I, Jonathan T. Sutton, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Biomedical Engineering.

It is entitled:  
Tissue Bioeffects during Ultrasound-mediated Drug Delivery

Student's name:  Jonathan T. Sutton

This work and its defense approved by:

Committee chair: Christy Holland, Ph.D.

Committee member: Shiva Kumar Shanmukhappa, Ph.D.

Committee member: T. Douglas Mast, Ph.D.

Committee member: Gail Jean Pyne-Geithman, Ph.D.

Committee member: Marepalli Rao, Ph.D.
Tissue Bioeffects during Ultrasound-mediated Drug Delivery

by

Jonathan Sutton

B.S; Physics, College of William and Mary (2008)

Submitted to the College of Engineering and Applied Sciences Biomedical Engineering Program in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Engineering at the UNIVERSITY OF CINCINNATI

April 2014

©University of Cincinnati
Tissue Bioeffects during Ultrasound-mediated Drug Delivery

by

Jonathan Sutton

Submitted to the College of Engineering and Applied Sciences
Biomedical Engineering Program
on March 27, 2014, in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy in Biomedical Engineering

Abstract

Ultrasound has been developed as both a valuable diagnostic tool and a potent promoter of beneficial tissue bioeffects for the treatment of cardiovascular disease. Vascular effects can be mediated by mechanical oscillations of circulating microbubbles, or ultrasound contrast agents, which may also encapsulate and shield a therapeutic agent in the bloodstream. Oscillating microbubbles can create stresses directly on nearby tissue or induce fluid effects that effect drug penetration into vascular tissue, lyse thrombi, or direct drugs to optimal locations for delivery. These investigations have spurred continued research into alternative therapeutic applications, such as bioactive gas delivery. This dissertation addresses a fundamental hypothesis in biomedical ultrasound: ultrasound-mediated drug delivery is capable of increasing the penetration of drugs across different physiologic barriers within the cardiovascular system, such as the vascular endothelium, blood clots, and smooth muscle cells.

Dissertation Supervisor: Christy K. Holland
0.0.1 Publications

The author of this dissertation has contributed to the following articles that are published in or submitted to peer-reviewed scientific journals:


0.0.2 Acknowledgments

General

Thank you to everyone who, in their own unique way, helped me successfully submit this dissertation. I entered the Biomedical Engineering Program at the University of Cincinnati in the late summer of 2008, and have been engaged in research from the day I walked in the door of the Medical Sciences Building. Over the past 5.5 years, I’ve worked with countless professors, post-doctoral fellows, graduate students, research techs, and undergraduates—all of which deserve more than my gratitude. Azzdine Ammi, Kate Hitchcock, Jonathan Kopechek, and Nik Ivancevich got me on my feet in the scientific world, and took me seriously when they probably shouldn’t have. Kevin Haworth maintained their momentum, and taught me so many of the technical and career skills which I used in this dissertation, and that I have and will apply in my life. Gail Pyne-Geithman and Danielle Caudell-Stamper have counseled me on the mysterious world of non-engineering, and kept my dissertation “relevant”. Deborah Vela and Kumar Shanmukhappa have unselfishly lent helping hands from the amazing world of pathology. Ken Bader and Guillaume Bouchoux have been bottomless sources of wise scientific and philosophic consultation. Kirthi Radhakrishnan has been right there all along, carving a path one step ahead. Jason Raymond taught me a boatload of technical skills, and how to make a (very) good graph. Steve Perrin, Matt Gruber, and Kyle Rich have been riding along with me from the beginning, and kept me sane. Shenwen Huang, the latecomer, for imparting pockets of sage medical

A gray vertical line indicates portions of this section published or submitted for publication in:
wisdom and her metaphysical and culinary musings. In general, all of you constantly remind me how fun it can be to walk one’s own path—Thank You.

I like to think that one of the most important characteristics of a scientific investigator is creativity. I have been fortunate enough to be counseled by a sage PhD advisor, Dr. Christy Holland, who values very strongly the idea of creative solutions to problems. Christy Holland has worked tirelessly throughout her career to enable this type of scientific approach, and has worked tirelessly to populate her workplace with a diverse array of unique people. Throughout my dissertation studies, Christy Holland has been at the center of this group of people. Christy Holland’s unique ability to congeal the thoughts, hypotheses, and discoveries of these myriad individuals over many years certainly came to light as my doctoral studies matured. This dissertation is much more a product of that ability, as it is my own scientific progress.

Finally, a dissertation is just as much an emotional endeavor as it is a professional and intellectual feat. Thus, thank you to those who have helped me outside the laboratory everyday. I’ve received countless intangible expressions of support over the years, both from my family and friends—without whom I would have not persevered. My parents, Kimberly and Michael, have instilled an attitude of “work hard, play hard” from the beginning: an emboldening balance that is crucial in and out of any “lab”. They instilled that attitude from the earliest of age in my brothers, Matthew, Brett, Peter, and Mickey, upon whom I’ve relied constantly over the years for emotional, intellectual, and philosophical support. My teachers (St. Pius X, Covington Catholic HS, College of William and Mary) were never awarded for their relentless tutelage. Hopefully this dissertation can be the start to my morsel of thanks.

Specific

In Chapter 3, I acknowledge Marepalli Rao, Ph.D., for his support with statistical analysis, Jason L. Raymond, M.S., for his assistance with flow system components, and Stehlin Meats (Colerain, OH, USA) for providing porcine carotid tissue. Financial support was provided by NIH R01 NS047603 and a grant from the UC
For Chapter 4, I gratefully acknowledge Shane Duffield (University of Cincinnati; Biomedical Engineering) for his assistance with size distribution measurements and the technical staff at the National Center for Physical Acoustics (University of Mississippi, Oxford, MS USA) for manufacturing the custom tissue bath reservoir for ultrasound studies. The authors would also like to thank Dr. Neil Weintraub (Medical College of Georgia) for useful discussions about vascular biology and implementation of the tissue bath system.

This work was supported by NIH R01 HL74002 and a Pilot Grant from the University of Cincinnati Cardiovascular Center of Excellence (CVCoE).

In Chapter 5 I would like to thank T. Douglas Mast, Ph.D. for the use of the Zonare imaging system for alignment of arteries, and Shiva Kumar Shanmukhappa, DVM, Ph.D. for pathology support. I also acknowledge funding from NIH R01 HL074002.

The typesetting of this dissertation would not have looked as crisp without the open-source efforts of the LaTeX community at the Massachusetts Institute of Technology, specifically Stephen Gildea, PhD who originated the format. Countless others have made tweaks along the way. You will likely never read this document, but your efforts are surely appreciated.
Contents

0.0.1 Publications .................................................. 3
0.0.2 Acknowledgments ............................................. 4

1 Introduction ....................................................... 11

1.1 Background and Significance .................................. 11
  1.1.1 History of drug delivery ................................. 11
  1.1.2 Contemporary approaches to drug delivery ............ 12
  1.1.3 Ultrasound-mediated drug delivery .................... 12

1.2 Gap in Knowledge .............................................. 13

1.3 Hypotheses and Specific Aims .............................. 14

1.4 Dissertation Overview ......................................... 15

2 Concepts in ultrasound-mediated drug delivery ............ 17

2.1 Ultrasound ...................................................... 17
  2.1.1 Imaging .................................................. 17
  2.1.2 Technology for Therapeutic Delivery and Monitoring .. 18

2.2 Ultrasound-mediated Drug Delivery ........................ 18

2.3 Cardiovascular Disease ....................................... 21
  2.3.1 Barriers to Drug Delivery .............................. 22
  2.3.2 Mechanisms of Cellular Delivery ...................... 24
  2.3.3 Delivery to Cardiovascular Tissue .................... 27
  2.3.4 Ultrasound-mediated Mechanisms of Vascular Tissue Delivery 30
  2.3.5 Paracellular Transport .................................. 30
  2.3.6 Damage-induced Transport ............................. 31
  2.3.7 Transcytosis ............................................ 32

2.4 Stroke .......................................................... 32
3 Clot retraction affects the extent of ultrasound-mediated thrombolysis in an \textit{ex vivo} porcine thrombosis model.  

3.1 Materials and Methods ................................................. 42
  3.1.1 Whole-Blood Clot Formation .................................... 42
  3.1.2 Histology and Image Analysis .................................. 43
  3.1.3 Scanning Electron Microscopy .................................. 44
  3.1.4 \textit{Ex Vivo} Carotid Perfusion System ....................... 45
  3.1.5 Experimental Treatment .......................................... 46
  3.1.6 Model Parameters ................................................ 48
  3.1.7 Ultrasound Therapy ............................................... 49
  3.1.8 Passive Cavitation Detection ................................... 50
  3.1.9 Data Analysis and Statistical Methods ......................... 52

3.2 Results ................................................................. 52
  3.2.1 Histology .......................................................... 52
  3.2.2 Scanning Electron Microscopy .................................. 53
  3.2.3 Clot Fluid Phase Estimation .................................... 54
  3.2.4 Ex Vivo Thrombolysis ............................................. 54
  3.2.5 Cavitation Detection ............................................. 56

3.3 Discussion and Conclusions .......................................... 57
4 Ultrasound-mediated delivery of nitric oxide-loaded liposomes to \textit{ex vivo} porcine carotid tissue

4.1 Introduction ................................................................. 62
   4.1.1 Ultrasound-mediated drug delivery ................................. 62
   4.1.2 Experimental confirmation of drug delivery ....................... 62

4.2 Materials and Methods .................................................... 63
   4.2.1 Tissue bath system .................................................. 63
   4.2.2 Bubble liposomes .................................................... 65
   4.2.3 NOBL Characterization ............................................... 66
   4.2.4 Hemoglobin ......................................................... 67
   4.2.5 Ultrasound Exposure & Cavitation Detection ....................... 67
   4.2.6 Treatment ............................................................ 69
   4.2.7 Tension analysis .................................................... 70
   4.2.8 Statistics ............................................................ 70

4.3 Results ............................................................................ 71
   4.3.1 Liposome Characterization .......................................... 71
   4.3.2 Cavitation .............................................................. 73
   4.3.3 Vasorelaxation .......................................................... 73

4.4 Discussion ....................................................................... 75
   4.4.1 Liposome Characterization .......................................... 75
   4.4.2 Ultrasound exposure and cavitation ............................... 77
   4.4.3 Vasorelaxation ........................................................... 78
   4.4.4 System Development .................................................. 78
   4.4.5 Smooth muscle relaxation .......................................... 80

4.5 Conclusions .................................................................... 80

5 Delivery of bevacizumab-loaded echogenic liposomes to porcine carotid tissue increases in the presence of stable cavitation

5.1 Introduction .................................................................... 81
5.2 Materials and Methods ........................................ 81
  5.2.1 Bevacizumab-Loaded ELIP (BEVELIP) ............. 81
  5.2.2 Atheromatous Carotid Arteries ...................... 83
  5.2.3 Normal carotid arteries ............................... 84
  5.2.4 Ex vivo porcine carotid perfusion system .......... 84
  5.2.5 Color-Doppler ultrasound exposure ................. 84
  5.2.6 Cavitation Detection .................................. 86
  5.2.7 Treatment Overview .................................. 88
  5.2.8 Quantification of bevacizumab delivery .......... 89
  5.2.9 Statistics .............................................. 91
5.3 Results ....................................................... 91
  5.3.1 Immunoreactivity of BEVELIP ....................... 91
  5.3.2 Ex vivo system parameter measurement ............. 91
  5.3.3 Bevacizumab penetration ............................. 92
  5.3.4 Cavitation detection ................................ 94
5.4 Discussion .................................................... 95
  5.4.1 Bevacizumab Penetration ............................ 95
  5.4.2 Cavitation Detection ................................ 97
  5.4.3 Carotid histology .................................... 98
  5.4.4 Immunohistochemistry ............................... 99
  5.4.5 Bioeffects ............................................. 99
5.5 Conclusions .................................................. 100

6 Conclusions and Future Directions 101
  6.1 Conclusions ................................................ 101
  6.2 Future Directions ......................................... 106
List of Figures

2-1 Ultrasound-mediated drug delivery pathways ........................................... 26
2-2 Proposed mechanisms of ultrasound-mediated drug delivery ................. 28
2-3 Cavitation mechanisms of sonothrombolysis ...................................... 34

3-1 In vitro models of clot retraction ......................................................... 43
3-2 Schematic of ex vivo thrombolysis system ........................................... 46
3-3 Ultrasound alignment and send/receive electronic configuration .......... 50
3-4 In situ acoustic pressure calibration ..................................................... 51
3-5 Histological depiction of clot retraction models .................................. 53
3-6 Scanning electron microscopy .............................................................. 55
3-7 Thrombolytic efficacy ........................................................................... 56
3-8 Cavitation energy ................................................................................. 57

4-1 Electronic configuration of the ultrasound tissue bath system ............. 65
4-2 In situ ultrasound beam profiles of the 1-MHz and 7.5-MHz transducers. 68
4-3 Procedural timeline used in ultrasound tissue bath experiments .......... 69
4-4 Temporal profile of vasorelaxation ....................................................... 71
4-5 Acoustic and size characterization of nitric oxide-loaded bubble liposomes 72
4-6 Spectral analysis of scattered acoustic signal from nitric oxide-loaded bubble liposomes ......................................................... 74
4-7 Summary of vasorelaxation data ............................................................. 75

5-1 Experimental Setup ............................................................................. 83
5-2 Directivity pattern of transmitted ultrasound field, histologic analysis 85
5-3 Duplex doppler ultrasound exposure ................................................... 86
5-4 Elevational pressure profile of the color-Doppler line ......................... 87
5-5 DAB staining in atheromatous, adherent blood clot, and neointima . 90
5-6 DAB staining area by treatment group within the intimal/medial tu-
nics of porcine carotid arteries. . . . . . . . . . . . . . . . . . . . . . 92
5-7 DAB staining in normal porcine carotid arteries . . . . . . . . . . . . 94
5-8 DAB staining area by treatment group within the adventitial tunic of
porcine carotid arteries. . . . . . . . . . . . . . . . . . . . . . . . . . . . 95
5-9 DAB staining in atheromatous porcine carotid arteries . . . . . . . . 96
5-10 Cavitation emissions . . . . . . . . . . . . . . . . . . . . . . . . . . . . 97
5-11 Temporal profile of cavitation emissions . . . . . . . . . . . . . . . . 98
List of Tables

2.1 Investigations of US-mediated drug delivery to cardiovascular tissue. 24
2.2 Comparison of drug vehicles. 37
3.1 Parameters measured in the \textit{ex vivo} thrombosis system. 48
Chapter 1  Introduction

1.1  Background and Significance

1.1.1  History of drug delivery

The use of drugs to treat human ailments is a practice that pre-dates classical antiquity. Paleoanthropological evidence suggests that Neanderthals, residing near the Zagros Mountains in present-day Iraq, implemented herbal medicines to treat common conditions over 60,000 years ago [6]. Since this initial recognition of medicinal remedies, a myriad of drugs has been developed, ranging in source from natural to genetically engineered, and in effectiveness from curative to fruitless.

Recently, life-saving drugs have been developed to treat almost any specific diseased cell. These treatments have been administered orally, topically, intrathecally, and intravascularly in an attempt to target the drug to diseased cells while limiting interaction with healthy cells. Often, meager drug specificity presents the clinician with a risk-benefit decision [7–9]: how much of and for how long should a drug be administered to offer maximal gains for the patient?

Our ability to rectify a disease condition depends on many factors. Firstly, the drug must be administered in a manner that permits specificity to the diseased tissue. While many topical treatments benefit from high specificity, the majority of disease conditions are subcutaneous or systemic, requiring surgical intervention or percutaneous drug infusions to interstitial tissue or the vasculature. Once in the bloodstream, a circulating drug will interact with a vast surface area within the human body [10]. Despite this apparent advantage, the human vasculature has evolved sophisticated barriers to prevent exogenous molecules from passing into interstitial tissue [11]. Secondly, circulating biomolecules degrade foreign agents that exhibit immunogenicity. This process results in drug decay that limits the vascular con-
centration of circulating drug both spatially and temporally [12]. Thirdly, a treatment must affect disease pathology once it reaches the targeted tissue. For many conditions, molecular pharmaceuticals offer a biochemical resolution to disease pathology. However, other conditions, such as acute thrombosis, may be more effectively treated by mechanical methods [13], and for which biochemical approaches offer little promise.

### 1.1.2 Contemporary approaches to drug delivery

Since the early 20th century, the medical community has made significant advances that seek to overcome these obstacles to vascular drug delivery and improve drug deposition into specific tissue beds. Surgical techniques [14], though relatively invasive, permit direct perfusion of drug into the tissue bed of interest. Magnetic techniques [15] exploit the magnetic properties of therapeutic molecules, allowing site-specific delivery to tissues such as clot [16] or tumors [17]. Transdermal [18] and hydrogel [19] approaches can affect the local deposition of drug upon stimuli such as heat, pH changes, or ultrasound exposure. In general, drug specificity has been improved substantially with discoveries such as the enhanced permeation and retention effect (EPR) [20], and the development of targeting antibodies to molecular biomarkers [21]. These advances help to “steer” the drug towards pathologic tissue. Drug immunogenicity has been mitigated somewhat by use of techniques such as pegylation [22], which shield the drug from degradation and prolong circulation time. Mechanical stent deployment has recently become a mainstream method to provide structural support, recanalize stenotic blood vessels, and elute drug into the local tissue bed [23], yet is plagued with severe successive reocclusion [24].

### 1.1.3 Ultrasound-mediated drug delivery

Ultrasound—traditionally a diagnostic medical imaging modality—has recently been investigated as a tool to augment therapeutic drug delivery. Many of the benefits of ultrasound as a diagnostic tool also apply to therapeutic ultrasound, such as utilization of a broad dynamic range, compact design, minimal invasiveness, and tissue contrast. By coupling the high resolution imaging of ultrasound with novel echogenic
drug vesicles, the **specificity** of drug delivery can be improved. These vesicles can be rendered echogenic by co-encapsulation with a bubble stabilized against dissolution, containing inert or bioactive gases. Bubble-loaded vesicles can temporarily disrupt the barriers to effective drug deposition within pathologic tissue if driven at a sufficient acoustic pressure to induce cavitation. At these acoustic pressures, vesicles tend to liberate their gas pockets which can be verified with conventional ultrasound imaging [4]. Further, drugs can also **avoid immunogenicity** by encapsulation within a vesicle and their release profiles confirmed ultrasonically. Finally, high resolution and intensity focusing of an ultrasound array can cause cavitation or exert acoustic radiation force, which can **elicit mechanical effects** within pathologic tissue, such as tissue or thrombus fragmentation.

### 1.2 Gap in Knowledge

Despite the potential to improve drug delivery, the mechanisms by which ultrasound elicits bioeffects remain unclear. Many demonstrations of ultrasound-mediated drug delivery (UMDD) have been performed *in vivo*, where mechanistic data is difficult to obtain, largely due to the lack of appropriate *in vitro* and *ex vivo* models [5]. Ultrasound-mediated drug penetration has been consistently demonstrated across the blood-brain barrier to treat a variety of neurologic conditions [25]. However, a paucity of data exist that demonstrate UMDD into the vascular tissue of large vessels—a haven for cardiovascular disease progression [26]—possibly due to the strong barriers to transport within these vessels [27]. Once delivered to vascular tissue, a drug must diffuse into or be endocytosed by pathologic cells to elicit a potent bioeffect. Within the field of sonothrombolysis, co-administration of thrombolytic agents combined with acoustically activated bubbles has been shown to increase vessel recanalization *in vivo* and improve clinical outcomes compared to conventional treatments [28]. However, thrombolytic treatments are ineffective on certain clinical subpopulations [29], possibly due to variation in blood clot composition [30]. The studies outlined in this dissertation aim to provide evidence to fill these gaps by investigating
the effectiveness of ultrasound at penetrating various barriers to drug transport in the human body. By examining the success of this technique alongside the specific morphological and biochemical properties of drug barriers, mechanistic conclusions can be drawn and the field of ultrasound-mediated drug delivery will be advanced.

1.3 Hypotheses and Specific Aims

The goal of the studies described in this dissertation was to characterize bioeffects induced in vascular tissue in the presence of acoustically activated microbubbles. Specifically, ultrasound can interact with microbubbles and promote enhanced delivery of a drug to nearby tissue through cavitational mechanisms. In large arteries, proteins can be released from acoustically activated vesicles and driven across major vascular barriers by ultrasound. Alternatively, bioactive gas release by ultrasound can cause a decrease in vascular tension—an effect associated with vascular permeability. In the presence of a thrombus, ultrasound can promote the penetration of thrombolytic drugs into the heterogenous fibrin mesh, expediting lysis. These effects, enhanced drug penetration and vasorelaxation, depend on the specific type of cavitation experienced by a microbubble. Thus, the central hypothesis of this dissertation is that acoustically activated microbubbles promote the delivery of drugs to vascular tissue across different physiological barriers.

Hypothesis 1: The permeability of whole blood clots affects lytic rate in the presence of acoustically activated microbubbles and a thrombolytic drug.

Specific Aim 1: Investigate the effect of whole blood clot composition on sonothrombolytic efficacy.

Specific Aim 1a: By modifying previously established methods, develop a whole blood porcine clot model that mimics the two extremes of in vivo thrombus retraction. Specific Aim 1b: Test the thrombolytic efficacy of low frequency ultrasound in combination with a thrombolytic drug and microbubbles on these two clot models.

Hypothesis 2: Nitric oxide delivery from bubble liposomes to porcine
smooth muscle cells increases in the presence of ultrasound.

Specific Aim 2: Investigate changes in vascular tension when carotid arteries are exposed to ultrasound and nitric oxide-loaded microbubbles.

