ABSTRACT

NYANKIMA, ANGE GLORIA. Designing Phase-change Contrast Agents for Safe Diagnostic Ultrasound Imaging and Enhanced Therapeutic Outcomes (Under the direction of Dr. Paul Dayton).

Phase-change contrast agents (PCCAs) are positioned to improve the reach of diagnostic and therapeutic ultrasound. These contrast agents, also known as nanodroplets, are formulated with liquid perfluorocarbon cores, which result in smaller mean diameter and increased in vivo stability, as compared to their microbubble (MB) counterpart. When needed for imaging or therapy, PCCAs are vaporized, or activated, into a gas, whereas MBs are formulated as such. With a gas core, both agents will produce unique backscattered signal as a result of oscillating in an acoustic field. In contrast to MBs, PCCAs have the potential to expand the reach of ultrasound contrast agents (UCAs) beyond vascular barriers. This has vast advantages in diagnostic and therapeutic applications of ultrasound technology.

The oscillating nature of UCAs can be utilized for more than just signal generation. Depending on the application, this phenomenon in vivo can induce biological effects that has the potential to injure surrounding tissue. For diagnostic purposes, imaging with UCAs should be optimized to minimize negative tissue response in the organ being examined. Alternatively, for therapeutic outcomes, an oscillating UCA is utilized to intentionally produce bioeffects to the surrounding tissue, including temporarily opening biological barriers that limit drug delivery.

In order to achieve these goals of safe diagnostic imaging and enhanced therapeutic outcomes, it is necessary to optimize PCCA formulation with respect to the desired in vivo response. I hypothesize that by characterizing in vivo responses as a function of PCCA formulation, one can arrive at the ideal formulation for the desired ultrasound application.
In my first objective, I will explore the use of PCCAs for diagnostic imaging. I will begin this objective by exploring potential bioeffects from MB-mediated contrast enhanced ultrasound (CEUS), which utilizes an imaging sequence involving high amplitude pulses which cause substantial microbubble disruption. With this understanding, I will proceed to investigate potential bioeffects from PCCA-mediated CEUS, particularly focusing on the vaporization phase of PCCA-mediated CEUS. In this study, bioeffects will be measured as a function of PCCA formulation.

My second objective will be to identify an optimal PCCA formulation for enhanced thermal ablation. The pursuit will require investigating the lesion size and heating efficiency from exposing various PCCA formulations to high intensity focused ultrasound (HIFU) in a tissue-mimicking phantom. We will conclude with future directions of PCCA technology as a drug-delivering vehicle. It is my hope that this work will be utilized to further the advancement of PCCA technology, towards in vivo applications in diagnostic imaging and therapeutic applications with ultrasound.
Designing Phase-change Contrast Agents for Safe Diagnostic Ultrasound Imaging and Enhanced Therapeutic Outcomes

by
Ange Gloria Nyankima

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Biomedical Engineering

Raleigh, North Carolina
2019

APPROVED BY:

Dr. Paul A. Dayton
Chair of Advisory Committee

Dr. Caterina Gallippi

Dr. Emily Chang
External Member

Dr. X. Sha Chang

Dr. Xiaoning Jiang
BIOGRAPHY

Ange Gloria Nyankima (known as Gloria) was born and raised in Auburn, AL. She attended Auburn University, where she received a Bachelor’s of Chemical Engineering in 2013. In the same year, she began her Doctor of Philosophy in the joint department of Biomedical Engineering at North Carolina State University and the University of North Carolina at Chapel Hill. In 2014, she joined the lab of Paul Dayton, where she took an interest in phase-change contrast agents. During her graduate career, she explored the safety of phase-change contrast agents as a diagnostic imaging tool. In 2016, she was awarded an NIH F31 grant to pursue the use of phase-change contrast agents as a therapeutic tool. Gloria plans to continue assessing safety of medical devices as an AIMBE Scholar at the U.S. Food and Drug Administration.

The following publications were authored or coauthored by Gloria Nyankima in peer-reviewed engineering journals, as well as conference proceedings and poster presentations, during her time in the University of North Carolina at Chapel Hill and North Carolina State University joint department of Biomedical Engineering.

PUBLISHED ARTICLES AND CONFERENCE PROCEEDINGS:


2. “Microbubble-mediated sonoporation to increase the uptake of refillable drug depots: A proof of concept” C. Moody, A. G. Nyankima, V. Papadopoulou, Y. Brudno, and P.A.


ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Paul Dayton, for all of his mentorship during my time as his graduate student. To my committee, I appreciate your guidance and support through this memorable and arduous journey. And to the members of the Dayton Lab, both past and present, I cannot express the amount of gratitude I have for each and every one of you. My graduate career would not have been the same without the hours of conversations, miles of running, and endless support I received from the members of the Dayton Lab. To Samantha Fix, who began as my colleague in the Dayton Lab, and became a true friend—it has been an honor watching you grow as a scientist.

I would like to thank my family and friends for the never-ending belief in me. Whether or not you understood the challenges I was facing, you still remained confident in my ability. To Kirsten Willis, Anaidra Reese, Myricka Brown, Leonard Baldwin, and Alex Chavis, our yearly vacations have been the rejuvenation I needed to continue this journey. To my mother and siblings, Sandrine and Chanel, so much of me is what I have seen in each of you and I am thankful for your never-enduring love.

Finally, I would like to give a special thank you to my father, Laurent. As a result of many hours in your laboratory, and your own dissertation sitting on our bookshelf at home, I began this research journey. For the seeds you planted, which culminated in this document, I would like to dedicate this work to you.
# TABLE OF CONTENTS

LIST OF TABLES ................................................................................................................... ix
LIST OF FIGURES ................................................................................................................. xi
ABBREVIATIONS ................................................................................................................ xv

## CHAPTER 1 Introduction ......................................................................................................... 1
  1.1 Medical Ultrasound ........................................................................................................ 1
  1.2 Ultrasound Contrast Agent .......................................................................................... 7
  1.3 Objective ....................................................................................................................... 12
  1.4 Dissertation Outline ....................................................................................................... 13

## References ........................................................................................................................... 15

## CHAPTER 2 Histological and Blood Chemistry Examination of the Rodent Kidney After Exposure to Flash-Replenishment Ultrasound Contrast Imaging ........................................... 21
  2.1 Introduction .................................................................................................................... 21
  2.2 Materials and Methods ............................................................................................... 26
  2.3 Results .......................................................................................................................... 32
  2.4 Discussion .................................................................................................................... 34
  2.5 Conclusions .................................................................................................................. 38
  2.6 Acknowledgements ....................................................................................................... 39

## References ........................................................................................................................... 40

## CHAPTER 3 An In Vivo Assessment of the Potential for Renal Bioeffects from the Vaporization of Perfluorocarbon Phase-Change Contrast Agents .................................................. 43
  3.1 Introduction .................................................................................................................... 43
  3.2 Materials and Methods ............................................................................................... 45
  3.2.7 Statistical Testing ..................................................................................................... 52
  3.3 Results .......................................................................................................................... 52
  3.4 Discussion .................................................................................................................... 60
  3.5 Conclusion .................................................................................................................... 62
  3.6 Acknowledgements ....................................................................................................... 62

## References ........................................................................................................................... 64

## CHAPTER 4 Utilizing the Potential Bioeffects of Phase-change Contrast Agents for Therapeutic Ultrasound ..................................................................................................................... 67
  4.1 Introduction .................................................................................................................... 67
4.2 Bioeffects of Ultrasound .............................................................. 68
4.3 Ultrasound Contrast Agents ..................................................... 70
4.4 Sonoporation ............................................................................ 71
4.5 Thermal Ablation with High-intensity Focused Ultrasound ......... 74
4.5 Influence of Ultrasound Parameters on Droplets ..................... 77
References ...................................................................................... 80

CHAPTER 5 In vitro assessment of lesion development from phase-change contrast agent enhanced thermal ablation as a function of acoustic and perfluorocarbon parameters ........ 85
5.1 Introduction ............................................................................. 85
5.2 Materials and Methods .......................................................... 88
5.2.1 Phase-change contrast agent Formulations ......................... 88
5.2.2 Lipid Solution Formulation .................................................. 88
5.2.3 Perfluorocarbon Preparation ............................................... 89
5.2.3 Phase-change Procedure .................................................... 90
5.2.4 Tissue-mimicking Phantom Procedure ............................... 91
5.2.5 Thermal Ablation Procedure .............................................. 92
5.2.6 Data Processing: Acoustic Characterization of Lesion Area ...... 94
5.2.7 Data processing: Temperature Measurement of Lesion Heating .... 95
5.2.8 Statistical Analysis ........................................................... 97
5.3 Results ................................................................................... 98
5.3.1 PCCA Size Distribution ..................................................... 98
5.3.2 Lesion Acoustic Characterization ....................................... 98
5.3.3 Thermal Measurement of Lesion Development .................... 101
5.4 Discussion ............................................................................ 103
5.5 Conclusions .......................................................................... 108
5.6 Acknowledgements .............................................................. 108
References ...................................................................................... 110

CHAPTER 6 Challenges of Optimizing Phase-change Contrast Agents for Extravasation 114
6.1 Introduction ............................................................................ 114
6.2 Data ....................................................................................... 119
6.3 Challenges of Observing Extravasation ................................. 139
6.4 Final Recommendations for PCCA Technology ...................... 142
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>References</td>
<td>144</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>147</td>
</tr>
<tr>
<td>APPENDIX A Supplemental Information for Chapter 2</td>
<td>148</td>
</tr>
<tr>
<td>A.1 Methods &amp; Materials</td>
<td>148</td>
</tr>
<tr>
<td>A.2 Results and Discussions</td>
<td>148</td>
</tr>
<tr>
<td>References</td>
<td>151</td>
</tr>
<tr>
<td>APPENDIX B Supplemental Information for Chapter 3</td>
<td>152</td>
</tr>
<tr>
<td>APPENDIX C Supplemental Information for Chapter 5</td>
<td>156</td>
</tr>
</tbody>
</table>
Table 3-1: Designated acoustic parameters for experimental groups i-v. For the mechanical index (MI), the maximum MI transmitted in the region of interest is reported. A focused pulse at 5 MHz with 5 cycles per pulse was transmitted in all subjects. For each group presented, an animal number (n) of 8 was used.

Table 3-2: Serum creatinine levels at all three time points for the 24 hour observation are presented by group: (i) Decafluorobutane (DFB) at max, (ii) Mix at max, (iii) Octafluoropropane (OFP) at max, (iv) Mix at min, (v) OFP at min. Data points indicated with a (*) at the 24 hour time point denote an increase of or greater than 150% (from baseline). This aligns with the serum creatinine requirements in the Acute Kidney Injury Network (AKIN) classification for indicating potential acute kidney injury. For a female Fischer rat, the 95% confidence interval for serum creatinine level is 0.2-06 mg/dL.

Table 3-3: The clinical chemistry and histology results of the recovery study show minimal bioeffects after two and four weeks of recovery. No red blood cell (RBC) casts were found in either the two- or four-week recovery subjects. Creatinine levels for one subject in each group showed an increase of >200%, which has been indicated with a (*). For a female Fischer rat, the 95% confidence interval for serum creatinine level is 0.2-06 mg/dL.

Table 6-1: Comparison of surface charge of commercially available UCAs and the contrast agents made in the Dayton Lab (In-House). In-House contrast agents were tested on a DLS (company, USA) with a total of 3 vials.

Table 6-2: Size distribution results of DFB-containing MBs and PCCAs formulated with 30% PEG. Data was gathered using an AccuSizer for 780A the MBs and an AccuSizer FX Nano for the PCCAs.

Table 6-3: Size distribution results of DFB PCCAs after being condensed by either pressurized air or nitrogen. Distributions were found using an AccuSizer FX Nano.

Table 6-4: Size distribution metrics for the size-sorting procedure demonstrate population similarities and differences between starting MB population, isolated NBs, and the resulting NDs. The averages are a result of two trials.

Table B-1: The parameters, histopathology, and serum creatinine for a preliminary investigation where Sprague-Dawley rats experience no PCCA vaporization. Instead, the activation pulses (at an MI of 2.7) were delivered to the experimental kidney. These parameters were not used in the study presented here. Both kidneys were assessed (by the same pathologist) and found to be normal.

Table B-2: Description of histopathology evaluations.

Table C-1: Area of lesion data for all four formulations, organized by mean, standard deviation (std. dev), and the number of lesions produced during HIFU treatment (N).
Table C-2: Area under the curve (AUC) data for all four formulations, organized by mean, standard deviation (std. dev), and the number of lesions produced during HIFU treatment (N). .......................................................................................................................... 156

Table C-3: Peak temperature data for all four formulations, organized by mean, standard deviation (std. dev), and the number of lesions produced during HIFU treatment (N). ........................................................................................................................................ 156
LIST OF FIGURES

Figure 1-1: A generalized block diagram of the transmit (bottom) and receive (top) processes that are implemented to generate an ultrasound image using a multi-element (total of N elements) transducer. In the diagram, a preclinical kidney with contrast agents flowing in the vessels results in the contrast-enhanced ultrasound image presented in the “Image Display”. Image was produced by my colleague, Dr. Kennita Johnson. 2

Figure 1-2: Diagram demonstrating the predicted received signal from one element in a multi-element transducer, when positioned to image a kidney. The transmitted signal, shown as a one cycle sinusoid, is reflected at the kidney boundaries and received by the transducer, corresponding to the upper and lower boundaries of the kidney and surrounding tissue. 4

Figure 1-3: Design components of PCCA technology, describing considerations for fabrication, shell composition, and inner core that will impact acoustic response and particle stability. The inner core of PCCAs can contain (but are not limited to) octafluoropropane (OFP), decafluorobutane (DFB), dodecafluoropentane (DDFP), perfluorohexane (PFH), and perfluorooctylbromide (PFOB). The information portrayed in this figure was reproduced from Lea-Banks et al [43] with permission from Elsevier. 12

Figure 2-1: Experiment Timeline. On the first day, animal BUN levels were measured prior to being imaged (t=0). For short term bioeffect assessment, blood and kidneys were collected for BUN and histopathology four hours post imaging. For long term bioeffect assessment, blood and kidneys were collected two weeks after imaging. This timeline was followed for animals exposed to 1.0 MI and 1.9 MI microbubble destructive pulses. 28

Figure 2-2: Imaging Setup. Animals were positioned such that only one kidney would be exposed to the ultrasound field. A) A water bath was placed above the animal so that the focus of the transducer would align with the kidney. B) The transducer was steered across one side of the animal, while the animal was administered isoflurane through a nose cone and a heating pad maintained a healthy body temperature (35.9-37.5°C). 31

Figure 2-3: Clinical Chemistry Results. Average baseline and endpoint data collected for each group were analyzed via paired T-test. The BUN levels after 2 weeks in the 1.9 MI pulse group were significantly higher than baseline levels. In addition, BUN levels of both groups analyzed at 4 hours were also found to be statically significant. P-values between 0.01 and 0.05 are indicated with the symbol ‘*’. Normal BUN range is indicated with dashed lines (15 to 24 mg/dL). 33

Figure 2-4: Histopathology Results. No bioeffects were observed after 1.0 and 1.9 MI pulse or at either time point. 34

Figure 3-1: Phase-change contrast agent (PCCA) size distribution (as a function of concentration) for all three formulations tested, including decafluorobutane
(DFB), octafluoropropane (OFP), and 1:1 DFB-OFP mixture. Size distributions represent averages from three vials.

**Figure 3-2:** Photomicrographs of kidneys from a rat in group iii. In the cortex (a) there are many red blood cell casts within proximal tubules (arrows). One tubule also contains sloughed cellular debris (circle). There is mild interstitial inflammation in response to the tubular injury. The glomerulus is normal. Within the medulla of the same rat (b), red blood cell casts can be seen in collecting duct lumens (arrows) and there is also sloughed cells within a duct lumen (oval). Scale bars (bottom right corner) in (a) and (b) represent a length of 20µm. Transmission electron micrographs of a rat from group iii revealed that tubules with intraluminal red blood cells (c). The red blood cells (R) were found in the lumens (L) of tubules with intact apical brush borders (BB). The epithelial cells (E) had scattered electron dense material within cytoplasmic lysosomes. The glomerular capillary walls of this rat (c) were normal. Podocytes had segmental foot process effacement (not shown). Parietal epithelial cells (PEC) had disruption of the cell membrane and marked mitochondrial swelling. Scale bars (bottom right corner) in (c) and (d) represent a length of 10µm.

**Figure 3-3:** Total red blood cell (RBC) casts presented as boxplots for all five groups observed (labeled with the perfluorocarbon and mechanical index used). Each group contains the experimental kidney (Exp) alongside its paired control (Con). Data is shown with the interquartile range as the box edges and the median data point is presented as a line inside the box. Circular points (‘o’) indicate outliers, defined as points beyond 1.5 times the interquartile range. Statistically significant values are indicated with an asterisk (*).

**Figure 5-1:** Schematic of the HIFU experimental setup. PCCA-mediated HIFU was conducted in an acrylamide-album hydrogel phantom. Phantoms were maintained at a temperature of 37ºC using a heated water bath. A phantom holder was manufactured for the two different phantom volumes. The TIPS transducer was positioned 10 mm away from phantom surface, and was guided across the phantom with an accompanying 2D motion stage (not pictured).

**Figure 5-2:** Flow diagram of data processing of acoustic characterization study. Ultrasound images from acoustic characterization study were analyzed through MATLAB. The chosen lesion was isolated from the rest of the image by placing an ROI around the lesion. A threshold was applied to identify lesion location from phantom background. The values were binarized for area calculation.

**Figure 5-3:** Example temperature recording. Thermal heating was measured using a fiber optic probe, which recorded temperature changes in real-time. An example of data from the probe is presented, showing the change in phantom temperature during a HIFU ablation. HIFU was performed on a 20 mL phantom containing PCCAs at a concentration of 1.0 µL/mL.

**Figure 5-4:** PCCA size distribution of the four PCCA formulation used throughout the study. Alongside the distribution is a table presenting the mean diameter and mean concentration of each PCCA formulation. Measurements were averaged from three samples per PCCA formulation.
Figure 5-5: Example B-mode images of sample lesions created in PCCA-embedded tissue mimicking phantoms. Images are presented (top to bottom) from the least stable PCCA-core, C3C4, to the most stable PCCA-core, C5. Lesions are organized in increasing transmitted pressure, beginning at 1.0 MPa (left) and ending at 4.0 MPa (right). These pressures are indicated above the lesion. All phantoms were made with a 1.0 μL/mL concentration of PCCAs.

Figure 5-6: Lesion area with respect to PNP, together with Spearman correlation testing results. Lesion area was shown to increase with increasing PNP for C3C4 (r = 0.93, p = 0.003), C4 (r = 0.96, p = 0.003) and C5 (r = 1.00, p = 0.0004), but not C4C5 (r = 0.75, p = 0.066).

Figure 5-7: Area of lesions produced at 1 MHz center frequency, 1 sec exposure, and 100% duty cycle. Data is presented in groups of all four PCCA formulations with respect to transmitted PNP, together with the statistically significant differences between formulations found by Tukey’s multi-comparison testing. A dashed red line represents the FWHM focal area of the 1 MHz TIPS transducer.

Figure 5-8: Peak temperature (left) and AUC (right) with respect to PNP for all four PCCA formulations. Thermal assessment of PCCA-embedded tissue mimicking phantoms was measured by a fiber optic probe. Temperature changes during HIFU treatment were quantified as (a) peak temperature and (b) area under the curve (AUC). Average and standard deviation data are presented for transmit PNP of 2.0, 3.0, and 4.0 MPa. Statistically significant differences between some formulations were found at 2.0 MPa (all statistically significant p-values from Tukey’s multi-comparison are depicted).

Figure 6-1: Cancer vascular environment is understood to be porous, allowing for nanoparticles to passively migrate outside tumor vessels. This characteristic of cancer can be utilized to target extravascular targets of cancer as shown in schematic.

Figure 6-2: Difference in size distribution as a result of PEG concentration demonstrates little difference between the two PCCA formulations.

Figure 6-3: Comparison of size distributions of pressure increases used to condense MBs with air. Data displayed resulted from measurements taken from an AccuSizer FX Nano (n=3).

Figure 6-4: Difference in size distribution as a result of pressure increases when condensing MBs with nitrogen. Input pressures ranged from 10 psi to 20psi, and measurements were taken from an AccuSizer FX Nano (n=3).

Figure 6-5: Images of vials demonstrating optical change of particle solution as isolating procedure progressed from MBs (A) to NBs from the infranatant (B) to PCCAs (C).

Figure 6-6: Average size distribution of size sorted DFB PCCAs (N=2). The protocol begins with a solution of MBs, and NBs are isolated after centrifugation. PCCAs are then formulated from the NBs. All size measurements were collected from an AccuSizer FX Nano.
Figure 6-8: Optimization of centrifuge speed was conducted using the AccuSizer 780A to measure size distribution (n=2/speed). Infranatants of each centrifuge speed were compared to one another and 1,700xRPM was isolated as a potential candidate, given lower mean and mode diameter and higher concentration.  131

Figure 6-9: Time optimization was conducted by measuring PCCAs isolated from infranatants centrifuged at 1,700xRPM. Size distributions of PCCAs were measured with the NanoSight.  134

Figure 6-10: In vitro ultrasound images of MBs vaporized from droplets, demonstrating the differences in the level of activation from non-sorted (top row) and size-sorted (bottom row) droplets. Both PCCA populations contained a PFC core of DFB. As a result of the smaller diameter PCCAs, the size-sorted droplets required the higher peak-negative pressures to vaporize at all visualized depths.  137

Figure 6-11: In vivo rat kidney (circled) ultrasound images of MBs formed from activated droplets. NDs were administered to rodent models, and imaging was conducted of the right kidney. The figure demonstrates a single animal, over a 20min period, after one injection of size-sorted NDs. Activation pulses for PCCAs vaporization were transmitted at 4.5 MPa (peak negative pressure).  138

Figure A-1: Clinical Chemistry Results. BUN levels are grouped by imaging parameters and endpoint. Results of paired T-test indicate a statistically significant increase in BUN levels for subjects in the high MI group after 2 weeks (p-value from 0.001 to 0.01 is marked with a ‘**’). No statistical significance was observed in the low MI groups. Average BUN values remained within normal range for female Fischer rat (15 to 24 mg/dL, dashed lines).  149

Figure A-2: Histopathology Results. Kidney for both low and high destruction pulses at 24 hours and 2 weeks. Data is displayed as the mean ± standard error of the mean (SEM).  150

Figure B-1: Representative histopathology images (cortex and medulla) of the imaged kidney from one animal per treatment group. Images of the cortex (60X magnification) are on the left and the medulla (40X magnification) are on the right. (1A-B) The representative image of decafluorobutane (DFB) at a mechanical index (MI) of 1.9 depicts red blood cell (RBC) casts (black arrows) and proteinaceous cellular debris in the lumen of a tubule (white arrow) in the cortex. RBC casts were not present in the medulla. (2A-B) Representative images of 1:1 mix at an MI of 1.9 show RBC casts in both the cortex and medulla (black arrows) and cellular and nuclear debris, likely from a necrotic tubular epithelial cell. (3A-B) In octafluoropropane (OFP) at an MI of 1.9 representative images, RBC casts and protein casts are indicated with black and yellow arrows respectively. Cellular debris was also seen in this animal with associated inflammation around the tubule (white arrow). (4A-B) Representative images of rats exposed to 1:1 mix at an MI of 1.35 show normal renal parenchyma. Rare RBC casts were seen in this group. (5A-B) Renal histopathology of the OFP at an MI of 0.85 treatment group was within normal limits, without RBC casts.  154
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D</td>
<td>Three-dimension</td>
</tr>
<tr>
<td>ADV</td>
<td>Acoustic droplet vaporization</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the Curve</td>
</tr>
<tr>
<td>C3C4</td>
<td>1:1 Octafluoropropane-Decafluorobutane</td>
</tr>
<tr>
<td>C4</td>
<td>Decafluorobutane</td>
</tr>
<tr>
<td>C4C5</td>
<td>1:1 Dodecafluoropentane-Decafluorobutane</td>
</tr>
<tr>
<td>C5</td>
<td>Dodecafluoropentane</td>
</tr>
<tr>
<td>CEUS</td>
<td>Contrast-enhanced ultrasound</td>
</tr>
<tr>
<td>DDFP</td>
<td>Dodecafluoropentane</td>
</tr>
<tr>
<td>DFB</td>
<td>Decafluorobutane</td>
</tr>
<tr>
<td>DSPC</td>
<td>1, 2-distearoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DSPE</td>
<td>1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced Permeability and Retention</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>HIFU</td>
<td>High-intensity focused ultrasound</td>
</tr>
<tr>
<td>MB</td>
<td>Microbubbles</td>
</tr>
<tr>
<td>MR</td>
<td>Magnetic resonance</td>
</tr>
<tr>
<td>NB</td>
<td>Nanobubbles</td>
</tr>
<tr>
<td>ND</td>
<td>Nanodroplet</td>
</tr>
<tr>
<td>OFP</td>
<td>Octafluoropropane</td>
</tr>
<tr>
<td>PCCA</td>
<td>Phase-change contrast agents</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEG2000</td>
<td>polyethylene glycol-2000</td>
</tr>
<tr>
<td>PFC</td>
<td>Perfluorocarbon</td>
</tr>
<tr>
<td>PNP</td>
<td>Peak negative pressure</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TIPS</td>
<td>Therapeutic Imaging Probe System</td>
</tr>
<tr>
<td>UCA</td>
<td>Ultrasound contrast agent</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 MEDICAL ULTRASOUND

Ultrasound is well known in the field of medical imaging for being portable, inexpensive, and its non-ionizing mechanism of action, making ultrasound is accessible to use in vulnerable patient populations and low resource settings [1]. Unlike magnetic resonance (MR) imaging, ultrasound can be quickly implemented by a patient’s bedside. Additionally, imaging can be performed in real-time, providing information from anatomical features from B-mode, vessel flow maps from Doppler imaging, and mechanical properties of tissue from elastography [2], [3]. Ultrasound does have limitations. Imaging has a limited field of view, particularly in comparison to modalities like MR and computerized tomography (CT). Neither does ultrasound have the resolution capabilities of optical modalities, like confocal imaging. Today, ultrasound imaging has expanded from the grainy image quality the technology is traditionally known for producing. Developments in ultrasound imaging span across hardware and software, including transducer material and design, as well as sophisticated imaging processing techniques to reduce noise and improve signal contrast. While ultrasound continues to develop in improving image quality, developers strive to maintain the major benefits previously discussed that separate ultrasound from the other imaging modalities.

