



Bassel Rifai

Lady Margaret Hall

Supervisors

Constantin-C. Coussios, Yiannis Ventikos

Cavitation-Enhanced Delivery of Therapeutics to Solid Tumors

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Abstract

Poor drug penetration through tumor tissue has emerged as a fundamental obstacle to cancer therapy. The solid tumor microenvironment presents several physiological abnormalities which reduce the uptake of intravenously administered therapeutics, including leaky, irregularly spaced blood vessels, and a pressure gradient which resists transport of therapeutics from the bloodstream into the tumor. Because of these factors, a systemically administered anti-cancer agent is unlikely to reach 100% of cancer cells at therapeutic dosages, which is the efficacy required for curative treatment.

The goal of this project is to use high-intensity focused ultrasound (HIFU) to enhance drug delivery via phenomena associated with *acoustic cavitation*. ‘Cavitation’ is the formation, oscillation, and collapse of bubbles in a sound field, and can be broadly divided into two types: ‘inertial’ and ‘stable’. Inertial cavitation involves violent bubble collapse and is associated with phenomena such as heating, fluid jetting, and broadband noise emission. Stable cavitation occurs at lower pressure amplitudes, and can generate liquid microstreaming in the bubble vicinity. It is the combination of fluid jetting and microstreaming which it is attempted to explore, control, and apply to the drug delivery problem in solid tumors.

First, the potential for cavitation to enhance the convective transport of a model therapeutic into obstructed vasculature in a cell-free *in vitro* tumor model is evaluated. Transport is quantified using post-treatment image analysis of the distribution of a dye-labeled macromolecule, while cavitation activity is quantified by analyzing passively recorded acoustic emissions. The introduction of exogenous cavitation nuclei into the acoustic field is found to dramatically enhance both cavitation activity and convective transport. The strong correlation between inertial cavitation activity and drug delivery in this study suggested both a mechanism of action and the clinical potential for non-invasive treatment monitoring.

Next, a flexible and efficient method to simulate numerically the microstreaming fields instigated by cavitating microbubbles is developed. The technique is applied to the problem of quantifying convective transport of a scalar quantity in the vicinity of acoustically cavitating microbubbles of various initial radii subject to

a range of sonication parameters, yielding insight regarding treatment parameter choice.

Finally, *in vitro* and *in vivo* models are used to explore the effect of HIFU on delivery and expression of a biologically active adenovirus. The role of cavitation in improving the distribution of adenovirus in porous media is established, as well as the critical role of certain sonication parameters in sustaining cavitation activity *in vivo*. After identifying safe conditions for sonicating mice with subcutaneously grown human breast cancer tumors, it is shown that following intratumoral or intravenous co-injection of ultrasound contrast agents and adenovirus, both the distribution and expression of viral transgenes are enhanced in the presence of inertial cavitation.

This ultrasound-based drug delivery system has the potential to be applied in conjunction with a broad range of macromolecular therapeutics to augment their bioavailability for cancer treatment. In order to reach this objective, further developmental work is recommended, directed towards improving therapeutic transducer design, using transducer arrays for treatment monitoring and mapping, and continuing the development of functionalized monodisperse cavitation nuclei.

Statement of Originality

I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at the University of Oxford or any other educational institution, except where due acknowledgement is made in the thesis.

Any contribution made to the research by others, with whom I have worked at the University of Oxford or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

Bassel Rifai.

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ACRONYMS

AMG	Algebraic Multigrid
BSA	Bovine Serum Albumin
CDS	Central differencing scheme
CFD	Computational fluid dynamics
CFL	Courant-Friedrichs-Lewy
CGS	Conjugate gradient squared
DC	Duty cycle
EPR	Enhanced permeability and retention
FDM	Finite difference method
FFT	Fast Fourier Transform
FVM	Finite volume method
HIFU	High-intensity focused ultrasound
IFP	Interstitial fluid pressure
PAA	Polyacrylamide
PCD	Passive cavitation detector
PLIC	Piecewise Linear Interface Construction
PRF	Pulse repetition frequency
PRFP	Peak rarefactional focal pressure
PRP	Pulse repetition period
RANS	Reynolds-averaged Navier-Stokes
SLIC	Single Line Interface Construction
TMM	Tissue-mimicking material
UCA	Ultrasound Contrast Agent
UDS	Upwind differencing scheme
VOF	Volume-of-Fluid



MOTIVATION, PHYSIOLOGY & DISEASE

Cancer is a group of over one hundred diseases characterized by uncontrolled, invasive, and metastatic cellular growth. As a leading cause of death in the developed world, there is significant interest in its characterization and treatment. In this chapter, the pathophysiological basis of cancer, the medical options available to cancer patients, their advantages and limitations are explored. Particular attention is paid to the structure of the solid tumor microenvironment and the physical barriers to molecular transport because these are largely responsible for the shortcomings of many modern therapies. Finally, the potential for a new technique which employs high-intensity ultrasound to propel therapeutics into under-treated areas is considered.

Around 2500 BCE, a high priest and physician of the Old Kingdom of Ancient Egypt set down on papyrus a treatise detailing the best medical practices of the time. Eighty years ago, the papyrus reached the hands of an American archaeologist, who translated it into modern English for the first time. In doing so, he revealed the previously unknown sophistication of the history of the practice of medicine (Breasted, 1991).

The four-thousand, five-hundred year-old document consists of forty-eight medical case studies, progressing from examination to diagnosis and treatment in a

rational and scientific manner. It contains the first known description of certain anatomical details of the nervous system, and it correctly prescribes sutures for open wounds and immobilization for spinal cord injuries.

Case forty-five, however, is an anomaly, being the only one which offers no useful guidance. The case title: "Instructions concerning bulging tumors on the breast." Advice to the practicing physician: "There is no treatment" (Allen, 2005).

1.1 EPIDEMIOLOGY OF CANCER

The experience of cancer has always been terrible, but until the last century, its mark on society has been light. Cancer is a "modern" disease, with increased incidence rooted in two factors: exposure to carcinogens (tobacco and various industrial chemicals standing out as glaring examples), and increased longevity. That is, as humans live longer, they become prone to diseases that express themselves at an older age (the median age for breast cancer diagnosis is 61; for prostate cancer 67). It is therefore no surprise that, as life expectancy at birth in the developed world has risen from 47 at 1900 to 78 today, cancer in those countries has risen to become the second leading cause of death (in low-income countries, it is not in the top ten) (NCI, 2010b). Nor is it a surprise that funding for cancer research has risen exponentially since the "War on Cancer" began with the US National Cancer Act of 1971, and stood at £12 billion globally in 2004 (Eckhouse et al., 2008).

Prior to analyzing the prospects for treatment, it is worthwhile to explore the basic properties of cancer, a group of more than one hundred diseases characterized by uncontrolled, invasive, and metastatic cellular growth due to changes in the genetic information of cells.

Cells and tissues are complex systems with critical checkpoints to ensure nor-

mal growth, development, and function. Normally, the division, differentiation, and death of cells are carefully regulated. All cancers begin as a single cell that has lost control of its normal growth and replication processes (AICR, 2007). This loss of control can be inherited (5-10 per cent of cancers result directly from inheriting genes associated with cancer), or can involve damage accumulated over time to the genetic material as a result of endogenous factors (such as hormones, oxidative stress) or exogenous factors (such as tobacco smoke, radiation, infectious agents, and various industrial chemicals).

Apart from hematological cancers (such as leukemia), this loss of control results in a tumor or mass. Left untreated, the tumor will take one of two paths: if the tumor contains only *benign* cells, it will stop growing untreated. However, if the tumor contains *malignant* cells, the tumor will continue to grow, divide, and possibly metastasize (spread from the primary site to other parts of the body), recruiting new blood vessels and imposing an elevated metabolic burden in the the infiltrated tissue until the organ fails due to nutritional starvation (Hanahan and Weinberg, 2000).

Thus, there exists an interest in detecting and eliminating cancer cells before they compromise the functionality of the host tissue. Early detection, defined as the identification of a disease before symptoms appear, began with the Pap test for cervical cancer, which, since its adoption by the American Cancer Society in the 1960's, reduced cervical cancer death rates in the US by over 70% (ACS, 2010). Recent decades have seen the advent of new screening techniques, including immunohistochemical tests such as that for prostate cancer, and advanced imaging techniques, such as positron emission tomography (PET), magnetic resonance imaging (MRI), computed tomography (CT), and sonography (ultrasound), which promise to further advance the drive to early detection.

1.2 CANCER TREATMENT

Upon identification of a cancerous solid tumor, treatment revolves around two principles: first, that symptomatic relief and improved life expectancy are associated with removal of large volumes of tumor; and second, that a single viable cancer cell, if left behind, has the potential to develop into a new tumor. Thus, the following therapies, each possessing unique strengths and weaknesses, are often combined serially or in parallel, to maximize the likelihood of eradicating the tumor.

Surgery: Treatment of most solid tumors begins with surgical excision. However, because of the venous processes that spread from a tumor, removing the main lump is evidently no guarantee that the cancer is extracted in totality, nor is there a threshold on the thickness of the margin of healthy tissue removed which decreases recurrence or improves prognosis (this finding led to certain procedures, such as the radical mastectomy, falling out of favor) (Shapin, 2010).

Radiation Therapy: In certain types of cancer, including those of the brain, breast, and prostate, hyperproliferative cells have a diminished ability, compared with normal tissue, to repair DNA damage induced by bombardment with ionizing radiation. Therefore, the primary cancer can be treated by either irradiating using external sources (photon or charged particle beams) or internally embedded sources (such as brachytherapy seeds) (NCI, 2010c).

Chemotherapy: Upon maturity, the vast majority of the body's cells (with the exception of some, such as hair growth cells and intestinal lining cells) divide very rarely. One strategy that has been exploited to target fast-dividing cancer cells is to develop cytotoxic (cell-killing) agents which impair cell-division - of which there are over 90 in clinical use today. These drugs may be admin-

istered orally or intravenously, on their own or in combination, and may be preceeded or followed by surgery and/or radiotherapy. Chemotherapy shows highest success rates on 'young' tumors, where vasculature and rapid cell division are more uniform across the tumor volume (Straw et al., 1974). Mechanisms of resistance to chemotherapy in 'mature' tumors are described in further detail below.

Hormone Therapy: In certain cancer cells, such as those of the breast, prostate and uterus, steroid hormones are powerful drivers of gene expression, and modulating hormone levels can suppress or even reverse tumor growth. Most hormone therapies seek to block hormone signalling through disrupting the signal transduction cascade. This has been achieved by administering hormone synthesis inhibitors or hormone receptor antagonists.

Although there are other options currently employed or in clinical trials, including, for example, microwave and ultrasonic ablation, treatment of cancer broadly centers around the combination of surgery, radiation therapy, and chemotherapy - notoriously termed "slash, burn and poison," - which aims to take the body to the brink of collapse and back again, and hope the cancer does not survive. However, despite the evolution of a multimodal treatment approach, eradication of cancer has been elusive. In the 1970's, about one in two people diagnosed with cancer were not alive five years later. Today, that figure stands at one in three (NCI, 2010a), marking progress, but not the revolution hoped for during the development of these techniques.

This is because each of the treatments described above possess intrinsic or technological challenges. A major limitation behind surgery and ablation, for example, is that the cancerous tissue must be clearly identifiable to the physician for treat-

ment planning and post-treatment evaluation. While modern imaging techniques show improved sensitivity and accuracy, flagging individual cancer cells remains an elusive goal. For the time being, therefore, surgery and radiotherapy remain coarse tools which are most useful on centimeter-scale tumor masses. In fact, surgical resection is only indicated in 25% of cases.

Radiotherapy, meanwhile, has a fundamental limitation in solid tumors. The responsiveness of a cancer to radiation is quantified by its radiosensitivity. Critically, oxygen is a potent radiosensitizer (Gray et al., 1953). The presence of hypoxic cells in the tumor core has long been recognized, and there is a large body of evidence indicating that hypoxic cells are relatively resistant to radiation treatment and can repopulate the tumour after radiotherapy - a significant obstacle to the effectiveness of radiation therapy.

Finally, a fundamental challenge in both chemotherapy and more advanced targeted therapies is poor drug penetration through tumor tissue. In order for an anti-cancer agent to be effective, it must efficiently reach all target cancer cells at therapeutic levels. However, most anticancer drugs are toxic to both tumor and normal cells, so the intravenous dose delivered is limited by healthy tissue tolerances. Additionally, drug delivery in solid tumors is highly heterogeneous. These two factors may contribute to clinical recurrence of tumors following chemotherapy.

In the next section, the physiology of the solid tumor, the consequences to effective drug delivery, and modern techniques to overcome the biochemical and mechanical barriers posed by solid tumors are explored.

1.3 THE SOLID TUMOR MICROENVIRONMENT

The tumor can be divided into three compartments: the blood vessels, interstitium (the space between cells, composed of interstitial fluid and extracellular matrix), and cells (normal tissues also contain lymph vessels, but tumors with a functioning lymphatic system have not been observed). Their respective volume fractions within the tumor vary dramatically depending on, among other factors, the host tissue. Blood vessels, for example, comprise anywhere from 1% to 28% of the tumor (Jain, 1988), while interstitium accounts for 13-60% (Jain, 1987a).

Drug delivery to the tumor involves three steps: (1) convective transport through tumor vasculature, (2) extravasation from vasculature into the interstitial fluid, and (3) convection/diffusion through the tumor interstitium to all target cells. The timescales of these processes must compete with the timescale of drug clearance by other organs in the body, which can vary dramatically from molecule to molecule. Immunoglobulin G (IgG), for example, has a blood plasma half-life of 3-4 weeks, while the fragment antigen binding (Fab) region of an antibody has a half-life of several hours (Nguyen et al., 2006).

1.3.1 Vascular Transport

In order for a tissue to remain viable, each cell within the tissue must have access to a constant supply of oxygen and nutrients. In small organisms, diffusion and other passive transport mechanisms are sufficient to provide this supply. However, larger organisms have evolved a complex, highly structured network for the delivery of nutrients and removal of waste products over longer distances - the cardiovascular system. Driven by the heart, this system has the objective of delivering nutrients and removing waste products by *convection* for any distance longer than a several tens of micrometers. Convective transport is vital because of the low interstitial diffusiv-

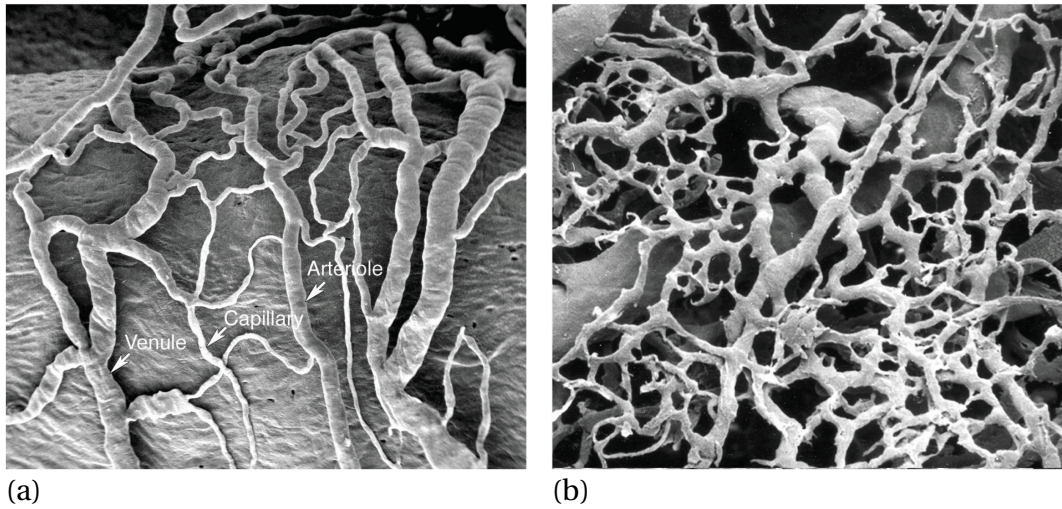


Figure 1.1: Scanning Electron Microscope (SEM) image of the polymer cast of **(a)** normal mouse vasculature and **(b)** xenograft of human tumor in nude mouse. Normal vasculature exhibits an organized progression, in which blood is fed from arterioles to capillaries to venules. Tumor microvasculature lacks organization, contains arterio-venous shunts, loops, and prematurely terminated (or ‘blunt-end’) vessels (McDonald and Choyke, 2003).

ity of many biologically important molecules. Thus, blood enters a normal organ through arteries, which branch into arterioles, then into capillaries, where metabolite exchange takes place. Capillaries then collect into venules, then veins, which send the blood back into circulation.

Only at the capillary bed level is diffusion an important contributor to solute transport. And even here, reliance on diffusion is mitigated by two factors: (1) most of the $\sim 10^{13}$ cells in the human body are within a few cell diameters of a blood vessel, and (2) a pressure gradient favors *extravasation*, or transcapillary transport, of solutes into the interstitium in healthy tissue (Minchinton and Tannock, 2006).

However, in solid cancers, homeostatic regulation of cell growth breaks down and cancer cells proliferate. The resulting metabolic stresses (such as low pH, low pO_2) induces the cells to release angiogenic molecules in order to recruit new blood

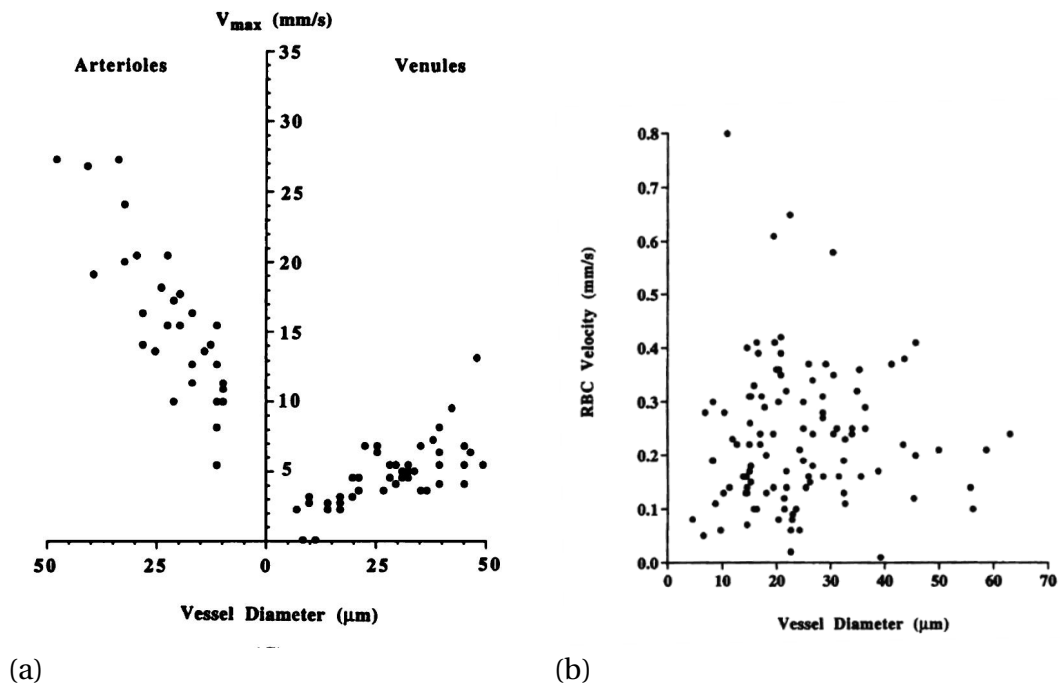


Figure 1.2: **(a)** Blood flow velocity in normal vasculature. Note the strong correlation between vessel diameter and velocity. **(b)** Blood flow velocity in tumor vasculature. Morphological and functional distinctions between arterioles and venules is reduced, and blood flow shows weak correlation with blood vessel diameter (Fan Yuan et al., 1994)

vessels. Perhaps due to an imbalance of angiogenic regulators, the resulting tumor vasculature is highly disorganized (Figure 1.1) (Kerbel, 2000). Branching patterns contain excessive loops and arteriolar-venous shunts, and are so irregular as to defy categorization (i.e. artery to capillary bed to vein), instead being collectively referred to as *tumor microvessels* (Trédan et al., 2007). The growth of cancer cells ‘outpaces’ blood vessel proliferation, and the rapid expansion of the tumor forces vessels apart, creating a population of cells distant from blood vessels, and compresses lymphatic ducts, resulting in insufficient draining of the interstitium (Minchinton and Tan-nock, 2006).

Because of these factors, blood flow in tumors is disorganized and variable. In

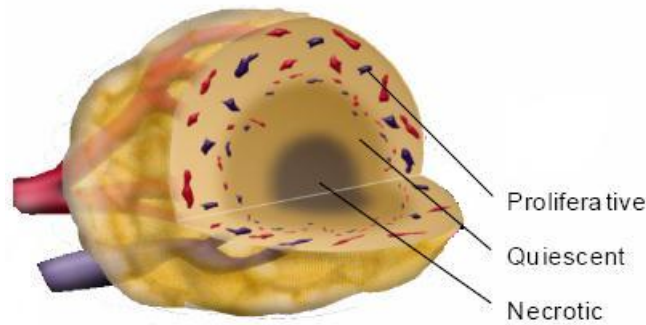


Figure 1.3: An idealized, spherical representation of the three regions of the solid tumor, corresponding to nutrient availability. (Helmlinger et al., 1997b) Clinically observed solid tumors may possess several such spheroids or alternate geometries, but the relationship between distance from cell to the nearest functioning blood vessel and cellular behavior (division, quiescence, or necrosis) is generally conserved.

normal vasculature, blood flow is proportional to vessel diameter and the pressure gradient between arterioles and venules, and inversely proportional to the geometric resistance in the vessel network (Figure 1.2). Tumor vasculature, however, exhibits a reduced pressure gradient (discussed below) and increased geometric resistance (due to the peculiar branching pattern of tumor vessels) (Baish and Jain, 2000). Additionally, vascular morphology is spatially and temporally heterogeneous. It has been observed that blood flows may accelerate, slow, or even reverse within the same tumor vessel at different time points.

Macroscopically, the growing tumor can be segmented into three regions, based on perfusion rates: an avascular, anoxic, necrotic region; a hypoxic, quiescent region; and a normoxic, hyperproliferative advancing front (Figure 1.3) (Endrich et al., 1979). Limited experimental data in humans has been consistent with the corresponding human tumor xenograft animal studies, demonstrating low perfusion in necrotic tissue, which gradually increases such that in the advancing front, perfusion rates are substantially higher than in adjacent healthy host tissue (Figure 1.4).

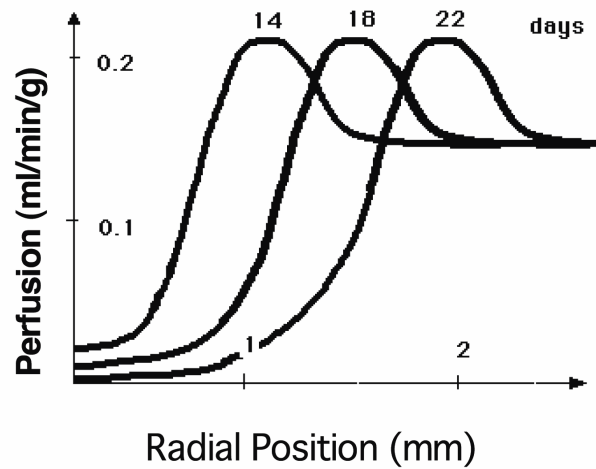


Figure 1.4: Tissue perfusion as a function of distance from tumor centroid in a canine lymphosarcoma. As the hyperproliferative region of a tumor expands with time, the necrotic core grows in its wake. Note that at all time points, the perfusion rate in the hyperproliferative region is higher than in the adjacent healthy tissue (Straw et al., 1974)

Is it therefore not surprising that there is a reduction in delivery and uptake of oxygen, nutrients, and therapeutic agents beneath the advancing front of the tumor, and that this effect becomes more pronounced as the tumor expands. Poor vascular organization (and a lack of functional lymphatics) also leads to the build-up of metabolic waste products, such as lactic acid, lowering interstitial pH, a phenomenon exacerbated by the existence of microvessels so narrow as to obstruct the passage of red blood cells (and the hemoglobin-bound oxygen they carry). These factors account for at least two mechanisms of tumor resistance to conventional chemotherapy: first, cells distant from blood vessels are exposed to lower concentrations of drugs, and second, many drugs – particularly protein-based therapeutics – have functionality which is highly sensitive to pH.

It should be noted, however, that tumor vessels are of two types: those recruited from the preexisting network of the host tissue, and those produced in response to cancer cell angiogenic factor release. The former are rarely infiltrated by the tumor,

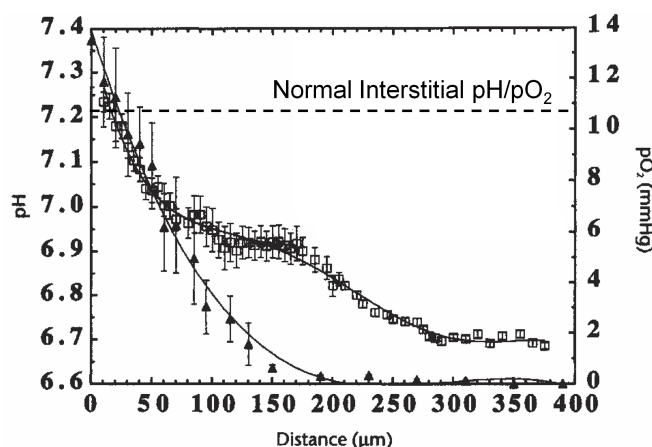


Figure 1.5: Mean interstitial pH and pO₂ profiles of a 27-day old tumor, with distance measured from the nearest blood vessel (Helmlinger et al., 1997b)

and so usually retain structural integrity, while the newly formed vessels tend to lack smooth muscle cells and thus respond differently to vasoactive agents (Jain, 1987a). The tumor response as a whole will therefore depend on the relative prevalence of the two types of vessels, which varies from one location within the tumor to another, and from one tumor to another.

Identifying compounds which improve tumor vasculature is an active area of research. VEGF Antagonist is one which has been shown to "normalize" blood vessels in the tumor (Tong et al., 2004). It may seem counterproductive to increase the flow of nutrients to the tumor, and thereby promote tumor growth, but conceptually speaking, normalizing the vasculature makes it more conducive to effective anti-cancer therapy. Additionally, vascular normalization reduces regions of hypoxia, augmenting the tumor response to radiation therapy (Tong et al., 2004). So far, much of the research is still pre-clinical, and has shown limited success by, for example, normalizing vasculature but only temporarily.

1.3.2 Extravasation

In order for molecules circulating within the bloodstream to reach cancerous cells, they must next cross the vessel wall. Fluid flux (J) from blood vessels to tissue interstitial fluid can be described by a modified form of Starling's law of filtration:

$$J = L_p S \Delta P \quad (1.1)$$

where L_p is the hydraulic conductivity of the capillary membrane, S is the surface area for exchange, and ΔP is the net pressure difference,

$$\Delta P = (P_{CAP} - P_{IF}) - \sigma(COP_{CAP} - COP_{IF}). \quad (1.2)$$

P_{CAP} and P_{IF} are the hydrostatic pressures in the capillaries and interstitium, respectively, COP_{CAP} and COP_{IF} are the colloid osmotic pressures of the capillaries and interstitium, respectively, and σ is the plasma protein reflection coefficient, which varies between zero and one depending on the permeability of the microvessel wall (a value of one corresponds to total impermeability of the vessel wall to the solute. Most plasma proteins have σ between 0.7 and 0.9) (Staverman, 1951). In human capillaries, the osmotic pressure gradient is about 20 mm Hg, favoring fluid retention in the capillaries. This is countered by a hydrostatic pressure gradient of approximately 23 mm Hg, resulting in a net outward filtration pressure from the capillaries into the surrounding fluid (Heldin et al., 2004).

This law is equally valid in tumor tissues and regular tissues. However, there are two unique features that must be accounted for in transvascular transport in tumor tissue.

First, microvascular permeability is, on average higher in tumor vessels than healthy ones. Healthy capillary walls consist of three complete concentric layers: the glycocalyx, the endothelium, and the basement membrane (Figure 1.6), which

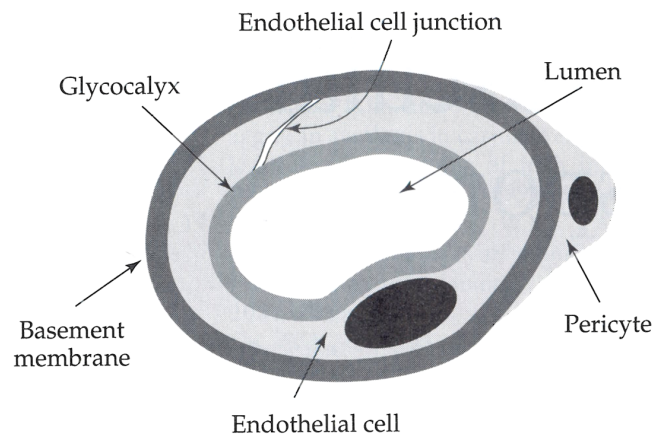


Figure 1.6: Structures of the normal microvessel wall, The glycocalyx is negatively charged, and hypothesized to be a molecular sieve that opposes the transport of macromolecules. The endothelial cell resists the transport of hydrophilic solutes, but contains many 50-70nm vesicles involved in transendothelial transport. The endothelial cell junction is formed by protein assemblies, and contains molecular transport channels. The basement membrane is a fiber-matrix layer containing proteoglycans, glycoproteins, and other structural components. (Truskey et al., 2004)

form a complex system for regulating the passage of molecules both passively (e.g. via a sieve-like extracellular matrix) and actively (e.g. via vesicle-mediated transport). Tumor microvessels, in contrast, often exhibit fenestrated endothelial cells (Figure 1.7) as well as incomplete basement membranes. The effect of these abnormalities is to raise the cutoff particle size to values as high as $2\ \mu\text{m}$ (compared to 20 nm for healthy tissue) (Figure 1.8) (Truskey et al., 2004).

An advantage of the leakiness of tumor vasculature is that therapeutic agents between 20 nm and $2\ \mu\text{m}$ in size will preferentially accumulate in the tumor, a phenomenon known as enhanced permeability and retention (EPR) (Maeda et al., 2000). Exploiting the EPR effect for drug targeting is an active area of research.

The second feature particular to tumor transvascular transport is that convection across the microvessel wall is negligible at the tumor core. This is a direct consequence of the increase in interstitial fluid pressure (IFP) in most tumors. The eti-

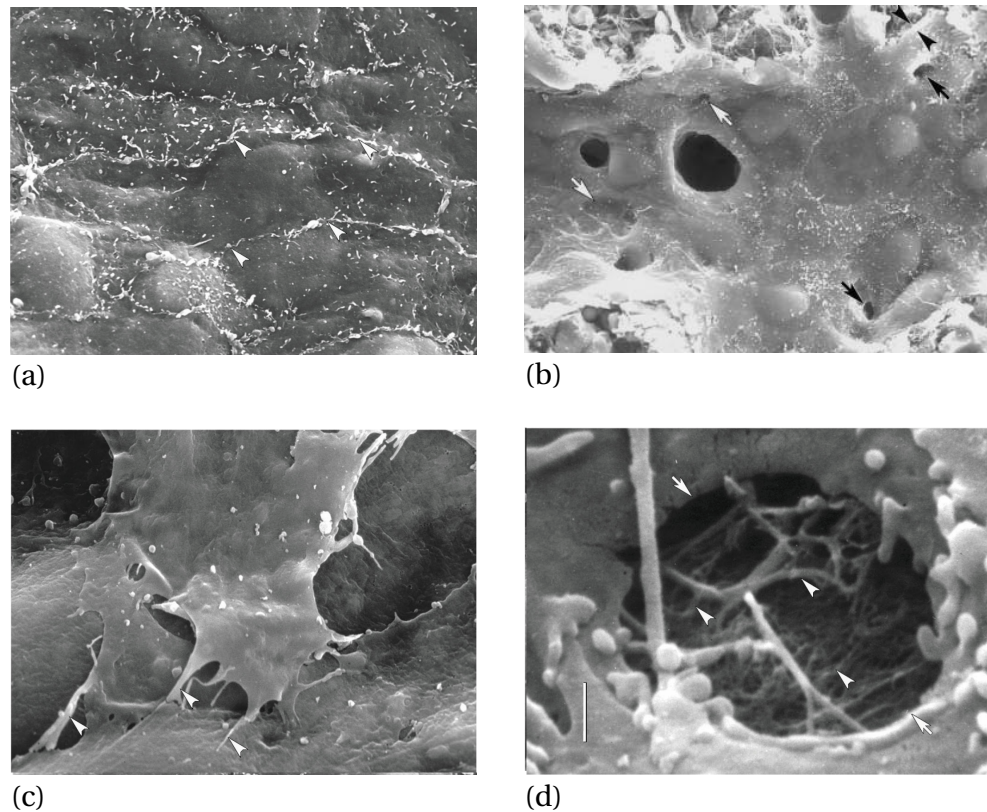


Figure 1.7: SEM images comparing luminal surface of a **(a)** normal blood vessel in a mouse mammary gland, containing smooth endothelial junctions (arrowheads), with **(b)** tumor vessel of a mouse mammary carcinoma, the well-defined lining layer (arrowheads) of which contains scattered defects (arrows), including fenestrated capillaries, overlapping endothelial cells, **(c)** cellular processes (arrowheads), and other abnormalities. **(d)** Magnification of endothelial gap (arrows) revealing basement membrane filaments (arrowheads). Scale bar in d applies to all panels (5 μm in (a) and (b); 2 μm in (c); 0.5 μm in (d)) (McDonald and Choyke, 2003)

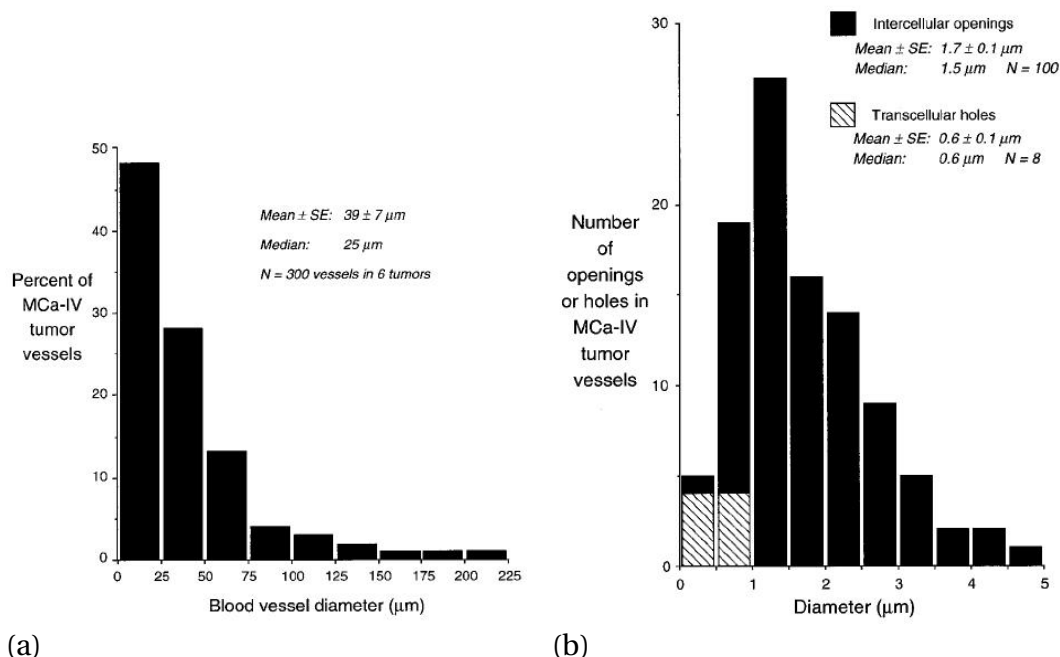


Figure 1.8: Blood vessel characteristics in MCa-IV, a mouse mammary carcinoma, that is known to have blood vessels on the high end of the range of leakiness for tumors, as evidenced by a functional pore size of 1200 to 2000 nm. **(a)** Size distribution of vessels. **(b)** Size distribution of intercellular openings and transcellular holes in lining cells in blood vessels (Hashizume et al., 2000)

ology of elevated IFP during early stages of tumor growth includes defects in the vessel wall (such as fenestrations, discontinuous basement membranes, absence of smooth muscle cells) as well as lymph vessel abnormalities (Boucher et al., 1996). More mature tumors exhibit elevated IFP due to the solid stresses generated by cell proliferation in a constrained space, and the increased blood flow resistance when this proliferation compresses blood vessels (Figure 1.9) (Helmlinger et al., 1997a). Increased IFP reduces the transmural pressure gradients, lowering convection across tumor vessel walls, thus compromising the transport of macromolecules (Figure 1.10).

The problem is compounded by transmural coupling between IFP and microvascular pressure due to high permeability of tumor vessels. That is, the pulsatile os-

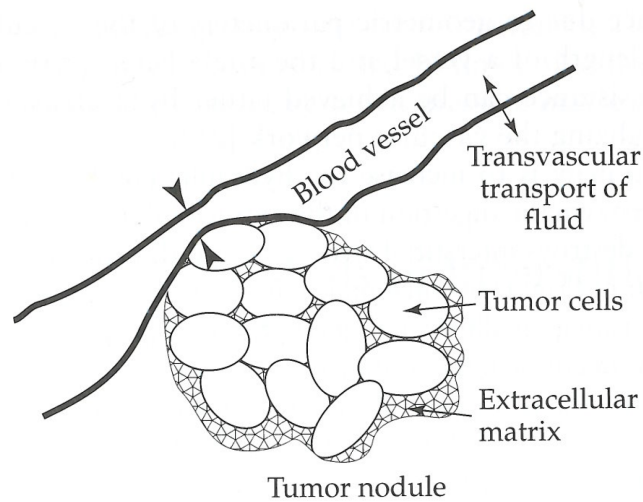


Figure 1.9: Proposed mechanism of Interstitial Fluid Pressure elevation. Tumor cell proliferation exerts stress on a nearby blood vessel, reducing its cross-sectional area and increasing resistance to blood flow (Yuan, 1997).

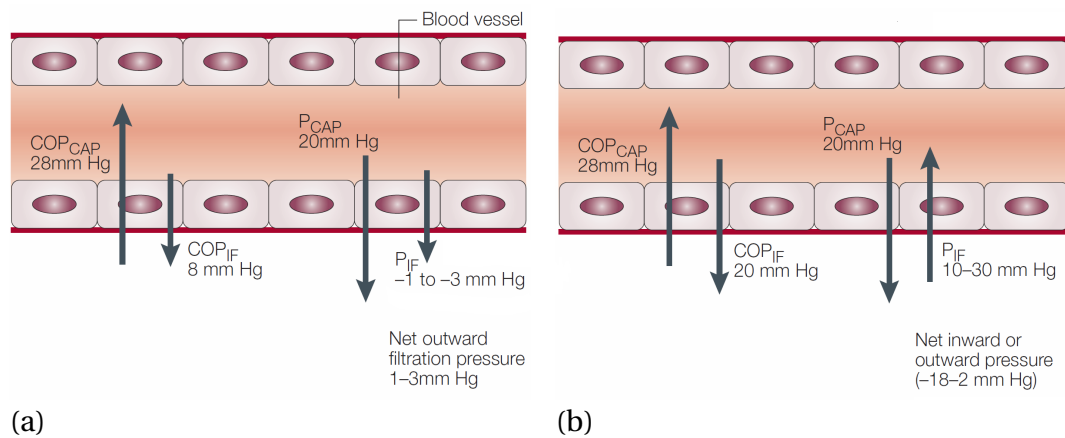


Figure 1.10: Transcapillary transport in normal tissues **(a)** and tumour tissues **(b)**. In normal human capillaries, colloid osmotic pressure COP_{CAP} is 28 mmHg, favoring fluid retention. This is opposed by a hydrostatic capillary pressure difference $P_{CAP} - P_{IF}$ of 21-23 mmHg and an interstitial colloid osmotic pressure COP_{IF} of 8 mmHg, resulting in a net outward filtration pressure from the capillaries in tissues of 1-3 mmHg. It is this pressure which encourages extravasation. Tumour tissues exhibit elevated COP_{IF} and P_{IF} , with the latter outweighing the former, resulting in a net *inward* pressure as high as 18 mmHg. (Heldin et al., 2004)

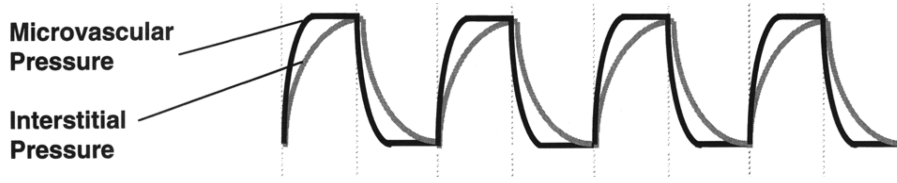


Figure 1.11: Interstitial response to cyclical changes in microvascular pressure (MVP). Quantitative studies have shown the time delay between microvascular pressure and IFP to be approximately 10 seconds, and that the integrated difference between MVP and IFP is negligible across an entire cycle, resulting in zero net fluid exchange

cillations in pressure are completely transmitted to the interstitial fluid (with an experimentally observed 10 second delay). As a result, extravasation during systole is counteracted by intravasation during diastole (Figure 1.11) (Netti et al., 1995). Additionally, transmural pressure coupling can lead to blood flow stasis in tumors, even without occluding the vessels (Jain, 2005). The extent of the increase in IFP for various cancer types is summarized in Figure 1.12(a).

1.3.3 Interstitial Transport

Once a molecule *has* extravasated, its movement through the interstitial space occurs by diffusion and convection. For neutral molecules in dilute systems, the transport of solutes, $\mathbf{N}$, is given by:

$$\mathbf{N} = -D_{\text{eff}} \nabla C + \mathbf{v}_f f C \quad (1.3)$$

Where D_{eff} is the effective diffusivity of the solute, $\mathbf{v}_f$ is the interstitial fluid velocity, f is the retardation coefficient (the ratio of convective velocity of solutes to interstitial fluid), and C is the concentration of solute.

The physicochemical properties of a molecule (for example, molecular weight, shape, charge and aqueous solubility) and the structure and composition of the interstitium determine the values for D_{eff} and f . Extensive experimental work has

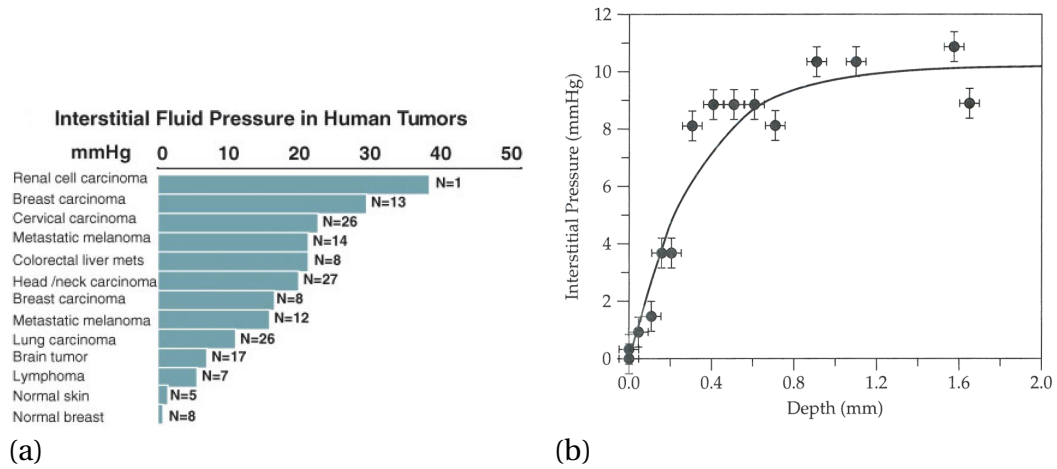


Figure 1.12: **(a)** Leaky vessel walls and lack of functional lymphatics contribute to increased IFP in human tumors (Fukumura and Jain, 2007b); **(b)** IFP drops sharply at the periphery of a tumor, resulting in oozing of interstitial fluid outward from the tumor center (Jain, 2005)

determined that these values are in fact not so different between healthy and tumor tissue (Jain, 1996). What, then, accounts for the non-uniformity of intravenously delivered macromolecules in the tumor? In fact, both diffusive and convective phenomena are at play.

Given a diffusivity of D_{eff} , the time constant τ for a molecule to diffuse a distance L is approximately $L^2/4D_{\text{eff}}$ (Truskey et al., 2004). For a typical macromolecule such as the aforementioned IgG, τ takes on a value of 1 hour for a 100 μm distance, several days for a 1 mm distance, and several months for a 1 cm distance. Such long interstitial travel distances exist only in tumors. Thus, for a 1 mm tumor, diffusional delivery would take days, and for a 1cm tumor, months. Diffusion can further be retarded by molecule-specific interactions with the extracellular matrix or other components of the interstitium. And of course the penetration depth will be determined by the balance between delivery and consumption. Cellular metabolism reduces drug penetration and build-up within the tissue, while binding and sequestration increase net drug levels within a tissue, but limit its penetration and activity.

This problem is not diminished by convective transport in the interstitium. As previously discussed, the tumor interior exhibits high interstitial fluid pressure. Moreover, this uniformly high pressure drops precipitously to normal values in the tumor margin (Figure 1.12(b)). This leads to fluid motion from the tumor's periphery into surrounding tissue. Experiments using *ex vivo* tumor tissue have estimated the outward-radiating fluid velocity to be 0.1-0.2 $\mu\text{m/s}$. It is hypothesized that the fluid velocity is an order of magnitude lower *in vivo* (Jain, 1987b). It is estimated that, where normal tissue would drain extravasated blood plasma through the venous and lymphatic system, 6-14% of plasma entering the tumor exits through the periphery (Jain, 1989), carrying macromolecules along with it. So in order to diffuse into a tumor, a macromolecule must overcome the convective flow.

Between the counterproductive convective transport, large diffusion distances, and binding of drugs to components of the interstitium, it is unsurprising that interstitial transport is one of the most difficult steps in drug delivery, particularly for large therapeutic agents.

Theoretical studies have suggested, however, that vascular spacing - not interstitial fluid flow - is chiefly responsible for the heterogeneous distribution of macromolecules in tumors, though of course the two are closely related pathophysiologically (Baxter and Jain, 1989).

1.3.4 Implications for Treatment

Based on the information above, the obstacles to the treatment of solid tumors can be understood. The effectiveness of anti-cancer agents depends upon their ability to access cells capable of regenerating a tumor (tumor stem cells). For a treatment to be curative, it must reach all such cells. Thus, any discussion about the effectiveness of proposed therapeutics must be couched in a measure of its capacity to reach

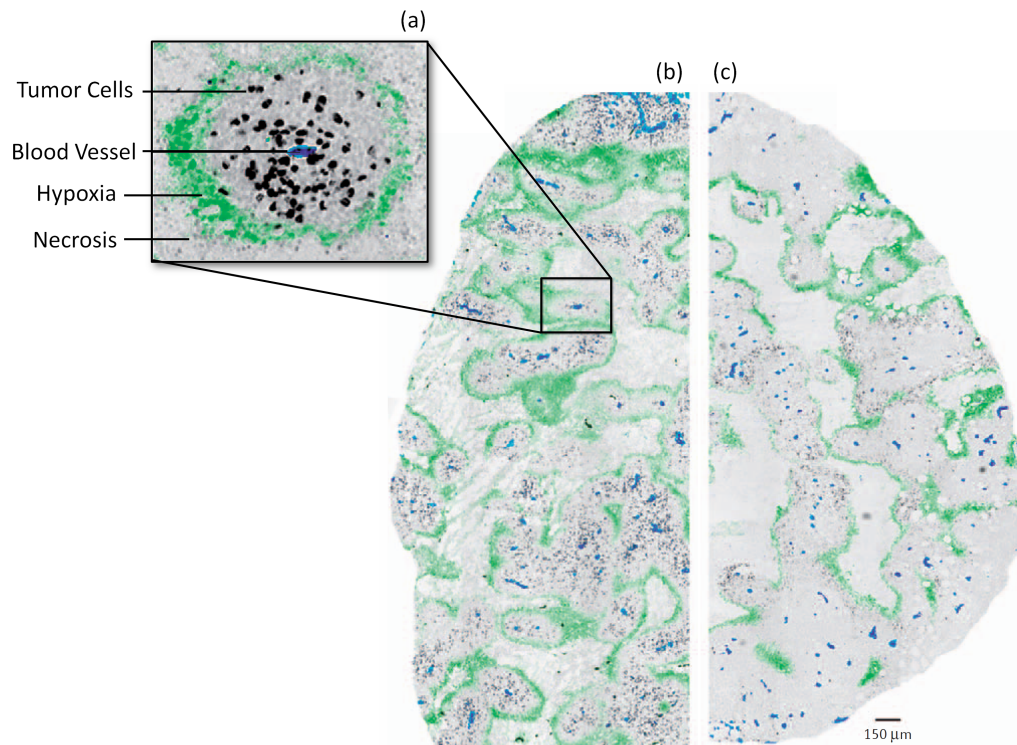


Figure 1.13: Histology of a human colorectal cancer xenograft. **(a)** Cells surrounding a blood vessel. S-phase (proliferating) cells appear in black, hypoxic cells in green, and blood vessel endothelial cells in blue. **(b)** In untreated tumor, S-phase cells are distributed around blood vessels. **(c)** 48 hours after treatment with gemcitabine, proliferation is suppressed proximal to blood vessels. However, it persists more distally to blood vessels, indicating that therapeutic levels of the drug were not attained throughout the replicating regions of the tumor (Minchinton and Tannock, 2006).

the tumor via the vasculature, extravasate into the tumor interstitium, and achieve therapeutic levels (via some form of transport - passive or otherwise) across all areas of the tumor.

The example of treating a human colorectal cancer xenograft with gemcitabine can be used to obtain an estimate of the transport properties of typical cancer drugs. Gemcitabine belongs to a class of chemotherapeutics called nucleoside analogs, which, when incorporated into DNA during the cell division process, prematurely terminate DNA replication and so induce cell death.

Forty-eight hours after intravenous injection into a mouse model, histological samples of the tumor volume reveal four distinct populations of cells, surrounding each blood vessel like the layers of an onion. First, cells nearest the blood vessels received sufficient nutrition to divide, and a therapeutically sufficient dose of gemcitabine, and so apoptosed during division. Progressing further from the blood vessel, cells received sufficient nutrition to divide, but *not* enough gemcitabine, so continued to divide. Beyond those existed hypoxic cells - that is, cells receiving insufficient nutrition to warrant division, but enough nutrition to survive in a quiescent state. Finally, furthest away from the blood vessel, cells received insufficient nutrition to survive, leading to necrosis (Figure 1.13) (Huxham et al., 2004).

This is an important example because the middle two populations of cells pose two very distinct challenges: one needs to be treated with a higher dose of chemotherapy, and the other, even more distal from the vessels, requires a therapeutic which operates on hypoxic, non-cycling cells.

This realization has given rise to a new strategy: complementing traditional chemotherapeutics with hypoxia-selective cytotoxins - that is, drugs whose cytotoxicity is activated in the low-pH, low oxygen environment in the tumor interior, and which target molecular pathways critical to cell survival in such environments. Specifically killing hypoxic cells has greater therapeutic potential than 'normalizing' vasculature to render hypoxic cells vulnerable to conventional radiation and chemotherapy, because the cytotoxin would be tumor-specific (because hypoxia is only found in tumors), and completely complementary to traditional treatments (Brown and Giaccia, 1998). The concept is illustrated in Figure 1.14.

Of course, the effectiveness of such a technique continues to depend on the ability of the hypoxic-selective cytotoxin to reach the target cells at sufficient concentration. And so, as often happens, such treatments fall short of the promise which

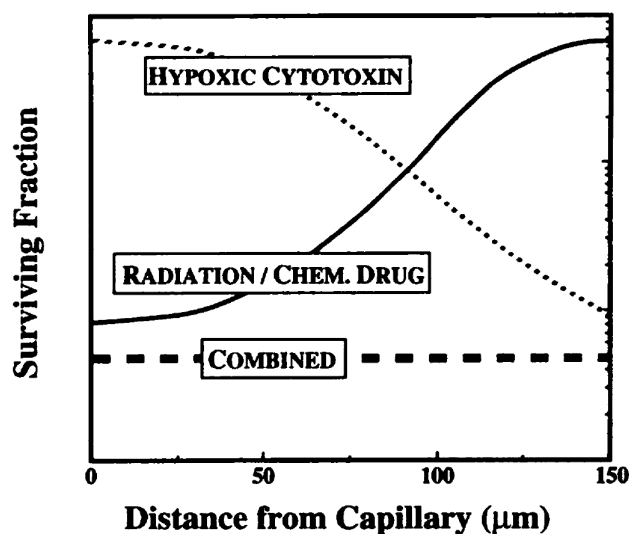


Figure 1.14: Conceptual treatment plan for tumors containing hypoxic regions. Radiation/chemotherapy alone would only kill cells proximal to blood vessels, while hypoxic cytotoxins would only kill cells distal to blood vessels. The combination results in low overall surviving fraction (Brown and Siim, 1996).

their specificity and potency suggest, because most research into the resistance of cancers to treatment has concentrated on molecular mechanisms of resistance, and not on drug delivery.

The birth of molecular medicine has both mitigated and exacerbated this problem. On the positive side, the search for a "silver bullet" cancer cure has given way to the realization that tumors are as varied as the tissues and circumstances which give rise to them. Sophisticated methods of characterizing tumors by their metabolic processes have yielded therapies which are highly selective for cancer cells, and largely avoid the deleterious side effects associated with systemic toxicity of first-generation chemotherapeutics. However, therapeutic particles have grown in size and complexity, often including targeting, activating, and imaging moieties in the 100 nm - 4 μm size range (Emelianov, 2008).

In all these, drug delivery has been neglected in favor of the conceptual ap-

peal of such therapies. This is perhaps unsurprising, given the drug discovery process, which often begins with the screening of drug candidates by applying them to single-layer cell cultures, where drug transport is immaterial.

1.4 ENHANCED TARGETING

Bridging the technological void between drug discovery and drug delivery has generally taken the form of injecting an inactive form of the drug into the bloodstream, and converting the drug to the active form at the target tissue only. This allows for increased injection dose, since the median toxic dose of an inactive drug is ordinarily higher than its active counterpart. Increased injection dose yields elevated drug concentration in the functioning tumor vasculature and surrounding tissue, potentially improving treatment and prognosis. Moreover, from a clinical perspective, localized activation and reduced systemic toxicity raise the effective *therapeutic index* of a drug, easing dose titration.

Localized activation may be achieved biochemically (as in the case of redox-activated chemotherapeutic prodrugs), or via extracorporeal devices. For example, intravenously injected metallic nanoparticles naturally accumulate in the tumor via the EPR effect. External laser sources, excited at the appropriate frequency, have been shown to selectively deposit energy in tissue containing the nanoparticles, leading to localized thermal ablation (Larson et al., 2007).

In other work, cytotoxic anti-cancer agents have been encapsulated into thermally sensitive liposomes and injected intravenously, where their activity is shielded as the liposomes accumulate in the tumor via EPR. Therapeutic ultrasound, generated from an external device and focused onto the tumor tissue, results in heat deposition, which has been shown to rupture the liposomes, causing them to release

their cargo (Mylonopoulou et al., 2010; Frenkel et al., 2006).

These techniques show particular promise where drug levels in the tumor taper due to cellular consumption. Their application would be well-complemented by a technology to transport therapeutics to less well-perfused areas.

1.5 ULTRASOUND-ENHANCED DRUG DELIVERY

High-intensity ultrasound can produce, in addition to its thermal effects, various *mechanical* effects relevant to the problem of delivering therapeutics into solid tumors. In particular, ultrasound can trigger - within a well-defined focal volume - the formation, oscillation, and collapse of microbubbles. Ultrasound-driven microbubble excitation - or *acoustic cavitation*, as it is known - is capable of an astounding range of bioeffects.

In the following chapter, the nature of acoustic cavitation and its known biomedical applications are explored. In subsequent chapters, original work on exploiting cavitation and its associated phenomena for the enhanced convective delivery of therapeutics within the solid tumor is presented.



BIOMEDICAL ACOUSTICS

Therapeutic ultrasound has shown promise in a diverse range of biomedical applications, from thermal ablation to thrombolysis. In many cases, cavitation has been implicated as a critical element responsible for the observed bioeffects. In this chapter, ultrasound wave propagation through the body, the circumstances under which cavitation can occur, and the variety of behaviors of cavitating microbubbles which underlie their therapeutic versatility are explored. Methods for the detection and post-processing of acoustic emissions radiated by cavitating microbubbles are investigated in order to enable monitoring of bubble activity. Finally, the therapeutic applications of ultrasound are presented, with an eye towards applying the insights from previous work towards the enhanced convective delivery of therapeutics within the solid tumor.

Biomedical ultrasound is rapidly emerging as a therapeutic tool, with extensive research activity aimed at both characterizing and controlling ultrasonically mediated bioeffects. At one clinical extreme, sound waves of amplitudes in the tens of megapascals impinge upon a millimeter-sized kidney stone, shattering it into pieces (Sturtevant, 1989). If the acoustic intensity is reduced a thousand-fold, there exists a regime gentle enough to reversibly open nanometer-sized gap junctions in the blood-brain barrier for the passage of large molecules, without any collateral damage (McDannold et al., 2006). To understand the design of various therapeutic ultrasound regimens and to identify critical treatment parameters, the physics of

ultrasound are explored hereafter, beginning with the fundamentals of linear sound wave propagation.

2.1 BASICS OF LINEAR SOUND WAVE PROPAGATION

Sound is a mechanical wave consisting of density variations in an elastic medium, propagating away from a source (Leighton, 1994). Density variations are propagated by oscillation of the constituent molecules (or particles) of the medium parallel to the direction of the wave.

Ultrasound is such a waveform oscillating at a frequency above that of the range of human hearing (20 kHz). The simplest description of ultrasonic interaction with a material is in terms of small amplitude waves that can be approximated by linear theory. By ignoring nonlinear terms in the equation of continuity, the momentum conservation equation, and the equation of state, one can obtain the linearized equation for propagation of an ultrasound wave, shown here in the one-dimensional case for simplicity:

$$\frac{\partial^2 p(x, t)}{\partial x^2} - \frac{1}{c^2} \frac{\partial^2 p(x, t)}{\partial t^2} = 0 \quad (2.1)$$

where $p(x, t)$ is the pressure disturbance, x is the spatial dimension, t is time, and c is the wave speed.