Specific Aim 2a: Develop a vascular tissue bath system to characterize changes in vascular ring tension during simultaneous drug perfusion and ultrasound exposure. Specific Aim 2b: Characterize changes in vascular tension during ultrasound-induced nitric oxide release and delivery to carotid tissue.

Hypothesis 3: Color-Doppler ultrasound exposure increases the penetration of bevacizumab-loaded echogenic liposomes across the vascular endothelium of normal and atherosclerotic vascular tissue.

Specific Aim 3: Correlate delivery of drug-loaded echogenic liposomes into porcine carotid tissue after color-Doppler exposure with the amount and type of detected acoustic cavitation.

Specific Aim 3a: Within an established ex vivo perfusion system, expose Yucatan miniswine porcine carotid segments, perfused with bevacizumab-loaded ELIP (BEVELIP) in plasma, to color-Doppler ultrasound. During treatment, detect the acoustic scattering from the flowing BEVELIP to verify differential amounts and types of cavitation occurring within the lumen. Specific Aim 3b: Develop immunohistochemical methods to determine the distribution of bevacizumab within porcine carotid tissue. Specific Aim 3c: Measure the distribution of bevacizumab within the porcine carotid tissue as a result of BEVELIP delivery from the treatments applied in Specific Aim 3a.

1.4 Dissertation Overview

The body of this dissertation describes experiments that investigate the ability of ultrasound to improve drug delivery across various physiological barriers. Chapter 2 outlines some of the theoretical concepts required to conduct research in this area of ultrasound-mediated drug delivery. Basic principles of acoustic propagation and mechanical effects elicited during acoustic phenomena are discussed and related to
physical and biochemical transport of drug carriers. To introduce the concept of sonothrombolysis, Chapter 3 reviews current literature and outlines commonly used experimental procedures within the field, such as clot models and metrics for thrombolytic efficacy. It continues to describe a study conducted to investigate the differential ability of oscillating microbubbles, driven by 120-kHz ultrasound, to expedite clot lysis in a porcine carotid thrombosis model. Chapter 4 describes a study that demonstrates the construction of a novel system to measure the release and delivery of nitric oxide from bubble liposomes to smooth muscle cells within the carotid media using pulsed 1-MHz ultrasound. In this study, the potent ability of nitric oxide to elicit arterial relaxation is measured as a function of ultrasound exposure. Chapter 5 discusses the delivery of an anti-angiogenic drug—bevacizumab—to porcine carotid tissue using a clinical diagnostic ultrasound system. For effective delivery to porcine carotid tissue, a drug must be transported across two major barriers to drug delivery: the vascular endothelium and internal elastic lamina. Together, these experiments demonstrate ultrasound-mediated drug delivery to three distinct targets across various physiologic barriers and further demonstrate ultrasound as a novel method to improve drug delivery.
Chapter 2    Concepts in ultrasound-mediated drug delivery

2.1    Ultrasound

2.1.1    Imaging

Since its development as a imaging strategy in the mid 1900s, ultrasound has grown steadily as a diagnostic modality to interrogate tissue properties. Ultrasound is a non-invasive method to produce image of body structures and is achieved real-time such that tissue motion can also be imaged. Acoustic pulses are emitted from a ultrasonic transducer into the body, which reflect off interfaces based on differences in acoustic impedance. Upon arrival back at the face of the ultrasound transducer, acoustic energy is detected as a function of time, which corresponds to differences in acoustic impedence, as a function of distance along the axis of acoustic propagation. Along one dimension, this technique is dubbed \textit{amplitude mode} and is often used for alignment of structures in ultrasonic experimentation. One of the earliest forms of ultrasound imaging and now the most ubiquitous, brightness mode (b-mode), pro-

---


duces a two-dimensional image of tissue structures. In the past 20 years, diagnostic ultrasound imaging has experienced waves of technical development. One such development, color-Doppler ultrasound, is a technique that produces information about the speed of the acoustic scatterers within an image plane by assessing the shift in frequency in the received signal scattered from a moving object. To achieve adequate velocity resolution, color-Doppler ultrasound uses acoustic pulses of greater duration than standard b-mode imaging, and thus more energy deposition into tissue, which is associated with bioeffects [31–33].

2.1.2 Technology for Therapeutic Delivery and Monitoring

A prime benefit of ultrasound imaging to the clinician is its non-invasive nature, guided in large part by the development of guidelines to prevent mechanical [34] and thermal [35] tissue damage. Over recent decades, as understanding of disease physiology, morphology, and genetics evolves, the idea of using ultrasound to deposit heat or induce mechanical stress within biological tissue becomes increasingly attractive. From this notion, therapeutic US has been developed to elicit beneficial bioeffects in the body by harnessing both thermal and mechanical mechanisms such as histotripsy, lithotripsy, ultrasonic ablation, sonothrombolysis, and ultrasound-mediated drug delivery. Research in these areas suggests that the amount of acoustic power required to achieve these therapeutic bioeffects (> 1 W) often exceeds that of standard imaging modalities (0.1 – 10 mW). Thus, a rigorous understanding of the mechanisms underlying these ultrasound-induced bioeffects is crucial to ensuring the evolution of ultrasound therapies alongside conventional and novel imaging strategies.

2.2 Ultrasound-mediated Drug Delivery

When US interacts with stabilized gas bodies, such as echo contrast agents, a localized breach in the endothelial barrier can be triggered [36]. Specific mechanisms mediating this response are actively under study. In addition, theragnostic agents—containing both gas and drugs—are under development; US activates the gas bubble
and causes the release of a therapeutic payload [37]. Interaction of US with the liberated gas can also transduce mechanical energy to local tissue, which elicits a biochemical response [38].

Acoustic cavitation is the formation and collapse of gaseous and vapor bubbles in a liquid due to an acoustic pressure field [39–53]. Cavitation is generally classified into two types: stable cavitation, which results in acoustic emissions at subharmonics of the fundamental frequency and associated ultraharmonics, and inertial cavitation, which is characterized by broadband acoustic emissions. When a bubble oscillates nonlinearly about its equilibrium radius, the radiated pressure wave may include subharmonic or ultraharmonic content [53–55]. Broadband emissions are generated when bubbles undergo large radial oscillations and collapse violently [56, 57]. This type of bubble motion is dominated by the inertia of the surrounding fluid, hence the label “inertial” cavitation. Stable cavitation can induce bubble-associated microstreaming [58, 59] and inertial cavitation can cause microjetting and pitting on solid surfaces [60–62]. Exploiting the acoustic impedance mismatch between microbubbles and blood, acoustic radiation force on microbubbles [63, 64] has been used to push drug-loaded agents in the bloodstream toward the endothelium for improved local deposition of the drug [65, 66]. Similarly, acoustic streaming—a steady fluid flow caused by an acoustic field—can increase mass transport of a therapeutic [67–69].

Acoustic cavitation has been shown to mediate many therapeutic US applications, including drug and gene release and delivery [70–79], and sonothrombolysis [2, 80–83]. Passive and active cavitation detection techniques have been developed to monitor acoustic cavitation [84, 85]. Passive schemes employ a transducer that listens passively (i.e. no transmit) to emissions from acoustically activated microbubbles. More recently, multiple-element arrays have been developed to allow for spatial resolution of bubble activity over a large area [86–89]. Active subharmonic imaging techniques for bubble detection using US arrays have also been implemented [90–92].

US can exert direct effects on tissue to enhance cardiovascular drug uptake. These mechanical effects can promote beneficial bioeffects by augmenting the bodys
natural mechanisms, as in the case of sonothrombolysis [81], or by creating new delivery pathways, as in the case of sonoporation where US induces pore formation in the cellular membrane [93, 94]. US is capable of facilitating delivery of bioactive macromolecules, gases, and cell nuclear material to a variety of vascular tissue and cell types, such as thrombus [81, 95], endothelial cells [96], smooth muscle cells [97], and cellular nuclei [98]. Table 2.1 (pp. 24) highlights some of these investigations.

Ultrasound-mediated drug delivery to these different tissue targets could involve disparate mechanisms and transport routes, which depend on specific biochemical responses to US. For example, acoustically active, drug-loaded vesicles can become tethered to the endothelial membrane via a vascular ligand [99]. Thereupon, vesicles extravasate (a) paracellularly, through the breakdown of tight junction proteins [100], (b) intracellularly by endocytosis [101] or through sonoporation [94]. Recent attempts to manipulate the elastic properties of drug-loaded vesicles have rendered their adhesion to activated endothelium in a similar way to immunoreactive leukocytes [99]. Alternatively, drug delivery to intraluminal thrombi is accelerated by US in a similar fashion to that of blood flow around and through a clot. The fluid dynamics at the surface of a thrombus replenishes fibrinolytic proteins within the clot [81], while also removing fibrin degradation products [102]. Ultrasound-mediated drug delivery seeks to promote these processes for the sake of delivering therapeutics to diseased tissue. These pathways, and the hypothesized mechanisms by which US could facilitate their manifestation are depicted in Figures 2-1 and 2-2, and discussed below.

The barrier between the bloodstream and vascular tissue presents a significant challenge to extracorporeal drug delivery. Macromolecules can accumulate on the luminal side of this barrier if appropriate ligands exist to facilitate affinity. Once bound, transport into the local tissue bed occurs if the body’s natural mechanisms permit permeability of the endothelium and other vascular barriers [103, 104]. Pharmacokinetic modeling, coupled with the development of targeted pharmaceuticals, has improved the specificity and efficacy of therapeutics. However, sensitizing the endothelium to increase local permeability remains an area of active research to
improve cardiovascular drug delivery [105].

2.3 Cardiovascular Disease

Currently in the United States, 83.6 million adults have been diagnosed with at least one type of cardiovascular disease (CVD) [106]. Within the United States, direct and indirect costs associated with CVD are projected to rise to $918 billion by 2030 [107]. Atherosclerosis is a vascular disease and the major underlying pathology that can transform into many other CVDs, and affects many large arteries within the vasculature [107].

Atherosclerosis is characterized by the build-up of lipids, cholesterol, and other substances in arterial walls and the formation of plaques [108]. These plaques elicit an immune response [109] and become unstable, shedding fatty deposits and calcified matter into the lumen [110]. The atherosclerotic plaque fragments subsequently occlude downstream small-diameter vessels, or become nuclei for thrombi, leading to myocardial infarction, pulmonary thromboembolism, or ischemic stroke. Atherosclerotic tissue is also prone to angiogenesis, or the proliferation of blood vessels, to feed hypoxic, nutrient-rich tissue. Carmeliet et al. [111] discuss the pathophysiology of angiogenesis and its prevalence within the vasculature. Vascular endothelial growth factor (VEGF) is a primary angiogenic signaling molecule, and a major target for cardiovascular, anti-angiogenic therapies [112].

The development of effective anti-inflammatory pharmaceuticals and statins has dramatically enhanced the quality and extent of care for atherosclerosis, but at an average cost of $800 million per drug [113]. Furthermore, the amount of drug that can be administered intravenously to a patient is often limited by its effect on healthy tissue, rather than the extent of disease [114]. One such drug—bevacizumab, an antibody to vascular endothelial growth factor (VEGF)—has been developed to inhibit angiogenesis, but its clinical use is stagnated by off-target effects [115, 116]. Non-invasive techniques to enhance the concentration of drugs reaching pathologic
tissue, thereby arresting the progression of atherosclerosis, would constitute a significant advancement in CVD care by improving clinical outcomes and reducing health costs.

### 2.3.1 Barriers to Drug Delivery

Many factors hinder the penetration of therapeutics from the vascular lumen into an arterial tissue bed, including blood flow, luminal concentration of drug, barrier permeability, pressure-induced convection, and intracellular transport rates [27, 117, 118]. The specific contributions of each factor within large vessels have been investigated using analytical models specifically developed to describe the transport of tracers into vascular tissue. Penn et al. used one such model to describe the in vivo penetration of horseradish peroxidase (HRP) into the tunica of hypertensive rats by treating the arterial bed as a system containing four compartments: the vascular lumen, the tunica intima, the tunica media, and the tunica adventitia [27]. Though the endothelium was the dominant barrier to penetration, the internal elastic lamina was responsible for up to 25% of penetration resistance. Another study by Penn et al. [117] showed that once the endothelial permeability exceeded a threshold, drug accumulated in the intima due to preferential pressure-driven convection. Analytical models of drug delivery are important due to their ability to describe the degree to which individual vascular properties contribute to vascular drug delivery.

The vascular endothelial barrier differs among organ types depending on the function and level of control required for optimal performance. Appreciable differences have been found in structure, signaling control, selectivity, and permeability [119]. The vascular endothelial barrier function and integrity can be compromised in many ways, such as bacterial toxins, oxidative stress, ischemia, immune function and dysfunction and vasculopathy as well as cancerous changes [120–124]. Increased understanding of these disease processes not only helps develop theragnostic strategies, but also gives insight into the normal function of the vascular endothelium, which can be manipulated for drug delivery.

Most research effort directed toward drug delivery from the lumen into the tis-
issue bed has been, and continues to be, focused on the blood-brain barrier (BBB). This bias is largely due to relatively recent understanding of the pathophysiology of many neurological diseases and the enormous potential benefit of effective therapeutic delivery. The BBB is a highly-selective and tightly-regulated endothelial barrier. A complex network of intercellular proteins form a tight junction where selective molecular passage is achieved by size exclusion. These proteins are coupled to intracellular structural and signaling proteins within the endothelial cells. Tight junction proteins are in contact with adjacent astrocytic endfeet, conferring a higher level of control and providing avenues for modulation. There is a large body of excellent literature describing this unique barrier [125], the challenges it presents [126] and the approaches to drug delivery currently under investigation [127].

Outside of the brain, transendothelial transport is less stringently regulated, but remains a significant barrier to drug delivery. Within the cardiovascular system, one may encounter endothelial barriers in conductance vessels, capillaries and neovascular tissue (e.g. tumors, ischemic damage) [128, 129]. Aside from differences in interaction with and regulation of vascular smooth muscle [130], variations in barrier structure and function can be discerned [131]. Conductance vessels, such as aorta, pulmonary and coronary arteries, typically possess robust endothelial layers that regenerate quickly after trauma. Also, when healthy, these cells are resistant to shear stress and oxidative damage necessary in a high-pressure, high-flow elastic system. Conductance vessel endothelial function is closely coupled to vascular regulation, monitoring oxygen and substrate levels and providing feedback to the smooth muscle via intrinsic relaxation agents (nitric oxide, bradykinin) or contractile agents (adrenergic β1-receptor agonists) to adjust bulk flow.

Cardiac microvascular endothelium is a unique barrier that also communicates with neuronal cells to synchronize and regulate heart rate and blood flow [132]. Accordingly, the microvascular endothelium is tightly controlled specifically with respect to electrochemical gradients and ion flow to preserve cardiac neurovascular coupling [133]. Disruption of this barrier could affect normal cardiac function and must be borne in mind when manipulating tight junction proteins in the heart.
Vessels formed during tumorigenesis, as well as in neovascularization of ischemic myometrium, tend to be tortuous and thin-walled. The endothelial layer is easily damaged, and not well regulated [124, 129]. Thus, the vascularization of tumors is characterized as leaky—a condition often promulgated by tumor cells to foster further pathological angiogenesis serving the tumor [111]. This phenomenon is both a useful avenue for somewhat specific drug delivery to the tumor tissue, and a target for reduction—by drugs or radiation—to starve the tumor [15].

2.3.2 Mechanisms of Cellular Delivery

Currently, a significant body of cardiovascular drug delivery work focuses on ultrasound-mediated drug delivery to individual cells. These in vitro investigations explore two important interactions vital to drug delivery: (a) drug transfer dynamics
from a delivery vesicle to a cell and (b) mechanical interactions between cells and oscillating bubbles. Acoustic pressure amplitude, bubble concentration, and the proximity of bubbles to cells [146] all play a role in drug delivery efficiency.

Hamster ovary cells exposed to pulsed US exhibited increased uptake of FITC-dextran, a fluorescent macromolecule unable to diffuse across plasma membranes [134]. Furthermore, the cells promoted the expression of luciferase plasmids added to the extracellular serum. At higher acoustic pressures (> 0.3 MPa), cells were unable to repair pores in the local membrane caused by acoustic cavitation, resulting in lysis and loss of viability. Mehier-Humbert et al. further investigated the extent of pore formation in a study examining the dependence of tracer size on sonoporative transport. Particles of increasing size up to 37 nm in diameter were able to penetrate the plasma membrane in the presence of phospholipid microbubbles exposed to US (0.57 MPa; 1.15 MHz) [138].

The degree of sonoporation, whether “reparable” or “lethal”, is also dependent on bubble-to-cell separation. In a flagship study on sonoporation, Ward et al. described a nonlinear increase in lethal sonoporation as the theoretical bubble-to-cell spacing decreased from 60 µm to below 20 µm for Optison™ microbubbles [147]. The authors posited that acoustic cavitation was responsible for this bioeffect. Based on the published acoustic parameters and experimental setup, recent cavitation simulations support this assertion, predicting that Optison™ microbubbles would nucleate both stable and inertial cavitation [53].

Acoustic cavitation has been the prime emphasis of ultrasound-mediated drug delivery, especially for delivery of drugs into cells. When a microbubble experiences an acoustic pressure wave near a biological membrane, bubble oscillations provoke desired [95] or deleterious bioeffects [147]. The type of fluid dynamics that result depend on parameters such as the frequency, amplitude, and duration of US [148]; the material properties of the microbubble, its shell, and the nearby surface [149]; and the degree of bubble proximity to that surface [150]. Several studies have investigated microbubble interactions experimentally [78, 148] and theoretically [151].
Figure 2-1: An overview of three penetration routes stimulated for ultrasound-mediated drug delivery. Sonoporation refers to the localized, mechanical disruption of a plasma membrane, which allows drugs and ions to diffuse passively. Transcellular pathways, such as endocytosis, involve active transport of drug via cytosolic vesicles. The paracellular route occurs when endothelial cells spread apart, either due to desquamation or by tight junction breakdown from bubble-induced shear stress. From: Sutton et al. Ultrasound-mediated drug delivery for cardiovascular disease. Expert Opin. Drug Deliv. Vol. 10. No. 5 pp. 573-592, 2013.

The microjet formation from inertially collapsing microbubbles can create pores mechanically in the plasma membrane [94]. Endocytosis and subsequent transcytosis can be stimulated through this localized mechanical destruction of the endothelial membrane. Depicted in Figure 2-1, cavitating microbubbles near a cell membrane can create a pore for increased membrane permeability to macromolecules, genes, and extracellular ions, which is repaired by cellular mechanisms within seconds [95, 101, 146]. Membrane permeability to extracellular ions upregulates endocytosis of perimembranous macromolecules due to the calcium flux into the cell [152]. Upon apical endocytosis, vesicular macromolecules can be expelled into the cytoplasm, or trafficked basolaterally for exocytosis into the intima, completing the transcytosis pathway [153].
The type of microbubble oscillations required to observe these effects have also been investigated. Inertially cavitating microbubbles near a membrane form 1 — 200 nm pores due to localized tensions [154], and are capable of resealing bifunctionally by either rapid phospholipid rearrangement or gradual, exocytosis-mediated membrane tension changes, which restores membrane integrity [94]. Microbubble-induced tissue damage correlates with the mechanical index at high acoustic pressures, evidenced by linear correlations with cell viability and capillary rupture above a peak rarefractional pressure of 0.61 MPa [155]. By definition [156], correlation of this bioeffect with the mechanical index further confirms that tissue damage is associated with inertial cavitation. Evidence of these effects at lower acoustic pressures exists as well. Juffermans et al. observed calcium influx into rat cardiomyoblast cells with Sonovue® (Bracco Suisse SA; Geneva, Switzerland) microbubbles at a mechanical index of 0.1, which is likely not sufficient to cause inertial cavitation [47]. Interestingly however, when intracellular hydrogen peroxide was not sequestered during the experiment, the extent of calcium influx increased, suggesting a biochemical mechanism at low acoustic pressures [143]. As the acoustic pressure amplitude is increased however, peroxide-dependent bioeffects are attenuated, as evidenced by Lawrie et al. (mechanical index 2.0) [71] and Lionetti et al. (mechanical index 1.2) [156].

2.3.3 Delivery to Cardiovascular Tissue

Investigations of drug delivery to tissue beds shed light on the extent of drug penetration and the risk of deleterious bioeffects. As illustrated in drug delivery studies to individual cells, a fine line exists between desirable cellular bioeffects and irreversible cell damage. Herbst et al. exposed atheromatous porcine carotid vessels to ICAM-targeted echogenic liposomes conjugated to vascular stem cells in the presence of continuous-wave US [143]. Stem cell delivery to the arterial tunics increased significantly within the intima when exposed to US at a peak-to-peak pressure amplitude of 0.15 MPa, but was absent beyond the internal elastic lamina. In *ex vivo* murine aortas, ICAM-targeted echogenic liposomes extravasated beyond the endothelium in the presence of 1-MHz continuous-wave US but did not extravasate
Figure 2-2: An overview of some proposed mechanisms for transcellular and paracellular ultrasound-mediated drug delivery. Transcellular: As a result of bubble-induced shear stress along the cell membrane, extracellular drugs can undergo caveolin-1-mediated endocytosis. Additionally, sonoporation can create “holes” in the cell membrane facilitating influx of ions or drugs. Paracellular: Shear stress from cavitation-induced microstreaming can cause caveolin-1 to detach from endothelial nitric oxide synthase (eNOS). Here, it converts arginine to nitric oxide, stimulating vasodilation and possible paracellular permeability. Alternatively, this shear stress deforms the actin cytoskeleton, which can cause conformational changes and breakdown of tight junction proteins (ZO-1, occludin). From: Sutton et al. Ultrasound-mediated drug delivery for cardiovascular disease. Expert Opin. Drug Deliv. Vol. 10. No. 5 pp. 573-592, 2013.

in the absence of US exposure [1]. The spatial distribution of delivery was confined to the intimal layer, with scant penetration beyond the first few elastic lamellae. At these modest acoustic pressures, no ultrasound-induced tissue damage was observed histologically.