1.1.1 Mechanism of Ultrasound Imaging

Ultrasound can be defined as mechanical waves of greater frequency than that of audible human hearing (20-20,000 Hz) [2], [3]. To produce the waves at these high
frequencies, piezoelectric material is encased in a device known as a transducer. A transducer can be manufactured with one piece of piezoelectric material (referred to as a single-element transducer), or multiple smaller sections that are operated together or separately (referred to as a multi-element transducer or array) [2]. The transducer is designed to efficiently convert electric pulses into sound waves, and respond to incoming sound waves by converting the mechanical stimulations of the received acoustic wave into electric signals. Through signal processing techniques, the received electrical signal is amplified, digitized, and spatially localized (in a process known as beamforming) in order to produce an image. This process can be completed on the order of milliseconds, enabling real-time imaging (see Figure 1-1).

**Figure 1-1:** A generalized block diagram of the transmit (bottom) and receive (top) processes that are implemented to generate an ultrasound image using a multi-element (total of N elements) transducer. In the diagram, a preclinical kidney with contrast agents flowing in the vessels results in the contrast-enhanced ultrasound image presented in the “Image Display”. Image was produced by my colleague, Dr. Kennita Johnson.

When ultrasound is transmitted into the body, the mechanical wave propagates through tissue. When the wave experiences a change in impedance, which describes the density and compressibility of a material, the wave is scattered in multiple directions, including reflected back to the transducer. The forward scattered wave continues in the transmitted direction (see Figure 1-2), but with decreased energy. The fraction of the wave
that is backscattered to the transducer is used to produce the ultrasound images.

Computational analysis of the returning sound waves takes into consideration the time it took the wave to leave the element of the transducer and return (known as time of flight) and then factors the estimated speed the wave traveled through a given medium. For tissue like the kidney, an estimated speed of sound is about 1,565 m/s, while in tissue like the lung (which contain air) and the skull, an estimated speed of sound is 600 m/s and 4,080 m/s, respectively [2].

For a multi-element transducer, the received signal for each element is amplified and digitized. Then, the spatial location of the returned signal is calculated using time of flight and speed of sound information. A final signal amplitude is found by summing the spatially corresponding signal across the elements of the transducer, and demodulating this signal through envelope detection. Finally, dynamic compression, which determines how bright a pixel will be in comparison to other pixels in the image, as well as any filtering techniques to improve image quality, are completed prior to displaying the image on the system’s monitor. This process can be completed multiple times in a second, resulting in real-time video of the physiological and anatomical state of an organ of interest.
The physical mechanism of ultrasound allows the modality to be used for more than just imaging. Sound waves can produce biological responses to tissue as a result of the mechanical nature of the wave. These mechanical effects range in severity, and are controlled by acoustic parameters, such as frequency, pressure amplitude, and exposure time. Ultrasound waves can be designed to push tissue, and subsequently monitor the displacement of the tissue. This field of imaging, known as elastography, can then be implemented to characterize tissue and the state of pathophysiology [4]. As well, ultrasound can be designed to manipulate gas bubbles. In an acoustic field, gas bubbles will oscillate, inducing
mechanical effects towards surrounding tissue [5]. Known as sonoporation, the phenomena can cause microstreams or microjets capable of puncturing cellular membranes and opening intercellular junctions [5]. Parameters of the wave can also be designed to transform the acoustic wave into a shock wave as it travels through tissue, capable of inducing tissue damage. This technique is known clinically as histotripsy, when targeting soft tissue, or lithotripsy when targeting calcified materials, like gallbladder and kidney stones [6].

The second way acoustic waves can affect biological tissue is by thermal effects. When ultrasound is transmitted, the wave is attenuated (decreased carrying energy) by way of the sound wave scattering as it experiences density changes, or energy absorption by the tissue. The main mechanism of energy absorption is from frictional heat loss. As the mechanical wave travels, molecules in the medium rub against one another, and the energy of momentum is transformed into heat [6], [7]. Like the mechanical effects, by controlling the intensity of the sound wave, the thermal effects can be kept minimal, to a few degrees, similar to a fever. This technique, known as mild hyperthermia, has been shown to improve drug delivery to cancerous tissue [8], [9]. Thermal effects from ultrasound can also be intensified to rapid thermal injury, in which cell death is induced in a matter of seconds. The latter is categorized as high-intensity focused ultrasound (HIFU) ablation.

These biological responses, both mechanical and thermal in nature, can be utilized for therapeutic purposes, but in diagnostic imaging, where the purpose is to observe and diagnose the physiological state of the organ of interest, it is imperative that any potential biological response by the diagnostic imaging modality be minimized. The United States Food and Drug Administration (FDA) supervises the approval of new medical technology,
and implementation of regulatory policies in order to maintain the safety of all medical
devices and therapeutic drugs.

1.1.2 FDA Regulations for Diagnostic Ultrasound Imaging

To address the sources of bioeffects from ultrasound imaging, thermal and mechanical, the FDA developed the thermal index (TI) and mechanical index (MI) as metrics to display on imaging systems to gauge the risk of inducing these bioeffects for a given imaging procedure. The limits the FDA places on ultrasound systems depend on the system’s intended use. Typically, the derated spatial-peak temporal average intensity ($I_{spta,3}$) cannot exceed 720 mW/cm$^2$ (see Eqn. 1-1) [10]. The spatial-peak temporal-average intensity is derated by a value of 0.3 dB cm$^{-1}$ MHz$^{-1}$ to account for wave attenuation through tissue. In the equation, $P_{PP2P}$ represents the peak-to-peak pressure (MPa), $T_{pulse}$ represents the time the transducer is transmitting (sec), $z$ is the acoustic impedance of the tissue, and $T_{prf}$ is the time interval of the pulses (sec). The TI is a ratio of the acoustic power output ($W_{output}$, in Watts) divided by the power necessary to raise the temperature of tissue one degree Celsius ($W_{deg}$) [11] (see Eqn 1-2). When acoustic parameters exceed a value of 1.0, it is necessary for the TI value to be displayed on the imaging system. The FDA regulates diagnostic ultrasound procedures to remain below 6.0. The MI, which may also be displayed on imaging systems, addresses the risk of cavitation of innate gas bubbles created in tissue. As Eqn. 1-3 shows, MI is the derated peak negative pressure ($PN_{der}$) divided by the square root of the transmit center frequency ($f_c$) [11]. The peak negative pressure is derated by 0.3 dB cm$^{-1}$ MHz$^{-1}$.

\[
I_{spta} = \frac{P_{PP2P}^2 T_{pulse}}{2zT_{prf}} < 720 \text{ mW/cm}^2 \quad \text{Eqn. 1-1}
\]

\[
TI = \frac{W_{output}}{W_{deg}} < 6.0 \quad \text{Eqn. 1-2}
\]
It is important to note that these guidelines apply to diagnostic ultrasound systems alone. The FDA is currently reviewing and approving uses of contrast agent technology for diagnostic ultrasound imaging, which means refining previously described acoustic limits when developing imaging protocols for the presence of contrast agents. In the next section, we will discuss the use of ultrasound contrast agents for both imaging and therapeutic applications.

1.2 ULTRASOUND CONTRAST AGENT

1.2.1 Microbubbles

Ultrasound contrast agents (UCAs) overcome a major limitation in imaging blood vessels with ultrasound. Doppler imaging is a technique in ultrasound that utilizes the shift in phase of received acoustic signal when sound waves are reflected from a moving scatterer [3]. With Doppler imaging, flow velocity color maps of vessels can be created in real-time.

While Doppler imaging has advanced as a technique for characterizing blood flow in real-time, and without the need for administering any contrast agents, the technology possesses shortcomings, mainly low sensitivity to blood velocity, and poor resolution, therefore, capillary networks are impossible to resolve. In addition, blood does not scatter sound waves very well. UCAs provide a large change in impedance in the anatomical field, resulting in substantial signal return. Predominant UCAs in commercial use are in the 1-10µm range, with a lipid, protein, or polymer shell, surrounding a gas-core, like perfluorocarbon (PFC) [12], [13]. These agents are categorically referred to as microbubbles
(MBs). As a result of their gas-core, MBs oscillate in an acoustic field, producing a broadband acoustic signal. This nonlinear property of MBs makes their signal response distinctly different from the linear response of tissue, enabling sophisticated signal isolation from tissue [12]. As a result of their size range, MBs are limited to vasculature, making them an ideal blood-marker. Applications of MBs in ultrasound imaging widely range in diagnostic imaging. Contrast enhanced ultrasound (CEUS) utilizes MBs to diagnosis and monitor diseases in the kidney [14]–[17], prostate [18], [19], breast [20]–[22], and even atherosclerotic plaque [23]–[25]. Clinically, the FDA has approved MBs use for echocardiography and hepatocellular carcinoma diagnosis [26].

Applications of therapeutic ultrasound have expanded with the inclusion of MBs. As a source of cavitation, MBs allow the same therapeutic targets previously described to be achieved at lower acoustic thresholds. In the absence of MBs, therapeutic ultrasound relies on innate gas bubbles to induce mechanical effects, which will vary greatly depending on tissue type. Administering MBs assures presence of cavitation seeds to aid in therapeutic efficacy. Current research includes increasing drug delivery beyond barriers like the blood-brain barrier [27], [28] and difficult to reach tumors [29], [30]. This use of MBs to modulate vessel and cellular membrane permeability is known as sonoporation. Other therapeutic ultrasound techniques have shown improved results in the presence of MB technology, including thermal ablation [31], [32] and sonothrombolysis [33].

MBs have their limitations. First and foremost, the half-life of MBs are on the order of minutes [34]. For applications in imaging and therapy, it can be advantageous to utilize a longer circulating UCA. Second, the typical size distribution of MBs limits extravascular applications. Molecular imaging is a technique in imaging in which biomarkers are attached
to a contrast agent. Over time, the contrast agent will passively attach to the desired target, allowing users to indirectly image the presence of target in an organ of interest [35]. A longer circulation time will increase the probability and density of targeted UCAs correctly binding to their target. In a tumor, where leaky vasculature can result in the passive escape of UCAs out of tumor vasculature, a smaller UCA has the potential to bind to targets previously inaccessible [36], [37]. These limitations of MBs also affect therapeutic ultrasound applications. Though MBs are capable of inducing heating in tissue, there are concerns with off target effects. As Moyer et al. [38] showed, ablating tissue with MBs not only increases the temperature at the focus, but also in the near-field tissue. In response to MBs gaseous form, wherever there is a sufficient acoustic field, the MBs present will cavitate. The potential for extravascular presence of UCAs could open therapeutic delivery and thermal ablation beyond vasculature. One solution for overcoming the limitations of MBs is through the use of phase-change contrast agents (PCCAs).

1.2.2 Phase-change Contrast Agents

Gas-filled UCAs have expanded the capability for the use of medical ultrasound in both therapy and diagnostic imaging. PCCAs, a liquid-filled counterpart to traditional UCAs, provide the same unique harmonic signatures of commercial UCAs, once vaporized to a gas-core, but their initial nanometer diameter range and particle stability (from the liquid-core) should allow them to enter extravascular spaces inaccessible to traditional agents. Additionally, in their liquid state, PCCAs are ineffective at oscillating in an acoustic field, until the agents are vaporized to a gas, in a process known as acoustic droplet vaporization (ADV). ADV can be accomplished through thermal means, or high-intensity acoustic pulses. It is important to note that similar liquid PFC core particles have been studied for use with
MR and PET [39], [40], where ADV is not necessary. For the rest of this discussion, PCCAs will be acknowledged for their use in therapeutic and diagnostic ultrasound.

PCCAs are often formulated similarly to MBs, meaning they include a lipid, polymer, or protein shell with an inert PFC core [41] (see Fig. 1-3). Their liquid-core allows for a variety of fabrication methods, including microfluidic device manufacturing, extrusion methods, sonication and agitation of lipid/PFC emulsions, and condensation of MBs to PCCAs [42], [43]. Initial formulations included PFCs, like perfluorohexane (PFH, C₆F₁₄, boiling temperature= 57°C) and dodecafluoropentane (DDFP, C₅F₁₂, boiling temperature= 29°C), which are liquid at room temperature. These particles can require pressure amplitudes (above the FDA MI limit of 1.9) to vaporize [44], [45]. To enable PCCA use for diagnostic imaging, our lab developed a condensation method that would enable low-boiling PFCs, like octafluoropropane (OFP, C₃F₈, boiling temperature= -37°C) and decafluorobutane (DFB, C₄F₁₀, boiling temperature= -2°C) to be formulated as MBs and converted to PCCAs [44], [46]. These particles maintain liquid-cores above boiling temperatures of their bulk PFC as demonstrated by in vivo stability studies conducted by Sheeran et al [47]. In this study, rodent kidney perfusion imaging demonstrated increased circulation half-life of PCCAs containing low-boiling PFCs as compared to MBs. PCCA in vivo stability can be explained by homogenous nucleation theory as shown by Mountford et al [48].

PCCA imaging sequences can include a high MI focused activation pulse (MI>1.0) that will specify a region of PCCA vaporization, followed by contrast imaging of the present bubbles (MI<0.1) [44], [49]. The spatial selectivity provided by the activation requirement with PCCA technology has potential advantages for perfusion imaging and aberration
correction, while PCCA in vivo stability and mean diameter range has advantages for molecular imaging, specifically targeted tumor imaging [37].

In therapeutic applications, the properties of spatial selectivity with PCCAs, in vivo stability, and decreased size distribution are compelling for utilization of PCCA technology. To date, therapeutic research for PCCAs spans from sonothrombolysis [37], [50], [51], gene and drug delivery [52]–[54], to blood-brain-barrier opening [55], histotripsy [56], [57], and HIFU [54], [58], [59]. As compared to diagnostic imaging, therapeutic ultrasound has an expanded range in acoustic output, enabling the use of the higher-boiling PFC as well as the low-boiling PFC formulations for PCCA use. Ultimately, more investigation is required to improve PCCA technology for the diverse applications in therapy and diagnostic imaging, beginning with the technology’s formulation and how this will impact the desired in vivo biological response.
Figure 1-3: Design components of PCCA technology, describing considerations for fabrication, shell composition, and inner core that will impact acoustic response and particle stability. The inner core of PCCAs can contain (but are not limited to) octafluoropropane (OFP), decafluorobutane (DFB), dodecafluoropentane (DDFP), perfluorohexane (PFH), and perfluoroocetyl bromide (PFOB). The information portrayed in this figure was reproduced from Lea-Banks et al [43] with permission from Elsevier1.

1.3 OBJECTIVE

PCCAs are uniquely positioned in their development to address multiple limitations in medical applications of UCAs (as described previously). As the technology progresses in each of these application areas, questions concerning ideal formulation given application requirements will arise, particularly when considering safety and efficacy. Through design considerations, some of which are shown in Figure 1-3, PCCA technology can be improved to better tailor PCCAs for each application in therapeutic and diagnostic imaging. What is

---

needed towards this goal is an understanding of the intersection of contrast formulation, acoustic parameters, and the resulting in vivo response. Whether the question is minimizing the in vivo bioeffects from PCCA-mediated contrast-enhanced ultrasound, or engineering PCCAs for an intended therapeutic outcome, it is vital to optimize PCCA formulation for improved in vivo bioeffects.

1.4 DISSERTATION OUTLINE

In this dissertation, I explored this intersection of PCCA formulation, acoustic parameters, and the desired in vivo response for the use of PCCAs in medical ultrasound. I hypothesized that by optimizing PCCA formulation and adjusting acoustic parameters, I could develop a technique that would generate the desired in vivo bioeffects for given applications in imaging and therapy. This work is split into two parts. In the first half, I will address reducing unwarranted bioeffects in diagnostic imaging with PCCAs. In chapter 2, I will present my work exploring potential bioeffects from a MB-mediated ultrasound technique, which utilizes a similar high MI imaging sequence as is used for PCCA imaging. This work will lay a foundation for the investigation of potential bioeffects of PCCA-mediated contrast imaging in chapter 3, which will focus on the in vivo tissue response to the vaporization phase of PCCA imaging.

In the second half, I will explore PCCA formulation and acoustic parameters for therapeutic ultrasound, beginning with an introduction to the mechanisms driving PCCA-mediate therapeutic ultrasound in chapter 4. In chapter 5, I will investigate the impact of PCCA formulation and acoustic pressure on thermal ablation lesion development in a tissue-
mimicking phantom. Finally, I will conclude by introducing the current challenges of formulating PCCAs as an extravasating, theranostic vehicle for drug delivery.
REFERENCES


2015.

CHAPTER 2

HISTOLOGICAL AND BLOOD CHEMISTRY EXAMINATION OF THE RODENT KIDNEY AFTER EXPOSURE TO FLASH-REPLENISHMENT ULTRASOUND CONTRAST IMAGING

2.1 INTRODUCTION

Contrast-enhanced ultrasound (CEUS) with microbubble (MB) contrast agents has been in use for decades outside the United States. However, use of FDA-approved ultrasound contrast agents (UCAs) has been limited to echocardiography in the United States until April 2016, when Lumason was approved for adult and pediatric imaging of the liver. There are several advantages of CEUS over contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI), including increased enhancement sensitivity due to lack of contrast extravasation outside of the vasculature, lack of contrast nephrotoxicity, lack of ionizing radiation, and the ability to capture real-time imaging[1], [2]. Of these, the lack of nephrotoxicity has made CEUS of particular interest for kidney imaging since CT and MRI contrast agents are sometimes contraindicated in patients with compromised kidney function [3].

During a low mechanical-index (MI) CEUS study, wash-in of a bolus of MBs into the area of interest is captured and saved as a cine loop [4]. However, this type of non-destructive MB imaging only permits visualization of the target area once during contrast

---

2 This chapter was published in Ultrasonics. The original citation is as follows: A. G. Nyankima, S. K. Kasoji, R. Cianciolo, P. A. Dayton, and E. H. Chang, “Histological and blood chemistry examination of the rodent kidney after exposure to flash-replenishment ultrasound contrast imaging,” Ultrasonics, 98, pp. 1-6, 2019.
wash in. Visualization of another region of interest or a repeat study requires waiting for several minutes until the contrast has cleared circulation and can be re-administered. Alternatively, CEUS studies can also be performed using a continuous infusion of MBs and delivering moderate to high MI pulses (0.7-1.9) [5], [6], to disrupt the MBs and clear the MB signal. This technique is referred to as destruction-reperfusion or flash-replenishment. It allows for multiple cycles of contrast wash-in to be achieved in rapid succession, providing the potential to image multiple planes of interest or to repeat visualization of flow dynamics in a target region [5].

Though flash-replenishment has clinical advantages, there are biological concerns with the use of higher MI imaging. When present in an acoustic field, the gas-filled agents can resonate at varying magnitudes depending on the intensity of the field. If the acoustic field is substantial enough, this cavitation can lead to mechanical bioeffects on the surrounding tissue. As a result of the potential for injury, it is necessary to ensure safety of the contrast agent in addition to determining clinical efficacy. In the case of CEUS imaging, there are years of clinical experience with hundreds of thousands of patients that support a negligible amount of severe adverse effects after administration [7]–[10]. However, the scope of clinical data is limited because human histology samples are often not available since biopsies are usually not performed at the time of imaging. Moreover, long-term clinical follow-up has not been collected. In contrast, preclinical studies have investigated bioeffects of CEUS extensively. Previous studies have observed bioeffects in organs like the lungs, liver, intestines, and heart [11]–[15], but for the study presented, we will limit our focus to the kidney.
Previous studies have assessed the potential bioeffects induced in the kidney during ultrasound imaging with MBs, particularly in the kidney of rodent models [16]–[22]. Although there are many variables to be considered, such as acoustic pressure, center frequency, duty cycle, attenuation, transducer focus, as well as bubble parameters such as size and concentration, it is understood that under some conditions acoustic excitation of MB contrast agents can cause renal hemorrhage. Miller et al. [19] demonstrated glomerular capillary hemorrhage (GCH) in rat kidneys from microbubble-mediated diagnostic ultrasound developed as a function of frequency and pressure of acoustic pulses delivered. In the aforementioned study, the authors observed frequencies of 1.0, 1.5, 1.5, 2.3, 2.5, 3.2, 3.5, 5.0, and 7.5 MHz with respective peak negative pressures of 1.9, 2.3, 2.7, 3.7, 2.6, 2.3, 3.9, 5.6, and 5.9 MPa. Kidneys were exposed to these parameters for a 1 min duration of time. Of these parameters, GCH was present in a range of frequencies from 1.5-3.2 MHz. Presence of GCH was absent when maintaining the parameter \( \frac{P_r}{f} < 0.5 \), where \( P_r \) is the peak rarefractional pressure (MPa) and \( f \) is the frequency of the pulse (MHz). It has also been shown that with the use of appropriate acoustic parameters, renal MB imaging can be performed while avoiding undesired bioeffects. In a rodent model, Johnson et al. [17] observed no bioeffects after exposing kidney volumes to one sec MB destruction pulses, transmitted at 7 MHz and peak negative pressure (PNP) of 5.03 MPa. These parameters correlate to a MI of 1.9; the clinical maximum allowed setting for diagnostic ultrasound imaging under the U.S. Food and Drug Administration (FDA). This imaging sequence was followed by low energy contrast imaging at 7 MHz and an MI of 0.18 (PNP of 476 kPa). The procedure was repeated three times across the kidney. At a lower frequency, Jiménez et al. [16] observed kidneys in a porcine model after circulating MBs in the kidney were exposed
to a one sec MB destructive pulse at 1.5 MHz and an MI of 1.9 (PNP of 2.1 MPa). The study included transmitting directly to the kidney in a single plane, and was repeated six times in a single plane. The authors reported that no bioeffects were seen in histology or in biochemical renal function parameters, and thus concluded that the ultrasound parameter ranges tested did not result in tissue damage. The MI metric used in both the Jiménez et al. [16] and Johnson et al. [17] study is different from the metric highlighted in Miller et al. [19], in that the peak negative pressure in the MI metric is derated by a value of 0.3 dB cm\(^{-1}\) MHz\(^{-1}\) and divided by the square root of the transmitted center frequency.