Equation (2.1) requires that fluid particle velocities are much lower than the speed of sound in the liquid and that pressure and density fluctuations are negligible compared to the equilibrium pressure and density (Morse and Ingard, 1987). Therefore, it is only valid for low-amplitude excitation. In such circumstances, the linearity of the solution is preserved - that is, the shape of the waveform is unchanged as it travels - even if the waveform amplitude is decreased by attenuation (or, in the case of non-planar waves, geometric spreading).

As an ultrasound wave travels, it is attenuated due to the combination of viscous dissipation and scattering arising from tissue inhomogeneities. Solutions to Equation (2.1) therefore are generally of the form:

$$p(x, t) = \Re\{P_o e^{i(\omega t - kx)} e^{-\alpha x}\} \quad (2.2)$$

where $c = \omega/k$, ω is the angular frequency of the wave, k is the wavenumber, and α is the amplitude attenuation constant. The frequency dependence of α generally takes the form $\alpha = \alpha_o (f/f_o)^\eta$, where α_o is the attenuation coefficient at f_o , $\eta = 2$ for water and $\eta = 1.1$ for many tissues of interest (Liu et al., 2006). Thus, higher frequencies are more readily attenuated than lower ones. From a clinical perspective, generation of high frequency components - and their subsequent viscous absorption - effectively converts acoustic energy into thermal energy, resulting in elevated heat deposition, which may cause unwanted tissue damage. Of course, while heating may be an undesirable side effect in certain circumstances, it has applications in the non-invasive thermal ablation of deep-seated cancerous tumors (Coussios and Roy, 2008). In this context, selecting an appropriate frequency inevitably leads to a compromise, since higher frequencies, though readily absorbed at the focus, are significantly attenuated along the propagation path, cause ‘prefocal’ damage (ter Haar and Coussios, 2007).

However, like many forms of therapeutic ultrasound, ablative ultrasound employs pressure amplitudes in excess of 1MPa, where linear theory ceases to represent physical reality because of the emergence of two nonlinear effects: distortion of the propagating sound wave due to non-linear propagation, and cavitation (Muir and Carstensen, 1980) (Starritt et al., 1985) (Khokhlova et al., 2001).

2.2 NONLINEAR SOUND WAVE PROPAGATION

In the limit of small P_o in Equation (2.2), the particle velocity is negligible relative to the wave velocity throughout the acoustic cycle. Under the approximation $v \ll c$, the entire wave can be approximated as travelling at the same bulk speed c . However, as P_o increases, two important physical phenomena begin to emerge. In the regime where v is no longer much smaller than c , particles in the compressive region of the wave travel at $c + v$, while particles in the rarefactive region travel at $c - v$. Additionally, the compressed region exhibits elevated bulk modulus and stiffness, and therefore speed of sound. These effects synergistically cause the pressure peak to travel faster than the pressure trough, distorting the travelling wave towards a sawtooth shape (Muir and Carstensen, 1980).

The extent to which a linear wave becomes distorted depends on the coefficient of non-linearity B/A , which is a material-specific property. Distortion increases as amplitude, frequency, and distance of propagation increase (Hamilton and Blackstock, 1998). Frequency-domain analysis demonstrates that wave distortion transfers energy from the fundamental frequency to its harmonics. In light of the frequency dependence of the absorption coefficient α , distortion enhances wave attenuation, which in tissues is a power law of frequency (Goss et al., 1978, 1980).

Returning to the example of non-invasive thermal ablation, the nonlinear phenomenon described above mitigates the correlation between focal heating and pre-focal damage in the following manner: acoustic excitation can be generated by a focused ultrasound transducer at lower frequencies than those typically required to generate ablative heating (1-10 MHz), resulting in reduced attenuation as the wave propagates through tissue. Only as the travelling wave converges within the focal volume does the pressure rise steeply and the wave distort, resulting in the genera-

tion of harmonics and the enhancement of absorption. Thus, apart from geometric focusing which can be used to guide heat deposition to the volume of interest, distortion of the propagating sound wave can improve the focusing of the resulting heat deposition compared to the acoustic intensity profile.

Yet of the two nonlinear effects that emerge at high values of P_o , it is not wave distortion, but *cavitation*, that most efficiently converts energy to higher frequencies, and in fact gives rise to a range of other thermal and mechanical mechanisms with diverse clinical applications.

2.3 CAVITATION INCEPTION

The term ‘cavitation’ loosely refers to the dynamic response of a bubble subjected to an external stress. This encompasses nucleation (bubble formation), bubble oscillation, and bubble collapse, under a variety of actuation mechanisms. The discussion below is however limited to acoustically driven cavitation, and the nucleation process is treated separately from the oscillation and collapse processes, despite their intrinsic interdependence (Brennen, 1995).

Cavitation has been implicated in a wide range of bioeffects associated with therapeutic ultrasound, including thermal necrosis (Kennedy, 2005; Leslie and Kennedy, 2007; Coussios et al., 2007), mechanical tissue homogenization (histotripsy) (Roberts et al., 2006; Xu et al., 2007), release of encapsulated therapeutics (sonodynamic therapy) (Kinoshita and Hynynen, 2006; Mylonopoulou et al., 2010), reversible opening of the blood-brain barrier (Hynynen et al., 2005; McDaniel et al., 2006), reversible permeabilization of the cell membrane (sonoporation) (Ohl et al., 2006a), and the dissolution of blood clots (thrombolysis) (Datta et al., 2006; Everbach and Francis, 2000). Each of these examples exploits a specific physi-

cal phenomenon associated with cavitation, such as viscous heating, fluid jetting or streaming in the vicinity of microbubbles.

The relative prominence of these phenomena depend, among other things, on the acoustic excitation parameters and on the pre-existence or absence of cavitation nucleation sites (Tho et al., 2007; Elder, 1959).

2.3.1 Homogeneous Nucleation

In the absence of a pre-existing nucleation site, statistical thermodynamics predict the spontaneous formation of bubbles. In a pure liquid, surface tension is the macroscopic manifestation of the intermolecular forces that tend to hold molecules together. The net energy, W_{CR} , that must be deposited in a liquid to form a bubble of radius R_C is

$$W_{CR} = \frac{4}{3}\pi R_C^2 \sigma \quad (2.3)$$

$$= \frac{16\pi\sigma^3}{3(\Delta p_C)^2} \quad (2.4)$$

where σ is the surface tension. Such bubble formation is a stochastic process whose behavior is governed by the probability that the thermal motions of the molecules would lead to a local energy perturbation of magnitude W_{CR} . For a bubble of this size to grow, the incident negative pressure needs to overcome the excess pressure inside the bubble which results from surface tension, $\Delta p_C = 2\sigma/R$. This quantity is essentially the tensile strength of the liquid at its ‘weakest’ point (the location of the bubble). Expansion of such a bubble is known as homogeneous nucleation. At physiologically relevant conditions, the size of stochastically generated cavities is on the order of Angstroms, yielding tensile strengths of ~ 10 MPa, and their emergence is both unpredictable and ephemeral.

2.3.2 Endogenous Nuclei

Cavitation *has* been observed at more modest pressure amplitudes than those required for spontaneous homogenous nucleation (ter Haar and Daniels, 1981; Holland et al., 1996), suggesting the pre-existence of bubble nuclei in tissue, attributable to a variety of mechanisms, including the crevice model (Harvey et al., 1944; Apfel, 1970; Crum, 1982), the skin surface model (Yount et al., 1984), spontaneous nuclear fission (Walder and Evans, 1974), cosmic radiation (Sette and Wanderlingh, 1962), and tribonucleation (Ikels, 1970).

From a clinical perspective, however, *in vivo* conditions such as density, perfusion, and dissolved oxygen vary significantly between subjects, tissues, and even within the same tissue (particularly - as described in Section §1.3.1 - in disease states such as the solid tumor), and little work has been done to explore or account for the pre-existence of nuclei and resulting nucleation threshold as a function of these variables (Rapoport et al., 2007).

2.3.3 Exogenous Nuclei

An alternative is to *introduce* nuclei into the physiological environment, which increases the reproducibility of cavitation activity at intensities below those required for heterogenous nucleation and irreversible tissue damage. In a clinical setting, this is usually achieved via intravenous injection.

This technique has its roots in contrast-enhanced ultrasound, a diagnostic procedure in which gas-filled microbubbles are injected into the bloodstream, where they scatter ultrasound pulses emitted by a diagnostic transducer array. The reflections, detected by the same transducer array and digitized, can be processed in increasingly sophisticated ways to yield high-contrast images of vasculature, perfu-

sion, and even blood flow velocities in the heart and other organs. Because microbubbles exhibit a unique response to ultrasound which improves the imaging contrast of the tissues they penetrate, they are termed ultrasound contrast agents (analogous to gadolinium-based compounds being termed MRI contrast agents) (Stride and Coussios, 2010).

For this purpose, it is insufficient to simply inject free bubbles into the bloodstream since the excess pressure which builds inside a bubble to balance its surface tension leads to dissolution. In fact, a free air microbubble in gas-saturated liquid completely dissolves in less than a second.

Therefore, over the past several decades, significant research has gone into developing synthetic nuclei with ‘optimal’ characteristics. Of course, clinical optimization is challenging because there exist a vast parameter space of both *inputs* - such as shell materials, cargos, and ultrasound parameters - and *performance* - such as persistence, biocompatibility, and acoustic response. The three major classes of synthetic nuclei, as well as their advantages and limitations, are summarized below.

2.3.3.1 Rigid-Shelled Microbubbles

The first stabilized microbubble to receive approval by the FDA and become commercially available was Albunex[®] in 1994 (Lindner, 2004). Albunex[®] bubbles, formed by sonication of a solution of human albumin, are air-filled bubbles with a mean diameter of 4.3 μm surrounded by a shell of aggregated albumin approximately 15 nm thick (Christiansen et al., 1994).

The encapsulating shell forms a barrier between the aqueous and gas phases, helping to sustain the gas cavity via two mechanisms: first by reducing surface tension, and second by providing physical resistance to gas leaving the core (Epstein and Plesset, 1950; Borden and Longo, 2002).

However, albumin shells tend to be rigid (Dayton et al., 1999), weakening their radial response to acoustic excitation (particularly their nonlinear response, which is critical to certain visualization algorithms and several therapeutic applications as well). Additionally, the use of animal-derived materials introduces associated immunogenicity issues (Ferrara et al., 2007).

Second-generation rigid microbubbles incorporate gases other than air into their interior. Suitable gases - frequently perfluorocarbons - have water permeation resistances several orders of magnitude higher than air, in addition to lower solubility and diffusivity in water. Low permeability is desirable because it reduces the rate at which gas in the bubble interior dissolves into the surrounding liquid while the shell is intact. Low solubility is desirable because it reduces the rate at which the bubble dissolves once the shell has ruptured. Both these characteristics therefore improve bubble stability and persistence in the bloodstream (but do not enhancing their acoustic responsiveness (Kabalnov et al., 1998a,b)).

2.3.3.2 Lipid-Shelled Microbubbles

Lipids are a class of amphipathic compounds containing hydro- or fluorocarbon chains attached to hydrophilic 'head' group, such as a phosphate group. Under appropriate conditions (concentration, pH, etc.), these compounds spontaneously self-assemble into monolayer coatings at a gas-liquid interface (Lee and Needham, 2001; Lee et al., 2001).

Included in this class is SonoVue[®], a phospholipid-shelled, sulphur hexafluoride-filled (SF_6) contrast agent with a mean diameter of 2.5 μm (Figure 2.1). SonoVue[®] was used as a cavitation nucleation agent in the *in vitro* and *in vivo* studies described in Chapter 5.

Though less stable than polymer- or albumin-shelled microbubbles, lipid-

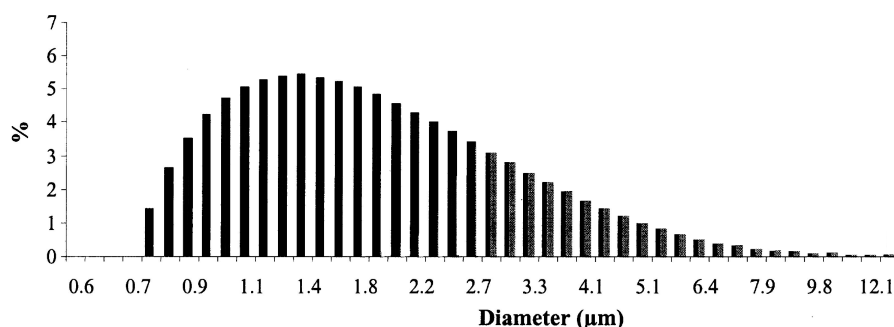


Figure 2.1: Size distribution of SonoVue microbubbles (Gorce et al., 2000)

shelled microbubbles are easier to form (frequently only requiring resuspension in water), and because the shell is only a single molecule thick, it is highly compliant, allowing for outstanding acoustic properties (Ferrara et al., 2007). Specifically, its elasticity renders it more echogenic than its albumin-shelled counterpart, and the lipid shell readily expands, compresses, and reseals in response to acoustic excitation (Dayton et al., 1999; Chomas et al., 2001; Bloch et al., 2004).

A further advantage is that phospholipid chemistry is well-developed, allowing various classes of functional ligands to be easily coupled to the bubble surface, before or after lipid resuspension, either through direct covalent linkage or through immunoconjugation (Klibanov, 2005). Antibodies attached to microbubbles, for example, can be used to enhance their retention near cells expressing specific surface receptors.

2.3.3.3 Hydrophobic Nanoparticles

An emerging class of nucleation agents includes nanoparticles which exhibit a combination of hydrophobicity and high surface roughness, and therefore entrap air particles on their surface when resuspended in water. These particles, inspired by the ‘crevice’ model of nucleation (Atchley and Prosperetti, 1989), are an active area of research, and though they have not yet achieved clinical use, experimental

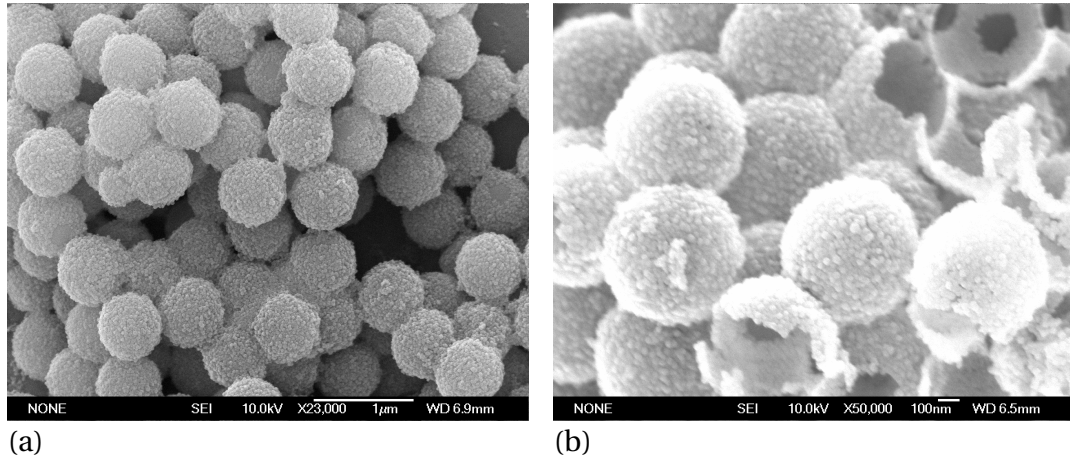


Figure 2.2: Scanning electron micrograph showing surface morphology and highly uniform size of **(a)** solid and **(b)** hollow hydrophobic nanoparticles produced by layer-by-layer surface deposition. These particles have been demonstrated to reduce cavitation threshold and increase cavitation activity *in vitro* (Wagstaffe et al., 2010).

work has demonstrated their capacity to dramatically lower the cavitation threshold under clinically relevant ultrasound parameters (Wagstaffe et al., 2010). Moreover, their size, which can be precisely tuned (Figure 2.2(a)) by adjusting synthesis conditions, makes them an ideal candidate for localizing cavitation within cancerous tissue since, as discussed in Section §1.3.2, sub- μm particles passively accumulate in tumor interstitium via the EPR effect, which would offer obvious benefits for targeted therapy and drug delivery. Second-generation hydrophobic nanoparticles may additionally encapsulate a gas pocket (Figure 2.2(b)), giving them the stability of rigid-shelled microbubbles, but of much smaller size.

The proof-of-concept *in vitro* work described in Chapter 3 uses off-the-shelf hydrophobic microparticles to enhance the presence and control the location of cavitation. It should be noted, however, that these particles served only to elucidate the physical phenomena explored. Because of their immunogenicity, they were not used in conjunction with living tissue or cells.

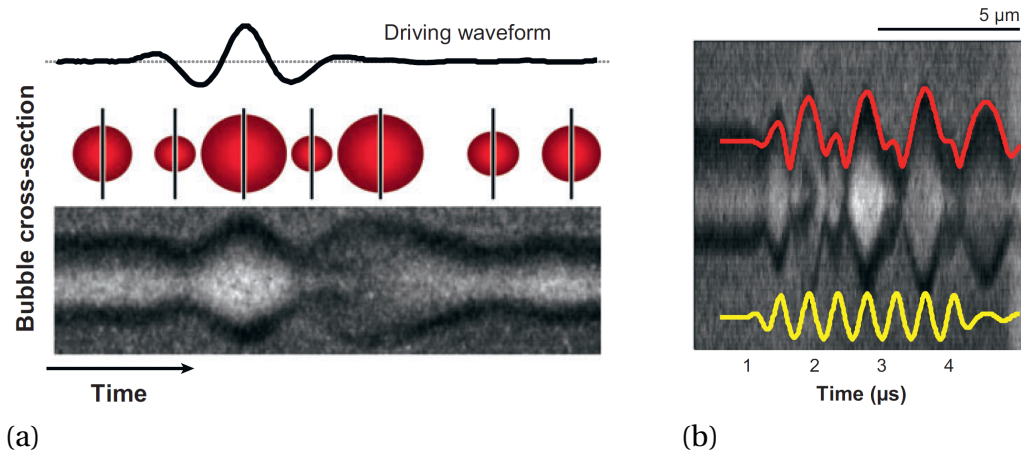


Figure 2.3: Lipid-shelled microbubble response to acoustic excitation. **(a)** To facilitate comparison between experimental results and 1-D (radius vs time) bubble models, an oscillating bubble is photographed, and the center-line of the image is extracted and displayed as a function of time. **(b)** Comparison between an experimental radius-time image of a bubble driven by a 2.25 MHz, 360kPa peak rarefactional pressure field (transmitted pulse shown in yellow) with the predicted radius-time curve (shown in red) shows strong agreement (Ferrara et al., 2007).

2.4 CAVITATION DYNAMICS

Having explored the means by which cavitation can be nucleated, the *dynamics* of cavitating microbubbles are explored - that is, their behavior in response to ultrasonic excitation. There are several justifications for focusing our efforts on the dynamics of *free* bubbles, rather than shelled or otherwise constrained bubbles. Of the two types of nuclei employed in our experimental work, the first - nanoparticles - is hypothesized to release free-floating bubbles from its crevices. The second - lipid-shelled agents - exhibits dynamics which are well-predicted by free-bubble models discussed below (Figure 2.3), for driving pressures beyond several hundred kilopascals (Morgan et al., 2000). This is perhaps unsurprising given the thickness and composition of the lipid shell.

2.4.1 Bubble Dynamics

In the case of a spherical bubble in an infinite medium, assuming an incompressible viscous liquid, and for bubble radius R much smaller than the driving wavelength, the radial response to a time-varying pressure field is given by the Rayleigh-Plesset equation (Plesset and Prosperetti, 1977):

$$R\ddot{R} + \frac{3}{2}\dot{R}^2 = \frac{p_g + p_v - p_\infty - p_{ac}}{\rho_L} - \frac{2\sigma}{\rho_L R} - \frac{4\nu_L\dot{R}}{R} \quad (2.5)$$

where p_g is the bubble gas pressure, p_v is the bubble vapour pressure, p_∞ is the ambient pressure, p_{ac} is the acoustic driving pressure, σ is the surface tension coefficient, ν_L is the kinematic viscosity of the liquid, and ρ_L is the density of the liquid. Because this equation assumes an incompressible medium, bubble oscillations of large amplitude and violent collapses are not accurately represented.

The Keller-Miksis equation (Keller and Miksis, 1980) incorporates compressibility of the medium by including the acoustic radiation pressure from the bubble:

$$\left(1 - \frac{\dot{R}}{c}\right)R\ddot{R} + \frac{3}{2}\left(1 - \frac{\dot{R}}{3c}\right)\dot{R}^2 = \left(1 + \frac{\dot{R}}{c}\right)\frac{p}{\rho} + \frac{R}{\rho c} \frac{dP}{dt} \quad (2.6)$$

where

$$p = p_g + p_v - p_\infty - \frac{2\sigma}{R} - \frac{4\nu_L\dot{R}}{R} \quad (2.7)$$

Of course, the physiological environment presents further complexities which lead to departures from these simplified models, including interaction with nearby bubbles as well as vessel walls, and viscoelastic tissue which constrain expansion and disrupt symmetry. However, the present work does not seek to precisely characterize the behavior of each bubble in a clinical setting, but rather to explore the variety of behaviors bubbles are capable of and the bioeffects which those behaviors are hypothesized to or have been reported to induce. This theoretical foundation is intended to inform the end goal of not only designing a system which induces a

specific type of bubble behavior, but which is supplemented by a real-time or quasi-real time measure of treatment progress based on the spatial extent, duration, and type of cavitation being observed.

Different types of cavitation arise because, at a given frequency, the nature of the response of a bubble to acoustic excitation changes depending on the pressure amplitude of the wave. Cavitation is generally divided into two categories:

Stable Cavitation: At lower acoustic amplitudes of excitation, the bubble is said to undergo stable cavitation, which involves the *periodic* (but not necessarily sinusoidal) oscillation of the bubble about an equilibrium radius (Figure 2.4, light curves). In the context of equation (2.5), the ‘pressure’ terms dominate, and the response of the bubble is governed by the compressibility of the bubble contents. Under certain conditions, stable cavitation has also been associated with shape oscillations and microstreaming (Leighton, 1994).

Inertial Cavitation: At higher acoustic amplitudes, the inertial ($\dot{R}$) term in equation (2.5) dominates, resulting in significant bubble expansion during the rarefactional phase of the acoustic field, followed by rapid collapse driven by the inward inertia of the surrounding liquid during the compression phase of the acoustic field (Figure 2.4, dark curves). During collapse, the bubble contracts to a fraction of its initial radius, yielding extreme pressures and temperatures within the bubble, which drive its subsequent expansion. Inertial cavitation is associated with fluid jetting, sonoluminescence, shock wave formation, and heating (Coussios and Roy, 2008).

Although there is a fundamental difference between the concepts of stable and inertial cavitation, it should be noted that the transition between the two is not

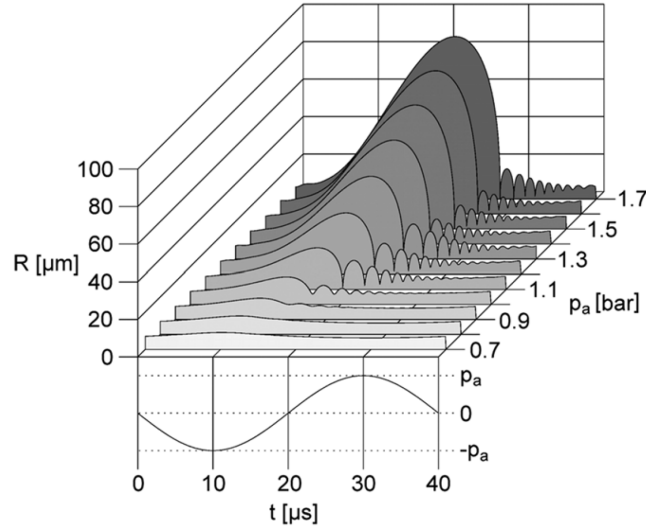


Figure 2.4: Numerically simulated radius-time curves of a $7\mu\text{m}$ bubble excited at 25kHz across a range of acoustic pressure amplitudes p_a . At low pressures, the bubble undergoes stable cavitation (light curves). As pressure is increased, the bubble begins to inertially cavitate, characterized by violent collapse and often including bubble rebounds (Lauterborn and Kurz, 2010)

abrupt - rather, it takes place through a continuum. Assuming constant frequency f_0 , as peak pressure amplitude is increased, a linearly pulsating bubble will begin to undergo nonlinear pulsations with frequency content containing integer multiples of f_0 . With further increases in excitation, the bubble will reach the so-called bifurcation zone, which involves the transition from pulsations of period $1/f_0$ to pulsations of period m/f_0 ($m = 2, 3, 4, \dots$), and eventually the stochastic zone, which involves chaotic pulsations and the disappearance of the periodic structure altogether Figure 2.5(a) (Ilyichev et al., 1989). Note that similar effects can also be observed by maintaining constant pressure P_0 and *decreasing* the excitation frequency Figure 2.5(b).

2.4.1.1 Inertial Cavitation Threshold

There is a great deal of interest in controlling which type of cavitation is incited, as the phenomena associated with each are distinct. Although the transition be-

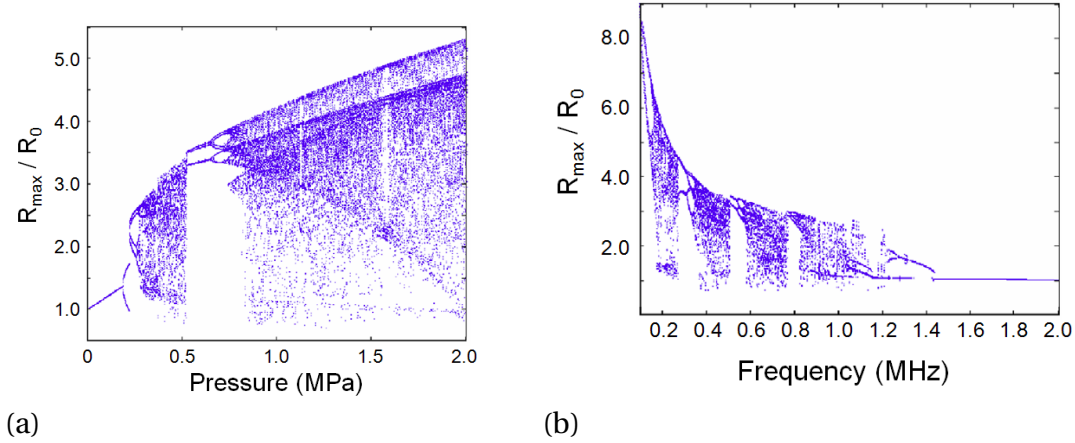


Figure 2.5: Bifurcation diagram plotting each local maximum of the radius-time curve of an oscillating bubble as a function of the x-axis variable, simulated using a Keller-Miksis model. The bifurcation diagram of a bubble with $R_0 = 10 \mu\text{m}$, with respect to: **(a)** pressure when $f_0 = 500 \text{ kHz}$, **(b)** frequency when $P_0 = 500 \text{ kPa}$ (Behnia et al., 2009). Note the onset of chaotic behavior at $R/R_0 > 2$ a heuristic threshold with importance in delineating between stable and inertial cavitation, discussed in Section §2.4.1.1.

tween the two is not sharp, various criteria have been proposed as inertial cavitation thresholds:

- **R_{max} threshold:** Maximum bubble radius equal to twice the initial radius (Flynn, 1975; Church, 2005)
- **T_{max} threshold:** Maximum temperature within the bubble during collapse equal to 5000 K (Flynn, 1975; Apfel and Holland, 1991)
- **$\dot{R}$ threshold:** Maximum bubble wall velocity greater than the speed of sound in air (Church, 2002)

Of course, selecting an appropriate threshold largely depends on the intended application. For example, the threshold for cavitation-enhanced thermal ablation may be most closely tied to $\dot{R}$, since most of the acoustic emissions associated with

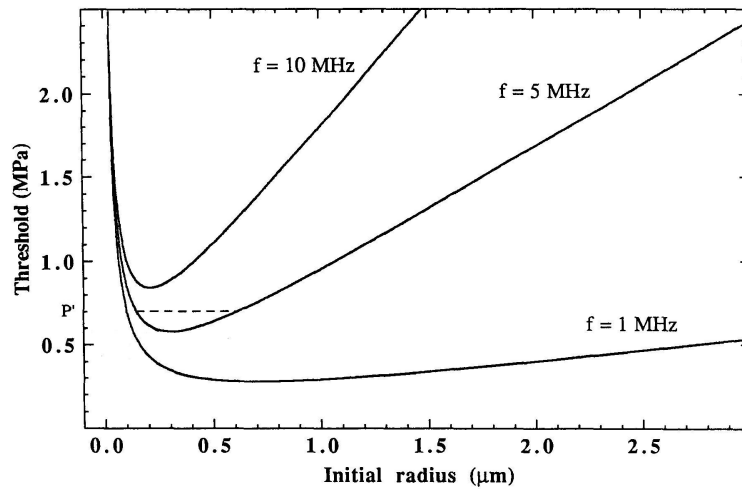


Figure 2.6: Cavitation threshold in water as a function of initial nucleus radius for three frequencies of sonication. Dotted lines indicate the range of bubble sizes that will inertially cavitate at 5 MHz when driven at pressure P' (Apfel and Holland, 1991).

cavitation-enhanced heating occur during collapse. In contrast, the R_{max} threshold might be most relevant to cavitation-enhanced drug delivery, because of its hypothesized dependence on microstreaming generated by *periodic* bubble oscillations (beyond $R_{max} > 2R_0$, bubbles transition from periodic to chaotic oscillations Figure 2.5(a)).

Independently of the method used, the threshold criterion does not yield a single pressure value applicable to all bubble sizes and all frequencies, since the threshold is dependent on both frequency and initial bubble radius. Using the maximum collapse temperature criterion of 5000 K, a family of inertial cavitation threshold curves was derived (Apfel and Holland, 1991), based on the isothermal growth and adiabatic collapse of pre-existing argon-filled bubbles in incompressible water subject to single-pulse ultrasound (Figure 2.6).

It is worth emphasizing that this study, designed to address safety concerns during *diagnostic* ultrasound, only applies to short pulses propagating through a pure liquid in which all bubble sizes are assumed to pre-exist. None of these assump-

tions hold during therapeutic ultrasound exposure of tissue. Furthermore, it has been shown that sub-threshold excitation can yield inertial cavitation several cycles into a longer ultrasound exposure (Flynn and Church, 1988).

When considering longer pulse lengths and biological tissues, rectified diffusion and the effect of viscoelasticity must also be considered, as discussed below.

2.4.1.2 Rectified Diffusion

In the absence of an applied acoustic field, the excess pressure within a bubble – generated to balance the surface tension of the liquid – raises the partial pressure of the gas within the bubble, causing a net flux of the gas into the liquid and dissolution of the bubble.

In the presence of an oscillatory pressure field, there may exist an asymmetry in mass flux across the bubble wall, which can cause the ‘equilibrium’ bubble size to change over time. This is known as rectified diffusion, and although numerical analysis of this effect must account for the coupling between the equation of bubble motion and the diffusion equation, a coarse understanding of the phenomenon can be achieved by considering the change of bubble mass as described by the transport equation:

$$\frac{dm}{dt} = 4\pi R^2 D \left. \frac{\partial C(r, t)}{\partial r} \right|_{r=R} \quad (2.8)$$

where m is the mass of gas within the bubble, D the diffusion constant of the dissolved gas, r is the radial coordinate, and $C(r, t)$ is the concentration of dissolved gas.

During the compression phase, gas pressure in the bubble increases, causing net flux into the liquid. During the rarefaction phase, the reverse is true. However, during the rarefaction phase, the surface area $4\pi R^2$ of the bubble is larger than in the

compression phase, causing an imbalance in mass flux which favors bubble growth.

Reinforcing this ‘area’ effect is the ‘shell’ effect: During bubble pulsation, the thickness of a hypothetical isovolumetric shell of liquid around the bubble will change - during rarefaction, the liquid shell thins, and during compression, it thickens. Shell thinning increases the concentration gradient $\partial C(r, t)/\partial r$, and therefore the flux of gas into the bubble, whereas shell thickening decreases the concentration gradient and outward flux.

Thus, the ‘area’ and ‘shell’ effects reinforce one another, resulting in ‘rectified diffusion’. As a consequence, small bubble nuclei undergoing stable oscillations in water over a number of cycles tend to grow, reach resonant size, oscillate violently, collapse and fragment (Leighton, 1994).

For a given bubble size and excitation frequency, rectified diffusion will only occur above a certain amplitude of insonification, which generally lies below the inertial cavitation threshold amplitude (Church, 1988). Thus, a bubble in such a field will undergo one of several processes.

- If the bubble initially lies below the threshold for rectified diffusion, it will dissolve.
- If the bubble lies between the thresholds of rectified diffusion and inertial cavitation, it will grow (travelling to the right on Figure 2.7).
- The bubble may grow and intersect the inertial cavitation threshold, at which point it will undergo inertial collapse.

Note that an inertially collapsing bubble can fragment, and these fragments can grow by the processes described above, so that the process of cavitation is positively reinforced.

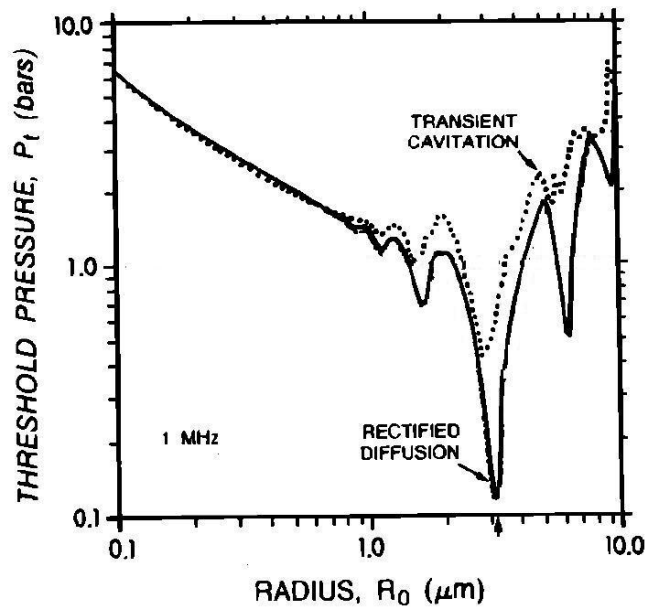


Figure 2.7: Thresholds for rectified diffusion (solid) and inertial cavitation (dotted) at 1MHz (Church, 1988). For combinations of initial bubble radius and pressure (R_0 , P_t) above the dotted line, the bubble will cavitate inertially. For combinations (R_0 , P_t) between the solid and dotted line, the bubble will grow through rectified diffusion.

As can be seen, cavitation at the single-bubble level in water is a largely unstable process. Long-lasting stable cavitation *has* been reported (Mestas et al., 2003), but this frequently involves trapping a bubble at the surface of different objects – a wire, a hydrophone, wax surface, etc. – in a very controlled manner, and is usually done as a means of studying stable oscillations rather than exploiting its associated phenomena (microstreaming, viscous heating, etc.).

Of course, the physiological environment mitigates unstable bubble behavior in several ways. First, it is an ideal setting in which to ‘trap’ bubbles, since their movement is impeded by vasculature and tissue. Second, the process of rectified diffusion is limited by the concentration of gas in the surrounding medium and its diffusivity through the medium, which is lower *in vivo* than in water (see Section §1.3.3).

There also exist *mechanical* characteristics which further contribute to bubble stability.

2.4.1.3 Viscoelasticity

Biological tissues show varying degrees of viscoelasticity, a non-Newtonian flow behavior exhibiting properties of both viscous and elastic materials in a frequency-dependent manner. Because of the elastic component of these tissues, the Rayleigh-Plesset and Keller-Miksis equations must be modified to include shear terms, which generally serve to reduce oscillation amplitudes and therefore increase the inertial cavitation threshold (Yang and Church, 2005). This effect on bubble dynamics is compounded by the elevated viscosity in blood relative to water, which further dampens bubble oscillation amplitudes.

2.4.2 Effects of the Cavitating Bubble on its Surroundings

In addition to the enhanced heat deposition which arises from the ability of the cavitating bubble to convert energy from the driving frequency to higher, more readily absorbed frequencies, various other bubble behaviors have been observed which can be exploited in a biomedical setting. Here, the physical basis and experimental observations for two of these behaviors that are relevant to the current work are discussed.

2.4.2.1 Erosion and Jetting

The study of collapsing bubbles was largely inspired by attempts to understand and minimize the damage to ship propellers by hydrodynamic cavitation (Rayleigh, 1917). Only several decades later was the field expanded to include the erosion that arises from acoustic cavitation, which is understood to result from two phenom-

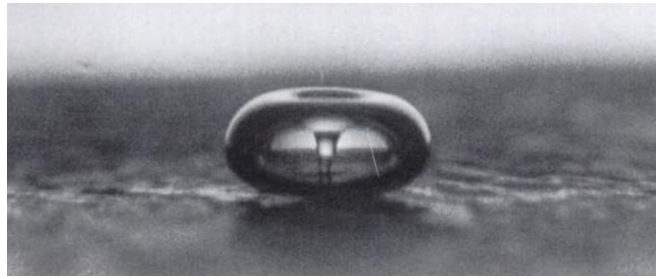


Figure 2.8: Fluid jetting during the collapse of 2 mm bubble subject to 60 Hz ultrasound at $4\text{--}5\text{ kPa}$. (Unpublished photograph by Professor L A Crum, University of Mississippi)

ena. The first is the shock wave emitted by a spherically collapsing bubble, which may have amplitudes up to 1 GPa . However, due to the rapid attenuation of this wave, only surfaces within approximately the initial bubble radius are affected.

The second phenomenon responsible for surface erosion occurs during collapse of a bubble near a solid boundary. Compared to a bubble collapsing in an infinite medium, fluid flow constraints cause asymmetrical collapse of the bubble, culminating in a fluid jet directed towards the solid surface Figure 2.8. Jet speeds of $\sim 150\text{ m/s}$ have been recorded, and are strongly dependent on the ratio of the bubble's distance from the wall to the maximum bubble radius (Vogel et al., 1989; Plesset and Chapman, 1971). This high-speed jet impacting the surface can crack tissue surfaces or cause 'pitting'.

2.4.2.2 Microstreaming

As discussed in Section §2.1, a propagating acoustic wave may be absorbed, creating an energy gradient which decreases in the direction of wave propagation. This gradient corresponds to a force which acts upon the liquid in the sound field. At clinically relevant ultrasound levels, this can create flows of up to 10 cm/s in water (Leighton, 1994), and is termed 'acoustic streaming'.

Another type of streaming occurs when sound waves are incident upon a small vibrating obstacle in the field, arising from the frictional forces between the obstacle and the medium. The temporal average of the velocity and pressure fluctuations arising from the friction is often non-zero, resulting in steady flow. This circulation, termed *microstreaming*, is generally confined to a boundary layer of thickness

$$L = \sqrt{2\mu/\rho\omega} \quad (2.9)$$

around the obstacle, where μ is the shear viscosity and ρ the density of the liquid. In the context of bubble oscillations in a sound field, this phenomenon is most pronounced when the driving signal is at the resonance frequency of the bubble. This effect has been exploited to disaggregate bacteria and biofilms (Everbach et al., 2008), shear DNA, cause hemolysis, and enhance thrombolysis (Datta et al., 2008). Additionally, the shearing action has been cited as a mechanism for sonoporation in the context of gene transfection experiments.

In the case of a static bubble, surface tension causes a bubble to adopt a spherical shape, minimizing surface energy. However, an acoustically cavitating bubble can undergo various modes of oscillation, affecting the observed circulation pattern in the surrounding liquid. Modes of oscillation are influenced by acoustic pressure amplitude, liquid viscosity, and contaminants present in the water (these tend to collect on the bubble wall and obstruct free tangential flow along the interface, effectively creating a ‘no-slip’ boundary, in contrast to the ‘free-slip’ boundary of clean bubbles) (Elder, 1959). Bubbles may undergo several modes of oscillation, and may change discontinuously from one to another.

Modes of oscillation can be broadly split into three categories: translational (Figure 2.9(a)-(b)), volumetric, and shape oscillations. A translational mode may simply be a linear harmonic oscillation of the bubble’s centroid along an axis, or slightly more complicated, such as a circular or elliptical orbital motion. The simplest volu-

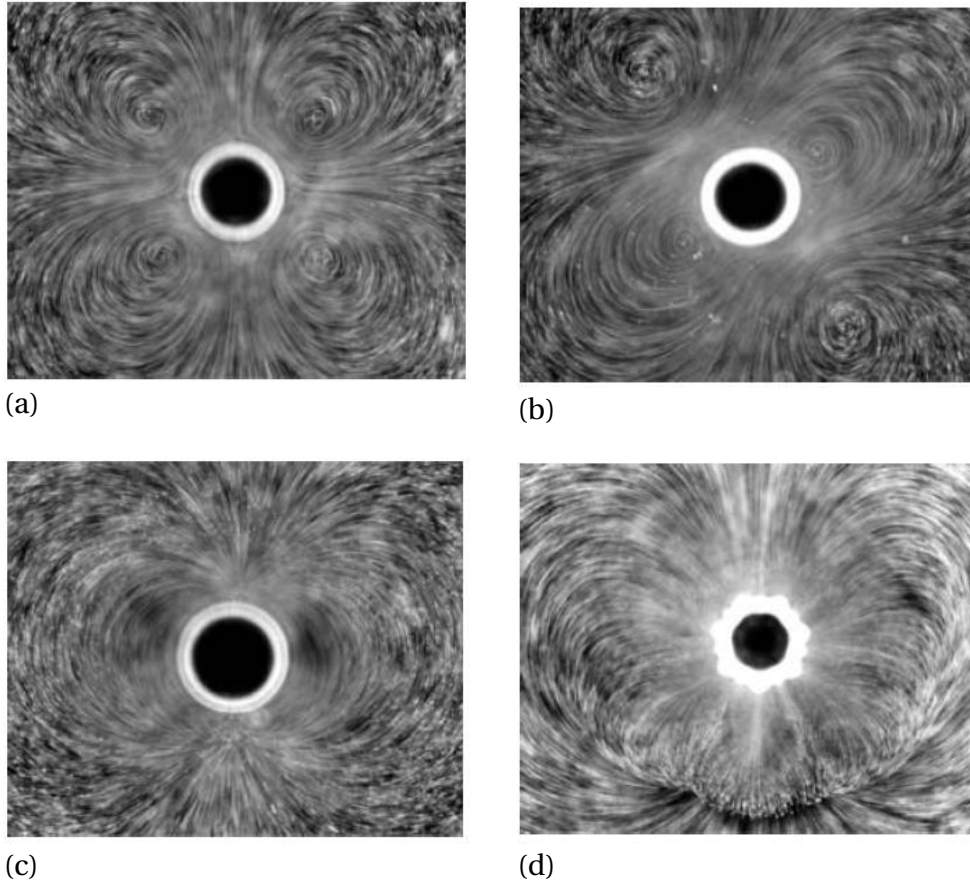


Figure 2.9: Streaming patterns around a $272\ \mu\text{m}$ radius bubble excited at various acoustic frequencies. (a) translational oscillation at $f = 2.4\text{kHz}$, (b) elliptical orbiting oscillation at $f = 2.1\text{kHz}$, (c) volumetric oscillation at $f = 8.7\text{kHz}$, (d) shape oscillation at $f = 11.2\text{kHz}$. Note that disruption of flow symmetry may occur even when the bubble wall motion appears to be radially symmetric. (Tho et al., 2007)

metric oscillation occurs at the resonant frequency f_M :

$$f_M = \frac{1}{2\pi R_0} \sqrt{\frac{3\gamma p_0}{\rho} - \frac{2\sigma}{\rho R_0}} \quad (2.10)$$

where R_0 is the bubble's equilibrium radius, γ is the ratio of specific heats of gas, p_0 is the hydrostatic liquid pressure, and σ is the surface tension (Minnaert, 1933). In this mode, the bubble expands and contracts spherically (Figure 2.9(c)), and is known as the breathing mode. At higher frequencies, bubbles can be excited to undergo shape oscillations (Figure 2.9(d)).

In an effort to experimentally characterize the various modes of oscillation and their effect on microstreaming patterns, Tho *et al* (Tho et al., 2007) used microparticle image velocimetry to trace 2 μm polystyrene particles in a chamber containing a pendant bubble attached to the upper wall of a microchamber and subject to acoustic excitation. The study concluded that the three main parameters affecting streaming flows were pressure amplitude, frequency, and bubble size. Small variations in bubble size did not induce dramatic variations in the streaming flow patterns. An increase in pressure amplitude only changed the streaming velocity, not the mode of oscillation - until a critical threshold, beyond which shape oscillations were induced. The streaming flows resulting from shape mode oscillations were several times faster than those observed in the translating and volume modes of oscillation. At optimal conditions, maximum streaming velocities reached 0.45 mm/s. (Elder, 1959)

Microstreaming also plays an important role in the rectified diffusion process. In the absence of microstreaming, as a bubble grows through rectified diffusion, it decreases the gas concentration in the surrounding liquid shells, which serves as a negative feedback mechanism, slowing the growth of the bubble until the dissolved gas in the liquid diffuses towards the bubble, which in general is the rate-determining step in the process. However, microstreaming circulates the surrounding liquid, refreshing the dissolved gas content in the shells surrounding the bubble wall. Thus, in the presence of microstreaming, growth rates through rectified diffusion tend to outpace the predictions of Equation (2.8). (Leighton, 1994)

2.5 CAVITATION DETECTION AND MONITORING

In the previous section, the dynamics of cavitating bubbles and various 1-D models which have been experimentally validated using high-speed video microscopy were

explored (Figure 2.3). However, discrepancies in bubble behavior between water and biological tissue are clearly anticipated. This presents the significant challenges of (1) not knowing exactly what type of bubble behavior occurs *in vivo*, and (2) being unable to *directly* observe bubble behavior, since biological tissue is optically opaque.

To overcome these obstacles, significant work has been done to monitor bubble behavior by using non-invasive acoustic techniques. Detecting cavitating microbubbles can either be done actively or passively.

A simple incarnation of Active Cavitation Detection (ACD) involves using traditional B-mode imaging to detect and localize backscatter from bubbles. However, this method has an important limitation. Because the intensity of a therapeutic ultrasound beam is several orders of magnitude higher than diagnostic ultrasound pulses, the therapeutic beam must be switched off during ACD (Owen et al., 2006). Thus, monitoring of the echogenicity of a bubble provides an instantaneous snapshot of its backscattering cross-section following therapeutic ultrasound exposure, rather than the dynamics which resulted in that cross-section during exposure. Moreover, under clinically relevant conditions, constructing the B-mode image by combining a sequence of A-lines can take several milliseconds (Szabo, 2004), during which time cavitating microbubbles, unless stabilized by boiling, may dissolve away.

An alternative to this technique is Passive Cavitation Detection (PCD), which involves monitoring the *secondary acoustic emissions* - that is, the pressure waves radiated by a bubble oscillating in response to therapeutic excitation, rather than the echoes of a diagnostic transmitted pulse. Passive cavitation detection has been successfully employed to non-invasively monitor heat deposition in the context of cavitation-enhanced thermal ablation of tissue (Coussios et al., 2006) as well as drug

delivery in the context of cavitation-enhanced thrombolysis (Datta et al., 2008).

For this method to be effective, it is necessary to detect acoustic emissions remotely, translate the acoustic ‘signatures’ into quantitative measures of various regimes of bubble activity, and do so at the necessary level of spatial resolution. Detection is achieved using ultrasound transducers which can either be focussed, providing sensitive measures of bubble activity in its focal region, or unfocussed, providing non-directional measures of cavitation activity within the field. Recently, the idea of using a linear array of passive cavitation detectors has been developed, with the goal of mapping bubble activity in two dimensions (Gyongy and Coussios, 2010).

Regardless of the detector used in passive cavitation detection, a major challenge in acoustic cavitation research is the interpretation of the acquired signal. This requires an understanding of the modes of emission expected from certain types of cavitation. Assuming no material attenuation, a bubble’s radiated pressure is given by:

$$P_{rad} = \frac{\rho R}{d} (2\dot{R}^2 + R\ddot{R}) + \mathcal{O}\left(\frac{1}{d^4}\right), \quad (2.11)$$

where d is the distance from the bubble center (Ilinskii and Zabolotskaya, 1992). Because the radiated pressure entirely depends on the bubble radial oscillations, monitoring and analyzing it can be useful in determining the nature of cavitation taking place. Detection of acoustic emissions is frequently done at a distance $d \gg R$, so near-field components (grouped as $\mathcal{O}(1/d^4)$ above) are often ignored in comparison to analytical and numerical solutions.

It is common to analyze acoustic emissions from cavitating bubbles in the frequency domain. Experimental work demonstrates that at low driving amplitudes, the power spectral content of the radiated pressure is concentrated at the excitation

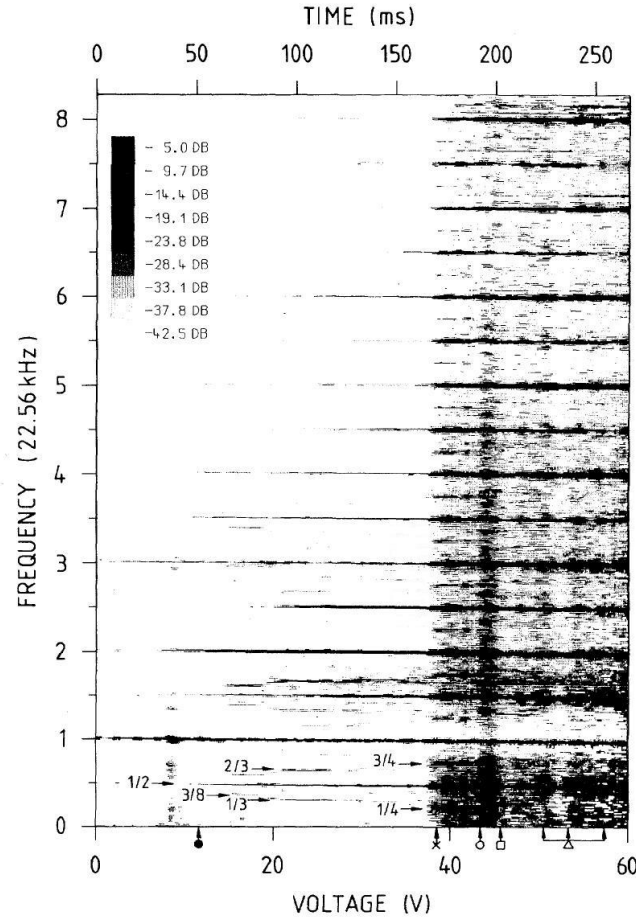


Figure 2.10: Sequence of the power spectra of secondary acoustic emissions as a function of the sinusoidal voltage driving an ultrasonic transducer (Lauterborn and Cramer, 1981). As the driving pressure is increased, harmonic emissions emerge first, followed by subharmonic emissions and their integer multiples (ultraharmonics), and finally broadband emissions.

frequency (Figure 2.10). As driving amplitudes increase, the spectral content becomes more complex, containing line-spectrum components as well as broadband signals (Lauterborn and Cramer, 1981).

The discrete peaks in the spectrum occur at multiples nf_0/m ($n = 1, 2, 3, \dots; m = 1, 2, 3, \dots$), where f_0 is the excitation frequency. The nomenclature applied to these components is as follows: fundamental (or principal) ($n=1, m=1$), harmonic ($n = 2, 2, 3, \dots; m = 1$), subharmonic ($n = 1; m = 1, 2, 3, \dots$), and ultraharmonic ($n= 2, 3,$

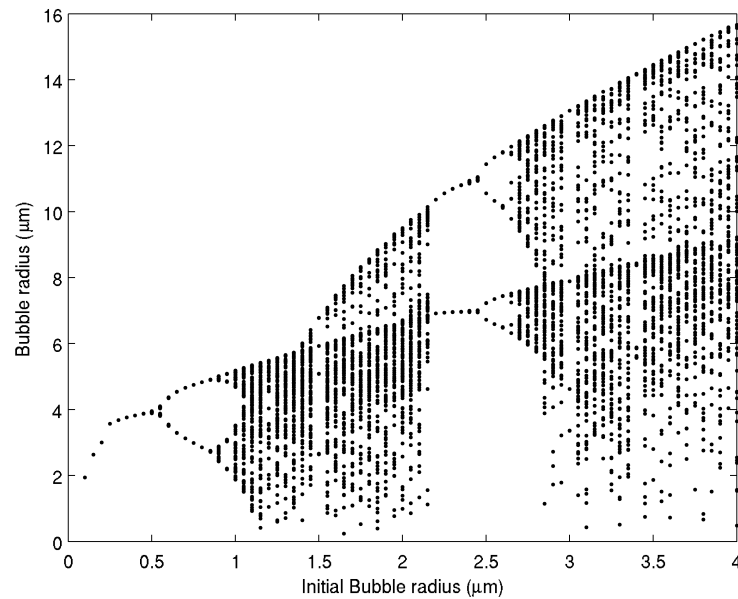


Figure 2.11: Bifurcation diagram showing the local radii maxima of bubbles of initial radius $< 4\mu m$ in water, for 0.3MPa excitation at 1MHz. (Collin, 2009). Because of the presence of linear pulsations, non-linear pulsations, bifurcations, and chaotic oscillations, the corresponding acoustic emission spectrum is rich with harmonics, ultraharmonics, and broadband emissions.

4, ... ; $m = 2, 3, 4, \dots$) (Ilyichev et al., 1989). Elevation of the 'noise floor' without distinguishable peaks is termed 'broadband'.

Note that although the radius-time curves of the bubble and acoustic emissions are not linearly related, there appears to be a qualitative correspondence between, for example, the emergence of subharmonics in the acoustic emissions (Figure 2.10) and the onset of period-multiplying bifurcations (discussed in Section §2.4 and depicted graphically in Figure 2.5(a)). However, of critical importance when interpreting PCD signals is that they represent the superposition of emissions emanating from within the focal volume, i.e. from a *population* of bubbles. Because these bubbles are likely to span a range of initial radii, a variety of behaviors can be expected even at constant excitation frequency and amplitude (Figure 2.11).

By distilling into a single signal an otherwise intractably complex system involv-

ing bubble populations of varying sizes at different positions in the sound field radiating acoustic energy through multiple layers of heterogeneous tissue, this signal represents a simplification of the behavior of the cavitation field, but one which can be empirically correlated with clinically relevant treatment metrics. To begin deconstructing a signal, the possible origin of various components of the emissions power spectrum must first be considered.

2.5.1 Emissions at the Fundamental

Because the intensities used in therapeutic ultrasound are orders of magnitude stronger than secondary acoustic emissions, inhomogeneities in the field commonly generate detectable scattering at the fundamental frequency (f_0). Clinical inhomogeneities include, for example, tissue-tissue interfaces, cells suspended in the bloodstream, as well as contrast agents. Because the relative contribution of these components is unknown, the f_0 component of the power spectra does little other than to indicate the intensity of the sonication and the extent of inhomogeneity. In practical situations, reflections at f_0 are often so overwhelming that the signal is filtered to remove them, allowing for improved vertical resolution at frequencies of greater interest in cavitation monitoring.

2.5.2 Harmonic Emissions

As discussed in Section §2.2, finite amplitude waves in a lossless medium tend towards a sawtooth shape, which in the frequency domain corresponds to increased content in the harmonics of the original excitation frequency. Generation of harmonic content in a homogeneous medium is increased at higher frequencies, higher sound amplitudes, and in media with a higher coefficient of nonlinearity. These harmonics in the *transmitted* waveform may be reflected by inhomogeneities

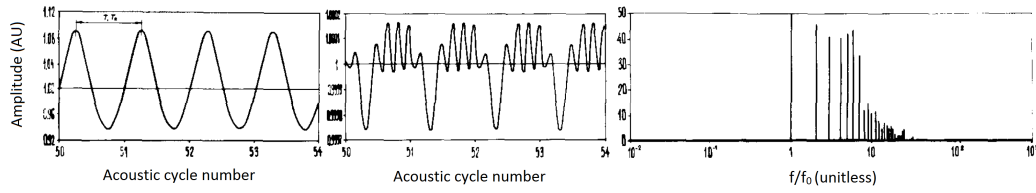


Figure 2.12: Bubble pulsations (left), secondary acoustic emissions (center) and emissions energy spectrum (right) of a $20\mu\text{m}$ bubble excited at 28kPa with a driving frequency of 31kHz, a combination which falls within a bifurcation zone. Note the dramatic nonlinearities present in the secondary emissions relative to the bubble pulsations (Ilyichev et al., 1989)

in the medium as described in Section §2.5.1 above.

In addition to the scattering of a nonlinear *driving* signal, harmonic emissions can result from the nonlinear secondary acoustic emissions of oscillating micro-bubbles. A power series expansion of the pressure-radius response of a bubble operating at high amplitudes demonstrates the potential for harmonic generation. Generally,

$$R(t) = R_0 + a_1 p(t) + a_2 p^2(t) + a_3 p^3 t + \dots \quad (2.12)$$

If $p(t) = P_0 \cos(\omega t)$, then higher power terms will produce responses at integer multiples of the driving frequency ω . (Leighton, 1994)

Finally, it should be emphasized that even an approximately sinusoidally oscillating bubble can, owing to the nonlinearity of Equation (2.11), produce secondary emissions at integer multiples of the driving frequency (Figure 2.12).

Thus, harmonics can be generated by (1) scattering of the harmonics generated by non-linear propagation of the excitation wave and (2) non-linear radial oscillations of the bubble. Note, however, that the signal detected from scattered harmonics is often not as prominent or confounding as that detected from the scattered fundamental - that is, mechanism (1) doesn't dominate the harmonic spectral content. Therefore, analysis of harmonics can still be useful in detecting the presence

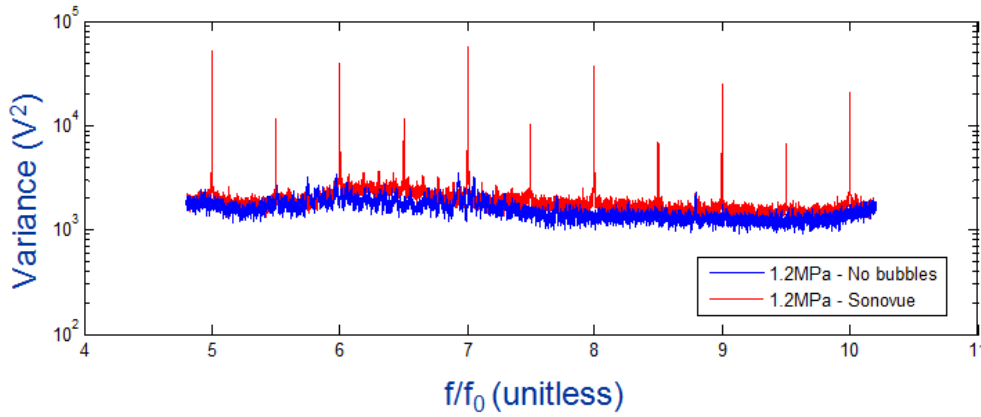


Figure 2.13: Power spectrum recorded by a PCD during 1.2MPa excitation at 0.5MHz in mouse tumor, in the presence or absence of the intravenously injected ultrasound contrast agent SonoVue[®]. Although the tissue is a nonlinear material, the combination of nonlinearity and inhomogeneities is insufficient to generate harmonics above the system noise floor (blue line). Thus, in this experimental setup, the presence of harmonics (red line) is a unique indicator of the presence of contrast agent within the PCD focal volume.

of microbubbles (Figure 2.13).