To promote delivery within the tunica media, Tiukinhoy-Laing et al. exposed porcine carotid arteries to α-actin-targeted ELIP labeled with calcein during 1 MHz US exposure at a peak-to-peak pressure amplitude of 0.23 MPa [97]. Considerable calcein was observed in the tunica intima and adventitia, suggesting a strong contri-
bution from transendothelial and adventitial delivery routes, respectively. Moderate calcein delivery was observed within the intact tunica media, depicting successful localization within smooth muscle cells.

Within descending porcine coronary arteries after balloon angioplasty, Phillips et al. observed increased transgene expression beyond the endothelium with intravascular US exposure in vivo [157]. The extent of delivery was again localized to the intimal layer, but endothelial desquamation that often accompanies balloon angioplasty was not investigated [158], opening the possibility of direct delivery to intimal cells aided by mechanical “pushing” from acoustic radiation force. Together, these investigations of ultrasound-mediated drug delivery consistently demonstrate that small macromolecules and cells can be safely delivered to the artery wall when circulating microbubbles are exposed to ultrasound with a moderate peak-to-peak pressure amplitude (0.15 – 0.5 MPa) and at frequencies between 100 kHz and 10 MHz.

Investigations of ultrasound-mediated drug delivery to the myocardium consistently demonstrate robust delivery of growth factors and genetic material for therapeutic benefit. Traditional techniques to revascularize diseased coronary artery tissue, such as percutaneous, transmyocardial administration of vascular endothelial growth factor (VEGF) can be improved up to eight-fold by administering the growth factor with 1-MHz color-Doppler US and microbubbles, as demonstrated by Mukherjee et al. [137]. Chen et al. delivered plasmid DNA to the rat myocardium using a combination treatment of 1.3 MHz, intermittent US and plasmid-loaded perfluoropentane microbubbles, and observed increased expression relative to untreated liver samples. Additionally, these investigators optimized their US exposures to deliver US bursts gated by the cardiac cycle, and demonstrated considerably higher therapeutic delivery compared to a continuously scanned US regime [139].
2.3.4 Ultrasound-mediated Mechanisms of Vascular Tissue Delivery

The biochemical and mechanical mechanisms that facilitate drug delivery to vascular tissue are consistent with those that occur during sonoporation. At least four postulated pathways of ultrasound-induced passage of materials from the luminal to the adventitial side of vascular tissue have been proposed in the literature: 1) paracellular widening of interendothelial clefts and tight junctions, 2) free passage through injured endothelial lining, and 3) transcytosis via fenestration and channel formation [159]. These mechanisms have been proposed and modeled [160] for the blood-brain barrier, but are also supported by studies in other tissue beds, including renal [161], prostate [162], and skeletal-muscle [163] tissue.

2.3.5 Paracellular Transport

Paracellular transport between functional endothelial cells is supported strongly in the drug delivery literature. Microbubble oscillations in small vessels are known to deform neighboring vessel walls mechanically by direct perturbation or fluid motion [164]. When a microbubble oscillates near a vessel wall, its shell is estimated to travel at speeds on the order of 250 m/s [165], either directly perturbing nearby structures or causing local fluid convection, dubbed microstreaming [166]. US exposure of intracranial vessels containing microbubbles showed reversible [167] (163) enhancement of endothelial opening [159], with neither acute nor chronic tissue damage [25, 168, 169]. When microbubble oscillation occurs in the cerebral microcirculation, the blood brain barrier is disrupted, causing drug extravasation due to breakdown of transmembrane tight junction proteins [170]—claudins, connexins and occludin [171–173]. VEGF is expressed in vascular endothelial cells in response to shear stress, mechanical disruption and response to immune cytokines in the circulation, or adjacent extravascular tissue. Shang et al. quantified three tight junction proteins (Claudin-5, Occludin, and ZO-1) at the mRNA and protein level before, during, and after a three hour exposure to 1-MHz US, in the presence or absence of Optison™ microbubbles. The three tight junction components were significantly

34
reduced (compared to control) by 1-MHz US at three and six hours post-exposure. Twelve hours after exposure, however, the protein expression and presence at the blood-tumor barrier had returned to normal levels. Temporary opening of the endothelial junctions via vascular-endothelial growth factor (VEGF) signaling is known as an intercellular route for molecule transport [103].

Depicted in Figure 2-2, nitric oxide, a short-lived but potent vasodilator, likely mediates paracellular permeability [174] via mechanotransduction of shear stress along the endothelium. Caveolin-1, a protein abundant within plasma membrane clefts, is known to bind endothelial nitric oxide synthase (eNOS) under normal shear conditions. Conformational changes in these proteins induced by high shear stress could liberate eNOS, leading to considerable activation of nitric oxide, vasodilation, and tight junction opening [175]. Depicted in Figure 2-2, ultrasound-enhanced, caveolin-1 mediated paracellular permeability is achieved due to activation of mechanosensitive caveolins in the vascular endothelium [175, 176]. Alternatively, paracellular permeability has been shown to diminish after periods of increased shear stress due to fluid flow in bovine brain microvascular endothelial cells [177]. These recent data on paracellular drug transport emphasize the need for continued research into the mechanisms of ultrasound-mediated drug delivery.

2.3.6 Damage-induced Transport

The most disruptive mechanism is the passage of molecules through a compromised endothelium. A recent ex vivo study [178] demonstrated that aortic smooth muscle exposed to pulsed color-Doppler and Optison™ microbubbles induced loss of receptor-mediated contractility and endothelium-mediated relaxation. Histologically, loss of endothelium and evidence of endothelial cell apoptosis were apparent. These observations were not seen in tissue treated with US but without Optison™. Chen et al. have performed a series of experiments observing microbubbles oscillating in venules using ultra high-speed microscopy [76, 164]. When Definity® microbubbles were exposed to short US pulses (2 µs), small microjets were directed away from the nearest surface, causing considerable invagination of the vessel wall and
partial endothelial desquamation. Capillary extravasation of cells [136, 179], dyes [180] and other particles [181] due to these large-amplitude bubble oscillations has been observed by a number of investigators, supporting its therapeutic potential for triggering localized, reversible [167] endothelial permeability.

### 2.3.7 Transcytosis

Microbubble cavitation can also provoke drug transport through a vascular endothelial cell. Transcytosis, in the context of drug delivery, consists of the drug being endocytosed at the luminal surface of the endothelial cell membrane, either in a receptor-mediated fashion [182] or by invagination caused by cavitation with US [183]. Subsequent transportation through several intracellular compartments and secretion from the cell at the basolateral aspect [184, 185] results in delivery to vascular tissue. Transcytosis is typically an alternative extension of the caveolar, endocytic pathway [186]. This is a receptor-mediated process, and provides a pathway for targeted drug delivery via receptor-targeted drug vesicles and ultrasound-enhanced endocytosis [187].

### 2.4 Stroke

#### 2.4.1 Acute Ischemic Stroke

Ischemic stroke is a deadly and debilitating condition that imposes a significant financial burden on health care systems worldwide. Internationally in 2008, 17.2% of all noncommunicable disease deaths were attributed to stroke. Two percent of all health care costs in the United States are attributed to the direct and indirect costs of stroke [188]. The lack of oxygen transport to brain tissue during stroke can impair motor and cognitive functions permanently. While alternative therapies are currently under investigation [189], intravenous (IV) administration of recombinant tissue-plasminogen activator (rt-PA) within 4.5 hours of symptom onset remains the only approved treatment for ischemic stroke [190]. In one clinical trial however, over
70% of patients receiving IV rt-PA did not recover completely after three months, most likely due to tissue damage prior to treatment or incomplete recanalization [191].

2.4.2 Sonothrombolysis

2.4.3 Background

Sonothrombolysis refers to the enhanced dissolution of thrombi due to US exposure. Early sonothrombolysis investigations demonstrated efficacy in the presence of a fibrinolytic drug, such as recombinant tissue-plasminogen activator (rt-PA) [192]. Rather than a direct mechanical or thermal mechanism, US affected the rate of plasminogen cleavage by rt-PA, hastening enzymatic fibrinolysis [193]. Datta et al. refined this hypothesis by promoting stable cavitation nucleated by an ultrasound contrast agent to enhance the penetration of both rt-PA and plasminogen into clots [81]. These results indicate that expedited fibrinolysis occurs due to increased availability of plasminogen binding sites for rt-PA. The mechanical and biochemical properties of the occlusive thrombus may also contribute to the degree of US enhancement of lysis [3, 194, 195].

2.4.4 In vivo thrombi

Thrombi from a host of origins and etiologies dislodge and travel to the cerebral vasculature, triggering hemostasis and further thrombosis [194]. Different thrombus subtypes are known to display unique qualities—possibly distinguishable by magnetic resonance imaging (MRI) [30]. Within a thrombus, the number of erythrocytes and amount of fibrin vary considerably [30, 196], strongly influencing susceptibility to rt-PA lysis. For example, thrombi of cardioembolic origin—richer in erythrocyte content [197]—are thought to be easiest to lyse with rt-PA, in contrast to thrombi from large vessel thrombotic disease formed under flow [198]. These differences in thrombolytic susceptibility are clinically evident and have been characterized extensively. In this dissertation, the term “thrombus” is used to refer to coagulated blood
Figure 2-3: Cavitation mechanisms in sonothrombolysis. As a result of local fluid dynamics around a cavitating microbubbles, the fibrinolytic enzymes recombinant tissue-plasminogen activator (rt-PA) and plasminogen penetrate deeper into the fibrin matrix. Additionally, fluid microstreaming removes fibrin degradation products from the surface of the clot, expediting the fibrinolytic process. From: Sutton et al. Ultrasound-mediated drug delivery for cardiovascular disease. Expert Opin. Drug Deliv. Vol. 10. No. 5 pp. 573-592, 2013.

formed in vivo and the term “clot” is used for coagulated blood formed in vitro. Other clot properties have also shown an effect on the lysis rate of clots in vitro and in vivo including fiber diameter, fiber orientation [196], and clot age or degree of retraction [195, 199].

The degree of retraction can have a significant effect on the structure, composition, and thrombolytic susceptibility of a thrombus. Following platelet activation, fibrin monomers form fibers, precipitate out of solution, and cross-link to form a matrix that entraps formed blood elements. Platelet filopodia, formed at the onset of coagulation, constrict adjacent fibrin fibers, reducing the total clot volume
and strengthening the matrix [199]. Also, hemodynamic factors, such as the degree to which thrombolytic drug is transported into the clot by local flow conditions, correlate strongly with thrombolytic efficacy [200].

2.4.5 Clinical Sonothrombolysis

Sonothrombolysis has been tested in clinical trials, but evidence to justify its universal use to treat ischemic stroke lacks consensus. The Combined Lysis of Thrombus in Brain Ischemia Using Transcranial Ultrasound and Systemic tPA (CLOTBUST) trial randomly exposed 126 patients with middle cerebral artery occlusions to intravenous recombinant tissue-plasminogen activator (rt-PA) and 2-MHz transcranial Doppler US and observed significant clinical improvement in 49% of patients, compared with 30% receiving rt-PA alone [28]. Molina et al. extended the clinical investigation of sonothrombolysis by implementing multiple IV microbubble infusions with US exposure [31]. Recanalization rates were significantly improved when Levovist, a contrast agent, was included with rt-PA and conventional transcranial Doppler US. However, these improvements were modest (54.5 vs 41% with rt-PA/US). Numerous investigators offer reasons for the absence of recanalization improvement in some patients, including inconsistent application of acoustic energy [201] and thrombus immunity to rt-PA lysis due to compositional factors [202, 203]. Other investigators cite differences between recanalization and reperfusion, a metric that correlates better with clinical outcome [204].

2.5 Vehicles for Enhanced Drug Delivery

The prospect of controlling drug concentrations within the vascular system by developing responsive physiological systems was outlined in 1986 by Kost and Langer et al. [15]. Since, US has been established as an effective external trigger for enhanced drug delivery by acoustically agitating vesicles containing encapsulated gas. Several vascular drug-delivery vehicles have been proposed to encapsulate therapeutics, such as nanogels [205, 206], micelles [207, 208], and perfluorocarbon droplet emulsions.
Drugs and bioactive gases have also been encapsulated in nanometer and micron-sized echogenic liposomes [21, 97, 212–215]. To be an effective vehicle, the agent must maintain stability in vivo while protecting the drug against endogenous agents. Second, the payload should be released at an optimal time and location. Table 2.2 highlights a few drug-delivery vehicles being investigated for cardiovascular therapy, and their associated drug encapsulation characteristics.

The design of therapy-loaded microbubbles targeting cardiovascular tissue is provided in excellent reviews by Lindner [216], Liu et al. [217], and Liang and McPherson [218]. Laing and McPherson explore the use of liposomes as targeted therapeutic delivery vesicles. Bekeredjian et al. outline the role of ultrasound contrast agent destruction as a particular mechanism for gene and drug delivery [219]. Bull et al. provide descriptions of microbubble designs and the fluid microdynamics induced by oscillating microbubbles [220]. Several relevant reviews on techniques for targeting drug-loaded vesicles to the endothelium have also been published [221, 222].

This chapter focuses on ultrasound-mediated drug delivery for the treatment of cardiovascular disease, both updating and expanding the aforementioned reviews and focusing on treatment of cardiovascular diseases. The complex interaction of US with microbubbles, including the gently effervescent activity of stable cavitation and the more violent volume pulsations of inertial cavitation are discussed. This focus allows for a detailed discussion of tissue-specific targeting techniques, drug-loaded vesicle design, and both physical and biochemical drug-delivery pathways.

Liposomal dispersions effectively encapsulate therapeutics [241] and have been granted FDA approval for clinical use to treat cancer and meningitis [21]. Liposomes either encapsulate the drug within the hydrophilic core or the hydrophobic bilayer [242], depending on the molecular structure of the drug. Amphiphilic macromolecules, such as proteins, intercalate within the lipid bilayer, exposing moieties to the extraliposomal space, while shielding other moieties internally. Gases stabilized by a lipid monolayer can also be encapsulated within these vesicles, rendering them echogenic and more physically responsive to US. Amphiphilic copolymer micelles,
Table 2.2: Comparison of drug vehicles.

<table>
<thead>
<tr>
<th>Author</th>
<th>Vehicle</th>
<th>Drug</th>
<th>[Vehicle]</th>
<th>[Drug] during Systemic Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kee et al. [223]</td>
<td>Liposome</td>
<td>Papaverine</td>
<td>1.85 mg/ml</td>
<td>3 mg/ml [224]</td>
</tr>
<tr>
<td>Buchanan et al. [214]</td>
<td>Liposome</td>
<td>NF-κB</td>
<td>2 mM</td>
<td>10 mM [225]</td>
</tr>
<tr>
<td>Rothdiener et al. [226]</td>
<td>Liposome</td>
<td>siRNA</td>
<td>500#</td>
<td>2.7 mM [227]</td>
</tr>
<tr>
<td>Fabilli et al. [209]</td>
<td>PFP droplet</td>
<td>Chlorambucil</td>
<td>3.12 mg/ml</td>
<td>20 μg/ml [228]</td>
</tr>
<tr>
<td>Fabilli et al. [210]</td>
<td>PFP droplet</td>
<td>Thrombin</td>
<td>3.1 IU/ml</td>
<td>1.3 IU/ml [229]</td>
</tr>
<tr>
<td>Huang et al. [230]</td>
<td>Liposome</td>
<td>Nitric Oxide</td>
<td>45 μM</td>
<td>1 mM [231]</td>
</tr>
<tr>
<td>Britton et al. [232]</td>
<td>Liposome</td>
<td>Xenon</td>
<td>150 μl/ml</td>
<td>3.2 mM [233]</td>
</tr>
<tr>
<td>Smith et al. [32]</td>
<td>Liposome</td>
<td>rt-PA</td>
<td>40 μg/ mg</td>
<td>2.8 μ/ mg [234]</td>
</tr>
<tr>
<td>Wang et al. [235]</td>
<td>Alginate</td>
<td>Diclofenac</td>
<td>7.5 mg/ml</td>
<td>2.05 mg/ml [236]</td>
</tr>
<tr>
<td>Wang et al. [237]</td>
<td>PFP droplet</td>
<td>Doxorubicin</td>
<td>304 mg/ml</td>
<td>1.8 mg/ml [238]</td>
</tr>
<tr>
<td>Jin et al. [239]</td>
<td>Nanogel</td>
<td>Urokinase</td>
<td>500 ng/ml</td>
<td>4 mg/ml [240]</td>
</tr>
</tbody>
</table>

Therapeutic concentrations calculated by assuming average human weight and blood volume. NF-κB: Nuclear factor-κB; PFP: Perfluoropentane; rt-PA: Recombinant tissue-plasminogen activator; siRNA: Small interfering RNA; #: mmole/mole lipid

which encapsulate drugs within hydrophobic micellar cores or hydrophobic shells, have also been investigated as drug vesicles [243].

Coating the drug delivery agent with biochemically inert substances can effect additional stability against immunogenic or enzymatic degradation that narrow the window of time for drug delivery. Effective drug shielding and increased solubility have been achieved through the use of cyclodextrins [244], which are supramolecular structures capable of forming inclusion complexes with drug molecules. The hydrophilic outer surface of cyclodextrin produces sufficient solubility in aqueous media, yet its interior binding region is hydrophobic, allowing for efficient encapsulation of insoluble organic compounds [21]. Covalent coating with ethylene glycol, or “PEGylation”, decreases a drugs kidney excretion rate, improves solubility, and mitigates immunogenicity [245]. Together, these techniques permit the encapsulation of therapeutics that would otherwise be unavailable for use systemically, thus broadening the potential scope of ultrasound-mediated drug delivery for treatment of cardiovascular disease.

Ultrasound-triggered drug release from the vesicle can occur via a variety of mechanisms. Acoustic cavitation is hypothesized to expel the contents of the vesicle within diseased portions of the vasculature, enhancing the therapeutic index and
mitigating nonspecific cytotoxicity. Gas-encapsulating liposomes are particularly suited for ultrasound-triggered release because the cavitation nucleation site is built into the vesicle [215]. Gas-free liposomes have also been shown to release their contents during acoustic cavitation [246]. Alternatively, thermally sensitive liposomes and copolymer micelles release their contents upon exposure to hyperthermia in tumor tissue or thermal deposition of US energy [247, 248].

2.6 Drug Targeting

Proposed cavitation-based mechanisms of drug delivery rely on the physical forces exerted by oscillating microbubbles. Since the distance over which these forces are exerted either by direct perturbation from bubble oscillation [93, 94] or fluid streaming [249]—is on the order of a bubble diameter, the microbubbles must be in close proximity with their tissue target. In capillary beds, this requirement is generally met due to the small vessel diameters (< 10µm) relative to microbubble size. However, in larger vessels such as the carotid, femoral, or peripheral arteries, the relative fraction of microbubbles near the thrombus or endothelium may not be sufficient to provoke a therapeutic response. Thus many groups have developed targeted delivery vehicles for efficient ultrasound-mediated drug delivery. The most common approach is to conjugate the microbubbles with vascular ligands, so that vesicle adherence to the diseased tissue is promoted. Molecularly targeted, acoustically active drug-delivery vesicles have been recently investigated for therapeutic application and diagnosis of inflammation, atherosclerosis, angiogenesis, intravascular thrombosis, and post-ischemic injury [250]. Several different antibodies conjugated to microbubbles are reported below.

Atherosclerosis and inflammation facilitate the adherence of both anti-ICAM-1 and anti-VCAM-1 conjugated microbubbles to plated cells and vessels [251–253]. The amount of adherence has been found to vary directly with the degree of atherosclerotic plaque formation, the number of molecular receptors present, or the activation state of endothelial cells [251]. The ability to target effectively depends
on the disease state, as demonstrated with early indication of cardiac transplant rejection in rats [254, 255], and initial inflammation of atherosclerosis in mice [252].

Another major approach to targeting endothelium is the use of anti-P-selectin conjugated microbubbles. Takalkar et al. showed that once anti-P-selectin conjugated microbubbles were adhered to P-selectin coated plates in a parallel plate flow chamber, shear stresses of 34 dyn/cm$^2$ were necessary to dislodge approximately half of the microbubbles [256]. To improve the probability of initial adherence, Rychak et al. [99] pressurized the microbubbles to cause a partial gas loss. This treatment resulted in an excess shell surface area of approximately 30%, allowing the microbubbles to deform from a spherical shape more readily. The deformable microbubbles adhered to the endothelium in the presence of higher shear stresses than non-deformable microbubbles. Ferrante et al. demonstrated an alternative means of improving adherence by conjugating the microbubbles with both anti-P-selectin and anti-VCAM-1 antibodies [257]. They found that the dual-targeted microbubbles adhered almost twice as efficiently in flow chambers compared to microbubbles targeted with either anti-P-selectin or anti-VCAM-1 alone.

Targeting to improve drug delivery to intraluminal thrombi has also been developed. Platelets, which exist in high concentrations on the surface of thrombi, can be targeted by conjugating a glycoprotein (GP) IIb/IIIa receptor inhibitor to the delivery vesicle [141, 258–261]. Investigations implementing this targeting strategy show enhanced thrombolysis in the presence of a thrombolytic and targeted microbubbles exposed to US. Culp et al. [141] and Xie et al. [258] both demonstrated that GPIIb/IIIa targeted microbubbles exposed to US expedite lysis more than non-targeted microbubbles in vivo. Hua et al. [259] demonstrated similar results in vitro using microbubbles conjugated to a tetrapeptide for targeting to activated platelets. Fibrin can be targeted by conjugating an inactive portion of rt-PA to the drug delivery vesicle. Klegerman et al. accomplished targeting to thrombus by intercalating the PPACK moiety of rt-PA, which exhibits strong fibrin affinity, within the phospholipid bilayer of ELIP [262].