Church and Miller [25] proposed that a combination of factors, specifically microbubble size at a certain frequency and pressure threshold, is necessary for the onset of bioeffects, such as GCH and petechial hemorrhage. Their kidney bioeffect model was tested by Miller et al. (2017) and shown to be valid at the frequencies tested—3.6 MHz and 5.5 MHz [26]. Variations in acoustic parameters, including the frequency, pulse repetition frequency of high MI pulses, acoustic focus, contrast dose, and exposure time, may be key factors resulting in the disparate degree of kidney injury in the different studies. In addition, protocol differences, including animal model, total exposure volume, and time of pathologic assessment make direct comparison of the model developed in Church and Miller [25] to models used in previous studies challenging [16], [17]. In general, there is a host of literature available which reports parameter ranges which are more likely to cause in-vivo bioeffects when microbubbles are exposed to ultrasound, (typically combinations of high pressures and low frequencies) as well as a range of parameters which are generally considered safe [16]–[22].
The success of CEUS safety in human patients can be attributed to the safety guidelines that are used both in the United States and internationally by leading institutions in ultrasound imaging [13]–[15], [27], which are largely founded on preclinical research. Generally, in regards to concern for mechanical bioeffects from administered contrast agent cavitation, it is recommended to maintain an MI below 0.4 during imaging [13]–[15]. However, in the Definity package insert [28], which includes FDA approved protocols, Lantheus recommends imaging at an MI of 0.8 or below. It is also noted that factors, such as contrast agent dose, transmit center frequency, PNP, acoustic exposure time, and transmit pulse repetition frequency, can alter the level of bioeffects observed when imaging above an MI of 0.4 [13], [15].

In light of these recommendations, we believe it is important to conduct bioeffect experimentation to assure safety, when imaging outside of clinical recommendations, taking into account specific imaging acoustic parameters (i.e. transmit frequency, MI, pulse duration, etc.) and accompanying contrast agent parameters (i.e. the formulation and the dosage). We therefore sought to replicate parameters similar to those used in high MI flash-replenishment imaging of humans to determine if these parameters would generate similar injury as seen in preclinical studies, i.e. GCH. Specifically, bioeffects were measured after an imaging sequence that included flash pulses, above an MI of 0.8 and limited to one second in exposure, followed by contrast imaging pulses (MI<0.4). Though the experiment included a pre-clinical dose regimen of contrast agents (much higher dose than is recommended for human use), the study was intended to assess the combination of the flash-sequence and the following CPS imaging. These sequences were repeated across the kidney volume to minimize risk of missing evidence of injury on histology due to sampling error.
We compared flash pulses at two MIs (both destructive to microbubbles): the FDA approved clinical maximum MI for non-contrast ultrasound of 1.9, the acoustic level at which previous studies demonstrated GCH [20], [21], and also a lower MI of 1.0, which is still sufficient to disrupt microbubbles and can therefore be used for flash-replenishment imaging. We hypothesized that injury might be inducible, but that it would be transient and would not cause long-lasting bioeffects.

This study used female Fischer 344 rat kidneys as a model for potential kidney bioeffects induced by imaging sequences that included MI pulses of 1.0 and 1.9 and assessed at 4-hours and 2-weeks post-imaging. Histologic examination of the kidney (gold standard of assessment) was performed by a veterinary expert in nephropathology. Blood urea nitrogen (BUN), obtained prior to CEUS and at time of necropsy, was assessed in combination with histology to determine kidney health.

2.2 MATERIALS AND METHODS

2.2.1 Animal Preparation

All animal procedures were certified by the University of North Carolina at Chapel Hill Animal Care and Use Committee. A total of 31 adult female Fischer 344 rats (150-250g) were used for the study (Charles River Laboratories International, Wilmington, MA, USA). During imaging, animals were anesthetized via nose cone administration of 2% isoflurane (Piramal Enterprises Limited, Mumbai, India) mixed with pure oxygen, flowing at a volumetric rate of 1.0 L/min. A 24-gauge tail vein catheter was inserted, which was used for blood collection and contrast agent injection. Animals were kept warm while under isoflurane anesthesia with a heating pad, and breathing was monitored for the duration of the
imaging procedure. For imaging preparation, hair was removed on the right flank of the animal with electric clippers (Pocket Pro Universal Trimmer, Wahl Clipper Corporation, Sterling, IL USA; 2.4oz and 4x2x1 inch in dimension) and hair depilation cream (Nair Hair Removal Lotion, Church & Dwight, Ewing Township, NJ, USA). Ultrasound gel was later applied to couple the transducer to the animal. The right kidney received CEUS imaging, while the contralateral kidney was only exposed to MBs. Further details on the imaging procedure can be found in the Imaging Procedure section.

A total of 31 animals was included in the study results. Animals were separated into four groups based on MI exposure and time of assessment of kidney histopathology. All animals were imaged once at either an MI of 1.0 (n=15) or 1.9 (n=16) at time 0. The study consisted of two time points within each MI exposure group—short- and long-term evaluations. Short term bioeffects were assessed 4 hours post imaging (n=16), while long term bioeffects were assessed 2 weeks post imaging (n=15). Figure 2-1 depicts a timeline for the study.
2.2.2 Microbubble Contrast Agent

FDA approved Definity (Lantheus, North Billerica, MA, USA) was used as the MB contrast agent in this study. The vials were activated in a vial shaker for 45 sec prior to use in imaging (Vialmix Shaker, Bristol-Myers Squibb, New York, NY, USA). As per the Definity package insert, activated Definity possesses a mean diameter range from 1.1 µm to 3.3 µm and a maximum concentration of $1.2 \times 10^{10}$ particles/mL. The contrast injection was prepared by diluting 200 µL of Definity into 400 µL of 0.9% saline. Prior studies have suggested that increased microbubble concentration may increase the likelihood of bioeffects [12], [18], [19]. The dose we used is over 10 times higher than the clinically recommended infusion concentration (1.3 mL of Definity in 50 mL of saline), and over 60 times higher than the recommended double bolus injection (single bolus is 10 µL/kg). We chose this higher concentration in order to test the limits of the parameter range which might be experienced in clinical use.
2.2.3 Ultrasound System

All imaging was performed on a Siemens/Acuson Sequoia 512 (Mountain View, CA, USA) using a 1-4 MHz 4C1 curvilinear transducer. The 4C1 transducer was chosen for this study as it is a common transducer used in human kidney imaging. B-mode imaging was performed at an MI of 1.5 at a frequency of 3 MHz and a frame rate of 21 Hz. Cadence pulse sequence (CPS) software was utilized for performing CEUS imaging at an MI of 0.21, a frequency of 1.5 MHz, and a frame rate of 14 Hz. The microbubble destruction (MBD) or flash pulse was set on the system and performed at an MI of 1.0 or 1.9, a frequency of 3 MHz, and a frame rate of 10 Hz. The duration of the pulse was set to 1 second. Reperfusion of contrast was captured for 5 seconds before the transducer was stepped to the next plane.

2.2.4 Full Volume Imaging Procedure

Rats were placed in the prone position and imaged in the sagittal plane (see Figure 2-2). Ultrasound gel was applied to the imaging area. A custom designed water bath, with an acoustically transparent bottom, was placed on top of the rat so that the ultrasound gel coupled with the bottom of the bath. The bath was filled with water to a height that allowed the focus of the transducer to be set at 60 mm, which is a clinically relevant depth of penetration. As a result of the 4C1 transducer design, and the size of the animal model used, the focus of the transducer covered a majority of the animal’s abdominal depth. The 4C1 transducer was clamped to a 3-axis motion stage and lowered into the water bath.

B-mode imaging was performed and the motion stage was manually manipulated to locate the center of the kidney. Once located, a custom LabVIEW (National Instruments, Austin, TX, USA) program which was synchronized with the ultrasound scanner was used to scan a 1.5 cm region in order to expose the entire kidney volume to the peak ultrasound field.
The scan region was restricted to the right side of the rat, and did not pass over the mid-line of the animal, in order to prevent exposure to the left kidney. Once the positioning of the rat and transducer was complete, microbubble injection began in 1.0 mL syringe (Norm-Ject®, Henke-sass Wolf of America, Dudley, MA, USA) at a rate of 40 µL/min (Definity recommends 4.0 mL/min in humans [27]), which was kept constant with a syringe pump (Pump11, Harvard Apparatus, Houston, MA, USA). After contrast arrival was detected in CPS mode, the 3D flash-reperfusion sequence was initiated.

The custom LabVIEW program (Texas Instruments, Dallas, TX, USA) was designed to mechanically move the transducer in the elevational dimension at a step size of 1 mm. A 1 mm step size was chosen to ensure that every region of the kidney was exposed to the MBD pulse at least twice. The elevational beam width of the transducer is ~2mm, so there was significant overlap of the high energy pulse at each location. At each position, the scanner was triggered by the LabVIEW program to output the MBD pulse, and then wait 5 seconds for sufficient contrast reperfusion. Each 3D scan lasted approximately 2 minutes, with a total infused volume of approximately 80-100 µL. Once complete, animals were continually monitored and kept warm until awake from anesthesia, at which point they were placed back into their respective cages.
Figure 2-2: Imaging Setup. Animals were positioned such that only one kidney would be exposed to the ultrasound field. A) A water bath was placed above the animal so that the focus of the transducer would align with the kidney. B) The transducer was steered across one side of the animal, while the animal was administered isoflurane through a nose cone and a heating pad maintained a healthy body temperature (35.9-37.5°C).

2.2.5 Kidney Blood Urea Nitrogen Chemistry Analysis

Serum BUN levels were used to observe kidney function prior to imaging and at the final time point. Blood preparation included separating the serum using a centrifuge at 1,000-2,000 x g for ~10 mins. Serum was stored in a -25°C freezer, and submitted to the UNC Animal Clinical Chemistry and Genetic Expression Laboratory for BUN analysis. Changes in BUN levels for each experimental group were assessed using paired T-test through MATLAB (MathWorks, Natick, MA, USA).

2.2.6 Histology

At the designated time point, animals were humanely euthanized, and both kidneys were immediately collected. Kidneys from the animals were fixed using formalin for 4-5
days (Azer Scientific, Morgantown, PA, USA) and then stored in 70% ethanol (Decon Labs, King of Prussia, PA, USA). The kidneys were routinely embedded in paraffin, sliced to 5 µm, and stained with hematoxylin and eosin (H&E) by the UNC Animal Histopathology Core. Three slices were chosen randomly by professionals in the histopathology core, and placed on a glass slide. Bioeffects evidenced in the kidney slices were blindly assessed by a veterinary nephropathologist (REC). Quantitative analysis included counting the total red blood cell (RBC) cast score. In this assessment, the number of RBC casts (including fragmented and intact RBCs in tubular lumens) were counted in ten sequential fields of view, across all three slices, at 10X magnification which allowed examination of the entire kidney cortex. The presence of these elements was taken as a sign of hemorrhage in the tissue prior to necropsy. The total value across the kidney sections is referred to as the total RBC cast score, and the average for each group is presented. Statistical assessment was also conducted in MATLAB. Additionally, 100 glomeruli were evaluated per kidney and any glomerulus with GCH (red blood cells within Bowman’s space) was counted.

2.3 RESULTS

2.3.1 Kidney Clinical Chemistry at 4-hours and 2 weeks post imaging

BUN levels 4 hours post imaging, in both the 1.0 and 1.9 MI groups, were significantly different from their baseline measurements (see Figure 2-3). Normal range of BUN levels for female Fischer rats is 19.18 ± 2.39 mg/dL (95% confidence interval of ~15-24 mg/dL) [29]. The 1.0 MI group increased from 23 ± 1 mg/dL to 24 ± 1 mg/dL (P=0.01), which is at the upper limit of the normal range. This reflects a mean percent change of 7%. The 1.9 MI group increased from 18 ± 1 mg/dL to 19 ± 1 mg/dL (P=0.02). Though this group
began with a lower BUN than the animals in the 1.0 MI group, the mean percent change for the 1.9 MI group was 9%, similar to the 1.0 MI group.

BUN levels 2 weeks post imaging in the 1.0 MI group showed no significant change. Average BUN levels in the 1.9 MI group 2 weeks post imaging increased from 17 ± 1 mg/dL to 19 ± 1 mg/dL (P=0.04), which reached statistical significance. This reflects a mean percent change of 12%. Except for the 1.0 MI 4-hour group, BUN levels remained well within normal range.

**Figure 2-3:** Clinical Chemistry Results. Average baseline and endpoint data collected for each group were analyzed via paired T-test. The BUN levels after 2 weeks in the 1.9 MI pulse group were significantly higher than baseline levels. In addition, BUN levels of both groups analyzed at 4 hours were also found to be statically significant. P-values between 0.01 and 0.05 are indicated with the symbol ‘*’. Normal BUN range is indicated with dashed lines (15 to 24 mg/dL).
2.3.2 Histopathology at 4-hours and 2-weeks

Histopathologic review of the kidneys at their final time points revealed no evidence of hemorrhage with any type of pulse transmitted at any time point (see Figure 2-4).

![Histopathology Results](image)

**Figure 2-4:** Histopathology Results. No bioeffects were observed after 1.0 and 1.9 MI pulse or at either time point.

2.4 DISCUSSION

In this study, we utilized clinically relevant acoustic parameters for CEUS imaging in order to investigate potential in vivo bioeffects. Given our results, it is important to consider variations in the protocol used to arrive at our findings, compared to previously published work. First, our experimental methods involved a volumetric scan of the kidney with 1mm steps such that each plane of imaging received at most 2 seconds of flash pulses as opposed to singular plane assessment used by Miller et al. [16], [18], [20], [21], [23] and Jimenez et
al. (2008) [25], in which all flashes were administered in the same plane. The imaging parameters used in this study (center frequency, frame rate, pulse repetition frequency, number of flash pulses per imaging plane, etc.) are more relevant for assessing what is clinically performed, as opposed to multiple (>5) seconds of flash pulses in a single plane which is more useful for determining the minimal threshold for bioeffects to occur. Contrary to this idea, we chose several imaging parameters with the intention of increasing the probability of inducing bioeffects, including increased microbubble dose and flow rate (compared to the clinical recommendation), and a 5 second wait time following flash pulses to increase contrast presence. This was done to focus observations on the clinical acoustic parameters, by testing them in an environment likely to develop bioeffects, as evidenced by previously cited preclinical kidney investigations, where Miller et al. showed that increasing the contrast agent concentration increases the presence of bioeffects [18].

Second, histopathologic assessment was conducted on a subset of kidney tissue, rather than the entire kidney, for each subject. Even though we only examined a subset of kidney tissue, because we performed a volumetric scan, any subset of tissue assessed would have been exposed to flash-perfusion imaging. To provide another measure of kidney function, we also quantified BUN, as a surrogate marker of whole kidney function at the same time points.

In regards to our findings, we observed some similarities to previous work. At the shortest time point, 4 hours post imaging, we observed no tubular injury or hemorrhage whether the kidney received an MI of 1.9 or 1.0, consistent with Jimenez et al. and Johnson et al. [24], [25]. However, we observed a mild elevation in BUN levels for both groups. Although BUN levels remained in the normal range, the statistically significant increase in
BUN levels suggest the possibility of some bioeffects not associated with visible hemorrhage. BUN is a non-specific marker of kidney injury and can be elevated due to a number of factors including high-protein diet, liver disease, and gastrointestinal bleed. In addition, we used the 4C1 probe, which is designed for human imaging, and given our model was a rat, we were unable to limit CEUS imaging to just the rat kidney so adjacent abdominal organs likely received the high MI pulses, which may also have contributed to the small increase in BUN. Histopathologic assessments for this study were conducted in the kidney only. It is also plausible that the elevation in BUN at 4 hours might have been a product of dehydration while recovering from the anesthesia during imaging or a direct result of anesthesia itself which can cause vasodilation and low blood pressures with resultant temporary decrease in organ perfusion.

A key difference between our study and many other previous publications is the inclusion of the longer time point. Miller et al. (2009) showed a decrease in histologic injury from 4 hours to 2 days post exposure to 1.9 MI pulses. By weeks 1 and 4, the location of imaging was difficult to find, indicating some recovery of the earlier seen injury. Histology did indicate signs of inflammation at the 1-week time point, and at 4 weeks, signs of fibrosis were found [20]. Similarly, at 2 weeks, we observed a statistically significant elevation in BUN levels, although still in the normal range, for the 1.9 MI but not the 1.0 MI group. Despite the increase in BUN in the 1.9 MI group, we found no evidence of RBC casts or other findings consistent with acute or persistent chronic injury via histologic examination. Based on these findings, we are unable to identify the cause of the increase in BUN in the absence of hemorrhage and cannot definitively state that the rise in BUN is kidney related. In a pilot study conducted by the authors in order to determine experimental parameters, RBC
casts were found in the tubules of only one group of the pilot study (n=4), the group receiving 1.9 MI pulses assessed 24-hours post imaging (see Supplemental Information located in Appendix A). The pilot differed from the study presented in three ways: (1) contrast imaging following flash pulses lasted for 1 sec, instead of 5 sec, (2) animals were positioned on their side, exposing both the experimental and control kidneys to the CEUS imaging, and (3) short term bioeffects were assessed 24 hours post-imaging. After observing hemorrhaging in the 1.9 MI group, the 24 hour time point was changed to 4 hours to assess if the RBC casts originated in the tubules, or formed in the glomeruli and migrated to the tubules over time. Additionally, with more contrast imaging time, microbubbles circulating would have more time to fill into capillary spaces before transmitting the next flash pulse.

In this pilot study, the hemorrhage was not accompanied by a significant increase in BUN. This could be explained by a low experimental population. It is important to note that both studies demonstrated an increase in BUN levels 2 weeks post imaging, when transmitting at an MI of 1.9. This could be indicative of a potential bioeffect in the clinical chemistry assessment 2 weeks post imaging that the histology assessment did not demonstrate 4 hours post imaging. One explanation for histology differences in the two studies may be a sampling error. A small subset of kidney tissue is being analyzed, in comparison to the total kidney volume. In anticipation of this fact, clinical chemistry was conducted to gather global kidney function. When combining the results of both the main and pilot study, it can be concluded that both studies show some sort of bioeffect (chemical or histologic) after exposure to 1.9 MI flashes. In the case of 1.0 MI, no histological evidence was ever found in either study. There was only the potential for kidney-related bioeffects shown in the BUN increase at 4 hours. Regardless of the study, no indications of bioeffects
were found in the 1.0 MI group being assessed 2 weeks after imaging. This indicates an acute and transient nature to microbubble-mediated CEUS bioeffects, and if there is low level injury occurring at an MI of 1.0 shortly after imaging, it can be avoided by implementing even lower MI MBD pulses during CEUS imaging (0.7 to <1.0).

We believe these findings can be used to guide clinical imaging parameter decisions in order to minimize potential kidney bioeffects. Using a clinical system and probe, clinically-approved microbubble agents, and imaging parameters similar to those that would be used clinically, we were able to assess the potential for bioeffects resulting from flash-replenishment CEUS of the rodent kidney. When considering translation of CEUS imaging parameters to human subjects, it is important to consider that although we focused the transducer at a clinically relevant depth of 6 cm, the ultrasound beam only traveled through less than 1 cm of attenuating tissue, in contrast to a clinical scenario where the ultrasound beam is attenuated by 6 cm or more of abdominal tissue. The MI value we used was based on the derated peak-negative-pressure transmitted to the focus of the transducer. It is likely that in a human patient, even less pressure will reach the focus, compared to the experimented rodent kidney models. Potentially, a given transmitted MI will generate lower levels of injury in human patients than what was observed in the study.

2.5 CONCLUSIONS

Microbubble destruction induced bioeffects during CEUS imaging were observed to be associated with mild elevations in BUN levels and, in the case of our pilot study, histologic indications of hemorrhaging in kidney tubules. The histologic signs of bioeffects were transient, and the BUN levels remained predominantly within the normal range for the
female Fischer rat. Most notably, both of these indications were manageable by implementing lower MI pulses (1.0). By maintaining an MI of 1.0 for MBD pulsing, we were able to eliminate short-term serum elevations in BUN after 2 weeks of recovery. Because there were mild but significant increases in BUN at the 2-week time point in the 1.9 MI group, and kidney injury is one of several potential causes, we suggest that until the possibility of persistent kidney effects can be completely eliminated, this be avoided by operating at a lower MI (such as 1.0 or less) when using MBD pulses for flash-replenishment imaging.

2.6 ACKNOWLEDGEMENTS

The authors would like to thank the staff of the UNC Animal Histopathology Core and UNC Animal Clinical Chemistry Core for their work with this project. Animal histopathology was performed in the LCCC Animal Histopathology Core Facility at the University of North Carolina at Chapel Hill with special assistance from Traci Raley and Amanda Brown. The LCCC Animal Histopathology Core is supported in part by an NCI Center Core Support Grant (2P30CA016086-40) to the UNC Lineberger Comprehensive Cancer Center. Gloria Nyankima was supported by the NIH F31 grant (5 F31 CA206602-03) during the time of the study. Finally, the authors would like to thank the North Carolina Translational & Clinical Sciences Institute for partially funding this project through the awarding of the NC TraCs IHHAR11503.
REFERENCES


CHAPTER 3

AN *IN VIVO* ASSESSMENT OF THE POTENTIAL FOR RENAL BIOEFFECTS FROM THE VAPORIZATION OF PERFLUOROCARBON PHASE-CHANGE CONTRAST AGENTS

3.1 INTRODUCTION

In Chapter 2, we explored the potential bioeffects of MB-mediated contrast-enhanced ultrasound (CEUS). This work, alongside the years of preclinical and clinical studies of microbubble (MB) contrast imaging, has formed the foundation of understanding in vivo biological responses for other developing ultrasound contrast agents (UCAs). For phase-change contrast agents (PCCAs), existing understanding of MB biological response addresses two factors of PCCA technology. First, as a phase-changing agent, the vaporized PCCA will behave similarly to MB contrast agents. Secondly, ultrasound imaging with PCCAs can require high MI acoustic pulses to induce vaporization, followed by low MI contrast imaging of the produced gaseous agent. This sequence is similar to the flash-replenishment imaging technique explored in Chapter 2.

The introduction of PCCAs to ultrasound has the potential to expand the capabilities of diagnostic and therapeutic ultrasound. These agents are formulated with a liquid-core, which enables formulation of smaller diameter contrast agents [1]–[3] as well as increased stability [4], [5] as compared to gas-core ultrasound contrast agents. Decreased size and

---

increased circulation time has foreseeable benefits in various ultrasound techniques, including focused ultrasound surgery and molecular imaging [6]. When utilized for these applications, acoustic pulses are transmitted to vaporize the liquid core in what is referred to as acoustic droplet vaporization (ADV) [7], [8]. Recently, there has been interest in PCCAs developed using lower boiling point perfluorocarbons (PFCs) that require less energy to vaporize (activate) than PFCs which are liquid at room temperature and pressure [9]. These lower boiling point PFCs include decafluorobutane (DFB, boiling point of -2°C) and octafluoropropane (OFP, boiling point of -37°C). Low boiling point PCCAs maintain their liquid phase, even at body temperature, as a result of homogenous nucleation physics and Laplace pressure [2], [5], [10]. The ability to customize droplet design and maximize the benefits of both high and low boiling point PFCs distinguishes PCCA technology from current UCAs. When optimized, PCCAs can be used in applications such as contrast imaging, drug/gene delivery, and therapeutic tissue ablation [1], [6], [11]–[16].

The need to evaluate parameter ranges for nanodroplet use without or with bioeffects has gained importance with advances in the technology’s application. In the gas state, the behavior of PCCAs can be compared to MB contrast agents. As discussed in Chapter 2, the biological response of MB-mediated CEUS varies with parameters, such as dosing, bubble size, acoustic center frequency, transmit pressure, frame rate, and acoustic exposure time. In the case of PCCA-mediated CEUS, there are also concerns of the phase-transition process, which is unique to PCCA technology. To date, little evidence has been presented on the in vivo bioeffects of PCCA vaporization. Investigations observing the phase-change of PCCAs revealed an overexpansion phase, as highlighted in Sheeran et al. [5]. Initially, as the PCCAs undergo a phase change from liquid to gas, the agents rapidly expand beyond the original
diameter before compressing and oscillating around a final diameter. Depending on the type of gas present in the PCCAs, this over-expansion can result in a bubble 6-8 times the initial diameter of the droplet [5]. There is a possibility that this initial expansion with a high wall velocity has the potential to result in additional bioeffects beyond cavitation. Caskey et al. [17] have observed that the oscillation of large microbubbles can affect the integrity of a microvessel endothelium. The presented study was designed to observe the potential for bioeffects during ADV of low-boiling point nanodroplets in the rodent kidney. By examining a range of formulations and acoustic parameters, we aim to understand parameters that would allow for safe use of PCCAs in clinical and preclinical applications.

