imaging of the human kidneys perfused with CF revealed punctate spots of signal darkening (Fig. 3.1 A and C). Each dark spot represents a single glomerulus and is caused by the accumulation of the superparamagnetic CF in the glomerular basement membrane. The control kidney showed no such spots (Fig. 3.1 B). The specific binding of CF to the glomerulus was confirmed with immunofluorescence microscopy (Fig. 3.1 D and E).

Our algorithm was able to identify labeled glomeruli in the MRI volumes of CF-inoculated kidneys (Fig. 3.1D). In this figure, glomeruli identified by the algorithm are assigned an arbitrary color for the purpose of distinguishing neighboring glomeruli from one another. The total number of regions defined as glomeruli by the algorithm was then tallied to yield total glomerular number (Nglomer). This yielded a count of 772,922 glomeruli for the kidney from the male, and 328,181 and 702,506 for the two kidneys from the females. These numbers are consistent with those reported in the literature (Nyengaard & Bendtsen, 1992).

Finally, the apparent volume of each glomerulus may be measured based on the number of voxels composing each region defined as a glomerulus multiplied by the voxel dimensions. Using this method, we report glomerular volumes of $8.0 \times 10^6 \pm 4.28 \times 10^6 \, \mu m^3$ for the kidney from the male, and $6.9 \times 10^6 \pm 3.9 \times 10^6 \, \mu m^3$ and $6.8 \times 10^6 \pm 2.9 \times 10^6 \, \mu m^3$ for the two kidneys from the females. These volumes are consistent with those reported in the literature (Nyengaard & Bendtsen, 1992).
Figure 3.1. MRI of a CF-labeled human kidney reveals punctate dark spots throughout the organ. Each spot represents a single glomerulus (A and C). A control kidney showed no such spots (B). Immunofluorescence confirmed the accumulation of CF (red) in glomeruli of CF inoculated kidneys (D). Naive control glomeruli remained clear of CF-related immunofluorescence (E). Each glomerulus may be identified and counted in the entire kidney (F).
Figure 3.2. Histogram of apparent glomerular volume distribution of a CF-infected kidney from a human male. Since the MRI-based method for counting glomeruli measures every labeled glomerulus in the kidney, this new method presents a unique opportunity to assess the distribution of glomeruli defined by their size. The average ± the standard deviation of glomerular sizes measured in this kidney was $8.0 \times 10^6 \pm 4.28 \times 10^6 \, \mu m^3$. 

![Histogram of apparent glomerular volume distribution](image)
DISCUSSION

In this work we set out to show that human glomeruli may be labeled with the MRI-visible, naturally derived cationized ferritin (CF) probe and subsequently visualized, counted and their sizes measured using MRI. Immunofluorescence confirmed that CF binds specifically to the glomerulus and $T_2^*$-weighted MRI confirmed that the labeled glomeruli are MRI-detectable. Once imaged with MRI, we were then able to identify, count, and measure the size of every glomerulus in these fresh human transplant kidneys. This MRI-based technique for measuring $N_{glomerulus}$ and $V_{glomerulus}$ in human kidneys also allows for assessment of the intra-renal glomerular volume distribution - a parameter exclusive to this technique that will allow glomerular populations defined by size to be correlated with renal and systemic diseases. As an example, a histogram of the intra-renal glomerular volume distribution in the kidney from the male donor is presented in figure 3.2.

In our previous works that first established this MRI-based technique we used the disector/fractionator method for validation (Beeman et al., 2011). We have elected to forgo this process in the current work because 1) the above mentioned work has already validated the technique in rodents and 2) our method is the only method to date that allows for identification of every glomerulus in the kidney. It is this second point that raises this potentially controversial question: Have prior histological techniques, which extrapolate $N_{glomerulus}$ and $V_{glomerulus}$ for only 30 - 40 sampled glomeruli, really yielded accurate results in human kidneys? Though the chances are slim, there exists a real possibility that these histological methods are inaccurate. We assert here that the MRI-based technique is the only way to identify, count, and measure every glomerulus in the kidney and should therefore not be bound by the assumptions made in prior histological techniques that merely extrapolate these
measurements. This work presents the unique opportunity to retrospectively assess the accuracy of the previous histological techniques for measuring Nglom and Vglom.

**Conclusions.** These results, along with previous in vivo detection of glomeruli and counts made in the rat, suggest the possibility of counting glomeruli in the clinic.
CHAPTER 4
A HIGH T₁-RELAXIVITY NANOPARTICLE FOR IN VIVO MRI-DETECTION OF GLOMERULI

INTRODUCTION

The goal of this work is to develop a non-invasive, MRI-based method for detecting kidney glomeruli in vivo. A single human kidney has between 200,000 and 2,000,000 nephrons which are responsible for maintaining homeostasis by balancing blood ion and glucose concentrations and blood pH levels. Each nephron begins with a single glomerulus that serves as a filter to retain blood cells and vital macromolecules in the blood stream while allowing smaller plasma constituents to pass from the capillary space into the nephron tubule. In the tubule small plasma molecules and water are either reabsorbed into the blood stream or expelled in the form of urine.

Diabetes, obesity, and hypertension are strongly linked to a low number of glomeruli (Nglom) (Brenner et al., 1988) and the volume of glomeruli (Vglom) has been linked to ~ 90% of chronic kidney diseases (CKD) (Puelles et al., 2012). Furthermore, recent evidence suggests that ~ 88% of patients with Type 2 diabetes (a patient population known to be at risk for CKD) also have a case of undiagnosed CKD. The insensitivity of current techniques to the early loss of healthy glomerular filtration surface area is a contributing factor to the poor rate of CKD diagnosis (Cullen-McEwen, 2003). It is likely that the number of patients requiring dialysis or kidney transplant could be greatly reduced if there existed a more robust method for detecting the early stages of CKD. There are already excellent histological techniques for measuring Nglom and Vglom but these techniques require resection and destruction of the entire organ (Bertram et al., 1992) - a procedure that is not clinically applicable.
Previous works have shown that Nglom and Vglom may be measured ex vivo using intravenous injection of the MRI-visible cationized ferritin (CF) nanoparticle and magnetic resonance imaging (MRI) (Beeman et al., 2011; Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008; Heilmann et al., 2012). When injected intravenously in vivo, CF will bind specifically to the anionic proteoglycans of the glomerular basement membrane (GBM). Each perfused glomerulus is rendered visible with T₂* -weighted MRI when CF-inoculated kidneys are imaged ex vivo. CF-labeled glomeruli may then be identified and their number and size measured from the MRI volumes using custom image processing software. This new technique is the first to measure all glomeruli in a kidney and has opened the door to the in vitro study of glomerular populations defined by their size and location in the kidney.

The ability to detect glomeruli in vivo would certainly serve a strong clinical purpose. CF has been shown to be non-toxic at MRI detectable doses (Beeman et al., 2012) and previous work has shown in vivo detection of glomeruli with CF and MRI to be possible, though quite difficult (Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008). It is difficult because the iron core of CF yields signal darkening when it accumulates in the glomerulus. When imaged in vivo, the contrast agent must compete against the blood background which also causes signal darkening. A paramagnetic agent that yields a bright signal but otherwise behaves in the same manner as CF when injected would certainly make in vivo imaging of the kidney glomerulus a simpler task and would potentially lower the required dose of contrast agent to detect glomeruli.

The ferritin protein is quite versatile when applied as a MRI contrast agent. It has been shown that apoferritin, the empty version of ferritin, may be loaded with iron in vitro to create a T₂ MRI contrast agent that is 100-times more
detectable than native ferritin (Bulte et al., 1994; Clavijo Jordan et al., 2010).

More recent we have developed a high relaxivity, paramagnetic T\textsubscript{1} MRI contrast agent inside of the ferritin protein shell, thereby allowing for brightened signal when imaged with T\textsubscript{1}-weighted MRI. This is accomplished by doping the iron core of the ferritin protein with tungsten. This tungsten-iron loaded (WFe) ferritin nanoparticle is detectable with T\textsubscript{1}-weighted MRI at concentrations as low as 25 nM. The goal of this work is to detect kidney glomeruli in vivo by cationizing WFe ferritin, injecting the cationized WFe ferritin (hereby referred to as paramagnetic cationized ferritin, or paraCF), and imaging the kidneys with T\textsubscript{1}-weighted MRI.

**METHODS**

**Loading the apoferritin core.** 48mM FeCl\textsubscript{2} was added at a rate of 12.5µl/min to a 2µM solution of apoferritin in an oxygen free environment using a syringe pump for a total of 140 minutes. 50 minutes into FeCl\textsubscript{2} pumping, 48mM Na\textsubscript{2}WO\textsubscript{6} was added at a rate of 12.5µl/min using a syringe pump for a total of 40 minutes. A total of 1.75ml of FeCl\textsubscript{2} and 500µl of Na\textsubscript{2}WO\textsubscript{6} was added to the protein solution. 200µl of 300mM sodium citrate was then added to the solution to chelate any remaining metal ions. The solution was the sonicated and centrifuged for 10 minutes at 957·g then dialyzed overnight against 3L of deionized water. Once dialyzed, the protein solution was filtered using 0.8µm and 0.2µm surfactant free cellulose acetate syringe filters to rid the solution of coarse non-specific metal oxides. A total tungsten and iron-loaded (WFe) ferritin protein concentration was obtained with a Coomassie Plus Bradford Assay Kit and inductively coupled plasma – optical emission spectroscopy (ICP-OES) was used to measure metal concentrations.

**Cationization of WFe ferritin.** WFe ferritin was cationized by coating its surface with amine groups via a carbodiimide conjugation of N,N-Dimethyl-1,3-
propanediamine (DMPA) to the carboxyl groups of the ferritin surface using 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). This process has been detailed by Danon, et al. (Danon et al., 1972) and in our previous works (Beeman et al., 2011; 2012). The resulting cationized WFe ferritin will hereby be referred to as paraCF. Once the cationization process was complete, the resulting paraCF solution was dialyzed against phosphate buffered saline (pH = 7.2) and concentrated to 11 mg paraCF per ml of PBS.

Animal preparation and imaging. ParaCF was injected intravenously to three male sprague-dawley rats at a dose of 4.4 mg/100 g. Three rats remained un-injected as naive controls. Rats were imaged in vivo at 7T with a 2D T₁-weighted gradient echo MRI pulse sequence using a custom built, kidney-specific radio frequency coil (TE/TR = 10/54 ms, resolution = 117x117x400 µm), then sacrificed and fixed by transcardial perfusion of PBS followed by 10% neutral buffered formalin. Right kidneys were be prepared for immunofluorescence histology.

Immunofluorescence. Once resected, right kidneys were bathed in 10% neutral buffered formalin for three hours, then washed in PBS overnight. Kidneys were then bathed in 15% sucrose, then 30% sucrose until fully infiltrated. Once cryoprotected by sucrose, kidneys were embedded in optimal cutting temperature (OCT) medium and rapidly frozen to -80 °C. Frozen kidney were sectioned at 35 µm on a cryostat (Leica Microsystems CM3050 S, Buffalo Grove, IL). Sections were thoroughly washed in PBS, permeablized, blocked in bovine serum, and incubated in a rabbit anti-horse ferritin (1:500) primary antibody (Invitrogen). Sections were then thoroughly washed and incubated in an Alexa594 goat anti-rabbit secondary antibody and 4',6-diamidino-2-phenylindole
Sections were mounted and imaged with a Zeiss 710 laser scanning confocal microscope.

**Data analysis.** Signal changes in the renal cortex due to the accumulation of paraCF in glomeruli was quantified by normalizing the mean signal magnitude in the renal cortex of paraCF-inoculated and naive rats to the mean signal magnitude of the muscle surrounding the rat’s spine. This is an effective method for quantifying the effects of CF accumulation as it does not accumulate in muscle. Normalized signal magnitude values in paraCF-injected rats was compared to that of naive controls using a two-tailed Student’s t-tests ($\alpha = 0.05$) (The Mathworks, Inc. Natick, MA).

**RESULTS**

**In vivo detection of glomeruli.** 1% agarose phantoms were used to establish the MRI-detectability of tungsten and iron loaded (WFe) ferritin. WFe ferritin was found to be detectable at concentrations as low as 25 nM (Fig. 4.1 a) and, when injected into the striatum, was found to be detectable in vivo at a concentration of 195 nM (Fig. 4.1 b and c) using a $T_1$-weighted gradient echo sequence at 7T. The presence of WFe ferritin reveals itself as bright signal in $T_1$-weighted MRI and shortens $T_1$ by approximately 22% compared to the contralateral control striatum.

WFe ferritin was then cationized (paraCF) and injected intravenously into healthy rats. $T_1$-weighted gradient echo MRI revealed signal enhancement in the renal cortex of paraCF-injected animals (Fig. 4.1 d). No such enhancement was seen in the renal cortex of naive controls (Fig. 4.1 e). An $\sim$ 50% increase in MRI signal magnitude was observed in the renal cortex of paraCF-injected rats compared to naive controls (when the signal magnitude of the renal cortex is normalized against that of muscle) (Fig. 4.1 f and g).
Figure 4.1. WFe nanoparticles are detected in vivo with $T_1$-weighted MRI. a) 2D Gradient Echo $T_1$-weighted image of a 1% agarose phantom. Increasing concentrations of WFe-apoferritin result in increased positive image contrast. b) 3D Gradient Echo $T_1$-weighted image of an in vivo inoculation of 8ul of 195nM WFe nanoparticles and native ferritin into a rat striatum. c) $T_1$ map color overlay showing an average $T_1$ for each ROI at the injection sites. The injection of WFe nanoparticles resulted in an average $T_1$ that was 22% shorter than the contralateral ROI. d) In vivo intravenous injection of paraCF labeling kidney glomeruli, spleen, and liver. e) Control image of a rat in vivo with no injection of agent. f) Thresholded image of paraCF labeled and, g) no injection of agent. Highlighted in red are pixels with intensities 50% higher than background muscle tissue, insets show cortex of paraCF kidney and control kidney. It is clear that the kidney is labeled with paraCF showing evident hyperintensities, while control did not show significant enhancement over background.
Figure 4.2. Confocal microscopy of a paraCF-labeled kidney glomerulus and a control glomerulus. ParaCF is labeled with a red fluorophore and cell nuclei are labeled with blue DAPI. A ribbon of red fluorescence was observed in the glomerulus in sections of paraCF-inoculated kidneys but not naive controls, indicating that paraCF is bound to the glomerulus. Scale bar = 20 µm.
**Immunofluorescence.** ParaCF-inoculated and naive control kidneys were sectioned and stained with an anti-ferritin antibody, which was then labeled with a red fluorophore. When imaged using a confocal microscope, a ribbon of red fluorescence was observed in the glomerulus in sections of paraCF-inoculated kidneys but not naive controls (Fig. 4.2). The ribbon of red observed in paraCF-inoculated kidneys is consistent with the distribution of the GBM in the glomerulus, indicating that intravenously delivered paraCF specifically targets the GBM. It is this accumulation of paraCF in the glomerulus that yields in vivo visualization of glomeruli when imaged with T₁-weighted MRI.