2.5.3 Subharmonic Emissions

There are four main sources of subharmonic noise generation by cavitating microbubbles. The first has been alluded to in the bifurcation diagrams previously shown. Consider, in Figure 2.11, a bubble of initial radius of $0.7\mu m$. In successive acoustic periods, it alternates between two maxima, and so repeats itself every two periods, yielding an $f_0/2$ component in the emissions spectrum.

Second, it has also been demonstrated that a bubble excited at twice its natural frequency is able to pulsate at the frequency of excitation *and* oscillate at its own natural resonance, which can be a source of subharmonic emissions (Lauterborn, 1976).

Third, it has been shown that at sufficiently high excitation amplitudes, inertial

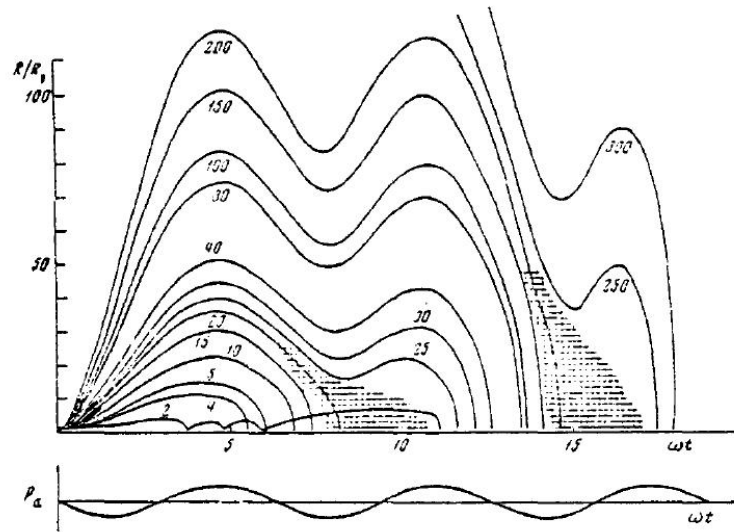


Figure 2.14: Radius-time curves for an air bubble in water. $R_0 = 1 \mu\text{m}$; $f = 500 \text{ kHz}$. Curve numbers refer to the relative excitation amplitude. The ‘arrested collapse’ over two or more acoustic cycles results in subharmonic emissions (Neppiras, 1980)

forces can cause an expanding bubble to ‘skip’ a contraction phase (known as ‘arrested collapse’) and continue its expansion for one or more cycles, resulting in an oscillation period at some multiple of the fundamental, corresponding to a subharmonic in the frequency spectrum, a phenomena known as period doubling, period tripling, etc (Figure 2.14). This is usually followed by inertial collapse and broadband signal generation.

Finally, it has been proposed that subharmonic emissions may be emitted by large amplitude bubble surface waves (known as Faraday waves) (Neppiras and Fill, 1969). However, subsequent work demonstrated that Faraday waves are inefficient sound sources, detectable only very close to the bubble, and so are unlikely to be responsible for the subharmonic signal detected in a clinical setting (Strasberg, 1956; Plesset and Prosperetti, 1977).

2.5.4 Ultraharmonic Emissions

Though the least understood of the spectral components, ultraharmonic signals are hypothesized to be traceable to two mechanisms. First, each of the sources for subharmonic emissions described above can yield ultraharmonic emissions as a result of the propagation of the subharmonic pressure wave through a nonlinear medium.

Second, in the same manner that the nonlinear relationship between radial motion and secondary acoustic emissions (Equation (2.11)) can yield harmonic emissions from sinusoidal oscillations, ultraharmonic emissions can be generated from subharmonic oscillations (Ilyichev et al., 1989).

2.5.5 Broadband Emissions

During a violent bubble collapse, wall velocities can approach the speed of sound in the liquid. The inertia of the in-rushing liquid results yields a very short time-scale collapse, producing a correspondingly short emission in the time domain, which is equivalent to a broad response in the frequency domain. This increase in the noise floor of the acoustic spectrum is uniquely associated with the presence of inertial cavitation. Although much of the resulting signal, particularly the higher-frequency components, is absorbed in the vicinity of the bubble, the remainder is easily detected. Perhaps of equal importance, the detected signal is proportional to the absorbed signal, facilitating remote detection and quantification of cavitation-enhanced heating (Collin, 2009).

2.5.6 Other Considerations

2.5.6.1 Quantitative Analysis of Acoustic Spectra

Bubble behaviors which may give rise to various spectral components were discussed in the previous sections. Of course, the inverse problem - reconstructing a source field from recorded emissions - is more complicated. Even with an infinite number of ideal receivers, mapping a source field from four dimensions (three in space and one in time) to three dimension (a treatment map) is an ill-posed problem. Yet under many circumstances, it has been shown that quantification of the energy contained in the broadband (Coussios et al., 2006; Z. Kyriakou and Coussios, 2011) or ultraharmonic (Datta et al., 2008) components of even a single PCD signal *can* be indicative of treatment progress. That these relationships happen to emerge should not be mistaken for a physical law. Because of the wide range of bubble sizes and behaviors, it is impossible to gauge from a single quantity whether, for example, an ultraharmonic peak arises due to a single powerful arrested inertial collapse, or several bubbles stably oscillating in the period-doubling bifurcation zone - hence the ill-posedness of the problem.

Additionally, comparing the ‘energies’ across different categories (for example, ultraharmonic versus broadband) is not meaningful; firstly, because they have different bandwidths of integration; and secondly, because the levels of sound emitted by an inertial cavitation event is not comparable to that emitted by a stable cavitation event. Thus, seeing twice as much energy in the broadband signal as in the ultraharmonic signal does not permit the conclusion that there was ‘twice as much’ inertial cavitation as stable cavitation. Energies can only be meaningfully compared within a category with respect to itself across various HIFU conditions.

Though the results presented in the following chapters once again demonstrate a quantitative correlation between acoustic emissions and specific measures of treatment success, it should be emphasized that the results are empirical and spe-

cific to the experimental setup - for example, the size range and concentration of nuclei - not universal. Achieving universal applicability of this technique may be possible in the future through combining source reconstruction algorithms with cavitation bioeffect models.

2.5.6.2 Effect of Sample History

It has been shown that the cavitation state of a liquid is *not* time-invariant, but rather depends on the history of the sample being exposed to ultrasound (Neppiras, 1980). By sequentially increasing then decreasing power amplitudes through a successive range of values, a 'hysteresis' effect was clearly observed (Frohly et al., 2000). Specifically, broadening of spectral components with increases in acoustic power were sustained even when acoustic power was decreased (Figure 2.15). Practically, this indicates that once generated, a cavitation state can be sustained with reduced driving power. This observation has given birth to the concept of instigating cavitation with a high-amplitude 'seeding' pulse, and then driving the cavitating microbubbles at lower pressures. This is hypothesized to minimize collateral damage resulting from migration of the bubble cloud to the pre-focal volume, and is an active area of research. The hysteresis effect has also been exploited in recent work to sustain cavitation using a real-time PCD signal-based controller (Hockham et al., 2010).

2.5.6.3 Absorption

From a monitoring perspective, any attempt to quantify cavitation based on secondary acoustic emissions must take into account the frequency-dependent absorption of the signal radiated by cavitating microbubbles. In a laboratory setting, this consideration is often immaterial, because the geometry of the experimental setup is often fixed and quantitative comparisons are relative rather than absolute.

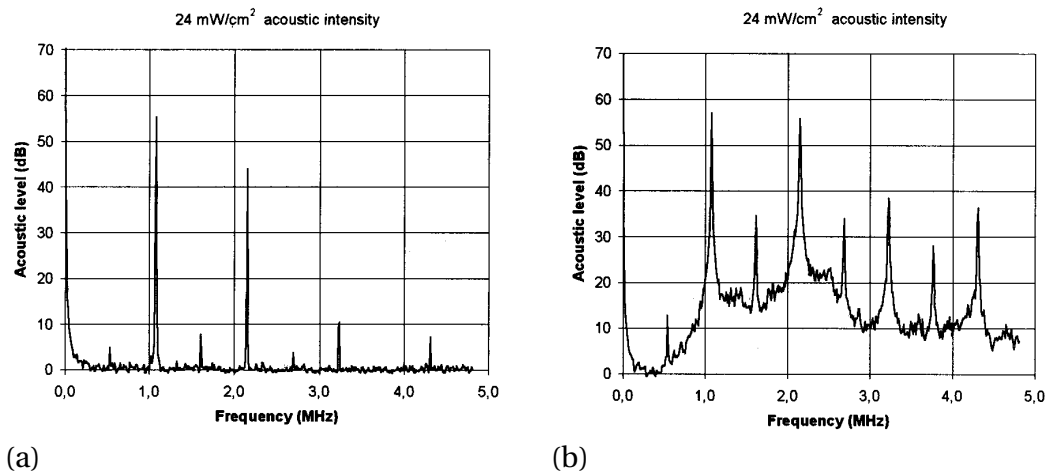


Figure 2.15: Cavitation noise spectrum distortion as a function of the acoustic intensity transmitted to the liquid, demonstrating the hysteresis effect. When an acoustic intensity of 24 mW/cm^2 is reached by gradually *increasing* the power, the resulting spectrum **(a)** is weaker in all components than that recorded at the same acoustic intensity achieved by gradually *decreasing* power **(b)** (Frohly et al., 2000). Although not shown here, the spectrum observed **(b)** contained more powerful harmonics, ultraharmonics, and broadband emissions than even a 60 mW/cm^2 reached by gradual power increase.

However, in a clinical setting, if the same course of treatment were applied at increased tissue depths, decreases in both the focal pressures and the returning signal can be expected. Moreover, comparing emissions across frequencies requires that the received signal is deconvolved with the frequency response of the medium.

2.6 THERAPEUTIC APPLICATIONS

Having explored the radial dynamics of bubbles in response to an ultrasound field and the various effects which they may have on surroundings, therapeutic applications of ultrasound, the acoustic parameter space employed in each application, and the role that cavitation is hypothesized to play are investigated. The objective is to apply the insights gained from research in other applications to the task of drug delivery in the solid tumor. The most commonly varied parameters are shown in

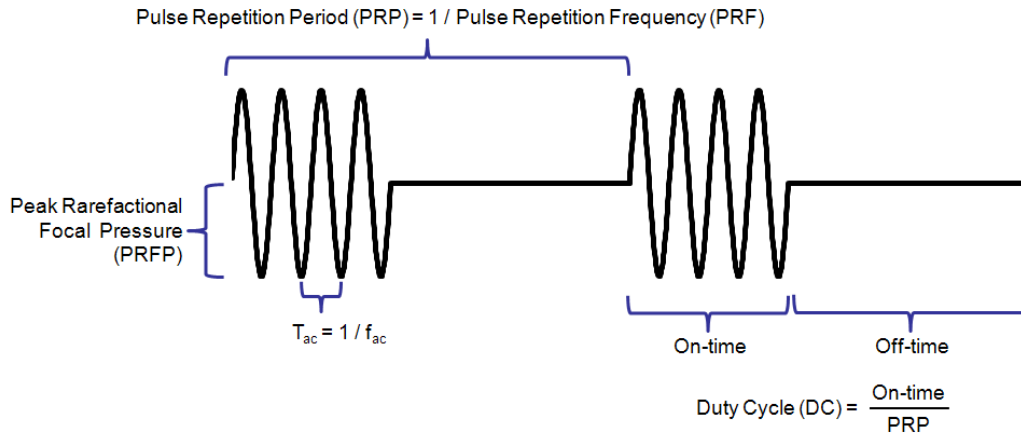


Figure 2.16: Idealized pressure-vs-time waveform elucidating various commonly cited acoustic parameters.

Figure 2.16, and include acoustic frequency (f_{ac}), peak rarefactional focal pressure (PRFP), duty cycle (DC), pulse repetition frequency (PRF), and total treatment duration.

2.6.1 Lithotripsy

The first clinically applied therapeutic ultrasound technology, extracorporeal shockwave lithotripsy (ESWL), deploys ultrasound pulses of microsecond-order timescales and peak pressures of tens of MPa to fragment kidney stones and gall stones (Sturtevant, 1989). Several hundred to over a thousand such pulses may be delivered in a typical clinical lithotripsy procedure, with a PRF generally below 2Hz, so that the time-averaged acoustic intensity remains low.

Although the original intended mechanism of action is simply the shearing action of the shock wave - generated extracorporeally by a spark at one focus of an ellipsoidal reflector, and guided by that reflector towards the stone at the other focus - it is now generally accepted that cavitation plays a role in stone fragmentation (Koch and Grunewald, 1989). As discussed in Section §2.4.2.1, the radiated shock

wave and fluid jetting from a collapsing bubble have been shown to erode solid surfaces.

2.6.2 Thermal Ablation

As shown in Sections §2.2 and §2.5.5, ultrasound, and in particular ultrasound-driven inertial cavitation, can locally deposit energy in a manner that has applications in thermal ablation (Coussios and Roy, 2008) of deep-seated tumors. This technique, known as High-Intensity Focused Ultrasound (HIFU), uses frequencies in the 1MHz range and continuous wave (CW) excitation at thousands of watts per square centimeter for up to several seconds to achieve temperature rises of tens of degrees in focal volumes comparable in size to a grain of rice (ter Haar and Coussios, 2007). Cavitation has been shown to yield local heating rates up to 330% higher than those predicted by acoustic absorption (Figure 2.17), in the context of the bioheat transport equation (Coussios et al., 2007).

However, several obstacles still exist to the widespread clinical use of HIFU for thermal ablation. First, a drawback of the treatment resolution offered by HIFU is that, for larger tumors, serial ablation of adjacent regions can take several hours, which is clinically undesirable. Additionally, physiological inhomogeneities can lead to deviation from heat deposition models, resulting in undertreated and overtreated areas. Spatially resolving treatment progress using an array of PCDs is an active area of research (Gyongy and Coussios, 2010).

2.6.3 Histotripsy

As an alternative to thermal ablation of unwanted tissue, histotripsy is a technology which applies HIFU to the *mechanical* ‘fractionation’ of tissue - that is, the destruction of cellular and supercellular structure within the treated volume, resulting in an

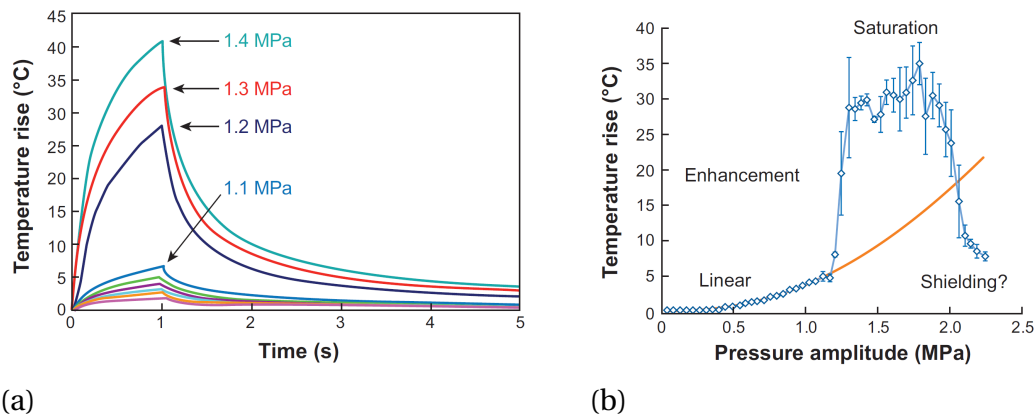


Figure 2.17: Temperature rise in a tissue-mimicking material resulting from 1-second CW excitation at various pressures. **(a)** Temperature curves across a range of peak rarefactional pressures. **(b)** Summary of experimental (blue) versus numerical (orange) temperature rise versus excitation pressure at $t = 1$ s. The numerical simulation does not account for the effects of cavitation. Note the abrupt increase in temperature rise at approximately 1.2 MPa, which corresponds to an increase in detected broadband emissions (not shown) (Coussios et al., 2007).

acellular liquid (Xu et al., 2004; Hall et al., 2007) that, irrigated away, reveals smooth cavities (Parsons et al., 2006).

It is hypothesized that at the acoustic focus, a microbubble cloud is generated from endogenous nuclei, and the mechanical stresses generated during the oscillation and inertial collapse of the cloud locally subdivide the targeted tissue, resulting in cellular destruction (Tran et al., 2003; Xu et al., 2005, 2007) and tissue homogenization. Studies to date have used ultrasound frequencies close to 750kHz. In order to achieve inertial collapses, high-intensity (20 MPa) short (3-30 cycle) ultrasound pulses are deployed with a pulse repetition frequency (PRF) ranging from 500Hz to 20kHz and sonication times of up to several minutes per treatment site (Xu et al., 2004; Roberts et al., 2006; Lake et al., 2008).

Treatment success is confirmed by the presence of anechoic regions in diagnostic ultrasound images and *ex vivo* by histological analysis. In both cases, the

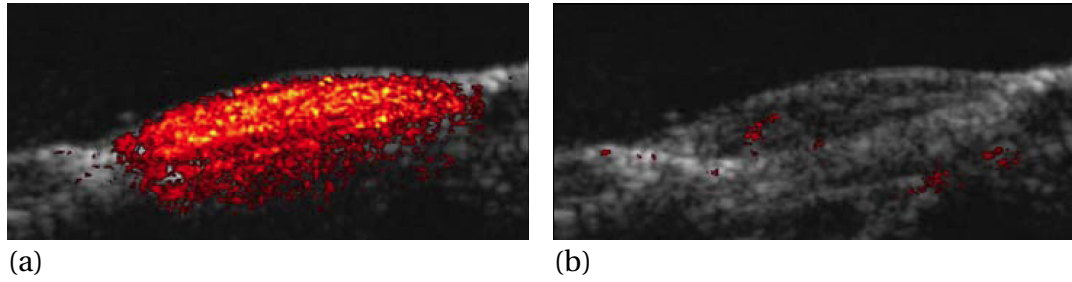


Figure 2.18: Blood perfusion in a 10 mm mouse colon adenocarcinoma, five seconds before **(a)** and ten seconds after **(b)** sonication (Chin et al., 2009)

demarcation between treated and untreated areas is sharp and visually apparent.

Under slightly milder sonication regimes and in the presence of exogenous nuclei, the possibility of limiting mechanical tissue disruption to the microvascular structure has been explored, with the paradoxically disparate objectives of either inducing hemorrhaging (Bohmer et al., 2010) or disrupting blood flow (Goertz et al., 2008). To achieve the former, a 1.2 MHz transducer was driven at 2 MPa in eleven 10,000-cycle bursts separated by 30 seconds. Although enhanced extravasation of a 66 kDa labelled macromolecules was observed, it was not found to be statistically significant. Enhanced extravasation in the muscle beneath the tumor, however, was significant, highlighting two challenges: first, that the axial focal depth of practical therapeutic transducers is large relative to preclinical tumor models, and second, that the healthy tissue surrounding a tumor is often comparatively rich in vasculature and therefore circulating nuclei. Both of these make it difficult to restrict cavitation and its bioeffects to the tumor volume and leave healthy tissue unaffected.

Using the same therapeutic transducer system, another group demonstrated that by applying three pulse ‘trains’ (separated by 20 seconds) of ten pulses at 5 MPa PRFP, 100000 cycles, and a PRF of 1Hz, transient reduction of blood flow was achieved (Figure 2.18), and daily treatment under this regime resulted in the deceleration of tumor growth and even reduction of tumor size (Chin et al., 2009). Inter-

estingly, the effect is hypothesized to be linked to an innate inflammatory response encouraged by the HIFU-induced damage, rather than the ischemia resulting from mechanical disruption of vasculature itself (Hu et al., 2007).

2.6.4 Thrombolysis

Datta *et al* developed a protocol for the *in vitro* formation of human blood clots to study mechanisms for enhancing the activity of recombinant tissue plasminogen activator (rt-PA), a thrombolytic enzyme (Datta et al., 2006). Diffusion of the enzyme into the clot is the rate-limiting step in the process, and this study demonstrated that the presence of cavitation in general, and stable cavitation in particular, can greatly enhance the rate rt-PA transport into the blood clot.

By co-injecting encapsulated bubbles in the 1-5 μm range and applying ultrasound, Datta, et al. were able to further enhance the microstreaming effect to supplement the low diffusive flux of rt-PA with enhanced *convective* mass transfer. Long treatments (30 min, 80% duty cycle) of ultrasound at 120 kHz were found to be most effective for this purpose. Three different peak pressures were tested, from 0.15 MPa to 0.35 MPa, chosen to induce either no cavitation, stable cavitation, or inertial cavitation. The study concluded that the presence of stably cavitating microbubbles (as identified by the presence of subharmonic emissions but absence of broadband emissions) yielded the most pronounced increase in penetration depth of rt-PA, as observed by fluorescent tagging and visualization (Figure 2.19), as well as the highest percent of clot mass loss (Datta et al., 2008).

Perhaps just as significantly, this study demonstrated that there was a quantitative correlation between the extent of clot mass loss and energy contained within the subharmonic emissions from the clot mass, which suggests that under certain circumstances, the progress of drug delivery may be predicted by the amount of

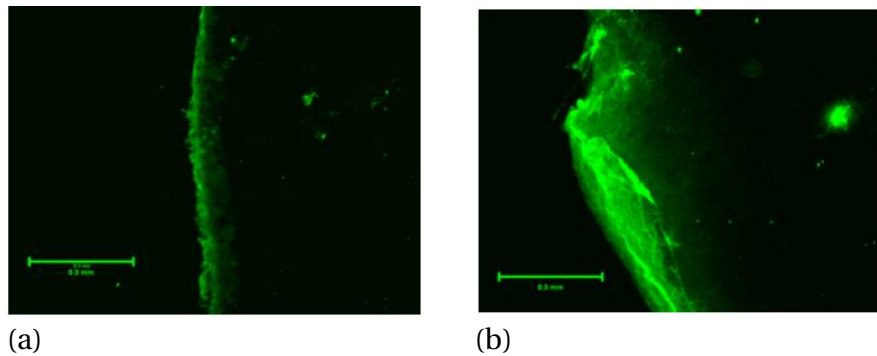


Figure 2.19: Distribution of fluorescent-tagged rt-PA into an *in vitro* thrombus model. (a) rt-PA and Ultrasound without microbubbles; (b) rt-PA, Ultrasound and microbubbles (Datta et al., 2008)

stable cavitation detected.

2.6.5 Gene Transfer

There have been a number of studies on ultrasound-enhanced transfer of genes and other macromolecules. A broad range of acoustic parameters have been tested, including continuous-wave 2 MHz excitation at up to 1 MPa for 60 seconds (Bao et al., 1997), to the very short, high-intensity lithotriptic pulses described in Section §2.6.1 (Ohl et al., 2006b).

It is hypothesized that sonoporation - the permeabilization of cell membranes by an acoustic field - is responsible for the delivery of large molecules into cells. Depending upon the extent of the rupture, this may result in irreversible cell damage (cell lysis) or reversible membrane damage, during which large molecules can leak into the cell before it is resealed. Reversible permeation of the cell membrane has been attributed to fluid jetting by collapsing bubbles (Ohl et al., 2006b) as well as the more gentle stresses developed during cavitation microstreaming (Collis et al., 2010a).

In the case of bubble collapse, it has proven challenging to achieve sonopora-

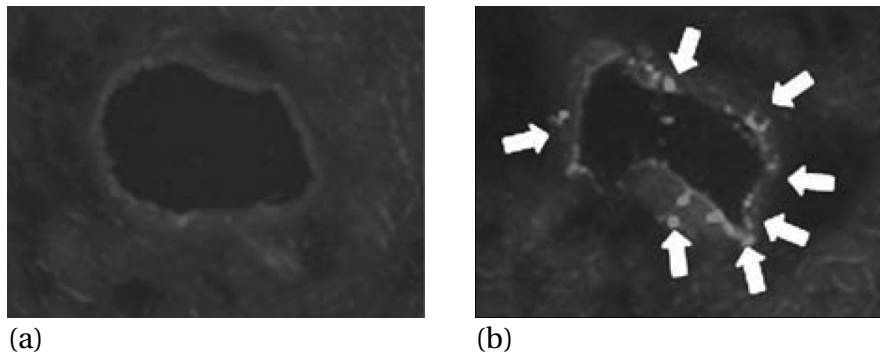


Figure 2.20: Fluorescence microscopy of coronary artery treated with (a) microbubbles and plasmid DNA or (b) microbubbles, plasmid DNA and ultrasound. Bright spots (arrows) indicate the presence of pDNA (Seip et al., 2010)

tion without significant collateral damage to nearby cells. One study of adherent cell monolayers identified four distinct regions of cellular response to a collapsing bubble: those closest to the bubble were detached from the substrate completely; adjacent to this region, cells remained attached but were irreversibly damaged; further still, cells were reversibly sonoporated; and finally cells furthest from the collapsing bubble were unaffected (Ohl et al., 2006b).

The combination of high sonoporative rates and limited cellular destruction has proven elusive. Moreover, the majority of these studies are focused on delivery to suspensions of cells (Bao et al., 1997; Rahim et al., 2006; Kim et al., 1996) or adherent cell monolayers, which are not representative of the physiological environment. One study *has* achieved delivery and expression of plasmid DNA *in vivo*, but reporter gene expression was limited to within $\sim 20 \mu\text{m}$ of the arterial wall (Figure 2.20), and sonoporative efficiency was not quantified (Seip et al., 2010).

2.6.6 Blood-Brain Barrier Permeation

The blood-brain barrier (BBB) is a system of tightly joined endothelial cells which separates blood from cerebrospinal fluid. This system resists the transport of most

macromolecules, which poses a significant barrier to treatment of many neurologic disorders (Rubin and Staddon, 1999).

Recent studies in mice have shown that transient disruption of the BBB is possible without causing undesired tissue damage, and that this objective is facilitated by the introduction of cavitation nuclei into the bloodstream (McDannold et al., 2006, 2008; Vykhodtseva et al., 2008). Ultrasonic frequencies of 0.5-1.5MHz have been employed, with peak rarefaction pressures of 0.5-3MPa, and low (10-20%) duty cycles, with treatment times of up to a few minutes (often in 30-second ‘shots’ interlaced with delays to allow heat dissipation).

Several mechanisms have been hypothesized for BBB permeation. One is that the bubble’s first-order motion in response to acoustic excitation triggers stretch-activated channels (van Wamel et al., 2004). Another is that the second-order cavitation microstreaming triggers these channels (McDannold et al., 2006, 2008). Lastly, it has been proposed that the permeation does not occur at stretch-activated channels at all, but rather between otherwise tightly-packed endothelial cells of the BBB (DePaola et al., 1999). Recent work (Choi et al., 2010) has suggested that short-burst ultrasound is equally effective in triggering permeation, lending credence to the first and third hypotheses, since microstreaming is unlikely to develop in very short time-scales.

2.7 IMPLICATIONS FOR THE PRESENT WORK

The objective of the present research is to use high-intensity focused ultrasound (HIFU) to improve the distribution of cytotoxic therapeutics in solid tumors. In light of the research into ablative (§2.6.2) and fractionative (§2.6.3) techniques for treating solid tumors, it is well worth considering the value of exploring yet *another*

ultrasound-based therapeutic regimen.

Both of these techniques rely upon extreme pressures impinging upon endogenous nuclei to generate violent cavitation activity and achieve the desired effect. As discussed in Section §2.3.2, with increased violence comes increased collateral damage, which can be undesirable in many circumstances. A useful analogy in this case is the work described in Section §2.6.6 on blood-brain barrier permeation. The historical use of *endogenous* nuclei to permeate the BBB was largely effective, but resulted in clinically unacceptable damage to surrounding brain tissue. By introducing microbubbles into the bloodstream, permeation could be achieved at significantly milder acoustic intensities. Furthermore, the technique no longer relied upon the coarseness of acoustic focusing for localization, but instead on its confluence with microbubble presence to produce the effect exactly where it is needed: at the interface between the blood plasma and the cerebrospinal fluid.

The same principle holds true regarding the treatment of cancer. In circumstances where collateral damage resulting from 20 MPa ultrasound is unacceptable, a milder ultrasound regime is required. And, as in the case of Alzheimers treatment (to take an example), reducing the peak pressures by an order of magnitude is mitigated by both the introduction of nuclei into the bloodstream, and the potency and specificity of the the therapeutics being delivered, which relieve the burden on ultrasound to treat all pathologic cells.

Analogies, of course, have their limitations. It may be tempting to, for example, blindly apply the ultrasound parameters used in BBB disruption to the treatment of cancer. However, this would fail to consider an important pathophysiological difference between the two: the transmural pressure gradient. While permeation of the BBB results in passive extravasation of macromolecules (owing to an otherwise intact vasculature and the resulting pressure gradient), solid tumor vasculature is of-

ten compromised to begin with, and therefore doesn't exhibit the pressure gradient which would promote passive extravasation (see Section §1.3.2, and in particular Figure 1.10).

This critical difference highlights the importance of understanding both the physiological environment under consideration and the variety of bioeffects therapeutic ultrasound is capable of. Based on this body of knowledge, it is hypothesized that by instigating and controlling cavitation in a manner that promotes convective transport, the penetration, activity, and efficacy of intravenously circulating therapeutics in solid tumors can be improved.

It is evident that controlled, localized cavitation requires the introduction of exogenous nuclei into the bloodstream. Moreover, it has been shown that stable cavitation can generate long-lasting microstreaming in the surrounding fluid (§2.4.2.2), which may be useful in improving the distribution of a therapeutic in blood vessels with stagnant flow (§1.3.1). However, based on the fluidics of porous media, it is unlikely that microstreaming will extend into the dense extracellular matrix of the tumor interstitium. Instead, it may be required to drive exogenous nuclei to inertial collapse, with the intent that the resulting high-speed liquid jets (§2.4.2.1) may propel therapeutics into the interstitium.

In the following three chapters, a simplified experimental model is first developed to demonstrate the feasibility of cavitation-enhanced drug delivery in a tumor-mimicking model. A novel numerical treatment of microstreaming is then presented, which for the first time couples the radial bubble oscillations to the streaming flow imposed on the surrounding fluid, and is used as a tool to optimize the combination of bubble size, excitation frequency and excitation amplitude required to maximize transport of therapeutic molecules. Lastly, the principle is tested in tumors implanted in small animals using a new generation of anti-cancer

agents - adenoviruses.



CAVITATION-ENHANCED MACROMOLECULE TRANSPORT IN VITRO

In this chapter, the ability of cavitation instigated by high-intensity focused ultrasound (HIFU) to increase convective transport of a model therapeutic is investigated in a cell-free *in vitro* tumor model. Transport is quantified using post-treatment image analysis of the distribution of a dye-labeled macromolecule, while cavitation activity is quantified by analyzing passively recorded acoustic emissions. The strong correlation found between inertial cavitation activity and drug delivery suggests both the mechanism of action of this phenomenon, and the clinical potential to develop a non-invasive system for monitoring ultrasound-enhanced drug delivery.

This chapter has been published in an alternative form in (Rifai et al., 2010).

In Chapter 1, it was shown that a significant obstacle to cancer therapy is the failure of tumor vasculature to facilitate distribution of therapeutic agents at sufficient concentrations throughout the solid tumor. Chapter 2 demonstrated the ability of high-intensity focused ultrasound (HIFU) to enhance convective mixing by a variety of mechanisms, including jetting and microstreaming.

To determine whether this technology meets the therapeutic need, a simplified experimental model has been developed. The model is based on an optically trans-

parent tissue-mimicking polymer molded to simulate certain aspects of the tumor vasculature, including the junction between a flowing vessel and an obstructed - or 'blunt-end' - vessel (see Section §1.3.1, and in particular Figure 1.1). After establishing flow of a dye-labeled macromolecule in solution through the flowing vessel and applying HIFU at the junction between the vessels, the extent to which the macromolecule is delivered to the obstructed vessel is optically measured. By testing various combinations of duration and intensity of sonication, an effective range within the parameter space is established.

In order to gain a further understanding of the physical mechanisms at play, and gauge the feasibility of extracorporeal monitoring of treatment progress, acoustic emissions are passively recorded and analyzed using numerical implementations of the concepts discussed in Section §2.5. Additionally, the role of exogenous nuclei on both cavitation activity and macromolecule transport is investigated, with the objective of furthering the understanding and control of the process.

The experimental design, setup, execution, and data acquisition and analysis are introduced below, with an eye towards developing an apparatus which can be translated into pre-clinical and eventually clinical use.

3.1 EXPERIMENTAL DESIGN AND SETUP

A schematic of the experimental setup used in this chapter is shown in Figure 3.1. The components can be divided into three groups: the tumor/blood flow model, the sonication equipment, and the treatment monitoring equipment.

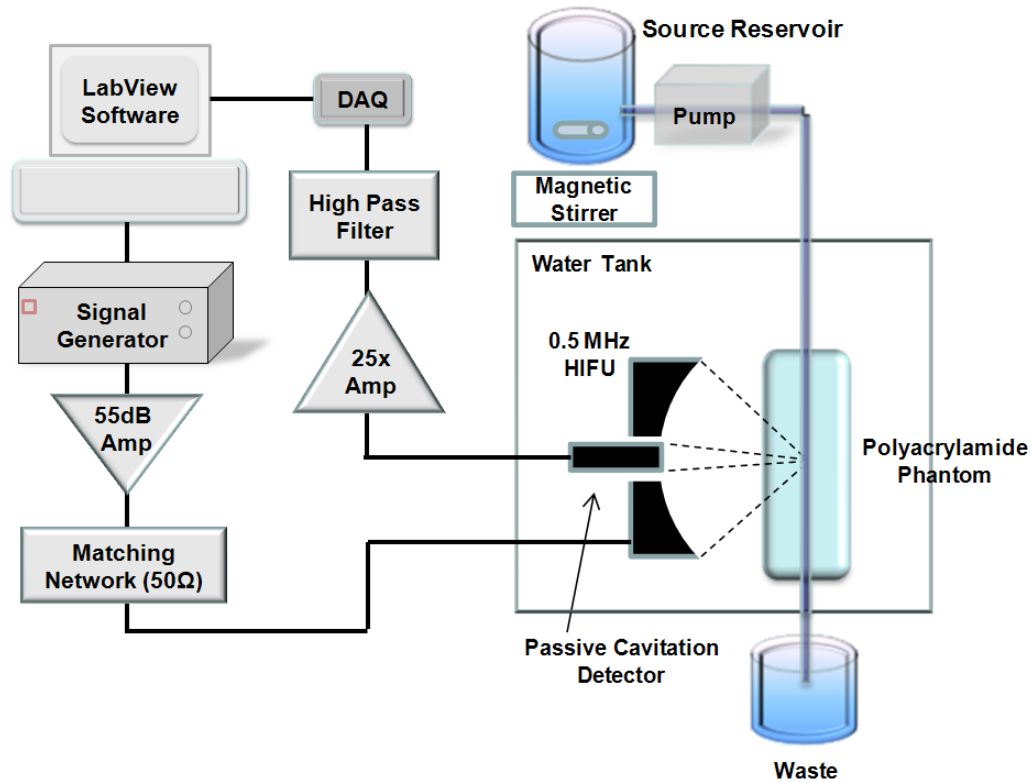


Figure 3.1: Experimental setup for ultrasound exposure of tumor phantom vessels. A 0.5MHz HIFU transducer is focused onto the junction between channel (1) (in which flow is driven by a peristaltic pump) and channel (3) (not shown, projecting out of the page). A confocally aligned PCD records acoustic emissions

3.1.1 Tumor Model

Tumors are inherently complex and inhomogeneous structures, varying in size, vascular distribution, and acoustic properties from one sample to the next depending on tissue of origin, maturity, and other attributes. In order to eliminate these variables as confounding factors, it is helpful to begin with a simplified setup and gradually move towards more sophisticated and clinically relevant configurations. Thus, rather than attempt to culture tumor spheroids or procure *ex vivo* tumor tissue for preliminary experiments, this series of experiments employs polymers which have tissue-like attributes, can be cast into a mold (often called a tissue ‘phantom’), and the manufacturing of which generates highly reproducible properties from one

sample to the next.

3.1.1.1 Tissue-Mimicking Material

Desirable characteristics of the tissue-mimicking material (TMM) include acoustic properties (such as speed of sound and attenuation) closely matched to those of tissue, optical transparency (for photography), and rigidity (because the TMM will be cast into a complex mold involving channels, which must not collapse or distort during experimentation). Because only vascular transport is considered in this chapter, the porosity and biocompatibility of the TMM need not be matched to physiologic values. However, in subsequent studies (§5.1) which sought to model extravasation, interstitial transport, and cellular response, the constraints of biocompatibility and physiologic porosity are enforced. The following candidate materials were considered for this study:

Agar: Previous work has used agar to mimic tissue Burlew et al. (1990). Purchased as a powder, agar molds are prepared by suspending the powder in water, heating to dissolve, and allowing to cool, at which point the gel stabilizes. By changing the concentration of agar in the solution, the material properties of the gel can be modulated. 3% agar provides material properties which are similar to tissue - a speed of sound of around 1540 m/s and a density of 1020 kg/m³. For soft tissues, speed of sound falls between 1450 m/s for fat and 1640 m/s for the eye lens (a value of 1540 m/s is built into most ultrasound scanners). Soft tissue densities vary between 920 kg/m³ for fat and 1070 kg/m³ for muscle. Agar therefore has a characteristic impedance (product of density and compressibility) similar to those of water and tissue, ensuring good transmission of ultrasound from the gel into an aqueous medium. Attenuation in agar is much lower than tissue (approximately 0.07 dB/cm/MHz), which poses a

problem in thermal ablation studies, but increases simplicity in a micromixing study, since thermal effects are not of interest and the low attenuation enables approximation of in situ pressures with the values obtained in the free field Burlew et al. (1990).

Polyacrylamide (PAA): Optically transparent PAA gel has been used in experiments as a tissue-mimicking phantom Lafon et al. (2005). Acrylamide monomer is dissolved in buffered solution and, upon the addition of a polymerizing agent and a catalyst, an exothermic polymerization reaction occurs. At 15% acrylamide, the speed of sound and density of the resulting gel are 1544 m/s and 1044 kg/m³, respectively. For the purposes of the present study, PAA exhibits two advantages over agar: first, because the reaction is chemically catalyzed, polymerization is rapid (requiring less than 30 minutes) and spatially uniform. Agar, in contrast, gels upon cooling, causing polymerization from the outside inward. This can take substantially longer times for proper setting. Second, PAA is more rigid and durable. Note that PAA is not biocompatible, and therefore cannot be employed in cell-embedding studies.

Biologically-Derived Cell Scaffolds: Progressing in complexity (and cost), biological interstitium extracts such as Matrigel[®] most faithfully mimic the extracellular environment (Kleinman and Martin, 2005). These gelatinous protein mixtures promote cellular behaviors and interactions that are otherwise not reproducible in laboratory conditions. However, when cast into molds with millimeter-sized vessels, Matrigel-derived phantoms were found to collapse under even moderate flow rates, and were therefore unsuitable for the present work.

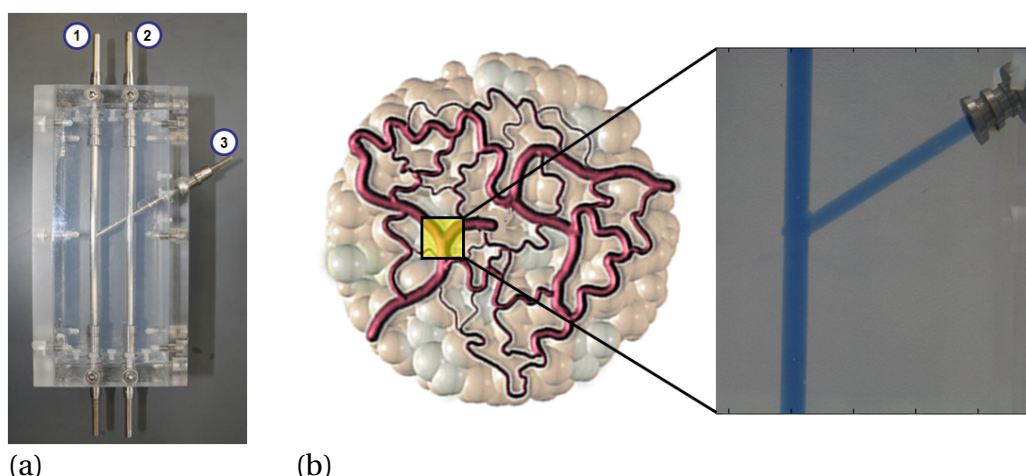


Figure 3.2: **(a)** Perspex chamber used to cast the PAA phantom. Upon polymerization, the metal mandrels can be removed, creating channels of radius 2.3 mm intersecting at a 60° angle within the phantom (In this series of experiments, only mandrels (1) and (3) were used). Additionally, the front and back perspex walls can be removed, allowing ultrasound excitation from the back (going out of the page) and image acquisition from the front. **(b)** Illustrative representation of tumor vasculature, including the branch between a normal vessel and a blunt-end vessel (yellow box). The magnified view of the optically transparent phantom immersed in water, with blue dye running through the channels, is intended to model this feature of tumor vasculature.

In light of its optical and acoustic properties, ease of handling, and robustness, PAA phantoms were used in this series of experiments. The polymer was cast according to established protocols (Appendix A) in the custom Perspex chamber shown in Figure 3.2.

3.1.1.2 Therapeutic Agent

Characteristics of a suitable model therapeutic include low diffusivity and optical contrast. Small molecule dyes are unsuitable for the task, as diffusion into vessel walls and into the obstructed channel introduce systematic sources of error and high baseline levels of transport. As an alternative, this study employs Bovine Serum Albumin (BSA) as a therapeutic analog. With a molecular weight of 60 kDa, the dif-

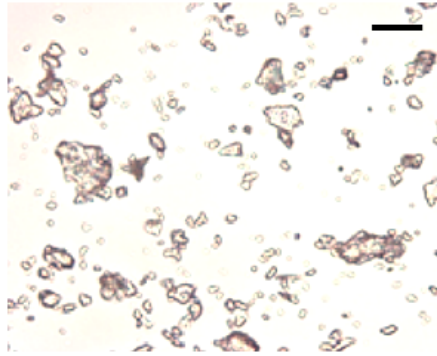


Figure 3.3: Off-the-shelf talc microparticles (optical microscopy) (Cox, 2007) Scale bar: 25 μm

fusion rate of BSA is negligible for the time scales involved in these experiments. Although BSA in solution is optically transparent, it has been shown that Evans Blue dye binds tightly to BSA in a non-reversible manner Patterson et al. (1992). Therefore, a 20x stock solution of 4% BSA and 0.67 mg/ml Evans blue dye was prepared in 1x Dulbecco's PBS buffer. This solution was diluted 1:19 for all experiments.

3.1.1.3 Cavitation Nuclei

One leg of the experiment sought to examine the effect of exogenous nuclei on both cavitation and drug delivery. In order to enable a direct comparison between exposure conditions without and with cavitation activity, it was decided to avoid the high scattering and attenuation associated with ultrasound contrast agents. Instead, off-the-shelf talc microparticles (Figure 3.3) were injected at a concentration of 0.5% (w/v). These particles are hydrophobic and have high surface roughness, two parameters that suggest (and experimentation confirms) that they both reduce the cavitation threshold (see Section §2.3.3.3) and increase cavitation activity. Note that these particles quickly settle in solution, and must be either resuspended immediately prior to use or continuously stirred.

3.1.1.4 Flow Control

The purpose of the flow system is to deliver the model therapeutic through the vertical channel (channel (1) in Figure 3.2(a)), which mimics a properly functioning blood vessel. By applying ultrasound from behind the phantom, directed out of the page and focused at the junction between channels (1) and (3), the objective is to propel therapeutic into channel (3), which is otherwise stagnant.

Therefore, the flow system was designed to deliver a constant, controllable flow rate of liquid through channel (1), and to allow the introduction of cavitation nuclei into the ‘bloodstream’ as necessary. The system consists of a peristaltic pump drawing from a source reservoir containing the solution of BSA and Evans Blue dye described above. The pump delivers the solution to an intermediate reservoir (to dampen flow pulsatility introduced by the peristaltic pump), which drains at a constant rate into tubing attached to the vertical channel (1). Just prior to the point of attachment, a 3-way valve is introduced, to allow for syringe injection of cavitation nuclei immediately prior to acoustic excitation. The outflow from channel (1) collects into a drain reservoir. The blunt-end vessel is mechanically clamped, to eliminate any net outflow from channel (1) into channel (3), and is flushed prior to each experimental run.

3.1.2 Sonication

Custom LabView software was developed, triggering a signal generator (Agilent 33220A) to output a continuous 483kHz sinusoid, which is transmitted to a power amplifier (E&I), through an impedance-matching network, and on to the HIFU device - a single-element spherically focused piezoceramic transducer (HIFU107, Sonic Concepts, Bothell, WA) with a 483kHz narrowband response and a circular opening to allow coaxial placement of a PCD (Figure 3.4). Needle-hydrophone cali-



Figure 3.4: Sonic Concepts 483kHz focused HIFU transducer with circular opening for coaxial PCD placement

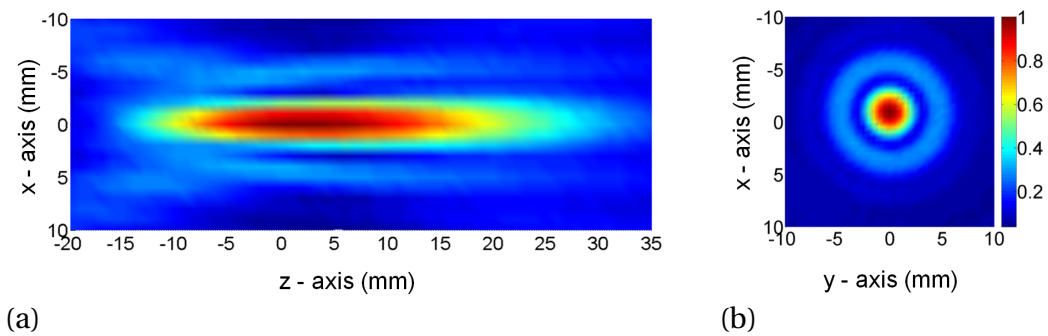


Figure 3.5: Longitudinal **(a)** and transverse **(b)** pressure profile surfaces of the HIFU transducer, shown on equal scales, with both surfaces intersecting the focal maximum. As a result of the low ratio of element size to focal depth, the focal volume is ‘cigar’-shaped, with strong transverse focusing and weak axial focusing. The pressure values in the figure are normalized to the peak value.

bration determined the transducer to have a 60 mm focal length, -6 dB beam diameter of 3 mm, and -6 dB focal depth of 10 mm (Figure 3.5).

The HIFU transducer was aligned such that the focus was coincident with the intersection of the two channels, and the direction of sound propagation was normal to the plane of the channels. This orientation was chosen to minimize the effect of acoustic radiation force, which acts in the direction of sound propagation, on transport of BSA.

3.1.3 Data Acquisition

3.1.3.1 Passive Cavitation Detection

Acoustic emissions at frequencies above than the fundamental were recorded by a broadband spherically focused PCD (Panametrics V319) with 15 MHz centre frequency and 69 mm focal length, mounted coaxially and confocally to the HIFU transducer. The received PCD signal was amplified 25-fold (Stanford Research Systems SR445A) high-pass-filtered at 800 kHz (Krohn Hite 3944) to suppress reflections at the driving frequency and allow increased vertical resolution at frequencies of interest (harmonics, ultraharmonics, broadband emissions), and digitized at a sampling rate of 25MHz (PicoScope 3205), and transmitted to custom LabView software for processing (§3.2).

Acoustic emissions at frequencies below the fundamental were recorded by an unfocused hydrophone (Panametrics V319) with a flat frequency response up to 1 MHz, mounted adjacent to (but out of the primary field of) the HIFU transducer. The received signal was amplified 25-fold (Stanford Research Systems SR445A), low-pass-filtered at 400 kHz (Krohn Hite 3944) to suppress reflections at the driving frequency and allow increased vertical resolution at subharmonic frequencies, and once again digitized at a sampling rate of 25MHz (PicoScope 3205).

3.1.3.2 Image Acquisition

A Canon EOS 400D Digital SLR camera was mounted co-axially with the HIFU transducer (but outside the water tank!) (in Figure 3.1, the camera is on the right but outside the field of view). Lighting conditions were controlled as much as possible to generate reproducible results suitable for quantitative analysis. In particular, human shadows can be sources of inconsistent lighting, so pictures were taken remotely or from an unintrusive angle. Firm mounting of the camera precluded in-



Figure 3.6: Screenshot of the custom-designed LabView interface used to carry out the experiment

incorporating a rigid registration algorithm in the image analysis software.

3.2 CONTROLLING SOFTWARE

Custom LabView software was developed to carry out all experiments. The interface is shown in Figure 3.6. The software includes the following features:

- Ability to generate complex excitation regimes, including, for example, a brief cavitation instigation pulse followed by a lower amplitude sustained pulse, or

repetitions of a cycle of pulses. These regimes could be relevant in light of the hysteresis effect discussed in Section §2.5.6.2.

- Real-time measures of received signal saturation, and ability to vary vertical range between experimental runs
- Signal post-processing to receive quasi-real-time power spectral analysis and temporally resolved signal variance.

Transmission and storage of the digitized PCD and hydrophone signals suffer hardware limitations. Specifically, data is recorded onto the buffer of the digital oscilloscope in 'blocks', then transmitted to the PC. Recording and transmission may not take place concurrently, and data transfer is the rate-determining step. When acquiring two channels of data at 25 MHz, the system is capable of recording approximately 4% of the total received signal (for example, 800 μ s recording, followed by a 19ms delay for transmission). Owing to this bottleneck, there is a trade-off between block length and acquisition interval. Various combinations were tested, before 'optimal' settings of 20,000 samples and 20 ms block separation were chosen. Thus, each 2 second experiment yielded 100 blocks, each of length 20,000 samples. Further reductions in the acquisition interval yielded statistically insignificant quantitative differences in the recorded energy spectra. Informally, the stochastic nature of cavitation activity, 'smoothed' over two seconds of data acquisition, yielded an 'ensemble average' which varied inappreciably at the millisecond time-scale.

An additional point should be made regarding the digitizer's vertical range. Because signals generated by acoustic emissions span several orders of magnitude (Figure 3.19(b)), so too do their electrically transduced counterparts, and therefore selecting a vertical range inevitably involves compromise. Setting it too high risks allowing weaker signal to go undetected, while setting it too low risks 'clip-

ping' stronger signals. Altering it from run to run, meanwhile, introduces artifactual changes in the calculated 'noise' floor, which is often used as a measure of inertial cavitation activity. In these experiments, the lowest vertical range which avoided clipping was chosen.

Notably, because the strongest signals arise from inertially collapsing bubbles, they closely resemble delta functions, so clipping them does not yield artifactual harmonics (as clipping a sinusoid would), but simply understates the magnitude of the inertial collapse, which may be acceptable in studies where only the presence of inertial collapse is monitored.

3.3 EXPERIMENTAL PROCEDURE

3.3.1 Alignment

The first step in each experiment is to confocally align the HIFU transducer and PCD. Briefly, a pulser-receiver (JSR DPR300) drives the HIFU in pulse-echo mode, with a 6 mm brass sphere approximately at the focus. Based on the water temperature, the speed of sound and the time delay corresponding to the pulse-echo path length is calculated (the focal length is determined during calibration). The pulse-echo received signal is displayed on an oscilloscope (LeCroy WaveSurfer 434). The sphere is attached to a 3-D positioning system, and is aligned depthwise using the appropriate time delay, and transversely by maximizing the reflected signal. The procedure is repeated for the PCD (with the HIFU fixed in place).

Once confocally aligned, the PCD is used to align the transducers with the phantom channel (because of its narrower beam width, manual alignment with the PCD is much more precise). The phantom is mounted on a 3-D positioning system, and all tubing is attached to minimize handling upon alignment. The channel is filled

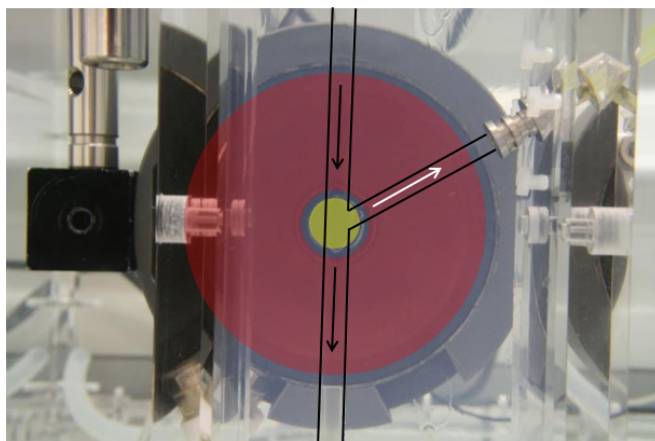


Figure 3.7: HIFU transducer (highlighted in red) and PCD (yellow) confocally aligned at the junction between the tissue phantom channels. Black arrows used to indicate direction of flow. The objective is to deliver therapeutic in the direction of the white arrow.

with air to maximize the acoustic impedance mismatch (and therefore the reflections). Appropriate delay times are used to ensure that the foci of the transducers lie at the midpoint of the front and rear channel walls. Once aligned (Figure 3.7), all remaining aspects of the experiment are controlled from outside the water tank. Camera alignment is performed visually.

3.3.2 Experimental Run

Flow is established by the peristaltic pump at 10 ml/min. If exogenous nuclei are to be injected, the 3-way-valve is switched to the particle-injection syringe, and a column of Evans Blue/BSA solution containing a suspension of particles is dispensed into the tubing and into the vertical channel. The valve is switched back to the flow loop to resume the controlled flow rate. The LabView software (with the relevant HIFU excitation and data acquisition parameters) is triggered, which synchronously activates the signal generator and records acoustic emissions. Once recording is complete, a white plastic backing is inserted between the transducer and the phantom (to increase the visual contrast between dye and background) and a photograph

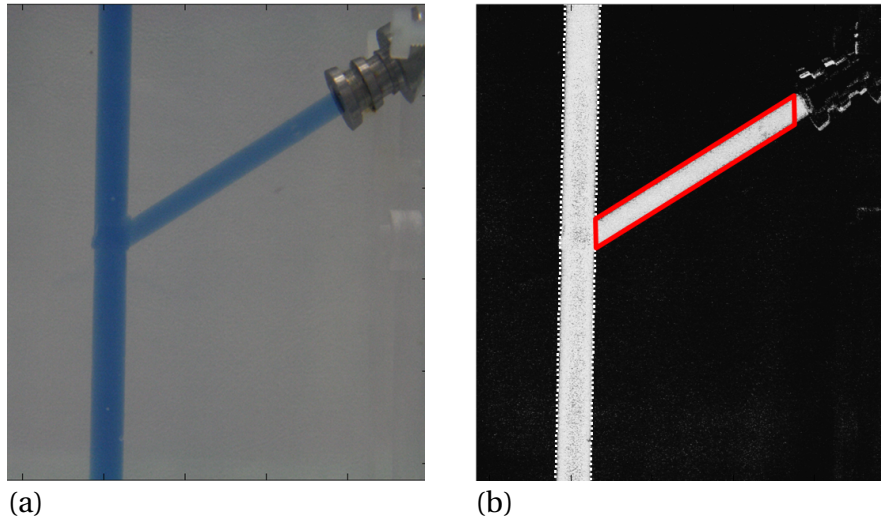


Figure 3.8: **(a)** Calibration photograph of the vessels perfused with BSA at its reservoir concentration (100%). **(b)** Gray-scale visualization of the 'red' RGB channel from the same photograph, after the background (0% BSA) image is subtracted. The edges of the obstructed vessel are outlined in red.

is acquired. This procedure is repeated across a range of pressures from 0.4 - 2.0 MPa and in the presence or absence of exogenous nuclei.

3.4 DATA ANALYSIS

3.4.1 Quantifying Drug Delivery

To quantify the delivery of BSA into the obstructed channel, the intensity value of each pixel along the obstructed vessel must be converted into a measure of BSA concentration at that point. Because lighting conditions vary spatially, and because of the asymmetrical three-dimensional structure of the phantom channels, it is necessary to create a calibration curve relating pixel intensity to BSA concentration at each pixel within the channel.

To accomplish this, serial dilutions of the BSA solution are prepared, and photographs are taken for each dilution level perfusing the channel. After subtracting

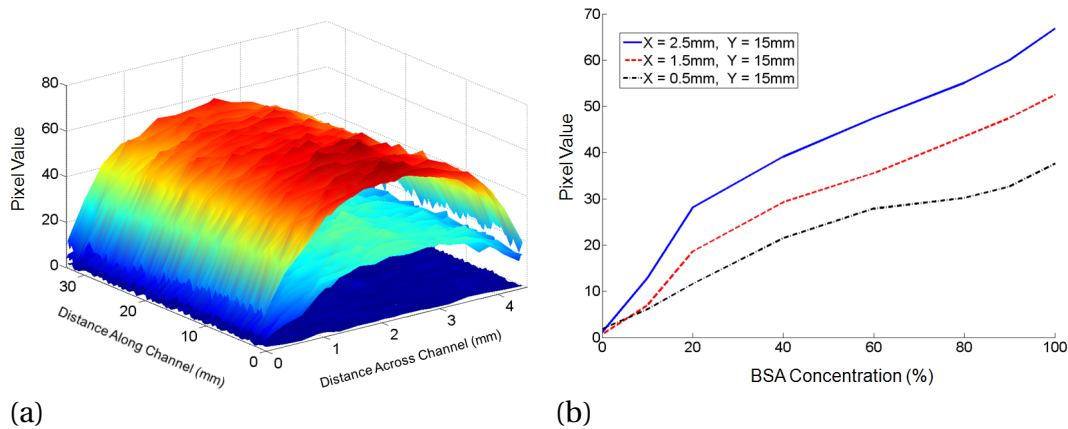


Figure 3.9: **(a)** Calibration plots, measuring pixel intensity at each point in the obstructed vessel for BSA concentrations of 0%, 40%, and 100%. **(b)** Representative calibration lines for three different locations in the obstructed vessel (X and Y correspond, respectively, to ‘Distance across channel’ and ‘Distance along channel’ in **(a)**), showing an approximately linear correspondence between BSA concentration and pixel value

the baseline (0% BSA) photograph, the vessel edges are identified using a simple edge detector Figure 3.8, and pixel intensity for each point in the obstructed vessel is measured (Figure 3.9(a)).