3.2 MATERIALS AND METHODS

3.2.1 Nanodroplet preparation

PCCA nanodroplets were prepared using a formulation and condensation procedure as described in [2]. The lipid-shell of the contrast agents used in this study were formed from 1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy (polyethylene-glycol)-2000 (DSPE-PEG2000) in a 9:1 molar ratio. The lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and were emulsified in a solution containing 80% (w/v) with phosphate-buffered saline (PBS), 15% (w/v) with propylene glycol, and 5% (w/v) with glycerol, resulting in a lipid concentration of 1.0 mg/mL. Sealed 3mL vials were prepared with 1.5 mL of the lipid solution for a gas exchange procedure, which replaced the air with PFCs. This procedure required connecting the vials to a vacuum line for several minutes, prior to filling the headspace with the PFC. Microbubbles at a concentration of $\sim 10^{10}$ bubbles/mL were
produced after shaking the vials for 45 sec in a Vialmix Shaker (Bristol-Myers Squibb, New York, NY, USA). The resulting microbubbles were condensed into nanodroplets using an isopropanol bath maintained between -7 ℃ to -12 ℃. The vial was swirled in the bath for 1 min, prior to venting the vial and continuing to swirl in the cold bath for an additional 1.5 mins. Following the temperature decline, the bubbles underwent a pressure increase via a custom-designed air-pressurizing device. The pressure in the headspace was increased until the microbubble solution noticeably changed, indicating the phase-change to nanodroplets. The inner core of these agents consisted of decafluorobutane (DFB, C4F10, boiling point of -2 ℃), octafluoropropane (OFP, C3F8, boiling point of -37 ℃), or a 1:1 mixture of the two PFCs. All PFCs used to make these droplets were purchased from FluoroMed (Round Rock, TX, USA).

Characterization of droplet diameter and concentration was completed using a NanoSight NS500 (Malvern Instruments, Westborough, MA, USA). Nanodroplets were diluted 1000-fold in 20nm-filtered, HPLC-grade water. Three vials for each nanodroplet type were measured. Data from all three vials were averaged and presented as mean ± standard deviation (SD).

3.2.2 Animal Preparation

The University of North Carolina at Chapel Hill Animal Care and Use Committee approved all experiments and procedures prior to experimentation. In the study, a total of 48 adult female Fisher rats (see Table 2.1), weighing 150-250g, were used (Charles River Laboratories International, Wilmington, MA, USA). The animals were housed four to a cage and were provided with standard chow and water ad libitum. The kidney was chosen as a model for its sensitivity to acoustic bioeffects, and it is traditionally used as a test tissue in
these studies. Animals were anesthetized with 2% isoflurane (Halocarbon Laboratories, River Edge, NJ, USA) mixed with oxygen delivered continuously to the rat through a nose cone. Animal temperature was maintained through a heated imaging platform. A 24-gauge catheter was inserted in the tail vein to administer a 120 µL bolus of nanodroplet-saline solution. This nanodroplet solution was prepared by taking 60 µL of the stock nanodroplet solution (concentrations shown in Figure 3-1) and 60 µL of saline. Hair removal via shaving and depilatory cream was required for ultrasound imaging. Ultrasound gel was used to couple the transducer to the animal. The transducer was positioned, such that the kidney was viewed in the transverse dimension. Activation was performed over the right kidney (unless otherwise specified), leaving the left kidney as a no ultrasound control. After imaging, animals recovered from anesthesia and were assessed before returning to animal housing. They were then placed back under anesthesia 4-6 hours later for a short period of time to collect blood. The initial study ended after 24-hours, where the animals were euthanized and the kidneys were removed for histopathology assessment. This procedure was done in accordance with the recommendations of the Institution of Animal Care and Use Committee at UNC. In a second study to assess long-term effects, animals were split into two groups (n=4) and one group was sacrificed at two-weeks and the other at four-weeks. After euthanasia, kidneys were collected and processed with the same method as the initial group, and histopathology was assessed in the same manner. No blood collection was conducted in the interim.
Table 3-1: Designated acoustic parameters for experimental groups i-v. For the mechanical index (MI), the maximum MI transmitted in the region of interest is reported. A focused pulse at 5 MHz with 5 cycles per pulse was transmitted in all subjects. For each group presented, an animal number (n) of 8 was used.

<table>
<thead>
<tr>
<th>Group</th>
<th>PFC Type</th>
<th>MI</th>
<th>Scan Length (Step Size) (units=mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>DFB</td>
<td>1.90</td>
<td>20 (1)</td>
</tr>
<tr>
<td>ii</td>
<td>DFB-OFP</td>
<td>1.90</td>
<td>20 (1.5)</td>
</tr>
<tr>
<td>iii</td>
<td>OFP</td>
<td>1.90</td>
<td>20 (1.5)</td>
</tr>
<tr>
<td>iv</td>
<td>DFB-OFP</td>
<td>1.35</td>
<td>20 (1)</td>
</tr>
<tr>
<td>v</td>
<td>OFP</td>
<td>0.81</td>
<td>20 (1)</td>
</tr>
<tr>
<td>Recovery</td>
<td>OFP</td>
<td>1.90</td>
<td>20 (1)</td>
</tr>
</tbody>
</table>

3.2.3 Imaging Procedure

A Verasonics research ultrasound system (Verasonics, Redmond, WA, USA) was used to drive an ATL L11-5 34mm linear array transducer composed of 128 elements (Phillips, Andover, MA, USA). Three pulse sequences were used: B-mode, contrast imaging, and activation. The B-mode imaging sequence was used for anatomical reference and consisted of plane-wave imaging with a 9 MHz, 1-cycle sinusoid at three different angles (-18°, 0°, 18°) for compounding [18]. Nanodroplets were activated using a technique described by Rojas and Dayton, (2016) [8]. This technique required drawing one ROI per slice of the kidney. From the ROIs, a map of focal targets in each plane was created. Each point was approximately 0.75mm axially and 0.5mm laterally apart (Rojas and Dayton, 2016). Activation pulses were separated by a 150 µsec transmit delay. Each pulse was focused, containing 5 cycles at 5 MHz. Activation pulse pressures were designed using an activation pressure matching method. For the clinical maximum activation groups, the transducer was driven such that a pressure resulting in a MI of 1.9 was delivered at each focal point. For this study, MI was defined as the peak negative pressure derated by 0.3 $dB \text{ cm}^{-1} \text{ MHz}^{-1}$ and divided by the square root of the center frequency of the transmitted pulse. For the activation
threshold energy groups, activation pressures were determined for each nanodroplet formulation in vivo by varying transmitted voltages. The lowest voltage with full coverage of the kidney was designated as the formulation’s minimum activation threshold acoustic parameter. During experimentation, that pressure was delivered throughout the region of interest (drawn using B-mode images). To accommodate for changes in depth, this minimum pressure was maintained by anticipating pressure attenuation and optimizing the voltage transmitted, using the activation pressure matching method. For DFB, the minimum pressure resulted in an MI above 1.9, and therefore this parameter was not used in the activation threshold study. Activation of droplets was verified using plane wave, pulse inversion, contrast imaging, transmitted at 4.5 MHz, at several different angles, with a pulse repetition frequency of 5 Hz. A preliminary study was conducted to assure imaging sequences alone would not cause renal bioeffects (see Appendix B, Table B-1). At acoustic parameters higher than what was implemented in this study, no bioeffects were observed.

In order to expose the entire kidney, a motion stage was controlled with a custom LabView (Texas Instruments, Dallas, TX, USA) script, and synchronized with the Verasonics to sweep the transducer in the cranial to caudal dimension and vaporize droplets in subsequent planes of the kidney. A 2-cm scan length was used, and a step size of 1mm was chosen for all groups except for group ii and group iii (the 1:1 mixture and OFP minimum activation groups). A step size of 1.5mm was used for these groups to minimize presence of vaporized microbubbles in adjacent planes.

3.2.4 Long-term Observation Imaging Procedure

In order to assess the presence of chronic bioeffects, a cohort of 8 animals were separated into two groups (n=4). One group was examined at two weeks, while the other was
assessed at four-weeks. OFP nanodroplets were vaporized at an MI of 1.9 in one kidney of each animal. Serum creatinine levels were assessed prior to imaging and at the time of necropsy. The formulation and acoustic parameters were decided for this study after observing the 24-hour histopathology of all five groups. As shown below, OFP at an MI of 1.9 developed the highest levels of bioeffects. All other imaging parameters and animal procedures were kept the same as with the 24-hour observation.

3.2.5 Serum Creatinine Analysis

Blood was collected from each rat before imaging (0 hours), 4-6 hours, and 24 hours after imaging. For the long-term recovery assessment, blood was only collected prior to imaging and at the time of autopsy. A 24-gauge tail-vein catheter was used for the collection of blood. Blood collected at the time of autopsy was taken from the abdominal artery. Collected blood was spun for 10 mins at 1,000-2,000 x g and the plasma was removed and kept frozen in a -25°C freezer. Samples were taken to the Animal Clinical Chemistry and Gene Expression laboratory at UNC for creatinine analysis.

To assess for kidney injury, the Acute Kidney Injury Network (AKIN) classification was applied. This system was designed for a clinical setting, where often healthy baseline kidney biomarkers are absent. The classification includes 3 stages, which correlate with increasing severity of acute kidney injury (Lopes and Jorge, 2013). A serum creatinine percentage increase of ≥150-200% indicates stage 1, ≥200-300% indicates stage 2, and ≥300% indicates stage 3 (Lopes and Jorge, 2013).
3.2.6 Histology

3.2.6.1 H&E-stained Tissue Analysis

Both left and right kidneys were sliced in half and fixed in formalin for 24 hours. After formalin fixation, both halves of both kidneys were routinely processed, embedded in paraffin, and sectioned. Samples were stained with hematoxylin and eosin and evaluated by a board certified veterinary nephropathologist (REC). For quantification of lesions, ten fields of the central portions of each kidney were examined at 10X magnification (as detailed in Appendix B, Table B-2). Additional pre-existing lesions (e.g. renal cortical cysts, chronic pyelonephritis) were noted in the event that they could be correlated with the ultrasound findings.

3.2.6.2 Transmission Electron Microscopy Analysis

One animal from each group, chosen at random, was used for ultrastructural analysis, and a 1-2mm cube of the right kidney was fixed in 3% chilled glutaraldehyde. The glutaraldehyde-fixed samples were post-fixed in 1% osmium tetroxide and then dehydrated serially. After infiltration in acetone / epoxy plastic, they were embedded in epon blocks for ultramicrotomy. One µm thick sections were then stained with toluidine blue to evaluate for the presence of glomeruli and tubules that contained red blood cell casts. Epon blocks that contained the regions of interest were then further sectioned at a silver-grey interference color of 55 to 60nm and the sections were places on copper mesh grids. Samples were stained with uranyl acetate and lead citrate. These samples were examined on OEL JEM-1400 transmission electron microscope (JOEL USA, Inc., Peabody MA, USA) and representative images of each specimen (glomeruli and tubules) were obtained with an
Olympus SIS Veleta 2K camera (Olympus Soft Imaging Solutions GmbH, Munster Germany).

3.2.7 STATISTICAL TESTING

A paired T-test was conducted on the quantitative assessment of histopathology in order to compare the experimental kidney, which received ultrasound exposure, to the control kidney. As well, an analysis of variance (ANOVA) test was conducted on the experimental kidneys of the groups exposed to the clinical maximum mechanical index. This test attempted to answer whether the formulation (or PFC core) produced differences in observed bioeffects. Statistical significance was decided to be an alpha value of 5% (p<0.05) a priori. All testing was conducted through MATLAB-generated scripts (MathWorks, Natick, MA, USA).

3.3 RESULTS

3.3.1 Nanodroplet Characterization

The three formulations of PCCAs were characterized for size distribution and concentration. As Figure 3-1 shows, nanodroplets are polydisperse, with peak diameters ranging from 100 to 300nm. Averaged mean diameters and standard deviations for PCCAs formulated with DFB, OFP, and 1:1 DFB-OFP were found to be 212.6nm ± 13.0nm, 186.5nm ± 28.2nm, and 212.6nm ± 22.6 nm respectively. In the same manner, the concentration of all three formulations were found to be 3.6x10^{11} ± 6.9x10^{10} particles/mL for DFB, 3.9x10^{11} ± 8.9x10^{10} particles/mL for OFP, and 3.7x10^{11} ± 5.2x10^{10} particles/mL for the 1:1 mixture nanodroplets.
Figure 3-1: Phase-change contrast agent (PCCA) size distribution (as a function of concentration) for all three formulations tested, including decafluorobutane (DFB), octafluoropropane (OFP), and 1:1 DFB-OFP mixture. Size distributions represent averages from three vials.

3.3.2 Serum Creatinine Levels

Creatinine was measured at three time points: 0, 4-6, and 24 hours post-imaging. Not all of these three time points were collected for each subject, as seen in Table 3-2, due to insufficient blood collection or separation of serum.
Table 3-2: Serum creatinine levels at all three time points for the 24 hour observation are presented by group: (i) Decafluorobutane (DFB) at max, (ii) Mix at max, (iii) Octafluoropropane (OFP) at max, (iv) Mix at min, (v) OFP at min. Data points indicated with a (*) at the 24 hour time point denote an increase of or greater than 150% (from baseline). This aligns with the serum creatinine requirements in the Acute Kidney Injury Network (AKIN) classification for indicating potential acute kidney injury. For a female Fischer rat, the 95% confidence interval for serum creatinine level is 0.2-0.6 mg/dL [19].

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Group i 0</th>
<th>Group i 4</th>
<th>Group i 24</th>
<th>Group ii 0</th>
<th>Group ii 4</th>
<th>Group ii 24</th>
<th>Group iii 0</th>
<th>Group iii 4</th>
<th>Group iii 24</th>
<th>Group iv 0</th>
<th>Group iv 4</th>
<th>Group iv 24</th>
<th>Group v 0</th>
<th>Group v 4</th>
<th>Group v 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>0.3</td>
<td>0.4</td>
<td>0.3</td>
<td>0.6</td>
<td>0.4</td>
<td>0.4</td>
<td>0.7</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>1.1*</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
<td>0.3</td>
<td>0.5*</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4*</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
<td>0.3</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.1</td>
<td>0.5</td>
<td>0.5*</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.7</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
<td>0.6</td>
<td>0.4</td>
<td>0.6</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
<td>0.3</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>7</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
<td>0.2</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.2</td>
<td>0.8*</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>8</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
<td>0.4</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

The results of the 24-hour observation showed rats with creatinine levels that could potentially be classified as acute kidney injury under AKIN. In Table 3-2, these results are indicated with a * to denote an increase of and above 150% (24-hr/0-hr x 100%). Of the five groups presented in Table 3-2, three of the groups expressed creatinine increases above 150%. In group ii (1:1 mix PCCAs activated at MI of 1.9), three animals increased by 200%. In group iii (OFP PCCAs at MI of 1.9), subjects expressed increases in creatinine levels ranging from 200% up to 500%. Finally, in group v (OFP PCCAs at a minimum activation level), one animal’s creatinine level increased above 150%.

In the long-term study (see Table 3-3), one animal in the two-week observation time point group and one animal in the four-week observation expressed an increase of 225%. Most of the other subjects in their respective groups showed little or no increase in creatinine.
Table 3-3: The clinical chemistry and histology results of the recovery study show minimal bioeffects after two and four weeks of recovery. No red blood cell (RBC) casts were found in either the two- or four-week recovery subjects. Creatinine levels for one subject in each group showed an increase of >200%, which has been indicated with a (*). For a female Fischer rat, the 95% confidence interval for serum creatinine level is 0.2-06 mg/dL [19]

<table>
<thead>
<tr>
<th>Subject No</th>
<th>Experimental RBC Cast</th>
<th>Control RBC Cast</th>
<th>Creatinine Levels (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>End-point</td>
</tr>
<tr>
<td>Two-Week</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>Four-Week</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
</tr>
</tbody>
</table>

3.3.3 24-hour Qualitative Assessment: Histopathologic and Ultrastructural Evaluation

In all animals, the non-imaged kidneys were within normal limits for adult rats. Red blood cell (RBC) casts were not observed, but some animals had minimal to mild interstitial inflammation and associated tubular basophilia, typical of the earliest lesion of chronic progressive nephropathy of rats. Some treated kidneys had varying degrees of red blood cell casts in both the cortex (Figure 3-2a) and medulla (Figure 3-2b). These casts range from intact red blood cells, to fragmented red cells admixed with hemoglobin crystals, to casts consisting of red blood cell components with, and increased frequency of pyknotic nuclei. At later time points there was evidence of tubular cytoplasmic basophilia and scattered mitotic figures, indicative of tubular epithelial restitution in a proteinaceous matrix. The epithelial cells in the cortical tubules which contained RBC casts were characterized by loss of the apical brush border (so-called “simplification”), cytoplasmic vacuolation.
Ultrastructural evaluation of 4 rats was also performed. Tubules containing erythrocytes within the lumen also had evidence of epithelial cell injury, consisting of loss of the apical brush border and mitochondrial swelling with loss of cristae (Figure 3-2c). The glomerular capillary walls were always within normal limits; however, there was evidence of podocyte and parietal epithelial injury. Podocytes had foot process effacement, whereas there was cytoplasmic rarefaction and mitochondrial swelling of both cell types (Figure 3-2d).
Figure 3-2: Photomicrographs of kidneys from a rat in group iii. In the cortex (a) there are many red blood cell casts within proximal tubules (arrows). One tubule also contains sloughed cellular debris (circle). There is mild interstitial inflammation in response to the tubular injury. The glomerulus is normal. Within the medulla of the same rat (b), red blood cell casts can be seen in collecting duct lumens (arrows) and there is also sloughed cells within a duct lumen (oval). Scale bars (bottom right corner) in (a) and (b) represent a length of 20µm. Transmission electron micrographs of a rat from group iii revealed that tubules with intraluminal red blood cells (c). The red blood cells (R) were found in the lumens (L) of tubules with intact apical brush borders (BB). The epithelial cells (E) had scattered electron dense material within cytoplasmic lysosomes. The glomerular capillary walls of this rat (c) were normal. Podocytes had segmental foot process effacement (not shown). Parietal epithelial cells (PEC) had disruption of the cell membrane and marked mitochondrial swelling. Scale bars (bottom right corner) in (c) and (d) represent a length of 10µm.

3.3.4 24-Hour Quantitative Assessment: Activation Threshold Group

Kidney bioeffects were quantified based on total red blood cell (RBC) cast found in randomly chosen sections of the kidney. Total RBC casts score for the activation threshold
groups (MI=0.81 for OFP and 1.35 for the 1:1 OFP-DFB mix) demonstrate absence of bioeffects (Figure 3-3). When analyzed against the control kidneys, paired T-test indicated no significant difference for either group. The mean and standard error of the mean (SEM) for total RBC cast for the 1:1 mixture and OFP nanodroplets were found to be $2 \pm 1$ casts and $1 \pm 1$ cast, respectively. Both groups also had control kidneys with an absence of RBC casts.

### 3.3.5 24-Hour Quantitative Assessment: Clinical Activation

The maximum energy activation pulses were applied to all three nanodroplet formulations. The results can be found in Figure 3-3. For DFB PCCAs, a mean RBC cast count of $19 \pm 4$ (Mean ± SEM) was observed for the experimental kidney. Using a paired T-test, this was found to be a significantly higher RBC cast mean than in the control kidneys ($p=0.002$). The total RBC cast score caused by 1:1 mixture PCCAs averaged $25 \pm 4$ casts. The experimental kidneys from these subjects were also statistically higher than the control kidneys ($p=0.0002$). The OFP PCCA cohort developed RBC cast with an average of $28 \pm 13$, the highest average of the three formulations, but also the highest SEM. Though it was found that the experimental kidneys were not statistically different from their control kidneys, the test was close at $p=0.06$, given an acceptance p-value of 0.05. The range of total RBC cast scored in this group was [0-100], with a median of 13.5. This wide distribution may explain the strong trend but lack of statistical significance. The control kidneys for all animals exposed to an MI of 1.9 showed no RBC cast.

The experimental kidneys of the three formulations in the clinical maximum group were compared using an analysis of the variance (ANOVA) and were found to be statistically the same ($p=0.68$). A Tukey-Kramer multiple comparison test resulted in the following individual results: OFP vs. 1:1 mix ($p=0.96$), OFP vs. DFB ($p=0.66$), and DFB vs. 1:1 mix.
(p=0.82). This means the type of PFC used does not seem to affect the amount of RBC cast presented 24-hours post imaging, when transmitting at a mechanical index of 1.9.

![Boxplot](image.png)

**Figure 3-3:** Total red blood cell (RBC) casts presented as boxplots for all five groups observed (labeled with the perfluorocarbon and mechanical index used). Each group contains the experimental kidney (Exp) alongside its paired control (Con). Data is shown with the interquartile range as the box edges and the median data point is presented as a line inside the box. Circular points (‘o’’) indicate outliers, (defined as points beyond 1.5 times the interquartile range). Statistically significant values are indicated with an asterisk (*).

### 3.3.6 Long-Term Bioeffect Assessment

Results for both the clinical chemistry and histology assessments can be found in **Table 3**. One subject at each time point showed elevated creatinine levels from 0.4 to 0.9 mg/dL. In the AKIN classification, this correlates to a stage 2 acute injury. When assessing histology for bioeffects, no RBC casts were found for any of the parameter ranges tested at two or four weeks.
3.4 DISCUSSION

Nanodroplet-induced bioeffects were assessed using two measurements—level of serum creatinine and histopathology of kidneys. In the moderate MI activation threshold groups, (MI=0.81 for OFP and 1.35 for the 1:1 OFP-DFB mix) creatinine level remained normal. Between the two formulations observed, only one animal (exposed to OFP nanodroplets) increased in creatinine, and under the AKIN classification, it was stage 1. The histopathology of the activation threshold groups also showed absence of bioeffects. Both groups of experimental kidneys exposed to OFP and 1:1 mixture droplets were statistically insignificant from their control kidneys, which experienced no intentional vaporization of PCCAs.

By increasing the mechanical index to 1.9, evidence of bioeffects began to emerge in all three formulations. The creatinine levels for subjects in the 1:1 mixture and OFP groups showed signs of acute injury. The rise in biomarker was as high as stage 3 in the OFP-exposed kidneys (group iii in Table 3-2). Histopathology also supports evidence of the acute injury. Total RBC casts scores for both the DFB and 1:1 mix PCCAs were found to be statistically higher than that of their control kidneys. Although we initially hypothesized that the differences in volatility of DFB and OFP would translate into different levels of bioeffects, a comparison between the experimental kidneys for all three formulations at the clinical maximum MI illustrated that the degree of bioeffects was not statistically different.

Variations in PFC volatility may have impacted the study in another manner. OFP nanodroplets vaporized at an MI of 1.9 demonstrated a wide range of total RBC casts score, as seen in Figure 3-3. This may be explained by the volatility of the PFC, which we understand can spontaneously vaporize at body temperature [10], [20]. Because this is a
phenomena harder to control, certain kidneys may have experienced more bioeffects as a result of bubble destruction than droplet vaporization. Further experimentation must be conducted to better understand the origins of the bioeffects observed in this study, in order to confirm this hypothesis.

When allowed to recover, our study showed that histopathology indications for acute injury observed after 24 hours were absent. This recovery was observed at both two- and four-week time points, and no residual evidence of injury was observed histologically at either time. There were two instances of elevated creatinine: one animal at two weeks and one at four weeks. It is difficult to determine the cause of the elevated creatinine based on histology alone. Further investigations would be necessary.

It is worth noting that some degree of hemorrhage also occurs with a renal biopsy, although the magnitude of this bioeffect compared to ultrasound-induced hemorrhage has not yet been studied.