Anti-α-integrins have been used to target microbubbles to neovascularization in
Of the total microbubble infusion however, only a small fraction adhere to the diseased endothelium. This physical limitation will be shared by all targeted UCA theragnostic. Acoustic radiation force exerts enough force to push non-targeted microbubbles within close proximity of endothelium in vivo by causing particle motion along the axis of acoustic propagation [63, 265]. When microbubbles were conjugated to targeting antibodies, the concentration of microbubbles along the vascular wall increased ex vivo [266] and in vivo [145]. Further, Liu et al. showed that acoustic radiation force can significantly increase adhesion in both arteries (using anti-CD34) and the microvasculature (using anti-ICAM-1) [63, 145].

2.7 Dissertation Emphasis

This dissertation documents cardiovascular delivery of three other drugs: (1) nitric oxide, a potent vasodilator, (2) bevacizumab, an anti-angiogenic antibody, and (3) rt-PA, an FDA-approved thrombolytic agent. Development of effective targeting and delivery methods for the treatment of cardiovascular disease would lead to a paradigm shift in the development of more effective, more economical, and less-invasive treatments for many types of cardiovascular pathologies.
Chapter 3  Clot retraction affects the extent of ultrasound-mediated thrombolysis in an ex vivo porcine thrombosis model.

As described in Chapter 2, Section 2.4, recombinant tissue-type plasminogen activator (rt-PA) is the only FDA-approved thrombolytic treatment for acute ischemic stroke—a deadly and debilitating cerebrovascular condition. Given the wealth of knowledge on clot susceptibility to rt-PA thrombolysis and the wide array of in vivo thrombus characteristics (2.4.4), we aimed to investigate the dependence of ultrasound-enhanced thrombolysis in the presence of microbubbles on clot composition. Using an established ex vivo model of thrombosis, we assessed the thrombolytic efficacy of rt-PA, 120 kHz continuous-wave (CW) ultrasound, and circulating microbubbles (Definity®; Lantheus Medical Imaging; Billerica, MA USA) on two distinct types of whole-blood clots. Further, we employed routine histological evaluation and scanning electron microscopy (SEM) to characterize differences in composition between the two clot types. These results, in the context of mechanistic and clinical investigations of ultrasound-enhanced thrombolysis (UET), will be discussed.

A gray vertical line indicates portions of this chapter published in:
3.1 Materials and Methods

3.1.1 Whole-Blood Clot Formation

Porcine venous blood was collected aseptically from donor hogs (Lampire Biologicals; Pipersville, PA USA), anticoagulated with a citrate-phosphate-dextrose solution (final concentration in blood: 16.1 mM Na-citrate, 17.6 mM d-glucose, 1.96 mM citric acid, and 2.3 mM NaH$_2$PO$_4$) and shipped on ice overnight to the University of Cincinnati. Porcine blood is a good alternative to human blood for thrombolysis research due to its availability, price, and biochemical similarity to human blood (Pond and Mersmann 2001). Before coagulation, the blood was placed on a laboratory stirrer for 15 minutes and incubated at 37.3°C for 20 minutes while exposed to room air to equilibrate gas content. To induce coagulation, the blood was recalcified with CaCl$_2$ to a final concentration of 16.1 mM and pipetted into glass Pasteur pipets Fisher Scientific; Pittsburgh, PA USA). The pipets were incubated at 37.3°C for three hours during coagulation, and placed in a laboratory refrigerator at 4°C for a minimum of three days before experimental treatment to allow for consistent clot retraction. These methods of clot formulation are similar to those implemented in other thrombolysis studies, and result in clots with appreciable lytic rates and consistent lytic susceptibility, which is imperative in mechanistic sonothrombolysis studies [79, 267–271].

The clot formation protocol was performed using two different types of Pasteur pipets, borosilicate and flint glass, to stimulate the coagulation cascade to different degrees. This resulted in different types of whole-blood clots (Figure 3-1), which were subjected to a combination treatment of intermittent, 120 kHz CW ultrasound with rt-PA and microbubbles. Borosilicate glass, a highly hydrophilic surface, produced stiff, retracted whole-blood clots, while flint glass produced softer, unretracted whole-blood clots. These qualitative observations are consistent with previous investigations of clot retraction by Faxalv et al. [272]. As discussed by Molina et al., clot retraction is a relevant process from a medical imaging perspective, as changes in
sensitivity and specificity of the susceptibility vessel sign (SVS) from gradient-echo MRI can occur as a result of differential clot retraction [194]. After the three-day retraction period, the interior of the Pasteur pipets contained the whole-blood clot (solid phase), translucent serum (fluid phase), and uncoagulated whole blood. The volume of the fluid phase was likely a result of syneresis, an effect first studied in blood clots by Pickering and Hewitt [273].


3.1.2 Histology and Image Analysis

Eighteen cylindrically shaped clots (nine retracted and nine unretracted) were produced in vitro as indicated above for microscopic image analysis. Each clot was removed from its respective Pasteur pipet, trimmed to 1 cm in length, and fixed in 10% neutral buffered formalin for 24 hours. Clots were submitted for paraffin
processing through a series of graded alcohol and xylene steps; all samples were subsequently bisected axially and paraffin embedded. Five µm-thick tissue sections were obtained from each paraffin block and stained with hematoxylin and eosin (H&E). Stained sections were subjected to qualitative and quantitative histologic evaluation under bright-field microscopy. Histologic sections were photographed at 20X magnification with an Olympus BX61 microscope equipped with a color digital camera (Olympus DP70, 12.5 megapixel resolution; Center Valley, PA USA). Image analysis data were processed with a custom-written MATLAB (The Mathworks Inc.; Natick, MA USA) image analysis routine that determined the total amount of open white space present in each image. Using binary pixel intensity thresholding, the total number of white pixels present in an image was summed, and this value was divided by the total number of pixels in the image to determine percent white space. The total number of white pixels was used to describe the extent of clot retraction and porosity.

3.1.3 Scanning Electron Microscopy

To characterize compositional properties, clots were processed in a similar manner to the method described by Weisel et al. [274] and subjected to routine SEM. All materials used for clot processing were obtained from Electron Microscopy Sciences (Hatfield, PA USA) and dilutions made with 0.2 µm-filtered deionized water. Following the three-day retraction period, clots were rinsed with cold phosphate-buffered saline (PBS, Sigma Aldrich Co.; St. Louis, MO USA) and axially sectioned for processing. The sections were fixed with 2% (v/v) glutaraldehyde in cacodylate buffer (#11650, Electron Microscopy, Hatfield, PA USA) overnight. Following fixation, the samples were rinsed twice with 0.1 M cacodylate buffer and post-stained with 1% (v/v) osmium tetroxide to enhance surface imaging contrast. Following two saline rinses, the clots were dehydrated with a series of 15 minute graded ethanol steps (v/v; 50%, 50%, 70%, 70%, 95%, 100%) and left in 100% ethanol overnight. Finally, the clots were chemically dried with pure propylene oxide and allowed to air dry in a fume hood. Immediately prior to imaging, the samples were sputter-coated
with gold-palladium under argon for 90 seconds to confer surface conductivity. SEM imaging was performed on axial sections from eight clot samples (four retracted and four unretracted clots from different porcine subjects). High-resolution (650–6500X magnification) images were captured of random positions on the axial section surfaces of each clot. In addition to differences in surface composition between the two clot models, qualitative differences were observed between the interior and the superficial surface (outside edge) of each clot. Thus, images of these surfaces were collected and analyzed separately.

Two blinded observers assessed the fibrin content and fiber diameter of each sample using routines written in MATLAB and ImageJ (National Institutes of Health; Bethesda, MD USA). To determine fibrin content, each observer selected a region of interest (ROI) on each image (3500X) devoid of red blood cells. Thereafter, linear contrast adjustment and binary thresholding were performed until the fibrin on the surface of each image was highlighted. Percent fibrin was calculated by dividing the number of highlighted fibrin pixels by the total number of pixels in the ROI. To determine fiber diameter, each observer was presented with a new, randomly chosen 150 × 150 pixel ROI. The diameter of the fiber closest to five randomly chosen locations within the ROI was measured and recorded by the observer (N = 83).

3.1.4 Ex Vivo Carotid Perfusion System

Thrombolysis experiments in the ex vivo arterial thrombosis system were conducted as previously described [2]. Briefly, carotid arteries were excised from mature hogs at an abattoir within one hour of exsanguination in accordance with University of Cincinnati Institutional Animal Care and Use Committee guidelines for post-mortem studies and transported to the University of Cincinnati for mounting in the ex vivo system. The perfusion system, depicted in Figure 3-2, consisted of a closed loop that circulated sodium-citrate anti-coagulated porcine plasma (Lampire Biologicals; Pipersville, PA USA) with a pulsatile blood pump (Harvard Apparatus; Holliston, MA USA). Porcine plasma was chosen as the perfusate because it contains the proteins vital to intrinsic fibrinolysis while avoiding the confounding effects that
erythrocytes introduce on ultrasound scattering and mass deposition within the clot sample.


3.1.5 Experimental Treatment

For each experiment, the sample holder was mounted within the system as follows. First, a carotid artery was trimmed to 4.5 cm and placed under physiologic pressure (90 – 100 mmHg) with PBS and inspected for side branches and gross leaks. If necessary, side branches were sutured with 4.0 braided silk suture (Deknatel; Research Triangle Park, NC USA). The distal end of the intact carotid artery was mounted to a 3 mm inner-diameter barbed, polypropylene cannula (Cole Parmer Instrument Company; Vernon Hills, IL USA) and secured with 3.0 nylon surgical suture (Look Surgical Specialties; Reading, PA USA). Next, a clot was gently washed with PBS, trimmed to 3.5 cm in length, blotted, weighed, and injected proximally.
into the carotid artery. The proximal end of the artery was secured to the upstream cannula with a nylon suture. Additionally, a small 30 Gauge hole was made in the downstream cannula prior to artery mounting to act as a flow bypass in the event that the clot completely clogged the downstream cannula during treatment. This scenario also occurs in vivo, as collateral branches of the thrombosed cerebral arteries often maintain residual flow of fibrinolytics into the ischemic region [189]. To provide the artery with a physiological perivascular fluid, the artery was bathed in degassed (35%), artificial cerebrospinal fluid (aCSF; in mM, 128 NaCl, 3.0 KCl, 1.0 MgSO₄, 23.5 NaHCO₃, 0.5 NaH₂PO₄ and 30 glucose [2]) contained separately from the tank water by an unlubricated latex condom.

The thrombolytic drug, rt-PA (Genentech; South San Francisco, CA USA), was reconstituted with 0.2 µm-filtered, deionized water to a concentration of 1 mg/ml, aliquoted into polypropylene cryovials, and frozen until use at −80 °C. Definity® vials were activated according to manufacturer instructions and used within 3 days. To preserve the composition of headspace gas, the Definity® vials were vented to octofluoropropane (C₃F₈) at 20 °C and one atmosphere of pressure as described by Prokop et al. [80].

At the beginning of each trial, a 10 mL syringe containing porcine plasma, porcine plasma with 7.88 µg/mL rt-PA, or porcine plasma with 7.88 µg/mL rt-PA and 0.70 µl/mL Definity® was infused proximally at a flow rate of 0.6 ml/min. A flow clamp, located distal to the sample holder, was adjusted manually throughout the trial to maintain a luminal flow rate of 1.5 ml/min. These infusion and luminal flow rates were selected to obtain a rt-PA concentration of 3.15 µg/mL [91, 270]. Definity® was infused to obtain a luminal concentration of 0.28 µl/mL, which is the highest concentration predicted theoretically assuming the manufacturer recommended venous bolus injection and a perfluorocarbon clearance rate of 5 µL/min. To obtain this concentration, a monoexponential function describing the half-life of Definity® in the body (Lantheus Medical Imaging; Definity® microbubble insert) was convolved with a function simulating a typical bolus injection regime. The microbubbles were assumed to disperse homogenously within a five liter blood volume, during an 11 sec-
ond bolus injection, which is consistent with clinical protocol for Definity® infusions performed at University Hospital in Cincinnati, OH for left-ventricular opacification [275].

Thrombolytic efficacy, determined by percent mass loss over the 30-minute treatment periods, was individually determined for retracted and unretracted clots. Although alternative metrics of thrombolytic efficacy have been proposed, such as d-dimer concentration [260], fractional clot width [270], and photoabsorbance [276], mass loss measurement is a simple experimental technique that is minimally susceptible to inter-sample variability, and is a chief concern for physicians treating acute ischemic stroke [268]. Additionally, alternative methods would have been difficult to implement in this ex vivo model—porcine d-dimer assay kits are not commercially available, and light absorbance by the ex vivo carotid artery would have confounded clot width and photoabsorbance measurements prohibitively.

### 3.1.6 Model Parameters

The physiologic parameters monitored during the course of experimental treatment (fluid pressure, temperature, oxygen concentration, flow rate, pH) did not vary significantly between trials \( (p > 0.05) \). Table 3.1.6 outlines these data, which are presented as the data mean ± SD. The inline flow rate was measured experimentally to be 1.42 ± 0.08 mL/min, which indicates an effective average luminal rt-PA concentration of 3.33 ± 0.18 µg/mL.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Plasma Alone</th>
<th>rt-PA Retracted</th>
<th>rt-PA Unretracted</th>
<th>rt-PA + Definity® + US Retracted</th>
<th>rt-PA + Definity® + US Unretracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature °C</td>
<td>37.53 ± 0.27</td>
<td>36.9 ± 0.37</td>
<td>30.09 ± 0.37</td>
<td>37.23 ± 0.40</td>
<td>37.23 ± 0.15</td>
</tr>
<tr>
<td>Pressure (mmHg)</td>
<td>99.1 ± 0.40</td>
<td>93.1 ± 6.2</td>
<td>95.5 ± 2.5</td>
<td>100.1 ± 4.8</td>
<td>98.1 ± 1.5</td>
</tr>
<tr>
<td>Flow (ml/min)</td>
<td>1.54 ± 0.03</td>
<td>1.78 ± 0.10</td>
<td>1.40 ± 0.13</td>
<td>1.39 ± 0.04</td>
<td>1.46 ± 0.04</td>
</tr>
<tr>
<td>([\text{rt-PA}]_{\text{calc}}) µg/ml</td>
<td>-</td>
<td>-</td>
<td>3.37 ± 0.24</td>
<td>3.39 ± 0.08</td>
<td>3.24 ± 0.08</td>
</tr>
<tr>
<td>([\text{Definity}]_{\text{calc}}) µl/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.29 ± 0.01</td>
</tr>
</tbody>
</table>

Rt-PA = recombinant tissue-type plasminogen activator; US=ultrasound
Data are expressed as means ± standard deviations. rt-PA and Definity® concentrations calculated based on the infusion pump flow rate and average flow rate through the artery.
3.1.7 Ultrasound Therapy

The ultrasound therapy was designed to mimic extravascular insonation of an intracranial thrombus located in the middle cerebral artery. The test segment was submerged in a tank filled with degassed, deionized water containing two circular single-element ultrasound transducers used for ultrasound therapy and passive cavitation detection. This experimental setup is depicted in Figure 3-3. Prior to each experiment, a calibrated 120-kHz therapy transducer (Sonic Concepts; Bothell, WA USA) was aligned orthogonally and confocally with a 2.25 MHz passive cavitation detector (Picker Roentgen GmbH; Espelkamp, Germany) using an ultrasonic pulser receiver (Panametrics 5077PR, Olympus NDT Inc.; Waltham, MA USA). The confocal point was positioned 8 mm downstream from the face of the clot for each experiment using a three axis-translation stage (Newport 423; Irvine, CA USA). This geometry was chosen to ensure that the ultrasound pressure amplitude across the clot profile was sufficient to induce stable cavitation, based on previous threshold measurements performed in this system [2] and on the in situ pressure profile of the ultrasound therapy transducer seen in Figure 3-4.

During experimental treatment, an RF function generator (Agilent Technologies Inc.; Santa Clara, CA USA) supplied a continuous-wave sinusoidal signal that was amplified (Amplifier Research, 750A250; Souderton, PA USA) and impedance-matched (custom matching network, Sonic Concepts Inc.) to drive the 120-kHz therapy transducer. Acoustic scattering from the sample holder and polycarbonate cannulae distorted the beam profile slightly, so an in situ pressure calibration was performed in the absence of an artery and clot. A broadband hydrophone (0.5 mm, Reson Inc; Goletta, CA USA) was positioned in the anticipated axial center of the artery and advanced incrementally downstream to the outlet cannula, passing through the natural focus of the therapy transducer in its transverse plane (Figure 3-4). Based on this calibration, the peak-to-peak acoustic pressure at the axial clot center was 0.48 MPa, which diminished to roughly 0.34 MPa at the upstream and
Retracted and unretracted clots were subjected individually to 120-kHz, continuous-wave ultrasound with intermittent pulsing. This scheme was designed to maximize the amount of stable cavitation occurring within the artery lumen during ultrasound treatment. Details describing the rationale and development of this technique were previously described by Hitchcock et al. [2].

3.1.8 Passive Cavitation Detection

Acoustic emissions detected within the sensitive region of a calibrated 2.25 MHz passive cavitation detector were amplified with a wideband voltage preamplifier (Signal Recovery Model 5185; Oak Ridge, TN USA) and saved on a digital oscilloscope (Lecroy Corporation; Santa Clara, NY USA). Twenty millisecond voltage traces were stored every second and post-processed in MATLAB to analyze spectral content, consistent with the methods of Datta et al. [79]. Ultraharmonic power, represented
in the signal at odd integers of half the insonating frequency, indicated the presence of stable cavitation, which is associated with fluid microstreaming [166, 277]. Broadband signal power indicated the presence of inertial cavitation, or bubble collapse from large radial excursions, which is associated with microjetting [76]. The energy contained within 15 ultraharmonic (175 – 185 kHz, 295 – 305 kHz, etc.) and 30 broadband (147.5 – 152.5 kHz, 207.5 – 212.5 kHz, etc.) frequency bands was summed within each power spectra. Stable and inertial cavitation correspond to acoustic emissions within these respective frequency ranges [278, 279], and have been the focus of a number of studies investigating the mechanism of ultrasound-enhanced thrombolysis [2] and drug delivery [1, 87, 169, 280–284].
3.1.9 Data Analysis and Statistical Methods

Statistical analysis was performed using the MATLAB Statistical Toolbox. Differences in means between experimental treatments were determined by one-way unbalanced ANOVA with an alpha level of 0.05. Subsequent unpaired Student t-tests with an alpha level of 0.025 were used to discern statistically significant differences between individual treatments. The data mean, standard deviation, p-value, t-statistic, and degrees of freedom (mean ± SD; $p$, $t_{\text{stat}}$, $DF$) are reported for each statistical comparison.

Two observers, blinded to the clotting technique, were chosen to conduct the analysis of scanning electron microscopy images. Each observer had previous experience in medical image analysis and clot characterization. Lilliefors test for normality was performed and p-values below 0.05 indicated significant deviations from normality. A Wilcoxon rank-sum test was performed to determine significant differences between medians at an alpha level of 0.05.

3.2 Results

3.2.1 Histology

Microscopic analysis of H&E-stained sections showed consistent qualitative differences in pattern between each clot type (from Section 3.1.2, Figure 3-5). Retracted clots produced in borosilicate pipettes appeared homogenous and displayed very densely packed RBCs allowing only scant amounts of fibrin to be visible. In some sections, fibrin was observed in thicker strands or accumulations. Conversely, unretracted clots produced in flint glass pipettes displayed loosely aggregated RBCs with a loose fibrin network often visible around the RBCs. In addition, this clot model showed considerable spacing (devoid of either fibrin or RBCs) relatively homogeneously distributed between the RBCs. Image analysis performed on H&E images revealed that this intercellular spacing comprised 3.45 ± 2.17% of unretracted clots,
and only 0.87 ± 0.95% of retracted clots, which was a significant difference ($p \ll 0.01$, $t_{\text{stat}} = 4.69$, $DF = 34$).

![Figure 3-5: Representative images of retracted (left column) and unretracted thrombi (right column) showing a cross-section (A, B), a longitudinal section (C, D) and high power 20x magnification. Stain: HE. Bar = 1 mm in A-D, 200 µm in E-F. At 20X magnification, unretracted thrombi (B) appear much more porous than retracted thrombi (E), as evidenced by the extent of space devoid of erythrocytes and fibrin. Image analysis of these images revealed that retracted thrombi contained 0.87 ± 0.95% of this space compared to 3.45 ± 2.17% within unretracted thrombi. From: Sutton J. T, Ivancevich N. M, Perrin S. R, Vela D. C, and Holland C. K. Clot Retraction Affects the Extent of Ultrasound-Enhanced Thrombolysis in an ex vivo Porcine Thrombosis Model. Ultrasound in Medicine and Biology. May 2013, 39(5): 813–824. doi: 10.1016/j.ultrasmedbio.2012.12.008]

3.2.2 Scanning Electron Microscopy

Qualitative and quantitative analysis of SEM images also revealed considerable differences in compositional properties between the two clot models. Representative SEM images of the interior of each clot type are depicted in Figure 3-5. These images
illustrate the disparity in fibrin content present within the clot interior. Superficial fibrin content was significantly greater \((p = 0.015)\) in retracted (73.8%) compared to unretracted clots (54.6%). Box and whisker plots of these data on the superficial surface of the clots, depicting the median, first and third quartile ranges, and data extrema are also depicted in Figure 3-6. Differences in fiber diameter in retracted and unretracted clots, respectively, were not significant. Superficial fibers had a mean diameter of 0.173 \(\mu m\) in retracted clots and 0.169 \(\mu m\) in unretracted clots \((p = 0.18)\). Interior fibers had a mean diameter of 0.232 \(\mu m\) in retracted clots and 0.253 \(\mu m\) in unretracted clots \((p = 0.97)\). Echinocyte formations were also observed in the erythrocyte population within SEM images. This is a known artifact of clot storage and aging, and is a result of dehydration, increased pH, and decreased ATP concentration [285].