**DISCUSSION**

In this work we have shown that tungsten-iron loaded (WFe) ferritin, a highly detectable T₁ nanoparticle, may be cationized to produce a new targeted nanoparticle called paraCF (paramagnetic cationized ferritin). Regular cationized ferritin is superparamagnetic and causes spin dephasing at the site of accumulation and a dark MRI signal. Unfortunately, this accumulation of CF is difficult to visualize in vivo in highly vascularized organs like the kidney because of the competing dark blood background. The paramagnetic nature of paraCF, on the other hand, causes signal brightening at its binding site. We have shown that paraCF can be used to detect glomeruli in vivo using T₁-weighted MRI and that its bright signal enhancement is easily visualized against the blood background of the kidney.

In vivo visualization of glomeruli with paraCF requires one quarter of the dose for detection compared to the studies in which glomeruli were labeled with regular CF and imaged in vivo. In fact, the high r₁ relaxivity of paraCF may allow for the dosage necessary to detect glomeruli in vivo to be reduced as much as 20-fold compared to regular CF. Though regular CF has already been shown to
have minimal effects on renal, hepatic, and immune function at MRI detectable doses (Beeman et al., 2012), the ability to reduce the dosage of contrast agent will only be beneficial to the translation of cationized ferritin-based agents from ex vivo applications to preclinical and clinical diagnostics.

The resolution required to image individual glomeruli in humans is approximately 117 µm\(^3\) (see Chapter 3). This is an unlikely in vivo resolution due to the lower signal-to-noise ratio of in vivo MRI. At a more likely resolution, say 1-2 mm\(^3\), there would be approximately 10 to 20 glomeruli within a single voxel. While outside of the scope of this work, T\(_1\)-weighted MRI may be used to dynamically monitor the uptake of paraCF in the glomerulus in vivo. By monitoring the rate at which paraCF accumulates in vivo in a single voxel containing multiple glomeruli we believe that MRI and paraCF can be used to measure either total glomerular filtration surface area or the total glomerular filtration rate (GFR) within a single voxel. This hypothesis is discussed further in Chapter 7.

Conclusions. The goal of this work was to develop a glomerulus-specific MRI contrast agent that can be easily detected in vivo. Regular cationized ferritin has a superparamagnetic core that is difficult to detect above the dark blood background in highly vascularized organs. We have shown that the metallic core of ferritin may be modified with a tungsten dopant that blocks the exchange coupling within the metallic core of the protein. This renders the tungsten-doped ferritin highly paramagnetic. We go on to show that the paramagnetic tungsten-doped ferritin may be cationized to form a new targeted MRI contrast agent called paramagnetic cationized ferritin (paraCF). We show that, when administered intravenously, paraCF binds to the glomerular basement membrane with high specificity and renders glomeruli MRI-visible. This highly detectable,
glomerulus-specific paramagnetic nanoparticle may be used to dynamically detect glomerular structure and function in vivo on a spatial scale that has previously been impossible.
CHAPTER 5
TOXICITY, BIODISTRIBUTION, AND EX VIVO MRI DETECTION OF INTRAVENOUSLY INJECTED CATIONIZED FERRITIN

INTRODUCTION

The extracellular matrix (ECM) plays a vital role in development and structural integrity of tissue throughout the body and is crucial for cell signaling, proliferation, and apoptosis (Adams & Watt, 1993; Boudreau, Werb, & Bissell, 1996; Rana, Mischoulon, Xie, Bucher, & Farmer, 1994; Sanes, 1983; 1989; Schindler et al., 2006). The ECM is exposed directly to circulation in a number of organs, functioning as a filter or as a site for macromolecular diffusion into the parenchyma. Sulfated, anionic proteoglycans, which are common molecular components of the ECM, associate with structural macromolecules such as collagen, forming part of the network that supports cells of varying types. Because of the functional nature of the ECM in development and maintenance of a healthy system, the ability to monitor the structure and function of the ECM may be important for basic noninvasive biological and toxicology studies and potentially in the clinic.

Ferritin, a superparamagnetic iron storage protein, is useful as an MRI contrast agent because of its small size (13 nm in diameter) and ease of functionalization. The payload of the ferritin molecule can be readily modified to increase the MRI relaxivity (Bulte et al., 1995; 1994; Clavijo Jordan et al., 2010; Meldrum, Heywood, & Mann, 1992). Cationized ferritin (CF), a chemically modified version of native ferritin, was originally proposed as an electron-dense label for electron microscopy of tissue (Danon et al., 1972) and has been further developed as a superparamagnetic contrast agent for imaging of the renal glomerular basement membrane (GBM) (Bennett, Zhou, Sumner, Dodd,
Bouraoud, et al., 2008) and measurement of whole-kidney nephron endowment with MRI (Beeman et al., 2011; Heilmann et al., 2012). CF has an equivalent relaxivity to unmodified native ferritin and is advantageous as an MRI contrast agent due to its small diameter, ease of functionalization and modification of the superparamagnetic core, and detectability using immunohistochemistry (IHC) for histological validation of labeling. In the kidney, the use of CF as a contrast agent may lead to a better understanding of renal function in the susceptibility to cardiovascular and kidney disease (Brenner et al., 1988; Hoy et al., 2008) and may yield a unique noninvasive measurement of renal glomerular function. CF has been also proposed as a contrast agent to detect the liver hepatic sinusoid in vivo (S. Beeman, mandarino, Rakela, & Bennett, 2011b), potentially allowing for assessment of liver disease. We have also shown that the distribution of CF is altered in a rat model of focal and segmental glomerulosclerosis (Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008), as detected with MRI, suggesting the possible use of CF as an MRI contrast agent to detect and evaluate early onset kidney disease. Cationized nanoparticles such as CF, when detected by MRI, may thus play an important future role in understanding the function of ECM in normal physiology and in disease.

The goal of this work was to establish the toxicity and biodistribution of CF after intravenous injection. Others have studied the biodistribution of magnetic nanoparticles and magnetoferritin after intravenous injection (Bulte et al., 1995), but cationization leads to distinct patterns of CF uptake related to the surface charge. This work is a step toward further in vivo studies of ECM using CF and MRI. The ability to detect ECM in vivo would improve monitoring of a variety of diseases in which changes to the ECM structure occur, including glomerulonephritis in the kidney (Harendza, Schneider, Helmchen, & Stahl, 1999)
and possibly fibrosis in the liver (Bedossa & Paradis, 2003). Furthermore, electron microscopy studies with CF have been useful to characterize outflow of cerebrospinal fluid in the brain ultrastructure (Voelz et al., 2007), and other basic biological processes (Brody et al., 1984; Danon et al., 1972; Schmidley & Wissig, 1986), raising the possibility of detecting similar processes dynamically with MRI.

In this work we report kidney and liver toxicology biomarkers, total leukocyte counts, and organ distribution of CF after intravenous injection using both ex vivo MRI and immunofluorescence. In addition to confirming the previously observed labeling of glomeruli in the kidney, we show that CF is also taken up into the hepatocytes, Kupffer’s cells, and endothelial cells of the liver, into macrophages in the red pulp of the spleen, and into alveolar endothelial cells of the lungs. To establish whether CF is toxic in MRI-detectable doses, we performed a blood panel for acute and chronic renal and hepatic toxicity after intravenous injection. We show that MRI-detectable doses (5.75 mg/100 g) of intravenously injected CF do not appear to have a detrimental effect on renal and hepatic function and do not significantly increase blood leukocyte counts. These results suggest that CF may be useful as an intravenous MRI contrast agent to detect ECM structure throughout the body.

METHODS

Synthesis of cationized ferritin. CF was synthesized according to Danon et al. (Danon et al., 1972). Commercial horse spleen ferritin (Sigma-Aldrich, Saint Louis, MO) was conjugated to N,N-dimethyl-1,3-propanediamine by adding 1-ethyl-3(3-dimethyl-aminopropyl) carbodiimide hydrochloride. Briefly, 2 mL of an aqueous solution of 2 M N,N-dimethyl-1,3-propanediamine was adjusted to a pH of 6 with appropriate volumes of 2 N and 0.2 N HCl and NaOH. We then added 0.5 mL of 55 mg/mL horse spleen ferritin to the solution followed
by 200 mg of 1-ethyl-3(3-dimethyl-aminopropyl) carbodiimide hydrochloride. The resulting solution was monitored for 2 h to maintain the pH at 6. The solution was then sealed and left overnight at room temperature. The solution was then dialyzed twice against 3.5 L of phosphate buffered saline (PBS) at 4°C for 12–24 h for each pass. CF was stored in PBS at 4°C.

**Animal preparation and blood collection.** *Toxicity and ex vivo MRI detection of CF.* Eighteen male Sprague-Dawley rats were used in the study of renal and hepatic toxicity as well as ex vivo MRI detection of CF. All animal protocols were approved by the Arizona State University Institutional Animal Care and Use Committee and performed in accordance with the NIH guide for the care and use of laboratory animals. Nine rats were anesthetized with a 5% isoflurane mixture in oxygen and administered 5.75 mg/100 g of CF in PBS via intravenous injection, in three doses spaced 1.5 h apart. The remaining nine rats did not receive any contrast injection (naive rats). Three injections were used because all previous studies with CF for MRI have used multiple injections, and we have not yet detected CF by MRI after a single injection. It is possible that concentrated CF could be delivered in a single bolus, but we have not evaluated whether concentrated doses result in CF aggregation. It is also possible that concentrated CF would lead to changes in how it labels the ECM. Another group of nine rats received no CF as controls.

Blood was collected from all 18 animals 1.5 h after the third injection, after which three animals from each group were sacrificed via transcardial perfusion and tissue was prepared for IHC and ex vivo MRI (tissue preparation described below). The remaining six animals from each group were used for chronic toxicity studies. The time points of the toxicity study were defined as acute (1.5 h after the last injection) and chronic (1 week after injections and 2 weeks after
injections). At each time point, 0.5 mL of blood was collected from each rat from the lateral saphenous vein via puncture with a 23-G needle. Blood was collected while animals were awake and manually restrained. Blood samples were immediately centrifuged to separate the serum from cells. Serum levels of calcium, blood urea nitrogen, creatinine, and albumin (ALB) were measured to assess renal function and alanine transaminase, aspartate transaminase, alkaline phosphatase, and total bilirubin to assess hepatic function. Analysis was performed by Charles River Laboratories (Wilmington, MA) in which biomarkers were measured by photometric absorbance using an ACE Alera Clinical Chemistry System (Alfa Wassermann, West Caldwell, NJ).

**Immune response.** A separate group of 12 male Sprague-Dawley rats was used to test the immune response of rats inoculated with CF. Eight rats were injected with CF as described above and four rats were not given any injection (naive). Blood was collected from the CF-injected rats via cardiac puncture 2 h (n = 4) and 1 week (n = 4) after injection. Blood was also collected via cardiac puncture from the naive rats. Whole blood samples were collected and shipped in heparinized Ethylenediaminetetraacetic acid (EDTA) tubes (Charles River). Total leukocyte counts were performed by Radil Research Laboratory using a Hemavet 950FS automated hematology analyzer.

**Biodegradation.** Biodegradation of CF in the liver and kidney was assessed by injecting four rats as described above and sacrificing via transcardial perfusion at 1.5 h, 2, 4, and 7 days after injection. Livers and kidneys were prepared for ex vivo MRI. The liver was chosen to assess biodegradation of CF based on the major role the liver plays in removing ferritin from the circulation via receptor mediated and reticuloendothelial system clearance (Bulte et al., 1995) and our observation that the detection of CF is robust in the liver.
Imaging. **Tissue Preparation.** Rats were given intravenous injections of CF or no CF as described earlier. Rats designated for IHC and ex vivo MRI were sacrificed 1.5 h after the third bolus injection via transcardial perfusion of PBS until liver and kidneys were visibly cleared of blood. The PBS was followed by perfusion of 10% neutral buffer formalin. Kidneys, livers, spleens, pancreas, and lungs were resected. Sections of each organ were placed in solutions of either 2% glutaraldehyde/0.1 M cacodylate solution for MRI or in 10% neutral buffered formalin for 2 h and then PBS for IHC.

**Immunohistochemistry.** IHC was performed on excised kidneys, livers, lungs, spleens, and pancreas to determine the distribution of intravenously injected CF in these organs. Tissues fixed for IHC were bathed in 15% sucrose for 1 day followed by 30% sucrose for 3 days, and rapidly frozen to -80 °C in optimal cutting temperature compound. Optimal cutting temperature-embedded tissues were sectioned at 35 mm in a cryostat (Leica Microsystems CM3050 S, Buffalo Grove, IL). Tissue sections were washed three times in PBS, permeabilized with 0.3% Triton X-100 (Sigma-Aldrich), and blocked for 1 h with Image-iT FX signal enhancer (Invitrogen, Carlsbad, CA) followed by CAS-block (Invitrogen) for 1 h. Sections were then incubated overnight at 4 °C in chicken antifibronectin (1:200) and rabbit anti-horse ferritin (1:500) primary antibodies (Invitrogen). Following primary antibody incubation, sections were washed three times with PBS, and incubated for 2 h at room temperature in Alexa488 goat anti-chicken and Alexa594 goat anti-rabbit secondary antibodies (Invitrogen). Sections were then incubated in 4',6-diamidino-2-phenylindole for 15 min to stain cell nuclei and then washed three times in PBS. Sections were mounted on slides with Vectashield fluorescent mounting media (VectorLabs, Burlingame,
Tissue sections were imaged with a Zeiss 710 laser scanning confocal microscope.

**Ex vivo MRI-detection of CF.** In order to determine whether labeling with CF was detectable with MRI, intact kidneys, livers, lungs, spleens, and pancreases were imaged ex vivo in PBS in either 20-ml syringes (livers) or 12-ml tubes on a Bruker 7T small animal imaging system (Bruker, Billerica, MA) with a 72-mm rat volume transmit coil and a rat brain surface coil (Bruker). All tissue was imaged using a T$_2^*$-weighted 2D gradient echo (FLASH) sequence with a 30° flip-angle, though each tissue type required a unique echo time/repetition time. A TE/TR of 20/28 ms was used for kidneys, 15/23 ms for liver, 20/80 ms for lungs, and 3.4/30 ms for spleen. Kidneys were imaged using a field of view of 1.5x1.5 cm and a matrix size of 200x200 (75x75 µm) and a slice thickness of 500 µm. Livers were imaged using a field of view of 2.1x2.1 cm and a matrix size of 300x300 (70x70 µm) and a slice thickness of 1,000 µm. Lungs, spleens, and pancreases were imaged using a field of view of 1.5x1.5 cm and a matrix size of 200x200 (75x75 µm) and a slice thickness of 1,000 µm.