Then, at each pixel location, a calibration line is constructed relating pixel intensity to BSA concentration. These lines generally differed from pixel to pixel due to variations in lighting and vessel thickness at each location (Figure 3.9(b)). Next, each post-insonation photograph is converted into a surface plot of BSA concentration (Figure 3.10) by interpolating calibration lines on a pixel-by-pixel basis. Finally, the total quantity of BSA delivered into the vessel is calculated by integrating the heat map across the volume of the vessel, taking into account the effect of vessel curvature on the volume corresponding to each pixel.

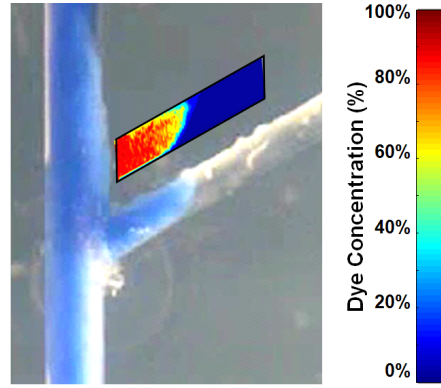


Figure 3.10: Typical photograph acquired after 2 s HIFU exposure. Inset: surface plot of BSA concentration, constructed using pixel-by-pixel interpolation of calibration images.

3.4.2 Quantifying Cavitation Activity

Cavitation activity was quantified on the basis of the frequency content of the acoustic emissions. Although the source of various spectral components is a subject of considerable complexity (see Section 2.5), in the present work it is assumed that the energy contained within broadband emissions corresponds to inertial cavitation activity, while the energy contained within ultraharmonic emissions corresponds to stable cavitation activity. For the purposes of quantifying these energies, the signal recorded from the PCD was analyzed. Since the 100 recorded data ‘blocks’ (§3.2) were not phase-locked, the total energy spectral density $\Phi(\omega)$ was computed as:

$$\Phi(\omega) = \frac{1}{2\pi} \sum_{k=1}^{100} \left| \sum_{j=1}^{20,000} f_k(j) e^{-i\omega j} \right|^2 \quad (3.1)$$

where $f_k(j)$ is the j -th sample of the k -th block of the digitized signal.

In order to arrive at a measure of the energy attributable to each spectral category (harmonic, ultraharmonic and broadband), custom-designed software is used to identify peaks within $\Phi(\omega)$ which fall within harmonic or ultraharmonic frequencies (Figure 3.11). The integral of the power spectrum is then calculated for each

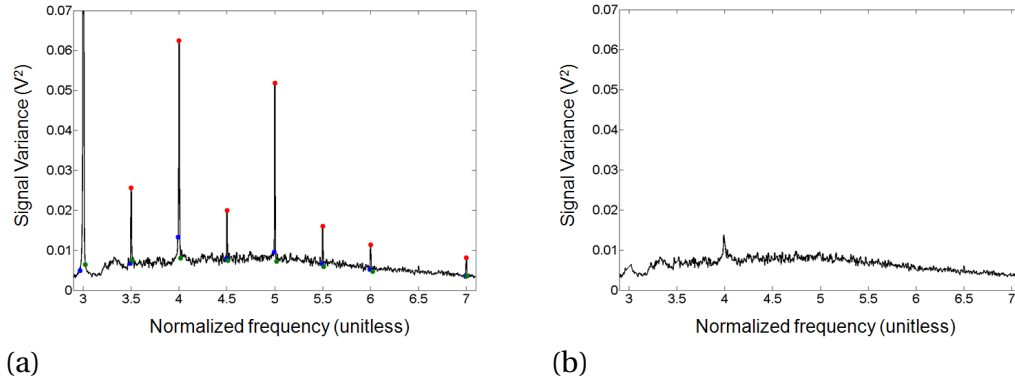


Figure 3.11: **(a)** Portion of a typical energy spectral density Φ from an experimental run, used to illustrate the effectiveness of the peak detection algorithm. The presence of a ‘peak’ (red dot) is determined by its magnitude relative to the signal variance, while its category (harmonic, ultraharmonic) is determined by its normalized frequency. **(b)** The broadband component of the signal as determined by the algorithm. Note that occasionally (as in the 4th harmonic), the shoulders of a peak are not fully removed, but the magnitude of these errors was found to be minor

peak, with the integration bounded from above by $\Phi(\omega)$ and from below by an imaginary line connecting the peak’s edges, implemented numerically as:

$$E_{peak} = \sum_{\omega=\omega_L}^{\omega_H} \left[\Phi(\omega) - \Phi(\omega_L) + \left(\frac{\omega - \omega_L}{\omega_H - \omega_L} \right) (\Phi(\omega_H) - \Phi(\omega_L)) \right] \delta\omega \quad (3.2)$$

Where ω_L and ω_H and the lower and upper frequency bounds of the peak, respectively, and $\delta\omega$ is the frequency resolution of the FFT. To determine the edges (blue and green dots in Figure 3.11(a)) of a peak, an algorithm was developed which applied a 10-point moving-average filter to Φ and identified the highest peak in the third derivative of the filtered signal, which was empirically found to correspond well with the visually identifiable edge of the peak (Figure 3.11(b)). Energies from each peak within a category are then summed together to arrive at the total energy attributable to that category. The remaining energy in the signal is classified as broadband.

For the purposes of this measurement, the portion of the spectrum between 400kHz and 800kHz was excluded for several reasons. Given the noise characteris-

tics of the available hardware, scaling the vertical range of the PCD signal digitizer to capture emissions at the fundamental would result in all other components of the signal (harmonics, subharmonics, broadband emissions) being indistinguishable from electrical noise. As discussed in Section §2.5.1, emissions at the fundamental do not uniquely indicate cavitation activity. Therefore, it was decided to filter the fundamental component of the spectrum so that spectral components which do indicate bubble activity could be resolved. The cutoff frequencies were chosen so that, under the highest pressures employed in the experimental setup, the power spectral density at the fundamental frequency was below that at the second harmonic.

3.5 RESULTS AND DISCUSSION

3.5.1 BSA Transport

To test the hypothesis that acoustic cavitation can promote delivery of BSA into an obstructed vessel, 2-second continuous-wave ultrasound was applied at 1.3MPa peak rarefactional pressure in the presence or absence of cavitation nuclei. Preliminary work demonstrated that in the absence of nuclei, the cavitation threshold is 1.7 MPa, while the presence of nuclei drops the threshold to 1.1 MPa. Therefore, at 1.3MPa, it is expected that phenomena associated with cavitation will be observed only in the experimental run containing nuclei, and qualitative video analysis confirms this hypothesis (Figure 3.12)

Having observed the increase in BSA delivery under cavitating conditions, the extent of BSA transport was quantified across a range of applied pressures. Figure 3.13 shows 'heat maps' of the spatial distribution of BSA in following 2-second ultrasound exposures at pressures from 0.4-2.0 MPa, in the absence Figure 3.13(a) and presence Figure 3.13(b) of nuclei. Each heat map is labeled with the corresponding sonica-

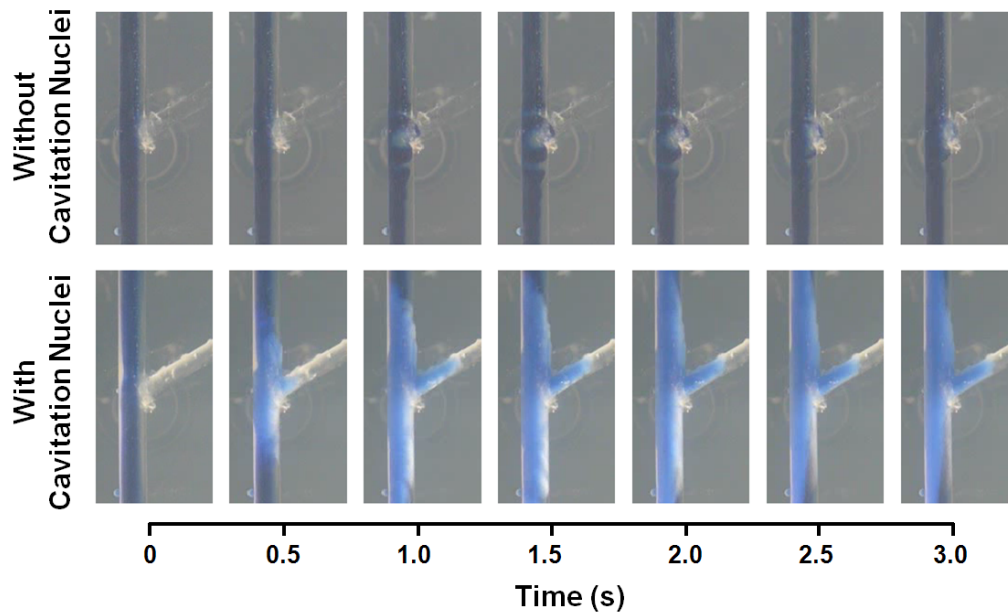


Figure 3.12: Snapshots taken every 0.5 s, of BSA distribution during treatment, in the absence (top row) and presence (bottom row) of cavitation nuclei. In each experiment, continuous-wave HIFU at 1.3MPa is applied between 0.5s and 2.5s. In the absence of cavitation nuclei, the radiation force is the dominant source of momentum transfer, and propels BSA particles against the far wall of the vessel, resulting in the blue ‘rings’ which correspond to the transverse pressure profile of the HIFU transducer (Figure 3.5). In the presence of nuclei, dye delivery into the obstructed vessel is dramatically increased

tion pressure. The parallelogram-shaped area (see Figure 3.10) has been ‘rectangularized’ for visualization.

From Figure 3.13(a), it is apparent that in the context of this experimental setup, applying ultrasound at up to 2.0 MPa in the absence of cavitation nuclei yields limited enhancement of drug delivery. In fact, the level of BSA delivered at sonication pressures below 1.3 MPa is largely a consequence of the shed vortex which develops in an analogous manner to the backwards-facing step geometry frequently encountered in fluid dynamics (Armaly et al., 1983). Although this produces an artifactual ‘baseline’ level of drug delivery, it also suggests a possibility for increasing BSA penetration by applying ultrasound at some point further along the obstructed channel

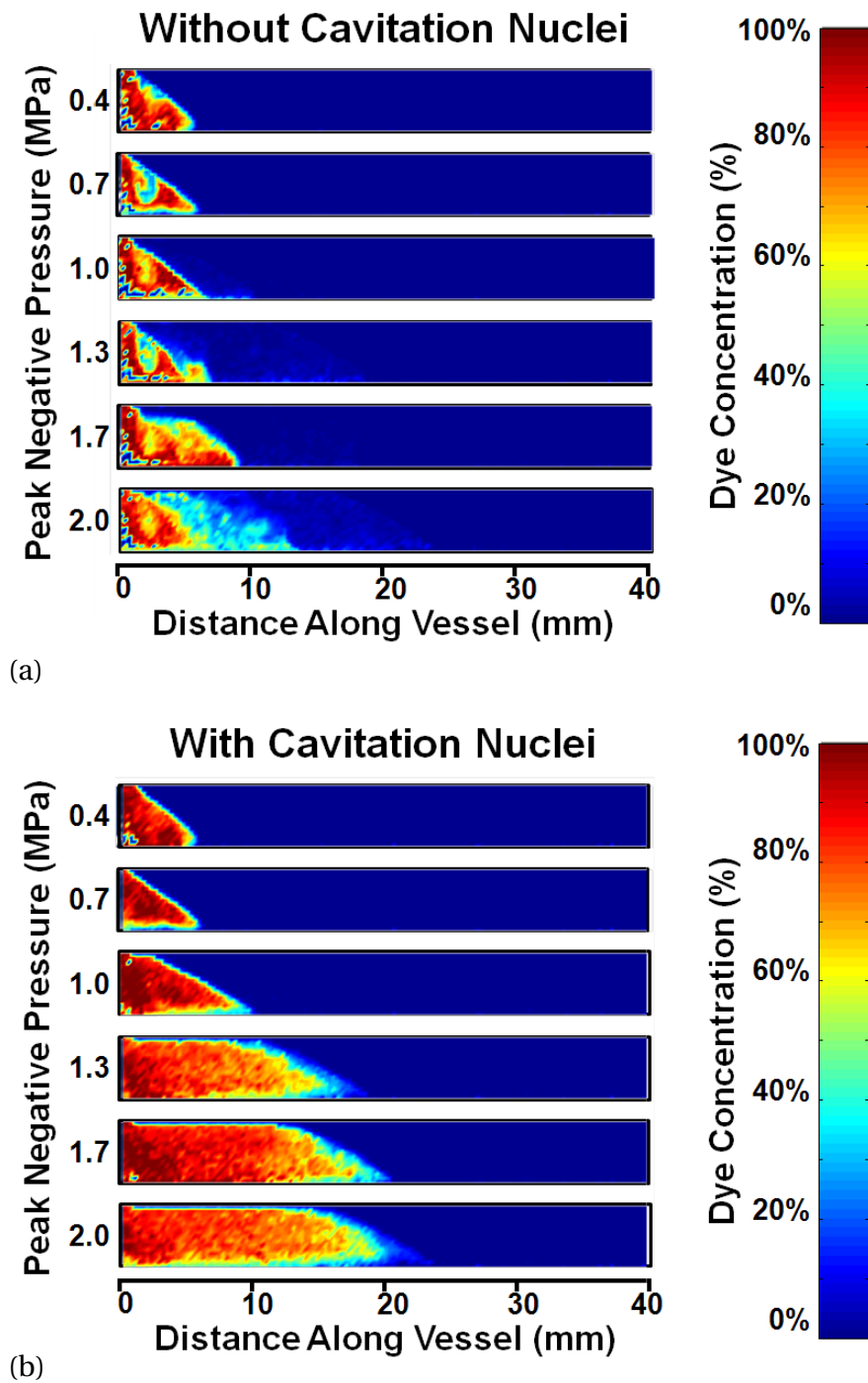


Figure 3.13: Concentration of BSA-EB in the blunt-end vessel following 2s HIFU at various amplitudes in the absence (a) or presence (b) of nucleating microparticles. 100% corresponds to the BSA concentration in the flowing vessel before ultrasound exposure.

rather than the junction between the channels.

In the presence of cavitation nuclei (Figure 3.13(b)), a sharp increase in drug delivery is observed at pressures above 1.0 MPa, an effect which saturates as the pressure is increased further. In all experiments, a sharp boundary is apparent between regions of high concentration and low concentration of BSA, confirming that convection rather than diffusion is the primary mechanism for enhanced drug delivery, which is consistent with the low diffusivity of this large molecule (if diffusion were the dominant mechanism, the concentration profile would resemble an exponential decay curve with increasing distance from the main vessel. However, the diffusive length scales of a sphere of equivalent volume to BSA over 2 seconds is on the order of tens of μm).

The relationship between peak pressure and total BSA delivered into the obstructed vessel is summarized in Figure 3.14. Note that several sources experimental variability exist, including inhomogeneous distribution of cavitation nuclei and the stochastic nature of cavitation activity. It is believed that experimental errors such as inconsistencies in phantom preparation, experimental procedure, or lighting do not contribute meaningfully to the experimental uncertainty observed.

3.5.2 Acoustic Emissions

3.5.2.1 Qualitative Observations

Up to this stage, discussion of the detection and interpretation of acoustic emissions has been limited to theoretical and experimental work found in literature. Thus, it is worth taking a moment to qualitatively explore the capabilities of the present experimental setup, and in particular two observations which highlight the sensitivity of the system.

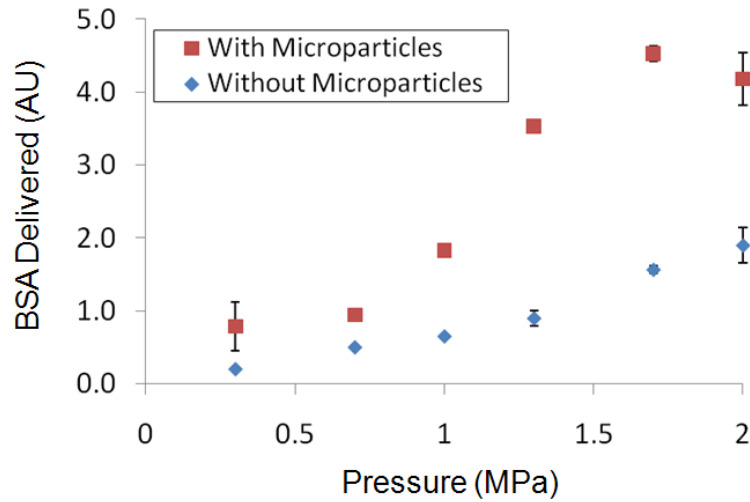


Figure 3.14: Total BSA in the obstructed vessel after 2 s HIFU exposure at various pressures, in presence or absence of microparticles. The presence of microparticles increases BSA delivery across all pressures, with the maximum value achieved at 1.7 MPa.

3.5.2.1.1 Period-Multiplying Bifurcations Versus Peak Pressure Section §2.4.1 discussed the tendency of cavitating bubbles subject to increasing pressure to transition from linear pulsations of period $1/f_0$ to nonlinear pulsations (still with period $1/f_0$), and to the ‘bifurcation zone’, in which pulsations of period m/f_0 ($m = 2, 3, 4, \dots$) develop. Although this transition is difficult to detect in the time domain, the subharmonic peaks that emerge in Figure 3.15 at successively higher pressures imply that period-multiplying bifurcations may be taking place. Specifically, as one would expect from, for example, Figure 2.11, as the excitation pressure increases, bubbles within a specific size range will transition to period-doubling, and in fact emissions corresponding to period doubling are observed (Figure 3.15(b)). Following another increase in pressure, emissions corresponding to period tripling emerge (Figure 3.15(c)), and finally, at 1.3 MPa excitation pressure, period quadrupling occurs (Figure 3.15(d)).

In fact, these results can even be used to estimate the size range of bubbles

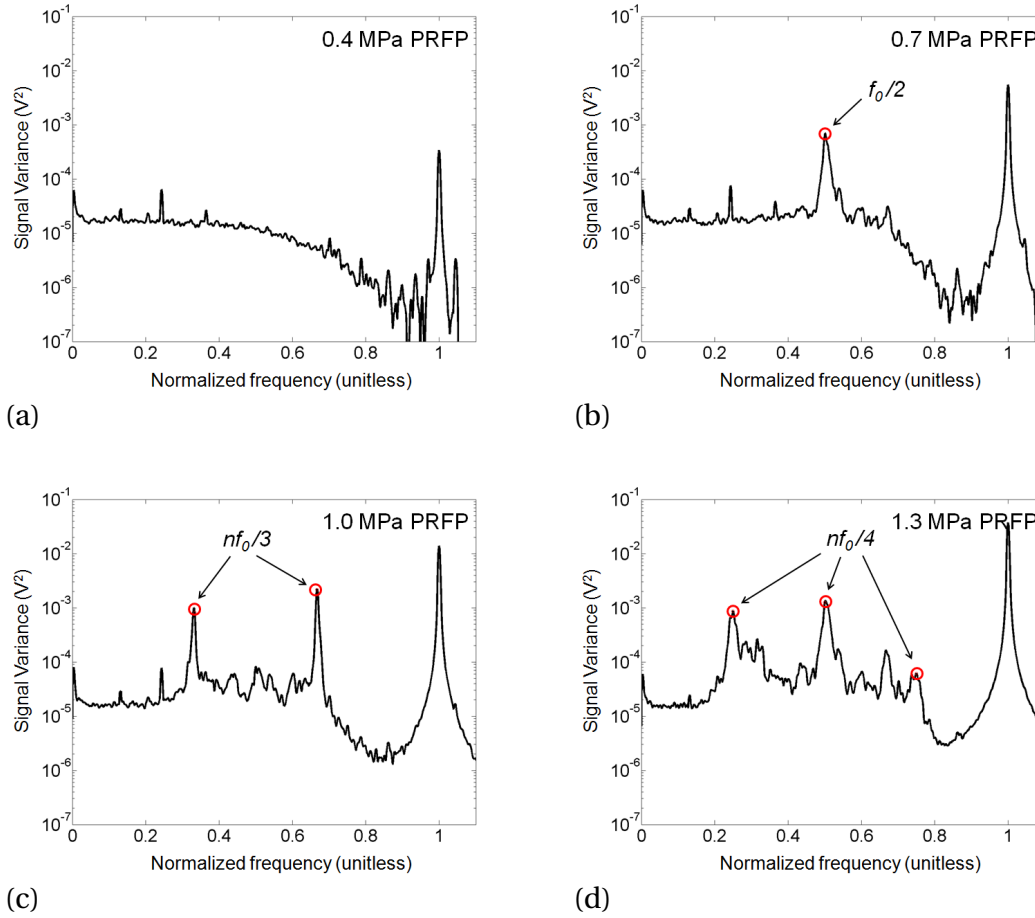


Figure 3.15: Period-multiplying effect captured by the hydrophone. As peak pressure is increased (progressing from figure (a) to (d)), period doubling, tripling, and quadrupling are observed in succession.

present in the acoustic focus, within the limitations of a free bubble model being applied to a crevice nucleation situation. Consider, for example, the bifurcation diagram of a $3 \mu\text{m}$ bubble at 483 kHz, with pressure varying on the x-axis Figure 3.16(a). The first bifurcation takes place at $\sim 1.1 \text{ MPa}$, so it is unlikely that bubbles of this size (or smaller) are responsible for the subharmonic observed in Figure 3.15(b). A $5 \mu\text{m}$ bubble, on the other hand, is in the period-doubling zone at 0.7 MPa, so the presence of a subharmonic peak at 0.7 MPa may indicate that bubbles in the $5 \mu\text{m}$ size-range are present. By similar reasoning at 1.0 MPa and 1.3 MPa, nuclei in the size range of $4\text{-}10 \mu\text{m}$ are hypothesized to be present in the flowing channel, which

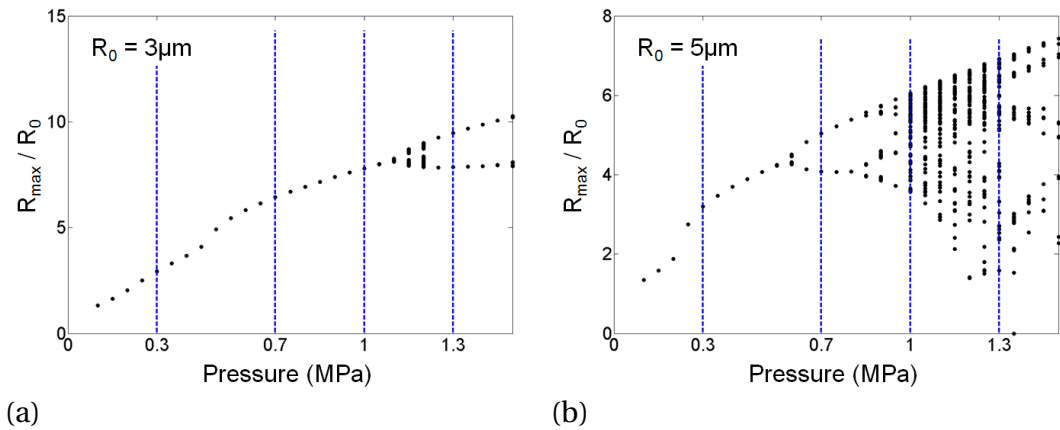


Figure 3.16: Bifurcation diagram plotting each observed local maximum of the radius-time curve of an oscillating bubble as a function of increasing pressure. Curves are obtained by numerically solving the Keller-Miksis equation with $f_0 = 483$ kHz for initial bubble radii of (a) $R_0 = 3 \mu\text{m}$ and (b) $R_0 = 5 \mu\text{m}$. The diagrams suggest that the experimentally observed subharmonic emissions at 0.7 MPa (Figure 3.15(b)) are more likely due to a $R_0 = 5 \mu\text{m}$ than a $R_0 = 3 \mu\text{m}$ since the former alternates between two local radii maxima at each acoustic cycle, resulting in an oscillation frequency of $f_0/2$ at this pressure. Blue dotted lines represent pressure values that were used experimentally

is reasonable given the dimension of microparticles in Figure 3.3, though of course the absence of evidence for bubbles outside this size range is not evidence of their absence.

An alternate hypothesis is supported by Figure 3.17, which shows a segment of the signal recorded by the PCD during the 1.3 MPa sonication. The sharp impulses, characteristic of inertial collapse, occur every four acoustic cycles during this portion of the treatment. This may be due to the ‘arrested collapse’ phenomenon discussed in Section §2.5.3. Note, however, that this periodicity was not regularly observed at the other excitation pressures.

3.5.2.1.2 Dramatic Effect of Exogenous Nuclei on Cavitation Activity Far less subjective are the components of acoustic emissions spectra that arise in the presence of exogenous nuclei. At relatively low pressures (0.4 MPa), sharp ultraharmonic peaks

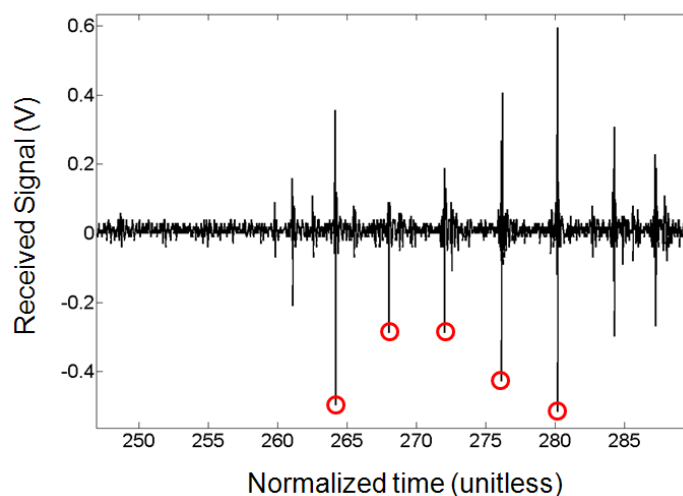


Figure 3.17: A portion of the signal recorded by the PCD during 1.3 MPa sonication, with acoustic cycle number (normalized time) along the x-axis. Inertial cavitation events are characterized by sharp impulses, of which several are visible in this window. Impulse minima labeled with red circles are separated by exactly four acoustic cycles. At high pressures, this can occur when expanding bubbles ‘skip’ a contraction phase under the inertial force of the surrounding water, and collapse one or more cycles later.

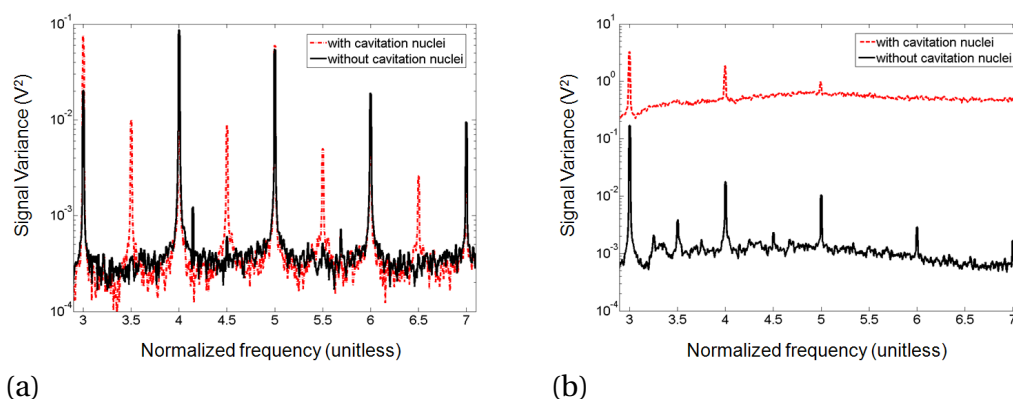


Figure 3.18: Frequency content of the PCD signal recorded in the absence (black, solid line) or presence (red, dotted line) of cavitation nuclei. **(a)** At 0.4 MPa, harmonics are observed in both signals, likely resulting from nonlinearities in the medium. The presence of cavitation nuclei results in very prominent ultraharmonic peaks, which likely indicate stable cavitation activity (0.4 MPa is too weak to cause inertial cavitation for the frequency and bubble sizes found in this system). **(b)** At 1.3 MPa, the noise floor is slightly elevated in the absence of nuclei, dramatically so in the presence of nuclei. Ultraharmonics are either absent or buried beneath the broad-band noise.

emerge in addition to the harmonic peaks arising from nonlinear propagation Figure 3.18(a). At higher pressures (1.3 MPa), the presence of nuclei is associated with an increase in the noise floor by a factor of several hundred, likely attributable to inertially collapsing microbubbles (Figure 3.18(b)).

3.5.2.2 Quantitative Analysis

In order to compare BSA transport to the various categories of acoustic emissions, the total energy spectral density $\Phi(\omega)$ is computed for each experimental run (Figure 3.19). In the absence of nuclei (3.19(a)), broadband energy rises approximately 10-fold at 1.0 MPa and saturates despite further increases in pressure. Ultraharmonics are detected at higher pressures, with $f_0/3$ multiples emerging at 1.0 MPa, $f_0/4$ at 1.3 MPa, and $f_0/6$ at 2.0 MPa. In the presence of nuclei (3.19(b)), $f_0/2$ multiples are visible at 0.4 MPa, but disappear at higher pressures. Broadband energy rises approximately 10-fold at 1.0 MPa, then increases a further two orders of magnitude at 1.3 MPa, beyond which it saturates. It appears therefore that the presence of nuclei increases ultraharmonic emissions at low pressures, decreases the inertial cavitation threshold, and increases the total amount of inertial cavitation observed, as indicated by broadband emissions.

Next, the peak detection algorithm is applied, and the received signal energy is allocated to either the harmonic, ultraharmonic or broadband category. The results are summarized in Figure 3.20. Ultraharmonic emissions, while monotonically increasing with pressure, show poor reproducibility from one run to the next (as indicated by the large standard deviation). Additionally, the influence of cavitation nuclei on ultraharmonic emissions is statistically insignificant (Figure 3.20(a)).

However, it appears that inertial cavitation activity, while erratic at the single-bubble level, is fairly consistent from one experimental run to the next, as indi-

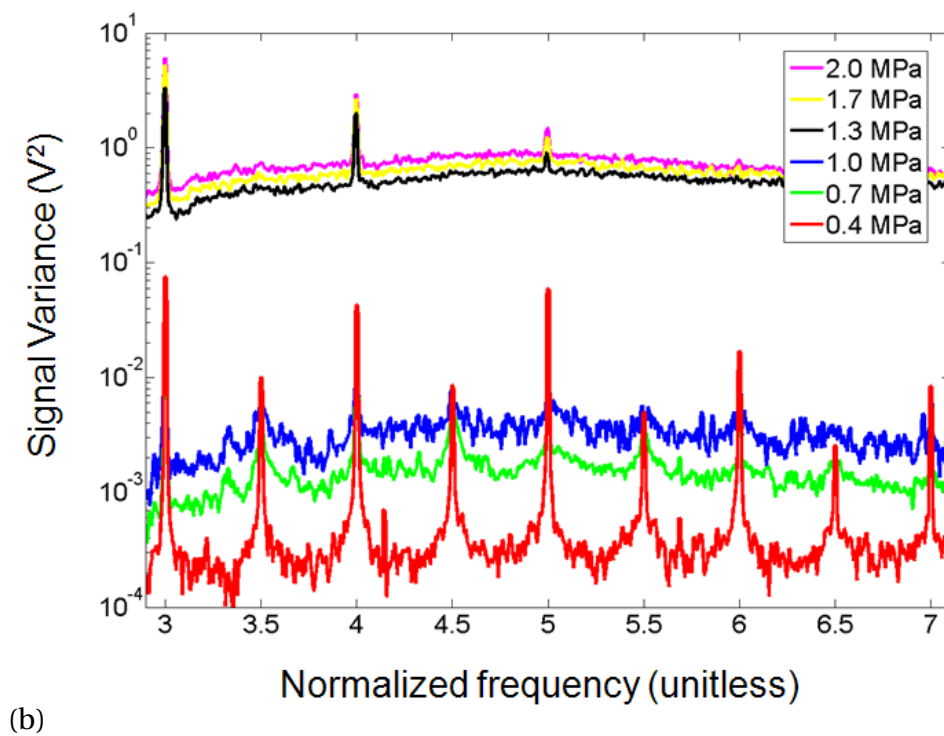
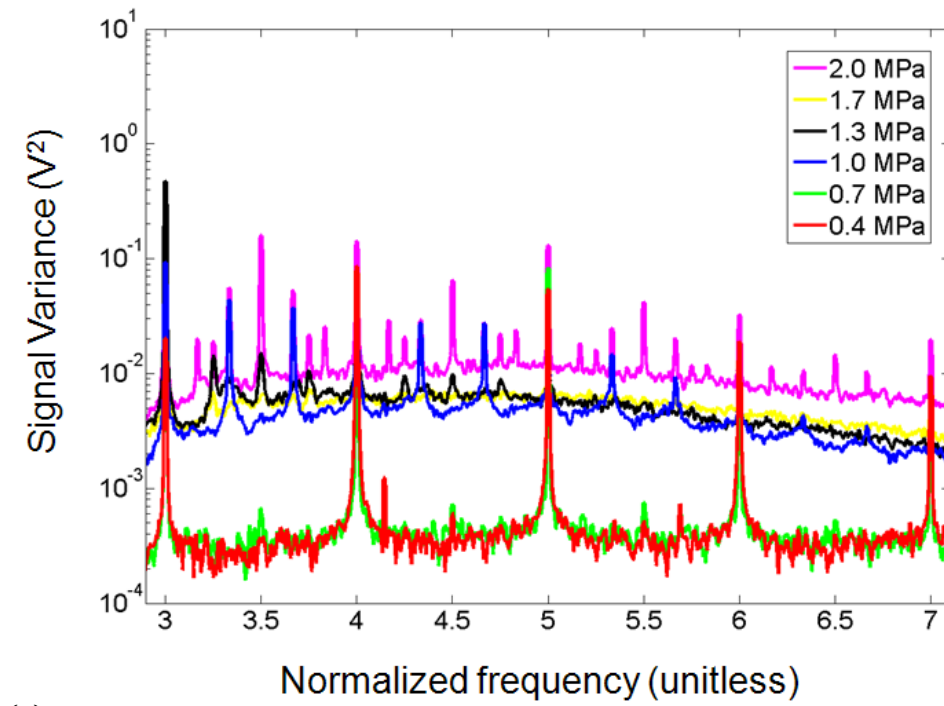


Figure 3.19: Frequency content of the PCD signal recorded in the absence **(a)** or presence **(b)** of cavitation nuclei across a range of pressures.

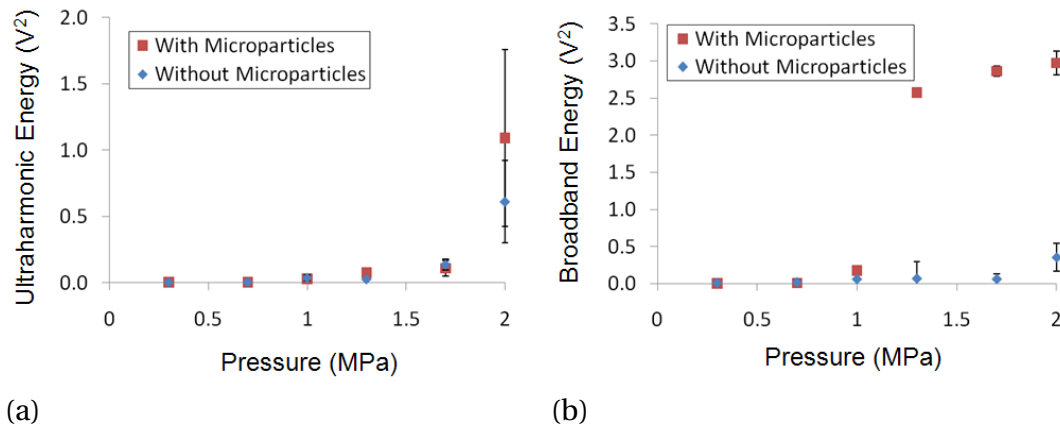


Figure 3.20: Total energy contained in ultraharmonic (a) and broadband (b) emissions at various pressures, in presence or absence of microparticles. Trends in ultraharmonic emissions are not apparent, show high variability from one run to the next, and are not materially affected by the presence of nuclei. Broadband emissions, in contrast, are increased dramatically and in a threshold-dependent manner by the presence of nuclei.

cated by the $< 5\%$ standard deviation in broadband emissions across all pressures (Figure 3.20(b)). Moreover, the effect of cavitation nuclei, as noted earlier, is pronounced, particularly at pressures above 1.0 MPa.

3.5.3 Relationship Between Cavitation and BSA Transport

As a final step, total BSA transport in each experimental run is compared to the categorized energy contained in acoustic emissions detected during that run Figure 3.21. The correlation between BSA transport and stable cavitation, initially hypothesized to be responsible for the convective mixing observed, is fairly weak. Although the onset of subharmonic and ultraharmonic peaks were clearly identifiable, the energy contained in these signals were poor indicators of total BSA delivered into the obstructed vessel. However, the energy contained in *broadband* emissions correlated very strongly with total BSA delivered ($r > 0.97$), suggesting that inertial cavitation is the dominant mechanism responsible for enhanced convective transport

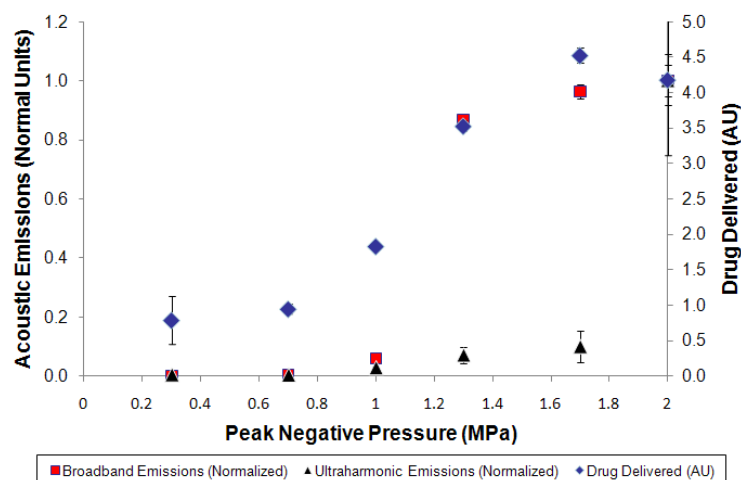


Figure 3.21: Graph of the energy contained in broadband emissions (squares, left axis), ultraharmonic emissions (triangles, left axis), and total BSA delivered into the obstructed vessel (diamonds, right axis) for 2 s exposures at various sonication pressures (x-axis). There is a strong correlation ($r > 0.97$) between BSA delivered and broadband emissions in the presence of nucleating microparticles. The correlation between ultraharmonic emissions and BSA delivered is much weaker ($r < 0.5$). Broadband and ultraharmonic emissions have been normalized to their respective maxima for visualization.

of BSA in the context of this experimental setup.

3.6 SUMMARY

This work has demonstrated the potential for ultrasound-induced cavitation to promote convective transport in a localized, non-directional manner. It was found that, under certain conditions, the presence of cavitation nucleation agents yielded a 7-fold increase in the convective transfer of dye-labeled macromolecule into an unperfused vessel, when compared to equivalent experiments conducted in the absence of nucleation agents.

Furthermore, it was for the first time demonstrated that a strong correlation exists between enhanced distribution of macromolecule and the energy contained within acoustic emissions characteristic of inertial cavitation, a finding which sug-

gests both the mechanism of action of this phenomenon, and the clinical potential to develop a non-invasive system for monitoring ultrasound-enhanced drug delivery.

While an inertially collapsing microbubble generates powerful streaming jets, this is not the only mechanism by which it can promote convection. In fact, although the two vessels in the phantom are oriented in a plane perpendicular to the HIFU acoustic axis, the radiation force on a microbubble can still result in a streaming and recirculation patterns. These will be sensitively dependent on the induced bubble dynamics - in particular the bubble's scattering cross-section - making it difficult to distinguish between collapse-driven and radiation force-driven streaming in a macroscopic setting. However, if it is the combination of the presence of bubbles and radiation force that creates the observed enhancement in drug delivery, then this enhancement should also be observed in the presence of larger, stably cavitating bubbles, which is not supported by the poor correlation between ultraharmonic emissions and BSA transport. This suggests that it is primarily inertial cavitation which is responsible for the observed enhancement.

Although ultraharmonic emissions were found to be poor indicators of convective transport, they may still have an important clinical application, one which is hinted at by the data in Figure 3.18(a). The presence of exogenous nuclei increases the strength of ultraharmonic peaks over a hundred-fold, and the persistence of this signal throughout low-pressure sonication suggests that these peaks may be a useful non-destructive indicator of the presence of cavitation nuclei in the acoustic focus. Though this may not be critical in a laboratory setting, where spatiotemporal nuclei concentration is predictable, replenishment of depleted nuclei in a clinical setting is dependent upon local perfusion rates, which can vary from tissue to tissue, particularly - as was discussed in §1.3.1 - in pathological tissues. An extracorporeal means

to monitor the reperfusion of cavitation nuclei before applying a therapeutic pulse may therefore be desirable.

This study has several limitations, primary among which is that the vessels considered in this experiment are too large to be found except in the tumor periphery. Microvessels in the tumor interior can be 1-2 orders of magnitude smaller, and the proximity of vessel walls and decreased linear flow rates in smaller vessels may affect streaming behavior. The *in vitro* experiments described in Chapter 5 employ a slightly smaller vessel, but challenges regarding the structural integrity of the wall limit further decreases in scale.

A further limitation of the present work is the use of talc microparticles as cavitation nuclei. Beyond their immunogenicity, which renders them unsuitable for *in vivo* experimentation, the microparticles are heterogeneous, resulting in a polydisperse population of bubbles, possibly up to tens of micrometers in diameter. Bubble dynamics depend strongly on initial radius and excitation parameters. Therefore, an important topic that needs to be considered is the size range of bubbles that are responsible for the observed bioeffect, and whether by optimizing the size range and ultrasound parameters, the bioeffect can even be enhanced. Of course, the time and costs of manufacturing a range of ultrasound transducers and developing the technology to synthesize monodisperse bubble populations are prohibitive, making an experimental approach to the problem unfeasible. Therefore, in the following chapter, a novel *numerical* technique is developed to predict the streaming effect that results from the oscillation of microbubbles of various sizes subject to a range of excitation frequencies and amplitudes.



COMPUTATIONAL MODELING OF CONVECTIVE TRANSPORT

In this chapter, several numerical methods are developed to quantify convective transport due to HIFU. First, the experimental work conducted in the previous chapter is reproduced numerically for cases where cavitation does not occur. Then, a flexible and efficient method to model the microstreaming fields instigated by cavitating microbubbles is presented. Accuracy in both microstreaming patterns and streaming velocities is demonstrated in two test cases with experimental analogues in the literature, actuated by either applied sound waves or electrowetting-driven harmonic wetting angle oscillations. The technique is applied towards quantifying convective transport of a scalar quantity in the vicinity of an acoustically cavitating microbubble of various sizes, subject to a range of sonication parameters, yielding insights useful to subsequent experimental work.

In the previous chapter, it was demonstrated that acoustic cavitation shows promise as a drug delivery tool and warrants further exploration. Specifically, there is an interest in discovering the combination of various experimental conditions, such as the size of cavitation nuclei, sonication frequency, and sonication amplitude, which optimizes convective transport.

Strictly speaking, ‘optimization’ involves identifying the extremes (the minima or maxima) of a closed-form expression. Unfortunately, many problems involving

flow and related phenomena are described by partial differential equations (PDEs) which are too complex to be solved analytically, except in special cases. It is convenient to tackle such problems using *computational fluid dynamics* (CFD) - the solution of equations of fluid mechanics using computers.

In this chapter, a straightforward simulation for the two-vessel system is developed, and macromolecular transfer is reproduced in the absence of cavitation. Once this is accomplished, the more challenging task of simulating the microstreaming arising from inertial cavitation is approached. A technique which accurately and efficiently reproduces the streaming patterns arising from periodically oscillating boundaries is developed. This method is validated against three very different scenarios: the two-dimensional flow of an incompressible viscous fluid enclosed between two coaxial circular cylinders, the three-dimensional flow surrounding an electrowetting-driven oscillating microbubble, and the three-dimensional flow around an acoustically cavitating microbubble. Finally, the technique is applied to the optimization, within clinically relevant parameters, of macromolecular transport surrounding acoustically cavitating microbubbles.

4.1 COMPUTATIONAL FLUID DYNAMICS

Below, the multi-physics software CFD-ACE+ (ESI Group, Paris, France) is used to simulate numerically the experimental conditions presented in Chapter 3, by sequentially incorporating into the model the effects of flow, ultrasound, and (in a simplified environment) cavitation. In all cases, macromolecular transport and distribution will be examined.

4.1.1 Mathematical Model

The starting point of a CFD solution is the mathematical model describing the system under consideration. It is here that the first approximation is made (and therefore the first systematic error is introduced). This makes it a critical step in development of the CFD solution. As an example, assume that a model is to be developed which studies the maximum velocities observed in the microstreaming of water surrounding a bubble in Figure 2.9. Should the temperature of the system be modeled? What about the dissolved gas concentration? Or the coupling between temperature and viscosity, between temperature and gas solubility, gas diffusivity, and the rectified diffusion process?

It is easy to see how such a model can become intractably complex, with countless coupled PDEs and the computational burden they impose, all to capture a bubble growth of 1% and its imperceptible impact on microstreaming velocities. Therefore, a model is usually chosen which omits physical processes assumed to be of negligible importance to the system. Of course this is often an iterative process in which layers of complexity are sequentially introduced into the model until it faithfully reproduces the desired elements of the physical system.

4.1.2 Algebraic Approximation

Once a mathematical model is chosen, a discretization method must be employed, of which the most common are the finite volume method (FVM), finite difference method (FDM), finite element method (FEM), particle method, and spectral method. These methods approximate differential equations by algebraic equations whose unknowns correspond to the values of each variable at a specified location in space and time.

The approximation of continuous differentials with discrete algebraic equations results in a systematic error known as the *truncation error*, because it usually arises from truncating the Taylor series expansion about a single point in space or time. If the first n terms of the Taylor series expansion are retained, the truncation error is proportional to $(\Delta x)^{n+1}$, and the method is referred to as n th-order accurate. Of course, this classification should be handled carefully, because the impact of a term depends exclusively on its magnitude and not on its mathematical order. But it happens that for many problems of interest in fluid dynamics, terms in the Taylor expansion diminish in magnitude with increasing order.

The present work uses the FV method, which uses the integral form of the conservation equation as its starting point:

$$\underbrace{\int_V \frac{\partial \rho \phi}{\partial t} dV}_{\text{transient}} + \underbrace{\int_S \rho \phi \mathbf{v} \cdot \mathbf{n} dS}_{\text{convection}} = \underbrace{\int_S \Gamma \nabla \phi \cdot \mathbf{n} dS}_{\text{diffusion}} + \underbrace{\int_V q_\phi dV}_{\text{source/sink}}, \quad (4.1)$$

where ρ is the density, ϕ is the conserved quantity, V is a differential volume element, $\mathbf{v}$ is the velocity vector, $\mathbf{n}$ is the unit normal to the surface S , Γ is the diffusivity of ϕ , and q_ϕ is the volumetric source/sink term. The two interior terms have been transformed from volume integrals into surface integrals by the application of Green's Theorem. Numerical approximation of each term in this equation is discussed below.

The solution domain is divided into a finite number of control volumes (see Section §4.1.3 below), with cell-averaged material properties and variables stored in the CV centroid. Surface and volume integrals in Equation (4.1) are approximated and applied to each CV. There exist a variety of methods for discretizing each term in Equation (4.1), depending on the order of accuracy required to achieve stability, convergence, and accuracy. Below, the methods employed in this chapter are discussed.

4.1.2.1 Transient Term

Just as a geometric domain is divided into CVs, so too must time be discretized. However, while the influence on a CV can be omnidirectional in space, it can only be historical in time (events at timestep t_{n+1} cannot influence behavior at timestep t_n). Beginning with an exact solution to the transient term,

$$\phi_{n+1} = \phi_n + \int_{t_n}^{t_{n+1}} \phi'(t) dt, \quad (4.2)$$

where $\phi_n = \phi(t_n)$, CFD-ACE+ offers the option to estimate the integral on the right hand side with either the explicit *forward Euler* method,

$$\phi_{n+1} = \phi_n + \phi'_n \Delta t, \quad (4.3)$$

or the semi-implicit *Crank-Nicolson* method,

$$\phi_{n+1} = \phi_n + \frac{\phi'_{n+1} + \phi'_n}{2} \Delta t. \quad (4.4)$$

The forward Euler method is only first-order in accuracy, but produces smooth solutions even for large Δt . The Crank-Nicolson method is second-order in accuracy, and is the method used in this study.

4.1.2.2 Convection and Diffusion Terms

Both the convection and diffusion terms require approximation of surface integrals, which is typically accomplished in two steps. First, the integral is approximated in terms of integrand values ($\rho\phi\mathbf{v}\cdot\mathbf{n}$ for the convective term and $\Gamma\nabla\phi\cdot\mathbf{n}$ for the diffusive term) at the cell face. Second, the integrand values at the cell face are approximated by interpolating those at CV nodes.

In the first step, the simplest integral approximation involves multiplying the surface area of the cell face by the value of the integrand at the center of the face. Higher-order schemes require values of the integrand at cell face corners as well.

Of course, to preserve the order of accuracy of the intergral calculation, the interpolation of cell face values must be of equal or higher order. *Upwind Interpolation* is numerically equivalent to using the Upwind Differencing Scheme (UDS),

$$\phi_f = \begin{cases} \phi_W & \text{if } \mathbf{v} \cdot \mathbf{n} > 0 \\ \phi_E & \text{if } \mathbf{v} \cdot \mathbf{n} < 0 \end{cases} . \quad (4.5)$$

where ϕ_f is the cell face value, ϕ_W and ϕ_E are the values at the cell nodes ‘west’ and ‘east’ of the cell face (in this one-dimensional example), and $\mathbf{n}$ is the outward-directed unit vector normal to the face f . This method is first order in accuracy, with the leading error term a diffusive flux. To see why, consider the estimate for ϕ_f by a Taylor expansion about ϕ_W :

$$\phi_f = \phi_W + (x_f - x_W) \left(\frac{\partial \phi}{\partial x} \right)_W + H \quad (4.6)$$

UDS retains only the first term, resulting in an artifactual diffusion term with numerical diffusivity proportional to Δx . Alternatively, a linear interpolation of ϕ_f between the nearest nodes is given by:

$$\phi_f = \left(\frac{x_f - x_W}{x_E - x_W} \right) \phi_E + \left(\frac{x_E - x_f}{x_E - x_W} \right) \phi_W, \quad (4.7)$$

which is second-order accurate. This corresponds to the Central Differencing Scheme (CDS). To obtain higher orders of accuracy, polynomial interpolation with several nearby node values is simple to implement, but comes at increased computational cost. The CDS method is used for simulations described in this chapter.

4.1.2.3 Source Term

Analogous to the approximation method for surface integrals, a volume integral can either be approximated by multiplication of the cell volume by the node center value, or by incorporating the interpolated value of nodes at other locations.

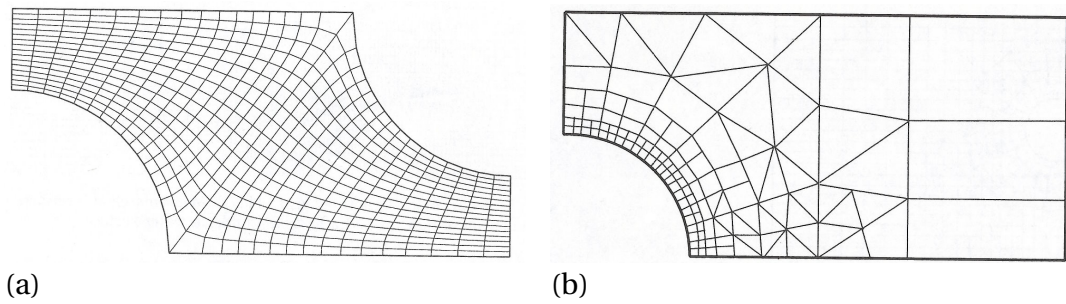


Figure 4.1: Examples of **(a)** structured and **(b)** unstructured computational grids

4.1.3 Geometric Discretization

The algebraic equations developed above are solved in each control volume (CV), or discretized element, of the geometric domain under consideration. Discretization is achieved by a numerical grid, which can either be structured (as in a cartesian grid, see Figure 4.1(a)), or unstructured (usually involving triangles in 2-D or tetrahedra in 3D (see Figure 4.1(b))). The advantage of structured grids is that their connectivity and regular structure results in simplified programming and matrix algebra. However, they are only suited to simple geometries. Arbitrary geometries are most easily handled by unstructured grids, though the irregularity of the computational matrix structure results in increased computational burden. Note that either grid type can be constructed so as to have fine grid resolution in some regions and coarser resolution in others, in order to focus computational power where it is required or of interest. In this chapter, structured grids are employed where geometry permits and computational cost is critical.

4.1.4 Numerical Solution

Approximation of the mathematical model yields an algebraic equation at each node which contains the variable at that node as well as at neighboring nodes, gen-

erally of the form:

$$A\boldsymbol{\phi} = \mathbf{Q} \quad (4.8)$$

where A is the coefficient matrix (which is usually sparse since the variable value at node ϕ_i is usually approximated using only a few neighboring nodes), $\boldsymbol{\phi}$ contains the variable values at the CV nodes, and $\mathbf{Q}$ contains terms with no unknowns.

Solution of this system can be performed using a variety of direct methods, including Gaussian Elimination, LU Decomposition, etc. However, the computational cost of direct methods are quite high, and the high level of accuracy they yield (limited only by the computer arithmetic error) is unnecessary given the discretization error already present. Therefore, several iterative methods have been developed which, when properly used, can be computationally less expensive than the direct method, and yield solutions of accuracy similar to that of the discretization scheme. One class of iterative methods is developed by recognizing that solving Equation (4.8) is equivalent to minimizing

$$F = \frac{1}{2} \boldsymbol{\phi}^T A \boldsymbol{\phi} - \boldsymbol{\phi}^T \mathbf{Q}. \quad (4.9)$$

By beginning with a starting guess $\boldsymbol{\phi}_0$, the *steepest descent* path $\mathbf{n}$ is identified (the minimum of $\mathbf{n} \cdot \nabla F$), the lowest value of F in the direction $\mathbf{n}$ identified, the corresponding $\boldsymbol{\phi}_1$ chosen as the new estimate, and the process repeated. Unfortunately, if the magnitude of F (conceptualized here as a surface plot in 3-D) has for example a narrow valley, guesses at $\boldsymbol{\phi}$ may oscillate between the walls of that valley, and convergence may be a slow process. This gave rise to the Conjugate Gradient Squared (CGS) method, in which each new search direction is required to be orthogonal to the preceeding ones, and which converges exactly when the number of iterations is equal to the size of the matrix (Golub and van Loan, 1990).

An alternative to the CGS method is the Algebraic Multigrid (AMG) method, the

basic idea of which is to use a hierarchy of grids, from fine to coarse, to solve a set of equations, with each grid being particularly effective for removing errors of wavelength characteristic of the mesh spacing on that grid. This method is particularly well-suited to unstructured meshes (ESI). Both the AMG and CGS methods, implemented in CFD-ACE+, were used in this chapter.

4.1.5 Visualization

The power of CFD lies in its ability to harness the computational power available today to minimize the three systematic errors - modeling, discretization, and iteration - to such a level that the resulting solution is of sufficient accuracy to guide engineering design decisions. Of course, the raw numerical solution itself is rarely useful. Rather, it is post-processing and visualization of the solution which yields accessible information and facilitates insight, for which purpose an ancillary piece of software, CFD-VIEW (ESI Group, Paris, France), was often used in this study.

4.2 OBSTRUCTED VESSEL SIMULATIONS

Section §3.3 describes an experiment in which solution containing dissolved dye-labeled BSA flows through a vertical channel, continuous-wave 483 kHz ultrasound is applied for two seconds across a range of peak pressures from 0-2 MPa at the junction between the flowing channel and an intersecting obstructed channel, and the quantity of BSA propelled into the obstructed channel is quantified optically.

It was found that in the absence of cavitation nuclei, BSA transport is still observed, albeit at significantly reduced levels. Here, a model is developed which attempts to replicate the experimental runs in which no cavitation nuclei are injected. Therefore, the model focuses on the fluid dynamics at the junction between the

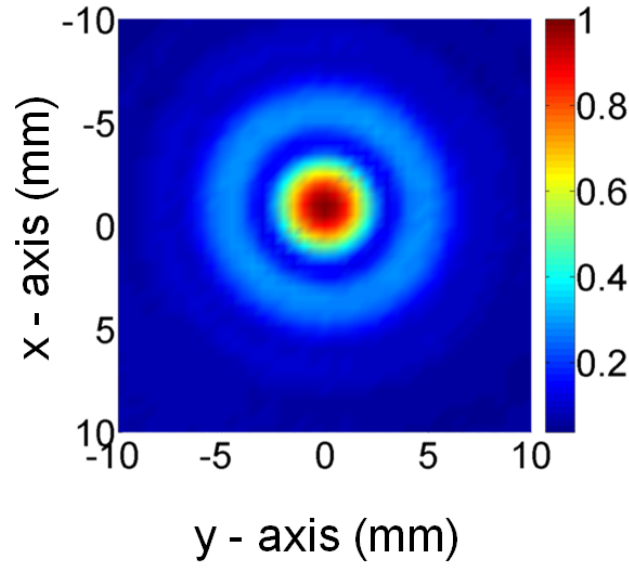


Figure 4.2: Transverse pressure profile surface of the HIFU transducer used to determine $p(t)$ in each computational cell

channels, as well as the momentum deposition resulting from attenuation of the propagating ultrasound wave. Acoustic scattering is ignored in this simulation.

4.2.1 Mathematical Model

4.2.1.1 Fluid Dynamics

Assuming liquid velocity $v \ll c$ and constant viscosity, the liquid flowing through the experimental setup is governed by a simplified form of the Navier-Stokes equation, for incompressible Newtonian fluids:

$$\rho \left(\frac{\partial \mathbf{v}}{\partial t} + \mathbf{v} \cdot \nabla \mathbf{v} \right) = -\nabla p + \mu \nabla^2 \mathbf{v} + \mathbf{f} \quad (4.10)$$

where ρ is the density of water, $\mathbf{v}$ is the velocity vector, p is pressure, μ is the dynamic viscosity, and $\mathbf{f}$ represents body forces acting on the fluid (such as gravity).