3.2.3 Clot Fluid Phase Estimation

To aid in clot characterization, the volume of the fluid phase (expressed as a percentage of total volume \(\pm\) one standard deviation) was measured in sixteen clots (eight retracted, eight unretracted) immediately after removal from each respective Pasteur pipet. For unretracted clots, this value was 58.2 \(\pm\) 6.7%, which was significantly greater than in retracted clots (10 \(\pm\) 8.7%; \(p \ll 0.05\); \(t_{\text{stat}} = 12.4\), \(DF = 14\)).

3.2.4 Ex Vivo Thrombolysis

During treatment, clots lysed in both the axial and radial directions. After treatment and blotting, visual inspections indicated that lysis was most apparent in the longitudinal direction. Considerable radial lysis occurred at the upstream portion of the clots, resulting in conical-shaped residual clots during the most efficacious treatments.

The thrombolytic efficacy of each treatment is depicted in Figure 3-7. Reported values correspond to the mean \(\pm\) one standard deviation (SD). Retracted clots subjected to control treatments (porcine plasma alone) lost a minimal amount of mass
Figure 3-6: Scanning electron microscopy images of the interior of representative unretracted (top left) and retracted clots (bottom left). Box and whisker plots (right column) indicate the median, 1st and 3rd quartiles, and extrema of data collected by blinded observation of fiber diameter (top right) and erythrocyte concentration (bottom right). Asterisks indicate significant differences between data medians. From: Sutton J. T, Ivancevich N. M, Perrin S. R, Vela D. C, and Holland C. K. Clot Retraction Affects the Extent of Ultrasound-Enhanced Thrombolysis in an ex vivo Porcine Thrombosis Model. *Ultrasound in Medicine and Biology.* May 2013, 39(5):813–824. doi: 10.1016/j.ultrasmedbio.2012.12.008

during the thirty-minute treatment period (8.8 ± 1.8%). Exposure to 3.15 µg/mL rt-PA increased mass loss significantly (p < 0.01; t_{stat} = 5.73, DF = 11) compared to control treatment (17.1 ± 2.6% vs. 8.8 ± 1.8%). However, when retracted clots were exposed to rt-PA, Definity®, and 120 kHz, no increase in mass loss (p = 0.28; t_{stat} = 1.14, DF = 10) occurred (17.1 ± 2.6% vs. 19.6 ± 4.7%). Unretracted clots lysed much more effectively during control treatments (44.9 ± 4.0%) than retracted clots. Exposure to rt-PA improved thrombolytic efficacy further (53.5 ± 4.5; p = 0.009, t_{stat} = 3.29, DF = 10). Co-administration of rt-PA, Definity® and ultrasound resulted in further significant increases in thrombolytic efficacy (73.2 ± 4.7%; p < 0.01, t_{stat} = 11.14, DF = 11). These data indicate that rt-PA improves thrombolytic efficacy significantly in both clot models compared to control treat-
ment. However, when rt-PA was augmented with Definity® and intermittent US exposures, thrombolytic efficacy only increased in the unretracted clots.


3.2.5 Cavitation Detection

Figure 3-8 depicts the detected energies from spectral analysis of acoustic emissions. In general, the amounts of stable and inertial cavitation, indicated by the spectral power at ultraharmonic and broadband frequencies, respectively, decayed in time during each 19.5-second intermittent CW pulse. Broadband activity was strong within the first acquired trace of each pulse. However, these emissions decayed rapidly, diminishing to baseline within the first few seconds. For rt-PA, Definity® + US treatments, the average amount of ultraharmonic and broadband energy detected by the PCD was greater in the retracted clots, however the differences were
insignificant ($p >> 0.05$; $t_{stat} \ll 1.1; DF = 11$). Since unretracted clots were, in general, larger in volume than retracted clots, the number of Definity® microbubbles flowing through the sensitive region of the detector was greater in the case of retracted clots. Acoustic emissions from the PCD were also processed for four clots (two plasma alone and two rt-PA treatments) to establish a baseline level of ultraharmonic and broadband activity when ultrasound was not applied. These energies were 0.35 and 0.39 $\mu V^2 s$, respectively.


### 3.3 Discussion and Conclusions

Retracted thrombi have increased resistance to rt-PA thrombolysis. We investigated this dependence in the context of ultrasound-enhanced thrombolysis. Thrombolytic enhancement, defined by an increase in clot mass loss, was observed in the
presence of microbubbles undergoing stable nonlinear oscillations, or stable cavitation. However, thrombolytic enhancement compared to sham treatment with rt-PA was observed only when unretracted whole-blood clots were exposed to rt-PA, 120 kHz ultrasound, and microbubbles. Within the two clot models, no significant differences were detected in the total amount of stable or inertial cavitation activity occurring within the artery lumina. Thus, the lack of thrombolytic enhancement within retracted clots was most likely caused by some difference in their composition, which prohibited a higher thrombolytic rate with US and microbubbles. These results imply a correlation between the compositional properties of clot and sonothrombolysis. Further investigation of this relationship could assist physicians in their clinical decisions about the use of rt-PA and sonothrombolysis.

Compositional analysis by SEM and H&E revealed that retracted clots contained a more-extensive fibrin network compared to unretracted clots that exhibited thrombolytic enhancement. Superficial fiber diameters were slightly smaller than those analyzed within the clot, regardless of clot model. This result could have been due to differences in thrombin concentration [286] or erythrocyte density [196] between the clot surface and interior. However, superficial and interior fiber diameters, respectively, were statistically identical in each clot model and likely did not contribute to sonothrombolytic susceptibility.

The dependence of sonothrombolysis on clot retraction has interesting mechanistic implications. Reduction in clot volume reduces pore size and expels blood plasma from pores within the fibrin network, resulting in a lower concentration of fibrinolytics such as t-PA and plasminogen within the clot [287]. Kunitada et al. [199], in their studies of platelet-rich clots, concluded that thrombolysis was inhibited by the degree of platelet-mediated clot retraction, which they attributed to a reduction in clot-bound plasminogen. In our study, a non-pharmacological technique was used to stimulate maximal platelet activation and clot retraction. Consistent with Kunitada et al., our results indicate that cavitation-enhanced thrombolysis could depend on clot-bound plasminogen. Using pulsed diagnostic ultrasound to accelerate clot lysis with Definity® microbubbles, Xie et al. [195] observed a reduction in throm-
bolytic efficacy as the clot aged from three to six hours. Plasminogen decreases in concentration within the clot with aging and necessitates an external source for further lysis. Combined, these results suggest that changes in clot architecture and plasminogen content affect the efficacy of oscillating microbubbles in accelerating thrombolysis.

Our results suggest three possible scenarios that may be necessary for thrombolytic enhancement with ultrasound. First, the concentration of plasminogen within a clot during lysis may have to exceed some critical threshold. This scenario is compelling because the kinetic rate of t-PA fibrinolysis decreases steadily with plasminogen concentration [276]. A plasminogen threshold would also depend on plasma rt-PA concentration, because higher concentrations of the fibrinolytic enzyme would not produce a higher rate of lysis. Secondly, the plasminogen distribution near the surface of the clot must be sufficiently deep to facilitate lysis in non-superficial layers. The plasminogen content in retracted clots during lysis localizes to the superficial fibrin layers less than 50 µm from the clot surface, and at much lower concentrations than in unretracted clots [288]. In this scenario, the presence of fibrin degradation products near the clot surface could limit rt-PA access to plasminogen within the clot. In our whole-blood clot model, the low thrombolytic rate observed in retracted clots most likely occurred due to a low rate of erythrocyte liberation, because a significant portion of a clot’s mass can be attributed to dense formed elements [30]. Therefore, micromixing by cavitating microbubbles must have contributed minimally to further fibrin degradation product removal in the presence of plasma flow. In the case of unretracted clots, the high rate of erythrocyte liberation may have congested the superficial binding sites for rt-PA and plasminogen. Microstreaming, known to occur near surfaces during bubble oscillation [166], could facilitate this removal and allow for accelerated fibrinolysis. Thirdly, the porosity of the clot must be sufficient to allow increased penetration of fibrinolytics by cavitation. Here, thrombolytic enhancement was observed when unretracted clots, composed of loosely aggregated fibrin and spaces devoid of particulate, were exposed to ultrasound, microbubbles, and rt-PA. Future studies should focus on studying each of these effects specifically
in appropriate \textit{in vitro} models.

The data presented here are also consistent with clinical and \textit{in vitro} thrombolysis trials. Molina \textit{et al.} [198], in their studies of thrombolytic efficacy and clinical outcomes as a function of clot subtypes, concluded that clots of acute cardioembolic origin lysed more easily and resulted in better clinical outcomes than clots from large vessel disease. Large vessel thrombosis is concomitant with significant clot retraction, due to accumulations of platelet aggregates [289, 290] on the clot surface. Both clot types in this study would likely occur \textit{in vivo} during ischemic stroke, as evidenced by the large variation in erythrocyte and fibrin composition in thrombi retrieved from middle cerebral artery occlusions [30]. Thus, the clot models tested in this study were likely similar to those formed in stasis within the fibrillating atrium, with varying degrees of platelet activation and adhesion.

In this study, both clot models were erythrocyte-rich, but differed in the extent of fibrin networking within their interior. Clots formed in highly hydrophilic borosilicate glass experienced significant clot retraction during \textit{in vitro} manufacturing. Retracted clots have been shown to lyse much less effectively, regardless of treatment, compared to unretracted clots [192, 291]. These results can likely be attributed to the lack of extensive fibrin matrix formation throughout unretracted clots (Figures 3-5 and 3-6), and the paucity of plasminogen present within retracted clots [287]. In unretracted clots subjected to plasma alone treatments, the small amount of t-PA present endogenously in porcine plasma (\(\approx 8\) ng/mL) [292] may have had a significant effect on lysis, compared to retracted clots. The lack of significant thrombolytic enhancement observed in sonothrombolysis trials with microbubbles may also be explained by the data presented here. Some clots, depending on their etiology and vascular origin, may be predisposed to sonothrombolytic susceptibility depending on the concentration of plasminogen bound within the fibrin matrix. Consistent with this hypothesis, \textit{in vitro} studies have proposed delivering plasminogen to the site of occlusion concomitantly with rt-PA [293].

Previous sonothrombolysis investigations are also consistent with the results presented here. Using clots manufactured in silicone-coated glass tubes, Datta [294]
observed significant thrombolytic efficacy enhancement in the presence of stable cavitation and rt-PA. Silicone is often used as a coating in blood collection tubes to prevent cell adherence by rendering the glass surface hydrophobic. Clots formed in these types of tubes experience incomplete clot retraction, and are most likely more susceptible to sonothrombolysis. In their *ex vivo* thrombolysis studies using clots manufactured aseptically after exsanguination at a local abattoir, Hitchcock *et al.* [2] observed significant thrombolysis enhancement in the presence of rt-PA and cavitating microbubbles. Based on past experience in this laboratory, clots formed in this manner are much softer and friable, and most likely do not experience robust clot retraction.

Though compelling mechanistic conclusions can be drawn from studies in this *ex vivo* thrombosis model, its limitations should be mentioned. As discussed by Hitchcock *et al.* [2], the cell signaling that occurs within the cardiovascular system of an organism during acute ischemic stroke is mostly absent in this *ex vivo* model. While intact vascular endothelial cells of excised carotid arteries remain capable of responding to strong vascular agonists [295], their ability to produce antithrombotic cofactors in response to ischemia or thrombosis diminishes after removal from the live animal and hypothermal storage [296]. To mitigate this effect, we chose to perfuse the *ex vivo* artery with porcine plasma saturated with oxygen (> 30 mg/L), which is greater than that in ischemic tissue [297]. Yet, these studies have also shown that endothelial cells, cultured in ischemic conditions, exhibit prothrombotic tendencies and increase permeability to the vascular tunics. Though this *ex vivo* model has limitations, it does provide a quasi-realistic environment to simulate thrombosis while testing the thrombolytic efficacy of well-characterized sonothrombolysis treatments.

In conclusion, the data presented here demonstrate that sonothrombolysis is difficult to achieve with retracted clots. The mechanism of this inhibition is unclear, but may be related to clot structure and/or biochemical composition. Future *in vitro* and *in vivo* studies should focus on these parameters when designing thrombolytic treatment strategies using rt-PA, microbubbles, and ultrasound.
Chapter 4  Ultrasound-mediated delivery of nitric oxide-loaded liposomes to *ex vivo* porcine carotid tissue

4.1  Introduction

4.1.1  Ultrasound-mediated drug delivery

Ultrasound has been investigated as a method to trigger enhanced drug delivery within the human vasculature [162]. Ultrasound-mediated drug delivery (UMDD), and its associated mechanisms, are described in Chapter 2. Nitric oxide is a molecule that plays a mechanistic role in UMDD (Section 2.3.5). Attenuation of nitric oxide production in the etiology of atherosclerosis progression [298] and diabetic vascular disease [299] further highlights the need for novel therapeutic nitric oxide modulation and delivery strategies.

4.1.2  Experimental confirmation of drug delivery

Strategies to study ultrasound-mediated drug release and delivery *in vitro* and *ex vivo* have involved optical and electrophysiological techniques [77, 142, 300, 301]. Optical techniques, such as fluorescence [142] or luminescence [300], take advantage of native optical properties of the therapeutic, or conjugation of tracer molecules.

---

A gray vertical line indicates portions of this chapter submitted for publication in:

**Sutton et al.** Pulsed ultrasound enhances the delivery of nitric oxide from bubble liposomes to *ex vivo* porcine carotid tissue. *Int. J Nanomedicine*. (Submitted).

66
Electrophysiological approaches, such as voltage-clamp techniques [301], directly assess the changes in membrane potential provoked during UMDD, but often require isolated cells cultured in vitro, where cellular processes can vary from in vivo conditions [302]. In vivo animal models of UMDD provide relevant bioeffect information, yet are costly and subject to considerable inter-subject variability. The ability to detect and monitor the response of intact, isolated vascular tissue in real time would constitute a significant advancement in the study of UMDD.

Isolated tissue bath perfusion systems have been used extensively to characterize contractility changes induced by a therapeutic [295, 303–305] in a variety of muscular tissue beds including gastric [306, 307], peripheral vascular [308], and cardiovascular [295]. In these systems, dose-dependent changes in active muscular tension can be characterized in response to vasorelaxing agents such as bradykinin [309], sodium nitroprusside [310], nitroglycerine [311], and nitric oxide [312]. Adaptation of the isolated tissue bath model to drug delivery studies could provide relevant, real time quantitative data on the drug release and delivery profiles triggered by ultrasound. The hypothesis of this study is that isolated tissue bath systems can be used to characterize nitric oxide-mediated changes in carotid vascular tone upon ultrasound exposure.

4.2 Materials and Methods

4.2.1 Tissue bath system

Porcine carotid arteries were obtained post-mortem from young Yorkshire pigs (≈ 20 kg) according to a protocol approved by the Institutional Animal Care and Use Committee at the Cincinnati Department of Veterans Affairs Medical Center (Animal Welfare Assurance Number A3446-01). Seven pigs were initially anesthetized with intravenous ketamine (30 mg/kg), followed by pentobarbital (35 mg/kg) for deep surgical anesthesia. Prior to artery excision, the animals were euthanized with saturated KCl solution ([KCl]_{in\ viva} ≈ 0.18 M). Segments of porcine carotid tissue were harvested immediately following sacrifice and stored in ice-cold, oxygenated
Krebs-Henseleit buffer (KHB; NaCl: 115.9 mM; KCl: 5.4 mM; MgSO₄·7H₂O: 1.2 mM; NaHCO₃: 25 mM; d-glucose: 11.1 mM; NaH₂PO₄: 0.5 mM) until use. The tissue was dissected free of loose adventitia and connective tissue, and segmented into rings. These rings were cut to a length between 3.95 and 4.05 mm; the mean wet mass after blotting was 20.5 mg with a standard deviation of 2.6 mg. Each segment was mounted on two rigid, stainless-steel wires. The bottom wire was fixed to the tissue bath, and the top wire was coupled to an isometric force transducer (Radnoti LLC; Monrovia, CA USA) for measurement of arterial tension. The electronic configuration is depicted in Figure 4-1. The tension signal was sampled digitally at 20 Hz (LabChart 6; AD Instruments, Colorado Springs, CO USA) and saved to a PC for post-processing. The carotid segment was submerged in a custom reservoir filled with KHB maintained at physiologic temperature with continuous bubbling of a 95% O₂ / 5% CO₂ gas mixture to maintain physiologic pH (Range: 7.35 – 7.45).

Following the mounting of the artery and submersion in physiologic KHB, healthy arteries underwent a brief contraction and relaxation cycle [304]. Artery rings not exhibiting this behavior were deemed unfit for experimentation and discarded. Prior to treatment, the arterial ring was pre-stretched to relieve the passive elastic component of arterial tension in a manner similar to that described by Herlihy and Murphy [304]. Briefly, the artery was stretched incrementally by translating the force transducer with a micropositioning stage (Radnoti LLC). To equilibrate the artery within physiologic buffer, the artery was alternately contracted and relaxed by serial infusions of KHB and KHB containing additional KCl, substituted in an equimolar fashion for NaCl. The artery was contracted initially to steady state using KHB containing 50 mM KCl after each increment. Following a KHB wash, this process was repeated until a maximum KCl contraction was achieved. Healthy arteries consistently responded to pre-contraction with 50 mM KCl-doped KHB, however the extent of pre-contraction was variable (Range: 200 – 330 mN). The basal ring tension that produced a maximum contraction tension was determined to be 75 mN. Thus, subsequent rings were manually pre-stretched to this basal tension upon mounting.
Figure 4-1: Two submersible ultrasound transducers are coupled to the reservoir: a transmit transducer (1 MHz, 3% duty cycle) focuses on the lumen of the artery, while a second receive transducer (7 MHz) detects cavitation emissions from nitric oxide-loaded bubble liposomes perfused within the vessel lumen via a 26 Gauge blunt injection needle. From: Sutton J. T, Raymond J. L, Verleye M. C, Pyne-Geithman G, and Holland C. K. Pulsed ultrasound enhances the delivery of nitric oxide from bubble liposomes to \textit{ex vivo} porcine carotid tissue. \textit{International Journal of Nanomedicine.} Submitted 2014

4.2.2 Bubble liposomes

Bubble liposomes containing octafluoropropane (C\textsubscript{3}F\textsubscript{8}) were manufactured according to Endo-Takahashi \textit{et al.} \cite{313} for bubble liposomes. This liposomal formulation consisted of dipalmitoylphosphatidylcholine (DPPC), N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium (DOTAP), polyethylene glycol (PEG) 2000, and PEG 750 in a 79:15:3:3 molar ratio. After manufacturing, 1 mL (1 mg lipid) of the liposomal emulsion was pipetted into glass vials, which were evacuated of headspace air with a laboratory vacuum, and stored at 4 °C until use. Immediately prior to use, vials were warmed to room temperature and 2.5 mL of gas was injected into the vial headspace through a rubber septum. For nitric oxide-loaded bubble liposomes (NOBLs) this gas consisted of a 50:50 volume ratio of nitric oxide (NO) gas (Sigma Aldrich; St. Louis, MO USA) and C\textsubscript{3}F\textsubscript{8} gas (Specialty Gases of America, Toledo, OH USA). To assess the effect of cavitation alone (without NO) on vasorelaxation, C\textsubscript{3}F\textsubscript{8} bubble liposomes (OFPBL) were also manufactured.
by injecting 2.5 mL of $C_3F_8$. Following this step, the vial was mechanically agitated by shaking vigorously for 45 seconds (VIALMIX, Lantheus Medical Imaging; North Billerica, MA USA). This agitation step heated the emulsion slightly, so the vial was allowed to equilibrate to room temperature. Immediately prior to experimental use, 500 µl of the liposomal emulsion was diluted into oxygenated ($pO_2$: 722 mmHg), room temperature KHB to a final concentration of 0.05 mg lipid/mL for infusion into the system.

4.2.3 NOBL Characterization

A sample of the NOBL suspension was diluted further (1:500) into room temperature, aerated phosphate-buffered saline (PBS 0.9% w/v; Sigma Aldrich) and the size distribution was measured using an impedance-based particle sizer (Beckman Coulter Multisizer 4; 30 µm aperture). Each measurement analyzed 100 µl of the diluted sample through a 20 µm aperture over 30 seconds into PBS. Each measurement produced a number density histogram with bins logarithmically spaced between 0.6 and 18 µm. This histogram was transformed into a volume-weighted distribution for further analysis. The results from three measurements, each using a fresh vial of NOBLs, were averaged to produce a final volume-weighted size distribution. The frequency-dependent attenuation coefficient, $\alpha(f)$ in dB/cm, was determined using a broadband substitution technique [314], the components of which were described previously in Raymond et al. [315]. Briefly, NOBLs were diluted into a reservoir containing room-temperature, aerated PBS, stirred, and allowed to flow by gravity into a sample chamber with acoustically transparent polycarbonate film windows (CLINIcell 25, Mabio, Tourcoing, France). A pair of broadband transducers (PI-20, Olympus NDT, Waltham, MA USA) was used to acquire the through-transmission spectrum over the frequency range 1 to 30 MHz (31 kPa peak negative pulse pressure; 33 dB dynamic range). The attenuation spectrum was computed from the received amplitude spectra in the absence (diluent alone) and presence of the BLs, respectively. The reservoir, sample chamber, and transducers were mounted in a test tank filled with distilled water maintained at 37 ± 0.5 °C using a circulating water
bath (Neslab EX, Newington, NH USA). Acoustic attenuation measurements were made in triplicate and a separate vial of NOBLs was used for each measurement.