**Ex vivo MRI to assess biodegradation.** Livers and kidneys prepared for the biodegradation study were imaged in 20-ml syringes using T$_2^*$-weighted 2D gradient echo (FLASH) sequence with a 30° flip-angle and a TE/TR of 15/23. T$_2^*$-maps of livers were also produced using a multi-gradient echo (MGE) sequence and echo times of 4, 11, 18, 25, 32, 39, 46, 53, 60, 67, 74, and 81 ms.

**Data analysis.** All statistical analyses were run as two sample, two-tailed Student’s t-tests in MATLAB (The Mathworks, Natick, MA) to test the null hypothesis that the mean difference between groups is zero at the significance level $\alpha = 0.05$. We compared all kidney and liver serum biomarker concentrations from CF inoculated animals against those of controls. Furthermore, to quantify
MRI signal differences in organs from CF inoculated and control animals, six randomly chosen regions of interest (ROIs) inside of the tissue were normalized against six randomly chosen ROIs in the surrounding buffer. We then compared the normalized signal values from CF inoculated animals against those of controls. T$_2^*$ values in the biodegradation experiments were calculated in T$_2^*$-map ROIs.

RESULTS

Toxicity. To determine renal toxicity of intravenously injected CF, serum levels of calcium (CAL), blood urea nitrogen (BUN), creatinine (CRE), and albumin (ALB) were measured 1.5 hours after injection, 1 week after injection, and 2 weeks after injection. The same measurements at the same time points were made in animals not administered CF. Serum levels of each kidney biomarker did not differ significantly from those of control animals at 1.5 hrs, 1 week, or 2 weeks after injection ($\alpha = 0.05$), indicating no acute or chronic renal toxicity after CF injection. The data are summarized in box plots in Figure 5.1. Central lines represent the median, the box edges represent the 25th and 75th percentiles, and caps represent the most extreme points not considered outliers. Stars represent these outliers.

To determine hepatic toxicity of intravenously injected CF, serum levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALK), and total bilirubin (TBIL) were measured 1.5 hours after injection, one week after injection, and two weeks after injection. The same measurements at the same time points were made in animals not administered CF. Serum levels of each liver biomarker did not differ significantly from those of control animals at 1.5 hrs, 1 week, or 2 weeks after injection ($\alpha = 0.05$). The data are summarized in box plots presented in Figure 5.2.
Figure 5.1. Blood serum measurements of calcium (A), blood urea nitrogen (B), creatinine (C), and albumin (D) in rats after intravenous injection of cationized ferritin (CF) or without injection (CTRL). The central line represents the median, the box edges represent the 25th and 75th percentiles, and the caps represent the most extreme points not considered outliers. Stars represent outliers. Serum measurements of all of biomarkers of renal function from CF-injected rats do not differ significantly from those from control animals (\( \alpha = 0.05 \)) at acute (n=9 per group) or chronic (n=6 per group) stages after CF administration.
Figure 5.2. Blood serum measurements of alanine transaminase (A), aspartate transaminase (B), alkaline phosphatase (C), and total bilirubin (D) in rats after intravenous administration of cationized ferritin (CF) or without injection (CTRL). The central line represents the median, the box edges represent the 25th and 75th percentiles, and the caps represent the most extreme points not considered outliers. Stars represent outliers. Serum measurements of all of biomarkers of liver function from CF injected animals do not differ significantly from those from control animals not administered protein ($\alpha = 0.05$), at acute ($n = 9$ per group) and all chronic time points ($n = 6$ per group) after injection of CF.
Figure 5.3. Total leukocyte counts. The total leukocyte counts of rats two hours (9.255 ± 1.89 x 10^{-3} cells/µL) and one week (9.33 ± 1.39 x 10^{-3} cells/µL) after CF-injection did not differ significantly (α = 0.05) from those of the control group (11.18 ± 2.06 x 10^{-3} cells/µL).
To determine whether there was an immune response to intravenously injected CF, we measured total leukocyte counts at two hours and one week after injection. Total leukocyte counts were also made in naive rats receiving no CF. The total leukocyte counts of rats (Fig. 5.3) two hours (9.255 ± 1.89 x 10^{-3} cells/µL) and one week (9.33 ± 1.39 x 10^{-3} cells/µL) after CF-injection did not differ significantly (α = 0.05) from those of the naive group (11.18 ± 2.06 x 10^{-3} cells/µL).

**Biodegradation.** Ev vivo T2*-maps of cationized ferritin inoculated livers revealed that T2* returns to baseline seven days after injection of CF. T2* in ROIs was 18.7 ± 0.45 ms 1.5 hours after injection, 13.9 ± 0.93 ms two days after injection, 12.3 ± 1.2 ms four days after injection, and 21.5 ± 0.4 ms seven days after injection. For comparison, liver T2* to be 20.9 ± 0.73 ms in naive rats. CF accumulation was not detectable in kidneys two days after CF injection.

**Tissue distribution studies.** We performed both immunofluorescence and ex vivo MRI to determine the tissue distribution and detectability of intravenously injected CF.

**Immunofluorescence.** Confocal imaging of kidneys from animals injected with CF suggests distinct localization of CF (red) to the glomerulus (Fig. 5.4A). Confocal imaging of kidneys from animals not injected with CF reveal minimal fluorescence associated with CF (Fig. 5.4E). This is consistent with previous observations that CF binds with high specificity to the GBM (Beeman et al., 2011; Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008; Heilmann et al., 2012).

Confocal imaging of livers from animals injected with CF showed substantial colocalization of CF (red) with fibronectin (green) surrounding the hepatic sinusoid, suggesting that CF binds to the extracellular matrix of Disse’s
Space. Furthermore, signal attributed to the presence of CF was observed inside of hepatocytes, endothelial cells of the hepatic sinusoid, and Kupffer's cells, suggesting internalization of CF by these cells (Fig. 5.4B). Minimal fluorescence associated with CF was observed in livers from animals not administered CF. (Fig. 5.4F).

Immunofluorescence from CF (red) was observed in the lung, as shown in Figure 5.4C. The distribution of fluorescence in these sections suggests that CF is bound to the surface of endothelial cells lining the lung capillaries and also endocytosed. Some red fluorescence was observed in the lung sections from animals not administered CF (Fig. 5.4G). We attributed this to nonspecific binding of IHC antibodies inside of alveolar macrophages.

Substantial red fluorescence was observed in spleen sections from animals in both the CF-injected and control groups, suggesting nonspecific binding of the antibody to cells in the spleen. Nonetheless, there were differences in the distribution of immunofluorescent signal between injected animals and control animals. Punctate red signal associated with CF accumulation was observed inside of the macrophages at the border of the white and red pulp (Fig. 5.4D), suggesting that these cells take up CF as blood filters through the red pulp.

While red fluorescence was observed in pancreases prepared for IHC, we were unable to discern a difference between the CF-injected and control cohorts. No pattern was observed in CF-inoculated sections that would specifically suggest accumulation of CF (data not shown). We attribute the red fluorescence observed in both CF-inoculated and control pancreases to non-specific binding of the antibodies.
Figure 5.4. Confocal imaging of perfused, excised kidneys (A and E), livers (B and F), lungs (C and G), and spleens (D and H) of rats after intravenous injection of cationized ferritin or no injection (control). Confocal imaging of kidneys from CF inoculated animals shows localization of red (CF) signal to the glomerulus (A). Kidneys from animals not injected with CF show minimal fluorescence on the red channel (E). Confocal imaging of livers from animals injected with CF showed colocalization of CF with the fibronectin (green) surrounding the hepatic sinusoid as well as punctate red signal inside of hepatocytes, endothelial cells, and Kupffer’s cells, suggesting internalization of CF by these cells (B). Minimal red fluorescence was observed in livers from control animals (F). Red fluorescence associated with the presence of CF is apparent in the lung sections prepared from animals injected with CF on confocal images (C). The distribution of red fluorescence in these sections suggests that CF is likely bound to the surface of and taken up into the endothelial cells lining the capillaries. Minimal red fluorescence was observed in the lung sections from control animals (G). Spleen sections prepared from animals injected with CF show punctate red fluorescence associated with CF accumulation (arrows) inside of the macrophages at the border of the white and red pulp (D). Red fluorescence was also observed inside of macrophages at the border of white and red pulp in the spleen sections from control animals due to non-specific binding of the anti-horse spleen ferritin antibody (H). Scale bar = 20 µm.
**MRI.** Gradient echo MRI (GRE-MRI) of kidneys excised from rats injected with CF revealed distinct spots of hypointensity throughout the cortex of the kidney (TE/TR = 20/28 ms), as shown in Figure 5.5A. MRI of kidneys from animals not administered CF showed no similar labeling (Fig. 5.5E). The distribution of hypointensity in CF-inoculated kidneys is consistent with the spatial distribution of glomeruli throughout the cortex and juxtamedulary regions of the kidney observed with immunofluorescence and with MRI results published in previous work at 9.4T, 11.7T, and 19T (Beeman et al., 2011; Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008; Heilmann et al., 2012). The voxel magnitude in six regions of interest (ROIs) in the cortex of the kidney were normalized to six ROIs in the buffer surrounding the tissue. The normalized MRI signal magnitudes were significantly different ($\alpha = 0.05$) between groups, with normalized magnitudes in CF inoculated and control kidneys of 0.86 ± 0.045 and 0.94 ± 0.02, respectively (Fig. 5.6).

GRE-MRI (TE/TR = 15/23 ms) of liver sections from rats administered CF showed striations of hypointensity throughout the liver (Fig. 5.5B), consistent with the known spatial distribution of the hepatic sinusoid. No similar pattern was observed in livers from control animals (Fig. 5.5F). The normalized MRI signal magnitudes were significantly different ($\alpha = 0.05$) between livers of CF-injected and control animals, with normalized magnitudes of 0.78 ± 0.04 and 1.26 ± 0.07, respectively (Fig. 5.6).

GRE-MRI (TE/TR = 20/80 ms) of the lungs of rats inoculated with CF showed substantial labeling throughout compared to lungs of control animals (figure 5.5C and G). IHC suggests that this labeling is due to the binding of CF to endothelial cell surfaces and internalization of CF into endothelial cells. The normalized MRI signal magnitudes were significantly different ($\alpha = 0.05$) between
groups, with normalized signal magnitude of CF-inoculated and control lungs of 0.64 ± 0.07 and 0.93 ± 0.02, respectively (Fig. 5.6).

Spleens from rats injected with CF had substantial labeling by CF in inoculated animals, compared to controls. The white pulp appears lighter than the surrounding red pulp in GRE-MRI (TE/TR = 3.4/30 ms), as shown in Figure 5.5D and 4H. IHC indicates that this may be due to the uptake of CF into the macrophages at the interface between the red and white pulp of the spleen (Fig. 5.5D and H). The normalized MRI signal magnitudes were significantly different (α = 0.05) in CF-inoculated and control spleen, respectively. The magnitudes were 0.43 ± 0.02 and 0.98 ± 0.05, respectively (Fig. 5.6).

No signal magnitude differences were observed between CF inoculated and control pancreas using GRE-MRI.
Figure 5.5. 7T GRE-MRI (TE/TR = 20/28 ms) of kidneys from rats inoculated with CF revealed distinct spots of hypointensity throughout the cortex of the kidney (A). The distribution of hypointense spots in kidneys of rats after CF administration is consistent with the spatial distribution of glomeruli throughout the cortex and juxtamedulary regions of the kidney. GRE-MRI of kidneys from control animals (no ferritin administration) are clear (E). GRE-MRI (TE/TR = 15/23 ms) of liver sections from rats administered CF showed striations of hypointensity throughout the parenchyma of the liver (B), likely due to the localization of CF to the ECM and cells surrounding the hepatic sinusoid. The entire parenchyma of the livers from control animals appear bright and without striation (F). GRE-MRI (TE/TR = 20/80 ms) of lungs from animals inoculated with CF show substantial darkening of the tissue (C) while lungs from control animals appear bright throughout the entire organ (G). GRE-MR images (TE/TR = 3.4/30 ms) of spleens of CF inoculated animals appear very dark (D) compared to controls (H). It appears that MRI-detectable CF accumulation is isolated to the red pulp of CF labeled spleens.
Figure 5.6. Normalized GRE-MRI signal magnitudes in organs of CF-injected rats and control rats with no injection. All CF-labeled organs are significantly darker than control tissue ($\alpha = 0.05$). The error bars represent a single standard deviation based on 6 randomly chosen regions of interest inside of the tissue divided by 6 randomly chosen regions of interest in the surrounding PBS.
DISCUSSION

In this work we investigated the tissue distribution and toxicity of intravenously injected cationized ferritin. The ferritin nanoparticle is useful as an in vivo label of the glomerular basement membrane (Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008) and can be used to count and measure glomeruli with MRI (Beeman et al., 2011; Heilmann et al., 2012). CF may be useful to study ECM structure and function throughout the body with MRI, and the data reported here is an important step towards using CF in future in vivo studies.

Measurements of serum levels of enzymes associated with kidney function indicated that intravenous injection of CF at MRI-detectable doses (5.75 mg/100 g) does not significantly affect renal function. Similarly, CF did not significantly affect liver function as measured by serum markers. Together, these results suggest that CF is not toxic to renal and hepatic function at doses that are detectable using MRI. Furthermore, the amount of CF required for in vivo MRI detection of the ECM may be decreased by creating cationized magnetoferritin, in which apoferritin is filled with as many as ~5,000 iron atoms, as compared to ~1,000 iron atoms inside of normal ferritin and has an increased particle and metal relaxivity (Bulte et al., 1995; 1994; Clavijo Jordan et al., 2010; Meldrum et al., 1992).

Confocal imaging suggests that CF specifically labels the glomerular basement membrane. Control kidneys did not show such labeling of the glomerulus. This result is supported by ex vivo MRI, which shows punctuate spots of hypointensity throughout the cortex and juxtamedulary regions of the kidney that are consistent with the spatial distribution of glomeruli. All previous work in detecting glomeruli with MRI was performed at 9.4T or greater. This is the first report of detection of glomeruli at 7T, suggesting that the techniques
described in previous studies are robust and practical using typical small animal MRI systems.

It is likely that the binding of CF to the glomerular basement membrane is due to an electrostatic interaction between the cationized ferritin and the anionic proteoglycans on the glomerular basement membrane. This interaction is facilitated by the large pressure gradient across the glomerular capillary wall, which shuttles the CF through the endothelial fenestration into direct contact with the glomerular basement membrane. Previous work has also suggested that some CF is internalized into podocytes and that some passes completely through the glomerular filtration barrier into Bowman’s capsule (Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008). There is still a need to characterize how CF accumulates dynamically in the kidney and how accumulation reports on renal function.