4.2.1.2 Momentum Deposition Due to Ultrasound

As discussed in Section §2.1, a travelling ultrasound wave attenuates due to the combination of viscous damping and scattering. This dissipation of acoustic energy flux permits gradients in momentum flux, which force acoustic streaming motions (Lighthill, 1978). The magnitude of the forcing term is given by:

$$F_{ac} = 2 \frac{\alpha I}{c} = \frac{2\alpha}{\omega^2 \rho c^2} \left\langle \left(\frac{\partial p}{\partial t} \right)^2 \right\rangle \quad (4.11)$$

where I is the acoustic intensity, α is the acoustic absorption coefficient ($\alpha \approx 5.8 \cdot 10^{-3}$ nepers/meter at 483 kHz, for distilled water at 20°C (Litovitz and Davis, 1965)), and the time average is carried out over a complete acoustic cycle (Pierce, 1981). At each computational cell with centroid $\mathbf{x}$, the peak amplitude of $p(t)$ is determined based on its position in the ultrasound field, using the transverse calibration in Figure 4.2. The longitudinal variation of peak pressure is less than 5% in this geometry, and is therefore neglected. Because the exact waveform $p(t)$ has not been characterized at different pressures, and because of the short propagation distances under consideration, the simplification is made to treat $p(t)$ as a sinusoid. Neglecting nonlinearities is likely to underestimate F_{ac} - particularly for higher peak pressures - though the results below suggest that under the conditions considered, nonlinearities do not significantly enhance momentum deposition. The source term F_{ac} is added to the z-component (directed out of the page) of $\mathbf{f}$ in Equation (4.10).

4.2.1.3 Tracking BSA Concentration

The spatial distribution of BSA from the flowing vessel into the obstructed vessel is also of interest. For dilute solutions, the movement of BSA obeys the general scalar transport equation:

$$\frac{\partial \phi}{\partial t} + \mathbf{v} \cdot \nabla \phi = \Gamma \nabla^2 \phi + S_\phi \quad (4.12)$$

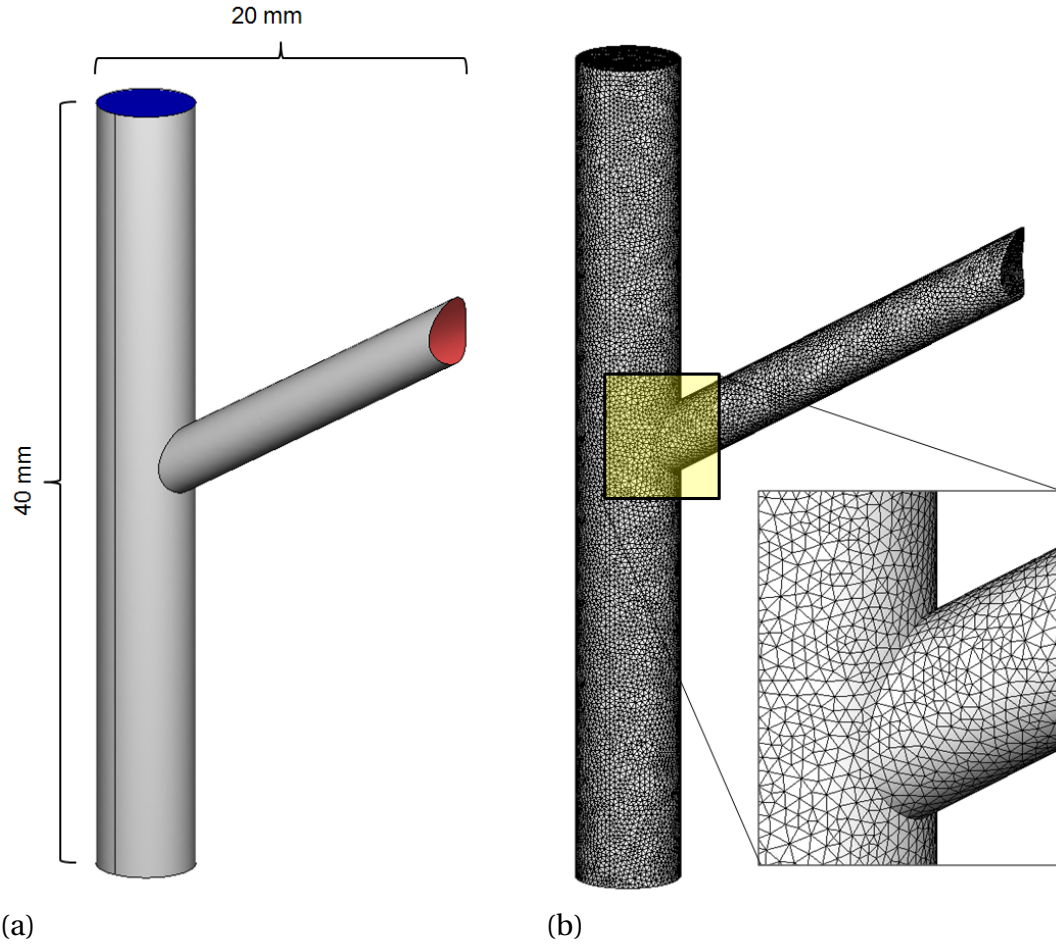


Figure 4.3: **(a)** Geometry of the computational domain under consideration. **(b)** Surface mesh resulting from numerical discretization of the domain

where ϕ is the scalar concentration (which for simplicity takes on a value of zero to one), Γ is the scalar diffusivity, and S_ϕ is the source or sink term (for example, the volumetric consumption of scalar by a chemical reaction). To isolate the convective transport effect, Γ was set to a value of zero in these simulations. This is not expected to be a source of deviation from the experimental work, given the short diffusive length scales estimated based on the experimental time scales and size of BSA (see Section §3.5).

4.2.2 Computational Domain and Discretization

The computational domain considered in this simulation extends 2 cm in each direction from the junction between the two channels (Figure 4.3(a)). It is necessary to divide the domain into a finite number of subdomains (or cells) over which the problem is to be solved. For stable numerical solutions, as the spacing between subdomains (the ‘grid spacing’) tends to zero, the discretization should approach the exact solution, but of course this comes at increased computational cost since the equations above must be solved (in their discretized form) at each cell. Therefore, it is customary to begin with a coarse grid and sequentially refine it to a point at which further refinement yields immaterial change in the solution. At this stage, the model’s truncation error is deemed tolerable, and the model is said to be grid-independent.

In unsteady flows, the fourth coordinate - time - must also be subdivided, and step size chosen to achieve timestep independence. Grid- and timestep-independence are related by the *Courant number* - the ratio of time step Δt to characteristic convection time $\Delta x/|v|$ (which is the time taken for a disturbance to be transmitted over the distance Δx). In many practical settings, an initial guess at the grid size is chosen to capture the desired flow field characteristics, then the timestep size is assigned such that the Courant number is below the threshold required for stability (the threshold is known as the Courant-Friedrichs-Lewy, or CFL condition, which is 1.0 for several explicit differencing schemes, but as low as 0.1 for the free surface methods used in Section §4.3.3.1 below.)

In this series of simulations, grid independence was achieved with spatial discretization into an unstructured grid containing $\sim 200,000$ tetrahedra (Figure 4.3(b)), each with a volume of $\sim 10^{-12} \text{ m}^3$, implying a grid edge scale of $\sim 100 \text{ }\mu\text{m}$. Timestep independence was achieved with step sizes of 50 ms. Convergence is defined as

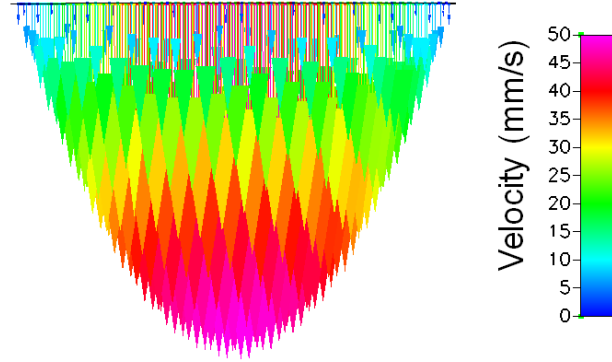


Figure 4.4: Velocity profile at the vertical channel inlet. Because several centimeters of channel upstream of the computational inlet are present in the experimental setup, it is assumed that flow at the inlet obeys Poiseuille's law.

the u -, v -, w -, and p -residuals reaching 10^{-10} of their values at first iteration of the solver.

4.2.3 Boundary Conditions and Initial Conditions

4.2.3.1 Boundary Conditions

In the experimental runs, the flow rate is fixed at 10 ml/min. It is expected that laminar flow is fully developed in the region of interest, which is tens of centimeters downstream of the physical flow inlet. Therefore, the fixed velocity condition applied to the boundary at the computational domain inlet takes the form:

$$v_z = \frac{2Q}{\pi R^2} \left(1 - \frac{r^2}{R^2} \right) \quad (4.13)$$

where v_z is the vertical component of velocity, Q is the volumetric flow rate, R is the diameter of the cylinder, and r is the radial distance from the center of the inlet (Figure 4.4).

A fixed pressure condition (0 Pa with respect to the reference pressure) was applied at the boundary outlet. The remaining domain boundaries, including the

cylinder walls and the tip of the obstructed vessel, were treated for simplicity as no-slip, no-flux rigid walls.

4.2.3.2 Initial Conditions

The fluid within the computational domain is initially set to have zero velocity. The domain is divided into two segments: the vertical channel and the obstructed channel. The scalar concentration in the vertical channel is set to 1, while that in the obstructed channel is set to zero.

4.2.4 Results

4.2.4.1 Velocity Fields

The fluid velocity at $t=1.0$ s is shown in Figure 4.5 for 0.0 MPa, 0.4MPa, and 1.0 MPa ultrasound. It is apparent that parabolic flow persists throughout the vertical channel in the absence of ultrasound, but becomes progressively more disturbed at the junction as sonication pressure is increased.

A closer look at the vessel junction (Figure 4.6(a)) reveals the shed vortex that results in the baseline level of drug delivery observed in the obstructed vessel even in the absence of ultrasound (Figure 3.13(a)). Of note, the maximum velocities in the presence of ultrasound are slightly downstream of the acoustic focus (Figure 4.6(b)), suggesting that convective transport may be further enhanced by applying ultrasound slightly upstream of the junction.

4.2.4.2 BSA Distribution Versus Sonication Pressure

The experimental and simulated distribution of BSA following each 2 second experimental run is shown in Figure 4.7 for no ultrasound (a-b), 0.4 MPa (c-d), and 1.0 MPa (e-f). Note the strong qualitative agreement between the dye distribution in all

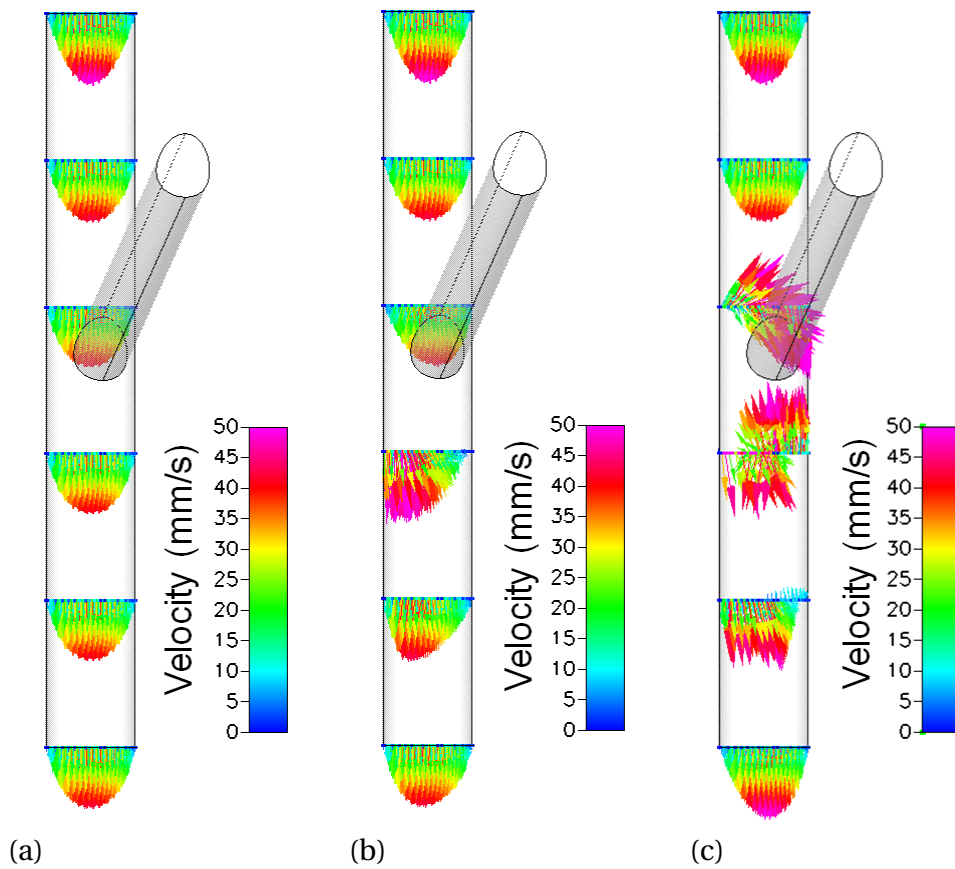


Figure 4.5: View from the right side of the flow phantom (ultrasound traveling from left to right in this image), showing circular cross-sectional velocity fields every 8mm along the path of the flow vessel at **(a)** 0 Pa, **(b)** 0.4 MPa, and **(c)** 1.0 MPa. At 0.4 MPa, flow becomes streamlined 16 mm downstream of the HIFU focus. At 1.0 MPa, vortical structures at the HIFU focus are much more pronounced. Arrow length corresponds to velocity magnitude.

cases. The ‘shed vortex’ at the junction between the two channels is clearly visible in both (a) and (b), and similar flow structures are visible in both (e) and (f). Differences between experimental and simulated results are likely due to a combination of experimental variability and the method used to visualize numerical results.

Quantitatively, the volumetric integral of BSA contained in the obstructed vessel for runs at 0 MPa and 0.4 MPa in the numerical results is within the standard deviation of the respective experimental results reported in Figure 3.14. However, at

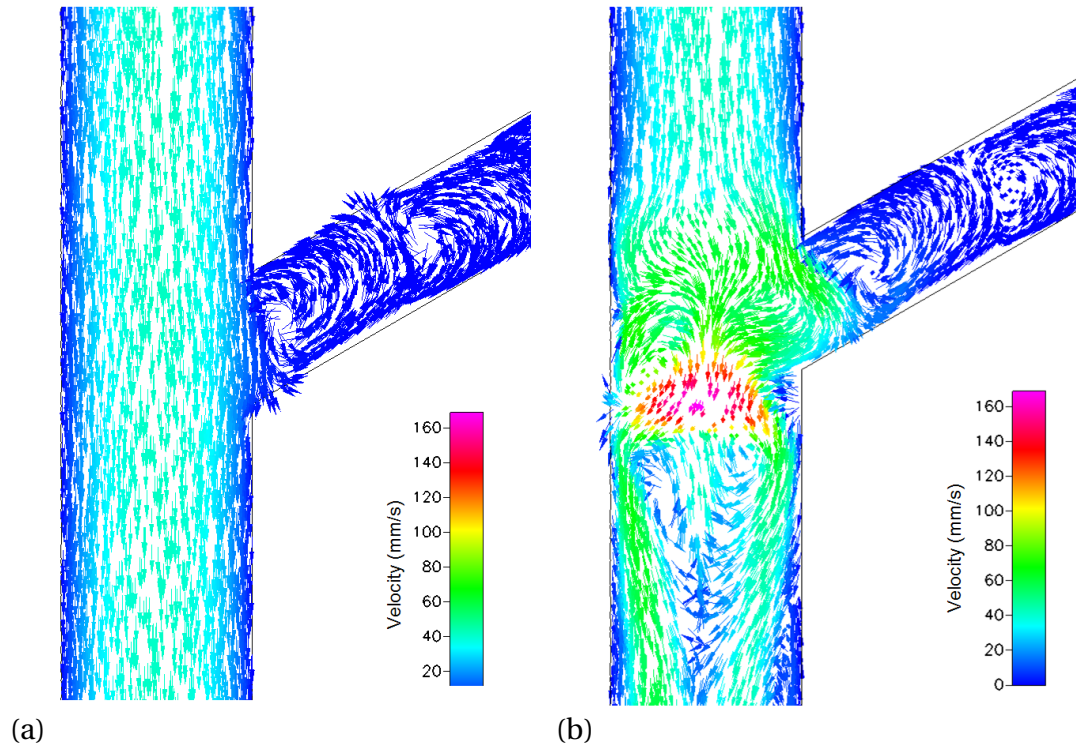


Figure 4.6: Transverse cross-section through the centerline of both vessels, showing the computed velocity field **(a)** in the absence of ultrasound. In order to visualize the shed vortex at the junction, velocity arrows are of uniform size, while color indicates magnitude. **(b)** Simulated velocity field at 1.0 MPa ultrasound, with the effect of acoustic attenuation modeled as a momentum source coming out of the page. Note that the maximum observed velocities are slightly downstream of the acoustic focus, which lies at the intersection of the vessel centerlines.

1.0 MPa, the numerical simulations show BSA levels approximately 2 standard deviations below the experimental values. Perhaps unsurprisingly, 1.0 MPa also marks the pressure at which acoustic emissions associated with inertial cavitation begin to be detected (blue line, Figure 3.19(a)), a finding which suggests that the incorporation of cavitation effects is critical to reproducing the experimentally observed enhancement of convective transport.

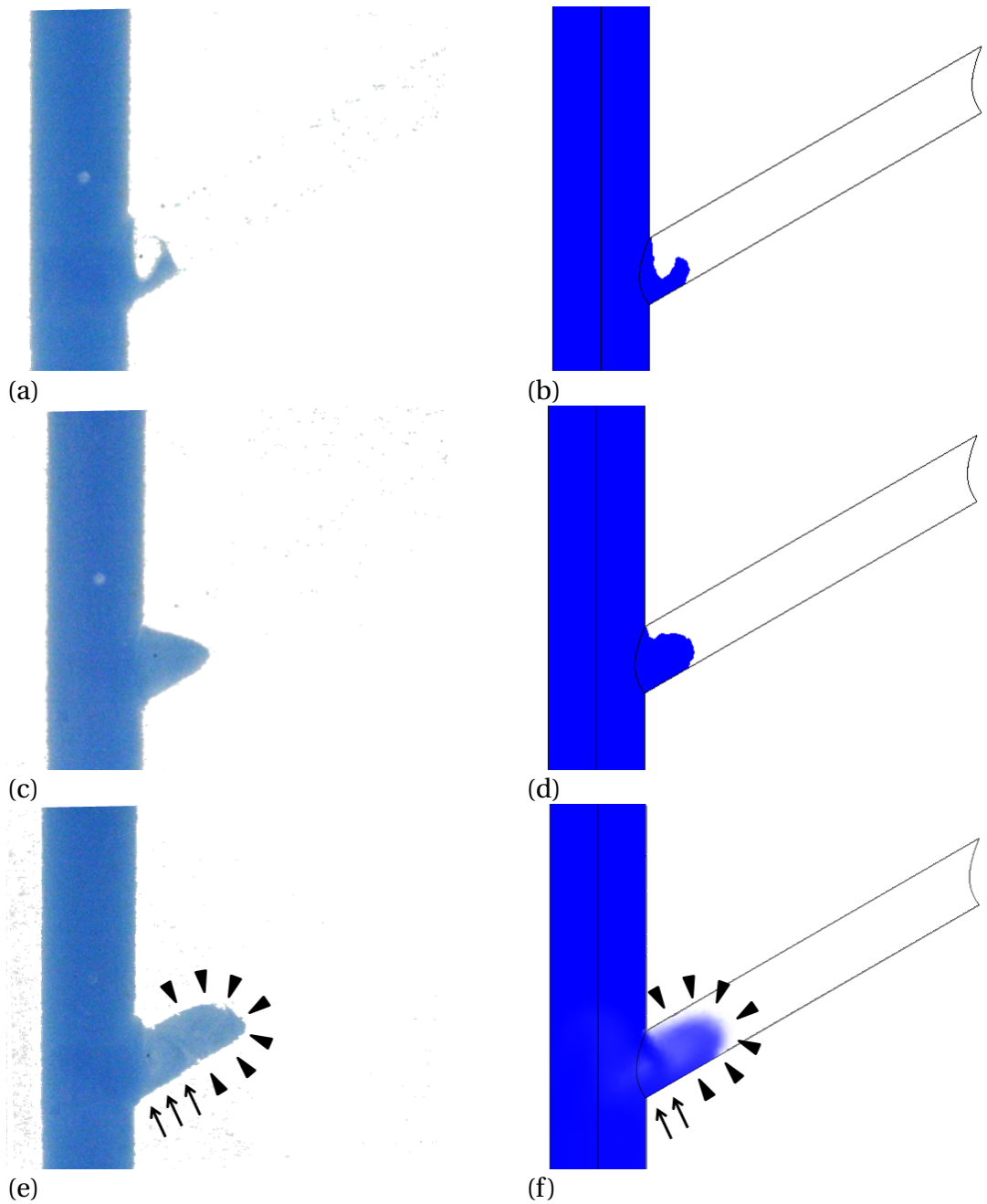


Figure 4.7: Experimental (left column) and simulated (right column) BSA distribution following 2 second ultrasound exposure at 0 MPa (top row), 0.4 MPa (middle row), and 1.0 MPa (bottom row). Ultrasound was simulated as a momentum source directed out of the page, with the magnitude and spatial profile consistent with the transducer characteristics and attenuation values for water. At 0 and 0.4 MPa, there is qualitative agreement in BSA distribution. At 1.0 MPa, an interesting feature begins to emerge: both the primary (arrows) and secondary (arrowheads) vortices are well-reproduced in the numerical simulation. However, the absolute magnitude of BSA transport is lower in the numerical simulation than the corresponding experiment.

4.3 CAVITATION-ENHANCED CONVECTIVE TRANSPORT

The introduction of cavitation nuclei into the experimental setup introduces significant complexity to the fluid dynamics. The nuclei span a range of initial radii, and interact with the sound field, one another, and vessel walls. Capturing these effects in a numerical simulation would require a mathematical model that incorporates two fluids and compressibility, and a discretization scheme that is accurate across several orders of magnitude in space (to capture both the sub- μm scale bubble motion and the millimeter-scale BSA transport) and several orders of magnitude in time (to capture the microsecond bubble oscillations across a 2 second experimental run), a combination which is not possible with the computer resources available. Moreover, the assumptions incorporated into the simulation - for example the concentration of nuclei and their interactions with one another - might not be valid in a physiological setting.

It may therefore be worthwhile to begin with a simpler case - a single oscillating microbubble - and to develop a numerical method which quantifies the convective transport of the liquid surrounding this bubble as a function of time, for given ultrasound parameters. The simulation can then be repeated across a range of initial bubble radii and at various ultrasound excitation frequencies and amplitudes, beginning with mild conditions where bubble behavior is most predictable, and progressing to more violent excitation. In this manner, a set of optimal acoustic parameters and bubble sizes can be identified for application in future experimental work on cavitation-enhanced drug delivery.

In fact, this method, if properly developed, has applications in optimization beyond drug delivery. Cavitation microstreaming has also been exploited to enhance mixing in biochips and microfluidic systems (Liu et al., 2002), disaggregate bacteria

and biofilms (Everbach et al., 2008), cause hemolysis, enhance thrombolysis (Hitchcock et al., 2010; Datta et al., 2006), and increase therapeutic extravasation (Bohmer et al., 2010). Additionally, the shearing action has been cited as a mechanism for sonoporation in the context of gene transfection experiments (Ohl et al., 2006b).

In all these applications, there exists the goal of optimizing, against a wide range of criteria, the physical phenomena responsible for the observed effect. In a DNA-sequencing biochip, for example, the desire for rapid homogeneous mixing may be constrained by limits on the maximum shear stress tolerable before DNA strands begin to degrade. Conversely, in the context of thrombolysis, the primary objective may be to limit the presence of internal stagnation zones, which could lead to untreated volumes of clot.

Thus, there is significant interest in the ability to model and predict the flow fields and other characteristics (such as shear stresses, vorticity, and dispersion) of cavitating microbubbles in a variety of geometrically constrained environments. Yet, of the significant body of theoretical work undertaken to characterize the flow field surrounding oscillating bubbles (Davidson and Riley, 1971; Longuet-Higgins, 1998; Wu, 2002), the majority is limited to the cases of spherical bubbles undergoing volumetric or translational oscillations in unbounded media. In instances where boundaries *have* been considered, theoretical treatment using Stokes flow singularity theory has shown promise in determining the magnitude and patterns of flow fields (Marmottant et al., 2006).

Below, a technique is developed which applies a type of Reynolds-Averaged Navier-Stokes (RANS) equation to account for streaming patterns arising from arbitrarily oscillating boundaries, and is applied to a subset of this class of problems - the streaming flows instigated by a cavitating microbubble. The method, first proposed in Packer et al. (2008), is refined and adapted to several systems in which

the Reynolds Stress field becomes dynamically stationary (time-periodic) over the course of a small number of actuation cycles, so that the net forces arising from spatial variations in Reynolds Stresses can be applied as driving terms in a steady simulation. Computational examples of two microstreaming scenarios, actuated by either applied acoustic fields or electrowetting-driven harmonic wetting angle oscillations, are presented and validated against experiments found in the literature (Tho et al., 2007; Ko et al., 2009). Finally, the method is applied to the problem of optimizing convective transport arising from contrast-agent sized microbubbles driven by HIFU.

4.3.1 Limitations of Conventional Numerical Techniques

Direct numerical simulation of the microstreaming fields surrounding a bubble undergoing stable oscillation about an equilibrium position presents a severe computational challenge. To see why this is the case, consider the time- and length-scales involved in modeling the streaming patterns in a typical MEMS-based acoustic micromixer. It has been shown that adjacent to an oscillating object, only a thin viscous layer close to the boundary is significant in driving streaming. The numerical grid must be fine enough to capture the flow field features contained within this boundary layer, which has thickness $\delta = \sqrt{2\gamma/\rho\omega}$, where γ is the shear viscosity and ρ the density of the liquid, and ω is the driving frequency (Lee and Wang, 1990) - a grid point separation on the order of μm .

A compressible two-fluid simulation with CFL number < 0.1 for the propagating sound wave thus requires a timestep on the order of nanoseconds, so in cases where the timescales of the driving source and evolution of steady streaming fields are separated by orders of magnitude, simulating the thousands of cycles over which steady flow develops requires $\sim 10^6$ timesteps, and $\sim 10^7$ computational cells, a com-

bination which is too expensive to be employed as a design tool, or at all, in many cases. Computational problems with a wide range of time scales are called *stiff*, and are one of the greatest difficulties faced in solution of differential equations.

4.3.2 Reynolds-Averaging and the RANS Equation

In situations where mean flow does not vary, the equations of fluid motion can be averaged over time. Beginning with the Navier-Stokes equation for incompressible fluids (Equation (4.10), reproduced here using Einstein notation):

$$\rho \frac{\partial u_j}{\partial t} + \rho u_i \frac{\partial u_j}{\partial x_i} = -\frac{\partial p}{\partial x_j} + \mu \frac{\partial^2 u_j}{\partial x_i^2}, \quad (4.14)$$

each velocity and pressure term can be decomposed into a mean (time-averaged) and fluctuating component:

$$\phi(\mathbf{x}, t) = \bar{\phi}(\mathbf{x}) + \phi'(\mathbf{x}, t), \quad (4.15)$$

where

$$\bar{\phi}(\mathbf{x}) = \lim_{T \rightarrow \infty} \frac{1}{T} \int_0^T \phi(\mathbf{x}, t) dt, \quad (4.16)$$

a technique known as Reynolds Decomposition. Averaging any linear term in this matter yields the averaged quantity of the same term. Averaging nonlinear terms, however, yields two terms:

$$\overline{u_i \phi} = \overline{(\bar{u}_i + u'_i)(\bar{\phi} + \phi')}, \quad (4.17a)$$

$$= \bar{u}_i \bar{\phi} + \overline{u'_i \phi'}, \quad (4.17b)$$

the second of which is only zero for uncorrelated variables, but otherwise cannot be represented in terms of their mean quantities. Applying Reynolds Decomposition to Equation (4.14), time-averaging (to eliminate the transient term), and rearranging, yields the RANS equation:

$$\rho \bar{u}_i \frac{\partial \bar{u}_j}{\partial x_i} = -\frac{\partial \bar{p}}{\partial x_j} + \mu \frac{\partial^2 \bar{u}_j}{\partial x_i^2} + F_j, \quad (4.18)$$

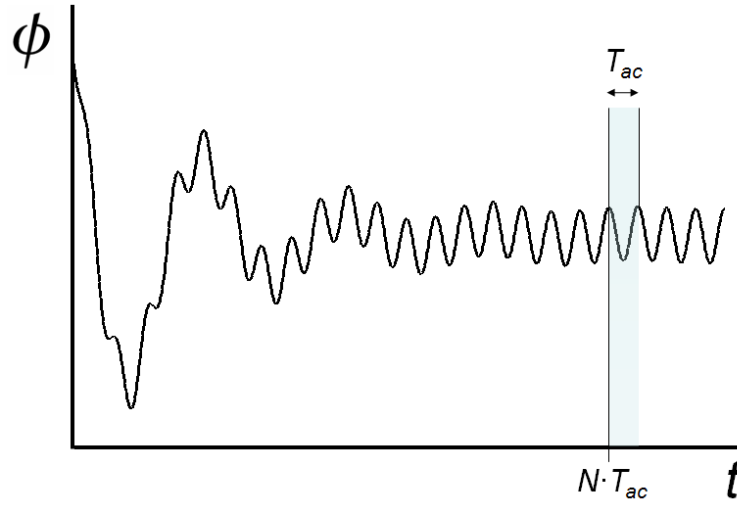


Figure 4.8: Time-averaging of the variable ϕ in Equation (4.20) begins after ϕ has converged to quasi-steady (time-periodic) behavior. The criteria for convergence used in this chapter is shown in Equation (4.27).

where all terms in the original Navier-Stokes equation are replaced with their time-averaged values, and there emerges a new term, F_j :

$$F_j = -\rho \frac{\partial \overline{u'_i u'_j}}{\partial x_i}, \quad (4.19)$$

which is the spatial rate of change of the Reynolds Stress.

Various attempts have been made to approximate F_j . For instance, Lighthill recognized that F_j , the net change of momentum in the j -direction per unit volume, is the force behind acoustic streaming, and developed relationships between F_j and a variety of sound wave attenuation mechanisms (Lighthill, 1978). Like acoustic streaming, cavitation microstreaming arises from second-order, nonlinear effects in which the temporal average of velocity fluctuations is non-zero. Although the time-independent circulation in cavitation microstreaming arises from frictional forces at the bubble/fluid interface rather than attenuation of a propagating wave (Leighton, 1994), F_j can be approximated in an analogous manner by recognizing

the time-periodic nature of F_j and replacing (4.16) with:

$$\bar{\phi}(\mathbf{x}) = \frac{1}{T_{ac}} \int_{nT_{ac}}^{(n+1)T_{ac}} \phi(\mathbf{x}, t) dt \Big|_{n=N} \quad (4.20)$$

where T_{ac} is the period of a single actuation cycle, and the time-averaging begins at cycle N , with N large enough that the Reynolds Stress field has converged (Figure 4.8). The criteria for such convergence are defined later in this section.

Employing this strategy, the general method directly follows:

Step 1. Unsteady simulation : Simulate the unsteady motion for a sufficient number of complete actuation cycles.

Step 2. Reynolds Stress Calculation : Calculate F_j over the N -th actuation cycle at each CV of the computational domain.

Step 3. Steady simulation : Apply the calculated F_j values as forcing terms driving a steady simulation to arrive at the cavitation microstreaming field.

Below, each step is described in further detail, as well as the numerical techniques, assumptions, and approximations applied to the validation examples in Section §4.3.6.

4.3.3 Step 1. Unsteady simulation

The desired output from Step 1 is the velocity $\mathbf{u}(\mathbf{x}, t)$ for each cell in the computational domain at each timestep in the N -th actuation cycle. Beyond this constraint, the design of the model in Step 1 is independent of subsequent steps, and can be developed to the level of complexity required to reflect the physical phenomena involved. Thus, a microbubble subject to a mild acoustic field may be adequately modelled by the radially symmetric translation of the boundary of a spherical wall

in an incompressible fluid, while an inertially cavitating microbubble model may require incorporating surface tension, air and water compressibility, heat transfer and other physics to faithfully reproduce streaming patterns.

The present work considers cases of microstreaming driven by either acoustic excitation or electrowetting-driven harmonic wetting angle oscillations. In cases of acoustically driven cavitation, it is assumed that the whole domain can be approximated to be under the same acoustic excitation pressure. That is, for a computational domain of characteristic length l and speed of sound in liquid c_l , $l \ll T_{ac} c_l$, and acoustic pressures are such that the Mach number $\ll 1$.

In the case of electrowetting-driven oscillations, it is assumed that bubble compressibility is secondary to contact angle behavior and surface tension forcing in driving microstreaming behavior. In all cases, it is assumed that $\rho_l \gg \rho_v$, where ρ_l is the density of liquid, ρ_v is the density in the cavity, so that there is negligible momentum transfer between the contents of the cavity and the surrounding liquid. Therefore, the motion of the air-water interface is of greater importance than the conditions (temperature, pressure, velocity) within the bubble in driving microstreaming in the liquid. Below, a numerical method which accounts for the motion of the interface between two fluids is discussed.

4.3.3.1 Free Surface Flows

In CFD, situations in which the position of a boundary between two fluids is only known at the beginning of the simulation are known as Free Surface flows. Neglecting phase changes at the free surface (an assumption which, incidentally, precludes the capture of rectified diffusion effects), two boundary conditions arise. First, that the normal component of fluid velocity at the surface is equivalent to the normal component to the surface itself. Second, that momentum is conserved at the free

surface. Implementing these boundary conditions requires knowledge of the location of the surface, which can be found using either of two techniques:

- *Interface-tracking methods*, in which the numerical grid is fitted to the boundary and advances with movements of the free surface
- *Interface-capturing methods*, in which the numerical grid is fixed, and the location of the free surface is determined by the fraction of each cell filled with each fluid

Among the interface-capturing methods is the *Volume-of-Fluid* (VOF) scheme (Hirt and Nichols, 1981; Rider et al., 1995), which is implemented in CFD-ACE+. In this scheme, a variable ψ is introduced, which takes on a value of 1 in cells that contain only water, 0 in cells that contain only air, and an intermediate value for cells containing a free surface. From the continuity equation, ψ must also satisfy:

$$\frac{\partial \psi}{\partial t} + \nabla \cdot \psi = 0. \quad (4.21)$$

In cells containing a mixture of water (fluid 1) and air (fluid 2), any extensive property ϕ_{ext} is approximated by:

$$\tilde{\phi}_{ext} = \psi \phi_{ext,1} + (1 - \psi) \phi_{ext,2}, \quad (4.22)$$

where $\tilde{\phi}_{ext}$ is the cell-averaged quantity. Intensive properties ϕ_{int} are approximated by:

$$\tilde{\phi}_{int} = \frac{\psi \rho_1 \phi_{int,1} + (1 - \psi) \rho_2 \phi_{int,2}}{\rho_{mix}}. \quad (4.23)$$

Of course, surface reconstruction is a prerequisite for determining the flux of ψ from one cell to the next. Several methods exist for determining fluid flux:

Homogeneous Foam In this zeroth order scheme, mixed cells are treated as homogeneous ‘foams’, and the orientation of the free surface is not determined. This has the effect of ‘smearing’ free surfaces over adjacent cells.

Single Line Interface Construction (SLIC) In the SLIC method, the flux through a face is determined by the flow direction and the upstream or downstream value of ψ . For example, if the upwind cell is partially water, and the downwind cell is all water, then the free surface is assumed to be parallel to the downwind cell, with water fluxed first. If both cells contain mixtures, then the surface is assumed to be perpendicular to the face, and the fluid in the upwind cell is fluxed as a mixture. This method is first-order accurate.

Piecewise Linear Interface Construction (PLIC) In this scheme, the free surface is assumed to be planar within the CV, and is uniquely defined by a vector normal to the infinite plane, and a point within the cell through which the plane passes (Kothe et al., 1996). The normal vector is determined by assuming that it is parallel to the gradient of ψ , which is calculated for the set of cells adjacent to and include the target CV. The location of the anchor point is chosen so that it truncates the liquid volume fraction ψ in the cell. PLIC is second-order accurate, and because each cell has a unique surface normal vector, the curvature from cell to cell can be computed. This allows surface tension effects to be incorporated.

Secondary Fluid Flux Calculations Once the interface shape and orientation have been determined, the flux of water is calculated by back projection from the cell face (Figure 4.9). This is an explicit computation based the liquid surface orientation in the previous timestep, and depends on information in the upwind cell only. For stability of this algorithm in solving Equation (4.21), the CFL number must be less than 1.0, but in practice, lower values are often required.

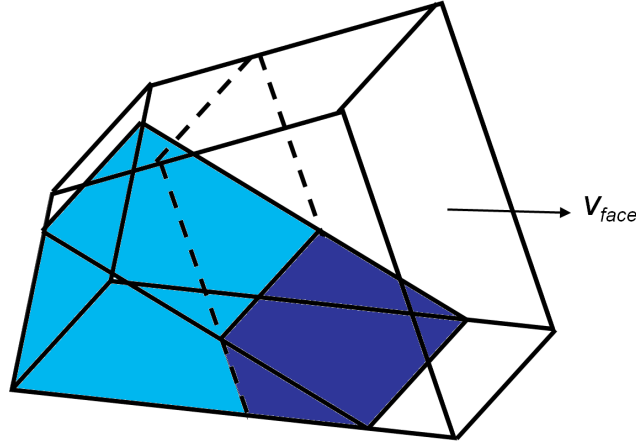


Figure 4.9: Computation of the secondary fluid flux in the PLIC method is achieved by back-projection of the cell face a distance $v_{face}\Delta t$ to its new location (dotted line). The liquid volume encompassed by back projection (dark blue) is allocated to the downstream computational cell. Reproduced from (ESI).

4.3.3.2 Bubble Motion

Because surface tension is of critical importance in the dynamics given the bubble sizes under consideration, the air-water interface is modeled using the PLIC interface capturing method, with timesteps chosen so that the CFL number < 0.1 . Bubble wall motion is generated, where appropriate, either through a mass/momentum source/sink within the bubble (examples in Sections §4.3.6.2 and §4.3.7), or through controlling the wetting angle boundary condition (example in Section §4.3.6.3). The spatial extent of the source/sink at a given timestep is confined to a sphere of 0.3 bubble radii positioned at the bubble centroid, and the magnitude is chosen to yield the experimentally characterized bubble motion. Note that this simplification of the Step (1) model is facilitated by the published experimental data which explicitly tracks bubble wall motion (and which offers an intermediate model validation step). In the case of a hypothetical or uncharacterized geometry, an oscillatory pressure boundary condition within a *compressible* Volume-of-Fluid model is required to generate bubble wall motion.

Fluid properties (density, viscosity, surface tension) for both air and water are kept constant at their standard temperature and pressure values across the computational domain, with the exception that capillary wave damping is implemented by increasing the viscosities of water and air by factors of 10 and 500, respectively, in the cells immediately adjacent to a fluid interface. This has the effect of increasing numerical stability, but suppresses higher-mode bubble shape oscillations (ESI). However, higher-mode oscillations were *not* experimentally observed at the excitation frequencies and bubble sizes presented, so capillary wave damping is not expected to materially affect simulated streaming patterns.

For discretization in space, Central Difference Scheme (CDS) is used, and an Algebraic Multigrid Solver (AMG) is used to accelerate convergence. Time advancement is performed using a second-order semi-implicit Crank-Nicolson scheme. This step of the algorithm is implemented in CFD-ACE+. However, the generality of the overall algorithm is emphasized: any other module or solver capable of handling the prevailing physics of the Step (1) problem can be used.

4.3.4 Step 2. Reynolds Stress Calculation

Step 2, implemented in Matlab, begins by calculating the various combinations of $\overline{u'_i u'_j}$, $i = 1 : 3, j = 1 : 3$ (of which there are six - $\overline{u'_1 u'_1}, \overline{u'_1 u'_2}, \overline{u'_1 u'_3}, \overline{u'_2 u'_2}, \overline{u'_2 u'_3}, \overline{u'_3 u'_3}$), at each point in the computational domain, by discretizing (4.20) with $\phi_{ij} = u'_i u'_j$:

$$\overline{\phi}_{ij}(\mathbf{x}) = \frac{1}{T_{ac}} \sum_{t=NT_{ac}}^{(N+1)T_{ac}} \phi_{ij}(\mathbf{x}, t) \left[\frac{\rho_l \psi(\mathbf{x}, t) + \rho_v (1 - \psi(\mathbf{x}, t))}{\rho_l} \right] \Delta t \quad (4.24)$$

where Δt is the (possibly variable) timestepping used in Step 1, $\psi \in [0, 1]$ is the liquid volume fraction in the computational cell centered at $\mathbf{x}$ (with $\psi = 1$ corresponding to a cell containing only water, and $\psi = 0$ corresponding to a cell containing only air). The term in brackets is an approximation of the fluid density in each computational

cell, and is included to account for stresses developed in cells through which the bubble surface passes during the course of the simulation. Note that absent the high densities created in the bubble during, for example, inertial cavitation, the term in brackets is well-approximated by $\psi(\mathbf{x}, t)$.

Because momentum fluxes decay rapidly and nonlinearly with distance away from the bubble (Figure 4.15), discretizing the spatial derivative $\partial \bar{\phi}_{ij} / \partial x_i$ in Step 2 is a critical element to resolving streaming patterns accurately. In this study, it was found to be computationally less expensive to use higher-order difference schemes in the calculation of $\partial \bar{\phi}_{ij} / \partial x_i$ for a given grid-independent result from Step 1, compared to further increasing grid resolution in Step 1 in combination with a forward- or backward- difference scheme for calculation of $\partial \bar{\phi}_{ij} / \partial x_i$. Therefore, third-order Taylor expansion of $\bar{\phi}_{ij}$ about $\mathbf{x}$ was used:

$$\bar{\phi}_{ij}(\mathbf{x} + \Delta \mathbf{x}) = \sum_{m=0}^3 \frac{1}{m!} \left[\Delta x_1 \frac{\partial}{\partial x_1} + \Delta x_2 \frac{\partial}{\partial x_2} + \Delta x_3 \frac{\partial}{\partial x_3} \right]^m \bar{\phi}_{ij}(\mathbf{x}) \Big|_{\mathbf{x}}. \quad (4.25)$$

Since the simulations considered in this thesis involved hexahedral structured grids, the 20 unknown coefficients in (4.25) were fitted in a linear least-squares manner using values at the 27-nodes contained in a 3x3x3-cell cube centered at node $\mathbf{x}$, rather than introduce a systematic, potentially directional approximation by exactly solving the system using 20 asymmetrically distributed nodes. In the case of unstructured grids, selection of neighboring nodes should take into account both proximity to the node under consideration, as well as the conditioning of the resulting linear system.

Once this computation has been performed at every node in the domain, the

forcing term at each node is given by:

$$\mathbf{F}(\mathbf{x}) = \begin{pmatrix} F_1 \\ F_2 \\ F_3 \end{pmatrix} = -\rho_l \begin{pmatrix} \frac{\partial \bar{\phi}_{11}}{\partial x_1} + \frac{\partial \bar{\phi}_{12}}{\partial x_2} + \frac{\partial \bar{\phi}_{13}}{\partial x_3} \\ \frac{\partial \bar{\phi}_{12}}{\partial x_1} + \frac{\partial \bar{\phi}_{22}}{\partial x_2} + \frac{\partial \bar{\phi}_{23}}{\partial x_3} \\ \frac{\partial \bar{\phi}_{13}}{\partial x_1} + \frac{\partial \bar{\phi}_{23}}{\partial x_2} + \frac{\partial \bar{\phi}_{33}}{\partial x_3} \end{pmatrix}. \quad (4.26)$$

In this study, the field $\mathbf{F}(\mathbf{x})$ is said to have converged at the N th oscillation cycle (henceforth $\mathbf{F}^{(N)}(\mathbf{x})$) if:

$$\frac{1}{V_{total}} \sum_{\mathbf{x}} V(\mathbf{x}) \frac{|\mathbf{F}^{(N)}(\mathbf{x}) - \mathbf{F}^{(N-1)}(\mathbf{x})|}{|\mathbf{F}^{(N-1)}(\mathbf{x})|} < \epsilon \quad (4.27)$$

where V_{total} is the total volume of the computational domain, $V(\mathbf{x})$ is the volume of the cell at location $\mathbf{x}$, and $\epsilon = 0.05$.

4.3.5 Step 3. Steady simulation

The object of Step 3 is then to apply these forcing values in an appropriate steady simulation. Of course, in a steady situation, an oscillating bubble wall must be replaced by a still boundary. Reasonable choices for bubble wall placement include its position at either extreme of the oscillation cycle, or its mean or initial position. Qualitatively, placing the bubble wall in its expanded state eliminates certain cells (and therefore both the forcing and viscous dissipation in those cells) from the computational domain, while placing it in its compressed state includes those cells. The steady simulations presented in this study define the bubble wall in its compressed, innermost state, but location was found to have minimal effect on the streaming patterns produced. This is unsurprising, as streaming vortex length scales were generally two orders of magnitude larger than bubble wall displacement length scales.

The settings and solver environment used for this third stage of the algorithm are very similar to those employed in the first stage, with the important difference that

this is a steady, thus more economical, computation. As with Step 1, the algorithm is fully modular; any methodology or solver that allows for a user-defined body force term can be used.

4.3.6 Validation and Test Results

In this section, numerical examples are provided to illustrate the performance of the new method. Grid independence is confirmed by achieving <10% difference in maximum observed flow rates between the results presented and those of simulations with computational cells 50% larger in each linear dimension, or one-half the number of timesteps. Note that in Section §4.3.7, these criteria are applied under one condition within the parametric study, while the remainder are assumed to be grid independent at the same CFL number.

4.3.6.1 Steady Flow of Fluid Enclosed Between Two Coaxial Cylinders

The first numerical example attempts to replicate the streaming patterns observed in Bertelsen et al. (1973), which examines the 2-dimensional flow of an incompressible viscous fluid enclosed between two coaxial circular cylinders of radii A and a , extending infinitely in the z -axis, with the larger cylinder oscillating laterally (in the x -direction) relative to the smaller cylinder with wall velocity $U = U_0 \cos(\omega t)$. Experimental and theoretical solutions have previously been obtained for cylinders of dimensions $a = 0.147 \text{ cm}$, $A/a = 2.78, 4.08, 7.14$, enclosing air of kinematic viscosity $\nu = 0.15 \text{ cm}^2/\text{s}$ subject to oscillations at $\omega = 2\pi \cdot 100 \text{ Hz}$ and $U_0 = 8.4 \text{ cm/s}$ (Bertelsen et al., 1973).

Figure 4.10 shows a *portion* of the discretized computational domain used to solve unsteady flow for $A/a = 4.08$ (for ease of visualization, only one quadrant is shown here and in subsequent figures, though the simulations were conducted

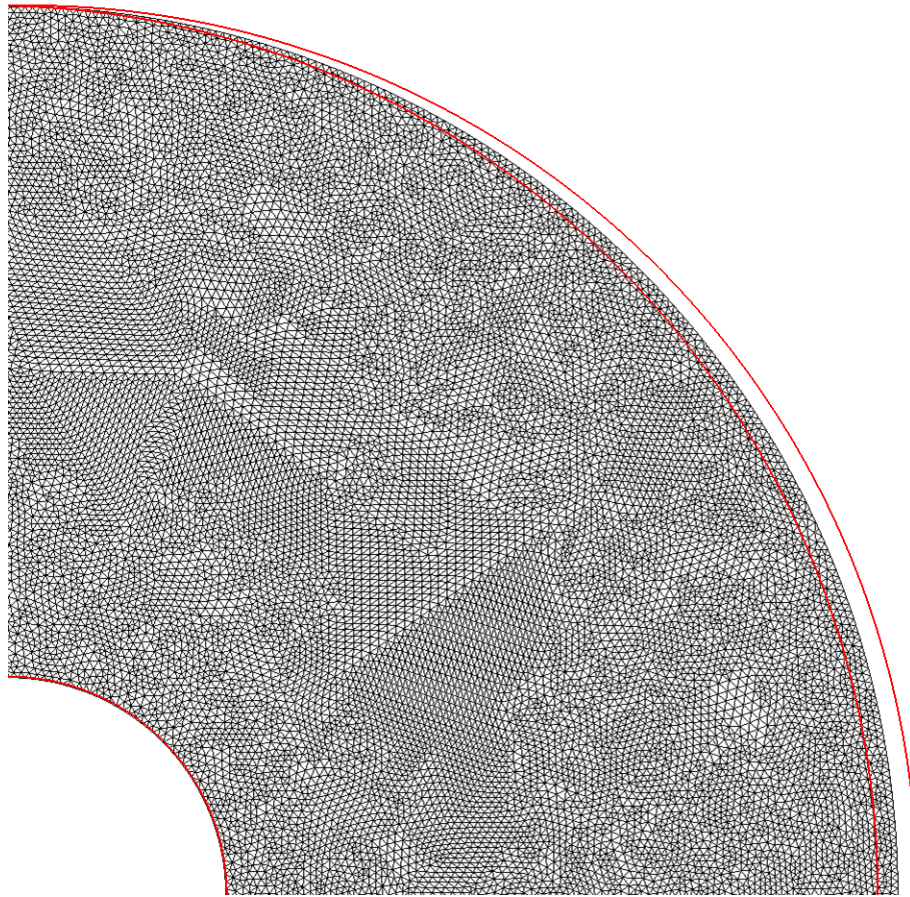


Figure 4.10: Geometry of one quadrant of the computational domain and discretization for $A/a = 4.08$. Also shown are outlines of the outer cylinder at either extreme of the sinusoidal oscillation (red). The inner cylinder remains stationary.

over the full 360° geometry). In contrast to subsequent validation examples which involved stationary computational grids and free surface flows, the computational grid in the two-cylinder scenario was allowed to deform as an elastic solid body in order to accommodate the translations of the outer cylinder. Therefore, in order to facilitate calculation of Reynolds Stresses at *fixed* points, both the velocity vector and the *location* of each node was recorded for each timestep. Grid-independence was achieved at 50,000-95,000 cells per simulation, depending on A/a , and at 100 timesteps per sinusoid.

In Step 2, Reynolds stresses at fixed points were calculated by first performing

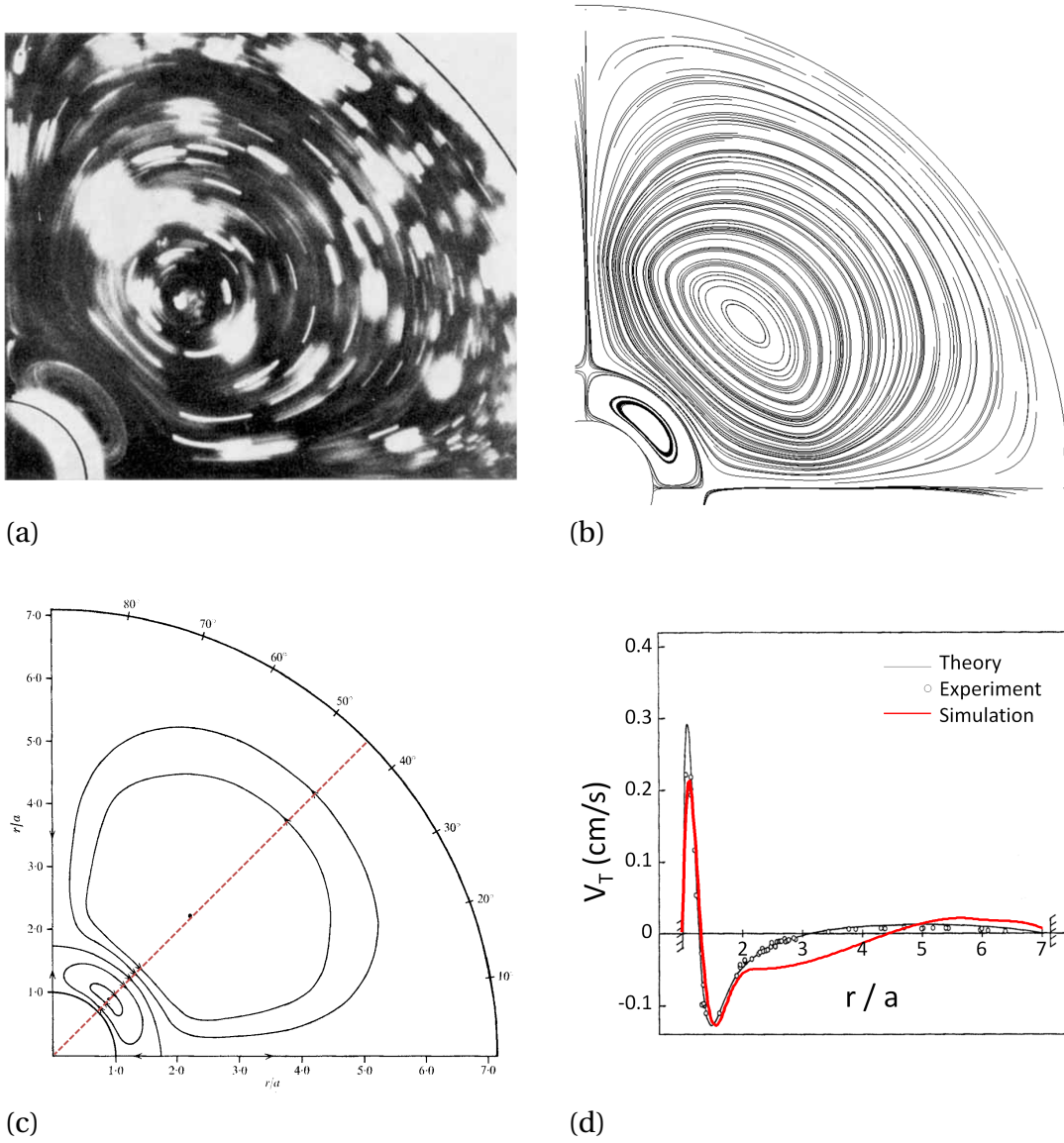


Figure 4.11: Steady flow between oscillating cylinders with $A/a = 7.14$. **(a)** Experimental streaklines. **(b)** Streaklines produced by numerical simulations. **(c)** Theoretical solution. **(d)** Theoretical (black line), experimental (circles), and simulated (red line) tangential velocity, as a function of the normalized distance r from the inner cylinder (along the red dotted line in **(c)**). **(a)** and **(c)** reproduced from (Bertelsen et al., 1973)

Delaunay triangulation at each time step and interpolating the velocity vector field at the location of each node in the subsequent steady simulation, then solving Equation (4.24). Convergence of $\mathbf{F}^{(n)}(\mathbf{x})$ is reached at $n = 5$.