4.2.4 Hemoglobin

The short half-life (< 1 s) of nitric oxide in the presence of hemoglobin (Hb) and O$_2$ in vivo limits the spatial extent over which nitric oxide can be effective as a signaling molecule [316]. In order to mimic the in vivo milieu more closely and to quench non-encapsulated NO, 1 g/L Hb (porcine, Sigma Aldrich) was added to the reservoir prior to each treatment. We observed that Hb concentrations greater than 1 g/L produced a negligible decrease in vasorelaxation during NOBL + KHB infusions and occasionally elicited contractions. Thus, 1 g/L Hb was used in all subsequent treatments.

4.2.5 Ultrasound Exposure & Cavitation Detection

To reveal the mechanism of ultrasound-mediated drug release and delivery of nitric oxide to vascular tissue, two ultrasound transducers were coupled to the tissue-bath reservoir. One transducer was used to deliver pulsed US to nucleate cavitation from the liposomes and a second transducer was used to monitor the acoustic emissions passively for evidence of acoustic cavitation. The experimental configuration is depicted in Figure 4-1. Prior to each experiment, a calibrated 1-MHz therapy transducer (Olympus Panametrics; Waltham, MA USA) was aligned confocally with a 7.5-MHz passive cavitation detector (PCD; Olympus Panametrics) using an ultrasonic pulser receiver (5077PR; Olympus NDT). The confocal point was positioned in the axial center of the carotid ring using a three-axis translation stage (Newport 423, Irvine, CA USA). This geometry was chosen to ensure that the US therapy field encompassed the entire carotid artery, while cavitation emissions were detected primarily from the lumen. During experimental treatment, a function generator (Agilent Technologies, Santa Clara, CA USA) supplied a sinusoidal signal (30 cycles, 100 Hz pulse repetition frequency) that was amplified (750A250; Amplifier Research,
Souderton, PA USA) to drive the 1-MHz therapy transducer. Acoustic scattering within the tissue bath reservoir affected the beam profile slightly. Therefore, an *in situ* pressure field calibration was performed in the absence of the artery ring and wires (Figure 4-2).

Figure 4-2: The −3 dB contours of the transmit field (blue) and receive sensitivity (orange) are depicted, along with the anticipated location of the artery (red). From: Sutton J. T, Raymond J. L, Verleye M. C, Pyne-Geithman G, and Holland C. K. Pulsed ultrasound enhances the delivery of nitric oxide from bubble liposomes to *ex vivo* porcine carotid tissue. *International Journal of Nanomedicine*. Submitted 2014

During ultrasound exposure, acoustic emissions were detected by the PCD, amplified (Model 5185, Signal Recovery; Oak Ridge, TN USA), and sampled at 20 MHz with an oscilloscope (LeCroy Model LT372, Chestnut Ridge, NY USA). Sequences of 128 voltage-time traces were saved serially to a PC for post-processing (see Section 3.1.8).

Ultrasound parameters were chosen to promote stable cavitation nucleated by the NOBLs within the lumen of the arterial ring over the 50-second treatment. A thirty-cycle therapy pulse was chosen to minimize standing waves in the reservoir.
To determine the optimal acoustic pressure to promote stable cavitation, a dose-escalation approach was employed. An artery was mounted within the reservoir filled with oxygenated KHB containing 1 g/L Hb. NOBL infusions (0.2 mL/min) with concurrent ultrasound exposure and cavitation detection were performed serially. Between each treatment, the reservoir was flushed with fresh Hb-doped KHB and the acoustic pressure increased incrementally. This process was repeated at peak-to-peak acoustic pressure amplitudes ranging from 0 MPa to 0.38 MPa. The peak-to-peak acoustic pressure amplitude that promoted maximal ultraharmonic energy over the 50-second ultrasound exposure (0.34 MPa) was chosen for all subsequent exposures.

4.2.6 Treatment

During treatment, the lumen of each artery was infused for 55 seconds with buffer alone or buffer + NOBLs using a 24-Gauge blunt hypodermic needle connected to a syringe pump (KD Scientific, #23; Canning Vale, AU). Additionally, infusions of the nitric oxide donor sodium nitroprusside (SNP, 12 µM Sigma Aldrich) was infused as a control agonist. After five seconds of infusion, ultrasound or sham exposure and cavitation detection commenced. This experimental procedure is diagrammed in Figure 4-3.

<table>
<thead>
<tr>
<th>Artery Preparation</th>
<th>Experimental Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artery equilibration</td>
<td>Hemoglobin (1 g/L) added to reservoir</td>
</tr>
<tr>
<td>Tension Measurement</td>
<td>Ultrasound exposure</td>
</tr>
<tr>
<td>KCl Contraction</td>
<td>Cavitation Detection</td>
</tr>
<tr>
<td>Treatment infusion</td>
<td>Time (s)</td>
</tr>
</tbody>
</table>

Figure 4-3: Repeated measurements were made in series following 35 mM potassium chloride washes, followed by tension equilibration. From: Sutton J. T, Raymond J. L, Verleye M. C, Pyne-Geithman G, and Holland C. K. Pulsed ultrasound enhances the delivery of nitric oxide from bubble liposomes to ex vivo porcine carotid tissue. *International Journal of Nanomedicine*. Submitted 2014
4.2.7 Tension analysis

Arterial ring tension was analyzed using a custom MATLAB script. Each tension curve was normalized to the maximum tension produced by 50 mM KCl KHB using Equation 4.1,

\[
\% \text{ Relaxation} = \frac{F_{\text{MAX}} - F(t)}{F_{\text{MAX}} - F_{\text{BASAL}}}
\]  

(4.1)

where \(T_{\text{MAX}}\) is the initial tension produced by KCl contraction, \(T(t)\) is the arterial tension as a function of time relative to the start of the treatment infusion, and \(F_{\text{BASAL}}\) is the basal tension after manual stretching (75 mN). A representative tension curve for each treatment is given in Figure 4-4. For each treatment, the minimum value of percent relaxation—\textit{maximal relaxation}—was used to compare across treatment groups. This metric has been used previously to compare treatments in nitric oxide-related vascular tension studies [317, 318].

4.2.8 Statistics

Statistical analyses were performed using MATLAB (Statistical Toolbox). Normal distributions were confirmed using Lilliefors test, at a threshold p-value of 0.05. Differences in means between treatment groups were analyzed using a one-way unbalanced analysis of variance (ANOVA). P-values less than 0.05 were considered statistically significant. Subsequently, pair-wise comparisons with a Bonferroni correction were performed to compare across individual treatments to minimize the familywise error rate for multiple comparisons.

Linear regression was performed to test for a correlation between maximal relaxation and (a) wet tissue weight, (b) 35 mM KCl tension, and (c) cavitation energy. A one-way unbalanced ANOVA was used to test for significant differences in data means. For these tests, the p-value \((p)\), f-statistic \((F_{\text{stat}})\), and degrees of freedom \((DF)\) are reported. Spearman’s rank correlation coefficients \((\rho)\) were computed to test for monotonic statistical relationships between maximal relaxation and spectral
Figure 4-4: Representative tension reduction (relative to the maximum KCl contraction tension) for buffer + US, buffer + NOBLs, NOBLs + US (peak-to-peak acoustic pressure amplitude: 0.34 MPa), and control agonist sodium nitroprusside (SNP). Each treatments minimum tension (maximal relaxation) was used for grouped analysis. \( T = 0 \) refers to the start of the treatment infusion. From: Sutton J. T, Raymond J. L, Verleye M. C, Pyne-Geithman G, and Holland C. K. Pulsed ultrasound enhances the delivery of nitric oxide from bubble liposomes to \textit{ex vivo} porcine carotid tissue. \textit{International Journal of Nanomedicine}. Submitted 2014

Cavitation emissions. The null hypothesis of no correlation was tested to produce a p-value; values below 0.05 were considered statistically significant.

4.3 Results

4.3.1 Liposome Characterization

Depicted in Figure 4-5(a), NOBLs have a broad, bimodal volume-weighted size distribution, ranging in diameter from approximately 1.5 to 15 \( \mu \text{m} \). The peak volume density occurred at a diameter of 2.5 \( \mu \text{m} \). A separate population of particles existed at 11 \( \mu \text{m} \), diminishing in diameter above 15 \( \mu \text{m} \).
Figure 4-5(a) shows the measured attenuation coefficient as a function of frequency for NOBLs ($N = 3$). NOBLs attenuate ultrasound across the −33 dB bandwidth of the system (1-30 MHz). A strong, broad resonance peak ($\alpha = 13.0$ dB/cm) was observed at 5 MHz. A second resonance peak ($\alpha = 12.3$ dB/cm) was observed near 28 MHz. NOBLs attenuated weakly between these two frequencies.

![Graph showing size distribution and acoustic attenuation](image)

Figure 4-5: (a; top) Size distribution ($N = 3$) of the NOBLs, weighted by volume (black), and number density (gray). The presence of particles > 4µm (by volume) agrees with Endo-Takahashi et al. [313]. Dots indicate one standard deviation. (b, bottom) Acoustic attenuation as a function of frequency, as determined using the system described in Raymond et al. [315]. From: Sutton J. T, Raymond J. L, Verleye M. C, Pyne-Geithman G, and Holland C. K. Pulsed ultrasound enhances the delivery of nitric oxide from bubble liposomes to ex vivo porcine carotid tissue. International Journal of Nanomedicine. Submitted 2014
4.3.2 Cavitation

In general, ultraharmonic and broadband acoustic emissions within the arterial lumen persisted throughout the fifty-second exposures in all eight ultrasound-treated arteries. Figures 4-6(b) and 4-6(c) show representative traces of the ultraharmonic and broadband acoustic power detected by the PCD as a function of time. A representative spectrum, averaged over the 50-second treatment, is depicted in Figure 4-6(a). Ultraharmonic energy was typically strong, yet variable, during the first few seconds of ultrasound exposure, and remained steady for the duration of the fifty-second exposure. Within the NOBL + US treatment group, no correlation between maximal relaxation and the average spectral emissions was observed (ultraharmonic: $\rho = -0.45$, $\rho = 0.21$; harmonic: $\rho = -0.46$, $\rho = 0.25$; broadband: $\rho = -0.50$, $\rho = 0.21$).

4.3.3 Vasorelaxation

Seven carotid rings from seven separate pigs were exposed to each experimental treatment, as shown in Figure 4-7. Carotid rings relaxed in response to treatments in a characteristic manner: a sharp tension decrease followed by steady restoration to the tension level induced by 35 mM KCl precontraction. Relaxation after 12 $\mu$M SNP infusions was significantly stronger (32%; S.D. 5%) than NOBL infusions ($p \ll 0.01$, $F_{stat} = 104.15$, $DF = 13$) and sham control treatments, which consisted of buffer and NOBLs (7%; S.D. 3%), buffer and US (1%; S.D. 2%), or OFPBLs and US exposure (2%; S.D. 1%). Arterial rings exposed to NOBLs + US experienced strong maximal relaxation (31%; S.D. 8%) that was significantly greater than buffer and NOBLs ($p \ll 0.001$, $F_{stat} = 29.01$, $DF = 13$) and statistically identical to 12 $\mu$M SNP treatment ($p = 0.18$, $F_{stat} = 2.06$, $DF = 13$). Across all experimental samples, no correlation was observed between the maximal relaxation and pre-contraction tension ($p = 0.82$, $F_{stat} = 0.05$, $DF = 21$) or wet tissue weight ($p = 0.33$, $F_{stat} = 0.98$, $DF = 21$).
Figure 4-6: (a; top) Averaged spectrum of detected cavitation emissions across a 50 second ultrasound exposure during nitric oxide-bubble liposome (NOBL) infusion into the lumen of a carotid artery (solid). Dotted black lines show the detected spectral emissions from the artery (background: buffer + US alone). Ultraharmonic ($\frac{3f}{2}$, $\frac{5f}{2}$, ...) and broadband frequency components of the transmitted fundamental frequency (1 MHz) were consistently observed, indicating strong nonlinear bubble activity. (bottom) Time evolution of ultraharmonic (b; middle) and broadband (c; bottom) frequency emissions from NOBLs during treatment. Circles indicate the mean of the local data group, bars indicate one standard deviation. From: Sutton J. T, Raymond J. L, Verleye M. C, Pyne-Geithman G, and Holland C. K. Pulsed ultrasound enhances the delivery of nitric oxide from bubble liposomes to ex vivo porcine carotid tissue. International Journal of Nanomedicine. Submitted 2014
Discussion

4.4.1 Liposome Characterization

The characteristics of the NOBLs can be compared directly with previously reported data on $C_3F_8$-loaded bubble liposomes manufactured using similar methods. Endo Takahashi et al. [313] reported a volume-weighted size distribution peak at 749 nm with a significant volume-weighted population greater than 4 µm. These bubble liposomes of Endo Takahashi et al.—containing only $C_3F_8$—are smaller than the NOBLs described in this study (Figure 4-5). NOBLs have a biomodal volume-
weighted size distribution with a primary peak at 1.5 and a smaller, variable peak at 11 \(\mu m\). This size distribution is appropriate for clinical translation of UMDD: NOBLs are small enough to pass through the pulmonary capillary bed [319] yet large enough to permit strong cavitation nucleation by 1 MHz ultrasound [53].

The size discrepancy between the primary peaks could be explained by the use of a NO/\(C_3F_8\) blend in this study, compared to \(C_3F_8\) alone used in Endo Takahashi \textit{et al.} [313]. NO is more soluble in lipid than in aqueous solution [320], yet reacts more readily with oxygen in these environments. NO is further soluble in perfluorocarbon, demonstrated by prolonged release profiles in an \textit{in vivo} hamster model [321]. In this study, efforts were made to ensure that the procedure used to manufacture NO/OFP-loaded liposomes mimicked closely the procedure outlined by Endo-Takahashi \textit{et al.}. Kim \textit{et al.} describe an alternative method to encapsulate nitric oxide within echogenic liposomes which minimizes oxidation and degradation of nitric oxide prior to experimental use [33]. Future work should implement these procedures and tailor the gas and lipid composition in NOBL formulations to achieve optimal release profile and size distribution to improve therapeutic benefit.

Using this liposomal formulation, the potential stability of NOBLs \textit{in vivo} is unclear. DOTAP—a cationic lipid—likely conferred an affinity to the anionic endothelial glycocalyx [322]. This strong affinity to the vascular walls would decrease circulation time \textit{in vivo} [323], possibly necessitating local infusion. Alternatively, the presence of long chain (PEG 2000) and short chain (PEG 500) polyethylene glycol has been used in liposomal formulations to mitigate potential immunogenicity and antigenicity, thus prolonging circulation time [324]. On the other hand, the lipid compositional properties of the NOBLs may affect their ability to promote potent vasorelaxation in the vasculature. In our model, bubble liposomes were injected directly into the lumen of the arterial ring, where they were exposed to thirty-cycle pulses of 1-MHz ultrasound at a peak-to-peak acoustic pressure amplitude of 0.34 MPa. Hb at \textit{in vivo} concentrations would completely quench NO produced within the lumen of a blood vessel, thus necessitating NO delivery in close proximity to the endothelium, according to [308, 325]. Future studies investigating ultrasound-
mediated nitric oxide release from liposomes should consider these issues.

4.4.2 Ultrasound exposure and cavitation

The data presented here demonstrate that strong relaxation by nitric oxide-loaded liposomes can be enhanced by ultrasound exposure at peak-to-peak acoustic pressures similar to those previously described (1.0 MHz continuous-wave, 0.30 MPa, [33]). In this study, vascular relaxation was triggered from NOBLs with pulsed ultrasound at a peak-to-peak acoustic pressure amplitude of 0.34 MPa at 1 MHz. Despite the detection of strong cavitation at this acoustic pressure, the precise mechanism of NO release and delivery remains unclear. Smith et al. describe acoustically driven diffusion at moderate acoustic pressures[326] and other investigators [53, 327] have postulated that the lipid shell of microbubbles must first be ruptured before cavitation nucleation can occur, liberating encapsulated gas during rapid fragmentation. At a 0.34 MPa pressure exposure, it is likely that NO was released from the bubble liposomes gradually over a number of acoustic cycles. Radhakrishnan et al[328] have detected loss of echogenicity from contrast agents at acoustic pressures below the stable and inertial cavitation thresholds. In their experiments using Definity® and echogenic liposomes, the onset of stable and inertial cavitation was concomitant with an 80% loss of echogenicity. Thus the release of NO from bubble liposomes could also occur at acoustic peak-to-peak pressures lower than 0.34 MPa utilized in the present study. The minimum pressure amplitude that triggers the delivery of NO to vascular tissue warrants further elucidation.

In this study, $C_3F_8$ stabilized NO within the bubble liposomes prior to activation by ultrasound. The different properties of these gases (e.g. molecular size, solubility), could promote differential diffusion profile during acoustically driven oscillation. Upon membrane distention during volumetric bubble expansion, NO would likely dissolve more readily in the surrounding aqueous medium due to its high solubility. Also, after NO liberation, the residual $C_3F_8$ bubble activity could promote microstreaming and enhanced convection and penetration into nearby vascular tissue. The diffusivity of NO, roughly 3300 $\mu$m$^2$ s$^{-1}$ in muscular tissue [325, 329], is high
enough to support a linear diffusion distance of roughly 250 µm (approximate radial depth of porcine carotid smooth muscle cells [330]) over several seconds. NOBL infusions with ultrasound exposure consistently caused sharp decreases in vascular tension—typically occurring on the order of tens of seconds—followed by a steady tension restoration period, which lasted several minutes (Figure 4-4).

4.4.3 Vasorelaxation

Arterial segments relaxed significantly more when treated with NOBL infusions in the presence of pulsed ultrasound, compared to infusions with NOBLs alone (Figure 4-4). The ability of ultrasound to promote the delivery of encapsulated nitric oxide to vasulcar tissue agrees with observations by previous investigators [33]. Kim et al, using nitric oxide-loaded echogenic liposomes (NO-ELIP), demonstrated increased vasodilation of rabbit carotids after exposure to 5.7 MHz color-Doppler ultrasound at a peak-negative acoustic pressure of 0.35 MPa [328]. Huang et al. used a mixture of NO and argon (Ar) gas to encapsulate nitric oxide and trigger effective release to porcine carotid tissue [230]. In this study, the release of NO from the liposome into the surrounding medium was decreased by roughly 65% using the Ar/NO mixture. At lower peak-to-peak pressure amplitudes (< 0.40 MPa), other encapsulated bioactive gases, such as xenon, have been shown to exhibit enhanced release and delivery profiles with ultrasound [232]. Other studies have demonstrated the feasibility of encapsulating nitric oxide or nitric oxide-yielding molecules to prevent physiologic degradation [331]. For example, McKinlay et al. demonstrated strong vasorelaxation of porcine coronary rings incubated with nitric oxide bound within a porous organic metal framework [331]. Future studies should focus on elucidating the mechanism of NO release from drug vehicles so that efficient exposure protocols can be developed to translate ultrasound-mediated nitric oxide delivery into the clinic.
4.4.4 System Development

Though the ultrasound tissue bath system is capable of providing real-time feedback on nitric oxide delivery to vascular tissue, it has a few limitations. Here, a volatile anesthetic was used to sedate the animals prior to carotid excision, which can reduce endothelium function significantly by inhibiting endothelial production of nitric oxide [310]. Cavitation near the vascular endothelium during UMDD [298] is hypothesized to liberate endothelial nitric oxide synthase enzyme (eNOS) from membrane-bound proteins, such as caveolin-1 [100, 332]. Increased cytosolic eNOS results in increased endothelium-derived NO [333]—an effect likely absent in our model. Future studies should employ sedatives that do not adversely affect endothelial response, or should use abattoir-derived vascular tissue.

During arterial precontraction, porcine Hb was diluted into the reservoir to a concentration of 1 g/L. At this concentration, arteries consistently re-established equilibrium contraction tension following NO-induced vasorelaxation. This was likely due the quenching of excess nitric oxide by Hb—a reaction well-documented in vivo [142, 334]. Because free Hb quenches nitric oxide a thousand time faster than Hb within intact erythrocytes [335], the presence of free Hb in the bath likely resulted in a weaker vasorelaxation than expected in vivo in the absence of hemolysis.

At higher Hb concentrations (> 40 g/L) or in whole blood (120 g/L [303]), arteries routinely experienced periodic contractions that prevented tension restoration to equilibrium. This effect was likely indicative of vasospasm, which has been documented in cerebral arteries as a result of subarachnoid hemorrhage [306]. While this effect was a limitation in our model, Fathi et al. [299, 336] describe a potential role of nitric oxide delivery to treat cerebrovascular tissue following subarachnoid hemorrhage. Also, using intact erythrocytes, Kim et al. describe a technique to release nitric oxide from loaded echogenic liposomes (NO-ELIP) within the rabbit carotid artery to reduce ischemic neurologic deficits [33]. Future applications of this ultrasound tissue bath model should implement hemoglobin in the form of intact erythrocytes to ease the transition to possible clinical applications, such as localized
ultrasound-mediated delivery of nitric oxide for arresting atheroma development or
induction of vascular hyper-permeability.

4.4.5 Smooth muscle relaxation

_In vivo_, nitric oxide-mediated modulation of smooth muscle tone is well described
by two biochemical pathways: (a) stimulation of guanosine 3:5 cyclic monophosphate
(cGMP) and adenosine 3:5 cyclic monophosphate (cAMP) and (b) direct modula-
tion of calcium-activated, potassium channel (K\text{Ca}^+) permeability [318]. Bolotina
_et al._ describe the effect of exogenous nitric oxide on cGMP and K\text{Ca}^+. These au-
thors observed that cGMP-mediated vasorelaxations from exposure to nitric oxide
were transient in nature and concentration dependent, as determined by quantifying
maximal relaxation [317]—the metric used here. According to Tare _et al_., the K\text{Ca}^+
pathway can affect the temporal nature of nitric oxide-mediated vasorelaxation. Ac-
cordingly, these authors integrated vasorelaxation over time to quantify integrated
relaxation [318]. Due to the potential for sensitivity to the K\text{Ca}^+-dependent pathway
of nitric oxide-mediated vasorelaxation, integrated relaxation could be considered as
a potential metric in future applications of this system.