There are factors to consider given limitations to the experimental design. We utilized computer controlled 3-D scanning to ensure the entire kidney was acoustically sonicated in each experiment. Although this is an improvement over many prior studies that exposed only a single plane of the kidney to ultrasound, our study remains limited in that only a subset of tissue was provided for histology assessment and quantification. However, kidneys were sectioned in a perpendicular direction to the movement of the motion stage, ensuring sonicated tissue/histology overlap. The AKIN classification used to analyze the creatinine results was designed for human patients and not validated in rodent models. Finally, the extent to which these factors effect healthy kidneys may not stand true when observed in a diseased model.
The ability to minimize or maximize bioeffects with PCCAs has benefits across the field of ultrasound. In therapy, PCCAs can be implemented as a dynamic tool at the discretion of the physician. Bioeffect regimes enhanced by nanodroplets could be advantageous for an ablative approach to treating tumors and unwarranted solid masses, as has been described previously [21]–[23]. With the same technology, treatment techniques, such as sonoporation or hyperthermia, may be performed utilizing acoustic parameters that would generate minimal or temporary bioeffects. In the area of diagnostic ultrasound imaging, systems can be designed for PCCA imaging without bioeffects.

3.5 CONCLUSION

There are three findings we can conclude from this study. (1) PCCA vaporization can be achieved in the rodent kidney with moderate mechanical indices (0.81-1.35) without inducing bioeffects. (2) Using a mechanical index of 1.9 with PCCA can cause bioeffects in the rodent kidney. (3) Observed bioeffects at MI=1.9 were acute, mainly a result of hemorrhaging in the renal tubules, and the kidney appears to recover from these bioeffects after two weeks, with no histopathologic evidence of the injury.

3.6 ACKNOWLEDGEMENTS

Funding for this work was provided in part by National Institutes of Health F31CA206602 (A.G.N), F31CA196216 (J.D.R.), as well as R21EB021012 with collaborator Dr. Charles Caskey, at Vanderbilt University, and R21EB021103, with collaborator D. Elisa Konofagou at Columbia University.
The authors would like to thank Dr. James Tsuruta for his work supplying the microbubble formulation for the included study. Additionally, authors would like to thank all core staff at the Animal Clinical Chemistry and Animal Histopathology for their services that aided this project. Animal histopathology was performed in the LCCC Animal Histopathology Core Facility at the University of North Carolina at Chapel Hill with special assistance from Traci Raley and Amanda Brown. The LCCC Animal Histopathology Core is supported in part by an NCI Center Core Support Grant (2P30CA016086-40) to the UNC Lineberger Comprehensive Cancer Center. Lastly, the authors would like to thank Alan Flechtner from the Comparative Mouse Phenotyping Shared Resource (Cancer Center Support Grant - P30 CA016058) for electron microscopy specimen preparation.
REFERENCES


CHAPTER 4

UTILIZING THE POTENTIAL BIOEFFECTS OF PHASE-CHANGE CONTRAST AGENTS FOR THERAPEUTIC ULTRASOUND

4.1 INTRODUCTION

The present standard of care for patients diagnosed with cancer includes surgery, radiation, and/or chemotherapy. Though mostly effective, these methods come with their own set of concerns and limitations. Chemotherapy and radiation are often accompanied with uncomfortable and painful side effects, while surgery has varying risk for complications for the patient. Considering an estimated 1.7 million people were diagnosed with cancer in 2018 in the United States alone [1], minimizing these issues when possible with alternative techniques can improve the quality of life of many future cancer patients. Furthermore, the growing concern of health care cost emphasizes the need for novel inexpensive technologies. Therapeutic ultrasound, or the broad use of acoustic energy for therapeutic outcomes, can be applied in support or replacement of existing technologies in cancer treatment, from the removal of diseased tissue as an alternative to surgery or facilitate the uptake of chemotherapy into impenetrable tumors.

Ultrasound has many advantages in tackling these challenges. Ultrasound as a medical imaging system is noninvasive, nonionizing, portable, and cheaper than most imaging systems. In terms of therapy, ultrasound can be directed to affect a small volume (sub-cubic millimeters depending on frequency), allowing for control of therapeutic activity and increased safety with treatment. The duality of ultrasound as a therapeutic and imaging system provides a simple mechanism for monitoring and guiding therapy, with few
challenges in aligning the two systems, when comparing the use of other imaging modalities for monitoring (e.g. MR-guidance).

4.2 BIOEFFECTS OF ULTRASOUND

In designing a strategy for therapeutic ultrasound, it is desirable to understand the innate physics of acoustic waves traveling through tissue. Studies exploring biological response of tissue to ultrasound date back as far as the mid-20th century [2], [3]. There are two main bioeffects, or biological responses of tissue, associated with an acoustic field traveling in vivo—thermal and mechanical. Thermal bioeffects are caused by the absorption of acoustic energy in the form of heat due to frictional losses as the wave travels through the viscoelastic medium. Ultrasound, being a mechanical wave, pushes the tissue, and the friction induced from tissue movement generates heat, which then dissipates and is absorbed by the surrounding medium [4], [5]. Mechanical bioeffects (or non-thermal bioeffects) are largely associated with the creation and oscillation of gas bubbles by the acoustic wave. As the bubbles oscillate, they push and pull surrounding fluid, exerting mechanical forces on tissue [6]. Mechanical bioeffects also include the generation of shock waves, which can be powerful enough to injure tissue and calcified materials [7].

Ultrasound is a unique tool in the field of therapeutic medicine because of its ability to manipulate tissue for desired outcomes. This property of ultrasound has resulted in the wide range of methods in the field of therapeutic ultrasound at varying levels of acoustic intensity. Sonoporation is a method in which ultrasound is used to temporarily, mechanically disturb cells, facilitating extravascular and intercellular delivery of therapies [8]–[10]. Sonoporation has enabled opening physiological barriers, such as endothelial tight junctions
and efflux transporters, which is characteristic of the vascular system of the brain. As well, sonoporation can be used to reduce off-target effects associated with whole body drug circulation [11], [12]. Thermally, researchers have seen promising results by inciting small degrees of temperature change, known as hyperthermia, to improve cellular activity and drug delivery [13], [14]. Alternatively, therapeutic ultrasound can be used to incur permanent damage, with techniques like histotripsy and thermal ablation. Histotripsy utilizes the generation of shock waves to mechanically fragment unwarranted soft tissue [15]. When treating calcified materials, such as kidney stones, this technique is known as lithotripsy. Thermal ablation rapidly heats tissue beyond the threshold of cellular survival, subsequently denaturing necessary proteins and inducing cellular death [16].

In order to generate these biological responses, investigators utilize acoustic parameters, including frequency, pressure amplitude, duty cycle, pulse repetition frequency (PRF), and total exposure time to increase or minimize therapeutic outcomes. These parameters vary significantly from one technique to another. Generally, when thermal bioeffects are desired, pulse length or duty cycle is extended and exposure time is increased. Depending on target location and the transducer used to conduct treatment, any generalization of acoustic parameter preferences may need to be readjusted. In the United States, the Food and Drug Administration (FDA) has set limits to minimize thermal and mechanical bioeffects during diagnostic ultrasound (see Chapter 1 for more detail). These regulations do not apply for therapy, and parameter guidelines are determined on a case-by-case basis during the FDA’s regulatory approval process.
4.3 ULTRASOUND CONTRAST AGENTS

While therapeutic ultrasound has shown promise, there can be challenges with the acoustic energy requirements to achieve desired therapeutic response. With increased acoustic requirements comes increased risk of off-target effects. Many researchers hypothesize these hurdles facing therapeutic ultrasound progression and adoption can be solved with ultrasound contrast agents (UCAs). Microbubble (MB) contrast agents are readily used in diagnostic ultrasound as a blood-marker. Since their size ranges from 1-10µm in diameter [6], they are retained in the circulatory system. For this reason, and a half-life on the order or minutes, researchers have looked to a liquid-based contrast agent for increased stability and vascular escape. These properties are advantageous for therapeutic applications. Phase-change contrast agents (PCCAs), or nanodroplets, possess properties ideal for the potential long surgical times of therapeutic ultrasound. As a result of their liquid core, PCCAs have an increased in vivo stability [17], and a spatial selectivity property lacking in MB technology. PCCAs require an activation stage, known as acoustic droplet vaporization (ADV), where acoustic pulses are transmitted to vaporize a PCCA liquid core to a gas, producing an agent similar to a MB. In this state, the agent can be utilized for sonoporation or thermal ablation. By designing the PCCA for a particular ADV threshold, the desired therapeutic activity can be limited to locations where ADV pulses have been transmitted, enabling a level of safety and precision missing in MB-mediated therapeutic ultrasound.

As a result of the many potential benefits, PCCAs have been explored for techniques, such as histotripsy [18], [19], photothermal therapy [20], [21], and sonothrombolysis [22]. In the field of oncology, the promise of opening barriers to enhance drug delivery has great promise [23], [24]. PCCA-mediated focused ultrasound is not limited to oncology. It is
important not to forget, PCCAs can be utilized in the field of diagnostic imaging. The therapeutic and imaging duality of PCCAs, known as theranostics, has potential for enabling the visualization of drug delivery, as both a vehicle for carrying drug/gene therapies and as an agent for opening barriers [25]–[27].

As the use of PCCAs in therapy develops, researchers will be faced with optimizing nanodroplet formulation and ultrasound parameters given disease pathophysiology and anatomical location. Ultimately, treatment protocols will be dictated by the in vivo tissue bioeffects desired for safe and effective outcomes. In the following, I will focus this discussion on sonoporation and thermal ablation, to set the foundation for studies detailed in following chapters. I will discuss mechanisms behind sonoporation and thermal ablation, and how PCCAs can improve safety and efficacy of these two techniques.

4.4 SONOPORATION

Sonoporation is a technique in which ultrasound is utilized to open physiological barriers for drug and gene delivery. The presence of UCAs in the acoustic field act as a necessary source for cavitation to produce mechanical effects. In the absence of UCAs, acoustic thresholds for sonoporation would need to be higher to generate gaseous bubbles in tissue to be the cavitation source [28]. As a theranostic agent, the use of UCAs for sonoporation also allows monitoring location of therapy with ultrasound imaging [29]. UCAs can be outfitted with the therapeutic agent, acting more as a vehicle for drug delivery (as opposed to co-administration of UCAs and therapy), or with molecular targets to enable biological specificity for therapy [25].
The mechanism behind sonoporation is not yet fully understood but several phenomena are associated, including stable and inertial cavitation, shear shock waves, microstreaming, and microjets capable of puncturing holes in nearby cellular membranes [30]. Acoustic microstreaming involves the vortical flow of liquid, imparting shear stress on cellular membranes. Microstreaming can be associated with stable cavitation of MBs in an acoustic field, where MBs oscillate in a linear and symmetrical manner [28]. It is thought that this is the predominant mechanism of action during sonoporation therapy. In addition to microstreaming, liquid microjets can form during sonoporation from the rapid collapse of a MB, known as inertial cavitation. This is often associated with an acoustic field of increased intensity from stable cavitation. Inertial cavitation can also produce shock waves that can generate high stresses on cellular membranes, leading to passages for drug delivery [28]. More investigation is needed to elucidate the direct mechanism behind sonoporation. It is likely that a combination of these actions are at play, and also, mechanisms depend on acoustic parameters [30].

By incorporating PCCAs for drug delivery, advantages of longer in vivo circulation and treatment spatial selectivity associated with PCCA-technology over MB contrast agents can be exploited. This has been demonstrated in preclinical prostate tumors [31], fibrosarcoma [32], and chemotherapeutic delivery of polymeric PCCAs [33], [34]. In terms of sonoporation, MBs do have an edge in terms of clinical translation. As a result of regulatory approval for MBs, clinical investigations have demonstrated potential efficacy in using sonoporation to increase drug uptake in pancreatic tumors, a prognosis with low survival rates [35].
Of all the physiological barriers, the blood-brain barrier (BBB) is of particular interest in the field of therapeutic ultrasound. The landscape of existing neurological medical technology for improving therapeutic outcomes is limited. Ultrasound has a unique potential to overcome these challenges. While there are small molecule drugs that can overcome the BBB, these drugs effect the entire brain. Focused ultrasound can target a specific area, allowing passage of therapies for several hours [36]. To date, the large attenuation from the skull is still a challenge for transcranial therapeutic ultrasound, but developments in the field are being made. PCCAs have been shown to advance BBB disruption, even in comparison with MBs [37], [38]. In terms of clinical translation, current trials are being conducted with regulatory approved MBs [39].

Similarly to the understanding of the mechanism behind sonoporation, the ideal acoustic parameters for safe and effective in vivo sonoporation is still being optimized. The acoustic parameters of interest are transmit frequency, pressure (specifically, peak-negative pressure), duty cycle/pulse length, and time between pulses [27], [40]. While this work was not conducted with PCCAs, it is expected that the vaporized PCCAs will possess the same trends as commercial MBs. In deciding acoustic parameters for sonoporation treatments, it is vital to make determinations based on optimal MB response. It is difficult to set delineations on MB cavitation. Often, mechanical index (MI) is used, but MI does not perfectly estimate MB response, particularly thresholds between stable and inertial cavitation. An MI of 0.8 has been recommended as the threshold to stay below to maintain stable cavitation during diagnostic imaging [40], but other parameters (exposure time, MB size) can impact this threshold. In regards to therapeutic outcomes, it is also important to consider treatment location when considering transmit frequency. While higher frequencies carry more acoustic
energy, they also attenuate faster, limiting the penetration depth for therapy. A survey of literature will elucidate sonoporation treatments being conducted around 1 MHz, though transcranial locations are typically treated at lower frequencies [40]. Qin et al. [27] notes a range of frequencies and pressures, which have demonstrated vasculature injury, including 1-5 MHz and 0.5-2.5 MPa.

Sonoporation is also conducted with longer pulse lengths of higher duty cycles [27]. This parameter allows for longer MB response in an acoustic field, leading to increased physiological responses. The trade-off for increasing pulse length is a worse axial resolution. By allowing for time in between pulses, Wible et al. [41] have shown increased vascular bioeffects when MBs are able to refill small capillaries prior to the transmission of subsequent acoustic pulses. In addition to these acoustic parameters, contrast agent parameters, like increased mean diameter and increased contrast agent dosage, should also be considered when designing sonoporation treatments to improve therapeutic delivery [27].

PCCAs have great potential as a tool for overcoming physiological barriers and as a vehicle for therapies. One remaining challenge is translating PCCAs for clinical implementation. This means optimizing PCCA fabrication for ideal size ranges to exploit the leaky vasculature of tumors. In Chapter 6, I will investigate methods for potentially achieving this goal.

4.5 THERMAL ABLATION WITH HIGH-INTENSITY FOCUSED ULTRASOUND

Ultrasound for thermal ablation, also known as high-intensity focused ultrasound (HIFU), has seen major achievements with FDA approval of the technique for prostate
cancer, uterine fibroids, essential tremors, and painful bone metastasis [42]. Advancements for HIFU have been hindered from wide-spread clinical implementation in the United States for several reasons [4], [43]. First and foremost, surgical time is long. A given ultrasound focus can be around a cubic millimeter. Depending on the size of the solid mass, or the number of masses being treated, surgical times can take several hours. Second, real-time monitoring of treatment is slowly progressing. In the United States, the main source of guidance is MR imaging, as a result of advancements in MR thermometry. Ultrasound guidance techniques are improving, particularly due to ease of alignment and calibration between the therapy and imaging elements. Additionally, ultrasound possesses real-time imaging capabilities, lacking in clinical MR systems. Third, there is a lack of standardization of dosimetry with the technology. The field has a wide-range of ways to characterize treatment parameters. Organizing and regulating this so that everyone is using the same language when describing treatment strategies is imperative, not only for clinical studies but in preclinical experiments leading to the clinic. Standardization of dosimetry should also impact the calibration and characterization of hardware associated with the treatment. Finally, the field is working to minimize off-target bioeffects. These off-target symptoms often occur in the near-field, along the path the beam travels to reach the focal point.

HIFU is driven by the absorptive property of tissue to irreversibly heat the target region. Injury to the target is mainly a result of coagulative necrosis—a type of necrosis characterized with maintained cellular architecture and absent nuclei [4], [16], [44]. In designing HIFU treatments, treatment frequency greatly impacts two major factors: absorption and penetration depth. Higher frequency acoustic waves are absorbed more readily than lower frequencies. As a consequence of increased absorption, higher frequency
acoustic waves have a shorter penetration depth. For this reason, HIFU treatment parameters are designed more so for the desired target location, with treatments ranging from several hundred kilohertz to 8 megahertz [16]. When treatments are designed for shallow targets, higher frequencies can be implemented for efficient heating. The lower frequency HIFU treatments can still be effective as a result of nonlinear acoustic generation in the form of shock waves at the beam’s focus [45]. As acoustic waves travel through tissue, the frequency bandwidth of the wave increases, potentially forming a shock wave. This is a result of the peak-positive (compressional) part of the wave, traveling faster than the peak-negative (rarefactional) part of the wave. The generation of higher frequency content is useful for improving ablation efficacy. Typically, HIFU parameters will produce these higher harmonics at the focus [4]. In addition to frequency, parameters like high amplitude and long pulse durations directly impact the degree of tissue heating during a HIFU treatment.

The major side-effect of HIFU therapy is off-target heating effects. The presence of MBs during thermal ablation provides a source of cavitation to enable heating of tissue at lower acoustic output than with HIFU alone. In the case of thermal ablation, there are benefits in both stable and inertial cavitation. Stable cavitation provides a continuous source of frictional heating, while inertial cavitation results in a rapid and destructive collapse of a bubble, capable of producing nonlinear acoustic waves [45]. Benefits of MB-mediated HIFU have been shown in studies like, Tran et al. [46] and Coussios et al [47]. Moyer et al. [48] demonstrated the major short-coming of HIFU with a non-specific UCA. With MBs, near-field heating along the treatment path was still an issue. Additionally, shadowing from MBs in the near field, suppressed heating potential at the treatment focus. PCCAs, as a result of their spatial selectivity, can mitigate these issues by remaining a liquid contrast agent along
the beam path, until ADV is achieved at the transducer’s focus. Further demonstrations of the use of PCCAs for HIFU include Kopechek et al [49], [50]. and Zhang et al [51].

With great promise of PCCAs enabling the further clinical implementation of HIFU, it is imperative to explore the impact PCCA formulation may have on the desired biological response of HIFU. In Chapter 5, I will investigate the role of PFC core on the resulting lesion developed from PCCA-mediated HIFU in a tissue-mimicking phantom. PCCA formulations were investigated for the acoustic and thermal characteristic of the lesions observed.

4.5 INFLUENCE OF ULTRASOUND PARAMETERS ON DROPLETS

PCCA technology is very similar to MB technology in many ways, particular in terms of mechanism of action for sonoporation and thermal ablation. Their major difference, the activation phase of liquid PCCAs into a gaseous contrast agent, is a vital consideration in designing therapeutic applications of PCCAs. There are multiple factors that can impact the ADV threshold, or pressure required to induce vaporization. These factors can be separated into three categories: (a) acoustic parameters, (b) PCCA properties, and (c) environmental factors.

(a) Acoustic Parameters

Typically, ADV threshold decreases with increasing transmit center frequency. This trend is explained by superharmonic focusing [52], where high frequency content of the transmitted acoustic wave focuses inside the droplet, producing a pressure profile much higher in magnitude than the transmit pressure. This trend may be contested as a result of pulse length [53]. Longer pulse lengths can reduce threshold. When investigating the impact
of acoustic parameters on ADV threshold, it is important to put results in the context of equivalent pulse length or exposure time. Equivalent cycle numbers will result in lower frequency activation pulses having longer “on times” versus higher frequencies, while equivalent exposure time, will result in higher frequency pulses subjecting PCCAs to more cycles during vaporization.

(b) PCCA Properties

PCCA properties can impact the ADV threshold in regards to the PFC core and the particle diameter. Lower boiling point PFCs will need less pressure to vaporize [54]. In regards to PCCA size distribution, smaller diameter particles result in higher ADV thresholds. This property can also be explained by superharmonic focusing, where larger diameter droplets focus acoustic waves more, increasing the pressure the droplet experiences during ADV [24].

(c) Environmental Factors

Finally, environmental factors, like surrounding media temperature and viscosity, can impact the ADV threshold. As the temperature of the surrounding media increases, the threshold for vaporization decreases. In regards to viscosity, PCCAs require more energy to induce ADV in blood than in water. In PCCAs made with low-boiling PFC cores, Sheeran et al [54]. and Rojas et al. [55] have demonstrated these properties, respectively. In summary, determining the acoustic parameters and PCCA formulation for improved therapeutic outcomes needs to be considered carefully, but there is a survey of literature to support these decisions. These properties should only be viewed as a foundation. Much more work is needed in advancing therapeutic applications of PCCAs, given anatomical location and
features, therapeutic ultrasound system design, and particular PCCA formulation and characteristics.
REFERENCES


5.1 INTRODUCTION

The medicinal use of extreme temperatures is a viable option for the removal of diseased solid masses. Radiofrequency ablation, laser ablation, and cryoablation are examples of such therapies, but may require laparoscopic guidance if the target is too deep to conduct treatment percutaneously [1]. High-intensity focused ultrasound (HIFU) utilizes high energy ultrasound to treat diseased tissue, by generating a rapid rise in temperature, beyond the point of survival [2], [3]. The heating-induced injury from HIFU has been described as a result of coagulative necrosis [4]. Additionally, ultrasound has advantages over the other alternative surgeries (radiofrequency ablation, laser ablation, cryoablation) due to the technology’s increased penetration depth and noninvasive property. As of lately, HIFU has been approved in the United States for treatment of essential tremors, painful bone metastasis, uterine fibroids, and prostate cancer, though many more targets are being explored in preclinical settings, as well as in human patients internationally [5].

Despite the clinical and technical growth of HIFU, the method faces challenges. First, surgical times can take several hours as a result of the small focal volume of traditional HIFU devices and the large tumor volume the focused beam needs to cover [1] [6]. Second, a prominent side-effect of HIFU is near-field heating, often demonstrated by way of skin burns [6], [7]. A strategy for overcoming these challenges may lie in the use of a cavitation
enhancing agent, such as gas-filled microbubbles (MBs) or liquid-filled phase-change contrast agents (PCCAs). Previous studies suggestion, these agents could decrease the therapeutic pressure requirements to induce coagulative necrosis [8]–[10].

MBs and PCCAs enhance therapeutic outcomes by providing a source of cavitation in the presence of a therapeutic ultrasound field. Within the field, the oscillating particle has the ability to generate bioeffects ranging from temporary endothelial openings [11] to permanent injury from heating of tissue during HIFU treatments [9], [12]. Through in silica models of MB-mediated HIFU, researchers have shown the source of increased thermal heating with the presence of MBs, as a result of viscous damping [13], [14]. In preclinical investigations, MBs have shown promise as an enhancing agent for HIFU [15]–[17]. And in terms of clinical translation of MBs for therapeutic applications, MBs have shown promise in drug delivery clinical trials across the blood-brain barrier [18] and through barriers surrounding pancreatic tumors [19], though there is still more work needed in regards to clinical-use with HIFU.

MB technology possesses shortcomings that have direct consequences in the field of therapeutic ultrasound. MBs have short half-lives, on the order of several minutes, which may hinder use in longer therapy procedures, like HIFU [20]. In contrast, PCCAs, also known as nanodroplets, have been shown to circulate longer in vivo [21]. Like MBs, PCCAs can be formulated with a lipid, protein, or polymer shell, except surrounding a liquid perfluorocarbon (PFC) core. But nanodroplets, are smaller in diameter than MBs, typically several hundred nanometers. PCCAs are beneficial for therapeutic use, in that they do not oscillate in an acoustic field until their core is vaporized through a process known as acoustic droplet vaporization (ADV). Once a gas, the agent will cavitate within the acoustic field,
capable of enhancing thermal delivery to the surrounding medium when the field is intensified to HIFU levels. As our lab has shown, the spatial selective nature of nanodroplets can be advantageous for HIFU, as seen by reduced off-target effects with PCCA-mediated HIFU [22]. This was achieved by designing the therapeutic acoustic parameters to reach the ADV threshold of the PCCA at the beam’s focus. PCCA cavitation events would be limited to where the focus is placed. Thus, thermal ablation could be minimized in the near-field of the beam, overcoming the off-target effects being seen in the clinic. There are several ways to control ADV threshold through PCCA formulation, including PFC core and PCCA size distribution [10]. Preclinical investigations seem to confirm the benefits of enhancing HIFU with PCCAs [23]–[27]. Ultimately, PCCAs possess the increased specificity and circulation time that would enable further translation of HIFU into expanding therapeutic targets.