Confocal and MR imaging suggest that most of the CF accumulation in the liver occurs in the ECM and cells immediately surrounding the hepatic sinusoids. Just as in the kidney, it is likely that the colocalization of CF with the ECM surrounding the hepatic sinusoid is due to an electrostatic interaction between CF and the anionic proteoglycans of the ECM. Furthermore, confocal microscopy shows that CF is also taken up into Kupffer’s cells (macrophages inside the hepatic sinusoid), endothelial cells surrounding the hepatic sinusoid, and hepatocytes.

Colocalization of CF with the endothelial cells lining the capillaries interfacing the alveoli was observed using confocal imaging. This accumulation was also detectable with MRI. This observation is consistent with reports in the electron microscopy literature of CF binding to the anionic endothelial cell surface as well as uptake of CF into endothelial vesicles (Brody et al., 1984).
Furthermore, CF uptake might be used to study lung disease with MRI, because substantial binding of CF to the anionic alveolar basement membrane has been observed with electron microscopy after severe damage to the endothelial lining (Barrowcliffe & Jones, 1987; Brody et al., 1984). In healthy lungs, CF should not pass the endothelial barrier. CF labeling to study lung function with MRI is a potential area for future investigation.

Splenic uptake of CF was observed in both MRI and immunofluorescence, with heavy labeling of the red pulp in CF inoculated spleens. There was also substantial background fluorescence in spleen histological sections prepared for confocal imaging, likely due to large number of monocytes and endogenous ferritin in the spleen. CF appears to be taken up into the macrophages at the interface between the white and red pulp.

Although TEM has shown that CF decorates the endothelial plasmalemma and fenestra of the mouse pancreas after intravenous injection (M. Simionescu, Simionescu, Silbert, & Palade, 1981), we were unable to see a difference in fluorescence between pancreases of CF-injected and control rats. It is likely that not enough CF accumulated in the pancreases observed in this study to produce a fluorescent signal above the signal from non-specific antibody binding. Furthermore, no signal differences in GRE-MRI were observed in pancreases in this study. We do not conclude from these results that CF does not bind in the pancreas, but instead that our experiment lead to no observable signal changes that may be attributed to the binding of CF in the pancreas.

Previously studies have shown that chronic exposure to CF yields elevated levels of anti-cationized horse spleen apoferritin in the plasma (Iskandar, Zhang, & Rodriguez, 1986), though the exposure is approximately 25 times the total dose used in this work over the period of a week. Antibody
reactivity might be minimized by producing recombinant ferritin to match the recipient species (Santambrogio et al., 1993; Uchida et al., 2008). Furthermore, rabbits have been shown to develop proteinurea after intravenous injection of CF (Batsford, Sasaki, Takamiya, & Vogt, 1983), though we have yet to observe proteinurea in rats used in our experiments. These are certainly avenues for further exploration as the use of CF as an MRI-visible contrast agent advances, though we are encouraged by our results that suggest minimal toxic effects of CF.

The goal of this work was to determine the toxicity and distribution of CF after intravenous administration. By measuring common toxicity biomarkers and total leukocyte counts, we found that CF is neither nephrotoxic nor hepatotoxic at detectable doses nor does it elicit a significant immune response. Furthermore, we have established that CF accumulates in a number of organs in the body, including liver, kidney, lungs, and spleen, likely by binding to the extracellular matrix and endothelial glycocalyx. The organs studied in this work were selected because they contain fenestrated capillaries and were therefore likely to be labeled by CF. We expect that whole-body, 3D MRI may reveal other potential targets of CF. However, as evidenced by the range of TE values required to image the organs presented in this work, whole-body experiments would have to carefully take into account the background $T_2^*$ of each organ.

CF, when used as a MRI-detectable contrast agent, has potential advantages because it is small enough (~13 nm) to fit through capillary endothelial fenestrations and has a highly affinity to exposed anionic basement membrane such as the glomerular basement membrane. The optimization of CF-delivery and MRI-acquisition in will further improve the specific detection of anionic structures both in vivo and ex vivo.
**Conclusions.** We have shown that CF is neither nephrotoxic nor hepatotoxic in healthy rats at MRI-detectable doses. Furthermore, total leukocyte counts suggest that there is not a significant immune response to intravenously injected CF. We detected CF accumulation in the kidney, liver, lungs and spleen using immunohistochemistry and MRI. These results suggest opportunities to study of physiological structure and function of the ECM in vivo through the use of CF as an MRI contrast agent.
INTRODUCTION

Chronic liver disease accounts for ~30,000 deaths per year in the United States (National Center for Health Statistics, 2011). Non-alcoholic-steatohepatitis (NASH), an advanced and chronic form of non-alcoholic fatty liver disease (NAFLD), is associated with type 2 diabetes and obesity and affects ~6 million people in the United States (Erickson, 2008). With increases in the incidence of type 2 diabetes and obesity, the number of people who will develop NASH is likely to rise. Left untreated, NASH may progress to cirrhosis. Several complications develop in the case of cirrhosis including hepatic encephalopathy, renal failure, and hepatocellular carcinoma (Bataller, 2005). Microstructural changes due to the deposition of excess extracellular matrix (ECM) and the loss of endothelial fenestrations (Farrell et al., 2008; Friedman, 2000; Iredale, 2007; Lough et al., 1987; Martinez-Hernandez & Amenta, 1995) reduce access of plasma molecules to the hepatic parenchyma and therefore reduce hepatic function (Benyon & Iredale, 2000; Friedman, 2000). A robust method to detect these microstructural and functional changes to the liver would be an important diagnostic tool.

Here we demonstrate a magnetic resonance imaging (MRI) technique to detect changes in macromolecular access to the perisinusoidal space of the liver due to NASH. The perisinusoidal space (Disse’s space) is the major site of molecular exchange between the blood plasma and hepatocytes in the liver. It is situated beneath a layer of fenestrated endothelia that serve as a sieve, allowing
passage of proteins and other plasma molecules into the perisinusoidal space while retaining cells in the hepatic sinusoid capillary space (Wisse, 1970). Beneath the fenestrated endothelial layer, inside the perisinusoidal space, is a layer of ECM that is critical to normal hepatic structure and function: impacting cell migration, proliferation, differentiation and gene expression (Bedossa & Paradis, 2003; Friedman, 2000; Iredale, 2007). Chronic liver disease often leads to a thickening of the ECM and a loss of endothelial fenestrations (Farrell et al., 2008; Friedman, 2000; Iredale, 2007; Lough et al., 1987; Martinez-Hernandez & Amenta, 1995) that ultimately reduce macromolecular access to the perisinusoidal space. This in turn leads to reduced plasma exchange with hepatocytes and altered hepatic function (Friedman, 2000; Iredale, 2007).

Rodent models are important for investigating the molecular and cellular mechanisms of NAFLD. There are a large number of genetic and dietary models of NAFLD, including the \( db/db \) and \( ob/ob \) genetic mouse models and high carbohydrate and high fat diets, though these specific genetic and dietary models often only result in steatosis (Farrell et al., 2008). The methionine-choline deficient (MCD) dietary model results in rodents developing hepatic changes that closely resemble those of NASH in humans. These changes include steatosis, focal inflammation, fibrosis, and necrosis of hepatocytes (George et al., 2003; Rinella et al., 2008; Teramoto, Bowers, Khettry, Palombo, & Clouse, 1993). Furthermore, it has been suggested that this model may also exhibit loss of endothelial fenestrations (McCuskey et al., 2004).

Typically, NASH is differentiated from NAFLD by biopsy. However, due to the diffuse nature of liver fibrosis, diagnoses based on liver biopsy can be subject to sampling errors (Bedossa et al., 2003; Siddique et al., 2003). Biopsy may also cause pain and major complications (Thampanitchawong & Piratvisuth, 1999).
Recently, imaging techniques have been developed to assess the extent of liver fibrosis throughout the entire organ using ultrasound elastography (Ophir, Céspedes, Ponnekanti, Yazdi, & Li, 1991). This method detects viscoelastic changes that occur during the development of fibrosis (Sandrin et al., 2003; Yeh et al., 2002). MRI also shows promise in detecting liver disease because it is non-invasive, three dimensional, high resolution, and provides excellent soft-tissue contrast. Magnetic resonance elastography (MRE) techniques have been developed based on applying mechanical waves to measure tissue stiffness (Huwart et al., 2006; Manduca et al., 2001; Muthupillai et al., 1995). Current MRE techniques are noninvasive and practical to detect gross changes in liver structure. Nonetheless, there remains a strong need to distinguish focal changes to microstructure and function that develop due to chronic liver disease.

A potential strategy to detect structural and functional changes in the liver due to steatohepatitis is to develop a MRI contrast agent that specifically targets the perisinusoidal ECM of the liver. Targeted MRI contrast agents have been developed to detect molecules and cells throughout the body (Cormode et al., 2010; Geninatti Crich et al., 2005; S. Zhang et al., 2003). Iron oxide nanoparticles of approximately 10-50 nm are one type of contrast agent that creates a detectable change in the MRI signal. Ferritin, a 13 nm protein-based iron oxide nanoparticle, has been proposed as a useful natural nanoparticle contrast agent because of its uniform size, biocompatibility, and ease of functionalization in both natural and modified states (Bennett, Shapiro, Sotak, & Koretsky, 2008; Bulte et al., 1995; Uchida et al., 2008). Because the ferritin nanoparticle has an iron oxide core, its accumulation creates a darkening in the MR image that can be detected using $T_2^*$-weighted MRI. It was previously shown that the ECM in the kidney glomerulus may be imaged using MRI after the intravenous injection of a
cationized ferritin (CF) (Beeman et al., 2011; Bennett, Zhou, Sumner, Dodd, Bouraoud, et al., 2008; Heilmann et al., 2012). This technique is based on the passage of CF through the endothelial fenestrations of the glomerular capillary wall and subsequent electrostatic binding of CF to the anionic proteoglycans of the ECM. Intravenous CF is also non-toxic at MRI-detectable doses (Beeman et al., 2012).

Here we investigate the use of CF to detect reduced macromolecular access to the perisinusoidal space in a rat model of NASH.

METHODS

Synthesis of Cationic Ferritin. Cationized ferritin (CF) was synthesized from native horse spleen ferritin per Danon et.al (Danon et al., 1972). 1-ethyl-3(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) was used to conjugate commercial horse spleen ferritin (Sigma-Aldrich, Saint Louis MO) to N,N-dimethyl-1,3-propanediamine (DMPA). This was accomplished by first adjusting a 2 M solution of DMPA to a pH of 6 using 2 N and 0.2 N HCl and NaOH. Once a pH of 6 was achieved, 0.5 ml of 55 mg/ml horse spleen ferritin was added to the solution, followed by 200 mg of EDC. The pH of the solution was monitored for 2 hours after addition of EDC to ensure the pH remained at 6. The solution was left covered at room temperature for 12 hours. The CF solution was dialyzed 2 times against 3.5 L of phosphate buffered saline (PBS) at a pH of 7.3 and 4 °C for 12-24 hours for each pass. The CF solution was stored at 4 °C. Native horse spleen ferritin has an iron oxide core and a per particle T<sub>2</sub> relaxivity of ~2,455 mM<sup>-1</sup> s<sup>-1</sup> (35). Cationized ferritin shares the same iron oxide core and relaxivity as native ferritin (data not shown).

Animal Preparation. A total of 30 male Sprague-Dawley rats were used in this study. All animal protocols were approved by the Arizona State University
Institutional Animal Care and Use Committee and performed in accordance with the NIH guide for the care and use of laboratory animals. The 30 animals were split into two groups – the first (12) to study CF-labeling of the perisinusoidal ECM in healthy animals, and the second (18) to study the use of CF and MRI in a rat model of non-alcoholic steatohepatitis (NASH). All injections were performed while the animals were anesthetized with a 5% isoflurane mixture in oxygen. A total CF dose of 5.75 mg / 100 g was given in three bolus doses (~0.5 ml per dose) spaced 1.5 hours apart.

The first group, consisting of 12 healthy rats weighing 293 ± 13 g, was used to demonstrate the labeling of the perisinusoidal ECM by CF in healthy rats administered intravenous bolus doses of CF. A group of three rats administered CF was compared to a group of three rats administered an equivalent dose of native ferritin (NF) and to a group of three naive rats receiving no contrast agent. The rats were imaged with MRI in vivo and then sacrificed via transcardial perfusion of PBS and formalin 1.5 hours after the last injection. Livers were resected and prepared for ex vivo MRI, histology, and TEM. The final three rats were prepared in the same way and perfused 2 days, 4 days, and 7 days after injection to observe the distribution of CF in the liver over time. Transmission electron microscopy (TEM), MRI, and histology are detailed below.

The second group, consisting of 18 rats, was used to study the use of CF in a rat model of non-alcoholic steatohepatitis (NASH). Nine rats were fed a methionine choline deficient (MCD) diet (George et al., 2003; McCuskey et al., 2004; Rinella et al., 2008; Teramoto et al., 1993) and nine were fed a control diet of the same composition supplemented with methionine and choline. All rats were weighed weekly, as animals on an MCD diet tend to lose weight (Fig. 6.4). Serum alanine transaminase (ALT) was measured by photometric absorbance
(Charles River Laboratories, Wilmington, MA) in six of the rats fed the MCD diet and six of the rats fed the control diet to determine disease maturity. All experiments took place after serum ALT in MCD-fed animals were approximately three times the serum ALT of animals fed the control diet (12). This occurred 14 weeks after starting the special diet. Six MCD fed rats and six control diet fed rats were imaged in vivo with MRI before and after injection of CF and sacrificed by transcardial perfusion of PBS and 10% neutral buffer formalin immediately after the post-injection MRI. Livers were resected and prepared for ex vivo MRI, histology, and TEM. The remaining three MCD fed rats and two control diet fed rats remained naive and were sacrificed by transcardial perfusion without being injected with CF. Livers from these animals were resected and prepared for ex vivo MRI, histology and TEM. One sample from the un-injected control group was removed due to failure to properly perfuse the animal. MRI, histology, and TEM are detailed below.

In vivo MRI. For in vivo MRI, rats were prepared as described above. 1.5 hours after the final injection of CF, rats were anesthetized with a 5% isoflurane mixture in oxygen by nose cone and imaged at 7T on a Bruker 7T/35 scanner (Bruker, Billerica, MA) using a 72-mm quadrature transmit/receive radio frequency coil (Bruker). A series of axial $T_2^*$-weighted 2D-gradient echo fast low angle shot (FLASH) sequences were acquired using a flip angle of 30°. Five different TE values were used (4, 5, 6, 7, and 8 ms). A TR of 60 ms was used for each scan. In vivo images were collected with a resolution of 234x234x1000 µm (field of view = 6x6 cm, matrix size = 256x256, scan time = 4 min. 36 sec.). $T_2^*$ maps were calculated using the series of FLASH images (data analysis described below). Twenty-four averages were collected for each scan to improve
SNR and reduce motion artifacts (the total scan time was 4 minutes 36 seconds per scan).