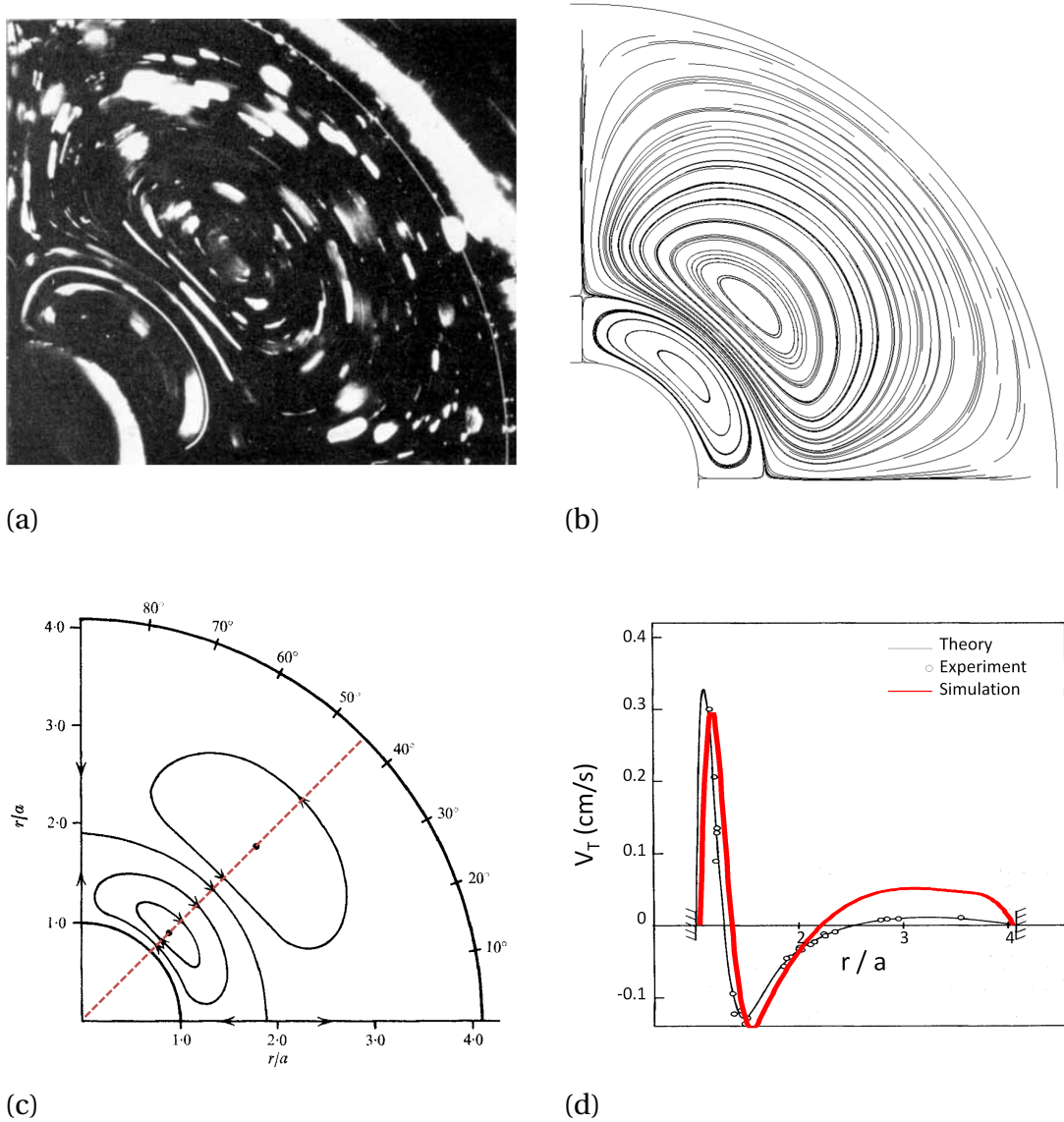


Figure 4.12: Steady flow between oscillating cylinders with $A/a = 4.08$. **(a)** Experimental streaklines. **(b)** Streaklines produced by numerical simulations. **(c)** Theoretical solution. **(d)** Theoretical (black line), experimental (circles), and simulated (red line) tangential velocity, as a function of the normalized distance r from the inner cylinder (along the red dotted line in (c)). **(a)** and **(c)** reproduced from (Bertelsen et al., 1973)

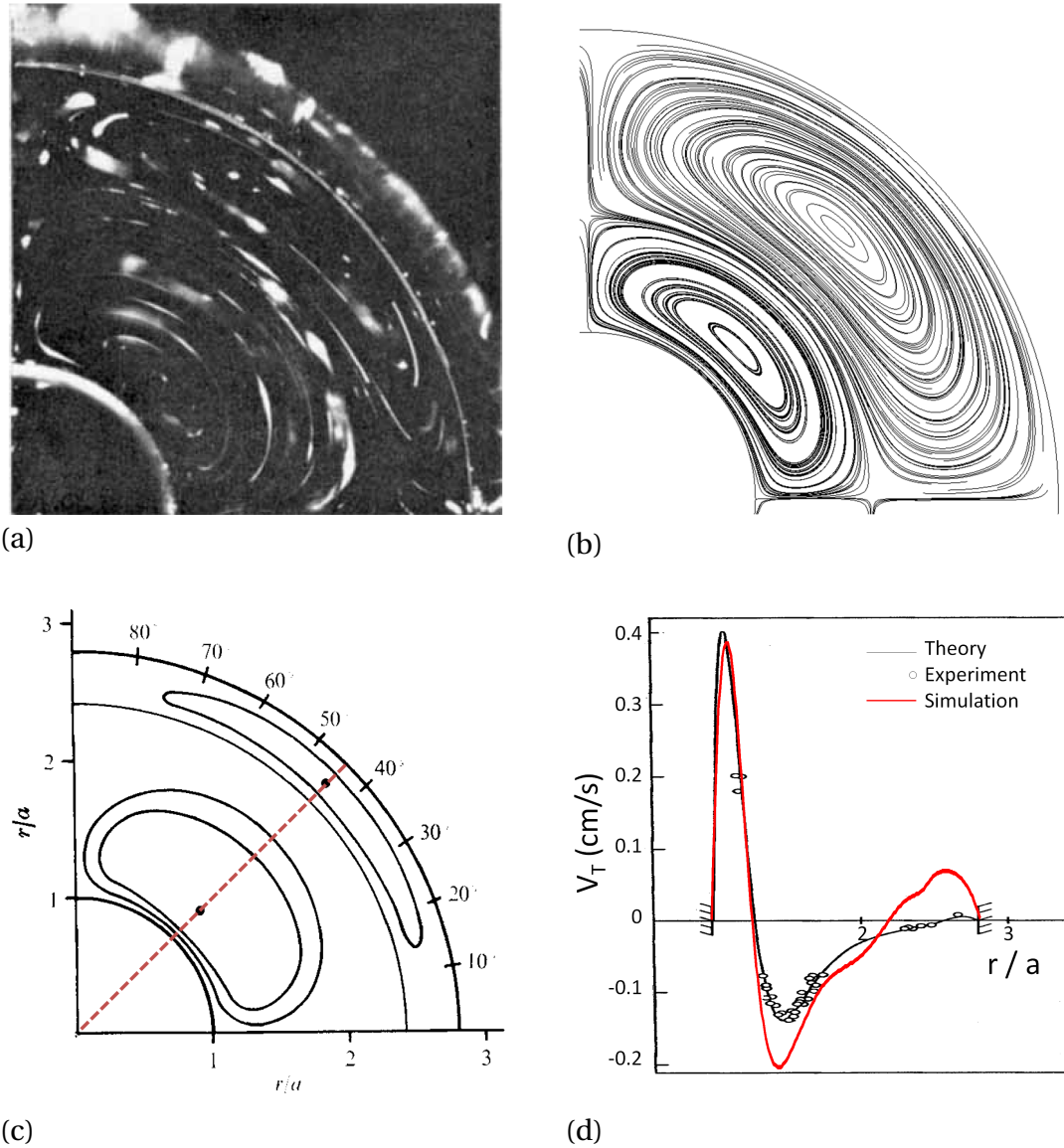


Figure 4.13: Steady flow between oscillating cylinders with $A/a = 2.78$. **(a)** Experimental streaklines. **(b)** Streaklines produced by numerical simulations. **(c)** Theoretical solution. **(d)** Theoretical (black line), experimental (circles), and simulated (red line) tangential velocity, as a function of the normalized distance r from the inner cylinder (along the red dotted line in (c)). **(a)** and **(c)** reproduced from (Bertelsen et al., 1973)

Figures 4.12-4.11 compare the theoretical, experimental, and numerical results for three different values of A/a . In all cases, the separation of flow into inner and outer vortices is reproduced. Additionally, the inner vortex shows very strong agreement in both shape and steady flow velocities. However, discrepancies begin to emerge closer to the outer cylinder. Specifically, tangential velocities near the outer cylinder are stronger than theory predicts. This has the effect of shifting the core of the outer vortex and, particularly for $A/a = 2.78$, exaggerating the tangential velocity between the cores of the vortices.

It is suspected that the numerical artifact is introduced with the interpolation technique used to estimate values at fixed coordinates, as the computational grid deforms. This technique, Delaunay Triangulation, is first-order, and therefore overestimates values along functions with positive higher-order terms (such as the Reynolds Stress field near the outer cylinder wall). However, because the intended application of this technique will not involve deforming grids, no further attempts were made to refine this simulation.

4.3.6.2 Microstreaming around an acoustically cavitating microbubble

The second test case attempts to replicate the experimentally observed streaming patterns in Section 4.1 of Tho et al. (2007). Briefly, a captive bubble of radius $240\ \mu\text{m}$ in a chamber of dimensions $30\ \text{mm} \times 30\ \text{mm} \times 0.66\ \text{mm}$ (the x- y- and z-axes, respectively) is subject to sinusoidal, $34\ \text{Pa}$ excitation at $2.4\ \text{kHz}$, resulting in an oscillation with maximum bubble wall displacement of $6.4\ \mu\text{m}$. Streaming patterns and velocity fields in the z-plane were recorded at various depths of focus using micro-Particle Image Velocimetry (micro-PIV). Note that the optical focal depth is relatively large compared to the bubble size, resulting in substantial depthwise velocity averaging, which is a significant source of experimental data uncertainty. The $2\ \mu\text{m}$ micro-PIV

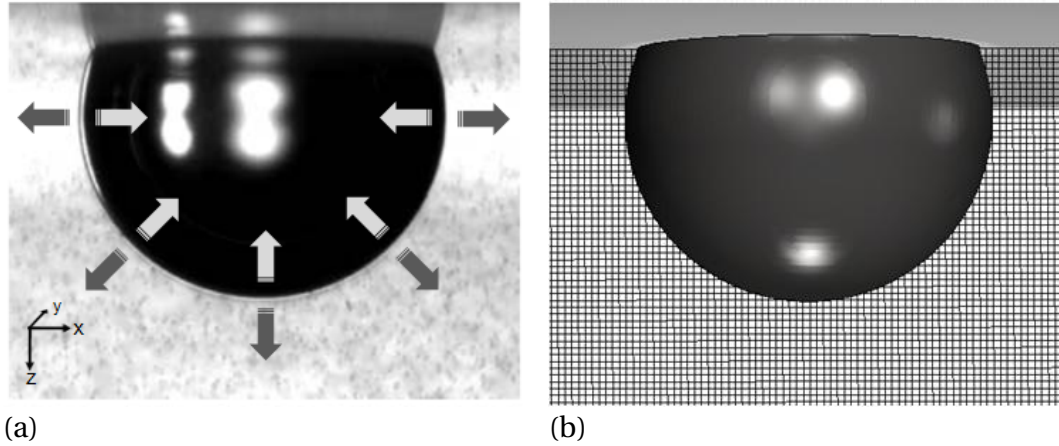


Figure 4.14: **(a)** Experimental and **(b)** numerical initial conditions, with a cross-section of the computational grid of $5 \mu\text{m}^3$ cells shown in **(b)** for scale. Note that the VOF PLIC method tracks the air/water interface as a plane within each hexahedral cell, allowed sub-grid bubble wall resolution. **(a)** reproduced from (Tho et al., 2007) with arrows added to indicate direction of bubble wall motion.

tracer particles used in this protocol aggregate at the air-water interface, decreasing the bubble wetting angle, an effect which is incorporated into the simulation. This 'surface skin' of particles also inhibits fluid motion over the bubble, resulting in a 'no-slip' boundary condition. Accordingly, the Step 3 steady simulation in this example is conducted with a no-slip bubble surface (Tho et al., 2007). Bubble growth due to rectified diffusion is negligible at the time scales considered in this simulation, and is therefore ignored (Eller and Flynn, 1963).

Figure 4.14 shows the experimental (a) and numerical (b) initial conditions of the bubble wall, including a cross-section of the computational grid, which consists of $5 \mu\text{m}^3$ computational cells. The full z-dimension (0.66 mm) of the experimental volume is simulated (and, as in the experiment, bounded by no-slip, no-flux walls above and below). The computational domain is truncated in the x- and y-dimensions from 30 mm to 4 mm, and perimeter walls are replaced by fixed-pressure boundary conditions, a modification justified by analogous two-

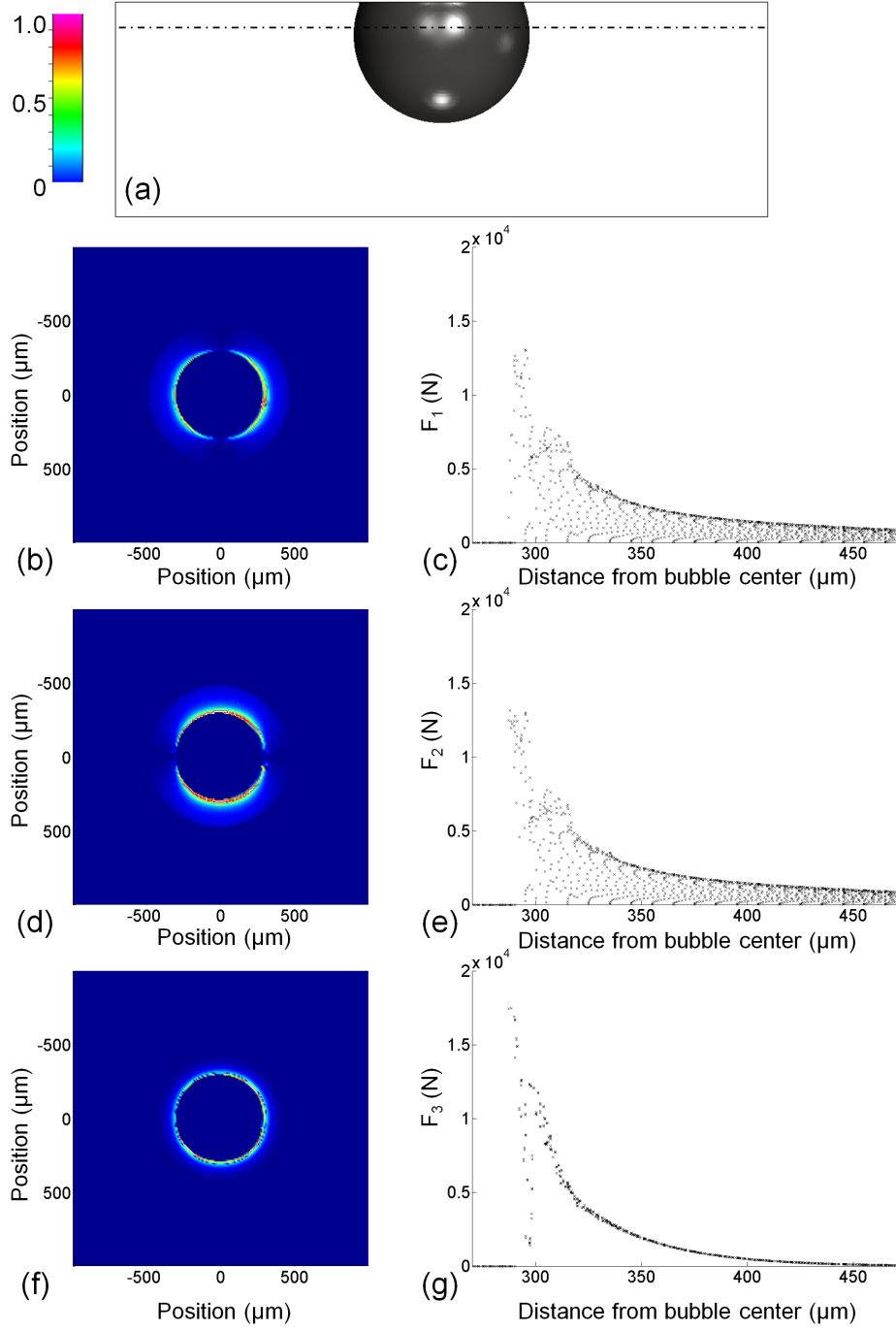


Figure 4.15: Reynolds Stresses along the z -plane with depth $75 \mu\text{m}$ from the wall on which the captive bubble rests (dotted line, (a)). Magnitude of the x -component of the Reynolds stress is displayed spatially (b) and as a function of distance from the bubble center (c). (d)-(e) y -component of the Reynolds stress. (f)-(g) z -component of the Reynolds stress. Note that the stress decays to $<10\%$ of its maximum value within $150 \mu\text{m}$ of the bubble surface.

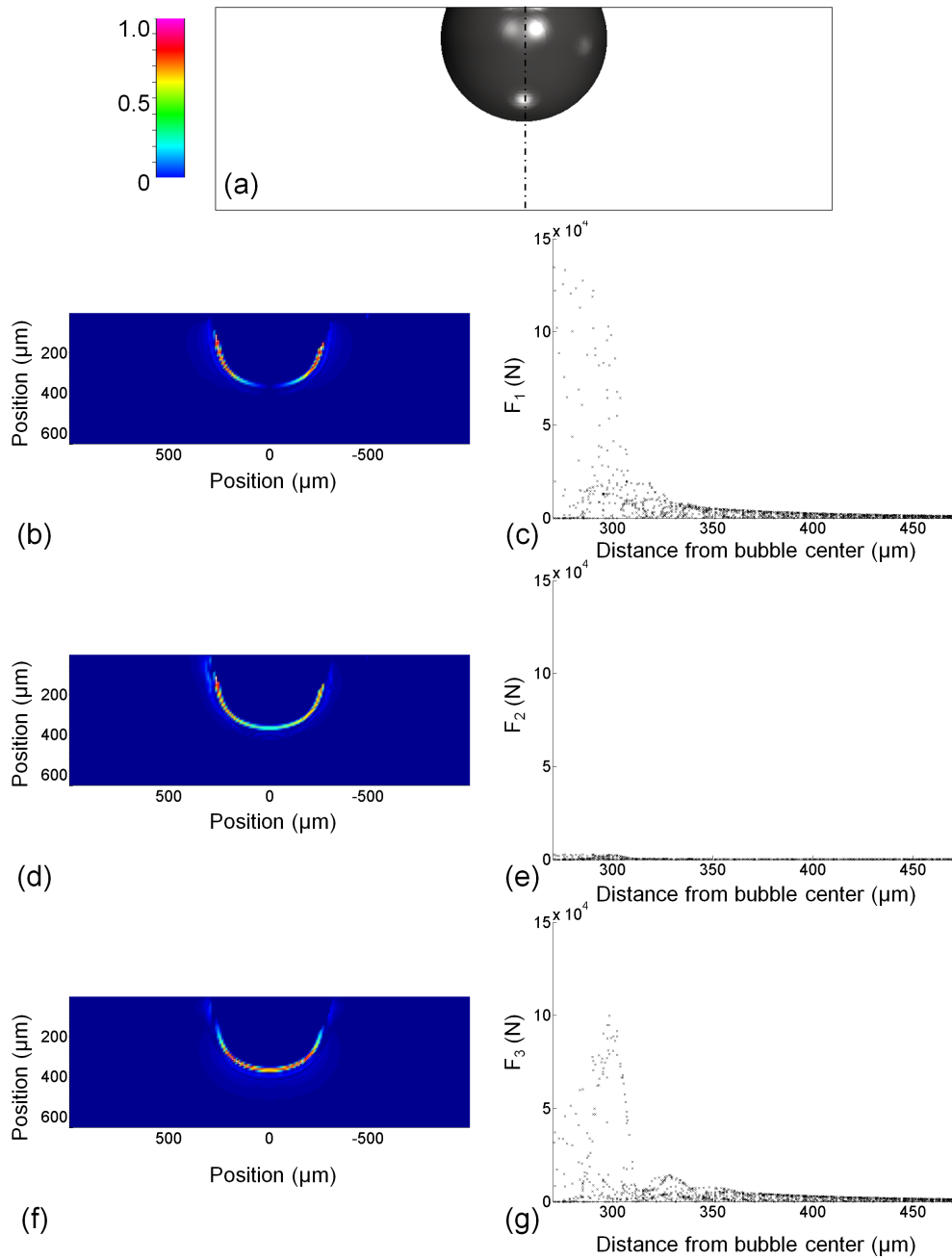


Figure 4.16: Reynolds Stresses along the y-plane cutting through the bubble center (dotted line, (a)). (b)-(c) x-component of the Reynolds stress. (d)-(e) y-component of the Reynolds stress. Note the absence of depthwise Reynolds stresses, a phenomenon which anticipates a plane of symmetry between the streaming patterns (f)-(g) z-component of the Reynolds stress. Note once again that the stress decays to <10% of its maximum value within $150 \mu\text{m}$ of the bubble surface.

dimensional simulations in which truncation was found to have negligible effects on the results. This is unsurprising for two reasons: (1) the distance from the bubble wall to the truncated boundaries is more than two orders of magnitude larger than the bubble oscillation length scales, and (2) the magnitude of the Reynolds Stress field, largest near the bubble surface, decays to <10% of its maximum value within 150 μm of the bubble surface (Figure 4.15 and Figure 4.16).

Because of the relatively mild wall displacements involved, the oscillation period of 410 μs produces timestep-independent results at 60 timesteps per period. The forces $\mathbf{F}^{(n)}(\mathbf{x})$ are computed across the entire computational domain for each oscillation cycle according to the methods described in Step 2. Convergence of $\mathbf{F}^{(n)}(\mathbf{x})$ is reached at $n = 6$.

The steady streaming fields are then computed by applying the source term $\mathbf{F}^{(6)}$ to a steady, single-fluid simulation in which the bubble is replaced by a rigid, no-slip wall. The results are then compared to those reported in (Tho et al., 2007). Figure 4.17 plots microstreaming fields in the z -plane 75 μm from the wall on which the captive bubble rests. Both the experimental (Figure 4.17(a)) and numerical (Figure 4.17(b)) results show qualitatively similar quadrupole streaming patterns at this depth. Quantitatively, it is important to note that the experimental micro-PIV analysis yields velocity vector fields in the xy -plane (Figure 4.17(c)). Velocity in the z -direction is not quantified, but can be inferred from the divergence of streaklines (Collis et al., 2010b). Therefore in Figure 4.17(d), only the xy components of velocity are plotted, and show similar patterns and maximum streaming velocities within a factor of two of the experimental results, which is reasonable given the uncertainty in experimental data acquisition.

Repeating the analysis at a focal depth 300 μm from the wall on which the captive bubble rests, similarities in both streakline patterns and even stronger agree-

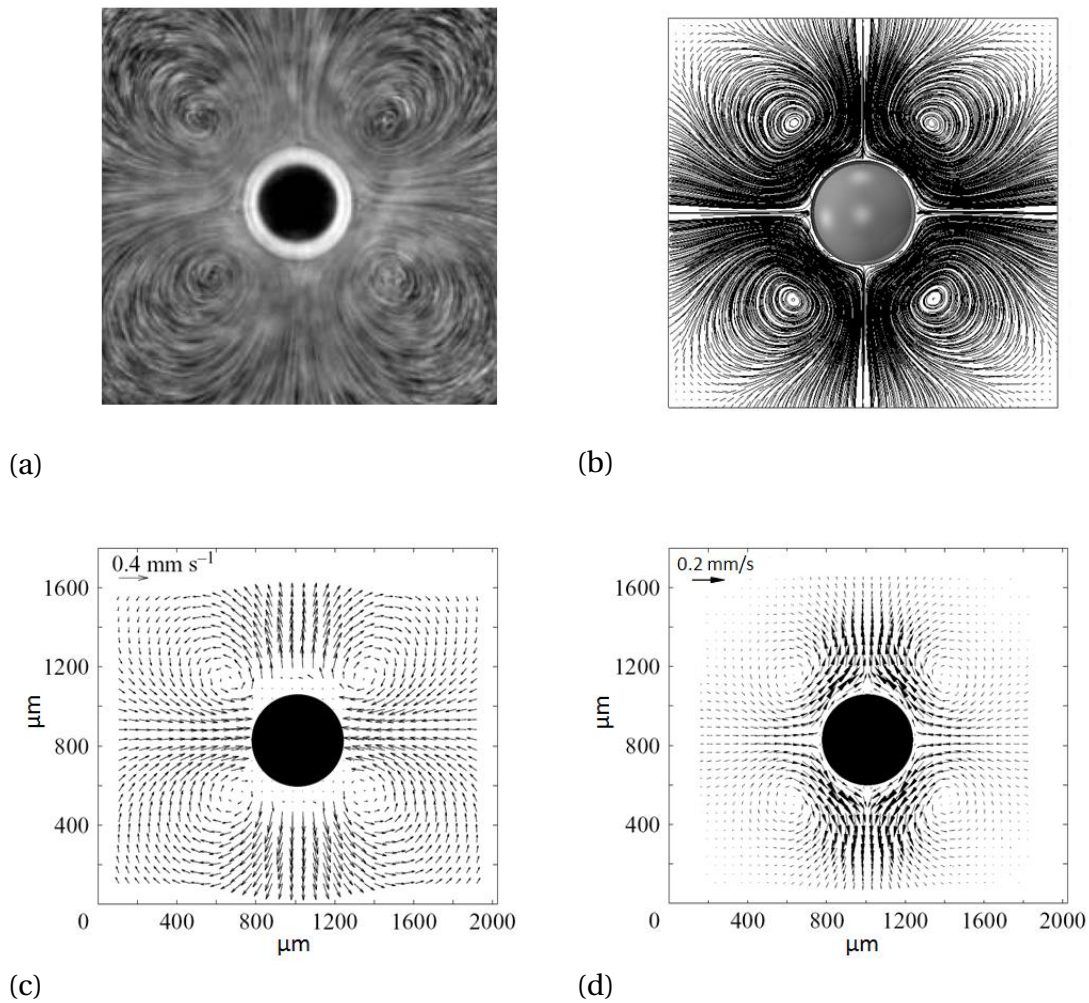


Figure 4.17: **(a)** Experimental and **(b)** numerical streaklines produced by oscillations of a $240\mu\text{m}$ captive bubble, observed at a depth of $75\mu\text{m}$ from the wall on which the captive bubble rests. **(c)** Experimental and **(d)** numerical vector flow fields. **(a)** and **(c)** reproduced from (Tho et al., 2007)

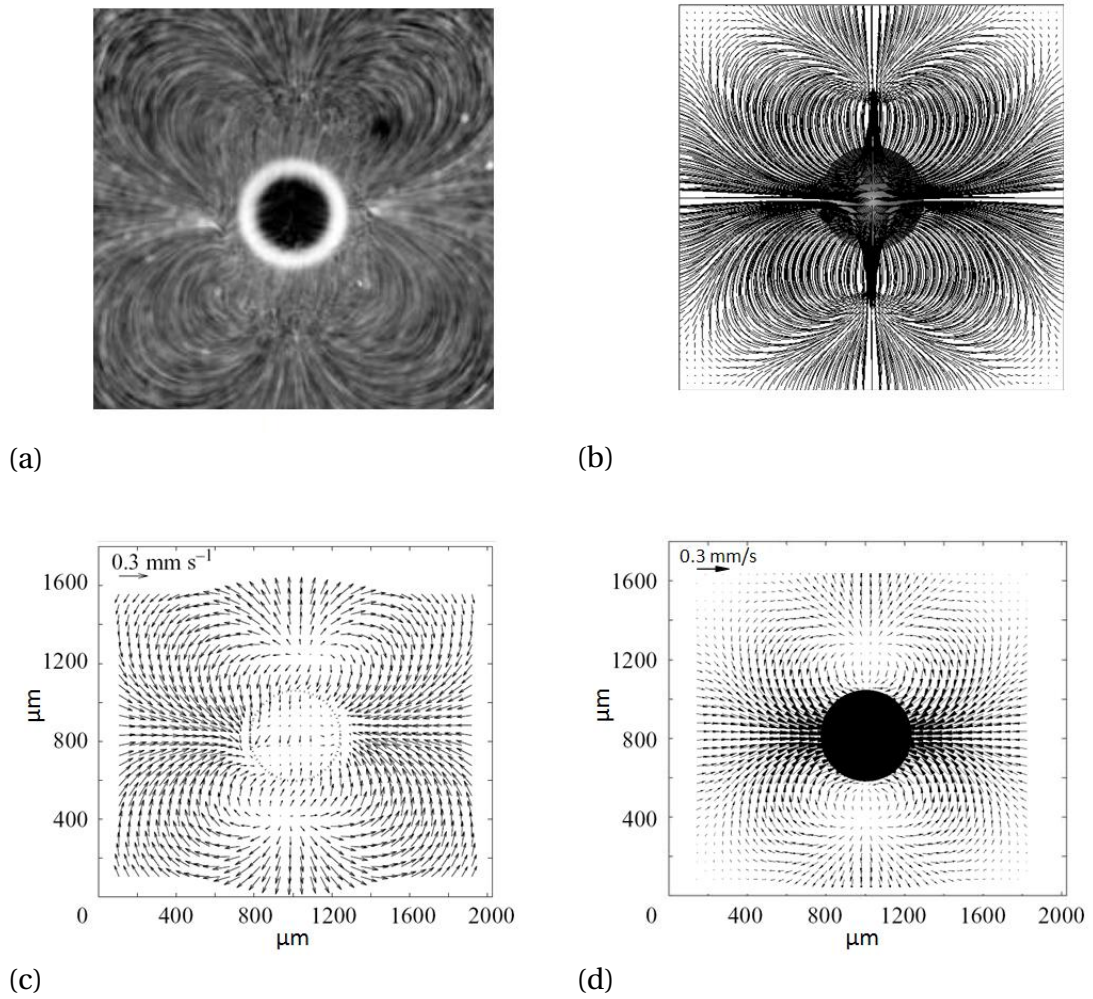


Figure 4.18: **(a)** Experimental and **(b)** numerical streaklines produced by oscillations of a $240\ \mu\text{m}$ captive bubble, observed at a depth of $300\ \mu\text{m}$ from the wall on which the captive bubble rests. **(c)** Experimental and **(d)** numerical vector flow fields. **(a)** and **(c)** reproduced from (Tho et al., 2007)

ment in maximum velocities are observed (Figure 4.18). Note that one discrepancy, the relative absence of streaklines which pass in front of the bubble in Figure 4.18(a) compared to Figure 4.18(b), may be attributable to optical filtering of streaklines outside the focal plane in the experimental results, a phenomenon which it was not attempted to replicate computationally.

As a final step, vertical cross-sectional flow fields are qualitatively compared to streaming patterns reported in Elder (Elder, 1959) for a captive bubble exposed to

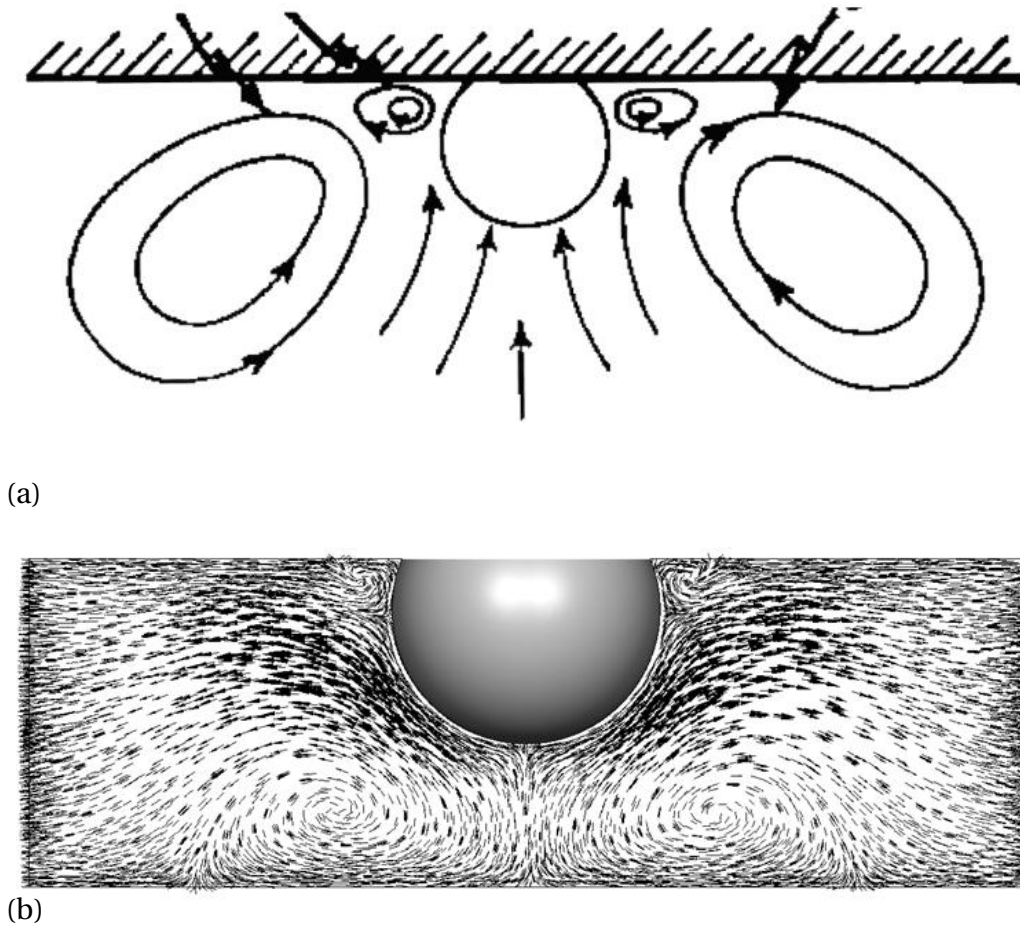


Figure 4.19: Qualitative comparison between **(a)** experimental and **(b)** numerical flow patterns observed in a vertical cross-section through the captive bubble. The presence of a wall at the bottom of **(b)** produces a flattening of the large streaming lobes. Smaller lobes near the bubble-wall attachment point are observed in both cases. **(a)** reproduced from (Elder, 1959)

ultrasound. Figure 4.19 shows good qualitative agreement in several flow features, including both a large recirculation lobe and a smaller one nearer the attachment point between the bubble and wall. In the numerical simulation (Figure 4.19(b)), the large lobes are flattened relative to the experiment (Figure 4.19(a)), a consequence of the wall bounding the liquid from below in the simulation (the depth of the experimental apparatus was large enough to be approximately unbounded).

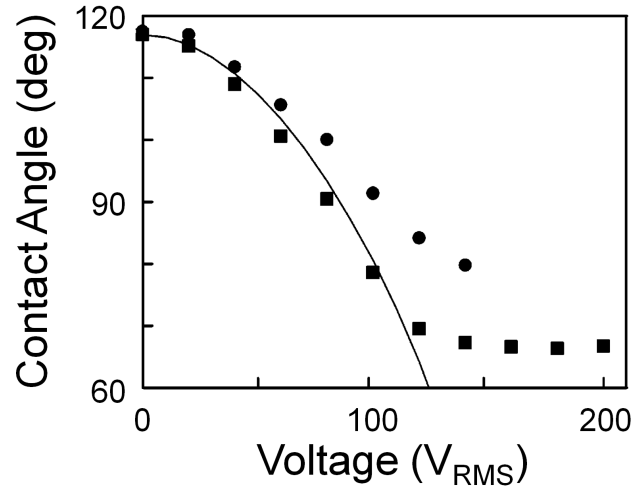


Figure 4.20: Dependence of contact angle on applied voltage in the case of DC (rectangles) and 10 kHz (circles). The solid line represents Equation (4.28), the approximation used in this study, which involves 200 Hz excitation at 60-100 V_{RMS}

4.3.6.3 Microstreaming due to electrowetting-driven bubble oscillations

In the third test case, the performance of the algorithm is tested against experimentally characterized flow fields induced by electrowetting-driven harmonic wetting angle oscillations (Ko et al., 2009). In the setup, a captive air bubble of volume $1\mu\text{L}$ rests on a dielectric wall, and an externally applied electrical potential source induces changes in the wetting angle between the bubble and wall. When a sinusoidal voltage source is applied, the wetting angle oscillates periodically, and the resulting bubble shape oscillations induce steady microstreaming in the surrounding aqueous solution. The streaming patterns are characterized using fluorescent tracer particles in suspension.

The relationship between electrical potential and bubble wetting angle under *equilibrium* conditions is given by:

$$\cos \theta = \cos \theta_0 + \frac{\epsilon V^2}{2\gamma d} \quad (4.28)$$

where, θ_0 is the contact angle absent the externally applied electrical potential, θ is

the contact angle under electrical potential V , γ is the interfacial tension between liquid droplet and surrounding fluid, and ϵ and d are the electric permittivity and thickness of the dielectric layer, respectively (Verheijen and Prins, 1999). Note that this equation is derived by minimizing free energy under thermodynamic equilibrium conditions. In the case of an unsteady, sinusoidally oscillating voltage source, departures from this equation increase with increasing excitation frequency and peak voltage. Specifically, deviation in the contact angle θ saturates at lower angles and lower voltages (Figure 4.20), phenomena which are neglected here.

These simulations are conducted over a 4 mm^3 computational volume bounded by a wall at the base and constant pressure boundary conditions on the remaining sides. The volume is discretized into $20 \mu\text{m}^3$ cells. With the $1 \mu\text{L}$ bubble positioned in the center of the base, the contact angle boundary condition is made to vary in accordance with (4.28) with $V = V_{rms}\sqrt{2}\sin(2\pi f t)$, $f = 200\text{Hz}$ and $V_{rms} = \{60, 70, 80, 100\}$.

The oscillation period of 5ms produces time-independent results at 160 timesteps per period (note that this refers to the excitation period, not the contact angle oscillation period, which occurs at $1/(2f)$ as a consequence of the V^2 term in (4.28)). Convergence of $\mathbf{F}^{(n)}(\mathbf{x})$ is reached at $n = 5$.

V_{rms} (Volts)	Jet Velocity (mm/s)	
	Experiment	Simulation
60	0.5	0.3
70	3.0	4.5
80	7.0	8.0
100	7.5	10.0

Table 4.1: Comparison between experimental and numerical jet velocities observed along a line passing through the center of the bubble and perpendicular to the dielectric wall, for various values of V_{rms} at $f = 200\text{Hz}$

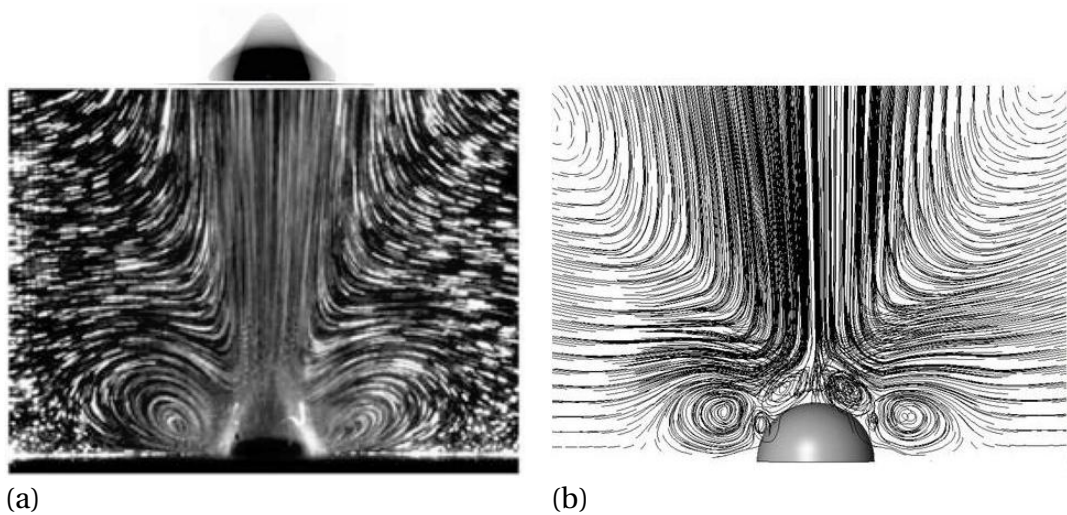


Figure 4.21: **(a)** Experimental and **(b)** numerical streaklines for a $1\ \mu\text{L}$ captive bubble oscillating in response to a 200Hz electric potential source with amplitude 80V (rms). Bubble oscillation pattern shown above **(a)**. **(a)** reproduced from (Ko et al., 2009)

The experimental (a) and numerical (b) streaklines are shown in Figure 4.21 for $V_{rms} = 80$. Note that both the synthetic jet and the recirculation lobes are captured in the numerical simulation. In the experiment, no attempt was made to resolve the velocity field completely. Rather, streaklines were captured in two dimensions, and the jet velocity along the center line was quantified. Table 4.1 compares the maximum velocities recorded experimentally and numerically in the region above the oscillating bubble. There is reasonable agreement in both trend and magnitude between experimental and numerical results, given the uncertainties present in the experimental data acquisition.

4.3.7 Scalar transport due to stable cavitation

Having demonstrated its ability to accurately replicate experimentally observed microstreaming fields, this method is now applied to the original objective of optimizing convective transport of a scalar quantity in the vicinity of an acoustically cavitat-

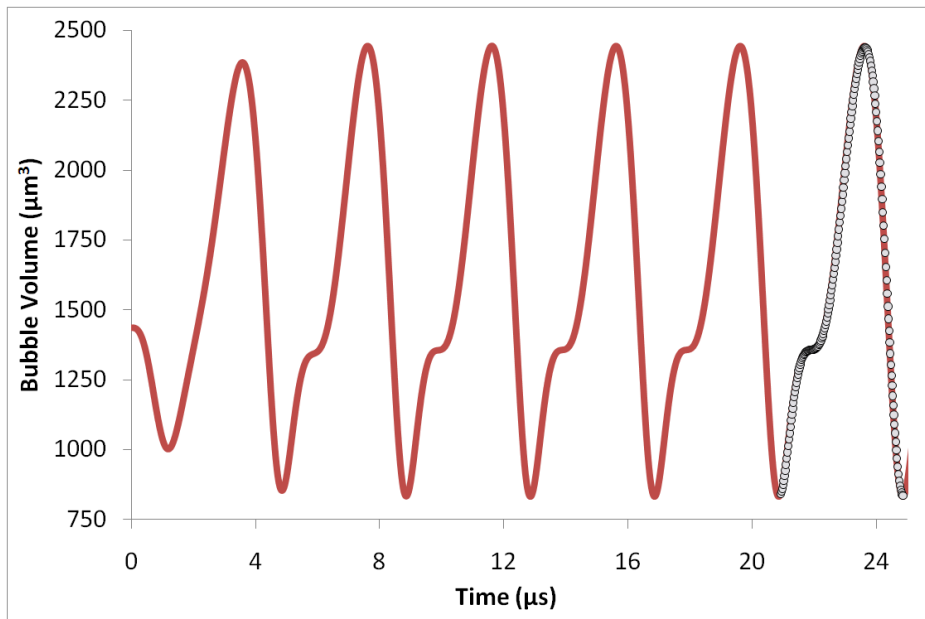


Figure 4.22: Plot of bubble volume versus time for a bubble of initial radius $7\mu\text{m}$ bubble subject to 50kPa, 250kHz ultrasound. The bubble size in the CFD simulation was controlled to match the output of a 1-D Keller-Miksis model (red line). After reaching time-periodicity, the Reynolds Stresses were calculated over a single acoustic cycle (black circles). Each circle represents the volume at the end of a timestep in the CFD simulation

ing microbubble. The application in improving the distribution of a low-diffusivity therapeutic agent in areas of poor blood flow has been discussed extensively. The envisioned treatment protocol, conceptually validated in Chapter 3, involves injecting cavitation nuclei in tandem with a therapeutic agent, and focusing ultrasound in the volume of interest within the body (Bohmer et al., 2010). The nuclei in this case might be biocompatible ultrasound contrast agents. Of particular interest is the range of initial bubble radii and sonication conditions which most effectively increase the length scales over which drug can be transported and decrease the required duration of ultrasound treatment.

Numerical analysis of the scenario begins with several simplifications, first of all by performing the simulation in the case of a single unshelled bubble oscillating in

an unbounded medium, avoiding the clinical complexities which arise in the presence of a nearby bubbles or vessel walls. Bubble expansion/contraction is achieved by injecting/removing mass from the bubble interior so that for the duration of the simulation, the bubble volume is such that a hypothetical spherical bubble of equivalent volume (black circles, Figure 4.22) satisfies the (1-D) Keller Miksis model for the given initial radius and ultrasound parameters (red line, Figure 4.22) (Keller and Miksis, 1980). Beyond this constraint, only surface tension controls the behavior of the bubble wall.

Given that the treatment protocol will likely involve exogenously injected shelled microbubbles, the relevance of simulating the behavior of unshelled bubbles may be disputed. Indeed, it may appear more realistic to introduce a bubble shell into the simulation with experimentally characterized mechanical properties. However, there are several limitations to such a technique. From a computational perspective, introducing a shell is a significant challenge, because the numerical grid would have to span a broad range of scales to accommodate a very thin (15 nm) shell and much larger (50 μm) microstreaming patterns. Moreover, accurately tracking this shell as it travels several μm requires extraordinarily fine timestepping to satisfy the CFL condition for numerical stability.

Regardless, at the pressures under consideration, shell rupture is likely to occur very early in the sonication. Indeed, it has been shown that at lower mechanical indices than those employed here, two-cycle ultrasound pulses separated by 1 ms induce bubble rupture within approximately 30-120 pulses, depending on initial bubble size (Caskey et al., 2007). Moreover, the process is not well-studied enough, nor are bubbles of a given size sufficiently uniform in composition or behavior, to predict oscillatory lifetime or shell destruction with sufficient accuracy to warrant inclusion in this numerical simulation.

4.3.7.1 Parametric Study Design

Recognizing the computational burden of the validation studies described earlier in this chapter, it is important to constrain the parameter space as much as possible before embarking upon a parametric study. Constraints include clinical compatibility as well as guidance from published therapeutic ultrasound studies (see Section §2.6) and from the experimental work described in the previous chapter.

Acoustic Frequency At frequencies above 1 MHz, heat deposition due to attenuation, which this work aims to avoid, begins to become important. As frequency is dropped, both the focal volume and focal depth increase if transducer geometry is kept constant. Though this is a slightly artificial, rather than intrinsic, limitation, pre-clinical and clinical considerations do limit transducer curvature. The lowest frequency examined here is 125 kHz, which would likely be a challenge to implement (particularly in small animal experiments).

Initial Bubble Radius Microbubbles of initial radius larger than 10 μm pose the clinical risk of becoming lodged in capillary beds and restricting blood flow. Therefore, clinically relevant ultrasound contrast agents are generally below this size. Although there is no limit to how small cavitation nuclei can be (in fact, experimental work is underway to develop cavitation threshold-lowering particles of only a few hundred nanometers in size), smaller nuclei generally have higher cavitation thresholds. Therefore, the smallest bubble simulated in this study has initial radius of 500 nm.

Peak Rarefactional Pressure Although it is desirable to explore a range of bubble radii and frequencies, with peak pressures up to 2.0 MPa, the dramatic oscillations under such peak pressures require considerable computational resources.

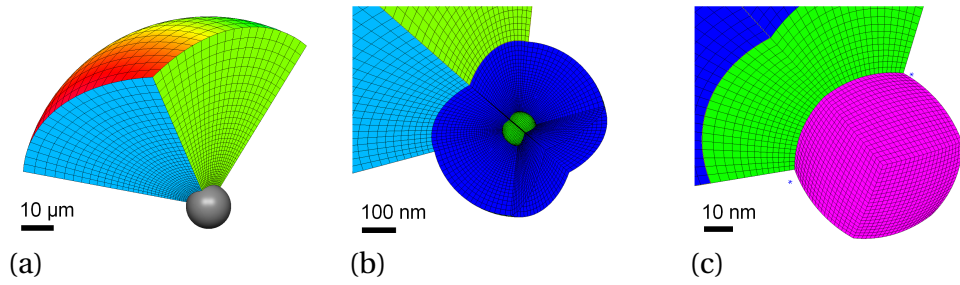


Figure 4.23: Discretization of the spherical computational domain of radius $50\mu\text{m}$ into a structured grid containing 1.6 million hexahedral cells, shown at various scales (scale bar at bottom left of each image), with the bubble surface (defined independently of the numerical grid locations) shown in gray in (a)

Therefore, the initial parametric study is conducted at both 50kPa and 100kPa. Further studies which explore the impact of ramping pressure are limited to a single frequency and two initial bubble radii (chosen based on the results of the lower-pressure studies).

In summary, simulations are performed for free bubbles of initial radius $0.5\text{--}10\mu\text{m}$, frequencies of 125–1000 kHz and, initially, pressures of 50–100 kPa.

4.3.7.2 Numerical Discretization

Computational grid Grid independence is achieved with spatial discretization of a sphere into a structured grid of radius $50\mu\text{m}$ containing 1.6 million hexahedral cells (Figure 4.23). The number of circumferential grid points was kept constant, so that the arc length of a CV scaled with radius from the center of the computational domain. The radial length of a CV grew in proportion to the arc length such that for each CV, its three linear dimensions were within a 4% range. This created CVs with volumes ranging from 0.3 nm^3 to $7.1\mu\text{m}^3$. Note that the relationship between Reynolds Stress fields and microstreaming is unconfirmed at these length scales, which are 1–2 orders of magnitude below those in Sections §4.3.6.2 and §4.3.6.3.

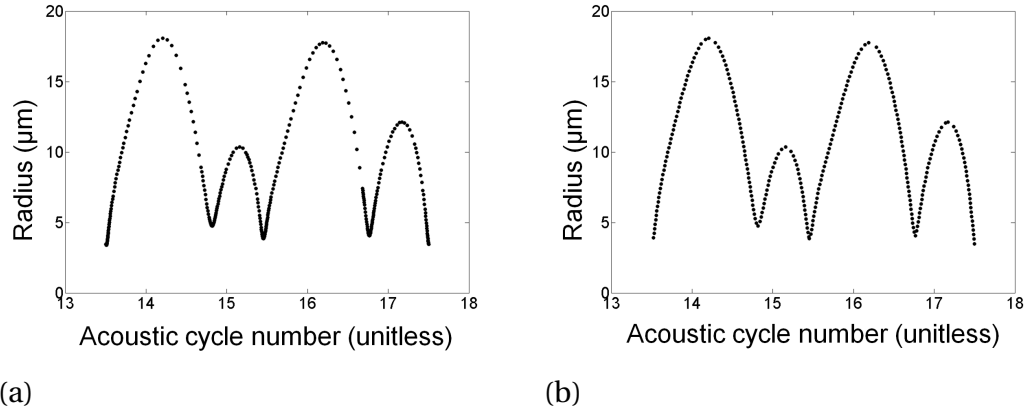


Figure 4.24: Solution points in the radius-time curve produced by the Keller-Miksis model (a), and the redistributed points employed in the CFD simulation (b). Timestepping is refined where velocities are high and the CFL condition would otherwise be violated. At the valleys of the curve, where fine timestepping was required in order to accurately resolve bubble collapse and rebound in the Keller-Miksis model, the CFD simulation can take advantage of the pre-determined solution and employ coarser time scales.

Temporal discretization In the validation examples considered in Sections §4.3.6.2 and §4.3.6.3, timestep-independence was achieved by discretizing each actuation cycle into 100 timesteps or less, of equal duration. However, in this parametric study, the oscillations considered are much more dramatic relative to the initial bubble radius and computational grid element size. Enforcing the CFL condition therefore requires substantially finer timestepping. Moreover, the timestepping that emerges from the 1-D Keller-Miksis model has been subject to very different convergence criteria than those applicable to a VOF CFD model.

Specifically, the Keller-Miksis model, which accounts for the compressibility of the surrounding fluid, requires extremely fine timestepping as an inertially collapsing bubble reaches its minimum radius and acceleration reaches its peak, while coarse timestepping is sufficient during the high-velocity, low-acceleration regions (Figure 4.24(a)). In contrast, the VOF technique requires that a free surface crosses

no more than approximately 10% of the width of a cell during each time step, a constraint that manifests itself during the explosive growth and collapse phases, where bubble wall velocity is high, but not at the collapse minimum, where only acceleration is high.

Therefore, the timestepping produced by the Keller-Miksis simulation was redistributed to produce a radius-time curve optimized for the CFD simulations. Because of the broad range of cell sizes spanned by the computational domain (Figure 4.23), this was accomplished by performing a cell size look-up at the initial bubble radius, determining the maximum free surface propagation distance permitted at a CFL number less than 0.1, and interpolating the time and radius values from the Keller-Miksis simulation to advance to the next time step, and repeating this process along the entire Keller-Miksis curve, to yield a CFD radius-time curve (Figure 4.24(b)).

R_0 (μm)	Frequency (kHz)			
	125	250	500	1000
0.5	5140	3316	2152	1401
1	2408	1578	1036	676
1.5	1463	972	642	430
2	920	628	457	336
2.5	872	600	428	302
3	829	600	421	280
4	808	578	378	248
5	803	532	324	192
6	776	490	353	155
7	751	472	332	131
8	718	416	266	115
10	634	524	193	92

Table 4.2: Timesteps required per acoustic cycle to achieve numerical convergence at 100 kPa Peak Rarefactional Pressure at various frequencies and initial bubble radii.

Again, each bubble was driven over 6 acoustic cycles. However, in contrast with the scenarios previously described in this section, up to 5000 timesteps were re-

quired per acoustic cycle to achieve numerical convergence (Table 4.2).

4.3.7.3 Centroid Tracking

In Sections §4.3.6.2 and §4.3.6.3, bubble wall motion is generated through mass sources and sinks within a sphere of 0.3 bubble radii positioned at the bubble centroid. Because the bubbles undergo mild oscillations, motion of the bubble centroid is neglected. In this study, and with an eye towards developing algorithms general enough to accommodate future studies involving multiple bubbles in an acoustic field, the bubble centroid is computed at the end of each timestep, and the mass sources are made to translate with the bubble centroid.

4.3.7.4 Convective Transport

After computing the forces F_j using data from the final oscillation cycle and modeling the steady streaming field, a further simulation quantifies convective scalar transport: with the bubble at its initial radius R_0 , a scalar $\xi(\mathbf{x}, t)$ of zero diffusivity is given initial distribution

$$\xi(\mathbf{x}, 0) = \begin{cases} 1, & R_0 < |\mathbf{x}| < R_0 + 1\mu m \\ 0, & \text{otherwise} \end{cases} \quad (4.29)$$

That is, the scalar ξ is initially confined to a $1\mu m$ layer surrounding the bubble. The steady streaming field is imposed, and the spatiotemporal distribution of scalar is solved over 1 second in 1 ms timesteps using a Central Difference Scheme in space and Crank-Nicolson scheme in time. To visualize the results, a new variable $\chi(r, t)$ is introduced which measures the fraction of scalar contained in a $1\mu m$ -thick shell centered at radius r at time t , implemented numerically as:

$$\chi(r, t) = \frac{1}{\xi_{total}} \sum_{\mathbf{x}} \xi(\mathbf{x}, t) V(\mathbf{x}) \Pi(|\mathbf{x}| - r) \quad (4.30)$$

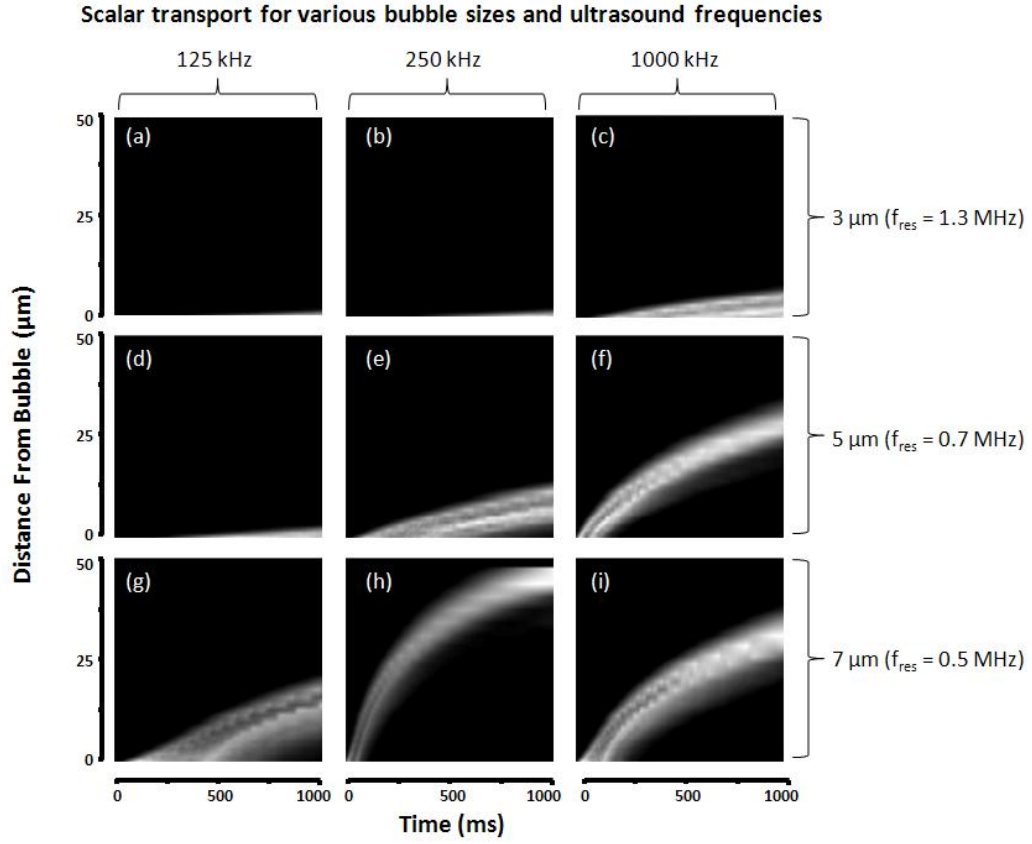


Figure 4.25: The radial distribution of a zero-diffusivity scalar, initially confined to a $1\mu\text{m}$ layer at the surface of a bubble, is shown as a function of time for bubbles of initial radii 3, 5, and $7\mu\text{m}$ subject to 100kPa excitation at 125kHz, 250kHz, and 1000kHz for a total duration of 1 second

where ξ_{total} is the total scalar in the computational domain at $t = 0$, V is the volume of the computational cell centered at $\mathbf{x}$, Π is the rectangle function, and the summation is carried out across every cell in the domain. Select results are shown in Figure 4.25, where $\chi(r, t)$ is plotted for three different initial bubble sizes at three different sonication frequencies (f_{res} , the theoretical resonant frequency, is given to the right of each bubble size).

In Figure 4.25 (a)-(c), as insonation frequency is increased towards the natural resonance of the bubble, convective length scales increase. Similarly, in vertical series (a-d-g) or (b-e-h), it can be seen that at a given frequency, increasing the bubble

size towards the resonant radius for that frequency enhances convective transport. However, this trend does not hold in the series (c-f-i). In fact, further analysis (not shown) indicates that although transport for a given bubble size is maximized at resonant frequency, transport for a given frequency is *not* maximized at the resonant bubble size. Instead, slightly larger bubbles are favored, which may have root in the inverse relationship between bubble size and surface tension, and the resulting propensity of larger bubbles to undergo shape oscillations, a phenomenon which has been shown to enhance microstreaming (Tho et al., 2007).

The results across the entire parametric study are summarized in Figure 4.26. Here, each plot corresponds to a different sonication frequency, and each line represents the ‘median’ distance that the scalar ξ is transported as a function of time. That is, each point on the line represents, at that time, the radius of a hypothetical spherical shell which encloses half the starting quantity of scalar.

From the plot, it is clear that scalar transport is maximized across the broadest range of bubble sizes at 250 kHz and 500 kHz. Therefore, if it proves difficult to synthesize monodisperse cavitation nuclei in an experimental setting, these frequencies are reasonable starting points. Furthermore, it is apparent that at these mild pressures, convective transport is generally higher for larger microbubbles.