4.5 Conclusions

The ultrasound tissue bath system described here demonstrates a novel, effective
technique to characterize ultrasound-mediated delivery of a bioactive drug to
vascular tissue. Ultrasound tissue bath systems can be used to monitor UMDD in
real time. The data presented here demonstrate that nitric oxide can be released
from bubble liposomes with 1 MHz pulsed ultrasound exposure and deposited into
vascular tissue. Nitric oxide penetration into tissue causes potent vasorelaxation,
which manifests as changes in isometric vascular tension. Future studies using this
technique may focus on monitoring the delivery of other bioactive agents that cause
vasorelaxation, such as proteins, cells, or nucleic material.
Chapter 5  Delivery of bevacizumab-loaded echogenic liposomes to porcine carotid tissue increases in the presence of stable cavitation

5.1 Introduction

Ultrasound has been investigated as a novel strategy to effect enhanced, site-specific vascular delivery of cardiovascular drugs (Chapter 2, Section 2.2), such as the anti-angiogenic drug, bevacizumab (Chapter 2, Section 2.3). The objective of the study described here was to investigate the effect of different modes of bubble activity from bevacizumab-loaded echogenic liposomes (BEVELIP) on the delivery profile of bevacizumab into normal and atheromatous porcine carotid tissue.

5.2 Materials and Methods

5.2.1 Bevacizumab-Loaded ELIP (BEVELIP)

Preparation

BEVELIP was manufactured for use in arterial experiments at the University of Texas Health Sciences Center-Houston (UTHSC) [337]. Briefly, BEVELIP were prepared by mixing the lipid components 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleyoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycerol-3-phosphor-rac-1-glycerol (DPPG), 1,2-dipalmitoyl-sn-glycero-3-phospho-ethanolamine (DPPE), and cholesterol (CH) at a 46:23:8:8:15 molar ratio in a round-bottom flask as a chloroform solution [212]. The chloroform was evaporated under argon
while spinning the flask submerged in a 50 °C water bath. The consequent lipid film was placed under vacuum for 4 hours at < 100 mTorr pressure for complete removal of the solvent. The dry lipid film was rehydrated with 0.2 M mannitol in 12.5-fold diluted 0.02 M phosphate-buffered saline, pH 7.4 (PBS), containing 0.8 mg/ml of bevacizumab (Genetech, South San Francisco, CA) to a concentration of 10 mg lipid/ml. The hydrated lipid was centrifuged at 13,200 RPM in a microfuge for 20 minutes to remove unencapsulated bevacizumab. Each aliquot of the suspension was placed in a −80°C freezer and lyophilized for 24 – 48 hours. Each lyophilized 5 mg dry cake was rehydrated with 0.5 ml room temperature, deionized (> 17.5 MΩ) and filtered (0.22 µm) water (Barnstead NANOPure system, Thermo Fisher Scientific Inc.; Waltham, MA USA) immediately prior to use. The NANOPure water had previously been vigorously shaken in a 250 mL bottle to bring the dissolved gas content to 100% relative to standard temperature and pressure.

**Immunoreactivity**

Reconstituted BEVELIP aliquots were centrifuged at 14,100g for 20 minutes at room temperature. Based on optical absorbance at 440 nm, which has been established as a measure of intact liposomal concentration within the submicron size range, centrifugal pellets contained 97.4 ± 2.1% (SD, N = 6) of the total ELIP [338]. The phospholipid content of centrifugal fractions was also measured by the colorimetric Stewart assay [339], which indicated that 96.2% of total liposomal phospholipid was recovered in the pellet. Aliquots of the liposomal suspension and the resuspended pellet in 0.5% Tween-20 (total BEV and total pellet BEV), as well as the supernatant and the intact resuspended pellet were assayed for immunoreactive bevacizumab with a direct enzyme-linked immunosorbent assay (ELISA) as previously described [340]. Immunoreactive bevacizumab in intact pellet suspensions was considered to be tightly associated with ELIP, while having epitopes exposed on the liposomal surface. Three separate batches of BEVELIP were prepared to complete the carotid artery experiments described herein. The immunoreactivity of each batch was assessed separately and reported.
Figure 5-1: An overview of the *ex vivo* carotid perfusion system. A peristaltic pump circulates porcine plasma containing bevacizumab-loaded echogenic liposomes (BEVELIP) through a viable porcine carotid artery. A phased ultrasound array exposes the arterial lumen to color-Doppler ultrasound, while scattered cavitation emissions are detected by a passive cavitation detector (PCD).

### 5.2.2 Atheromatous Carotid Arteries

A porcine carotid model of atheroma (ATH) was developed as previously described [341] at the University of Health Sciences Center in Houston (UTHSC). Briefly, ten Yucatan miniswine were housed and treated following a protocol approved by the Institutional Animal Care and Use Committee at UTHSC. The right common carotid artery endothelium of ten pigs was removed by balloon denudation by passing a 4F Forgarty embolectomy balloon catheter within a five centimeter section of the lumen. Following the denudation, miniswine were fed a high cholesterol diet to induce atheroma. After 45 – 60 days of atheroma progression, the right femoral artery was catheterized and the pig was euthanized with saturated KCl under general anesthesia with 3% isoflurane. Prior to excision of the left and right common carotid arteries, the sutures maintained the physiologic length of the vessel, which was immediately placed in Krebs-Henseleit buffer at 4 ± 2 °C. These atheromatous arteries
were shipped overnight to the University of Cincinnati and used within 24 hours of excision.

5.2.3 Normal carotid arteries

To serve as a control group, the carotid arteries from ten miniswine not subjected to endothelial denudation and high-cholesterol diet (normal) were excised and shipped directly to the University of Cincinnati in an identical manner to that described for atheromatous arteries.

5.2.4 Ex vivo porcine carotid perfusion system

A custom flow system was used to mimic blood flow in an ex vivo porcine carotid artery. This setup (Figure 5-1) enables a viable carotid artery to be mounted within physiologic flow while precise control of ultrasound parameters and cavitation detection is maintained. Upon arrival, the carotid arteries were segmented into three centimeter segments and cannulated with polycarbonate hose barbs (US Plastics; Lima USA). The technique for mounting the carotid arteries in the flow system is similar to that described in Chapter 3 in the absence of a clot. Modifications to that protocol are summarized below. In cases where the inner diameter of the lumen was small (< 2 mm), a blunt 14 Gauge hypodermic needle was used for cannulation. Each cannulated carotid segment was mounted in a custom artery holder, and the perivascular space was filled with degassed phosphate-buffered saline (PBS; Sigma Aldrich; dissolved O$_2$: < 3 mg/L) retained by a latex condom (Durex, Parsippiny, NJ USA). This sample holder was inserted within the ex vivo perfusion system and submerged in a lucite water tank containing degassed, deionized water prior to experimentation.

5.2.5 Color-Doppler ultrasound exposure

A phased imaging array driven by an ultrasound imaging scanner (P4-1, HDI5000; Philips Healthcare, Andover USA) was chosen to expose carotid arteries to scanned mode ultrasound and effect cavitation along the length of the carotid artery. The field
Figure 5-2: The directivity pattern of the first color-Doppler line was aligned three millimeters downstream from the entrance cannula for each treatment. Five-micron thick sections were cut serially for hematoxylin and eosin and immunohistochemistry at the anticipated focus of the first color-Doppler line relative to the imaging frame.

of the phased array was calibrated in a large acrylic, degassed water tank with a calibrated PVDF needle hydrophone (0.2 mm; Precision Acoustics; Dorchester UK). Figure 5-3 depicts example pulse waveforms and frequency spectra for the color-Doppler and interleaved B-mode pulses. The pulses were focused to 28 unique color-Doppler locations, or lines, along the azimuth of the phased array, spaced by roughly 200 µm.

At the image depth corresponding to the anticipated center of the arterial lumen (3.5 cm from the array face), the −3 dB transverse beam width of the focused color-Doppler lines was 6.0 mm (Figure 5-2) and the −3 dB elevational beam width was 8.0 mm (Figure 5-4). These widths were large enough to encompass the entire lumen of all arteries exposed to ultrasound treatment. In general, twelve color-Doppler pulses and two B-mode pulses were transmitted to each color-Doppler line sequentially during each imaging frame (frame rate: 15.43 Hz, color-Doppler pulse repetition frequency: 8 kHz).

During treatment, the arterial lumen containing flowing BEVELIP was exposed to duplex color-Doppler ultrasound at one of four peak rarefactive pressure ampli-
Figure 5-3: Interleaved color-Doppler (a, b) and b-mode (c, d) pulses focused in lumen of the carotid arteries to nucleate cavitation from flowing bevacizumab-loaded echogenic liposomes. Red: output from phased array at MI setting of 0.80, Blue: MI 0.40, Black: 0.06

Amplitudes. Trial groups were delineated by the presence or absence of flowing BEVELIP through the arterial lumen and the ultrasound exposure peak rarefactional pressure amplitude. The color-Doppler peak rarefactional pressure amplitudes [0 MPa (MI 0.0), 0.04 MPa (MI 0.06), 0.23 MPa (MI 0.40), 0.42 MPa (MI 0.80)] were chosen based on preliminary experiments to nucleate three distinct modes of bubble oscillation: (a) harmonic cavitation (b) stable cavitation, and (c) inertial cavitation, respectively. At these output levels, the corresponding peak rarefactional pressure amplitudes of b-mode pulses were 0, 0.02, 0.10, and 0.18 MPa.

5.2.6 Cavitation Detection

To detect cavitation, a single-element unfocused passive cavitation detector (3.5 MHz, Diagnostic Sonar, Livingston UK) was aligned confocally with the first color-Doppler
Figure 5-4: Elevational pressure profile at the azimuthal focus of color-Doppler line #1, 3.5 cm away from the face of the array ($z = 3.5 \text{ cm}$).

line, with respect to the image frame, using an radiofrequency pulser receiver (Panametrics 5077PR; Olympus NDT, Waltham, MA, USA). During treatment, RF signals from scattered pulses were gated by their travel time to the PCD, amplified (Comlinear Microcircuits; Loveland USA), filtered below 35 MHz (TTE, Los Angeles USA) and digitized by an oscilloscope (Teledyne LeCroy; Chestnut Ridge USA). These data were transferred to a PC for spectral analysis using a custom MATLAB (The Mathworks, Natick USA) routine. Briefly, scattered pulses emanating from the first color-Doppler lines were transformed into the frequency domain. To account for large variation in scattering from each artery, an averaged power spectrum from 15 seconds of background data—acquired for each respective artery with flowing plasma alone—was subtracted from each treatment power spectrum. Background-subtracted
treatment power spectra were averaged over three image frames (≈ 200 ms) and saved to a PC. Ultraharmonic (2.55 – 2.70 MHz and 4.30 – 4.45 MHz) and inharmonic (2.14 – 2.21 MHz, 2.99 – 3.06 MHz, 3.89 – 3.96 MHz, 4.74 – 4.81 MHz) frequency bands of the power spectra were summed, normalized by bandwidth, and associated with stable cavitation and inertial cavitation emissions, respectively, as introduced in Section 3.1.8. This process was repeated for the remainder of the 3.5 minute ultrasound exposure. Average ultraharmonic and broadband power spectral densities were computed for each arterial treatment and compared across treatment groups.

5.2.7 Treatment Overview

After mounting of the artery, five milliliters of perfusate, containing a combination of anticoagulated porcine plasma (Lampire Biologicals; Pipersville USA) and reconstituted BEVELIP (2 mg lipid/ml plasma), were pumped at 1.0 ml/min (±0.2 ml/min) with a peristaltic pump (Rabbit, Mettler Toledo; Columbus USA) through the ex vivo carotid artery. At the onset of each treatment, BEVELIP echogenicity was assess with the P4-1 probe operated in b-mode at a low output amplitude (MI 0.04) to ensure robust echogenicity in BEVELIP-treated arteries. Arterial segments receiving ultrasound exposure were exposed to duplex color-Doppler ultrasound at diagnostic acoustic pressures for 3.5 minutes. During each treatment, physiologic parameters were monitored with a temperature probe (Pendotech; Princeton USA), pressure meter (Pendotech), and flow transducer (Transonic Systems Inc.; Ithaca USA). This experimental setup is depicted in Figure 5-1.

After each treatment, the arterial lumen was flushed with 10 mL PBS and the sample holder removed from the water tank. The artery was fixed at its physiologic length in 10% neutral-buffered formaling (Sigma Aldrich) solution for three hours, then segmented for assessment of bevacizumab penetration. From each artery, a ring—roughly 2.25 mm in length—was segmented to ensure co-registration with the first color-Doppler line of the imaging frame (see Figure 5-2).

During treatment, arterial segments were exposed to duplex color-Doppler ultrasound at diagnostic acoustic pressures for 3.5 minutes. Before treatment, the carotid
artery was coregistered with the color-Doppler region of interest (ROI) of a phased array driven by an ultrasound imaging scanner (P4-1, HDI5000; Philips Healthcare, Andover USA). To characterize the anticipated acoustic field and timing pattern of color-Doppler and b-mode pulses, the phased array was calibrated in a large acrylic, degassed water tank with a broadband needle hydrophone (0.2 mm; Precision Acoustics; Dorchester UK). Figure 5-3 depicts example pulse waveforms and frequency spectra for the color-Doppler and b-mode pulses. The pulses were focused to 28 unique color-Doppler locations, or lines, along the azimuth of the phased array, spaced by roughly 200 µm (Figure 5-2). In general, twelve color-Doppler pulses and two b-mode pulses were transmitted to each color-Doppler line sequentially during each imaging frame (frame rate: 15.43 Hz, color-Doppler pulse repetition frequency: 8 kHz).

5.2.8 Quantification of bevacizumab delivery

Using a custom immunohistochemistry protocol, the distribution of bevacizumab within the porcine carotid arteries was determined by assessing the distribution of brown chromogenic substrate using brightfield microscopy. Briefly, the segmented artery ring was subjected to a series of graded alcohol and xylene dehydration steps and then embedded in paraffin. Two five-µm-thick radial paraffin sections were obtained from the axial center of the embedded arterial ring. One carotid section was incubated in the presence of an antibody against the Fc portion of human IgG (goat anti-human Fc IgG, Abcam; Cambridge UK), conjugated to horseradish peroxidase (HRP). The brown reaction product was produced by reaction of 3,3'-diaminobenzidine tetrahydrochloride (DAB) with HRP in the presence of peroxide. This were also counterstained with hematoxylin to provide tissue contrast. The section immediately adjacent to the IHC-stained section was stained with routine hematoxylin and eosin for histological analysis.

Digital brightfield images of the stained carotid sections were obtained using a high speed digital slide scanner (Aperio AT2, Leica Biosystems; St. Louis USA) at 40X magnification. The spatial distribution of DAB-positive pixels was determined using a custom MATLAB (The Mathworks, Natick MA USA) script implementing
an open source algorithm (NIH ImageJ; Bethesda, MD USA) based on the work of Ruifrok and Johnston [342] to decompose each image into separate DAB- and hematoxylin-specific images. Portions of tissue exhibiting artifactual signal due to tissue folding or mis-mounting, as determined by a blinded pathologist, was excluded from the analysis. DAB images were subjected to grayscale binary thresholding by an veterinary pathologist blinded to experimental treatments to produce a binary map of DAB-positive pixels to assess bevacizumab penetration. There was no statistically significant difference between the mean grayscale thresholds chosen across three separate immunohistochemistry staining batches, so an averaged threshold value across all batches was chosen. For each arterial section, the total tissue area that was positive for bevacizumab within two arterial compartments—(a) intima/media and (b)
adventitia—was calculated reported as a function of treatment for normal and athero-
matous arteries. These compartments were determined using a custom MATLAB
routine which required manual segmentation for the medial/adventitial boundary.
Portions of neointima, adherent clots, and lesions encroaching upon the lumen were
considered part of the intimal layer and included in the analysis (see Figure 5-5).

5.2.9 Statistics

Statistical analyses were performed using the MATLAB Statistical Toolbox. Differ-
ences in BEVELIP immunoreactivity and bevacizumab-positive area within treatment
groups were analyzed using a one-way unbalanced analysis of variance (ANOVA). P-
values less than 0.05 were considered statistically significant. Total DAB-positive
area was analyzed pairwise between individual treatments after a Bonferroni correc-
tion was performed to minimize the familywise error rate. Normal distributions were
confirmed using Lilliefors test, at a threshold p-value of 0.05.

5.3 Results

5.3.1 Immunoreactivity of BEVELIP

ELISA measurements performed at UTHSC revealed that all three batches of
BEVELIP were immunoreactive. The degree of immunoreactivity differed signifi-
cantly (p = 0.008), as determined by ANOVA. Batch #1 yielded 82.4 ± 17.5 µg
(N = 4) immunoreactive bevacizumab, compared to 102.4 ± 20.9 µg (N = 6) in
Batch #2, and 176.0 ± 0.0% µg (N = 1) in Batch #3.

5.3.2 Ex vivo system parameter measurement

Across all treatments, the mean flow rate of perfusate through the lumen of the
carotid arteries was 1.07 ml/min (max: 1.31, min: 0.8, stdev: 0.1 ml/min). The
mean fluid pressure was 95.2 mmHg (min: 84.1, max: 104.5, stdev: 6.6 mmHg). The
mean temperature of the perfusate was 37.1 °C (min: 36.3, max: 38.4, stdev: 0.6
°C).
5.3.3 Bevacizumab penetration

In general, DAB signal was observed in all three arterial tunics in both atheromatous and normal porcine carotid arterial sections. The intensity of staining ranged from weak and diffuse (Figure 5-7) to strong and particulate (Figure 5-9). Strong particulate staining was predominantly observed within adventitial tissue and atheromatous tissue of arteries treated with BEVELIP or BEVELIP + US. Weak, diffuse staining was observed throughout all the arterial tunics, with the exception of the deep medial layers (Figure 5-9).

Figure 5-6: DAB staining area by treatment group within the intimal/medial tunics of porcine carotid arteries. Error bars indicate standard error of the mean. Asterisks indicate statistically significant differences ($p < 0.05$).
**Normal arteries**

In normal arteries, DAB staining was strong in arteries treated with plasma alone (negative control) in adventitial tissue, indicating non-specific binding of the IHC (anti-Fc-IgG) antibody in this region. Within the intima and media, DAB-positive area varied across all samples within a treatment. DAB-positive area in arterial sections treated with BEVELIP and US (0.42 MPa) was significantly greater than any other treatment ($p < 0.01$). In general, DAB-positive area was predominantly observed near the endothelium and internal elastic lamina, irrespective of the angular location relative to the direction of US propagation (Figure 5-7). DAB-positive area was not observed within the deep elastin layers of the media. As observed on H&E-stained sections, there was evidence of some scant neointimal hyperplasia in normal carotid sections.

**Atheromatous arteries**

In general, the amount of DAB-positive area in atheromatous arteries was significantly higher than normal arteries. Within the intima/media, arteries treated with plasma alone (negative control) and plasma + US (0.06 MPa) demonstrated relatively weak DAB staining compared to arteries treated with BEVELIP. Ultrasound increased the extent of delivery, but only within the BEVELIP + US (0.06 MPa) group. BEVELIP + US (0.23 MPa) and BEVELIP + US (0.42 MPa) did not increase the amount of drug penetration significantly.

Within the intima and media, DAB staining did not exhibit an angular dependence, but rather was consistently observed in locations of early-to-mid stage atheroma, characterized by neointimal thickening with strong cell reorganization and immune cell accumulation (Figure 5-9). These areas of strong DAB staining often co-localized with fibrin clots, as visualized on adjacent five-µm sections stained with H&E (Figure 5-9). In arteries exposed to plasma alone and plasma + US, areas colocalizing with neointima and fibrin did not exhibit DAB-positive staining (Figure 5-9).
Figure 5-7: DAB staining in normal porcine carotid arteries. Top row: Representative low magnification brightfield images of arterial sections treated with plasma alone (I), plasma and bevacizumab-loaded echogenic liposomes (BEVELIP) (II), and plasma, BEVELIP, and ultrasound at a peak-rarefactional pressure of 0.42 MPa (III). Bar = 2 mm Middle row: Immunohistochemical (IHC w/ hematoxylin counterstain; blue) and hematoxylin and eosin (H&E) stains for intimal/medial tissue. Bar = 0.2 mm. Bottom row: Immunohistochemical/(H&E) and eosin stains for adventitial tissue. Bar = 0.2 mm.

5.3.4 Cavitation detection

Strong ultraharmonic and cavitation energy was detected within normal and atheromatous arteries exposed to color-Doppler ultrasound at peak-negative acoustic pressures of 0.42 and 0.23 MPa (Figure 5-10). In normal arteries, more ultraharmonic and broadband energy was detected in arteries exposed to a peak-negative pressure of 0.42 MPa than at 0.23 MPa. A minimal amount of ultraharmonic and broadband energy was detected in arteries exposed to peak-negative pressures of 0.04 MPa. Within atheromatous arteries, the trends in detected cavitation power were consistent across treatment groups. Temporally, cavitation persisted throughout the 3.5 minute color-Doppler exposures as seen in Figure 5-11.
Figure 5-8: DAB staining area by treatment group within the adventitial tunic of porcine carotid arteries. Error bars indicate standard error of the mean. Asterisks indicate statistically significant differences ($p < 0.05$).