**Ex vivo MRI.** Perfused, excised livers were imaged on a Bruker 7T/35 scanner and a 72-mm rat volume transmit coil and a rat brain surface coil (Bruker). A high resolution 70x70x1000 µm (field of view = 2.1x2.1 cm, matrix size = 300x300) T$_2^*$-weighted 2D-gradient echo fast low angle shot (FLASH) sequence was collected on each liver with a TE/TR = 15/23 ms and a flip angle of 30° (100 averages, scan time = 8 min. 36 sec.). The TE/TR used to image the perfused, fixed livers ex vivo is different than that used to image livers in vivo because the presence of blood in vivo shortens T$_2^*$. To measure T$_2^*$, a 2D multi-gradient echo sequence was used to collect 26 images with 26 evenly spaced echoes times between 2.48 ms and 42.54 ms. A TR of 1000 ms was used with the multi-gradient echo sequence along with a flip angle of 30° and a resolution of 280x280x1000 µm (field of view = 2.1x2.1 cm, matrix size = 75x75, no averaging, scan time = 57 sec.). T$_2^*$ maps were calculated from 26 gradient echo images (data analysis described below).

**Immunofluorescence.** Immunofluorescence (IF) confocal microscopy was performed on excised livers to determine the distribution of injected CF. Tissue fixed for IF was bathed in 15% sucrose overnight followed by 3 days in 30% sucrose. Sucrose-infiltrated tissue was rapidly frozen to -80 °C in optimal cutting temperature (OCT) compound. Embedded tissue was sectioned at 35 µm at -20 °C in a cryostat (Leica Microsystems CM3050 S, Buffalo Grove, IL). Sections were washed three times in PBS, permeabilized with 0.3% triton, and blocked in Image-iT FX signal enhancer (Invitrogen, Carlsbad, CA) and goat serum for one hour each. Sections were then incubated overnight at 4 °C in chicken anti-fibronectin (1:400) and rabbit anti-horse spleen ferritin (1:500).
primary antibodies (Invitrogen). Sections were then washed and incubated at room temperature for two hours in Alexa488 goat anti-chicken and Alexa594 goat anti-rabbit secondary antibodies (Invitrogen). Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) for 15 minutes to stain cell nuclei and then washed three times in PBS. Sections were mounted on slides with Prolong Gold anti-fade reagent (Invitrogen). The slides were imaged with a Zeiss 710 laser scanning confocal microscope.

**Transmission Electron Microscopy.** Pieces of liver tissue ~1 mm$^3$ were collected from CF-injected and naive rats fed the MCD and control diets. The specimens were placed in a 2% glutaraldehyde/0.1 M cacodylate solution. Tissue was dehydrated with a series of ethanol mixtures ranging from 70% to 100% and infiltrated with and embedded in epoxy resin. Sections were cut at a thickness of 70 nm and lightly stained with 0.2% osmium tetroxide in 0.1 M cacodylate buffer for 2 hours. Osmium tetroxide precipitates were removed via a 12-minute digestion with 1% periodic acid. Images were acquired on a Phillips CM12 transmission electron microscope.

**Inductively Coupled Plasma Optical Emission Spectroscopy.** ~1 g of liver tissue from each of the 18 rats in the NASH model study was digested in 9 ml nitric acid + 1 ml hydrochloric acid. After 15 min of pre-digestion at room temperature in the acid solution, the samples were fully digested by microwave heating to 200 °C at 400 W (CEM corporation, Matthews, NC). Once completely digested, iron content of each liver was measured using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Thermo Scientific, Waltham, MA).

**Data Analysis.** In vivo $T_2^*$ values were calculated from the five $T_2^*$-weighted FLASH gradient echo images (see ‘In vivo MRI’ above) using MATLAB.
Ex vivo $T_2^*$ values were calculated from the 26 gradient echo images of the multi-gradient echo sequence. Because ex vivo $T_2^*$ values obtained using the multi-gradient echo sequence are the same as those obtained from a series of ex vivo FLASH images (data not shown), a multi-gradient echo sequence was used ex vivo to collect more echoes and reduce scan total scan time across the entire ex vivo sample set. $T_2^*$ values were calculated from the image series by fitting the each voxel magnitude value at each time point to the equation $S(t)=S_0e^{-t/T_2^*}$, where $S(t)$ is the voxel signal magnitude value at the given TE, $S_0$ is the extrapolated signal magnitude value at TE = 0 ms, and $t = TE$.

Average signal magnitude and $T_2^*$ values we obtained by manually segmenting the livers from the MRI images or $T_2^*$ maps and averaging the remaining voxels. Average liver signal magnitude was compared across animals my normalizing to the signal magnitude of the muscle surrounding the spine in the same image (a circular region of interest was manually drawn to obtain the signal magnitude value of the muscle). $T_2^*$ histograms we produced from the segmented $T_2^*$ maps and intra-sample voxels were binned by their $T_2^*$ value.

Statistical analyses were run in MATLAB as two-sample, two-tailed Student’s t-tests to test the null hypothesis that the mean difference between groups is zero ($\alpha = 0.05$). All values in the text are represented as the inter-sample mean ± the standard mean error (SEM).

RESULTS

Intravenous CF labels the healthy perisinusoidal ECM and is detected with MRI. We first investigated whether intravenous cationized ferritin (CF) can be used to detect the perisinusoidal space in the liver using MRI. We injected CF into healthy Sprague-Dawley rats. For comparison, we also injected...
healthy rats with native ferritin (NF) and used a group of naive, un-injected rats as negative controls (n = 3 per group). We imaged rats in vivo 1.5 hours after injection on a Bruker 7T MRI scanner using a gradient-echo pulse sequence. In the resulting MR images (Fig. 6.1 A-C), we observed dark striations throughout the livers of CF-injected rats, consistent with the distribution of hepatic sinusoids in the liver. The livers of NF-injected rats appeared dark in the images, and livers from naive controls were bright. The average normalized MRI signal magnitudes in livers from CF- and NF-injected rats and naive control rats were all significantly different (alpha = 0.05, Fig. 6.1 D). These in vivo results suggest that CF uniquely labels the liver and that the labeling is detectable in vivo using MRI.

We used ex vivo MRI of the perfused, excised livers to further explore the differences in labeling between the CF-injected, NF-injected, and naive rats (Fig. 6.1 E-G). Dark striations were visible with ex vivo MRI in CF-inoculated livers, consistent with in vivo MRI. No striations were visible in livers from NF-injected or naive control rats. Line profiles through the images (Fig. 6.1 H) revealed an ~50% difference in signal magnitude between hepatic sinusoids and liver parenchyma in livers from rats injected with CF. We used immunofluorescence and transmission electron microscopy (TEM) to confirm the MRI results and to determine the location of injected CF in the liver. In immunofluorescence (Fig. 6.2 A-C), CF (red) was co-localized with fibronectin (green), suggesting that CF was bound to the ECM. Little ferritin-related fluorescence was seen in the livers of naive rats (Fig. 6.2 G-I). In TEM, CF was also observed in the perisinusoidal space (Fig. 6.8 E). NF was mostly found in Kupffer's cells and endothelial cells of the hepatic sinusoid (Fig. 6.2 D-F). Taken together, our immunofluorescence and TEM results thus confirmed that CF accumulated in the perisinusoidal space of the liver.
Figure 6.1. CF-labeling of the ECM of the hepatic sinusoid is detectable using MRI. In vivo with MRI (A-C) revealed detectable differences in contrast between rats injected with CF (A), NF (B), and naive control animals (C). Image signal magnitude in the liver was normalized to surrounding muscle (D). The differences in average normalized signal magnitude were statistically significant between all groups ($\alpha = 0.05$). Ex vivo MRI (E-G) revealed dark striations in CF-labeled livers (E). Livers of NF-inoculated rats show diffuse signal darkening throughout the entire liver (F) whereas those of naive rats were bright (G). Striations in the images of CF-labeled liver are consistent with the distribution of hepatic sinusoids. Line profiles in images of the ex vivo livers show discernible MRI signal decrease in the hepatic sinusoid of CF-labeled livers as compared to NF and unlabeled control livers (H). n = 6 per group. Error bars equal ± the standard error of the mean.
Figure 6.2. CF labels the perisinusoidal ECM 1.5 hours after intravenous injection, as shown in 63X immunofluorescence imaging. A-C show that CF (red) and fibronectin (green) are co-localized, consistent with ECM labeling with CF. No co-localization of NF with fibronectin is observed (D-F); instead NF appears to be taken up into macrophages and endothelial cells. Minimal ferritin immunofluorescence was observed in the unlabeled control liver (G-I). Scale bar = 20 µm, cell nuclei are stained with DAPI (blue).
To better understand the timing of CF uptake in the liver, we performed ex vivo MRI on livers that were perfused, fixed, and excised 1.5 hours, 2, 4, and 7 days after CF-injection. $T_2^*$-weighted MRI images revealed that the dark striations due to the accumulation of CF are apparent 1.5 hours injection (Fig. 6.3 A) but become diffuse throughout the liver 2 and 4 days after CF-injection (Fig. 6.3 B-C). CF was no longer detectable in the liver after 7 days (Fig. 6.3 D). We also performed immunofluorescence microscopy on livers perfused and fixed 1.5 and 3 hours after CF-injection. Immunofluorescence shows that CF was taken into hepatocytes, Kupffer’s cells, and endothelia by 3 hours after injection (Fig. 6.3 E-F). These data suggest that labeling of the perisinusoidal ECM is transient and most evident with MRI 1.5 hours after injection of CF. Furthermore, CF appears to be cleared from the liver within 7 days.
Figure 6.3. Specific, transient labeling of the perisinusoidal ECM. CF is moved into the liver parenchyma three hours after injection and is cleared from the liver by one week after injection. Immunofluorescence imaging of the liver from a healthy rat sacrificed 1.5 CF injection (A) shows that CF (red) is specifically labeled to the ECM (green). Immunofluorescence of the liver from a healthy rat sacrificed 3 hours after injection shows that CF has been internalized into hepatocytes, Kupffer's cells, and endothelia (B). Cell nuclei are stained with DAPI and fluoresce blue. Scale bar = 20 µm. T2*-weighted MRI 1.5 hours after injection reveals a spatial pattern of CF-accumulation consistent with the distribution of hepatic sinusoids. CF distribution, observed with MRI, appears diffuse throughout the liver parenchyma at 2 days and 4 days after injection (C - E). CF is no longer detectable in the liver 7 days after injection (F).
Perisinusoidal ECM labeling by CF decreases with steatohepatitis.

We performed experiments to establish whether CF-accumulation in the perisinusoidal space is altered by the development of non-alcoholic steatohepatitis (NASH) in a rat model. Rats were fed a methionine choline deficient (MCD) diet, which has been shown to precipitate NASH-like symptoms (George et al., 2003; McCuskey et al., 2004; Rinella et al., 2008; Teramoto et al., 1993). The rats began to lose weight as soon as one week after the start of the MCD diet (Fig. 6.4 A) but showed no other signs of disease. Serum alanine transaminase (ALT) measurements indicated that the dietary NASH model was established by 14 weeks in rats fed the MCD diet (Fig. 6.4 B). At this point, MCD-fed and control rats were imaged in vivo with MRI before and after injection of CF. Before CF injection, the livers of rats fed the MCD diet had decreased MRI signal magnitude compared to controls (Fig. 6.5 A-D), indicating a decrease in $T_2^*$ with the development of NASH. Importantly, the average in vivo signal magnitude (Fig. 6.5 E) of the livers of rats fed the MCD diet did not darken after CF administration ($\alpha = 0.05$).
Figure 6.4. Rats fed a methionine choline deficient (MCD) diet begin to lose weight as soon as one week after the start of the diet (A). ALT values in rats fed the MCD diet were significantly higher ($\alpha = 0.05$) than those from rats fed the control diet, indicating that NASH had been established in rats fed the MCD diet (B). $n = 6$ rats per group. Error bars equal $\pm$ the standard error of the mean.
Figure 6.5. Reduced CF-labeling of the perisinusoidal ECM of the liver is detectable in vivo in the rat model of non-alcoholic steatohepatitis (NASH) using T$_2$*-weighted MRI. In vivo MRI shows darkening in the images of MCD liver tissue before injection of CF compared to naive control (A, C). Signal magnitude did not change in liver tissue of MCD rats after injection of CF (B), while substantial darkening of liver tissue occurred in control rats after CF injection (D). To quantify the group differences, the liver signal was compared to surrounding muscle (E). Liver to muscle signal ratios from MRIs of pre- and post-injection control rats are statistically different, but pre- and post-injection ratios from rats fed the MCD diet are not ($\alpha = 0.05$). $n = 6$ for each group. Error bars equal ± the standard error of the mean.
The in vivo MRI signal magnitude differences were consistent with in vivo liver $T_2^*$ measurements (Fig. 6.6 A-D). Histograms (Fig. 6.6 E-F) showed a broad shift in $T_2^*$ in control rats after the injection of CF. No such shift was seen in MCD-fed rats. The average in vivo $T_2^*$ (Fig. 6.6 G) in rats fed the MCD diet was 5.7 ± 0.7 ms before injection of CF and 5.6 ± 0.5 ms after injection, which was not significantly different ($\alpha = 0.05$). The average in vivo $T_2^*$ in control rats was 12.3 ± 0.8 ms before injection of CF and 7.5 ± 0.8 ms after injection. There was a significant decrease (~40%, $\alpha = 0.05$) in $T_2^*$ of age-matched healthy rats after CF-injection.