4.3.8 Scalar transport due to inertial cavitation

In order to assess the impact initial bubble size at higher pressures, scalar transport is evaluated at a fixed frequency for two different bubble sizes as a function of pressure. Although a broader parametric study is desirable, the computational power required to accommodate larger expansion ratios and more dramatic collapses is prohibitive. Therefore, at a frequency of 500 kHz, a 2 μm and a 5 μm bubble are chosen, and driven up to 1.3 MPa peak rarefactional pressure. Despite narrowing

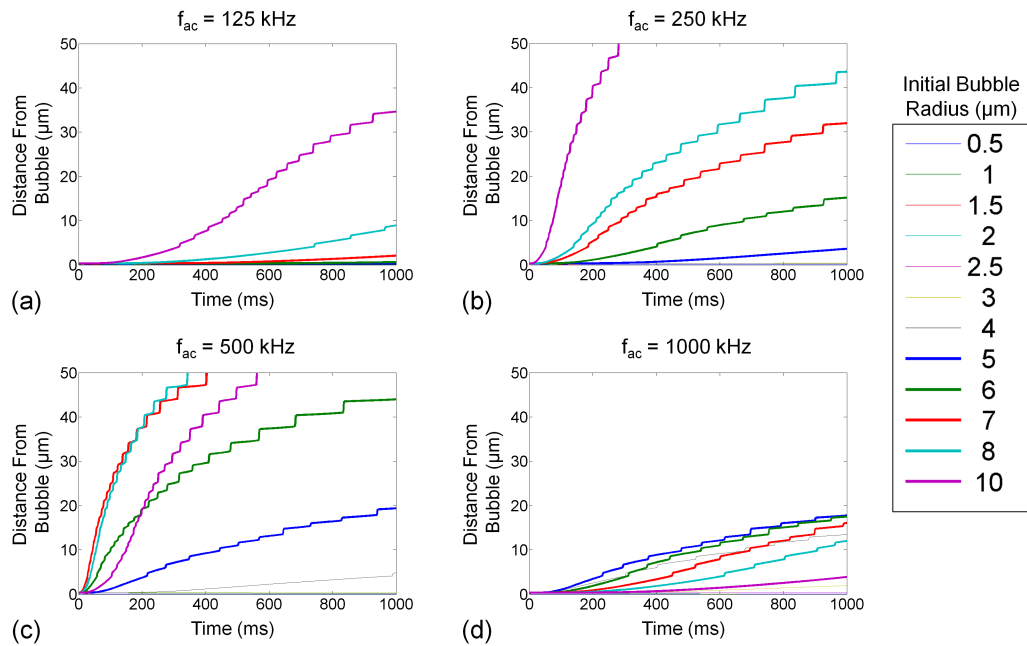


Figure 4.26: Median scalar transport distances - defined as the radius of a hypothetical shell which encompasses half the initial quantity of scalar present - across a range of bubble radii (legend), plotted at (a) 125 kHz, (b) 250 kHz, (c) 500 kHz, and (d) 1000 kHz.

the parametric space, further assumptions and modifications, which may affect the accuracy of the results, are required in order to yield computationally tractable conditions.

Modified Bubble Collapse Profile First, a significant modification is made to the radius-time curves produced by the Keller-Miksis model in order to facilitate CFD simulation. As previously discussed, high bubble wall velocities combined with a strict CFL condition require fine timestepping. At the bottom of an inertial collapse, where the computational grid is finest (Figure 4.23), bubble wall velocities can greatly exceed the speed of sound, resulting in timesteps on the order of pico- or even femto-seconds.

Under the hypothesis that expansion ratios are more important than bubble collapse in liquid convection, the radius-time curve is modified by identifying the pe-

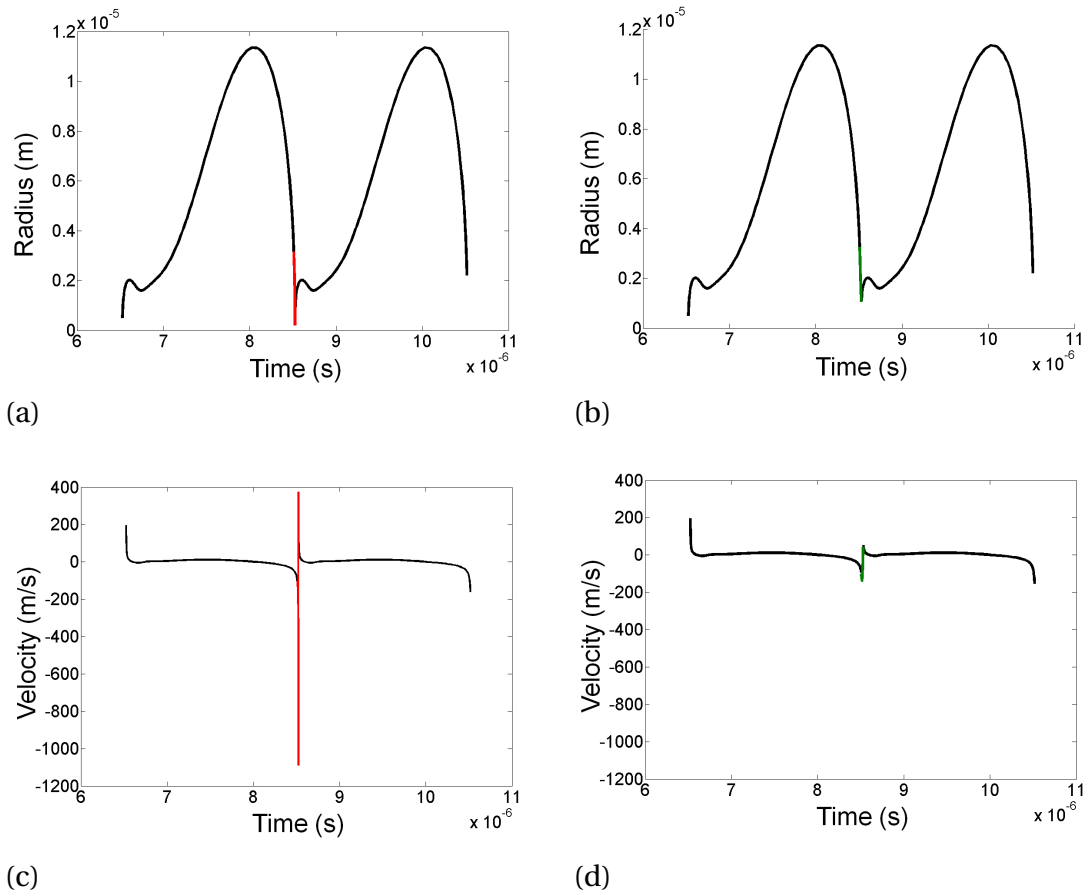


Figure 4.27: Modification of the Keller-Miksis radius time curve to yield computationally tractable CFD timestepping. **(a)** Original radius-time curve for a $5\ \mu\text{m}$ bubble subject to 700 kPa ultrasound at 500 kHz, showing a sharp collapse and rebound, where bubble wall velocities **(c)** can exceed the speed of sound (highlighted in red). The radius-time curve is modified **(b)** by replacing the region in red with a cubic spline connected the edges of the region (highlighted in green). Here the maximum bubble wall velocities do not exceed 150 m/s. **(d)**

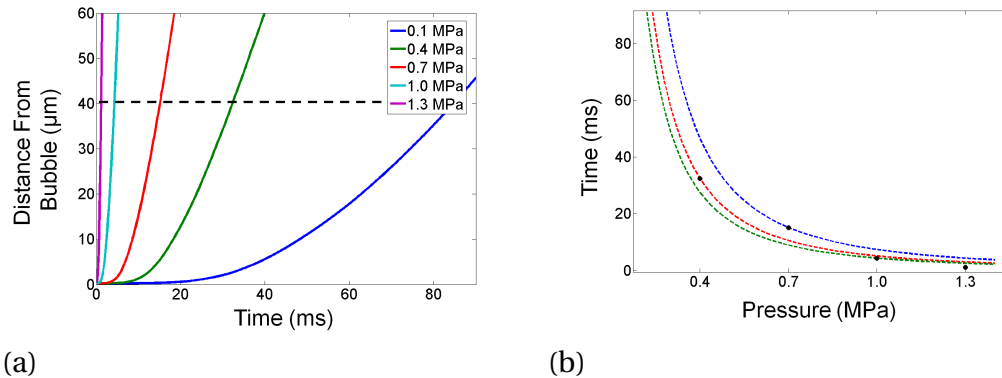


Figure 4.28: **(a)** Median scalar transport distances for a $5\ \mu\text{m}$ bubble subject to 500kHz sonication at various pressures. The dotted line is arbitrarily chosen as the ‘treatment objective’ for determining the relationship between total acoustic energy deposited and convective transport. **(b)** Treatment time required to achieve $40\ \mu\text{m}$ median scalar transport at various peak pressures (black dots). Dotted lines passing through each point indicate the ‘isodose’ curves, or curves corresponding to constant energy. (see Section §4.3.8.1 for further interpretation)

riod in which bubble wall velocity exceeds 200 m/s (Figure 4.27(a) and (c)) and replacing it with a cubic spline connecting the edges of the period which has more moderate velocities (Figure 4.27b and (d)). Although this modification further compromises the faithfulness of the simulation to physical reality, the results may still be useful if the underlying hypothesis proves reasonable.

Incompressibility Second, the assumption of incompressibility of the surrounding liquid is enforced, even as bubble wall velocities are driven to half the speed of sound of the gas. Therefore, the roles of energy deposition into liquid compression and subsequent viscous dissipation are not accounted for.

4.3.8.1 Convective Transport

Convective transport is analyzed in a manner identical to Section §4.3.7.4, with the exception that the total simulated duration is refined to account for the shorter

period over which scalar escapes the computational domain (Figure 4.28(a)). As expected, streaming velocities and scalar transport length scales increase with increasing pressure.

Relationship Between Acoustic Dose and Convective Transport The question arises as to whether the enhanced transport at higher pressures comes at an increased cost in terms of the total acoustic dose - that is, the product of acoustic intensity and duration of treatment (acoustic intensity is proportional to the square of pressure, so if the peak pressure is doubled, the treatment duration must be quartered in order to keep acoustic energy constant). In order to explore this, the duration required to achieve 'treatment success' - in this case arbitrary chosen to be a 40 μm median scalar transport distance, indicated by the dotted line in Figure 4.28(a) - is plotted against peak pressure (Figure 4.28(b)). In addition, isodose curves are plotted passing through each point. So, for example, any point along the blue dotted line has acoustic energy equal to the 0.7 MPa treatment. Any points lying below and to the left of this blue dotted line are more efficient than the 0.7 MPa treatment at converting acoustic energy into convective transport. These results suggest that the 1.3 MPa treatment is most efficient, though beyond 1.0 MPa improvements in efficiency with increasing pressure appear to plateau.

Effect of Initial Bubble Radius at High Pressures Finally, it is examined whether the similar relationships between bubble radius and sonication frequency hold at elevated peak pressures. At low pressures, it was observed that at 500 kHz, scalar transport around a 5 μm bubble is significantly higher than a 2 μm bubble (Figure 4.26(c)). However, as pressure increases, this effect becomes less pronounced. Specifically, the transport curves of a 2 μm (Figure 4.29, dotted lines) appeared to converge towards those of a 5 μm bubble (Figure 4.29, solid lines) as pressure is in-

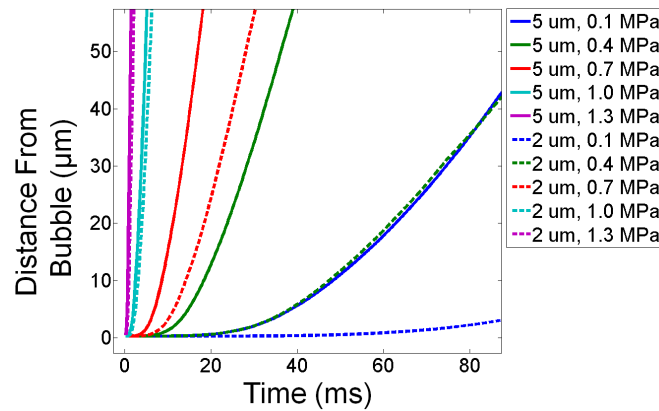


Figure 4.29: Median scalar transport distances for 5 μm bubble (solid lines) and 2 μm bubble (dotted lines) subject to 500kHz sonication at various pressures. Lines of the same color correspond to equal pressure. Enhanced transport by the larger bubble at low pressures (blue lines) becomes less pronounced as pressure increases, as indicated by the convergence of the solid and dotted lines at higher pressures.

creased. This suggests that at high pressures, where acoustic energy conversion is most efficient (§4.3.8.1), bubbles of sub-resonant size driven to inertial cavitation may be as efficacious as resonant bubbles in convective transport.

4.4 SUMMARY

In this chapter, several numerical methods are developed to quantify convective transport due to HIFU. First, the experimental work conducted in the previous chapter was reproduced numerically for cases where cavitation does not occur. Then, a flexible and efficient method to compute the steady streaming fields induced by a periodic actuator was presented. The method is general enough to be applied to *any* arbitrary geometry and underlying actuation mechanism, and is adaptable to *any* solver which accurately characterizes the flow. The principal factor governing the suitability of the technique is the tendency of the Reynolds Stress field to converge to a dynamically stationary (time-periodic) value over the course of a small number of actuation cycles under the simulation conditions. A formula for testing

this criterion is proposed, and the resulting numerical validation examples demonstrated good agreement with experimental reference data from literature in cases of both acoustic cavitation and electrowetting-driven bubble oscillations.

This technique was applied to the optimization of convective transport around an acoustically cavitating microbubble. It was discovered that at low pressures, resonant or near-resonant bubbles were most effective at enhancing the transport of a hypothetical zero-diffusivity scalar. However, the impact of initial bubble size becomes less pronounced as pressure is increased and large expansion ratios are observed. In this case, off-resonance bubbles are nearly equally effective at enhancing convective transport, a finding which has important implications in terms of the exogenous nuclei employed in future experimental work. Specifically, if sub-MHz frequencies are employed to minimize heat deposition and reduce cavitation thresholds, the use of clinical ultrasound contrast agents - which generally have a mean radius of $\sim 2 \mu\text{m}$ - may be warranted. Moreover, it was found that treatment at higher pressures had the desirable attributes of both reducing treatment time and reducing total acoustic energy necessary to achieve the desired bioeffect. *In vitro* and *in vivo* studies conducted at various pressures while maintaining constant acoustic energy confirm this finding, and will be described in the following chapter.



CAVITATION-ENHANCED VIROTHERAPY

In this chapter, the *in vitro* and *in vivo* models used to explore the effect of HIFU on delivery and expression of a biologically active adenovirus are presented. The role of cavitation in improving the distribution of adenovirus in porous media is established, as well as the critical role that pulse repetition frequency plays in sustaining cavitation activity. After identifying safe sonication conditions for mice with subcutaneously grown human breast cancer tumors, it is shown that after intratumoral or intravenous co-injection of ultrasound contrast agents and adenovirus, both the distribution and expression of viral transgenes are enhanced in the presence of inertial cavitation.

Chapters 3 and 4 were limited to investigating the convective transport in water of either a biologically inert macromolecule *in vitro* or a hypothetical zero-diffusivity scalar *in silico*. However, as discussed in Section §1.3, successful drug delivery requires not only vascular transport, but extravasation and interstitial transport. Confident in the conditions under which cavitation-enhanced micromixing is likely to be observed within vasculature, an agar-based, cell-embedding phantom with porosity matched to tumor interstitial porosity is introduced to enable investigation of the parameters under which ultrasound promotes both extravasation and transport through this viscoelastic medium.

A biologically active adenovirus is used as a therapeutic agent, and either the

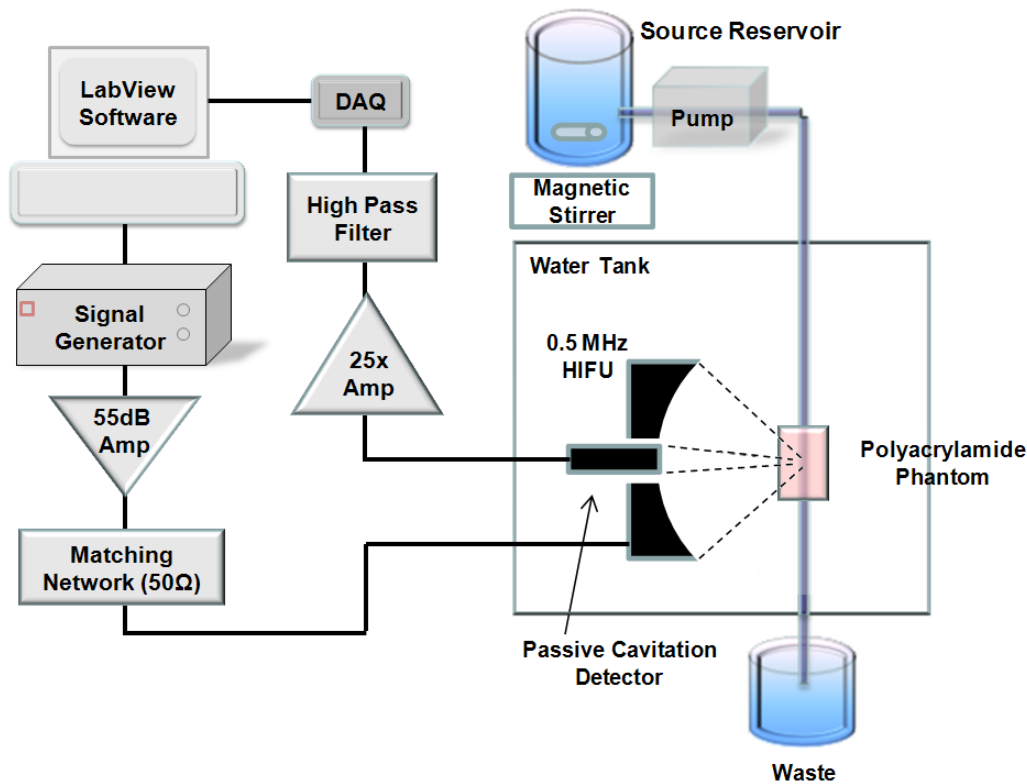


Figure 5.1: Experimental setup for ultrasound exposure of cancer cell phantoms. A 0.5MHz HIFU transducer is focused onto the centroid of phantom. A confocally aligned PCD records acoustic emissions

expression of its genome (for a reporter virus) or its impact on cell viability (for an oncolytic virus) is quantified under various cavitation regimes. Finally, the cumulative insight gained in the *in vitro* and numerical studies is applied towards the *in vivo* delivery of an oncolytic adenovirus in a human breast cancer xenograft animal model.

5.1 VIROTHERAPY IN A CANCER CELL PHANTOM

The principle underlying virotherapy differs from that of most other anti-cancer agents: viral particles are only able to infect intact cells, and the viruses used in this study have been engineered so as to only be able to replicate within cancer cells

(Bazan-Peregrino et al., 2008b). If an adequate number of viral particles reach cancer cells over a sufficiently large area, the replicating properties of the adenovirus will enable increasing rates of infection of cancer cells over a period of days. Virotherapy is therefore more dependent than any other cancer therapy on successful extravasation and uniform spatial distribution of the therapeutic agent, and as such constitutes an ideal experimental model in which to study cavitation-enhanced transport. Furthermore, because viral particles require an intact cellular membrane to infect cells, the effects of increasing drug transport can be readily distinguished from cavitation-induced sonoporation of cellular membranes, the latter effectively inhibiting viral infection.

In the experiments described in this section, the setup (Figure 5.1), hardware, and software are similar to those in Chapter 3, with modifications described below. The experimental procedure and subsequent biological assays were carried out in collaboration with two highly skilled and involved researchers, Drs. Miriam Bazan-Peregrino and Costas Arvanitis. In particular, Dr. Bazan-Peregrino's development and characterization of the oncolytic adenovirus employed in this study, as well as her expertise with molecular biology techniques, were instrumental to the project's success.

5.1.1 Hardware, Materials & Methods

5.1.1.1 Ultrasound Delivery and Detection

HIFU was generated by a single-element spherically focused ultrasonic transducer (H-107D, Sonic Concepts, Bothell, WA) with a slightly different center frequency (508 kHz) than previously used (483 kHz). The HIFU was controlled by custom made software using LabView 8.5 (National Instruments, Newbury, UK). The computer controlled a waveform generator (33250A, Agilent Technologies, Santa Clara, CA) whose signal was amplified by 55 db using a 300 W RF power amplifier (A-300, Electronic Navigation Industries). The power amplifier was then connected to the matching network of the HIFU transducer. This transducer was operated only at its center frequency. The aperture and the geometric focus of the transducer are 64 mm and 63 mm respectively. The beam profile of the HIFU focus has a full-width half-maximum of 30 mm in the axial direction and 3 mm in the transverse direction. The focus of the HIFU was acoustically aligned to the center of the flow vessel.

Acoustic emissions were recorded passively with a 15 MHz spherically focused PZT transducer (V319, Panametrics, Essex, UK) placed in the central opening of the 508 kHz transducer, as in the previous study. The PZT and the HIFU transducer were confocally and coaxially aligned. The raw signal from the PZT transducer was amplified 25-fold (SR445A, Stanford Research Systems, Sunnyvale, CA) and high-pass filtered (Krohn-Hite 3944, Brockton, MA) at 1 MHz. Data was recorded at sampling rate of 20 MHz with an 8-bit digitizer (Picoscope 3205, Pico technology, St Neots, UK).

5.1.1.2 Cell-embedding Agar Phantom

Breast cancer BT474-GFP cells were grown in RPMI, 10% foetal calf serum (FCS) (PAA, Somerset, UK), penicillin (25 U/ml), streptomycin (25 U/ml) and 10 μ g/ml

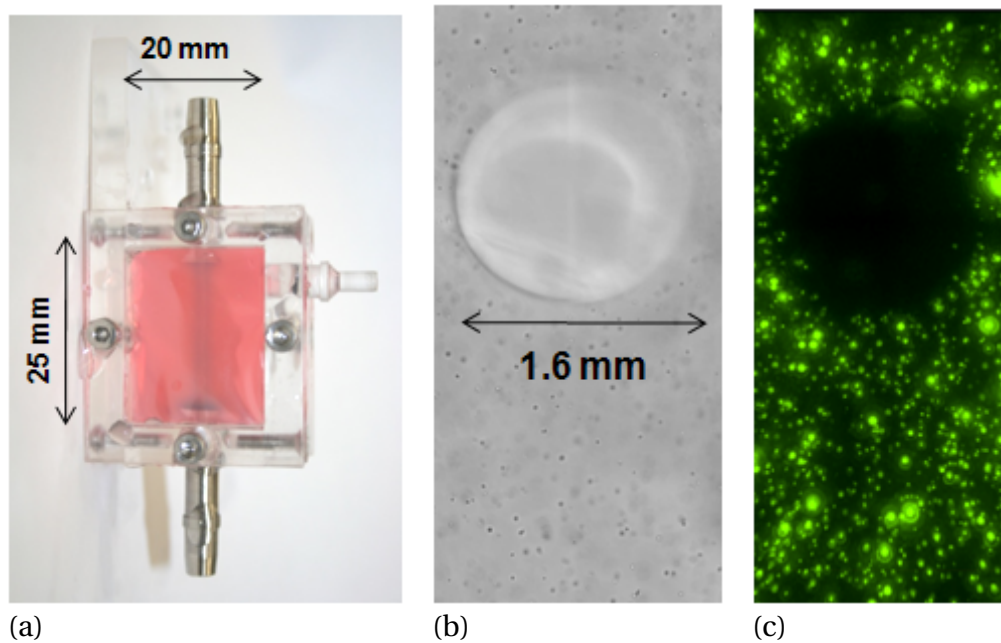


Figure 5.2: **(a)** Photograph of the 0.5% agarose gel containing breast cancer cells expressing GFP. The container was traversed with a metal rod which was removed after the gel set, leaving a 1.6 mm diameter vessel. Acoustically transparent windows seal the gel front and back, allowing the HIFU beam to propagate without interference. **(b)** Phase-contrast microscopic cross section of the phantom. **(c)** Fluorescent microscopic cross section of the phantom showing live (GFP-expressing) cells in green.

bovin insulin (Sigma-Aldrich, Dorset, UK). Mouse B lymphocytes A9 were grown in Dulbecco's modified Eagle's medium (DMEM) and 10% FCS. The A9-CAR cells (Mayr and Freimuth, 1997) were maintained in the same medium with 400 $\mu\text{g/ml}$ G418. UltraPure Agarose-1000 (Invitrogen, Paisley, UK) was microwaved in PBS, cooled to 37°C and then mixed with culture media and cells to give a final concentration of 0.5% agarose in 2% FCS, RPMI. BT-474-GFP cells were incorporated in the agarose liquid at a final concentration of 8.5×10^4 cells/ml. This suspension was poured into a phantom cast (internal dimensions 20 x 25 mm, with a cylindrical metal mandrel of diameter 1.6 mm) Figure 5.2(a) and allowed to set for 30 min at 4°C before removing the mandrel to reveal a 1.6 mm flow vessel Figure 5.2(b). The resulting gel has pore diameter of approximately 600-1200 nm (Fukumura and Jain, 2007a), which is

similar size to the endothelial gap of subcutaneous tumors (Maaloum et al., 1998). The front and back sides of the container were sealed with acoustically and optically transparent 15 μm thick Mylar (PMX980, HiFi, Hertfordshire, UK) to allow the entrance and exit of the ultrasound field.

5.1.1.3 Adenovirus preparations

Oncolytic adenoviruses are genetically programmed to preferentially replicate within cancer cells, and are designed to either lyse infected cells or to deliver genes encoding anti-cancer proteins. They are generally engineered for tumor selectivity, so that they spread to infect adjacent cancer cells, while leaving normal cells undamaged. Several viruses are employed in this study:

AdEHE2F: A replicating oncolytic adenovirus designed to kill breast cancer cells that have common features such as over-expression of estrogen receptor, elevated E2F activity and/or hypoxia (Hernandez-Alcoceba et al., 2002).

AdEHE2F-Luc: A replicating adenovirus which includes in its DNA the gene encoding for *luciferase*, a protein which catalyzes the bioluminescent oxidation of the pigment substrate *luciferin*. This virus was previously characterized (Bazan-Peregrino et al., 2008b) by pico green and TCID₅₀, with 2.6×10^{12} viral particles (vp)/ml and 3.16×10^{10} plaque forming units (pfu)/ml.

Ad-Luc: A non-replicating adenovirus expressing luciferase, with 4.5×10^{12} vp/ml and 7.59×10^{10} pfu/ml.

Ad-RFP: A non-replicating adenovirus expressing red fluorescent protein, purchased from Vector Biolabs (Philadelphia, PA) with a titre of 2.5×10^{11} vp/ml and 1×10^{10} pfu/ml.

5.1.1.4 Cell viability assay and luciferase expression *in vitro*

An excised cuboid ($4 \times 5 \times 6 \text{ mm}^3$) from the gel at the HIFU focus containing approximately 10,000 cells was transferred to a 96-well plate and 100 μl of 2% FCS RPMI media was added. After 24 h or 10 days, cell viability was measured by adding 40 μl of CellTiter 96[®] Aqueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Southampton, UK). 100% cell viability is defined by a control sample exposed to neither ultrasound nor adenovirus. After 24 h, luciferase expression was measured by adding 50 μl of 5x lysis buffer to each well, freezing at -80°C for at least 30 min, performing luciferase assay using 25 μl of sample and 25 μl of luciferin (Promega, Southampton, UK) and measuring relative luminescence by luminometry (Lumat LB 9507, Berthold Technologies, Redbourn, UK). To normalize against protein content, 3 μl of sample was mixed with 300 μl of advanced protein assay reagent (APAR) and protein content was measured following manufacturer's protocol (Cytoskeleton, Inc, Denver, CO).

5.1.1.5 Quantification of Adenoviral Particles

An excised cuboid from the gel was transferred to an eppendorf tube and 50 μl of QXL (QIAGEN, West Sussex, UK) was added to dissolve agarose and warmed at 70°C for 10 min. Then DNA extraction was performed using GenElute Mammalian Genomic DNA miniprep Kit (Sigma-Aldrich, Dorset, UK) following the manufacturer's protocol. The qPCR reaction used for measurement of adenoviral particles used primers as previously described (Bazan-Peregrino et al., 2008a).

5.1.1.6 Microscope imaging

An excised cuboid from the gel was transferred to an 8-well chamber slide system (Sigma-Aldrich, Dorset, UK). 300 μl of 2% FCS RPMI-phenol free media (PAA,

Somerset, UK) was added to each well. 24 h after ultrasound exposure, dead cells were stained using 1 μ M Sytox Blue (Invitrogen, Paisley, UK). Sytox Blue was imaged using a filter cube for Aqua (Exciter D436/20x, Dichroic 455DCLP, Emitter D480/30m) from Nikon UK Limited (Surrey, UK). A Nikon filter cube for TRITC (Exciter HQ545/30x, Dichroic Q570LP, Emitter HQ620/60m) was employed for RFP visualization. A Nikon filter cube for FITC (Exciter 465-495, Dichroic 505, Emitter 515-555) was employed for GFP visualization.

5.1.2 Experimental Procedure

The sonication procedure is similar to that described in Section §3.3.2. Briefly, flow is established through the vessel at a rate of 4 ml/min using a peristaltic pump (Minipulse 2, Gilson, Middleton, Wisconsin), which draws upon a reservoir containing either PBS alone (degassed for 5 min prior to each run), PBS mixed with adenovirus (reconstituted as described above to a final concentration of $1-2 \times 10^8$ vp/ml), or PBS mixed with virus and SonoVue[®] (Bracco, Italy) ultrasound contrast agent (UCA) (to a final concentration of 2000 bubbles/ μ l, measured by hemocytometer). Contrast agents were chosen to seed cavitation for this work because no biocompatible hydrophobic nanoparticles were available at the time of these experiments. Custom LabView software (with the relevant HIFU excitation and data acquisition parameters) is triggered, which synchronously activates the HIFU transducer and records acoustic emissions for post-processing. At the end of each experimental run, the vessel was flushed with PBS for 2 minutes.

5.1.3 Results

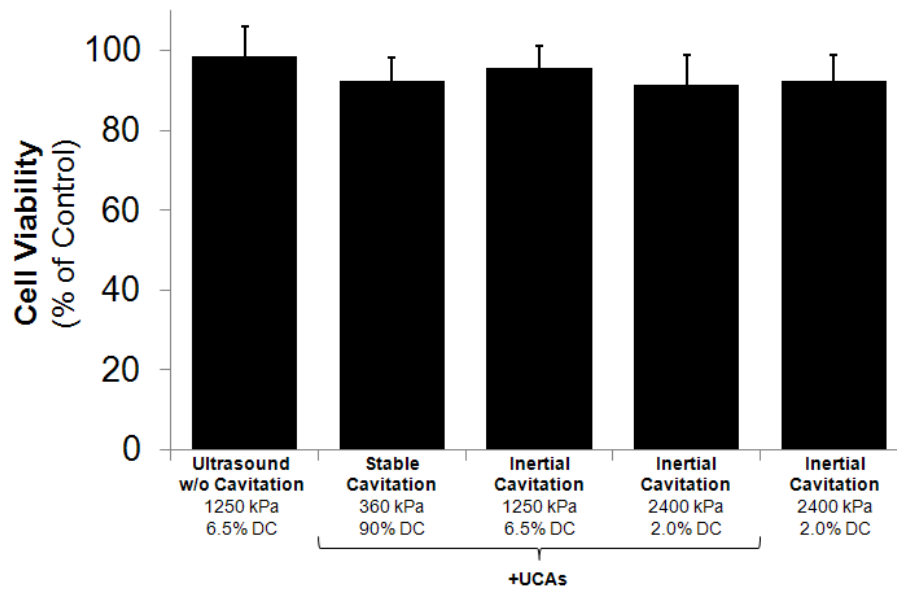


Figure 5.3: Results of an MTS assay performed 24 h post ultrasound exposure, showing the effect of ultrasound at various amplitudes and duty cycles, in the presence or absence of UCAs, on cell viability (quantified as percentage of untreated control). N=5 for each group.

5.1.3.1 Optimal Acoustic Parameters

In this series of experiments, acoustic dose (the product of acoustic intensity and duration of treatment, discussed in Section §4.3.8.1) was kept constant by varying duty cycle (DC) with the inverse square of peak rarefactional focal pressure (PRFP).

In the presence of microbubbles, the maximum ultraharmonic emissions (generally associated with stable cavitation) were seen at 360 kPa PRFP, 10 Hz PRF, and 90% DC; strong broadband emissions (generally associated with inertial cavitation) were seen at 1250 kPa PRFP, 10 Hz PRF, and 6% DC. In the absence of microbubbles, cavitation could not be sustained, and reproducible broadband emissions could be achieved only at 2400 kPa PRFP, 10 Hz PRF and 2% DC.

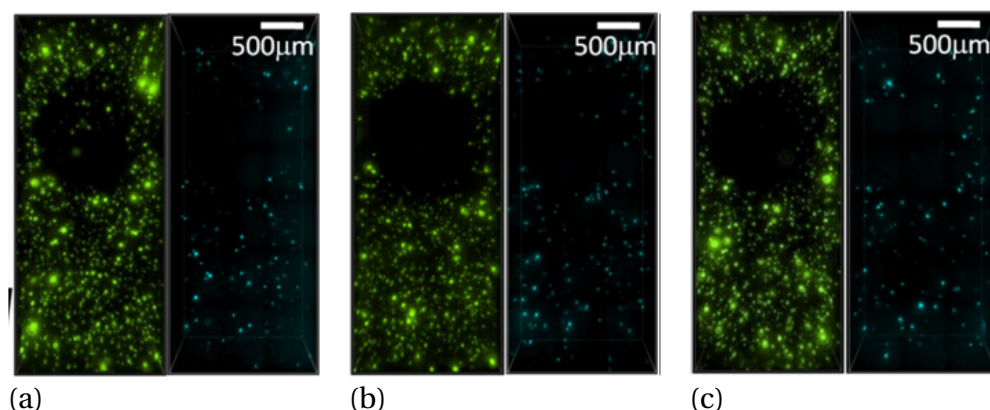


Figure 5.4: Cross section of the phantom, showing live cells in green (GFP-expressing cells) and dead cells in blue. Dead cells were stained with Sytox Blue 24 h post-sonication. **(a)** No Ultrasound. **(b)** Stable cavitation. **(c)** Inertial cavitation.

5.1.3.2 Effect of Ultrasound on Cell Viability

In order for virotherapy to be effective, the virus must be able to replicate within host cells, induce lysis, and infect neighboring cells. Therefore, the sonication conditions should be chosen so that they have minimal effect on cell viability. Post-exposure cell viability was evaluated using the CellTiter 96 AQueous MTS assay to measure metabolically active cells. No statistical difference in cell viability was observed under any ultrasonic parameter tested (Figure 5.3).

Additionally, Sytox-Blue was used to stain dead cells and observe their distribution (Figure 5.4). However, no increase in cell death was observed under any ultrasound exposure condition tested. These results suggest that if ultrasound induced-cavitation promotes extravasation of the oncolytic virus, the virus should encounter living cells in which to replicate.

5.1.3.3 Virus Distribution and Transgene Expression

In order to assess the distribution of viral infected cells after ultrasound exposure, AdeHE2F-Luc was co-delivered with non-replicating Ad-RFP. Only when a cancer

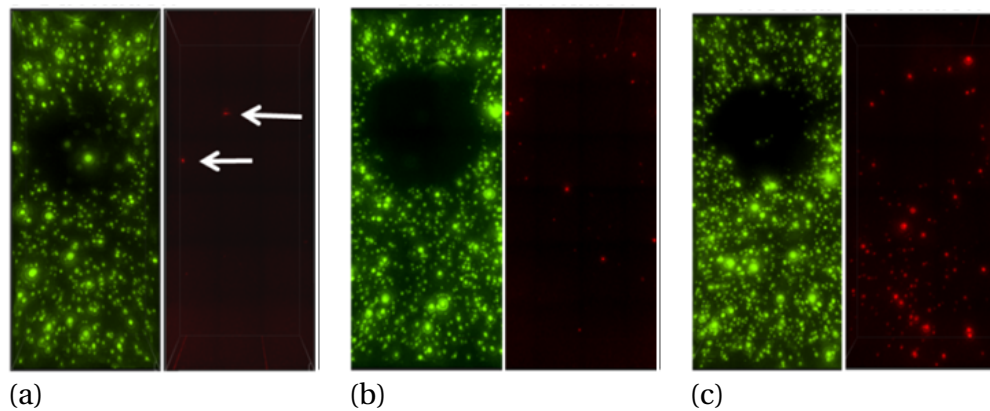


Figure 5.5: Cross section of the phantom, showing live cancer cells in green and viral infected cells in red (white arrows) following different sonication regimes. **(a)** No Ultrasound. **(b)** Stable cavitation. **(c)** Inertial cavitation. In the presence of inertial cavitation, infection rates are highest in the direction of ultrasound propagation (downward from top of page).

cell is co-infected with both viruses will Ad-RFP be replicated and the red fluorescent signal amplified. The co-infection model of an oncolytic adenovirus and a non-replicating adenovirus expressing a reporter gene has been studied previously with reproducible results (Guse et al., 2007). Representative pictures of red fluorescence at 3 days post ultrasound delivery are shown in Figure 5.5.

In the absence of detectable cavitation activity, few virally infected cells (white arrows) were observed around the vessel-like structure (Figure 5.5(a)). Stable cavitation increased the number of infected cells around the vessel-like structure and in the direction of the ultrasound beam (propagating downward from top of page) (Figure 5.5(b)). Inertial cavitation dramatically increased the number of viral infected cells around the vessel and in the direction of the ultrasound beam, including very large distances of up to 2 mm away from the vessel (Figure 5.5(c)).

Analysis of the viral particle content by quantitative real time polymerase chain reaction (qPCR) demonstrated that in the presence of stable cavitation, the viral concentration in the region of the agarose gel adjacent to the vessel approximately

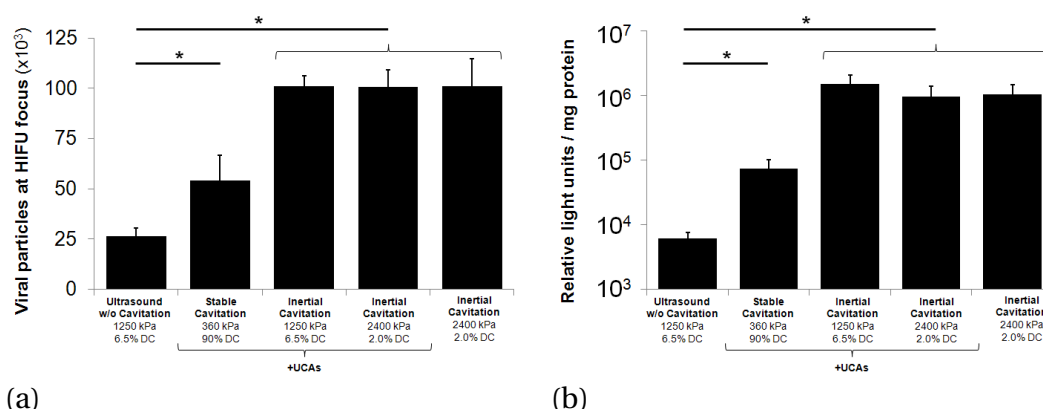


Figure 5.6: **(a)** Viral particle in the excised cuboid, measured by qPCR 30 min post-sonication. **(b)** Luciferase expression per mg of protein in the excised cuboid 24 h post-sonication. Luciferase activity indicates successful infection and transgene expression. Note the linear y-axis in **(a)**, in contrast to the logarithmic y-axis in **(b)**. N=5 for each group.

doubles (Figure 5.6(a)). When inertial cavitation was instigated either in the presence or the absence of microbubbles, then an approximately four-fold increase in viral particle concentration was observed.

Infectious activity of the delivered viral particles was analyzed using luciferase transgene expression 24 h post-ultrasound. In the model, the doubling in viral concentration at the HIFU focus induced by stable cavitation resulted in a 10-fold increase in luciferase expression compared to ultrasound in the absence of cavitation (Figure 5.6(b)). The quadrupling in viral concentration caused by inertial cavitation resulted in a 200-fold increase in transgene expression. These results show that with only a minor increase in viral delivery, a major increase in transgene expression can be achieved. Owing to the lack of reported research that cavitation exerts an influence on the transcriptional activity of tumor cells, it is hypothesized that the strongly nonlinear increase in transgene expression may be due to the broader spatial distribution of virus, and therefore viral infected cells, throughout the tissue phantom.

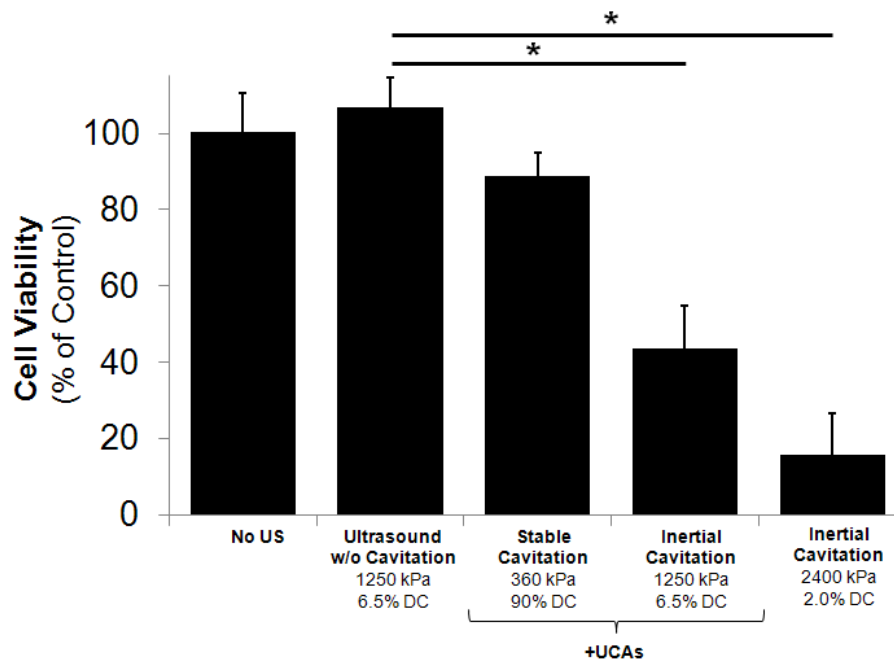


Figure 5.7: Cell death induced by AdeHE2F-Luc after cavitation-enhanced virus delivery, determined by MTS assay performed 24 h post ultrasound exposure (quantified as percentage of untreated control). N=5 for each group.

5.1.3.4 Cell Death by Virotherapy

Cavitation-enhanced delivery and improved distribution of AdeHE2F-Luc should result in cell death by virotherapy. In the absence of cavitation, AdeHE2F-Luc was unable to kill cells within the phantom. In the presence of microbubbles, stable cavitation did not significantly increase cell death by virotherapy, whereas inertial cavitation resulted in the death of approximately 55% of the cells in the model (Figure 5.7). For the same total acoustic dose, inertial cavitation thus proved to be more efficient at increasing virotherapy cell death compared to stable cavitation, a result which was anticipated by the numerical results discussed in Section §4.3.8.1.

In the absence of microbubbles, one further sonication regime was studied which delivered 3.7 times more energy than previous regimes, with parameters of 2400 kPa PRFP, 2.0% DC, and 10 Hz PRF. At this exposure level, in which ultrasound

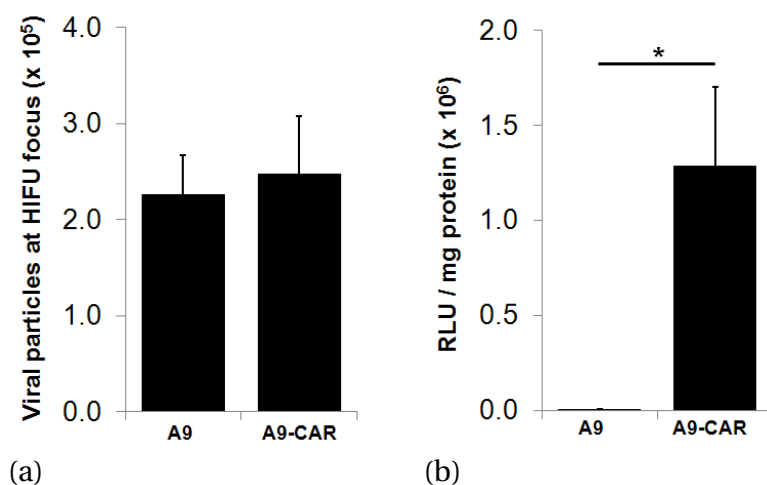


Figure 5.8: Evaluation of mechanism of enhanced transgene expression by inertial cavitation. Mouse B lymphocytes lacking adenoviral receptors (A9) or expressing Coxsackie and Adenovirus Receptor (A9-CAR) were evaluated for **(a)** viral particle delivery (measured by qPCR) and **(b)** transgene expression (measured by luciferase activity) of Ad-Luc following inertial cavitation. N=4 for each group.

alone did not affect cell viability (Figure 5.3), cell death induced by virotherapy reached up to 80%. This demonstrates that a significant increase in virotherapy *can* be achieved in the absence of ultrasound contrast agents, though a higher acoustic dose is required.

5.1.3.5 Mechanism of Action

The mechanism traditionally associated with inertial cavitation-promoted gene delivery is sonoporation, whereby collapsing bubbles create transient holes in the cell membrane, through which DNA enters the cell. This technique is effective for 'naked' DNA, which needs only to enter the cell nucleus in order to be expressed. However, the virus employed in this study requires direct binding of capsid proteins with cellular receptors (i.e. Coxsackie and Adenovirus Receptor, CAR) to enter the cell, release its DNA payload, and express transgenes. Therefore, cavitation-driven sonoporation - whereby collapsing bubbles create transient holes in the cell mem-

brane through which therapeutic agents enter the cell - is not expected to contribute significantly to viral genome expression.

To test this hypothesis, the transgene expression of Ad-Luc delivered by inertial cavitation was compared in two different cell types: A9 and A9-CAR. A9 is a mouse lymphoblast that lacks expression of CAR and other adenovirus receptors, and is therefore very difficult to infect with adenovirus. A9-CAR is the same cell line as A9, but made to express CAR constitutively for efficient adenovirus infection and transgene expression.

With the same number of viral particles delivered to both cell lines (Figure 5.8(a)), A9 cells had almost undetectable levels of transgene expression compared to A9-CAR (Figure 5.8(b)). This strongly suggests that enhanced adenoviral transgene expression promoted by inertial cavitation is due to enhanced extravasation rather than membrane permeabilization.

5.2 VIROTHERAPY IN A SMALL ANIMAL MODEL

In the experiments described above and in Chapter 3, the development of models which simulated certain aspects of tumor physiology was sought, including, for example, poorly functioning vasculature or dense interstitium. However, there are several simplifications inherent in these models which limit their clinical relevance, and which, though justifiable during preliminary experimental work, preclude their usefulness in refinements of the technology.

Specifically, the treatment of the extravascular space as a homogeneous hydrogel fails to account for the multiple barriers which a virus needs to cross between the lumen of a vessel and a cancer cell in neighboring tissue. These barriers are more complex and are differently affected by ultrasound compared to a hydrogel.

It is difficult to predict whether ultrasound regimes which increase the permeability of the hydrogel model system would likewise increase the permeability of vascular endothelium, the remainder of the vessel wall, and the extracellular space of the tumor. Moreover, the intratumoral IFP gradient, which is a significant barrier to molecular transport, was not reproduced in the experimental models. Finally, the millimeter-scale vessel diameters in the experimental models are orders of magnitude larger than the tumor microvessels. This is expected to alter the behavior of cavitating microbubbles, which is strongly influenced by nearby boundaries (Caskey et al., 2007).

Encountering the limitations of an experimental model is, of course, natural, and provides the impetus to move towards more clinically relevant models. Biomedical device development customarily evolves from physical characterization to *in vitro* experimentation, followed by small animal testing, large animal testing, and finally clinical trials. Below, a small animal model is presented and used to investigate the safety and efficacy of various sonication regimes following either intra-tumoral (IT) injection or intravenous (IV) injection of a combination of PBS, adenovirus, and microbubbles.

Once again, the substantial involvement of Dr. Bazan-Peregrino in developing and maintaining the animal models, performing various molecular biology-based analyses, and training in several of these, is gratefully acknowledged.

5.2.1 Materials & Methods

5.2.1.1 Animal model

All animal experimentation was performed in accordance with the terms of UK Home Office guidelines and the UKCCCR Guidelines for the Welfare of Animals in

Experimental Neoplasia.

ZR75.1 human epithelial mammary gland ductal carcinoma cells (provided by Prof. Adrian Harris, University of Oxford, UK) were injected subcutaneously in both the left and right flank (2×10^6 per site in a mixture 1:1 of matrigel:RPMI) and allowed to form tumors in 5-6 week old mice (Harlan, UK) given 17β -estradiol (Sigma) at a final concentration of $5 \mu\text{g/ml}$, 0.1% Ethanol and 0.04% Baytril (Bayer) in drinking water.

Tumor growth rates can vary from mouse to mouse, and between the left and right tumors of the same mouse. In this series of experiments, mice were grouped by tumor size, and treatment was administered when the fastest growing tumors reached 200 mm^3 in volume (estimated by caliper measurements of the three spheroid dimensions).

5.2.1.2 Ultrasound Treatment

A schematic of the experimental setup used for HIFU therapy is shown in Figure 5.9.(a) Prior to ultrasound treatment, the mouse is placed onto a Perspex stage, gently fixed in the supine position using water-proof clinical tape attaching each paw to the stage, and administered light anesthesia with isofluorane (3% for induction, 1% for maintenance) in a mixture of 50% nitrous oxide and 50% oxygen by face mask. The center of the tumor is visually identified and indicated with a black marker.

For intravenous (IV) injection studies, the mouse tail is then cannulated using a makeshift catheter consisting of an insulin needle attached to microtubing (Figure 5.10(b)). Injection is not administered until ultrasound treatment begins.

For intratumoral (IT) injection studies, the injection is administered 2 minutes

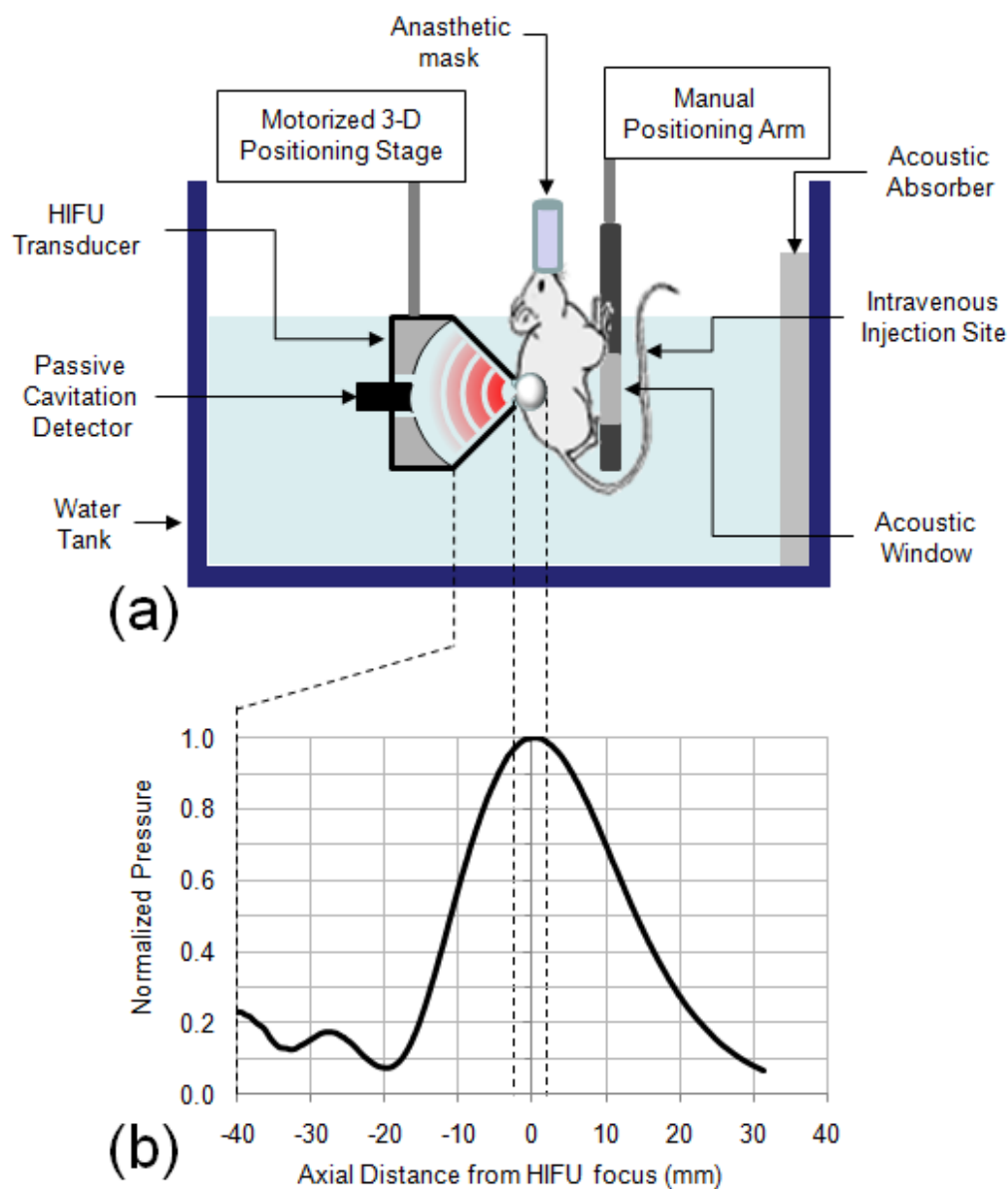


Figure 5.9: **(a)** Experimental setup for HIFU treatment of human xenograft tumors in mice. The mouse is mounted onto a perspex frame and immersed in water, keeping the head above water and masked with anesthetic dispenser. A conical applicator is mounted onto the 0.5MHz HIFU transducer, and an acoustically transparent latex sheet at the tip of the applicator is marked with a crosshairs indicating a point 2 mm pre-focus, so that when it is visually aligned adjacent to the tumor surface, the HIFU focus is located 2 mm into the tumor. A confocally aligned PCD records acoustic emissions. **(b)** Axial pressure profile of the HIFU transducer. The poor axial focusing can result in unwanted cavitation at the skin-water interface and deeper within the mouse body (see Section §5.2.2.1).

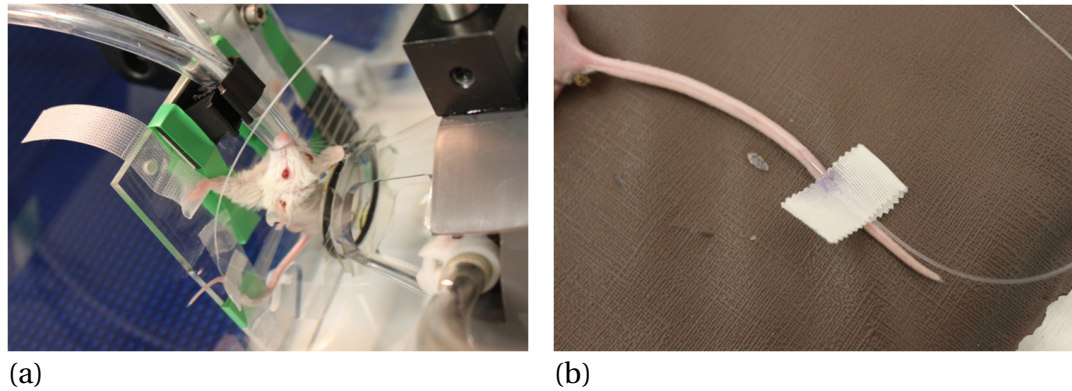


Figure 5.10: **(a)** Photograph of the mouse just prior to sonication, with HIFU transducer (gray) on the right, and conical applicator guiding alignment. **(b)** Cannulation of the mouse prior to immersion. The catheter is fixed with clinical glue to the tail and taped to the perspex stage, so that the tubing emerges from the water. The tubing is kept as short as possible to minimize 'dead' volume.

prior to ultrasound treatment.

A manual positioning arm is used to vertically immerse the stage into the water tank (maintained at 37°), so that the ears of the animal remain above water (Figure 5.10(a)). Each mouse bears contralateral tumors, of which one is in the treatment group, while the other is in the control group. The tumor in the treatment group is visually aligned to the focus of the ultrasound treatment system, indicated by the crosshairs marked on an acoustically transparent latex sheet affixed onto a perspex cone attached to the HIFU transducer encasement.

The HIFU transducer and PCD described in Section §5.1.1.1 were employed in this study. Because the HIFU transducer has a full-width half-maximum dimension of only 3 mm, the transducers were mounted onto a motorized 3-D positioning stage (ThorLabs, Newton, New Jersey) in order to allow treatment of multiple sites within the same tumor (which can have cross-sectional dimensions of up to 10 mm). The controlling LabView software was modified to automatically traverse several sites during the course of tumor treatment. Although sound in principle,

it emerged that these particular motorized stages introduce electrical noise which compromises the quality of the digitized PCD signal. The stages were therefore removed for the final series of intravenous studies in which acoustic emission data are presented, but future experiments will incorporate less electrically noisy motors.

Intratumoral injection protocol Following anesthesia, 25 μl of SonoVue microbubbles (SonoVue, Bracco, Italy), mixed with either 25 μl of Evans Blue dye (used for experiments testing only the safety of sonication parameters) or 25 μl of AdEHE2F-GFP, was injected into each of the two tumors using a syringe. Upon removal of the syringe, clinical sealant was applied at the injection site to prevent leakage. Two minutes after injection, the tumor was sonicated (sonication parameters tested are detailed in Section §5.2.2.2 below). The animal was then removed from the water tank and returned to its cage.

24 hours after treatment, fluorescence was measured using an *in vitro* imaging system (IVIS, Xenogen). The mouse was placed in the supine position, and excitation/emission filters were set to a GFP-sensitive range. The unshaved areas of the mouse were shielded with black fabric to suppress autofluorescence. The acquired fluorescence images were visualized and analyzed using custom-designed software in Matlab. This software identified regions of interest (ROIs) of equal size corresponding to the treated tumor, untreated tumor, or background fluorescence (a tumor-free section on the mouse). Within each ROI, the software counted the number of pixels at intensity levels five standard deviations above mean background fluorescence. The pixel count is used as a measure of the spatial extent of virus expression within the tumor.

Intravenous injection protocol 24 hours prior to treatment, clodronate liposomes, which have been shown to extend the half-life of adenovirus in the blood-

stream (Schughart et al., 1999), are injected intravenously.

Upon alignment of the tumor and HIFU transducer, sonication is administered for 240 seconds (parameters detailed in Section §5.2.2.3 below), with bolus intravenous injections of 25 μ l AdEHE2F-Luc (5×10^9 viral particles) mixed with 25 μ l UCAs (from a Sonovue vial resuspended in 2 ml saline) at $t=0$ s and $t=120$ s. The animal was then removed from the water tank and returned to its cage.

24 hours after treatment, luminescence was measured using an IVIS machine. Mice were injected intraperitoneally with 100 μ l potassium luciferin (Promega, Southampton, UK) dissolved at 15.8 mg/ml in PBS. The mouse was placed in the supine position in an IVIS chamber. The body of the mouse was shielded with duct tape to suppress luminescence emanating from the liver, which can alter background noise levels and result in processing artifacts. Luminescence images were acquired 5 minutes after intraperitoneal injection. The acquired raw luminescence data were visualized and analyzed in a method similar to that described above, with the difference that only total luminescence, rather than pixel count, was measured in each ROI.

5.2.1.3 *In vivo* and *ex-vivo* luciferase assays

Two hours after IVIS imaging, tumors were harvested, frozen, thawed and homogenized. tumor homogenates were analysed for luciferase activity using luciferin and relative luminescence was measured by luminometry (Lumat LB 9507, Berthold Technologies, Redbourn, UK). Relative light units were normalized against total tumor mass.