5.4 Discussion

5.4.1 Bevacizumab Penetration

From these data, it is reasonable to suggest that the distribution of bevacizumab could be affected by three factors: (a) ultrasound, both cavitation and non-cavitation effects, (b) specific binding of bevacizumab to matrix components of atheroma and/or adventitial extracellular matrix, or (c) the presence of vasa vasorum internae, a direct
Figure 5-9: DAB staining in atheromatous porcine carotid arteries. Top row: Representative low magnification brightfield images of arterial sections treated with plasma alone (I), plasma and bevacizumab-loaded echogenic liposomes (BEVELIP) (II), and plasma, BEVELIP, and ultrasound at a peak-rarefactional pressure of 0.42 MPa (III). Bar = 2 mm. Middle row: Immunohistochemical (IHC w/ hematoxylin counterstain; blue) and hematoxylin and eosin (H&E) stains for intimal/medial tissue. Bar = 0.2 mm. Bottom row: Immunohistochemical/(H&E) and eosin stains for adventitial tissue. Bar = 0.2 mm.

conduit for luminal drugs to the adventitia that bypasses major barriers of transport. It is important to note that, while DAB-positive area was quantified in this study, the intensity of DAB-staining (weak, diffuse vs. strong, particulate) varied significantly across the experimental samples.

More sophisticated image analysis strategies could be implemented to distinguish spurious staining, evidenced by DAB-positive area in plasma alone-treated vessels (Figures 5-8, 5-6) from weak and strong bevacizumab penetration. However, strategies to enable more robust and spatially resolved histological analysis should exercise caution regarding the non-linear optical density of DAB [343]. Furthermore, DAB-positive area was qualitatively observed between adherent atheromatous clots and the internal elastic lamina, suggesting differential permeability of these two tissue struc-
Figure 5-10: Top: Mean ultraharmonic and broadband cavitation energy detected by the 3.5 MHz passive cavitation detector in normal carotid arteries. Bottom: Cavitation energy detected in atheromatous arteries. All differences in respective cavitation energies between treatment groups were statistically significant ($p < 0.05$).

5.4.2 Cavitation Detection

Ultraharmonic and broadband energy, indicators of stable and inertial cavitation, were consistently detected at peak-negative acoustic pressures of 0.23 MPa and 0.42 MPa in the presence of BEVELIP. The amount of energy decreased with decreasing acoustic pressure, with very little energy being detected at a peak-negative acoustic pressure of 0.04 MPa. Interestingly, ultraharmonic and broadband energy...
was detected in plasma treatments with US exposure at 0.42 MPa in the absence of BEVELIP. This is likely due to an imperfect background subtraction, since the fundamental scattering tended to increase slightly during treatment.

To compare bevacizumab delivery to detected acoustic emissions from the PCD, bevacizumab penetration was only assessed at the PCD focal location. After fixation, one five-µm section of each carotid ring located at the anticipated color-Doppler focal zone was analyzed for bevacizumab penetration (see Figure 5-2). Further analysis should be conducted to determine if the interaction of cavitating microbubbles with the vascular endothelium elicits a bioeffect that occurs in locations along the axis of the artery downstream from the focus of the first color-Doppler line. These effects could also be correlated with cavitation activity occurring as a result of acoustic exposure to color-Doppler lines downstream of the line analyzed here.

5.4.3 Carotid histology

In this study, we choose to quantify the total area of DAB-positive tissue. This choice allowed us to determine, on a relative scale, the amount of BEV delivered to the tissue

Figure 5-11: Top: Ultraharmonic and broadband cavitation power as a function of time, as detected by the 3.5 MHz passive cavitation detector in normal carotid arteries exposed to color-Doppler ultrasound.
and this amount changed based on the artery's exposure to combinations of BEVELIP and ultrasound. However, it was also noted that vessel pathology influenced the amount of BEV delivered. Thus vessel pathology creates a confounding factor in determining the importance of BEVELIP and ultrasound. A future study could potentially incorporate vessel pathology by further segmenting the artery into more refined compartments such as neointima, fibrin, normal media, etc. Furthermore, the fraction of the area of each of these components that is DAB-positive could be computed. This normalized approach could help isolate the relative importance of vessel pathology versus ultrasound exposure. Multiple regression analysis could also be used to elucidate the relative roles of vessel pathology and ultrasound exposure.

5.4.4 Immunohistochemistry

The immunohistochemical strategy implemented here to assess the spatial distribution of bevacizumab in porcine carotid tissue is a promising technique for future porcine ex vivo vascular investigations. Noticeable levels of DAB staining were observed in the tunica adventitia in arteries not treated with BEVELIP. This scenario suggests non-specific binding of the Fc-IgG-HRP antibody to adventitial material, or non-specific oxidation of DAB. Future studies seeking to validate and assess the distribution of drugs and tracers within porcine vascular tissue should consider this approach.

5.4.5 Bioeffects

Recent investigations suggest a paramount role for a functional endothelium in ultrasound-mediated drug delivery [25, 100]. Experimentally, an intact endothelial barrier is necessary to assess the validity of ex vivo ultrasound-mediated drug delivery and its potential mechanisms [4]. In the data described herein, porcine carotid arteries were excised immediately post-mortem, shipped overnight in cold physiologic buffer, and used in experiments roughly 20 hours post-excision. H&E visualization of the intima and endothelium permitted qualitative assessments of endothelial presence, but more robust methods of visualizing and confirming the function of ex vivo
endothelium are needed. Alternatively, recent \textit{ex vivo} \cite{5} and \textit{in vitro} \cite{94} investigations discuss methods to evaluate ultrasound-mediated drug delivery by assessing real-time vasoreactivity and intracellular signaling—benchtop methods which assess bioeffects rather than the presence of a drug or tracer molecule.

5.5 Conclusions

The data presented here document increased penetration of bevacizumab, a humanized monoclonal antibody to vascular endothelial growth factor, from echogenic liposomes into porcine carotid tissue in the presence of color-Doppler ultrasound. Strong bevacizumab penetration, measured as DAB-positive area, was observed in arteries treated with BEVELIP and combinations of color-Doppler ultrasound, especially in those arteries exhibiting extensive atheromatous lesions and neointima. From these observations, the direct effect of ultrasound and cavitation on the delivery profile of bevacizumab into porcine vascular tissue is unclear. Future investigations seeking to develop methodology to quantify the spatial distribution of neointimal factors with affinity to BEV will aid in characterizing the specific contributions of ultrasound-mediated BEV delivery.
Chapter 6  Conclusions and Future Directions

6.1  Conclusions

The aim of the work described in this dissertation was to assess the ability of ultrasound to promote cardiovascular drug delivery across different physiological barriers. This is a worthy biomedical endeavor, given numerous shortcomings of traditional techniques to deliver drugs and treat diseased tissue. In Chapter 3, sonothrombolysis in unretracted clots suggests that ultrasound is capable of enhancing drug delivery through porous, permeable fibrin. In Chapter 4, increased vasorelaxation resulting from exposure to pulsed ultrasound and nitric oxide-loaded microbubbles suggests that ultrasound can enhance delivery to smooth muscle cells. And in Chapter 5, increased bevacizumab deposition into intimal and medial tissue in the presence of color-Doppler ultrasound suggests that ultrasound in capable of enhancing delivery across the endothelium of porcine carotid arteries.

In Chapter 3, the ability of continuous-wave ultrasound to enhance the delivery of recombinant tissue-type plasminogen activator to blood clots was investigated. By varying the degree of retraction, two blood clot models were tested which represented two thrombolytic extrema of erythrocytotic clots that may be encountered in vivo. Retracted clots were tough, non-porous, and fibrous while unretracted clots were friable, porous, and serum-filled. A thrombolysis model was constructed to examine the thrombolytic efficacy of these clot models under low flow during sonothrombolytic therapy. The results indicated that both models lysed effectively in the presence of

Portions of this chapter were published in:
rt-PA alone, compared to plasma alone. To assess the effect of sonothrombolytic therapy, continuous-wave ultrasound and bubbles were combined with rt-PA treatment. While rt-PA treatment lysed retracted clots effectively, unretracted clots lysed much more effectively in the presence of ultrasound and microbubbles. This result suggests that the mechanism of accelerated thrombolysis in the presence of cavitating bubbles is dependent on the compositional structure of the thrombus. One compelling explanation for this observation is the effect of acoustically activated microbubbles entrained within the fibrin mesh of unretracted, porous clots. An increased flux of microbubbles through the fibrin mesh would result in bubble oscillations in close proximity or directly against, nearby fibers. Stable bubble oscillations in this manner could assist in removing fibrin degradation products during lysis, cleave the mesh into smaller fibrin degradation products for efficient rt-PA lysis, or promote rt-PA penetration into an already plasminogen-rich clot.

The results gleaned from Chapter 3 regarding the propensity of a blood clot to experience sonothrombolysis have implications experimentally and clinically. They demonstrate the importance of using suitable blood clot models in sonothrombolysis experiments. Given the wide array of clot composition found in vivo, it is important to tailor a study of sonothrombolysis to one anticipated type of clot (e.g. from carotid arterial disease), rather than creating a “general model” of in vivo thrombus. The literature on this topic is exhaustive and comprehensive, and centers around the extensive work of John Weisel, PhD (University of Pennsylvania)[344]. These results could also have implications for the treatment of acute ischemic stroke using sonothrombolysis. When comparing benefits of a microbubble-guided, transcranial ultrasound exposure strategy alongside catheterization reteval options, clinicians should consider the source and etiology of the occlusive thrombus whenever possible.

In Chapter 4, a tissue bath model was constructed to investigate ultrasound-mediated delivery of an encapsulated vasodilating gas—nitric oxide (NO)—to porcine carotid tissue. Bioactive gases are attractive in the context of ultrasound-mediated drug delivery due to their ability to act not only as a therapeutic, but also as a cavitation nucleation agent. Further, bioactive gases such as NO, often are small
in molecular size and readily diffusible, which allows for more effective dispersion throughout the lumen, and unhindered passage through ultrasound-mediated pore formation. In this model, NO-loaded bubble liposomes were infused directly into the lumen of a porcine carotid artery ring, while being exposed to pulsed ultrasound at a low duty cycle. During acoustic exposure, the vascular tension of the artery was monitored to assess the extent of NO delivery into the thick tissue layers of the carotid artery, which contained viable smooth muscle. When exposed to an acoustic pressure sufficient to promote stable cavitation, carotid rings relaxed more strongly compared to rings exposed to NOBLs alone. The degree of ultrasound-mediated vasorelaxation was comparable that elicited by sodium nitroprusside, a nitric oxide donor and clinical vasodilating drug.

In Chapter 5, the construction of an experimental ex vivo flow system was described in order to survey the ultrasound-mediated penetration profile of bevacizumab (BEV)—an anti-angiogenic antibody—across the major barriers of the normal and atheromatous carotid artery. During treatments mimicking physiologic conditions, BEV-loaded echogenic liposomes were exposed to three acoustic pressures, chosen to nucleate three distinct modes of bubble activity. The hypothesis of this investigated was that cavitation by BEV-ELIP would promote antibody release from ELIP and subsequent delivery into porcine carotid tissue. A custom immunohistochemistry stain revealed strong BEV penetration in ultrasound-treated arteries compared to sham treatments. Within ultrasound-treated arteries, the extent and type of cavitation did not affect the degree of BEV penetration across the vascular endothelium, or in vasa vasorum-rich adventitial tissue. These results indicate that the presence of bubble oscillations was a strong promoter of increased BEV penetration in this large artery model. Also, BEV penetrated into atheromatous tissue more strongly than into normal carotid tissue. Possible explanations for this effect include upregulation of VEGF (antigen to bevacizumab) production in atheromatous tissue, and increased endothelial permeability associated with formation of neointima and atheroma progression.

This drug delivery model, using an ex vivo porcine carotid, is useful to study
mechanistic effects of ultrasound and bubbles on drug delivery into vascular tissue. However, *ex vivo* arterial systems are limited in their ability to mimic the pathophysiology of atherosclerosis. To improve this model further, some adaptations could be developed that would enable more robust and sensitive *ex vivo* drug delivery investigations in different tissue beds relevant to cardiovascular disease. Firstly, the health and integrity of the major barriers to drug transport are imperative to maintain. The endothelium of the carotid artery diminishes precipitously in the hours following excision from the animal. With this loss, the hypothesized mechanisms of ultrasound-mediated drug delivery, which rely heavily on the functional integrity of this barrier, become very difficult to investigate.

Future investigators should thus attempt to conduct experiments within a few hours of excision, or risk losing the functional integrity of this barrier. Secondly, this carotid model of vascular perfusion belies the complex paracrine and endocrine signaling upon which the endothelium and other cardiovascular cells rely to function normally or pathologically. For example, recent studies into the nature of atherosclerosis progression suggest that paracrine signaling from interstitial adipose tissue fuels disease progression. To overcome this limitation, other tissues could be investigated such as whole organ perfusion models, which may retain paracrine and endocrine more effectively *ex vivo*.

Thirdly, mechanistic studies of biomedical systems are most effectively studied when a response variable (i.e. bevacizumab penetration) can be measured with reasonable temporal resolution. Often, the hypothesized physical models describing the complex behavior of ultrasound-mediated drug delivery phenomena are strongly dependent on time. For example, diffusion and convection are phenomena—outlined by physical models [345]—that affect ultrasound-mediated drug delivery. The diffusion of a molecule across a permeable barrier is strongly dependent on parameters such as drug concentration, diffusivity, permeability, and time. The strength of a model’s ability to measure these variables with adequate temporal resolution determines greatly its ability to elucidate physical mechanisms.

Two of the studies in this dissertation investigated ultrasound-mediated drug de-
livery to porcine carotid vascular tissue (see Chapters 4 and 5). Contiguous four-millimeter common carotid segments from young Yorkshire pigs, immediately adjacent to the aortic bifurcation, were used to assess nitric oxide delivery to smooth muscle cells. Pathologic interpretation of H&E-stained sections of these arteries indicate a predominantly smooth muscle phenotype. However, occasional striations and intercalated discs were observed in the muscles at the exterior border of the tunica media, and in the tunica adventitia, consistent with cardiac type of musculature. In Chapter 5, three-centimeter sections of the common carotid artery of Yorkshire miniswine were used to assess ultrasound-mediated bevacizumab penetration. In these arteries, signs of cardiac phenotype were nearly absent in H&E sections. The presence of this phenotype in a carotid segment, and similarly its proximity to the heart [346], can affect the viscoelastic properties of its passive mechanical behavior. In Chapter 4, all segments were pre-stretched to 75 mN to relieve the passive elastic component of arterial tension. Small differences in cardiac phenotype and proximity from the heart across carotid sections thus likely confounded the measurement of active tension, and should be considered in future studies using this system.

In summary, ultrasound-mediated drug delivery is a promising strategy to improve the way drugs are delivered to diseased cardiovascular tissue. Backed with sound experimental evidence, this method relies on the ability of US to focus mechanical energy on target tissue beds to manipulate barrier properties, or actuate biochemical pathways leading to increased drug penetration into the cell or tissue of interest. Acoustic cavitation facilitates this drug delivery through mechanical effects and by releasing therapeutics from drug-loaded vesicles. Targeting methods, such as radiation force and biomolecular ligands, may be used in future studies to localize drug-loaded vesicles near the endothelium for efficient delivery. Sonoporation has been demonstrated as a means of cellular therapeutic delivery through ultrasound-induced pores. Delivery to the vascular wall and beyond relies on permeabilization of the endothelium, which can be enhanced by paracellular transport, transcytosis, or frank breach of the endothelial layer. Drug delivery to thrombi can be augmented by sonothrombolysis, which has been established as an effective method to recanalize
cerebral vessels in clinical trials [28, 31]. The mechanisms of ultrasound-mediated drug delivery outlined in this dissertation remain debated. However, future conclusions drawn from ongoing investigations will both add to the understanding of ultrasound-mediated cardiovascular drug delivery and improve the efficacy of treatments based on these concepts. The ability to provide localized efficacious treatment to vascular beds to reverse endothelial dysfunction, treat cardiomyopathy, or accelerate thrombolysis would markedly change the clinical approach to the treatment of cardiovascular disease and stroke.

6.2 Future Directions

Noninvasive strategies for ultrasound-triggered local therapeutic gas delivery for cerebral ischemic injury are being developed. As shown in Chapter 4, bioactive gases such as xenon and nitric oxide are promising neuroprotective agents [347] with minimal adverse effects due to rapid vascular scavenging by hemoglobin. The results of preliminary studies [232, 348] suggest that this bioactive gas, once released from a vesicle, provides neuroprotection for ischemic brain tissue quite efficiently. In the context of this dissertation, the technique to deliver nitric oxide to vascular tissue described in Chapter 4 may be extended to the study of bevacizumab delivery in Chapter 5. If the delivery of nitric oxide to local tissue beds also promotes increased vascular permeability, then it will be an attractive adjuvant to ultrasound-mediated drug delivery.

In Chapter 5, the encapsulation efficiency (≈ 25%), and resulting suboptimal immunoreactivity, may have slightly impeded the ability to observe bevacizumab penetration immunohistochemically. Currently, the efficiency of drug encapsulation techniques may limit the extent to which ultrasound-mediated drug delivery can elicit beneficial bioeffects in vivo as well. One promising strategy using a perfluoropentane (PFP) emulsion formulation encapsulated up to 300 µg/mL of doxorubicin drug, with an approximate 50% release profile once exposed to US [349] (Table 2.2). This release profile compares to the systemic pharmacodynamic doxorubicin concentration near 17 µg/mL [350]. Targeting approaches will likely increase drug concentration near
Nevertheless, future improvements in encapsulation techniques will augment ultrasound-mediated drug delivery substantially.

In the ultrasound-mediated drug delivery literature, there is a paucity of studies demonstrating drug transport from the lumen into medial tissue. In their studies of calcein delivery to porcine carotid tissue, Tiukinhoy-Laing et al. demonstrated strong calcein delivery to smooth muscle tissue, however the route of transport could not be determined due to the considerable presence of vasa vasorum within porcine carotid medial tissue [97]. Within these studies, drug penetration seems to have occurred through and between the endothelial cells of the vasa vasorum into smooth muscle cells, and not via direct penetration through luminal endothelium. The internal elastic lamina (IEL) likely obstructed this route, which, along with endothelium, is a significant barrier to drug transport [27]. The mechanical forces required to manipulate this vascular layer—rich in dense elastin fibers—may be vastly different than those exerted on the endothelium. The IEL is relatively impermeable even to water, except for small, sparsely distributed fenestrated pores on the order of 1 \( \mu \text{m} \) in diameter [351]. Future studies should investigate the specific mechanical interactions necessary to manipulate this barrier to increase the efficacy of ultrasound-mediated drug delivery.

Also in Chapter 5, a strategy to detect cavitation from bevacizumab-loaded echogenic liposomes nucleated by color-Doppler imaging pulses was implemented. Given the wide array of ongoing interdisciplinary studies on future ultrasound-mediated drug delivery research, from vascular biology to computational simulation of bubble dynamics, the development of techniques to facilitate successful clinical translation of ultrasound-mediated drug delivery is expected. For example, determination of optimal drug delivery schemes will spur a new US modality for theragnostic applications. Molecularly targeted, theragnostic agents can be used to image the functional extent of disease using US with high spatial sensitivity and specificity prior to clinical therapeutic intervention in a cost-effective manner. The ability to confirm disease status and trigger delivery of a therapeutic with the same agent would help ensure that only potentially responsive patients would be treated, which
in turn would streamline clinical trials of these agents. Patil et al. describe one such example to track flowing microbubbles accumulating along a vessel border with harmonic imaging [352]. In their study, three consecutive pulses (two inversion pulses, one radiation force pulse) were used to cancel linear tissue and bubble effects while pushing microbubbles to the perimeter of the lumen. Their results revealed an optimum frequency range for microbubble radiation force, which can be harnessed for future investigations. Hitchcock et al. [2] and Goertz et al. [353] demonstrated that for a fixed treatment period, the cavitation dose and thus beneficial bioeffect can be maximized by inserting quiescent periods in the US exposure. The quiescent periods were dependent on the vascular flow rate.

Real-time methods to monitor drug-delivery are also under investigation. MRI guidance has been critical to the clinical translation of US thermal ablation. It is expected that analogous advances will be made for ultrasound-mediated drug delivery. Passive cavitation imaging techniques have been developed to monitor microbubble-enhanced US thermal ablation [86, 87, 354]. These techniques have been applied to differentiate stable and inertial cavitation from contrast agents in flow [89, 355]. Furthermore, the image quality has been shown to be independent of the US insonation parameters [87]. Investigations to enhance and monitor drug-delivery efficacy will increase in frequency as investigators continue to reveal the proficiency of ultrasound-mediated drug delivery in treating cardiovascular pathology.

Finally, variability in the local cellular environment and the temporal dynamics of cavitation make individual responses to drug delivery therapy a challenge. In their investigation of cellular sonoporation in flow fields, Park et al. observed significant reduction in drug delivery in vitro when cells were cultured under shear stress (219). Throughout the human vasculature, cells are exposed to varying amounts of shear stress depending on pathology, patient age, and cardiovascular health. In vivo microbubble activity is also difficult to control, in part due to the use of polydisperse microbubble populations [356], acoustic pressure in situ, and vascular characteristics [357, 358]. Understanding the manner in which these factors affect the cellular and mechanical mechanisms of ultrasound-mediated drug delivery will be imperative to
successful treatment of cardiovascular disease.

Bibliography


[100] Sheikov N, McDannold N, Vykhodtseva N, Jolesz F, and Hynynen K. Cellular mechanisms of the blood-brain barrier opening induced by ultrasound in


Committee A. T. S. *Methods for specifying acoustic properties of tissue mimicking phantoms and objects.* American Institute of Ultrasound in Medicine, Laurel, MD, Jan 1995.