The in vivo MRI results were confirmed by our ex vivo liver $T_2^*$ measurements (Fig. 6.6 H-K). Histograms (Fig. 6.6 L-M) of $T_2^*$ in the entire liver confirmed a decrease in $T_2^*$ in control rats after injection of CF. No such decrease was observed in MCD-fed rats. The average ex vivo $T_2^*$ (Fig. 6.6 N) in rats fed the MCD diet was 6.3 ± 0.4 ms before injection of CF and 6.7 ± 0.3 ms after injection, which was not significantly different ($\alpha = 0.05$). The average ex vivo $T_2^*$ in control rats was 14.3 ± 1.3 ms before injection of CF and 7.3 ± 0.9 ms after injection. There was a significant decrease (~50%, $\alpha = 0.05$) in $T_2^*$ of healthy rat livers after CF-injection in ex vivo MRI.
Figure 6.6. Reduced CF-labeling of the perisinusoidal ECM of the liver in the rat model of NASH is detectable in vivo and ex vivo by generating maps of measured T$_{2}^{*}$ (A-D, H-K). Histograms (E-F and L-M) of T$_{2}^{*}$ in the entire liver show a broad shift in T$_{2}^{*}$ in control rats after injection of CF, both in vivo and ex vivo. No such shift was seen in MCD-fed rats. A significant (α = 0.05) reduction in average in vivo and ex vivo T$_{2}^{*}$ (G, N) was observed after CF injection in livers from rats fed the control diet. T$_{2}^{*}$ did not change significantly after CF injection in livers from rats fed the MCD diet. n = 6 per group for in vivo T$_{2}^{*}$ measurements. n = 2 for the ex vivo pre-injection control group. n = 3 for the ex vivo pre-injection MCD-fed group. n = 6 for both ex vivo post-injection groups. Error bars equal ± the standard error of the mean.
We used immunofluorescence, TEM, and inductively coupled plasma optical emission spectroscopy (ICP-OES) to investigate the mechanism of CF-labeling in MCD-fed and control rats. There were distinct differences in the spatial distribution of intravenously delivered CF between the livers of healthy rats and rats fed the MCD diet, as observed with immunofluorescence microscopy. Reduced ferritin immunofluorescence in the livers from rats fed the MCD diet compared to controls suggested reduced CF-labeling with NASH (Fig. 6.7 B). The redistribution of fibronectin immunofluorescence suggests thickening of the ECM and tissue displacement by lipid droplets. With TEM we observed CF in the perisinusoidal ECM in livers of both control and MCD-fed rats (Fig. 6.8 E, F). While it was difficult to quantify CF content using TEM due to the limited field of view, in the sections we examined there was less CF bound in the perisinusoidal space of livers from MCD-fed rats. We also observed lipid droplets displacing the hepatic parenchyma in livers from rats fed the MCD diet (Fig. 6.7 A-B, and Fig. 6.8 B). TEM revealed wide (~150 nm) fenestrations in the healthy endothelium (Fig. 6.8 E). In contrast, endothelia of livers from rats fed the MCD diet appear abutted against one other (Fig. 6.8 F), suggesting reduced exposure of blood to the perisinusoidal space. This reduced exposure of the blood to the ECM may account for the decrease in CF uptake we observed in MRI, immunofluorescence, and TEM. The reduced accumulation of CF in the livers of MCD-fed rats is further supported by liver iron concentration measurements made using ICP-OES (Fig. 6.9). Liver iron concentrations in MCD-fed rats increased 98 µg/g liver after injection of CF versus 217 µg/g liver in healthy rats. The increase in liver iron concentration after CF injection is exclusively due to the iron core of the accumulated CF. These data indicate that MCD-fed rats bind 55% less CF in the liver than their healthy counterparts.
Figure 6.7. Distinct differences in the spatial distribution of intravenously delivered CF are apparent between the livers of healthy rats and rats fed the MCD diet (C, D), as observed with immunofluorescent microscopy. Reduced ferritin immunofluorescence in the livers from rats fed the MCD diet compared to controls indicated reduced CF-labeling with NASH. The ECM (green) pattern in MCD livers appears redistributed compared to control livers due to thickening and displacement by lipid droplets (A, B). Tissue was collected from animals three hours after CF administration. Cell nuclei are stained with DAPI and fluoresce blue. Scale bar = 20 µm.
Figure 6.8. CF is localized to the perisinusoidal space in control and MCD-fed rats (E, F). The perisinusoidal space of rats not inoculated with CF is clear of CF (C, D). Wide (~150 nm) endothelial fenestrations are visible in livers from control rats (E) while endothelia appear abutted in livers from rats fed the MCD diet (F). 2.65 kx transmission electron microscopy images show large lipid droplets inside of hepatocytes in livers from rats fed the MCD diet (B). No such droplets were seen in livers from rats fed the control diet (A). White scale bars = 5 µm, black scale bars = 200 nm.
Figure 6.9. Liver iron concentrations were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES). Liver iron concentrations in MCD-fed rats increased 98 µg/g liver after injection of CF versus 217 µg/g liver in healthy rats. The increase in liver iron concentration after CF injection is exclusively due to the iron core of the accumulated CF. These data indicate that MCD-fed rats bind 55% less CF in the liver than their healthy counterparts. Pre-injection MCD n = 3, pre-injection CTRL n = 3, post-injection MCD n = 6, post-injection CTRL n = 6. Error bars equal ± the standard error of the mean.
Taken together, our data indicate that CF specifically labels the perisinusoidal ECM and that this labeling is detectable in vivo using MRI. The labeling of the perisinusoidal ECM was transient, peaking within three hours of injection. There was reduced CF-labeling of the perisinusoidal ECM in rats fed the MCD diet, as observed with in vivo and ex vivo MRI, immunofluorescence, and TEM. ICP-OES indicated that the CF-labeling of the perisinusoidal space was 55% less in MCD-fed rats compared to their healthy counterparts. TEM suggested that circulatory access of CF to the perisinusoidal space is blocked in disease by abutted endothelia. We conclude that the reduced perisinusoidal labeling by CF is thus due to excessive ECM deposition and loss of endothelial fenestrations associated with the advancement of NASH.

DISCUSSION

This work demonstrates that cationic iron oxide nanoparticles (CF) may be used to target and detect the ECM of the hepatic sinusoid using MRI, and that the binding of cationic nanoparticles may be used to detect structural and functional changes in the hepatic sinusoid in a rat model of non-alcoholic steatohepatitis (NASH).

It is likely that CF passes through the endothelial fenestrations of the normal hepatic sinusoid and bind to the perisinusoidal ECM through an electrostatic interaction between the cationic nanoparticle and anionic proteoglycans that comprise the ECM. The binding of CF to the perisinusoidal ECM appears to be highly specific and transient (Figs. 6.2-6.3). Immunofluorescence microscopy suggests that the cationic nanoparticles used in this study are internalized into the liver parenchyma after three hours.

One potential concern about the use of CF as a contrast agent is whether it is toxic in MRI-detectable doses. To address this, we have recently
found that CF has minimal acute or chronic toxicity at these doses, measured by renal and hepatic enzyme markers and lymphocyte counts (Beeman et al., 2012). In the future, the dose of CF used here may likely be reduced ~100-fold via modification of the metal core of the apoferritin protein to increase ferritin relaxivity (Bulte et al., 1995; Clavijo Jordan et al., 2010; Uchida et al., 2008).

We have shown that the amount of specific binding of CF to the perisinusoidal ECM is reduced in a rat model of NASH. This reduced binding of CF to the perisinusoidal ECM is observable with MRI (Figs. 6.5-6.6). One concern for in vivo imaging is the fact that the echo time (TE) is on the order of the $T_2^*$ in the liver in the animals with NASH. This raises the possibility that CF-accumulation did occur but was not detected because of the reduced dynamic range of the $T_2^*$ measurement. This is not the case, however, because ex vivo imaging with a higher background $T_2^*$ and a greater range of TE values showed the same reduced CF-accumulation. The reduced accumulation of CF in the livers of MCD-fed rats was also confirmed with ICP-OES, which revealed 55% less iron added to the tissue after CF injection in MCD-fed rats compared to their healthy counterparts. Since the increase in liver iron concentration after CF injection is exclusively due to the iron core of the accumulated CF, these data indicate 55% less CF binding in MCD-fed rats.

The reduced binding of CF to the perisinusoidal ECM in rats fed the MCD diet is likely caused by changes to the perisinusoidal ECM and loss of endothelial fenestrations (Farrell et al., 2008; Friedman, 2000; Iredale, 2007; Lough et al., 1987; Martinez-Hernandez & Amenta, 1995). Changes to the structure of and macromolecular access to the perisinusoidal ECM were identified in our immunofluorescence and TEM images, which show distinct differences in perisinusoidal fibronectin distribution and thickness (Fig. 6.7), reduced
accumulation of CF in the perisinusoidal space (Figs. 6.7-6.8), and suggest a loss of endothelial fenestrations (Fig. 6.8). We propose that the deposition of scar tissue in the hepatic sinusoid and the loss of endothelial fenestrations blocks macromolecular access to the perisinusoidal space. Detecting the reduced accumulation of CF in the perisinusoidal space with MRI might therefore serve as a molecular imaging biomarker for changes in macromolecular access to the hepatic parenchyma in chronic liver disease.

**Conclusions.** The goal of this work was to establish a non-invasive, ECM-specific molecular imaging biomarker to detect NASH-related changes in macromolecular access to the hepatic parenchyma. We have shown that intravenous injection of cationized ferritin (CF) allows for MRI detection of the perisinusoidal ECM through accumulation of the particles in the extracellular matrix. Furthermore, there is an MRI-detectable reduction in binding of CF to the perisinusoidal ECM in a rat model of NASH. The reduced binding of CF to the perisinusoidal ECM in the NASH model is likely caused by reduced macromolecular access to the perisinusoidal space due to fibrosis and loss of endothelial fenestrations. To our knowledge, this is the first report of an in vivo targeted imaging agent that is sensitive to NASH-related changes in macromolecular access to the perisinusoidal space. The reduced, transient accumulation of intravenously injected cationic nanoparticles may report on changes in macromolecular access to the liver parenchyma in chronic liver diseases such as non-alcoholic steatohepatitis.
CHAPTER 7
SUMMARY AND FUTURE DIRECTIONS

The work presented in this volume has focused on the development of a non-toxic MRI probe to detect tissue microstructure. Specifically, we set out to develop a cationic probe that specifically targets and renders visible the extracellular matrix (ECM) underlying fenestrated endothelia. It was with this goal in mind that five projects were defined and executed. These five projects will be summarized and potential future applications for this cationic MRI probe will be explored in the coming pages.

SUMMARY

Detecting kidney glomeruli in vivo and ex vivo. The kidney glomerulus is the first line of filtration in the nephron. A single human kidney has as many as 2,000,000 nephrons that are responsible for maintaining blood ion and glucose concentrations as well as removal of metabolic waste and regulation of blood pH. Changes in the number (Nglom) and size (Vglom) of glomeruli have been linked to a host of renal and systemic disease (Brenner et al., 1988; Hoy et al., 2008; Puelles et al., 2012), though techniques to measure Nglom and Vglom require resection and destruction of the entire kidney (Bertram et al., 1992). Furthermore, these methods merely extrapolate measurements from a small sample population of approximately 40 glomeruli. A method that measures every glomerulus in the kidney and is non-destructive would prove vital in preclinical research and potentially in clinical diagnostics.

In the work presented in Chapter 2 we injected rats intravenously with the naturally derived, MRI-visible cationized ferritin (CF) nanoparticle. We then perfused the rats, resected their kidneys, and imaged the kidneys ex vivo with T₂*-weighted MRI. We show that CF specifically labels the glomerulus of the rat
kidney and that this accumulation of CF renders each glomerulus visible when the kidney is imaged with $T_2^*$-weighted MRI. Furthermore, we show that labeled glomeruli may be identified, counted, and their sizes measured from the $T_2^*$-weighted MRI volumes. We compare the measurements of $N_{glom}$ and $V_{glom}$ made with the MRI-based technique with those made using the gold-standard acid maceration and disector/fractionator methods and find that the MRI-based method yields similar results to the histological methods. Furthermore, we show that the MRI-based method may be used to measure the intra-renal glomerular volume distribution - a measurement that was previously unobtainable using histology. In this work we conclude that the presented MRI-based technique yields comparable measurements of glomerular volume and size while providing the unique intra-renal glomerular volume distribution measurement.

Chapter 3 introduced an important extension of the work of Chapter 2. In this work we inoculated fresh human transplant kidney with CF with the goal of measuring $N_{glom}$ and $V_{glom}$ with MRI. This work is presented as a proof of concept that human glomeruli may be targeted with CF and subsequently visualized with MRI. We show that this is also possible to measure the number and size of human glomeruli using this technique. As with the study performed in rat kidneys, the intra-renal glomerular volume distribution may be measured - a measurement never before obtained in human kidneys. This technique may provide an opportunity to assess the viability of transplant kidneys before they have been grafted into a recipient and may be a big step towards measuring $N_{glom}$ and $V_{glom}$ in the clinic.

Finally, in Chapter 4 we introduce a novel ferritin based nanoparticle with a highly paramagnetic core for improved in vivo detection of glomeruli. Unmodified CF is a superparamagnetic nanoparticle that causes spin dephasing,
rendering a darkened signal at the site of CF accumulation. This provides excellent contrast in perfused, ex vivo organs that lack a dark blood background. Unfortunately, this dephasing is quite difficult to distinguish in vivo in highly vascularized organs with a dark blood background. It is for this reason that we have set out to develop a nanoparticle that behaves in the same way as CF when injected, with the exception that it creates a bright signal contrast instead of dark. In this chapter we show that the iron core of ferritin may be doped with tungsten. The tungsten dopant prevents exchange coupling within the metallic core of the protein. This reduces the r₂ of the nanoparticle while retaining its paramagnetic properties and effectively yields a large, paramagnetic nanoparticle with a high longitudinal relaxivity (r₁). We go on to show that the tungsten doped ferritin nanoparticle can be cationized, yielding a novel targeted nanoparticle called paraCF that may be used to detect anionic ECM components that underly fenestrated endothelia. We also show that paraCF may be used to detect kidney glomeruli in vivo based on the accumulation of paraCF in the glomerular basement membrane. The binding of paraCF to the glomerulus is highly detectable against the strong blood background of the kidney with T₁-weighted MRI. This highly detectable MRI contrast agent may be used to detect glomerular structure and function in vivo with a spatial resolution that has previously been impossible.

**Nanoparticle toxicity and biodistribution.** The results presented in Chapters 2-4 suggest that CF may eventually be used for routine assessment of glomerular integrity via intravenous injection and subsequent MRI. The goal of the work presented in Chapter 5 was to establish the nephro-, hepato- and immunotoxicty of intravenously injected CF as well as the clearance rate of accumulated CF. We also investigate other organs with a fenestrated
endothelium for CF-accumulation using MRI and immunofluorescence microscopy. We find that intravenously injected CF does not affect renal function, hepatic function, or change total leukocyte counts at the MRI-detectable doses and report a CF clearance rate of ~ 7 days. Furthermore, we find that, in addition to labeling the glomerulus, CF labels the extracellular matrix of the hepatic sinusoid of the liver, the extracellular glycocalyx of the alveolar endothelia, and the macrophages of the spleen. While there is still substantial work to be done to prove that CF is completely non-toxic, we conclude from this work that CF may at least serve as a useful, minimally toxic contrast agent to detect a number of structures in the body.

**Detecting microstructural changes in chronic liver disease.** In Chapter 5 we showed that CF labels the ECM of the perisinusoidal space in the liver and that this accumulation is detectable with MRI. We expanded upon this observation in Chapter 6, presenting a CF-based MRI technique to detect microstructural changes to the hepatic sinusoid in chronic liver disease. The perisinusoidal space is the site of molecular exchange between the blood plasma and the liver parenchyma (the hepatocytes). The perisinusoidal space lies between a fenestrated endothelium and the hepatocytes inside of the hepatic sinusoid. Chronic liver diseases such as non-alcoholic steatohepatitis (NASH) are known to cause major microstructural changes to the liver, including excessive deposition of ECM in the perisinusoidal space as well as a loss of endothelial fenestrations in the hepatic sinusoid (Farrell et al., 2008; Friedman, 2000; Iredale, 2007; Lough et al., 1987; Martinez-Hernandez & Amenta, 1995). In this work we demonstrate that the ECM-specific CF may be used to detect healthy perisinusoidal ECM in vivo with MRI. We further show that the binding of CF to the perisinusoidal ECM is reduced by 55% in a rat model of NASH, and
that this reduced binding is detectable in vivo with MRI. Immunofluorescence and electron microscopy indicated that the reduced binding is due to limited macromolecular access to the perisinusoidal space caused by NASH-related microstructural changes. The reduced accumulation of intravenously injected CF may report on changes in macromolecular access to the liver parenchyma in chronic liver diseases such as NASH.