To further the development of PCCA technology towards enhancing HIFU, it is vital to optimize the formulation of PCCAs for use in thermal ablation. From the shell composition to the inner core, considerations for the intended role should be taken when designing PCCAs for a clinical setting. In parallel, it is vital to address the acoustic parameters for optimal ADV and subsequent bubble oscillation. The relationship between PCCA formulation and ADV threshold has been studied previously in the context of imaging applications [28]: Sheeran et al. demonstrated the increase in ADV as the boiling point temperature of the PFC-core increased, but also how ADV threshold increased with decreasing PCCA diameter. As a result of the relationship between PCCA formulation and ADV threshold, it seems advantageous to explore the degree of lesion development and accompanying thermal increase differences as a function of PCCA formulation and transmitted pressure, but in the context of HIFU-related acoustic parameters.
In the following study, we observed the effects of PFC core and transmit pressure on the acoustic and thermal response of PCCA-mediated HIFU in an in vitro tissue mimicking phantom. The acoustic and thermal properties of the lesions developed during PCCA-mediated HIFU were defined by the following metrics: (1) area of lesion developed as captured by B-mode ultrasound images collected post treatment, and temperature curves, which were quantified as (2) area under the curve (AUC) and (3) peak temperature. We hypothesize that in the context of HIFU therapy, PCCAs formulated with lower boiling point PFCs would produce therapeutic effects at lower acoustic outputs. By optimizing PCCA-mediated HIFU, an ideal formulation for a given acoustic parameter may be identified, which could guide future in vivo PCCA-mediated HIFU therapy studies.

5.2 MATERIALS AND METHODS

5.2.1 PHASE-CHANGE CONTRAST AGENT FORMULATIONS

Four formulations of PCCAs were manufactured in-house based on a similar protocol as is published in Sheeran et al [29], before incorporating into tissue mimicking phantoms. The manufacturing procedure is detailed below.

5.2.2 LIPID SOLUTION FORMULATION

A 1 mM lipid solution comprised of 90 mole % 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 10 mole % 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (P2K) was formulated in phosphate buffered saline (PBS) containing 15 % (v/v) propylene glycol and 5 % (v/v) glycerol. Aseptic lipid Aliquots of 1.5mL of the aseptic lipid solution were made and sealed into 3 mL glass vials.
5.2.3 PERFLUOROCARBON PREPARATION

The lipid solution-containing vials were vacuumed for at least thirty minutes, and the headspace was replaced with a PFC gas. In the study, three types of PFCs were tested: octafluoropropane (C₃F₈, T_{boil} = -37°C), decafluorobutane (C₄F₁₀, T_{boil} = -2°C), and dodecafluoropentane (C₅F₁₂, T_{boil} = 29°C). These three PFCs will be referred to as C₃, C₄, and C₅, respectively. C₃ and C₄ were acquired in gas cylinders from Fluoromed (Round Rock, TX, USA), while C₅ was acquired as a liquid from Exfluor Research Corporation (Round Rock, TX, USA). These three PFCs were used to create four formulations of PCCAs: (a) 1:1 mixture of C₃ and C₄ (C₃C₄), (b) pure C₄, (c) 1:1 mixture of C₄ and C₅ (C₄C₅), and (d) pure C₅. The procedure for preparing PFC for addition to lipid solutions will be described in order of increasing boiling point temperature of PCCA core:

(a) C₃C₄ nanodroplets

The PCCAs made with a C₃C₄ core required a gas mixing apparatus, made by connecting two 60mL syringes. The gas mixing apparatus held equal amounts of C₃ and C₄, for a total gas volume of 120mL, both added to the apparatus at 6-8 psi. The apparatus was connected to the vacuum line, and injected into the vial headspace, after air removal.

(b) C₄ nanodroplets

For PCCAs made with pure C₄, a pressurized gas cylinder containing C₄ was directly connected to the vacuum line and added to the vials at a pressure of 6-8 psi.

(c) C₄C₅ nanodroplets
PCCAs formulated with a C4C5 core began with 60 mL of C4 gas in the gas mixing apparatus, filled at a pressure of 6-8psi. The apparatus was brought to -20°C, and once C4 gas condensed to a liquid, liquid C4 was mixed with 0.5mL of liquid C5. The mixture vaporized into a gas, a hot plate set to 60°C for about 30 minutes. Once vaporized, the gas-filled apparatus was connected to the vacuum line, and added to the headspace of the lipid solution-containing vials.

(d) C5 nanodroplets

To formulate PCCAs with a C5 core, 0.5mL of liquid C5 was injected into the gas mixing apparatus. The syringe was left on a hot plate set to 60°C until C5 fully vaporized (approximately 30mins in time). The apparatus was connected to the vacuum line to replace the vacuumed headspace of lipid-solution vials with C5.

5.2.3 PHASE-CHANGE PROCEDURE

The vials containing PFC headspace were shaken in a vial mixer (Lantheus, Billerica, MA, USA) for 45 seconds. Vials were submerged and swirled in a water bath maintained at a temperature between -11 and -13°C for a total of 2 mins. During this time, a needle connected to a nitrogen tank (set to 20 psi) was pierced through the septum cap. Once the line was open for 2 mins, the vial remained in the chilled water bath for another 30-45 seconds. The PCCAs were portioned into 200 µL aliquots and kept frozen at -20°C until thawed for tissue-mimicking phantom fabrication. Prior to incorporating PCCAs into a phantom solution, the size distribution of a given PCCA aliquot was measured using an AccuSizer FX Nano (Particle Sizing Systems, Port Richey, FL, USA).
5.2.4 TISSUE-MIMICKING PHANTOM PROCEDURE

The tissue-mimicking phantom procedure is a modified version of a protocol by Takegami et al. [30] and Phillips et al. [7]. The procedure begins with a solution of 40% acrylamide/bis-acrylamide in a 29:1 ratio (Fisher Scientific, Waltham, MA, USA). A 4.0mL volume of the acrylamide solution was mixed with 3.5mL of an albumin source and 2.5mL of deionized water (Fisher Scientific, Hampton, NH, USA) to create a 10 mL solution. For this study, we used egg whites separated from an egg yolk, which was prepared prior to beginning the phantom procedure. The solution was gently agitated, then placed under vacuum at 40°C for one hour. To remove any last air bubbles in the solution, the solution was centrifuged at 2,000 RPM (581 xG) for one minute. An aliquot of thawed PCCAs was injected into the solution, at a concentration of 1.0 µL of PCCAs per mL of phantom solution. Following PCCA addition, a 100 µL of a 10% (w/v) aqueous solution of ammonium persulfate (Sigma Aldrich, St. Louis, MO, USA) was added for every 10mL of acrylamide-albumin solution. Solutions were then placed into a mold, in which the hydrogel would polymerize. The polymerization process was activated with the addition of a 40 µL volume of tetramethylethylenediamine, or TEMED (Thermo Fisher Scientific, Waltham, MA, USA) for every 10 mL of phantom solution. The solution was stirred, then placed into a water bath maintained between 0°C and 10°C. Phantoms remained in the water bath until solidified.

For the acoustic characterization of lesions, a 120 mL phantom was made in a plastic box (dimensions: 13cm x 9cm x 1.5cm). With this phantom, three rows of lesions were produced, each row made from transmitted pressures ranging from 1.0 to 4.0 MPa (increasing in 0.5 MPa increments). Lesions in the row were 7mm apart. The phantom
solution was assembled as described before gently pouring into the plastic box. Once a single 120 mL solution, the PCCAs, ammonium persulfate, and TEMED were added in that order, at 12x the volume previously mentioned.

For the thermal characterization, 20 mL phantoms were made in 30 mL angled cylinder cups (dimensions: 3cm diameter base, 4.5cm diameter top, and 3cm in height). A different phantom volume was necessary to allow for insertion of the fiber optic probe. These phantoms were fabricated in 10mL batches, meaning the bottom half of the phantom was allowed to polymerize first, before adding the second 10mL phantom solution to polymerize on top. Any phantoms, for either study, resulting in holes, or signs of incomplete polymerization were disposed prior to the study. Before experimenting with the tissue mimicking phantom, any phantom containing excess, non-polymerized chemicals, was removed via rinsing water over phantoms and properly disposing the waste.

5.2.5 THERMAL ABLATION PROCEDURE

Thermal ablation was completed using the Therapeutic Imaging Probe System (TIPS) (Philips Research North America, Briarcliff Manor, NY, USA). The spherical focus of this transducer was positioned 80mm from the transducer face. The system included a 1.2 MHz seven-element annular array (Imasonic, Voray-sur-l’Ognon, France), with a full-width half maximum (FWHM) focal volume of 1 x 1 x 6 mm³. Thermal ablation was completed at 1 MHz, 100% duty cycle, one second exposure (for the acoustic characterization study) or ten second exposure (for the thermal characterization study), and a range of pressures from 1.0 to 4.0 MPa (peak-negative pressure). Lesion population for each pressure and PCCA formulation varied from 3 to 12 lesions (see Supplemental Information, Appendix C). For the thermal characterization study, only three pressures were observed: 2.0, 3.0, and 4.0 MPa. A
total of 4 lesions per pressure were collected for the thermal study. HIFU lesions were defined as the produced bubble cloud from droplet vaporization. The area of lesion development was measured using B-mode images with a 15L8 linear array, driven by an Acuson Sequoia 512 (Siemens, Mountain View, CA, USA). B-mode images were taken at 14 MHz and a mechanical index between 0.2 and 0.25. For the thermal characterization study, exposure time was increased from 1 sec to 10 sec. Additionally, only thermal change was only tested for three pressures—2.0, 3.0, and 4.0 MPa.

The experimental set-up included a water bath, which was maintained at a temperature throughout the study to achieve a 37°C phantom, using a sous-vide (Anova, San Francisco, CA, USA). Phantoms were placed in a holder fixed inside the water bath as shown in Figure 5-1. The 120mL phantoms were left for approximately 30 mins to equilibrate to ~37°C, while the smaller volume phantoms did not need as much time. Phantoms were coupled to the transducer housing using degassed water. Once HIFU exposure was complete, the TIPS was replaced with the 15L8 to capture post-ablation images on the Sequoia system. The 20mL phantoms were used one exposure at a time.
Figure 5-1: Schematic of the HIFU experimental setup. PCCA-mediated HIFU was conducted in an acrylamide-album hydrogel phantom. Phantoms were maintained at a temperature of 37°C using a heated water bath. A phantom holder was manufactured for the two different phantom volumes. The TIPS transducer was positioned 10 mm away from phantom surface, and was guided across the phantom with an accompanying 2D motion stage (not pictured).

5.2.6 DATA PROCESSING: ACOUSTIC CHARACTERIZATION OF LESION AREA

Images of lesions were analyzed with custom-made MATLAB software (MathWorks, Natick, MA, USA). A rectangular region of interest (ROI) was positioned around the lesion. The ROI was fixed in size to 0.5 sq. cm. A threshold of 25% of maximum pixel value was applied to denote lesion location from background, a priori. From this, the area of the lesion was calculated (see Figure 5-2 for diagram of this process). Scale bars were created using the caliper tool on the Sequoia, and setting the conversion in ImageJ, an open access software [31].
Figure 5-2: Flow diagram of data processing of acoustic characterization study. Ultrasound images from acoustic characterization study were analyzed through MATLAB. The chosen lesion was isolated from the rest of the image by placing an ROI around the lesion. A threshold was applied to identify lesion location from phantom background. The values were binarized for area calculation.

5.2.7 DATA PROCESSING: TEMPERATURE MEASUREMENT OF LESION HEATING

Temperature measurements within the phantoms during thermal ablation were assessed using a fiber optic thermometer (STF Probe, LumaSense Technologies, Santa Clara, CA, USA). The thermometer was inserted through the wall of the phantom cups and into the phantom by threading the fiber through a path perforated by a 16 gauge needle. With the fiber optic thermometer inserted into the phantom, the cup was placed in the holder for the phantom, in order to submerge the phantom into the water bath. Degassed water was poured on top of the phantom to couple the TIPS to the phantom. Guidance of thermometer location and TIPS positioning was aided by imaging with the Acuson Sequoia. The fiber optic probe was positioned 1 cm from the transducer casing (65cm from the transducer face), which was estimated to be the approximate location of the start of the lesions observed in the acoustic characterization study.
Accompanying thermometry software (True Temp 2.0, LumaSense Technologies, Santa Clara, CA, USA) recorded measurements of phantom temperature versus time for a total period of 100 seconds. Around the 10 sec time point, the HIFU treatment was started, and transmitted for 10 seconds. Temperature curves were processed in MATLAB (see Figure 5-3) to extract peak temperature and area under the curve (AUC). Peak temperature was defined as the maximum temperature reached during the temperature measurements. AUC was found from integrating the baseline-corrected temperature curve, where the baseline was taken as the temperature average from the first 5 seconds of the recording.

**Figure 5-3:** Example temperature recording. Thermal heating was measured using a fiber optic probe, which recorded temperature changes in real-time. An example of data from the probe is presented, showing the change in phantom temperature during a HIFU ablation. HIFU was performed on a 20 mL phantom containing PCCAs at a concentration of 1.0 μL/mL.
5.2.8 STATISTICAL ANALYSIS

All statistical analyses were conducted using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Statistical significance was set a priori at $p<0.05$ and the following convention is used for graphical depictions: (*) for $p<0.05$, (**) for $p<0.01$, (***) for $p<0.001$, and (****) for $p<0.001$. Data are presented as mean ± standard deviation throughout this work.

To assess whether acoustic pressure and PCCA formulation influenced the lesion properties of interest (respectively lesion area, AUC and peak temperature), a two-way repeated-measures analysis of variance (ANOVA) was conducted, followed by Tukey’s multi-comparison test. The two-way repeated-measured ANOVA was conducted with respect to PNP and PFC core composition.

For each PNP, the lesion area was also compared between all four PCCA formulations via Tukey’s multiple comparisons testing. With the same lesion area data, the PNPs were compared for a given PFC core via Tukey’s multiple comparison testing. The same procedures (ANOVA with Tukey’s post-test) were used to assess the effects of PNP and PFC core composition on the thermal therapeutic outcome (AUC and peak temperature).

The relationship between PNP and lesion properties of interest was investigated using correlation testing. For all three cases, the respective Spearman correlation coefficients ($r$) and $p$ values are reported.
5.3 RESULTS

5.3.1 PCCA SIZE DISTRIBUTION

The size distribution for all four formulations of PCCAs was measured prior to their addition to phantom solutions during fabrication. A total of three trials per PCCA formulation were averaged to produce the distributions shown in Figure 5-4, including the mean diameter and concentration from the measured distributions.

![PCCA Size Distributions](image)

<table>
<thead>
<tr>
<th>PCCA Formulation</th>
<th>Mean Diameter (μm)</th>
<th>Concentration (parts/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Std. Dev.</td>
</tr>
<tr>
<td>C3C4</td>
<td>0.20</td>
<td>0.01</td>
</tr>
<tr>
<td>C4</td>
<td>0.20</td>
<td>0.01</td>
</tr>
<tr>
<td>C4C5</td>
<td>0.19</td>
<td>0.00</td>
</tr>
<tr>
<td>C5</td>
<td>0.20</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Figure 5-4: PCCA size distribution of the four PCCA formulation used throughout the study. Alongside the distribution is a table presenting the mean diameter and mean concentration of each PCCA formulation. Measurements were averaged from three samples per PCCA formulation.

5.3.2 LESION ACOUSTIC CHARACTERIZATION

The lesion was defined as the vaporized contrast signal captured in B-mode images after HIFU treatment in the PCCA-embedded phantoms. Lesion development characterization investigated the differences in area between PCCA formulations measured from the captured images. HIFU treatments were performed at increasing PNPs (1.0 to 4.0 MPa). Figure 5-5 depicts B-mode images of a sample lesions from each PCCA formulation.
In the absence of PCCAs, tissue mimicking phantoms resulted in no hyperechoic area at any pressures tested (not depicted).

**Figure 5-5:** Example B-mode images of sample lesions created in PCCA-embedded tissue mimicking phantoms. Images are presented (top to bottom) from the least stable PCCA-core, C3C4, to the most stable PCCA-core, C5. Lesions are organized in increasing transmitted pressure, beginning at 1.0 MPa (left) and ending at 4.0 MPa (right). These pressures are indicated above the lesion. All phantoms were made with a 1.0 μL/mL concentration of PCCAs.

Correlation testing demonstrated a significant r value of 0.93 for C3C4 (p=0.007), 0.96 for C4 (p=0.003), and 1.00 for C5 (p=0.0004), indicating a direct relationship between
the area of the lesions observed and increasing PNP (see Figure 5-6). Correlation testing for C4C5 was close to being significant with an r value of 0.75 and a p value of 0.066. In accordance with these results, two-way ANOVA analysis was conducted and demonstrated that PNP had a greater influence on area data variability (54%, p<0.0001) than PCCA formulation (6%, p<0.0001).

![Area of Lesion](image)

**Figure 5-6:** Lesion area with respect to PNP, together with Spearman correlation testing results. Lesion area was shown to increase with increasing PNP for C3C4 (r = 0.93, p = 0.003), C4 (r = 0.96, p = 0.003) and C5 (r = 1.00, p = 0.0004), but not C4C5 (r = 0.75, p = 0.066).

In Figure 5-7, the area of lesion data is presented with all four PCCA formulations side-by-side for a given PNP. The red line in Figure 5-7 indicates the FWHM focal area of the TIPS transducer. From 2.0 to 4.0 MPa, PCCA-mediated HIFU resulted in a lesion area greater than the focal area of the TIPS transducer. Following two-way ANOVA, multi-comparison tests were conducted to compare the PCCA formulation results for each transmitted PNP group. Significant values have been indicated in Figure 5-7. Areas for lesions developed at 4.0 MPa, 3.5 MPa, and 2.0 MPa demonstrated at least one significant comparison between PCCA formulations. When exposed to 4.0 MPa, C5 PCCAs developed
a mean area of 0.26 ± 0.04 sq. cm. These lesions were statistically larger than lesions
developed by C3C4 PCCAs, (0.16 ± 0.04 sq. cm, p<0.0001), C4 PCCAs (0.19 ± 0.07 sq. cm,
p<0.0001), and C4C5 PCCAs (0.18 ± 0.04 sq. cm, p<0.0001). At a pressure of 3.5 MPa, C5
(0.22 ± 0.03 sq. cm) demonstrated significant increase in lesion area as compared to both
C3C4 0.13 ± 0.03 sq. cm, p=0.028) and C4C5 0.12 ± 0.01 sq. cm, p=0.014). At the lower
pressure of 2.0 MPa, the only significance found was the increased lesion development of
C4C5 PCCAs (0.13 ± 0.04 sq. cm) as compared to C3C4.( 0.08 ± 0.05 sq. cm, p=0.045).

![Area of Lesion](image)

**Figure 5-7:** Area of lesions produced at 1 MHz center frequency, 1 sec exposure, and 100% duty cycle. Data is presented in groups of all four PCCA formulations with respect to transmitted PNP, together with the statistically significant differences between formulations found by Tukey’s multi-comparison testing. A dashed red line represents the FWHM focal area of the 1 MHz TIPS transducer.

**5.3.3 THERMAL MEASUREMENT OF LESION DEVELOPMENT**

Similarly, thermal properties of PCCA-embedded tissue mimicking phantoms were
investigated for the influence PNP and PCCA formulation had on AUC and peak
temperature. Spearman correlation testing was completed to investigate the influence increasing PNP had on observed temperature curves. Correlation testing proved to be insignificant as a result of the insufficient amount of groups tested (three pressures observed—2.0, 3.0, and 4.0 MPa). Two-way ANOVA analysis found that for AUC, acoustic pressure was a greater source of data variability (64%, p<0.0001) than PCCA formulation (6%, p=0.0676). For peak temperature, acoustic pressure and PCCA formulation were fairly equivalent in variability contribution, with acoustic pressure at 23% (p<0.0001) and PCCA formulation at 26% (p<0.0001).

Following two-way ANOVA, Tukey multi-comparison test was used to compare PCCA formulations with respect to transmit PNP. For both AUC and peak temperature, significant formulations were found during HIFU treatments transmitting 2.0 MPa (see Figure 5-8). For peak temperature, C4C5 PCCAs achieved a significantly higher peak temperature (45.8±3.5°C) than C3C4 (39.9±1.1°C, p=0.0438) and significantly lower peak temperature than C4 PCCAs (47.5±1.2°C, p=0.034). As well, C5 PCCAs achieved a significantly higher peak temperature (56.3±5.6°C) than C3C4 (39.9±1.1°C, p<0.0001) and C4 PCCAs (47.5±1.2°C, p<0.0001). Multi-comparison test conducted on AUC showed significance between C5 (394.4±105.5 a.u.) and C4 (296.8±52.8 a.u., p=0.021) PCCAs.
Figure 5-8: Peak temperature (left) and AUC (right) with respect to PNP for all four PCCA formulations. Thermal assessment of PCCA-embedded tissue mimicking phantoms was measured by a fiber optic probe. Temperature changes during HIFU treatment were quantified as (a) peak temperature and (b) area under the curve (AUC). Average and standard deviation data are presented for transmit PNP of 2.0, 3.0, and 4.0 MPa. Statistically significant differences between some formulations were found at 2.0 MPa (all statistically significant p-values from Tukey’s multi-comparison are depicted).

5.4 DISCUSSION

It was hypothesized that the formulation of the PCCAs, and more specifically, the PFC core, would alter the degree of lesion development and thermal heating during HIFU treatments. As a result of differences in PFC boiling point temperature, the PCCAs containing PFCs with lower boiling point would more readily vaporize, and therefore, produce a larger lesion area. With increased tendency towards vaporizing, this would translate into increased heating and lesion size with lower boiling point PCCAs. In regards to pressure, it was hypothesized that with increased transmit pressure, lesion size and thermal response would also increase. The first objective was to validate these relationships between the independent variables: PFC core and transmitted PNP, with the observed lesion properties of area, AUC, and peak temperature. This was followed by determining if a single formulation could be isolated as a better performing formulation at a given acoustic pressure.
In the acoustic characterization study, the data showed increased lesion areas above the natural FWHM focal area of the TIPS transducer. This could be advantageous for improving treatment time. By increasing the treatment volume with the use of PCCAs, a given diseased volume could be treated faster than with the ultrasound beam alone. These results must be taken in the context of the HIFU treatment parameters used: 1 MHz, 100% duty cycle, 1 sec exposure. In addition, the phantoms were maintained at 37°C. Decreasing the phantom temperature would result in a higher ADV, potentially impacting PCCA lesion development [29], [32], [33].

Correlation testing demonstrated direct relationships between increasing PNPs and the area of lesion developed. The strong influence of acoustic pressure on lesion development was further affirmed in two-way ANOVA, where testing determined acoustic pressure was a greater source of variability in area data than PCCA formulation. This can be explained by the ADV requirements for utilizing PCCAs. Lesion area was measured via the bubble presence in ultrasound images post-thermal ablation. The minimum pressure necessary to vaporize PCCAs acts as a binary all-or-nothing threshold for measuring lesion area. Consequently, acoustic pressure heavily influenced the presence and resulting size of PCCA-produced lesions.

Two-way ANOVA of AUC data determined acoustic pressure was also the major source of variability, but the two factors were about the same for peak temperature. Though PCCA formulation accounted for a smaller percentage in the case of lesion area and AUC data, and was about equal in percentage for peak temperature data, the source of variability was significant, meaning PCCA formulation did influence variability of observed lesion...
properties. In the case of AUC, the influence of PCCA formulation on AUC variability was not found to be significant, but at a p-value of 0.067, PCCA formulation was close.

The final aim of this study was to identify a formulation that would be ideal for a given HIFU parameter. Multiple comparison testing between the four PCCA formulations, at respective PNPs, was completed to isolate the better performing formulation. Significance between PCCA formulations, in both the acoustic and thermal characterization studies, were limited to certain transmit PNPs (4.0, 3.5, and 2.0 MPa for the acoustic study and 2.0 MPa for the thermal study). Where significance was found, multi-comparison testing demonstrated the PCCA formulations that resulted in increased lesion area and thermal response were the two more stable PCCAs—C4C5 and C5. This was not always the case. In the peak temperature data, at 2.0 MPa, C4 PCCAs reached a higher maximum value than C4C5. In only one case was there a formulation that outperformed the other three. This was in the acoustic characterization study, at a PNP of 4.0 MPa, C5 was identified as statistically greater lesion area than the other three formulations. This trend was counter to our hypothesis that the PCCAs with a lower boiling point PFC-core would therefore be easier to vaporize into a microbubble, and thus, producing larger lesions. This could be explained by several theories. The phantom preparation process, which can take several minutes to polymerize, followed by the time the phantoms equilibrate in the heated water bath. By the time HIFU treatment is started on the PCCA-embedded phantoms, the PCCAs with the lower boiling PFC-core may have vaporized. Another theory is that the 1 sec, 100% duty cycle pulse destroyed more of the more volatile PCCAs. Further testing would be needed to explain the rejected hypothesis. The result that C5 and C4C5 PCCAs performed better in this in vitro investigation does have translatable meaning to an in vivo study. These PCCAs may be more
likely to stay in circulation, and therefore contribute to HIFU treatment throughout the
therapeutic procedure.