**FUTURE DIRECTIONS**

*T₂⁻ susceptibility volume versus the actual volume of glomerulus.* In Chapters 2 and 3 we have demonstrated that glomerular volume (Vglom) may be measured from MRI volumes of kidneys based on the volume of susceptibility artifact caused by the accumulation of CF in the glomerular basement membrane. While we have empirically shown this technique to be accurate, the nature of superparamagnetism should also be considered when making such measurements. The accumulation of a superparamagnetic probe can cause a susceptibility artifact that reaches far beyond the region in which the probe has accumulated. This appears to not be the case when glomeruli are labeled with CF, but we would like to prove that the volume of susceptibility artifact caused by the accumulation of CF in the GBM is indeed an accurate representation of glomerular volume.

We intend to accomplish this by taking a very small biopsy from a CF-labeled kidney (~ 1 mm³) and performing a T₂⁻-weighted gradient echo on the sample. We will then measure Nglom and Vglom from the MRI volumes in the same manner as Chapters 2 and 3. Once, the MRI-based measurements are complete we will prepare the biopsy for disector/fractionator stereology and measure Nglom and Vglom of the biopsy by sampling every glomerulus in the biopsy. Because the biopsy is so small, we will be able to co-localize glomeruli
visualized with MRI with the same glomeruli in our histological section. This will allow us to directly compare our MRI-based measurements of individual glomerular volumes with those made using stereology. Furthermore, this experiment will allow us to directly test the ability of our MRI-based method to identify glomeruli. With our MRI volume co-registered with the histological volume we will be able to compare false-positives and false-negatives from the algorithm.

**Glomerular populations.** One of the strong advantages of the MRI-based technique is its ability to sample every glomerulus in the kidney. This is something that has never been possible due to the huge number of glomeruli in a single kidney. It is our belief that the complete sampling of kidney glomeruli may be exploited to define unique glomerular populations in healthy and diseased kidneys.

We believe that unique populations of glomeruli may be defined by their CF-labeling intensity (the amount of CF that has accumulated in a glomerulus), and location in the kidney. We have run initial analyses on the images of CF-labeled rat kidneys to extract such data. A three dimensional histogram of glomerular location (normal distance from the surface of the kidney) versus relative glomerular labeling intensity (as measured by the ‘darkness’ of the glomerulus with MRI) suggests two distinct populations of glomeruli (Fig. 7.1). These populations are separated mostly by their labeling intensity. It is likely that the amount of CF that binds to the GBM is proportional to the plasma filtration rate across the glomerular capillary wall and/or the amount of GBM that is exposed to the capillary space. Future work will certainly be directed towards relating the labeling magnitude of glomeruli to glomerular health (which will be further discussed below).
Figure 7.1. A three-dimensional histogram of relative glomerular labeling intensity (defined as the ‘darkness’ of the glomerulus as measured by with MRI) versus the normal distance of a glomerulus from the surface of the kidney. The color of each cell represents the number of glomeruli binned at the coordinate of the cell. This histogram suggest two distinct populations of glomeruli separated by their labeling intensity.
Assessing glomerular health by dynamically measuring CF-uptake.

A potential method to assess glomerular health would be to dynamically track the accumulation of CF in the glomerulus using a fast MRI sequence. The manner in which the glomerulus filter macromolecules such as ferritin is still quite contentious (Deen & Lazzara, 2004; Deen, Lazzara, & Myers, 2001; Smithies, 2003). We would like to avoid this decades-long debate and simply take the two facts that we have developed in the foregoing text - that cationized ferritin binds to the GBM and that the accumulation of CF in the GBM renders glomeruli detectable with MRI - to assess the health of nephrons.

The exact metric for assessing glomerular health with MRI will depend on an in vitro experiment that we must conduct. We propose to perfuse a fresh rat kidney with an isolated organ perfusion pump. Using this pump we will regulate the intra-capillary pressure and the intra-capillary CF concentration. This experiment is designed to answer one important question - Is the accumulation of CF in the GBM driven by diffusion or by plasma flow across the capillary wall (GFR). The rate of CF accumulation in the GBM will be assumed to be proportional to the rate at which a voxel containing a glomerulus grow darker. If this experiment reveals that the accumulation of CF is independent of intra-capillary pressure then it stands to reason that the accumulation of CF is strictly diffusion driven. If the accumulation of CF in the GBM increases with intra-capillary pressure then its accumulation has a flow component. Either result is acceptable but each leads to a unique conclusion. If we find CF accumulation to be strictly diffusion driven then we are effectively measuring the exposed glomerular filtration surface area by dynamically monitoring CF uptake. the exposed filtration surface area is a very important metric that has strong implications about the state of a glomerulus. An abnormally large amount of GBM
is exposed to the intra-capillary space suggests that the glomerulus is growing in size to account for filtration deficits. This process may ultimately lead to hyperfiltration that is damaging to the glomerulus and ultimately leads to sclerosis of the whole nephron (Puelles et al., 2012). If we find that there is a flow component, then we have found a method to measure the single nephron glomerular filtration rate (snGFR) of every perfused glomerulus in the kidney. This would have major implications on the way kidney disease is diagnosed and monitored.

The experiment described above would take place in vitro, allowing for a resolution that is not attainable in vivo. This means that in vivo measurements of snGFR, Nglom, and Vglom are not likely in the ways that we have described in vitro. In vivo we are likely to have approximately 10-20 glomeruli in a 1-2 mm³ voxel but, by dynamically monitoring CF accumulation in vivo, we can still make physiologically important measurements. Based on the results of the experiment described above, we will either be able to measure the total glomerular filtration surface area within a voxel - a measurement that may report on whether the local glomeruli are hyperfiltering, or we will be able to measure the GFR of the 10-20 glomeruli in a single voxel - a measurement that provides approximately 10,000 times more information than the bulk GFR measurements currently made in the clinic (assuming one million glomeruli across both kidneys of a human).

**Tubules**

Another future goal of this work is to identify the tubules of the nephron based on the binding of CF that has leaked through the glomerulus. We have noticed in the MRI volumes of healthy rat kidneys that there are dark striations originating at a labeled glomerulus that point towards the center of the kidney.
Immunofluorescence microscopy suggests that these striations may be visualization of CF-labeled proximal tubules (Fig. 7.2).

We have identified these tubules (Fig. 7.3) based on their signal magnitude values and an origin at the glomerulus. We then measured the volume and the relative labeling intensity (defined as the amount of signal darkening caused by CF-accumulation) of each tubule and its attached glomerulus. This process was done ex vivo in a healthy rat kidney and in a kidney from a rat with glomerular sclerosis. It turns out the the ratio of glomerular volume to tubular volume is 0.5816 ± 0.3429 in the healthy rat and 0.4780 ± 0.2702 in the rat with glomerular sclerosis and the ratio of glomerular labeling intensity to tubular labeling intensity is 0.9067 ± 0.0631 in the healthy rat and 0.8033 ± 0.0362 in the rat with glomerular sclerosis. It is likely that the volume ratio is lower in the rat with glomerular sclerosis not because the glomerulus is smaller in the sclerotic kidney but instead because more of the tubule was labeled due to excessive leakage of CF into the proximal tubule. Conversely, most labeling of the proximal tubule is prevented by the glomerulus in the healthy kidney. The same can be said for the differences in labeling intensity. These preliminary data suggests that the ratio of glomerular volume and labeling intensity, as measured with MRI, may be related to the progression of kidney disease.
Figure 7.2. CF-labeling of the proximal tubule. Dark striations originating at labeled glomeruli and pointing towards the center of the kidney are visible in gradient echo MRI volumes of kidneys inoculated with CF (B). No such striations are visible in the control kidneys (A). Immunofluorescence microscopy (C) suggests that these dark striations are caused by CF-labeling (red) of the proximal tubule. An area of CF-related fluorescence in a tubule is identified by the arrow.
Figure 7.3. CF-labeled tubules may be identified based on their signal magnitude value and an origin at a glomerulus. Glomeruli are overlain with red and tubules are overlain with blue.
Translation to humans. Finally, Chapter 3 has strong implications regarding the use of CF in humans. It was shown that CF does indeed specifically bind to the GBM of the human glomerulus and that the accumulation of CF in the glomerulus is MRI-detectable. Furthermore, our initial study of CF toxicity and biodistribution suggests that CF does not adversely affect renal function. This is promising evidence if CF is ultimately going to be applied as a diagnostic tool in the clinic. Of course, this data merely suggests that CF is non-toxic at MRI detectable doses. Substantial preclinical work needs to be done before the agent can ever be injected into a human.

We believe that the first step towards translation of CF to the clinic is to recombinantly produce MRI-detectable human ferritin in bacteria. This has already been shown to be possible by Uchida, et al. (Uchida et al., 2008). An MRI contrast agent for use in humans that is based on human ferritin should minimize biocompatibility issues. The recombinant production of human ferritin and the cationization will have to conform to good manufacturing practice. We have established a relationship in the biomanufacturing industry to set up facilities that conform to good manufacturing practices.

Once the process of synthesizing and cationizing human ferritin has been developed in accordance with good manufacturing practices, we will confirm that the cationized recombinant human ferritin has the same targeting- and signal-darkening effect as CF and begin thorough toxicity screening in a rodent model. During the toxicity testing we will test a minimum of three doses on adult rodents. The acute toxicity studies will take place within 2 hours of exposure to the agent, sub-chronic testing will take place over a period of 90-days after exposure, and chronic toxicity studies will take place over a period of 12-24 months after exposure. Carcinogenicity and reproductive, developmental, dermal, ocular,
neuro-, and genetic toxicity will also be assessed during this time. All preclinical research protocols are subject to change based on consultation with the U.S. Food and Drug Administration’s Pre-investigational new drug consultation program.

Once all of the preclinical toxicity data has been collected, an investigational new drug (IND) application will be filed. If cleared we will interface with physicians, clinical facilities, and industry experts to begin a phase 1 clinical trial.

CONCLUSIONS

The goal of the work presented in this volume was to develop a cationic, MRI-detectable probe for non-invasive detection of kidney glomeruli and the perisinusoidal space of the liver. The preceding chapters have shown that the specific binding of intravenously injected cationized ferritin renders individual glomeruli of the kidney MRI-visible ex vivo. Detection of individual glomeruli with MRI has allowed for comprehensive measurements of glomerular number and size - parameters that have broad implications about systemic health but have previously been unattainable in the clinic due to the destructive nature of established techniques. We also demonstrate in vivo detection of glomeruli and detection of glomeruli in human transplant kidneys. These results have strong implications about the potential use of cationized ferritin in the clinic to diagnose renal diseases. Translation of cationized ferritin to human will be the focus of future work.

We have also shown in this work that cationized ferritin specifically binds to the extracellular matrix of the perisinusoidal space - the site of exchange between the hepatic parenchyma and the blood plasma. Chronic liver disease is detrimental to hepatic function in part because it blocks macromolecular access
to the perisinusoidal space. We have shown that cationized ferritin is a marker for macromolecular access to the hepatic parenchyma and may be a useful tool for monitoring chronic liver disease.

Finally, we have shown an initial study on the toxicity of intravenously injected cationized ferritin which reports minimal impact on renal, hepatic, and immune function. Furthermore, we show that cationized ferritin binds in a number of other organs.

The data presented in this volume suggests that cationized ferritin may find broad application in detecting and monitoring kidney and liver diseases in both preclinical and clinical settings.


APPENDIX A

PERMISSIONS
PERMISSIONS FROM SCIENTIFIC JOURNALS

Chapter 2 is a published article in the American Journal of Physiology - Renal Physiology and Magnetic Resonance in Medicine. No permissions are required for reproduction of this article (see below).

Permission Not Required
Permission is not required for this type of use.

Logged in as: Scott Beeman
Account #: 3000583459

Copyright © 2011, American Physiological Society
Chapter 5 is a published article in Magnetic Resonance in Medicine. The License for this article is presented here.

Chapter 3, 4, and 6 have not been accepted for publication and I therefore retain all copyrights to this material.
PERMISSIONS FROM CO-AUTHORS

All contributing authors to the works presented in this volume are aware of and have consented to the use of these works in this volume.
For each individual, describe the individual’s training and years of experience with all listed species and procedures they will be conducting under this protocol.

Kevin Beeman: Kevin is an Assistant professor in SBHSE. He has experience with both rat and mouse behavioral studies and surgical procedures. He has conducted numerous experiments involving surgical procedures and has developed a strong foundation in experimental design. He has been involved in studies that require the use of surgical techniques such as microinjections and intubation. He is currently overseeing a study on the effects of exercise on brain function in rats.

Bradley Hann: Bradley is a graduate student in the School of Biological and Biomedical Sciences at ASU. He has 4 years of experience in UG and grad research with rats, including surgical procedures and experimental design. He has conducted numerous studies involving surgical procedures, with a focus on understanding the effects of environmental factors on brain function.

Joseph Georges: Joseph is a graduate student in the School of Life Sciences since 2008. He has experience with all the viral synthesis protocols conducted in this research, as he has been involved in the development of several protocols.

Kevin is an Assistant professor in SBHSE. He has experience with both rat and mouse behavioral studies and surgical procedures. He has conducted numerous experiments involving surgical procedures and has developed a strong foundation in experimental design. He has been involved in studies that require the use of surgical techniques such as microinjections and intubation. He is currently overseeing a study on the effects of exercise on brain function in rats.

Bradley Hann: Bradley is a graduate student in the School of Biological and Biomedical Sciences at ASU. He has 4 years of experience in UG and grad research with rats, including surgical procedures and experimental design. He has conducted numerous studies involving surgical procedures, with a focus on understanding the effects of environmental factors on brain function.

Joseph Georges: Joseph is a graduate student in the School of Life Sciences since 2008. He has experience with all the viral synthesis protocols conducted in this research, as he has been involved in the development of several protocols.

Kevin is an Assistant professor in SBHSE. He has experience with both rat and mouse behavioral studies and surgical procedures. He has conducted numerous experiments involving surgical procedures and has developed a strong foundation in experimental design. He has been involved in studies that require the use of surgical techniques such as microinjections and intubation. He is currently overseeing a study on the effects of exercise on brain function in rats.

Bradley Hann: Bradley is a graduate student in the School of Biological and Biomedical Sciences at ASU. He has 4 years of experience in UG and grad research with rats, including surgical procedures and experimental design. He has conducted numerous studies involving surgical procedures, with a focus on understanding the effects of environmental factors on brain function.

Joseph Georges: Joseph is a graduate student in the School of Life Sciences since 2008. He has experience with all the viral synthesis protocols conducted in this research, as he has been involved in the development of several protocols.
The goal of our previous work was to develop an MRI-visible contrast agent that is specifically targeted to liver stellate cells (LSCs), which are involved in hepatic fibrosis. The activity of LSCs results in the deposition of collagen, leading to the formation of fibrosis. We have recently shown that a cationic particle can selectively bind to LSCs and accumulate in the liverPost-staining with the primary antibody and visualization of the bound primary antibody with a fluorescent secondary anti-rabbit antibody revealed the localization of the primary antibody to the cytoplasm of the cells. The secondary antibody bound to the primary antibody and was visualized with a fluorescent dye. The bound antibody was then visualized with a fluorescence microscope. The localization of the antibody to the cytoplasm of the cells indicated that the targeted contrast agent was specifically bound to LSCs and accumulated in the liver. The results of this study suggest that the cationic particle can be used as a targeted contrast agent for MRI visualization of LSCs in the liver.
Part III

Total Number of Rats for Part II: 45
A total of 45 rats will be needed for the in vivo transfection study (5x3 reps) of empty vector vs AQP1 positive Aquaporin overexpression vs...

Total Number of Mice for Part I = 151
Total Number of Rats for Part I = 112
Part I, I.C: Measure whole

Part I, I.B: E.

D.

Species which that procedure was applied; that is, pain in excess of that caused by injections or other minor procedures.

The USDA Regulations define a “painful or distressful procedure” as any procedure that causes more than momentary or slight pain or distress to the animal. A painful or distressful procedure must be performed under appropriate anesthetic or other procedures for relieving pain or distress. Painful or distressful procedures include, but are not limited to:

- Removal of organs or tissues
- Injection of irritating chemicals
- Administration of irritating chemicals
- Intracranial administration of tumor

If the procedure is requested as a public document, the protocol may cause more than momentary or slight pain or distress to the animal. Anesthesia may be administered to relieve these symptoms. The anesthesia must be administered under appropriate conditions. Anesthesia will be used to relieve pain or distress.

No. Proceed to section IV.

Justification for sample size is the same as for the in vivo transfection study. Examples of appropriate databases to search include PubMed and Web of Science. http://www.ncbi.nlm.nih.gov/pubmed

Date that search was conducted (Must be within 60 days of the IACUC review date):
Database used:

Online search of appropriate databases to search included PUBMED and Web of Science. http://www.ncbi.nlm.nih.gov/pubmed

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:

Date that search was conducted:
Database used:
VI. PROTOCOL

A. ANIMALS

1. Will animals need any special husbandry considerations? (e.g., veterinary care, including but not limited to single housing [for species that cannot be housed together because of social structure], housing, imaging, and handling procedures; pharmaceutical care, including but not limited to dosing regimen for anesthetics or analgesics required to perform the procedures; medical monitoring for high risk interventions or any procedures requiring monitoring and drug regimens; diet, including but not limited to any dietary requirements or a restricted or modified diet; temperature, including but not limited to those with temperature dependent proteins or the use of decreased or increased temperature for tissue collection; water, including but not limited to water restriction or exposure to high temperatures in connection with the animal use covered under this protocol; length of stay and associated costs; collection site considerations, including but not limited to sites that are not available in a pharmaceutical grade equivalent; and program costs. Additional information should be entered here.)

2. Will animals undergo surgery? (Yes/No)  

3. Will animals be exposed to trauma, injury, burning, freezing, electric shock or any other form of physical or psychological stress? (Yes/No)  

4. Will animals undergo radiation? (Yes/No)  

5. Will animals be exposed to chemicals or other potential hazards? (Yes/No)

B. CHEMICALS AND OTHER POTENTIAL HAZARDS

1. Will any animals require the use of DEAE (if you answer yes to any of the following questions, this information may be forwarded to another institution) 

2. Does this project involve the use of any other chemicals or hazardous materials? (Yes/No)

C. WILL ALL ANIMALS BE TERMINALLY KILLED?

1. What is the restriction parameter? Provide scientific justification.

2. How will you monitor for negative effects of food restriction (include information on how you will assess for animal growth)?

D. WILL ANIMALS BE WATER RESTRICTED?

1. What is the restriction parameter? Provide scientific justification.

2. How will you monitor for negative effects of water restriction (include information on how you will assess for animal growth)?

E. WILL ANIMALS BE EXPOSED TO HIGH OR LOW TEMPERATURES?

1. What is the restriction parameter? Provide scientific justification.

2. How will you monitor for negative effects of high or low temperatures (include information on how you will assess for animal growth)?

F. WILL ANIMALS BE EXPOSED TO ENVIRONMENTAL STRESSORS (e.g., non-acclimated temperature exposure, prolonged transport conditions)?

1. What is the restriction parameter? Provide scientific justification.

2. How will you monitor for negative effects of environmental stressors (include information on how you will assess for animal growth)?

G. WILL ANIMALS Undergo MARKING PROCEDURES?

1. What is the restriction parameter? Provide scientific justification.

2. How will you monitor for negative effects of marking procedures (include information on how you will assess for animal growth)?

H. WILL ANIMALS Undergo ANESTHETIC OR ANALGESIC PROCEDURES?

1. What is the restriction parameter? Provide scientific justification.

2. How will you monitor for negative effects of anesthetic or analgesic procedures (include information on how you will assess for animal growth)?

I. Will any animals need to be euthanized? (Yes/No)

J. Will any animals need to be humanely euthanized? (Yes/No)
I. PROVIDE THE Radiation Safety Committee approval #:

II. End point criteria

A. What clinical signs will be used as a basis for removal of an animal from the study?

B. Will animals possibly experience clinical signs intentionally or as a possible side effect of the study?

C. Will tissues be used to assure euthanasia?

D. If using a chemical method for euthanasia, what physical means (e.g., thoracotomy) will be used to assure each location is inspected semi-annually?

E. Will animals be immunized for antibody production?

F. Will animals need any special husbandry considerations (e.g., dietary changes, housing constraints, water restrictions, exercise, restraint, forced exercise)?

G. Performed in section II. E.

H. Will tissues be used for antibody production (other than for antibody production)?

III. ESTIMATES

A. Provide the Radiation Safety Committee approval #:

B. How will you monitor for negative effects of food restriction (include information on how you will account for animal growth)?

C. Does this protocol involve the use of DEA classified substances (e.g., lidocaine, phenylbutazone, thioridazine)?

D. Yes: Proceed to section II. F.

E. No: Proceed to section II. G.

F. Will drugs or chemicals be used in animals other than as control for nephron function?

G. Yes: Proceed to section II. H.

H. No: Proceed to section II. I.

IV. CHEMICALS AND OTHER POTENTIAL HAZARDS

A. How will you monitor for negative effects of food restriction (include information on how you will account for animal growth)?

B. Will animals need any special husbandry considerations (e.g., dietary changes, housing constraints, water restrictions, exercise, restraint, forced exercise)?

C. Does this protocol involve the use of DEA classified substances (e.g., lidocaine, phenylbutazone, thioridazine)?

D. Yes: Proceed to section II. F.

E. No: Proceed to section II. G.

F. Will animals need any special husbandry considerations (e.g., dietary changes, housing constraints, water restrictions, exercise, restraint, forced exercise)?

G. Yes: Proceed to section II. H.

H. No: Proceed to section II. I.

V. END POINT CRITERIA

A. What clinical signs will be used as a basis for removal of an animal from the study?

B. Will animals possibly experience clinical signs intentionally or as a possible side effect of the study?

C. Will tissues be used to assure euthanasia?

D. If using a chemical method for euthanasia, what physical means (e.g., thoracotomy) will be used to assure each location is inspected semi-annually?

E. Will animals be immunized for antibody production?

F. Will animals need any special husbandry considerations (e.g., dietary changes, housing constraints, water restrictions, exercise, restraint, forced exercise)?

G. Performed in section II. E.

H. Will tissues be used for antibody production (other than for antibody production)?

I. Will animals need any special husbandry considerations (e.g., dietary changes, housing constraints, water restrictions, exercise, restraint, forced exercise)?

J. Yes: Proceed to section II. H.

K. No: Proceed to section II. I.

VI. ESTIMATES

A. Provide the Radiation Safety Committee approval #:

B. How will you monitor for negative effects of food restriction (include information on how you will account for animal growth)?

C. Does this protocol involve the use of DEA classified substances (e.g., lidocaine, phenylbutazone, thioridazine)?

D. Yes: Proceed to section II. F.

E. No: Proceed to section II. G.

F. Will drugs or chemicals be used in animals other than as control for nephron function?

G. Yes: Proceed to section II. H.

H. No: Proceed to section II. I.

VII. CHEMICALS AND OTHER POTENTIAL HAZARDS

A. How will you monitor for negative effects of food restriction (include information on how you will account for animal growth)?

B. Will animals need any special husbandry considerations (e.g., dietary changes, housing constraints, water restrictions, exercise, restraint, forced exercise)?

C. Does this protocol involve the use of DEA classified substances (e.g., lidocaine, phenylbutazone, thioridazine)?

D. Yes: Proceed to section II. F.

E. No: Proceed to section II. G.

F. Will drugs or chemicals be used in animals other than as control for nephron function?

G. Yes: Proceed to section II. H.

H. No: Proceed to section II. I.

VIII. END POINT CRITERIA

A. What clinical signs will be used as a basis for removal of an animal from the study?

B. Will animals possibly experience clinical signs intentionally or as a possible side effect of the study?

C. Will tissues be used to assure euthanasia?

D. If using a chemical method for euthanasia, what physical means (e.g., thoracotomy) will be used to assure each location is inspected semi-annually?

E. Will animals be immunized for antibody production?

F. Will animals need any special husbandry considerations (e.g., dietary changes, housing constraints, water restrictions, exercise, restraint, forced exercise)?

G. Performed in section II. E.

H. Will tissues be used for antibody production (other than for antibody production)?

I. Will animals need any special husbandry considerations (e.g., dietary changes, housing constraints, water restrictions, exercise, restraint, forced exercise)?

J. Yes: Proceed to section II. H.

K. No: Proceed to section II. I.

IX. ESTIMATES

A. Provide the Radiation Safety Committee approval #:

B. How will you monitor for negative effects of food restriction (include information on how you will account for animal growth)?

C. Does this protocol involve the use of DEA classified substances (e.g., lidocaine, phenylbutazone, thioridazine)?

D. Yes: Proceed to section II. F.

E. No: Proceed to section II. G.

F. Will drugs or chemicals be used in animals other than as control for nephron function?

G. Yes: Proceed to section II. H.

H. No: Proceed to section II. I.

X. CHEMICALS AND OTHER POTENTIAL HAZARDS

A. How will you monitor for negative effects of food restriction (include information on how you will account for animal growth)?

B. Will animals need any special husbandry considerations (e.g., dietary changes, housing constraints, water restrictions, exercise, restraint, forced exercise)?

C. Does this protocol involve the use of DEA classified substances (e.g., lidocaine, phenylbutazone, thioridazine)?

D. Yes: Proceed to section II. F.

E. No: Proceed to section II. G.

F. Will drugs or chemicals be used in animals other than as control for nephron function?

G. Yes: Proceed to section II. H.

H. No: Proceed to section II. I.
V. END POINT CRITERIA

1. What criteria will be used to determine the termination of the study?

2. Will other tissues or body fluids be collected after death?

3. Will blood be collected from the saphenous vein?

4. What tissues will be fixed?

5. What tissues will be flash-frozen?

6. Will any post-mortem procedures be conducted?

7. How will animals be physically restrained for tissue collection?

8. Will any post-mortem procedures be conducted after death?

IV. EXTREMITIES

1. Name(s) and qualifications of person(s) performing surgery

2. Name(s) and qualifications of person(s) performing euthanasia

3. Name(s) and qualifications of person(s) performing tissue collection

V. APPENDIX 1: ANTIMICROBIAL SPECIMEN COLLECTION

1. Name:

2. Will antimicrobial specimen collection be done?

3. How will antimicrobial specimens be collected?

4. Antimicrobial specimen collection:

a. What antimicrobial specimen collection will be done?

b. How will the specimens be handled?

APPENDIX 2: SURGICAL PROCEDURES

I. GENERAL INFORMATION

A. Species:

B. Surgical Procedures:

C. Qualifications:

II. METHODS

A. Name:

B. Site:

C. Method:

D. Purpose:

E. Complications:

F. Alternative Methods:

G. Requirements:

H. Description:

I. Precautions:

J. References:

K. Appendix Conclusion:

L. Appendix References:

M. Appendix Notes:

N. Appendix Figures:

O. Appendix Tables:

P. Appendix Appendix:

Q. Appendix Appendix:

R. Appendix Appendix:

S. Appendix Appendix:

T. Appendix Appendix:

U. Appendix Appendix:

V. Appendix Appendix:

W. Appendix Appendix:

X. Appendix Appendix:

Y. Appendix Appendix:

Z. Appendix Appendix:
Evidence of infection at incision

Inability to right itself, unresponsive, or peppered

Stimulated.

Burrowing or not moving unless... as active as normal (e.g., Somnolent).

Animal no longer active (somnolent).

Culture the wound, clean the site.

Weigh the animal and compare surgical weight (matricide 28).

A glass bead sterilizer will be used for any subsequent sterilizing that may be needed. If animal is still in pain after that time, they will be removed from the study and euthanized.

What is the maximum duration of surgery?

2 hours.

Who will administer these drugs?

Buprenorphine twice daily for 3 days.

Anesthetic regimen:

1. Is postoperative pain or distress anticipated?
   - Yes. Proceed to section C.
   - No. Proceed to section D.

Will any animals recover from surgery?

No. This involves terminal, or non-survival, procedures.

Who will provide special care and what are their qualifications?

Yes. Provide medical and technical support and what are their qualifications on recovery from surgery. The surgeon will plan the day of surgery as a 24-hour day. Animals will be observed for the first postoperative day. If, for example, anesthesia is done at least two hours prior to the end of the experiment, the surgeon will provide all care and monitoring and care, including pain relief.

D. Will any animals receive from surgery?
   - Yes. The animal should be monitored for at least 24 hours (up to 48 hrs if needed).
   - No. Proceed to section D.

Who will provide special care and what are their qualifications?

For how long will special care be needed?

Who will provide special care and what are their qualifications?

Yes. Complete Section III.

Appendix 2 is complete.

Yes. Complete Section III.

Yes.

No. Appendix 2 is complete.

Yes. Provide medical and technical support and what are their qualifications on recovery from surgery. The surgeon will plan the day of surgery as a 24-hour day. Animals will be observed for the first postoperative day. If, for example, anesthesia is done at least two hours prior to the end of the experiment, the surgeon will provide all care and monitoring and care, including pain relief.

Table 1. Interventions and Expected Table 1. Interventions and Expected