The thermal characterization study demonstrated significant PCCA response at the
lowest PNP tested (2.0 MPa). This may be because treatment parameters of 3.0 and 4.0 are
over activating all formulations, erasing any differences between the formulations. The
thermal response also showed differences between the peak temperature metric and the AUC.
While there were several instances of PCCA formulations significantly increased in the peak
temperature data, these differences did not translate to the AUC data set. This may be
explained by the spatial sensitivity of the peak temperature metric. As a result of the limited
assessment volume of the fiber optic probe, peak temperature is more sensitive to
misalignment, while AUC represents a more global metric of thermal change in the tissue-
mimicking phantom.

In regards to the experiment, there were shortcomings of the setup that should be
addressed. PFC core is not the only parameter that can effect lesion development. Shell
composition and PCCA size distribution could have also been tested to improve PCCA-
response to HIFU therapy. In the preclinical space, researchers are investigating various
formulations, including shells of lipid, polymer, and protein in nature, as well as
manufacturing methods that would give increased control of size distribution [34]. The
study’s model also possessed shortcomings. The tissue-mimicking phantom had an
attenuation coefficient of about 0.3 dB cm\(^{-1}\) MHz\(^{-1}\) [7], which is less than tissue like liver (0.5
dB cm\(^{-1}\) MHz\(^{-1}\)) and muscle (1.2 dB cm\(^{-1}\) MHz\(^{-1}\)) [35]. Also, the PCCA-embedded phantom
model lacks the dynamic nature of an in vivo environment. In an in vivo model, factors of
attenuation, vessel locations, and vascular flow would contribute to the degree of lesion development, and likely alter the results found in our in vitro investigation.

Ultimately, an in vitro characterization study was the ideal setup for comparing PCCA formulations for HIFU therapy. When conducting optimization investigations, an in vitro experiment allows researchers to easily modify conditions, such as temperature, PCCA concentration, and sample size. Despite the shortcomings of the in vitro model, our findings can be used to plan future investigations with PCCA-enhanced HIFU therapy. For an in vivo investigation, there are several factors researchers should further consider when deciding on a PCCA formulation for enhanced therapeutic outcomes that could not be accounted for in an in vitro optimization. These factors include circulation time, in vivo activation threshold for PCCAs, and potential for extravasation. Circulation time, or how long PCCAs will remain viable during HIFU therapy, can be impacted by the PCCA’s in vivo stability and propensity for immune recognition. Our in vitro study maintained a phantom temperature of 37°C in order to take in vivo temperature stability into consideration. In regards to immune recognition, our PCCA formulation contained 10% polyethylene glycol (PEG), a stealth polymer used in pharmaceutical science to reduce immune recognition. Contrary to this idea, our lab has shown with repeat dosing, this technique becomes ineffective [36]. The in vivo ADV threshold may also impact the PCCA needed to enhance HIFU therapy. Our lab has shown that there are differences between in vitro and in vivo activation thresholds for PCCAs made with low boiling PFCs as a result of vessel size and blood viscosity [37]. Lastly, extravasated PCCAs may be beneficial for heating tissue beyond tumor vasculature. The potential for PCCAs to extravasate is dependent largely on particle size and circulation time. By optimizing PCCAs for these in vivo factors, meaning increased circulation time
(improved in vivo stability, reduced immune recognition), achievable acoustic activation with therapeutic ultrasound system, and potential for extravasation, PCCA-mediated HIFU therapy could advance as a viable clinical option for noninvasive surgeries.

5.5 CONCLUSIONS

In conclusion, acoustic pressure and PCCA formulation were both demonstrated to affect lesion size and heating in an in vitro tissue mimicking phantom experiment. As such, PCCA formulation and therapeutic acoustic parameters can be used to optimize PCCA-mediated HIFU therapy. As expected, acoustic pressure was shown to be a stronger influence on lesion size and thermal response. This reflects the necessity to induce PCCA ADV for therapeutic use. Nevertheless, PCCA formulation was shown to significantly affect the lesion properties as well. In limited instances, the more stable PCCA formulations, containing PFC-cores of higher boiling point temperatures, resulted in lesions with improved acoustic and thermal properties compared to those of lower boiling point temperatures. With this understanding of how HIFU parameters and PCCAs can be optimized for PCCA-mediated HIFU, researchers can better design preclinical investigations to advance PCCA technology towards overcoming the current challenges of HIFU therapy.

5.6 ACKNOWLEDGEMENTS

We would like to thank Brian Velasco for providing the lipids necessary to make the PCCAs. We would also like to acknowledge two National Institute of Health grants that funded this work: the R21EB021012, which was funded in collaboration with Dr. Charles
Caskey (Vanderbilt University) and the F31 training grant (F31CA206602), which supported A.G.N. throughout this study.
REFERENCES


CHAPTER 6

CHALLENGES OF OPTIMIZING PHASE-CHANGE CONTRAST AGENTS FOR EXTRAVASATION

6.1 INTRODUCTION

In the past several decades, the field of oncology has experienced a growth of nanotechnology to exploit the leaky vasculature associated with cancer. Therapeutic nanotechnology has the potential to reduce patient side effects of current chemotherapies, and increase treatment distribution to diseased tissue, both primary and metastatic sites. Drug delivery vehicles are designed to protect the non-diseased locations from therapy, and proportionally distribute to sites of interest, and release the needed therapy. The field has explored a plethora of mechanisms for releasing therapy from drug delivery vehicles in a manner that enables disease specificity. One strategy of doing so is to rely on unique properties of tumor environments to release the drug, including drug-to-vehicle linkers that will be released as a result of pH change, or overexpressed enzymes unique to the disease [1]. Another innovative strategy utilizes exterior stimuli to release therapy, including light, heat, magnetism, and even, ultrasound [2]. Not only could this decrease the issue of drug side-effects, but drug-delivery vehicles could increase efficacy, by increasing the amount of drug reaching target locations.

Though nanoparticles can be injected directly to the targeted tissue, which would minimize the issue of side-effects, most therapies rely on intravenous (IV) administration, in order to expose treatment to non-identified metastasis, or difficult to reach tumors. With IV administration, nanoparticles are reliant on a classic understanding of cancer. The hallmarks
of cancer, as noted by Hanahan and Weinstein in 2000 and 2011 [3], [4], include sustaining proliferative signaling, circumventing cell death, eluding growth suppressors, and most notably for drug delivery, inducing aggressive angiogenesis.

The enhanced permeability and retention (EPR) effect is a potential property of cancer, as a result of aggressive angiogenesis. The EPR effect describes the leaky vasculature, which can release particles through pores in the vascular network, and a dysfunctional lymphatic system that is incapable of draining the particles away from the extravascular space of tumor tissue. Therapeutic nanoparticles are designed to be small enough to accumulate in tumors, as a result of the EPR effect, and large enough to remain in the circulatory system when passing through healthy tissue.

![Enhanced Permeability and Retention (EPR) Effect](image)

**Figure 6-1:** Cancer vascular environment is understood to be porous, allowing for nanoparticles to passively migrate outside tumor vessels. This characteristic of cancer can be utilized to target extravascular targets of cancer as shown in schematic.

As a nanoparticle vehicle, PCCAs have an advantage many other drug delivery systems lack. In addition to carrying therapeutics where they are needed, PCCAs can be
visualized by ultrasound, allowing clinicians to image where the treatment is located. Ultrasound can also be used as the drug-releasing mechanism. By increasing the intensity of the transmitted pulses, and destroying the particle, the therapy is released exactly where it is needed.

As promising as this strategy seems, there are challenges. Clinically, the EPR effect is not as consistent as seen in preclinical research. The heterogeneity of the EPR effect is not only between tumor types, but also within the tumor itself. This may be explained by injectable xenograft tumor models, which grow more chaotic than clinically-identified tumors. Even allograft tumors don’t fully recapitulate their human counterpart cancer types. There are several factors concerning the tumor that influence the presence of EPR. First, the tumor type, size, location, and whether the tumor is a primary tumor or metastatic tumor will influence the EPR effect. Second, the vascular bed and surrounding stroma from which the tumor will grow, can influence EPR, as well as the presence or absence of a functional lymphatic network. Additionally, vascular changes as a result of co-medication, particularly drugs that influence blood pressure, can have an effect on the level of EPR in a tumor. It is important to note, there are ways to test tumors prior to treatment to gauge the level of EPR, which can guide clinicians on patients who will benefit the most from nanoparticle therapy [5].

PCCA extravasation has been studied and potentially shown by researchers like Rappaport [6], [7] and Kopechek [8]. In these studies, small animal models with chaotic vasculature were utilized to do so. Though work in preclinical models is important, and demonstrates early proof of concept, as discussed above, these models are not representative for a majority of clinically-observed tumors. Additionally, the PCCA formulations used in
these studies included high boiling point perfluorocarbons, which are more difficult to utilize with imaging ultrasound systems. Fortunately, the field of nanotechnology has investigated the ideal design of nanoparticles for clinical efficacy for decades. In the wide spectrum of presence and extent of EPR effect, there are properties of the nanoparticle that will increase in vivo circulation, limit kidney elimination, and reduce immune recognition—all of which are necessary for treatment success regardless of tumor type. Properties of effective nanoparticles include slightly negative to neutral surface charge, which will reduce protein opsonization for immune recognition, and a mean diameter of 100nm. Our in-house PCCAs are made with DSPC and DPPE-PEG2000. The PEG2000 is incorporated to decrease the recognition of the immune system. As Table 1 shows, the surface charge of our droplets is not too far from commercially available technology, and within the range of an ideal design. In the case of our in-house PCCAs, the latter parameter, a mean diameter of approximately 100nm, was absent.
Table 6-1: Comparison of surface charge of commercially available UCAs and the contrast agents made in the Dayton Lab (In-House). In-House contrast agents were tested on a DLS (company, USA) with a total of 3 vials.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-House</td>
<td>-2.7</td>
</tr>
<tr>
<td>SonoVue¹</td>
<td>-28.3</td>
</tr>
<tr>
<td>Definity¹</td>
<td>-4.2</td>
</tr>
<tr>
<td>Optison¹</td>
<td>-9.5</td>
</tr>
<tr>
<td>Albumin (bovine)²</td>
<td>-17.6:-24.2</td>
</tr>
</tbody>
</table>

There are two main reasons why a 100nm size distribution is unfavorable for an ultrasound contrast agent (UCA). First, as PCCAs decrease in size, they require more energy to activate. This can be explained by homogenous nucleation theory as well as acoustic focusing by the transmitting pulse changing due to size [9], [10]. Secondly, smaller droplets produce smaller bubbles. When imaging, these vaporized bubbles are often far from resonant bubble diameter for the imaging transmit frequency, decreasing the image quality of preferred contrast imaging sequences For example, a 10 MHz transmit pulse has a resonant bubble diameter of ~3µm [11].

Despite this, PCCAs formulated around 100nm would broaden the types of tumors we could treat via PCCA drug delivery, and the spatial distribution of treatment in those tumors. The following work pursued this goal of a 100nm PCCA-protocol that could be compared to the existing polydispersed PCCA formulation in regards to in vivo circulation time and extent of extravasation. I hypothesized since the monodispersed formulation would have a higher concentration of extravasation-capable nanoparticles, it would prove to be a
better candidate for consistently demonstrating extravascular imaging and extravascular drug delivery.

6.2 DATA

6.2.1 Experimental Overview

There are several protocols for formulating PCCAs. These include sonication, agitation, microfluidics, and condensation [12]. The ideal method for controlling particle diameter is a microfluidic device, because it can allow for superior control of PCCA parameters. The major challenge with this method is the time and yield, particularly when considering the volume necessary for an in vivo investigation. For this reason, I focused on optimizing our current PCCA protocol for a 100nm-centered size distribution. This batch-based condensation method, begins with forming microbubbles, and requires stages of decreasing temperature and increasing pressure to condense the core to a liquid (see Materials and Methods of Chapter 3 and Chapter 5). In the following, we will discuss three experimental trials to achieve 100nm PCCAs with the condensation procedure. These include (1) increasing the mole percentage of PEG2000, (2) increasing the endpoints of the pressurization stage, and finally, (3) isolating the smaller microbubbles, known as nanobubbles (NBs) before the condensation procedure. We will review the procedure used to achieve 100nm PCCAs and give conclusions on the sizing results. We will conclude with future directions of the work, and potential techniques currently in development that will greatly aid in progressing clinical application of PCCA technology.
6.2.2 Increasing Mole Percent of PEG: Introduction

One important consideration when making smaller particles, is the stability of the particle with decreased diameter. It was hypothesized that to create a stable 100nm lipid shell micelle, I needed to increase the surfactant, which would theoretically decrease the Laplace pressure on the PCCAs, by decreasing the surface tension on the particle (see Eqn. Y-1). In the equation, the Laplace pressure ($\Delta P$) is defined by the difference in pressure inside the particle ($P_{in}$) and the ambient pressure outside the particle ($P_{out}$). The Laplace pressure is dependent on the radius of the particle ($R$) and surface tension ($\sigma$). The current protocol involved a 10 mole percent (mol %) of PEG. By increasing it to 30 mol%, it was hypothesized that the smaller droplets would be stable, and therefore, potentially create more of them in our PCCA distribution, where otherwise, these particles disappeared soon after production.

$$\Delta P = (P_{in} - P_{out}) = \frac{2\sigma}{R} \quad \text{Eqn. Y-1}$$

6.2.3 Increasing Mole Percent of PEG: Methods

The protocol for creating a lipid solution for this study can be found in Chapters 3 and 5. The only change that was made to the lipid solution procedure was the ratio of DSPC: DSPE-PEG2000, which was decreased from 9:1 to 7:3. Specifically, 63 mM of DSPC was combined with 27 mM of DSPE-PEG2000 and emulsified in a solution of 15% (v/v) propylene glycol and 5% (v/v) glycerol in an 80% (v/v) 1x PBS solution. A 1.5mL aliquot was poured into septum-sealed 3 mL glass vials. Headspace of these vials were filled with DFB prior to shaking the vials into MBs and condensing them into PCCAs. The condensation process was similar to that described in Chapter 3, where vials were maintained
at a temperature between -7°C and -12°C, via an isopropanol bath. After swirling the vials in the water bath for 2 mins, the pressure in the vial headspace was increased to sufficiently convert MBs into PCCAs.

Size distributions of PCCAs made with 30% PEG and 10% PEG were measured using an AccuSizer FX Nano (PSS Particles, Entegris, Port Richey, FL, USA). Three vials were used to compute an average mean and concentration for each PCCA type, and statistically tested against one another using GraphPad Prism 8 (San Diego, CA, USA).

6.2.4 Increasing Mole Percent of PEG: Results & Discussion

A table is shown to display the concentration and mean diameter of PCCAs made with 30% and 10% PEG lipid shell (see Table 6-2). No statistical difference was found after increasing the PEG shell concentration to 30%. The mean diameter and particle concentration were tested using a two-sample T-test. A mean diameter (number-weighted average) of 0.19 was found for both PCCA formulations. Similarly, the resulting PCCA distribution concentration of the 30% PEG lipid shell showed no difference from the original protocol. The similarities in distributions can also be seen in Figure 6-2.

| Table 6-2: Size distribution results of DFB-containing MBs and PCCAs formulated with 30% PEG. Data was gathered using an AccuSizer for 780A the MBs and an AccuSizer FX Nano for the PCCAs. |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Size Distribution (n=3) | 30% PEG-PCCAs | 10% PEG-PCCAs |
| Mean Diameter (µm) | 0.19 ± 0.004 | 0.19 ± 0.004 |
| Concentration (particles/mL) | 9.62x10^{10} ± 2.08x10^{10} | 7.26x10^{10} ± 1.31x10^{10} |
Figure 6-2: Difference in size distribution as a result of PEG concentration demonstrates little difference between the two PCCA formulations.

As data shows, the increase in PEG did not have the hypothesized effect of increasing the population of 100nm PCCAs. Additionally, there were questions in regards to the stability of the particles. In terms of simple handling of the 30% PEG-PCCAs, the high rate of spontaneous vaporization was unrealistic for in vivo use. This occurrence is partially supported by work conducted by Abou-Saleh et al. [13], where PEG concentration of MBs were increased and in vitro characteristics of the contrast were studied. Authors saw a decrease in stability as the PEG concentration increased. It was predicted that different results could have been seen with PCCAs, as a result of the size difference, but this was not shown to be the case.

The principle idea of improving surface tension to create smaller MBs does seem to hold when poloxamer (or Pluronic) is added to the shell. Pluronic is a block copolymer that includes two hydrophilic ends made of polyethylene oxide and a hydrophobic center made of
polypropylene oxide. Krupka et al. [14] describes a formulation for developing nanobubbles (NBs) successfully, while maintaining stability and acoustic signal output. In the discussions, authors explain that the presence of Pluronic not only decreases surface tension, but increases the fluidity of the shell, which aids acoustic response during imaging [14].

6.2.5 Increasing Pressure for Condensation: Introduction

The creation of PCCAs includes a condensation phase, where we start by placing the microbubble solution in a cold bath before increasing the pressure in the headspace of the vial. Our second hypothesis for creating smaller droplets involved increasing the pressure applied to the microbubbles during the condensation phase of PCCA fabrication. This hypothesis is not without precedent: in the development of PCCAs using microfluidic techniques, temperature and pressure is manipulated to optimize size distribution [15], [16].

6.2.6 Increasing Pressure for Condensation: Methods

In the following work, I present two studies that explored changes in DFB PCCA size distribution as a result of increased pressure. In the first, the gas used for condensation was ambient pressure air. To produce the necessary pressure inside the vial, a syringe-based air-pressurizing device was utilized. As a result of the syringe, pressures were noted using volume changes, specifically 20mL of air, 30 mL of air, and 40 mL of air. In the second study, condensation of microbubbles was performed using compressed nitrogen. Unlike the first method, the second procedure does not reintroduce the vials back to atmospheric environment prior to compressing vial headspace. For this reason, droplets are compared separately.

The following study utilized two methods with inert gases, air or nitrogen. For the air comparison, a human-operated air compressor was utilized to increase pressure. Pressure is
measured in increments of volume added in milliliters. In the second comparison test, individual vials were connected to a compressed tank of nitrogen. Pressure added to the vial headspace is measured in pounds per square inch (psi). Each test included 3 vials of droplets. Size distributions were measured using the AccuSizer FX Nano.

### 6.2.7 Increasing Pressure for Condensation: Results & Discussion

The study using air to condense droplets tested three pressures measured in volume: 20mL, 30mL, and 40mL of air. The air used in this study began at atmospheric pressure. Average mean diameter and concentration are presented in Table 6-3, and the resulting size distribution from all three groups can be found in Figure 6-3. Average mean diameter and concentration was statistically tested using nonparametric, one-way ANOVA. No statistical difference was found between all three volumes of air used to condense the MBs for either the average mean diameter or the average particle concentration.

For the study comparing droplets condensed with nitrogen, three pressures were also tested: 10psi, 15psi, and 20psi (see Figure 6-4). These three pressures do not correlate with the pressures tested with air. Nonparametric, one-way ANOVA was used to compare the average mean diameter and average distribution of the groups condensed with nitrogen. Like the air condensed PCCAs, the distributions were statistically similar in regards to these metrics. The procedures tested with these six parameters did not partially increase the fabrication of 100nm PCCAs.

Though unsuccessful in this experimentation, the hypothesis of utilizing increased pressure to decrease PCCA still stands. Microfluidic devices have been engineered to manufacture PCCAs that result in controlled size ranges, from precise optimization of fabrication factors, like temperature and pressure [15], [16]. Unfortunately, these PCCAs
often suffer from limitations in yield. Through explorations in industrial manufacturing, PCCA fabrication could combine the precision and control of microfluidic device fabrication with the yield of sonication and condensation methods.

**Table 6-3**: Size distribution results of DFB PCCAs after being condensed by either pressurized air or nitrogen. Distributions were found using an AccuSizer FX Nano.

<table>
<thead>
<tr>
<th>Size Distribution of PCCAs (n=3)</th>
<th>20 mL Air</th>
<th>30 mL Air</th>
<th>40 mL Air</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Diameter (µm)</td>
<td>0.19 ± 0.004</td>
<td>0.20 ± 0.007</td>
<td>0.20 ± 0.004</td>
</tr>
<tr>
<td>Concentration (particles/mL)</td>
<td>7.26x10^{10} ± 1.31x10^{10}</td>
<td>5.70x10^{10} ± 1.01x10^{10}</td>
<td>6.61x10^{10} ± 1.01x10^{10}</td>
</tr>
<tr>
<td>Mean Diameter (µm)</td>
<td>0.20 ± 0.004</td>
<td>0.20 ± 0.008</td>
<td>0.21 ± 0.005</td>
</tr>
<tr>
<td>Concentration (particles/mL)</td>
<td>9.54x10^{10} ± 2.35x10^{10}</td>
<td>9.59x10^{10} ± 1.56x10^{10}</td>
<td>8.21x10^{10} ± 2.21x10^{10}</td>
</tr>
</tbody>
</table>
Figure 6-3: Comparison of size distributions of pressure increases used to condense MBs with air. Data displayed resulted from measurements taken from an AccuSizer FX Nano (n=3).

Figure 6-4: Difference in size distribution as a result of pressure increases when condensing MBs with nitrogen. Input pressures ranged from 10 psi to 20psi, and measurements were taken from an AccuSizer FX Nano (n=3).
6.2.8 Size-sorting PCCAs: Introduction

Finally, we attempted isolating the smaller MBs (or NBs), by size-sorting larger MBs from the NBs, which we hypothesized would condense into smaller droplets, ideally around 100nm. Based on previous work [17], we predicted a five-fold size decrease from a bubble to a droplet, which would mean isolating NBs around 500nm. We chose to use a centrifugation method in order to separate the bigger MBs from the NBs. In the protocol, we use an IEC Centra CL2 centrifuge (Thermo Electron Corporation, Waltham, MA, USA), which was capable of achieving speeds of 0 to 8,500xRPM (or 0 to 9,302xG).

6.2.9 Size-sorting PCCAs: Methods

In order to create a batch of the isolated NDs, we begin with two vials of the 1.5mL lipid solution (10% DSPE-PEG2000, 90% DSPC). Once headspace was replaced with the preferred PFC, the vials were shaken as previously described. MBs were combined in a 5mL syringe (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and diluted with 1 mL of 15% (v/v) propylene glycol, 5% (v/v) glycerol, and 80% (v/v) 1x PBS (or PGG solution). This MB solution was centrifuged for 5 min at an RPM of 1,700. This setting was found via optimization, which will be discussed in Section 5.2.4.2. Centrifugation of MB solution produced two phases. The lightest phase, at the top of the syringe, will be called the cake and it includes the larger diameter MBs. The bottom phase, mostly made of NBs, will be denoted as the infranatant. The infranatant was separated from the cake, and placed into a new vial. The NBs were condensed into droplets in a water bath set between -7°C to -11°C, and an air volume of 20mL.
6.2.10 Size-sorting PCCAs: Size Distribution Results & Discussion

Photographic evidence of the particle population changes are presented in Figure 6-5. For all data included in this study, DFB was the chosen PFC for the formulated PCCAs. Figure 6-6 depicts the size distribution of the three stages, which was measured from averaging size distributions from two trials of size-sorting PCCAs. In the graphs, the data depicts the MBs, the NBs, and the resulting PCCAs (see Figure 6-6). In the corner, a close-up of the NB and PCCA distributions are depicted. A table of values is presented to highlight the mean diameter and concentration averages from the graph (see Table 6-4). The measurements taken from AccuSizer FX Nano did not highlight much change in the diameter, though there is evident loss in particles, most notable in the 150nm to 200nm range.

Figure 6-5: Images of vials demonstrating optical change of particle solution as isolating procedure progressed from MBs (A) to NBs from the infranatant (B) to PCCAs (C).
Figure 6-6: Average size distribution of size sorted DFB PCCAs (N=2). The protocol begins with a solution of MBs, and NBs are isolated after centrifugation. PCCAs are then formulated from the NBs. All size measurements were collected from an AccuSizer FX Nano.

Table 6-4: Size distribution metrics for the size-sorting procedure demonstrate population similarities and differences between starting MB population, isolated NBs, and the resulting NDs. The averages are a result of two trials.

<table>
<thead>
<tr>
<th>Size Distribution- ASFX (n=2)</th>
<th>Microbubbles</th>
<th>Nanobubbles</th>
<th>Nanodroplets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean diameter (µm)</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Concentration (parts./mL)</td>
<td>7.12x10^{11}</td>
<td>2.16x10^{11}</td>
<td>4.02x10^{10}</td>
</tr>
</tbody>
</table>
6.2.11 Size-sorting PCCAs: Optimization of Centrifugation Parameters

Experimental testing was conducted to optimize the size-sorting parameters of centrifuge speed (RPM) and centrifuge time. Multiple parameters were tested between 1,000xRPM to 3,000xRPM for a 10 min exposure (see Figure 6-8). For each parameter shown, two trials were conducted. Size distributions of the NBs in the infranatant were measured on an AccuSizer 780A (PSS Particles, Entegris, Port Richey, FL, USA). No statistical testing was conducted on the data, but based on a comparatively high concentration and low mode, a speed of 1,700xRPM was chosen. From this test, centrifuge times of 5min, 10min, 15min, and 20min were tested. These times were tested by measuring the PCCA size distributions with a NanoSight NS500 (Malvern Instruments, Westborough, MA, USA). Figure 6-9 demonstrates the mean and mode of the particle distributions of each PCCA, given the time used to separate the MB distribution. No statistical testing was conducted, but distributions were generally similar to one another. Given the similarities, the shortest time of 5min was chosen for the centrifuge spin speed of 1,700xRPM.

---

4 The study was completed by Giancarlo Vasselli, but guided by A. Gloria Nyankima
**Figure 6-8:** Optimization of centrifuge speed was conducted using the AccuSizer 780A to measure size distribution ($n=2$/speed). Infranatants of each centrifuge speed were compared to one another and 1,700xRPM was isolated as a potential candidate, given lower mean and mode diameter and higher concentration.
Figure 6-9: Time optimization was conducted by measuring PCCAs isolated from infranatants centrifuged at 1,700xRPM. Size distributions of PCCAs were measured with the NanoSight.
6.2.12 Size-sorting PCCAs: In vitro and In vivo Imaging Results & Discussion

An in vitro analysis supported previously mentioned challenge of vaporizing smaller PCCAs. Size-sorted PCCAs were formulated (using the decided parameters in the optimization study) and placed in a 37°C water batch. Images were collected using a Verasonics V1 system (Kirkland, WA, USA), driving a L11-5 linear array transducer. While contrast images were developed at 9 MHz, the activation pulses were driven at 4.5 MHz. The peak negative pressures ranged from 2.25 MPa to 4.25 MPa. As Figure 6-10 demonstrates, non-size-sorted droplets have a lower acoustic threshold than the size-sorted droplets. Given these results, it was predicted that by formulating the size-sorted PCCAs with OFP, a lower boiling point PFC and thus, decreased acoustic threshold, we could achieve PCCAs with a smaller size distribution, but with comparable ADV thresholds to that of the non-size-sorted. The size-sorting procedure proved to be unsuccessful at fabricating working PCCA distributions with an OFP core.

With the understanding of the pressures required to vaporize the size-sorted PCCAs, there were concerns that due to tissue attenuation, these droplets would be difficult to image in vivo. An in vivo imaging study was conducted in a female Fischer 344 rat kidney (150-250g, Charles River Laboratories, Wilmington, MA, USA) to assess the signal generation of size-sorted PCCAs in vivo. The kidney was chosen as a model due to its function as a blood filtering system. PCCAs being too large to filter out of the kidney, the particles would circulate through the kidney before being filtered through other systems. Since the entire kidney can fit in an imaging plane, this organ is ideal for UCA in vivo circulation studies. After anesthetizing the animal, a solution of 60 µL size-sorted DFB PCCAs and 60 µL of 0.9% saline was injected into a tail vein catheter. PCCA images of the kidney were taken
similarly to the image sequence described in Chapter 3, where contrast images were collected before and after the transmission of activation pulses.

Figure 6-11 depicts the contrast images of the size sorted PCCAs at time points of 0, 5, 15, and 20 mins post injection of PCCAs. In the figure, the kidney has been circled, while a white arrow denotes the presence of MBs prior to activation pulses being transmitted. This seemingly indicates the uptake of vaporized MBs by the liver, or the circulation of the contrast to surrounding tissue. Based on the study conducted in Figure 6-10, a peak negative pressure of 4.5 MPa was used to conduct all activation sequences throughout the 20 min time period. Although at the higher limits of the L11-5’s recommended pressure output, it was possible to generate MBs from the size-sorted NDs at 4.5 MPa. Unfortunately, with subsequent studies, this was not the case, especially with repeat studies in a subcutaneous tumor model—where these PCCAs were designed to be utilized.

The difficulty in imaging the smaller droplets was not unexpected. It is understood that smaller droplets would increase activation threshold requirements [18]–[20]. Furthermore, my colleague Dr. Juan Rojas has demonstrated the increase in ADV during in vivo PCCA imaging even in non-sorted droplets [21]. Rojas et al. tested and showed the impact of vessel size and fluid viscosity on the increase of droplet vaporization threshold.
Figure 6-10: In vitro ultrasound images of MBs vaporized from droplets, demonstrating the differences in the level of activation from non-sorted (top row) and size-sorted (bottom row) droplets. Both PCCA populations contained a PFC core of DFB. As a result of the smaller diameter PCCAs, the size-sorted droplets required the higher peak-negative pressures to vaporize at all visualized depths.
Figure 6-11: In vivo rat kidney (circled) ultrasound images of MBs formed from activated droplets. NDs were administered to rodent models, and imaging was conducted of the right kidney. The figure demonstrates a single animal, over a 20min period, after one injection of size-sorted NDs. Activation pulses for PCCAs vaporization were transmitted at 4.5 MPa (peak negative pressure).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Pre-Activation</th>
<th>Post-Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>5</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>15</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>20</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

In summary, our method of size-sorting MBs to isolate NBs, and then condense them into PCCAs demonstrated one way of isolating the smallest population of MBs for the
manufacturing of PCCAs. As Figure 6-6 shows, the process does result in a loss of <200nm particles, as seen in the decrease of the 150nm peak from MBs to NBs to NDs. More work could be done to improve the isolation of this size-range particles, or more useful, the sole production of these particles. By simply formulating 100nm PCCAs, or 500nm NBs to convert to PCCAs, the protocol would likely reduce the time and waste produced in our described size-sorting method.

With our method, we were able to demonstrate the possibility of receiving acoustic signals from the smaller diameter PCCA distribution. As Figure 6-10 highlighted, size does impact the acoustic threshold but in an in vitro experiment, and within the bandwidth of the transducer, it was possible to generate MBs at the higher pressure outputs. More work is needed to improve the signal response in an in vivo application of the size-sorted PCCAs. In light of these challenges, there is still a potential to achieve the ideal 100nm PCCA distribution and utilize the technology for extravascular delivery of therapeutics. In the next section, we will discuss this potential and suggested techniques to propel PCCA technology in that direction.

6.3 CHALLENGES OF OBSERVING EXTRAVASATION

There are technical challenges in successfully identifying extravasated droplets. Mainly, the inverse relationship between size and ADV threshold, which may not be a problem except for the output pressure limits of the diagnostic ultrasound transducer that was used for this work. Additionally, the idea of using OFP, a lower boiling PFC, may be theoretically promising, but was practically unsuccessful under the conditions used in our fabrication procedure. Despite the inability to progress this work to in vivo tumor testing, it is
helpful to consider the potential challenges of experimentally testing the potential for extravasation with a responsive size-sorted PCCA distribution. An ideal demonstration of PCCA extravasation includes high resolution fluorescent optical imaging, which would be able to map tumor vasculature, alongside fluorescently-labeled PCCAs (hopefully located outside vasculature), as was accomplished by previous studies [6], [22]. Another experimental design that would support PCCA extravasation is the work of Yin et al.[23]. In the study, the experimented particle was administered to rodent models, and after a given time, animals were perfused, removing all blood, prior to histological analysis of tissue. In either case, these experimental setups would confirm the presence of PCCAs beyond tumor vasculature, but not the acoustic response of the identified particle. This is an important distinction to make, as result of the high potential for false-positives from fluorescently-labeling PCCAs. There is a risk of labeled free lipid or empty-micelles being mistaken as an extravasated PCCA.

In order to properly discern viable PCCAs from useless particles, it will be imperative to overcome the acoustic issues previously demonstrated. In overcoming these challenges of imaging PCCAs of small diameter and a core with high boiling point PFC, there are possible solutions developing in novel techniques of ultrasound research. Guidance of therapeutic ultrasound is moving towards cavitation detection to monitor therapeutic treatments in real-time, a feature much more difficult to achieve in magnetic resonance (MR) imaging, the gold standard for guiding therapeutic ultrasound in the United States. With cavitation detection, systems are designed with two types of elements: (1) low frequency elements for therapy, and (2) higher frequency for imaging. The imaging elements are intended to receive contrast signal during treatment for the harmonic response of bubbles in the treatment field. When
localized, treatment guidance can be improved to either minimize off-target effects or enhance target effects. This technique could deliver high enough pressures to activate extravasated PCCAs (given the therapeutic elements), and image their location beyond the vasculature. There are example devices on the market from companies like SonaCare Medical (Charlotte, NC, USA) and EDAP TMS, SA (Vauix-en-Velin, France), that use ultrasound-guidance to monitor treatment.

It is important to note that as promising cavitation detection is for therapeutic monitoring, the technique is often conducted at lower frequencies than the bandwidth usually associated with diagnostic transducers, which would introduce limitations on resolving PCCA extravasation. A possible solution for overcoming this particular challenge may lie in a novel technique known as super resolution. Super resolution is a growing imaging technique in ultrasound, in which highly resolved images are produced by localizing the spatial and temporal location of ultrasound contrast signals, beyond the resolution limitations of the system [24]. With this technique, the UCAs can be followed through space and time, and with this information, flow patterns of UCAs can be assembled together, creating a detailed image of vasculature size and blood velocity. Our lab, led by the work of Dr. Rojas, may have found a unique way of producing super resolution images [25]. After facing challenges with imaging droplets in an in vivo environment, Dr. Rojas developed a technique of imaging the signal response of droplet activation. However, the frequency content of droplet vaporization is low (~1.5 MHz), meaning low image resolution. Super resolution is a technique that can salvage the information from droplet activation to create images with improve resolution via the spatial and temporal localization techniques. With successful implementation of cavitation detection, super resolution image processing, and droplet
vaporization detection imaging, not only would this technique be able to identify viable
droplets and distinguish them from microbubbles (or micelles), by receiving the low
frequency droplet activation signals, but the resolution capabilities of super resolution may
be able to separate PCCAs located in tumor vessels from PCCAs located exterior to vessels.

6.4 FINAL RECOMMENDATIONS FOR PCCA TECHNOLOGY

In conclusion, as the ultrasound community continues to develop PCCA technology,
it is vital to consider the end-result of PCCAs. As described by Lea-Banks et al., translation
of PCCA is dependent on demonstrating (1) safety, (2) cost-effectiveness and ease of
handling, and (3) advantage and efficacy over currently FDA approved UCAs. Our lab has
completed work striving to understand the potential in vivo response of PCCAs as a
diagnostic imaging tool [26]. However, much more work should be done to understand the in
vivo response of PCCA imaging, including other organs, transmit frequencies, and imaging
sequences, than from what I have shown in Chapter 3. For a given application, droplets need
to out-perform microbubbles, which means focusing the clinical application of PCCAs to
targets where there exists a gap in efficacy from current commercial applications—reaching
beyond the vasculature and maintaining in vivo circulatory presence long beyond circulation
times of commercial UCAs.

The promise of PCCA technology is evident in the vast range of applications in both
therapy and diagnostic imaging. In therapy, from histotripsy to HIFU, droplets are being
investigated to decrease energy requirements for treatment. In imaging, molecular imaging
and super-resolution might be promising directions for PCCA-use in diagnostic imaging. It
should be clear that the same droplet formulation for one application (from composition,
formulation protocol, and shell design) may not be the same. As I hope my work has shown, optimization studies can identify safe and effective PCCA formulations and acoustic parameters for desired PCCA applications in both imaging and therapy. The most effective droplet for a given application must be designed with target outcomes in mind. I was able to develop a PCCA formulation that minimized bioeffects in the rodent kidney, but formulating the core with OFP or a 1:1 mixture of DFB-OFP. Activation pulses less than an MI of 1.9 were necessary to minimize bioeffects observed 24 hours post-imaging. I was also able to develop PCCAs that could produce enhanced lesion properties from a variety of formulations, under a range of therapeutic parameters. It is hoped that with both of these optimization investigations, research in PCCA technology can build from his work and continue to develop PCCAs for given clinical applications, guided by the influence PCCA formulation and acoustic pressure has on desired bioeffects.

As the challenges of developing an extravasating PCCA formulation show, there is still more work to do. Like any early-stage technology, PCCAs require continued innovation and optimization to produce a safe and effective, commercially-available, liquid-based UCA, capable of minimizing bioeffects during diagnostic imaging, enhancing thermal effects during HIFU, and reaching beyond the vasculature as a drug delivery vehicle.
REFERENCES


*Cell.* 2000.

*Cell,* vol. 144, no. 5, pp. 646–674, 2011.

biological barriers to drug delivery,” 

nanoemulsions as tumor-targeted drug carriers: Insight through intravital imaging,” 

ultrasonic tumor imaging and targeted chemotherapy,” 
*J. Natl. Cancer Inst.* vol. 99, 

“Accumulation of phase-shift nanoemulsions to enhance MR-guided ultrasound-mediated tumor ablation in vivo,” 

superheated fluorocarbon phase-change agents,” 
*Adv. Colloid Interface Sci.* vol. 237, 

interactions,” 


APPENDIX A

SUPPLEMENTAL INFORMATION FOR CHAPTER 2

A.1 METHODS & MATERIALS

A pilot study was conducted with an N of 16 (n=4/group) as a first round assessment of microbubble-induced bioeffects. This study was conducted with the same instrumentation and animal subjects as the final study presented, but with a few parameter changes. First, the animals were placed on their side while imaging the sagittal plane. Second, contrast imaging in between bubble destruction pulses was set to 1 second prior to stepping to the next plane. In this set up, the control kidney was exposed to ultrasound and bioeffects were observed in both. To address these issues, changes were made for the full study, including re-positioning the rat to reduce control kidney exposure.

A.2 RESULTS AND DISCUSSIONS

A.2.1 Preliminary Observation: Kidney Clinical Chemistry at 24-hours and 2-weeks

The preliminary observation included a total of 16 animals. Eight of these animals received a destructive pulse at an MI of 1.9. The other half received an MBD pulse at an MI of 1.0. The short-term and long-term endpoints were 24-hours and 2-weeks post imaging respectively. Blood samples prior to imaging (baseline) and at the time of necropsy (endpoint) were measured for BUN. For subjects exposed to an MI of 1.0, there was no significant change in the BUN levels from baseline to their endpoint (see Figure A-1).
In the 1.9 MI group, no significant change was observed after 24 hours. At the 2-week endpoint, the BUN of the 1.9 MI group had significantly increased from 17 ± 1 mg/dL to 23 ± 2 mg/dL (P=0.002).

Normal range for female Fischer rats is 19.18 ± 2.39 mg/dL (95% confidence internal ~15-24 mg/dL) [1]. As indicated in Figure A-1, mean BUN levels of all four groups remained in this range.

**Figure A-1: Clinical Chemistry Results.** BUN levels are grouped by imaging parameters and endpoint. Results of paired T-test indicate a statistically significant increase in BUN levels for subjects in the high MI group after 2 weeks (p-value from 0.001 to 0.01 is marked with a ‘**’). No statistical significance was observed in the low MI groups. Average BUN values remained within normal range for female Fischer rat (15 to 24 mg/dL, dashed lines).

**A.2.2 Preliminary Observation: Histopathology at 24-hours and 2 weeks**

In rats exposed to 1.0 MI pulses for CEUS imaging, no evidence of hemorrhage was found at either the 24-hour or 2-week time points (see Figure A-2). In the 1.9 MI group, total
RBC score averaged 13 ± 9 (mean ± SEM) when assessed after 24 hours. These signs of hemorrhage are absent on histopathologic examination after 2 weeks (see Figure A-2). As a result of the imaging setup, the signs of hemorrhage were observed in both the right and left kidney, removing a control from this study. It was this finding in the 1.9 MI at 24 hours that led to the final (and larger) study. To eliminate the chance of ultrasound exposure in the control kidney, the animals were imaged on their back, and the transducer mechanically-steered over the chosen imaging side. To determine if RBCs were originating from the glomeruli as a result of GCH, an earlier time point, similar to that used by Miller and colleagues, was chosen.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal No.</th>
<th>No. RBC casts (in ten 10X fields)</th>
<th>No. glomeruli with RBC (out of 100 glomeruli)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 MI at 24 hours</td>
<td>3</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>1.0 MI at 2 weeks</td>
<td>4</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>1.9 MI at 24 hours</td>
<td>4</td>
<td>13 ± 9</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>1.9 MI at 2 weeks</td>
<td>4</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

**Figure A-2: Histopathology Results.** Kidney for both low and high destruction pulses at 24 hours and 2 weeks. Data is displayed as the mean ± standard error of the mean (SEM).
REFERENCES

Table B-1: The parameters, histopathology, and serum creatinine for a preliminary investigation where Sprague-Dawley rats experience no PCCA vaporization. Instead, the activation pulses (at an MI of 2.7) were delivered to the experimental kidney. These parameters were not used in the study presented here. Both kidneys were assessed (by the same pathologist) and found to be normal.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Kidney</th>
<th>Type of Gas</th>
<th>MI</th>
<th>RBC Cast Score</th>
<th>Description</th>
<th>Creatinine 0 hours</th>
<th>Creatinine 4 hours</th>
<th>Creatinine 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Exp</td>
<td>None</td>
<td>2.71</td>
<td>0</td>
<td>Normal</td>
<td>0.1</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Con</td>
<td>None</td>
<td></td>
<td>0</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Exp</td>
<td>None</td>
<td>2.71</td>
<td>0</td>
<td>Normal</td>
<td>0.4</td>
<td>--</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Con</td>
<td>None</td>
<td></td>
<td>0</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Exp</td>
<td>None</td>
<td>2.71</td>
<td>0</td>
<td>Normal</td>
<td>0.3</td>
<td>--</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Con</td>
<td>None</td>
<td></td>
<td>0</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table B-2: Description of histopathology evaluations.

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Method of evaluation</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intratubular red blood cell casts</td>
<td>Examination of ten 10X fields</td>
<td>Total number of casts</td>
</tr>
<tr>
<td>Glomerular capillary hemorrhage</td>
<td>Examination of 100 glomeruli</td>
<td>Percent of glomeruli affected</td>
</tr>
<tr>
<td>Tubular degeneration and necrosis</td>
<td>Examination of ten 10X fields and semi-quantitative assessment of injury</td>
<td>Minimal-Vacuolar degeneration in one tubular profile</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mild-Vacuolar degeneration in 2 to 5 tubular profiles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderate-Degeneration and necrosis of tubular epithelial cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Severe-Large regions of coagulative necrosis of tubules</td>
</tr>
<tr>
<td>Interstitial inflammation</td>
<td>Examination of ten 10X fields and semi-quantitative assessment of injury; dominant</td>
<td>Minimal-1 to 3 inflammatory cells</td>
</tr>
<tr>
<td></td>
<td>cell type(s) was also noted</td>
<td>Mild – small aggregates of inflammatory cells which do not disrupt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>the tubulointerstitial parenchyma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderate- medium-sized aggregates of inflammatory cells which</td>
</tr>
<tr>
<td></td>
<td></td>
<td>separate the tubules</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Severe – Large aggregates of inflammatory cells with replace normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tubulointerstitial parenchyma</td>
</tr>
</tbody>
</table>
**Figure B-1:** Representative histopathology images (cortex and medulla) of the imaged kidney from one animal per treatment group. Images of the cortex (60X magnification) are on the left and the medulla (40X magnification) are on the right. (1A-B) The representative image of decafluorobutane (DFB) at a mechanical index (MI) of 1.9 depicts red blood cell (RBC) casts (black arrows) and proteinaceous cellular debris in the lumen of a tubule (white arrow) in the cortex. RBC casts were not present in the medulla. (2A-B) Representative images of 1:1 mix at an MI of 1.9 show RBC casts in both the cortex and medulla (black arrows) and cellular and nuclear debris, likely from a necrotic tubular epithelial cell. (3A-B) In octafluoropropane (OFP) at an MI of 1.9 representative images, RBC casts and protein casts are indicated with black and yellow arrows respectively. Cellular debris was also seen in this animal with associated inflammation around the tubule (white arrow). (4A-B) Representative images of rats exposed to 1:1 mix at an MI of 1.35 show normal renal parenchyma. Rare RBC casts were seen in this group. (5A-B) Renal histopathology of the OFP at an MI of 0.85 treatment group was within normal limits, without RBC casts.
APPENDIX C

SUPPLEMENTAL INFORMATION FOR CHAPTER 5

Table C-1: Area of lesion data for all four formulations, organized by mean, standard deviation (std. dev), and the number of lesions produced during HIFU treatment (N).

<table>
<thead>
<tr>
<th>PNP (MPa)</th>
<th>C3C4</th>
<th>C4</th>
<th>C4C5</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>N</td>
<td>Mean</td>
</tr>
<tr>
<td>1.0</td>
<td>0.000</td>
<td>0.000</td>
<td>3</td>
<td>0.015</td>
</tr>
<tr>
<td>1.5</td>
<td>0.018</td>
<td>0.005</td>
<td>3</td>
<td>0.072</td>
</tr>
<tr>
<td>2.0</td>
<td>0.084</td>
<td>0.050</td>
<td>9</td>
<td>0.115</td>
</tr>
<tr>
<td>2.5</td>
<td>0.072</td>
<td>0.023</td>
<td>3</td>
<td>0.142</td>
</tr>
<tr>
<td>3.0</td>
<td>0.127</td>
<td>0.052</td>
<td>8</td>
<td>0.147</td>
</tr>
<tr>
<td>3.5</td>
<td>0.126</td>
<td>0.028</td>
<td>3</td>
<td>0.145</td>
</tr>
<tr>
<td>4.0</td>
<td>0.158</td>
<td>0.042</td>
<td>9</td>
<td>0.186</td>
</tr>
</tbody>
</table>

Table C-2: Area under the curve (AUC) data for all four formulations, organized by mean, standard deviation (std. dev), and the number of lesions produced during HIFU treatment (N).

<table>
<thead>
<tr>
<th>PNP (MPa)</th>
<th>C3C4</th>
<th>C4</th>
<th>C4C5</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>N</td>
<td>Mean</td>
</tr>
<tr>
<td>2.0</td>
<td>233.603</td>
<td>35.437</td>
<td>4</td>
<td>296.763</td>
</tr>
<tr>
<td>3.0</td>
<td>551.226</td>
<td>50.471</td>
<td>4</td>
<td>468.622</td>
</tr>
<tr>
<td>4.0</td>
<td>792.651</td>
<td>194.284</td>
<td>4</td>
<td>581.142</td>
</tr>
</tbody>
</table>

Table C-3: Peak temperature data for all four formulations, organized by mean, standard deviation (std. dev), and the number of lesions produced during HIFU treatment (N).

<table>
<thead>
<tr>
<th>PNP (MPa)</th>
<th>C3C4</th>
<th>C4</th>
<th>C4C5</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>N</td>
<td>Mean</td>
</tr>
<tr>
<td>2.0</td>
<td>39.895</td>
<td>1.072</td>
<td>4</td>
<td>47.460</td>
</tr>
<tr>
<td>3.0</td>
<td>48.550</td>
<td>3.252</td>
<td>4</td>
<td>53.023</td>
</tr>
<tr>
<td>4.0</td>
<td>54.858</td>
<td>5.198</td>
<td>4</td>
<td>50.385</td>
</tr>
</tbody>
</table>