5.2.1.4 Diagnostic Ultrasound Imaging

Power Doppler was performed using a Vevo 770 imager (Visualsonics) with a 40MHz transducer, power 100%, RF cycles 2, Gain 20dB, velocity slow, wall filter 2.5, scan speed 2 and display settings: range max 24, range min 13.67 and priority 100%. Power Doppler analysis to calculate the percentage of vascularity in tumors was performed with Visualsonics software as described before (Hwang and Crum, 2009).

5.2.1.5 Analysis of acoustic emissions

Cavitation activity was quantified on the basis of the frequency content of the acoustic emissions, as described in Section §3.4.2. Briefly, the total energy spectral density was computed using custom-designed software in Matlab, and broadband energy was used as an indicator of inertial cavitation (IC), while ultraharmonic energy was used as an indicator of stable cavitation (SC).

5.2.2 Results and Discussion

5.2.2.1 Identifying Safe Sonication Parameters

It is known that HIFU can damage living tissue via several mechanisms, including ablation and fractionation. In the context of these experiments, tissue damage via ultrasound is undesirable. It is therefore important to identify the upper safety limits on a variety of parameters, including peak pressure, frequency, duty cycle, and treatment duration. A broader parameter space than the efficacious parameters described in the *in vitro* experiments above was tested.

It emerged that one of the foremost challenges in this experimental model is suppressing cavitation outside the tumor volume (i.e. at the skin-water interface and deeper within the mouse body). As is evident from Figure 5.9, the poor ax-

ial focusing of the transducer results in very little difference between peak pressures within the tumor and pressures only a few millimeters pre- or post-focally. Focusing HIFU on the tumor centroid in a superficial tumor model therefore inevitably leads to high pressures, and possibly cavitation, at both the skin/water interface and in the muscle beneath the tumor. Avoiding cavitation outside the tumor was the principle reason to inject nuclei intratumorally, where they were hypothesized to decrease the cavitation threshold in a very localized manner.

However, it was found that at lower acoustic frequencies (256 kHz), it was not possible to achieve enhanced extravasation of a macromolecule without also causing skin damage and/or leg impairment (likely the result of cavitation in the muscle). In fact, any extravasation observed was coincident with hemorrhaging in the tumor volume (Figure 5.11). Additionally, at DC values as low as 5%, inertial cavitation resulted in skin burns within 5 minutes, even where extravasation was undetectable.

It should be noted, however, that the proximity of the subcutaneous tumor to the skin made the exposure intensity difference between the two quite small (Figure 5.9). It is possible that, if the treatment protocol is translated to a larger mammal where the tumor is more deeply situated, a highly focused ultrasound may achieve tumor therapy while sparing the skin due to the ultrasound focusing geometry.

However, for the current experimental setup, despite the increased likelihood of cavitation and cavitation-mediated phenomena at 256 kHz, the remainder of the experiments were conducted at 508 kHz, and at this frequency, the spatial peak temporal average acoustic intensity (I_{SPTA}) was limited to 6 W/cm^2 . Under this constraint, parameter combinations which yielded enhanced viral distribution and gene expression without observable unwanted side effects were identified. These results are summarized below.

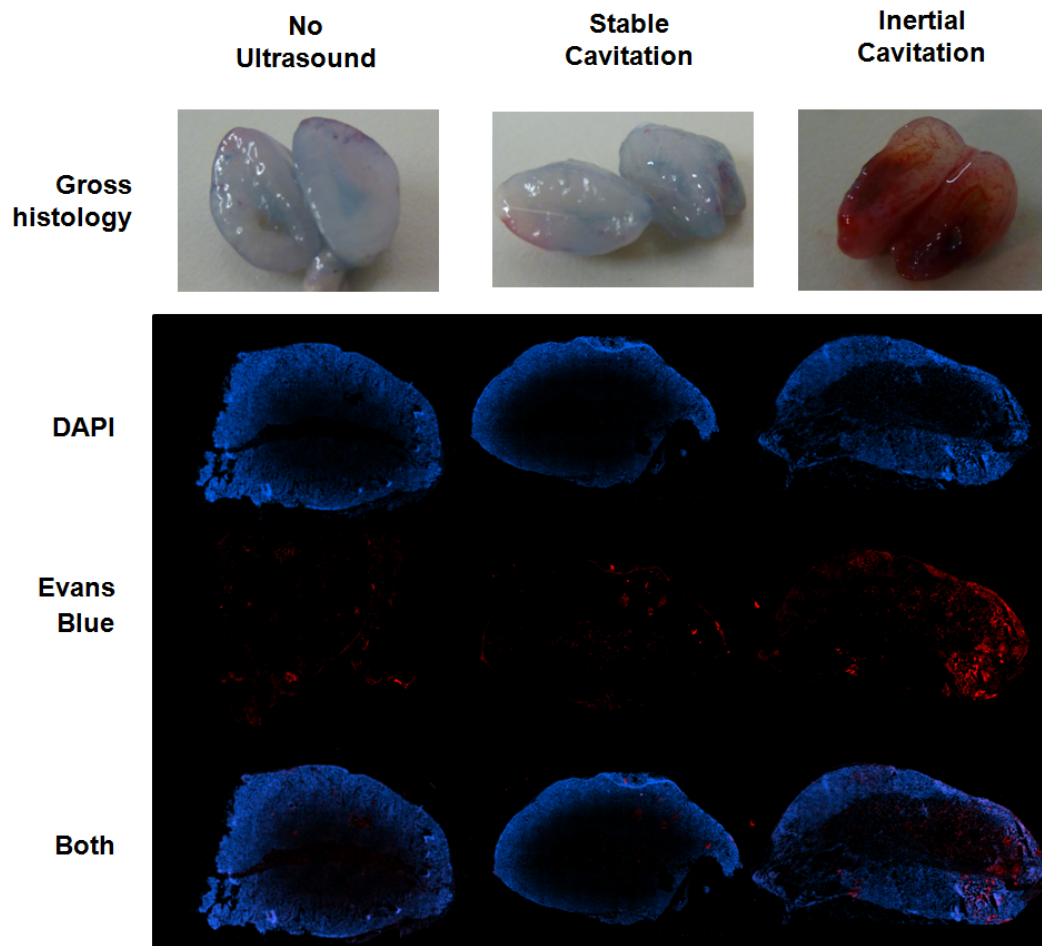


Figure 5.11: Gross histology and sectioning after no ultrasound (first column), stable cavitation (256kHz, 435 kPa, 90% DC, 5Hz PRF, 5 min treatment, second column), and inertial cavitation (256kHz, 750 kPa, 5% DC, 5Hz PRF, 5 min treatment, third column). The necrotic core is identifiable by the absence of DAPI, which stains live cells. Extravasation of Evans Blue (which spontaneously and irreversibly binds to serum albumin, the macromolecule of interest) is only detected following inertial cavitation, and is co-localized with regions in which hemorrhaging is observed.

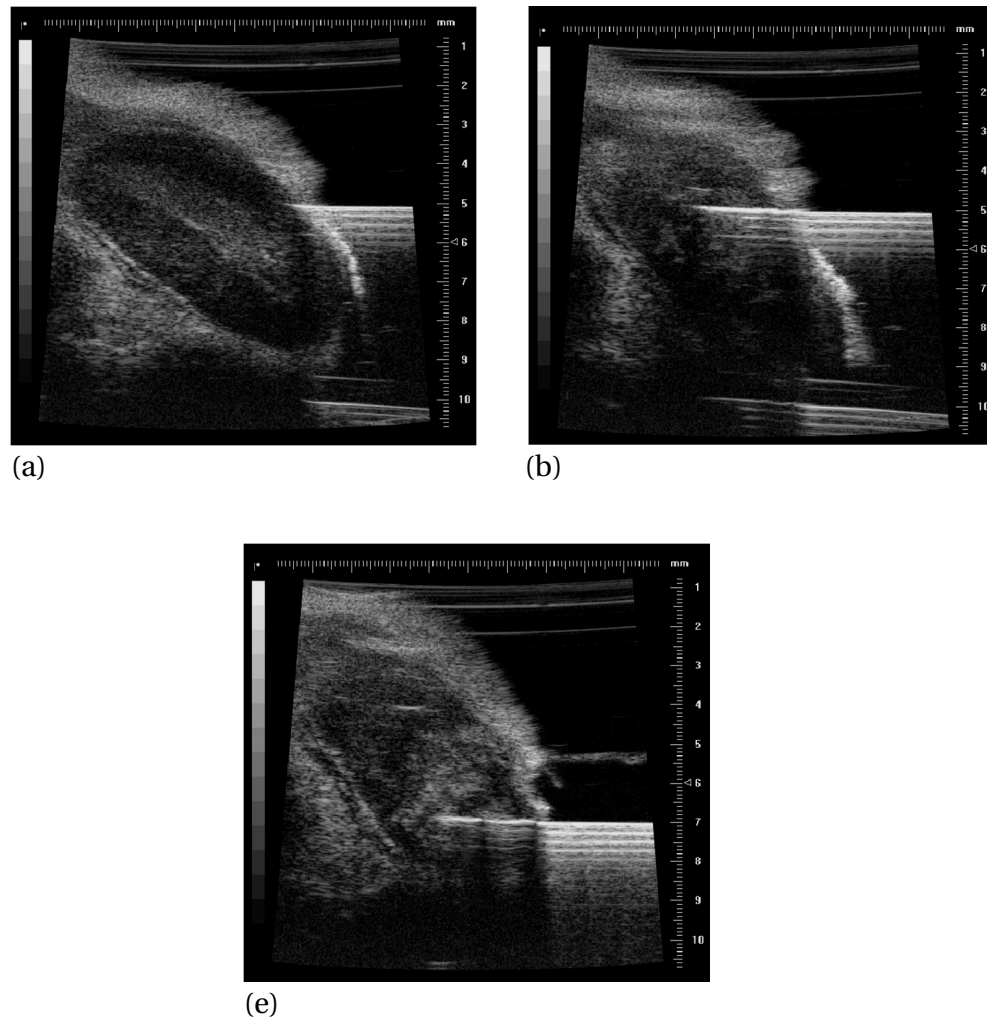


Figure 5.12: B-mode ultrasound image **(a)** before and **(b)** during intra-tumoral injection of Ad-GFP and UCA. The needle appears as the bright shaft entering the field from the right (its reflections are aliased as additional shafts appearing below), and the injectate produces a hyper-echoic region within ~ 3 mm of the injection site. **(c)** Multiple injections can improve the distribution of Ad-GFP and UCA, but care must be taken to ensure that prior injections are well-sealed (with clinical glue) to prevent rupture and escape of the injectate.

5.2.2.2 Effect of ultrasound exposure on gene expression following IT injection of adenovirus and UCA

In order to test the hypothesis that cavitation improves viral distribution and expression within the tumor, non-replicating adenovirus encoding for red fluorescent

protein (Ad-GFP) was co-injected with ultrasound contrast agent directly into each tumor.

Although the clinical relevance for localizing cavitation by direct IT injection of nuclei is questionable, it should be emphasized that future incarnations of this technology may not face the same obstacles to localization. Firstly, HIFU transducers can be custom-designed to have more powerful geometric focusing, which precludes the need for high concentrations of nuclei to locally reduce the cavitation threshold. Secondly, a drawback unique to this animal model is the superficiality of the tumor and therefore its proximity to the skin-water interface, which is particularly susceptible to entrapping nuclei within pores and other surface irregularities. Treatment of deep-seated human tumors within the human body will not present this problem, though of course other tissue-tissue interfaces (particularly involving bone or fat, which have the highest impedance mismatch to most organs) may function as cavitation nucleation sites.

Figure 5.12 shows a sequence of B-mode ultrasound images before (a), and during (b) injection, where hyperechogenicity is clearly visible and persists for up to 5 minutes following injection. This is unsurprising, given the poorly functioning vasculature and lymphatic system within the solid tumor. The hyperechogenicity was limited to within a radius of a few millimeters of the injection site, and was not always homogeneously distributed. Uniformity can be improved by performing multiple injections throughout the tumor (Figure 5.12(c)). However, for simplicity the present work involves a single injection.

Following injection, HIFU treatment was applied to the left tumor only at 500 kPa PRFP, 90% DC, 10 Hz PRF, for 120 seconds. These parameters were found to maximize cavitation activity (as determined by quantification of acoustic emissions) compared to various other combinations of parameters which involved the same

total acoustic energy deposition. The right tumor (control) was not exposed to ultrasound.

Representative images of transgene expression 24 hours post-HIFU treatment are shown in Figure 5.13, where fluorescence data has been ‘superimposed’ upon bright-field photographs of the mice (algorithmically, this was achieved by superimposing the bright-field photograph upon the fluorescence data, and varying the transparency of the photograph with fluorescence intensity).

In all cases, elevated levels of fluorescence in the sonicated (left) tumors compared to control (right) tumors were visually apparent. This corresponds to increased levels of delivery and expression of the payload, in this case the viral genome encoding for green fluorescent protein. Additionally, patchy genome expression is observed in the control tumor, while the sonicated tumor shows more uniform expression. Fluorescence above background levels was observed in all five sonicated tumors, but only three of five control tumors. This may be a consequence of the injectate having been completely confined to an area of unviable tissue (the necrotic core) in the control tumors, or may be due to an experimental error (though this is not likely, as IT injection is a fairly straightforward procedure, and unsuccessful injections are immediately apparent because the injectate escapes from the needle wound).

Quantitatively, in the absence of ultrasound, injections of AdGFP and SonoVue yielded fluorescence activity above background level ($2.55 \pm 0.93 \cdot 10^6$ Relative Light Unit (RLU) for control tumor, versus $6.69 \pm 0.69 \cdot 10^5$ RLU for background). The addition of ultrasound significantly enhanced GFP expression ($1.90 \pm 0.66 \cdot 10^7$ RLU, $P \approx 0.028$ vs no ultrasound, which was a 6.5-fold increase vs no ultrasound). Note that the increase in GFP expression is modest relative to that observed in the presence of inertial cavitation *in vitro*. This is likely attributable to the low concentration

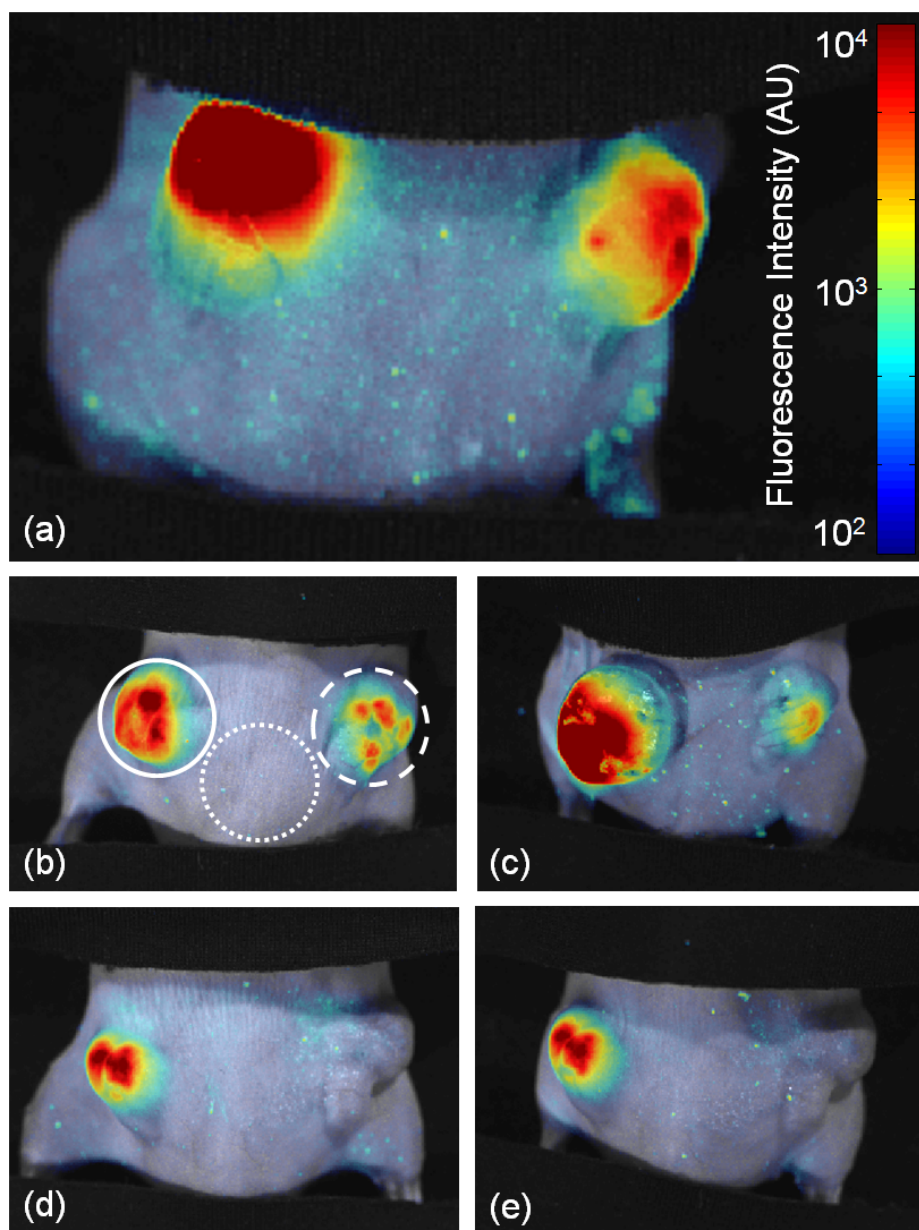


Figure 5.13: Transgene expression recorded 24 hours following HIFU treatment, indicated by fluorescence intensity. **(a)** Left (sonicated) tumors show elevated levels, as well as improved spatial distribution, of viral genome expression, relative to right (control) tumors (note that the color map range is chosen for to highlight both background noise levels and fluorescence signal spreading in the treated tumor. Raw fluorescence data was not saturated). **(b)** Regions of interest defining the sonicated (solid line), control (dashed line), and background (dotted line) for quantitative analysis. **(d)-(e)** Viral genome expression is not detectable in the control tumor, possibly due to tumor physiology or experimental error. $N=5$.

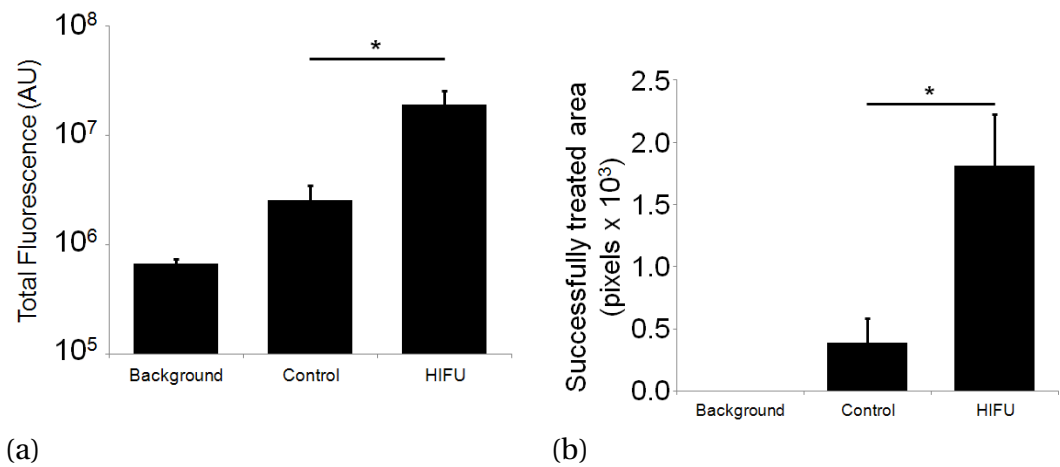


Figure 5.14: Quantitative analysis of transgene expression 24 hours post-treatment. Analysis of IVIS images show a statistically significant increase in **(a)** GFP expression and **(b)** GFP distribution in sonication vs control tumors. N=5 for each group.

of nuclei and therapeutic reaching the target in *in vivo* experiments relative to *in vitro* experiments, in which the supply of therapeutic and nuclei is constantly being replenished. These results are summarized in Figure 5.14(a).

The addition of ultrasound also enhanced GFP distribution within sonicated tumors, as defined by the number of pixels at intensity levels five standard deviations above mean background fluorescence ($1,813 \pm 416$ pixels for treatment group vs 390 ± 199 pixels for control group, $P \approx 0.014$).

5.2.2.3 Effect of ultrasound exposure on gene expression following IV injection of contrast agent and adenovirus

Although intratumoral injection is straightforward, there are several disadvantages. First and foremost, there exists significant morphological and functional variability within each tumor, and from one tumor to the next. The resulting uncertainty regarding the proximity of the injection site to viable tissue and functioning vasculature can have a substantial impact on cavitation activity, virus expression, and

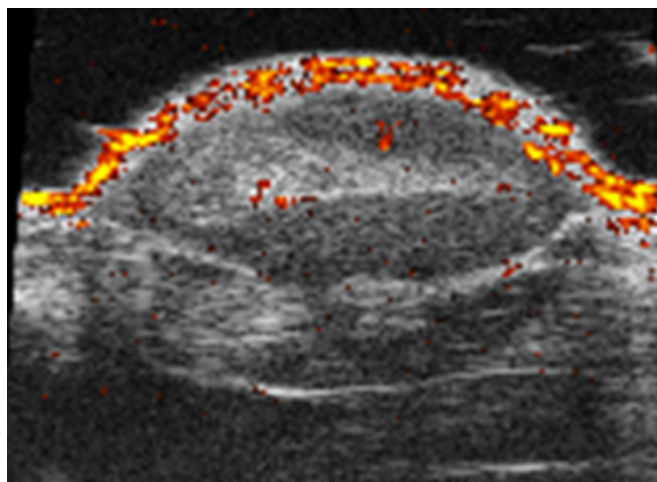


Figure 5.15: Power Doppler image showing a B-mode image of the tumor volume and surrounding tissue, superimposed with a map of functioning vasculature. Note that the skin is relatively well-perfused, with only sparse vasculature within the tumor volume.

clearance rates. These technical issues may explain the high variability in GFP expression between animals subjected to nominally identical treatments using the intratumoral injection method.

To circumvent these problems, a more well-defined delivery route was explored - via the tail vein - which, in addition to being more directly translatable to clinical practice, ensures virus/microbubble delivery to all functioning vessels within the tumor. IV injection is not, however, without challenges. As discussed in the IT study, localizing cavitation within the tumor given the geometry of this experimental model is not trivial. Exacerbating the situation in the IV study is the poorly vascularized tumor (a recurrent theme in our experimentation) relative to surrounding healthy tissue (Figure 5.15), which makes it difficult to deliver cavitation nuclei to begin with. IV injection can therefore preferentially promote cavitation in well-perfused tissue, such as the skin above the tumor or the muscle beneath it.

The most obvious consequence of this is the damage that can arise in healthy tissue. However, there is a second, more subtle, effect. The spatial pressure pro-

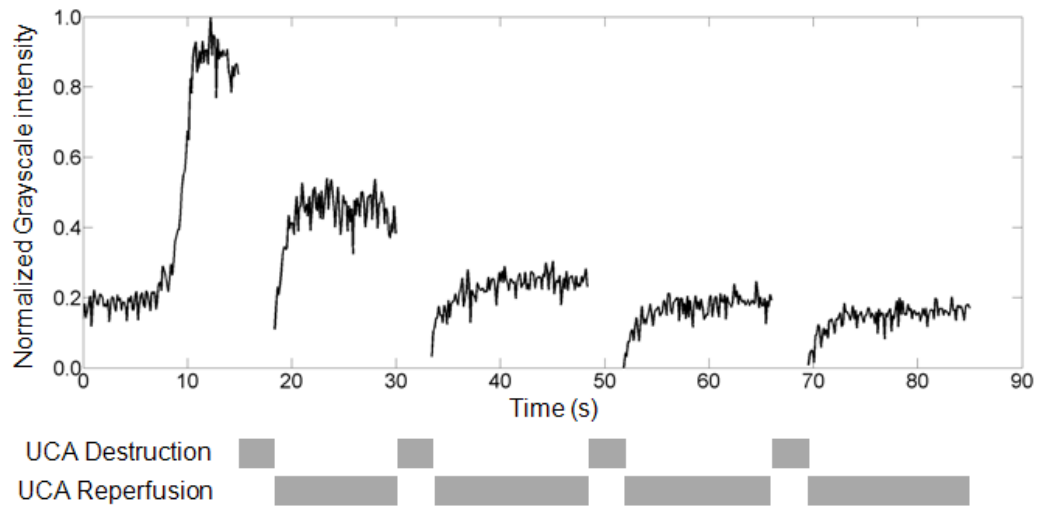


Figure 5.16: Grayscale intensity of B-mode ultrasound image recorded in the tumor volume as a function of time, normalized to maximum and minimum values observed. High intensities correspond to the presence of scatterers such as UCAs. Bubble-destroying ultrasound pulses are delivered every 15 s. Under the acoustic intensities employed in HIFU treatment, nuclei are likely to be destroyed. The delay between HIFU pulses should be sufficient to allow replenishment of nuclei. Half-reperfusion times were found to be approximately 4 s.

file of the HIFU transducer (Figure 3.5) is calibrated in the free-field - that is, in the absence of obstacles. However, if UCA concentration in the skin vasculature is sufficiently high, the pre-focal bubble cloud may impede the propagation of the treatment pulse, a phenomenon known as ‘shielding’. Shielding is a particular problem in cavitation-enhanced thermal ablation, and is hypothesized to reduce focal heating rates at pressures well-above the cavitation threshold (Figure 2.17(b)).

On a more practical level, tail vein cannulation is a barrier to high-throughput experimentation, requiring several minutes per mouse and very careful handling to ensure that the cannula is not dislodged (failure to cannulate becomes apparent when resistance is encountered during injection).

Pulse Repetition Frequency In our *in vitro* model, it was hypothesized that lower pulse repetition frequencies (PRF) would increase treatment efficacy by allowing longer quiescent periods between pulses during which new virus and UCA could enter the vessel and replenish those destroyed by the preceding acoustic pulse. In fact, the best PRF for maximal broadband noise (inertial cavitation) matched the half-reperfusion time, whereas for stable cavitation, changes in the PRF were irrelevant (perhaps because the UCAs are not destroyed at these pressures).

Although these findings directly contradict those published in (Shen et al., 2008), in which modifications in PRF were found to have little importance in intraportal delivery, it should be emphasized that the published study did not explore reperfusion times above 0.33 s, which was a serious limitation of this study.

UCA reperfusion time in ZR75.1 tumors was evaluated by applying destructive pulses with a diagnostic transducer and monitoring grayscale intensity as a function of time in the tumor area. This was done with the Vevo 770 imager under contrast mode. A sequence of frames was captured over 90 seconds with destructive pulses occurring every 15 seconds, and the grayscale intensity in a region of interest comprising the tumor was quantified over time using Matlab software (Figure 5.16). The results indicate that the UCA reperfusion half time is approximately 4 seconds. Thus, a half-reperfusion time of 2 s was employed, implying a PRF of 0.5 Hz for *in vivo* studies.

Injection Timing Using the same Vevo system, it was discovered that even in the absence of bubble-destroying pulses, the half-life of UCA in the bloodstream is approximately 80 seconds (Figure 5.17), similar to the pharmacokinetics of AdLuc (discussed below). Therefore, rather than administer the entire bolus of UCA at the beginning of treatment, boli of AdLuc and SonoVue were delivered at 0s and 120s

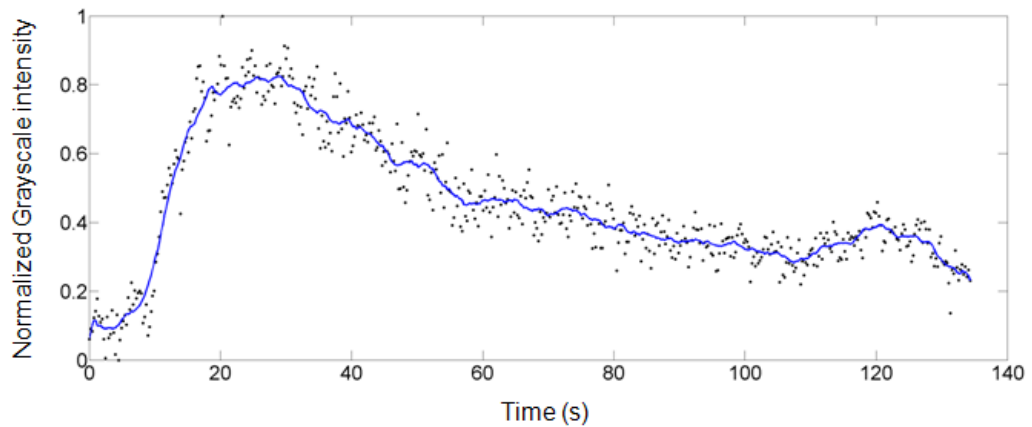


Figure 5.17: Grayscale intensity of B-mode ultrasound image recorded in the tumor volume as a function of time, normalized to maximum and minimum values observed. In the absence of bubble-destroying pulses, UCA concentration in the tumor drops to half its maximum value within 80 seconds.

(the beginning and midpoint of ultrasound treatment). Of course, continuous infusion may prove even more effective at sustaining cavitation activity.

Sonication Parameters The HIFU treatments were designed to match two parameter sets in the *in vitro* experiments above, including stable cavitation-promoting HIFU at 360 kPa, 90% DC, and inertial cavitation-promoting HIFU at 1.2 MPa, 6% DC. The PRF was revised to 0.5 Hz based on the physiologic half-reperfusion time, and total treatment was limited to 4 minutes (this is partially constrained by limitations on the total injectate volume allowable under the animal license). Only the right tumor was sonicated.

Gene expression 24 hours post-sonication Representative images of transgene expression 24 hours post-HIFU treatment are shown in Figure 5.18 and Figure 5.19 for inertial and stable cavitation regimes, respectively. As in the IT results, luminescence data has been superimposed upon bright-field photographs of the mice. These images contain several features of note.

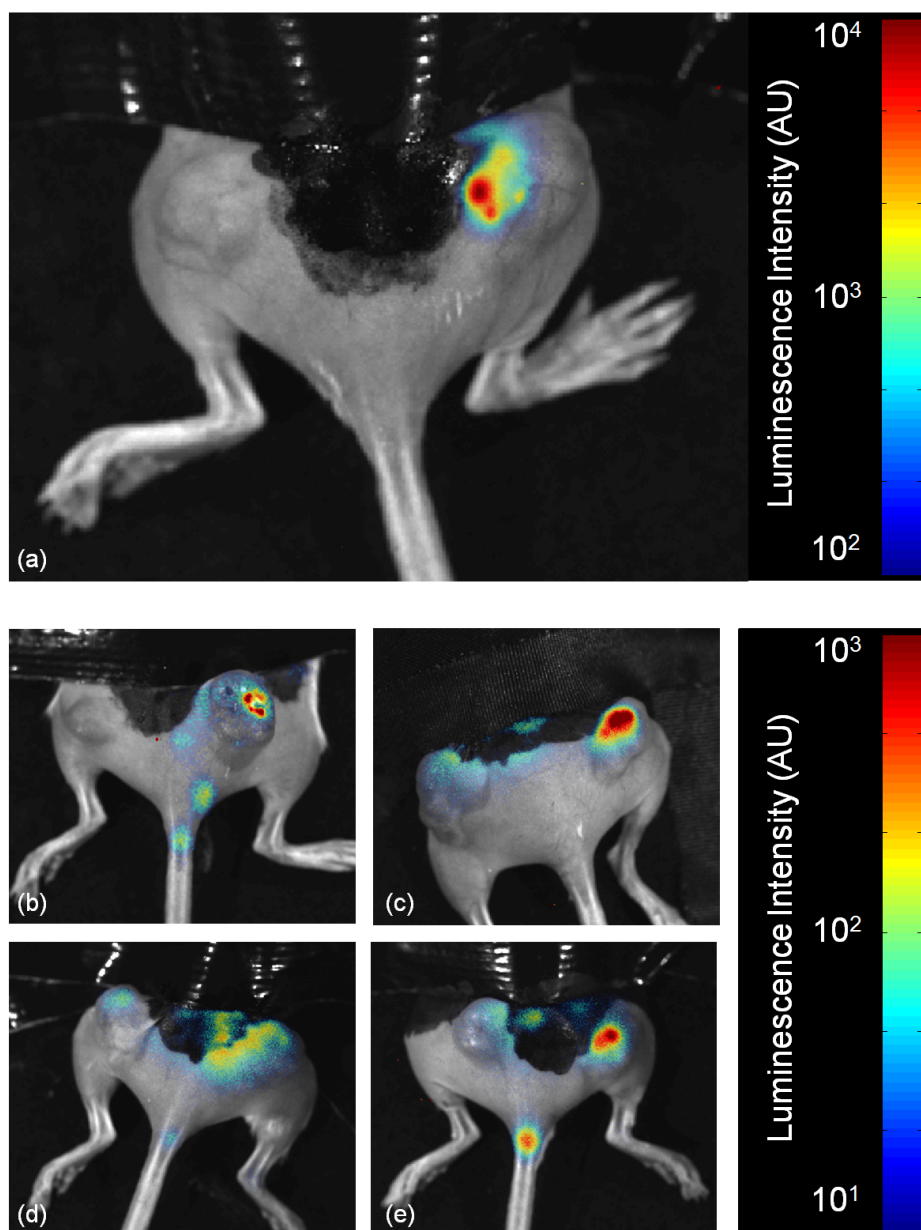


Figure 5.18: Transgene expression in several mice, recorded 24 hours following 1.2 MPa HIFU treatment. **(a)** The right (sonicated) tumor shows elevated levels of viral genome expression relative to the left (control) tumor. **(b)-(e)** Processing artifacts. Patches of luminescence in the feet and tail are occasionally observed. Luminescence emanating from the liver is significant. Despite attempts to shield the camera from this effect, the signal ‘spills’ into the tumor regions (see the treated tumor in **(d)**, for example). However, the signature ‘hot spots’ in 4 of these 5 photographs suggest successful delivery and expression of the viral genome. $N=5$.

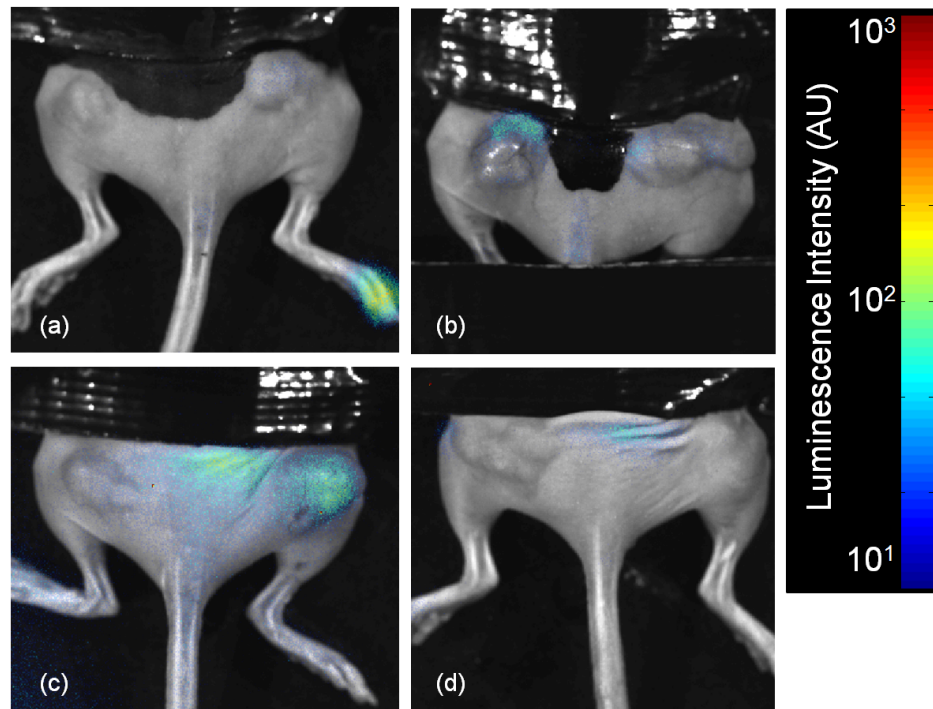


Figure 5.19: Transgene expression in several mice, recorded 24 hours following 360 kPa HIFU treatment. A mild increase in transgene expression is observed only in (c). N=4.

First, the virus, though designed to be selectively expressed in breast cancer cells, has a ‘leaky’ promoter region, which results in genome expression within the liver as well. Because the liver sequesters the virus with high efficiency, this results in strong luminescence emanating from the liver which, if unsuppressed, saturates the IVIS camera signal. Therefore, light emitted from within the liver is shielded from the camera by the combination of covering the mouse body above the liver with black fabric and duct tape, and applying water-soluble black ink to the skin surface in an iterative process until the signal is suppressed enough that it does not govern the camera exposure settings.

Despite this artifactual level of luminescence, which compromises the quantitative interpretation of IVIS data, ‘hot spots’ of luminescence do appear in 4 of 5 tumors exposed to 1.2 MPa HIFU, compared to 0 out of 4 tumors exposed to 360 kPa

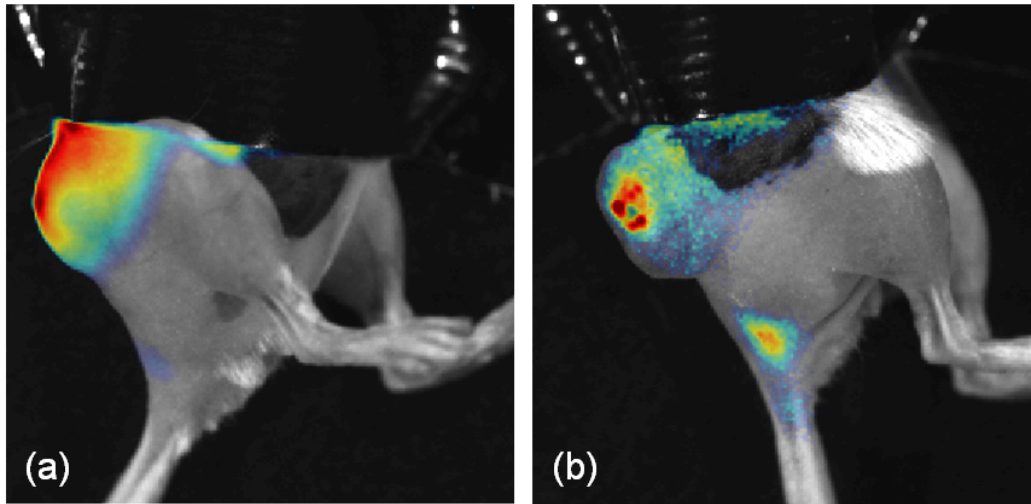


Figure 5.20: Lateral perspective of two mice treated with 1.2 MPa HIFU, demonstrating the confinement of transgene expression to the tumor volume (particularly the region closest to the skin surface) rather than the muscle beneath the tumor, where maximum extravasation was reported in (Bohmer et al., 2010)

HIFU and 0 out of 9 control tumors. However, these areas of elevated luminescence vary considerably in shape, size and absolute magnitude of gene expression (the luminescence scale in 5.18(a) is an order of magnitude higher than in (b)-(e).

Side views of several tumors (Figure 5.20) suggest that transgene expression was limited to the tumor and not the muscle beyond the tumor, demonstrating the specificity of combining targeted ultrasound technology and a selective oncolytic adenovirus, particularly when only one or the other does not provide sufficient targeting.

Quantitatively, intravenous administration of AdLuc and SonoVue yielded detectable but insignificant increases in luminescence in control tumors relative to background levels ($1.39 \pm 0.24 \cdot 10^5$ RLU for control tumor, versus $9.06 \pm 1.59 \cdot 10^4$ RLU for background). Sonication at 360kPa did not significantly enhance luciferase expression ($1.49 \pm 0.49 \cdot 10^5$ RLU per tumor). However, sonication at 1.2MPa significantly enhanced luciferase expression compared to control tumors ($4.21 \pm 0.47 \cdot 10^5$

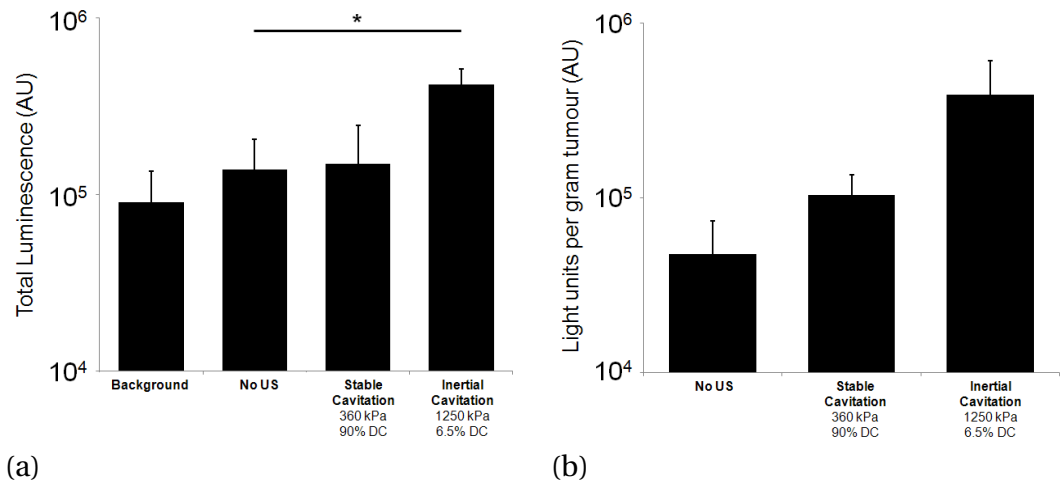


Figure 5.21: Quantitative analysis of transgene expression 24 hours post-treatment. **(a)** Analysis of IVIS images show a statistically significant increase in luciferase expression at 1.2 MPa only. **(b)** *Ex vivo* quantification shows a three-fold increase in luciferase expression following 360 kPa treatment, and a ten-fold increase in luciferase expression following 1.2 MPa treatment. N=5 for inertial cavitation group, n=5 for stable cavitation group, n=5 for no-US group, and n=4 for background group.

RLU per tumor, $P \approx 0.018$ vs control tumor, which was a 3-fold increase control tumor). These results are summarized in Figure 5.21(a).

Ex-vivo analysis of the tumors showed that at 360 kPa, transgene expression was increased by three fold compared to those non-ultrasound treated controls; whereas while employing 1.2 MPa HIFU, transgene expression was increased by an order of magnitude (Figure 5.21(b)).

Finally, the pharmacokinetics of the injected virus were studied to ensure that the treatment timing is reasonable, and that sonication in one tumor does not affect the blood virus levels encountered by the contralateral tumor. AdDNA in the bloodstream was quantified by taking blood samples from the tail vein at 0, 5, 15 and 30 minutes post-injection and performing qPCR on the samples. A study of 12 mice shows that even after 5 minutes, blood virus concentrations can vary by a factor of 5 from mouse to mouse, while after 15 minutes the range spans over an order

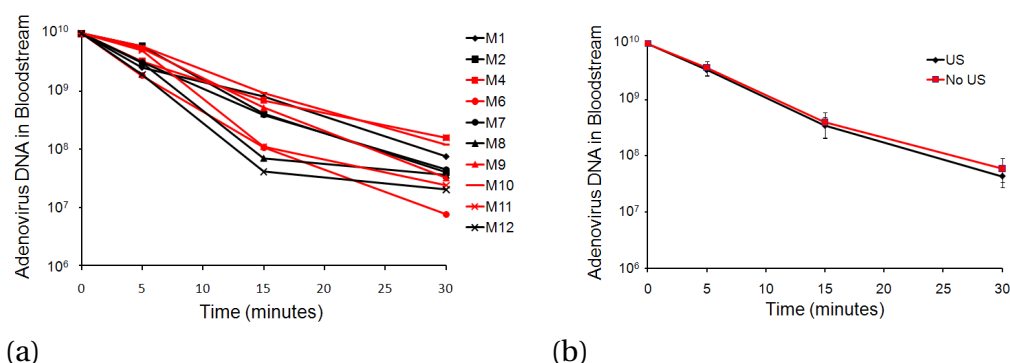


Figure 5.22: Quantification of bloodstream AdDNA based on qPCR of blood samples taken from each mouse at 0, 5, 15, and 30 minutes post-injection. Although significant variability in adenovirus half-life is observed from mouse to mouse (a), aggregate data suggests that these differences are not due to HIFU treatment (b). Therefore, pooling transgene expression data from contralateral control tumors in mice sonicated under different regimes is justified. N=12.

of magnitude (Figure 5.22(a)), which may be a significant source of variability in the luminescence results.

Pooling the data into treated and control groups confirms the initial hypothesis, that the quantity of virus delivered to the tumor does not materially affect the blood virus concentration (Figure 5.22(b)), and therefore the use of a bilateral tumor model, and comparison between treated and control tumors within the same mouse, are justified.

5.2.2.4 Analysis of acoustic emissions¹

In vivo experimentation introduces several complexities not present in an *in vitro* setting that influence the interpretation of acoustic emissions. Primary among

¹As discussed in Section §5.2.1, the PCD data acquired during the HIFU treatments presented above were compromised by electrical noise arising from the motorized positioning stages. The data presented in this section were acquired during a *subsequent* experiment with the same design and with my considerable initial involvement and guidance, but largely carried out by Drs. Robert Carlisle and James Choi, who will continue to develop this technology upon my departure. This experiment involved IV injection only, and did not include a 360kPa treatment group (since this sonication regime was not found to enhance viral expression).

these is the challenge of *mapping* cavitation activity. Although the focal width of the PCD is narrow relative to the HIFU transducer, its axial focusing is weak. For HIFU pulses beyond a few cycles, the recorded PCD signal therefore represents a depth-wise integration of the cavitation activity across the entire HIFU focal volume of 30 mm. This makes it impossible, in the context of this experimental setup, to resolve cavitation activity in the tumor from cavitation activity in the pre-focal skin or post-focal muscle, both of which are likely to contain higher concentrations of cavitation nuclei.

In spite of this limitation, temporal patterns in cavitation activity and patterns from mouse to mouse are identifiable, and may yet prove useful in gauging treatment success. In Figure 5.23, the energy spectral density of the PCD signal is plotted as a function of time, so that the color of each pixel represents the magnitude of the energy density at the corresponding frequency and time, each vertical strip of data corresponds to the FFT of the PCD signal recorded for that HIFU burst, and strips are separated by the 2-second pulse repetition period. High-amplitude streaks in the vertical direction correspond to broadband noise emissions, while those in the horizontal direction correspond to sustained harmonic or ultraharmonic emissions.

Immediately apparent is that broadband emissions are most pronounced immediately following the injections of UCA (and adenovirus) at 0 s and 120 s, begin to taper immediately, and return to baseline levels within 20-50s. Harmonic and ultraharmonic emissions, however, are sustained for the entire treatment, particularly in 5.23(a) and 5.23(b). The broadband signal in 5.23(d) inexplicably and abruptly drops after approximately 200 s of treatment.

The most unusual pattern of cavitation activity, however, is shown in 5.23(e), where the noise floor is highest to begin with, broadband emissions are observed only intermittently, and ultraharmonic emissions are not observed at all. Interest-

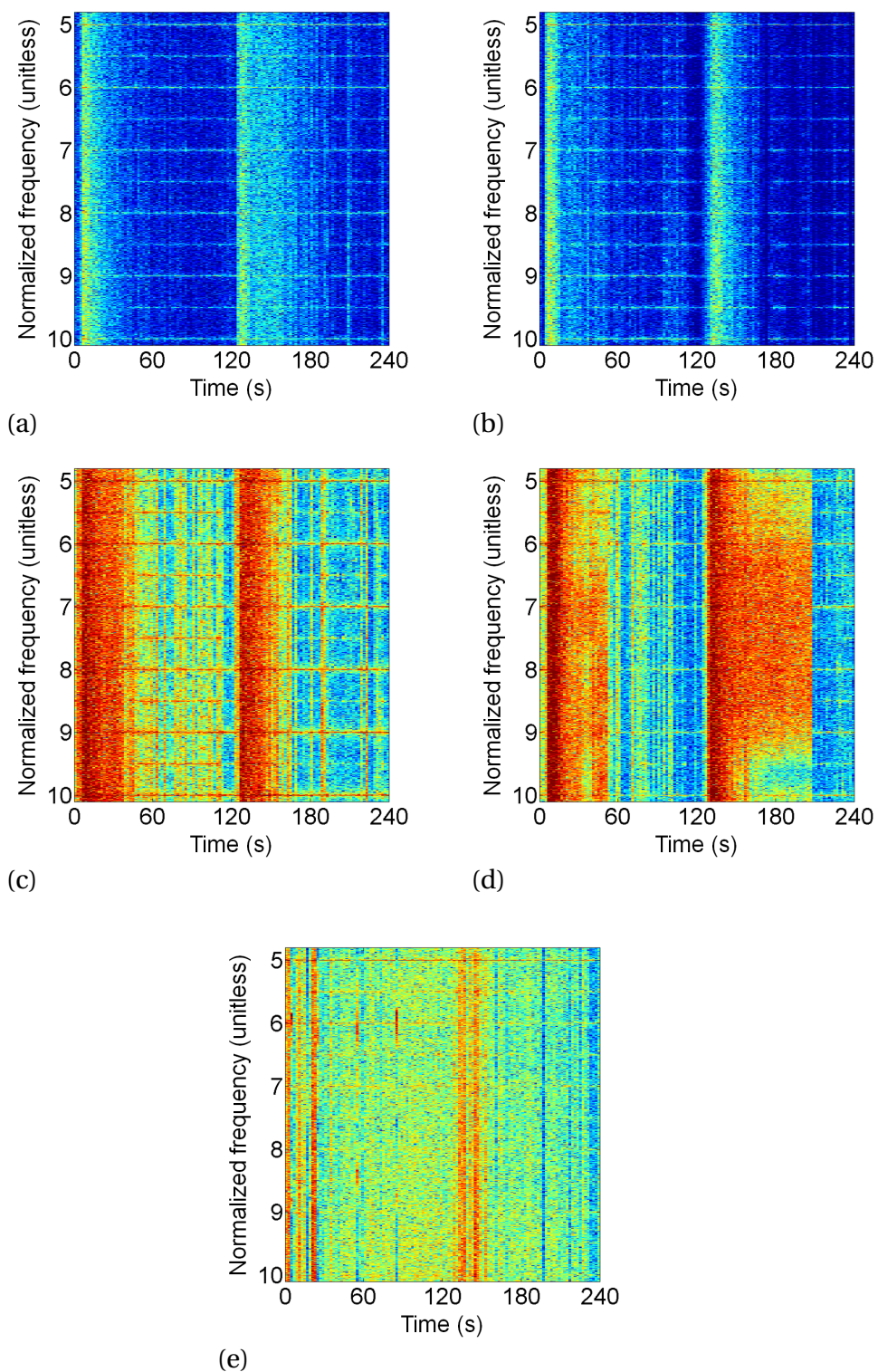


Figure 5.23: Energy spectral density of the PCD signal as a function of time in five mice identically treated with 1.2 MPa ultrasound. Pixel color represents magnitude of energy density.

ingly, the *ex vivo* luciferase expression in Mouse E was found to be 86% lower than the average of Mice A-D (Figure 5.25). This has several possible interpretations.

First, a procedural error, such as poor cannulation, may have resulted in poorer delivery of UCA and adenovirus into the bloodstream. This is at least partially supported by the lower levels of AdDNA observed in the *liver* of Mouse E relative to Mice A-D, though the reduction in liver AdDNA is not as dramatic as the reduction in tumor AdDNA (data not shown). Because the liver is responsible for removing virus from the bloodstream, normalizing tumor AdDNA levels against liver AdDNA levels may help correct for such errors.

The explanation is perhaps further supported by the recorded acoustic emissions. In the 20 s following UCA injection, the spectrogram in 5.23(e) looks qualitatively similar to the spectrogram at later time points in 5.23(c), where reduced bubble concentration is expected. This suggests that Mouse E may have been exposed to reduced UCA levels in the bloodstream to begin with. However, further work characterizing acoustic emissions at various initial UCA concentrations, as well as at different time points, is necessary to support this hypotheses.

A second interpretation is that the functionality of tumor vasculature in Mouse E is reduced, relative to Mice A-D. This would explain the modest rise in broadband emissions following injection, as well as the intermittence of inertial cavitation, which would be expected if reperfusion times were long relative to PRP.

The PCD signal variance as a function of time is plotted for Mice A-E in Figure 5.24(a). Interestingly, the baseline noise floor in Mice A and B is two orders of magnitude lower than the noise floor in Mice C and D. The anomalous and erratic cavitation activity in Mouse E is again apparent. Differences in noise floor and maximum increase in broadband emissions may reflect variability in absolute vascular-

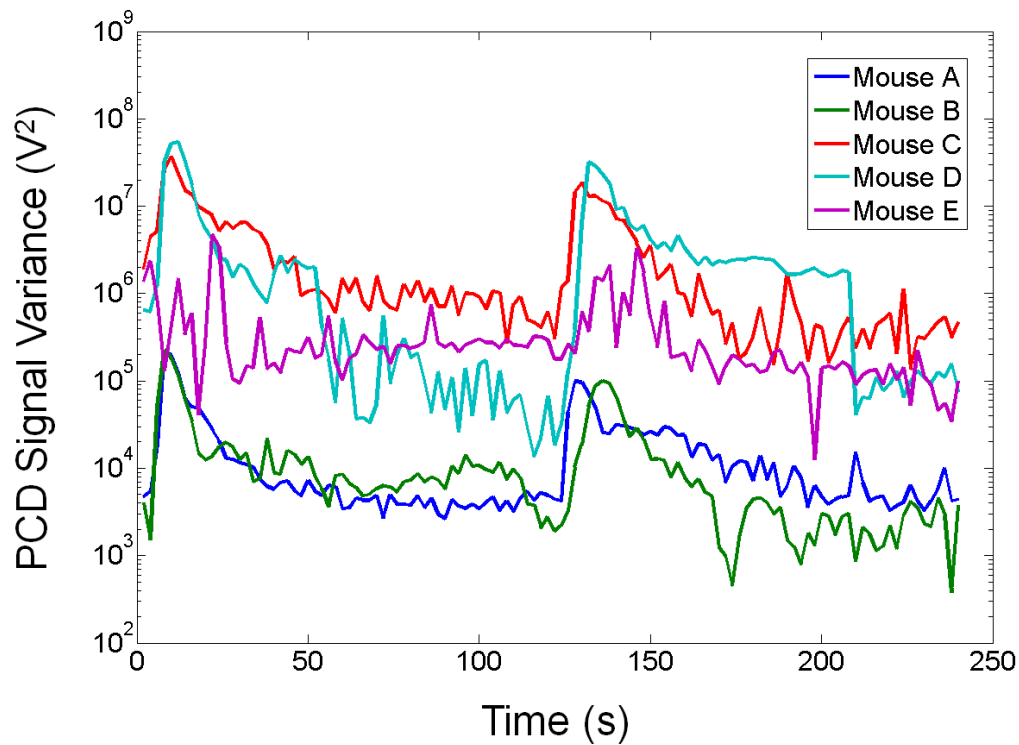


Figure 5.24: PCD signal variance plotted as a function of time for five mice (labels correspond to the subfigure letter in Figure 5.23). There is significant variability between samples in both the noise floor (compare, for example, the variance immediately preceding reinjection in (a) vs (c)) and the absolute magnitude of the rise in emissions following injection (data plotted on a logarithmic scale)

isation of the tumours, while the persistence of broadband emissions may reflect tumor reperfusion rates. For example, in 5.23(d), broadband emissions alternately rise and fall each successive pulse between 50 s and 120 s, which may indicate that 4 s are required for nuclei to re-populate the HIFU focal volume.

5.3 SUMMARY

In this chapter, both *in vitro* and *in vivo* models were presented which permitted exploration of the effect of HIFU on delivery and expression of a biologically active adenovirus.

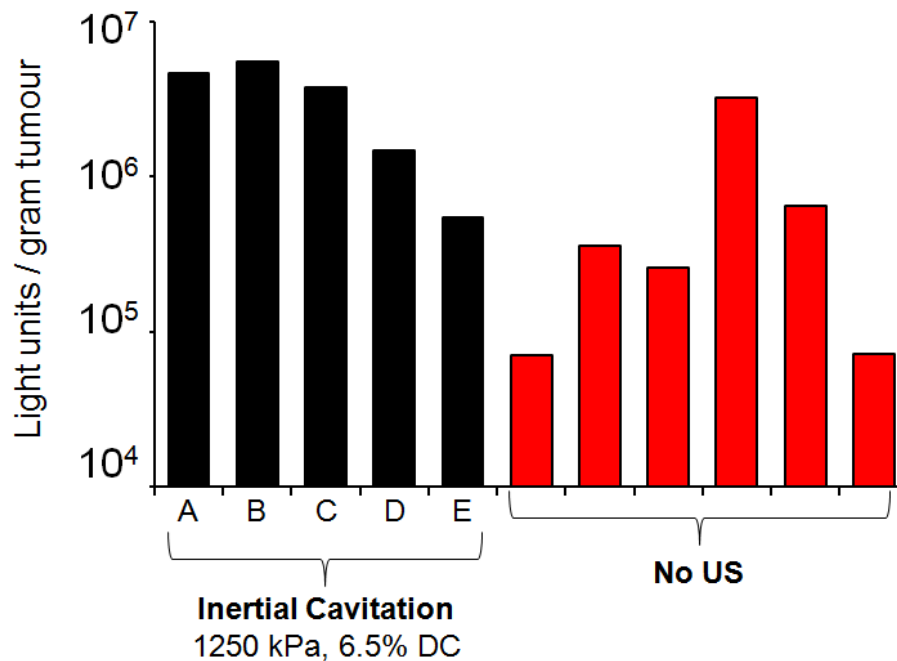


Figure 5.25: Luciferase expression per tumor as assayed *in vitro* post-cull (labels in sonicated group correspond to the subfigure letter in Figure 5.23). Although a general trend exists (with, for example 4 out of 5 sonicated tumors above 10^6 light units per gram tumor, compared to 1 out of 6 untreated tumors above the same level), the small sample size precludes statistical support for the impact of HIFU on transgene expression.

The *in vitro* work identified sonication parameters under which virus extravasated from a vessel into a cell-embedding agar gel with porosity similar to solid tumors, and the viral genome was expressed in infected cells. It was found that inertial cavitation at 1.2 MPa was most effective in this regard, yielding a 4-fold increase in extravasated viral particles, and a 100-fold increase in transgene expression. This effect was somewhat directional, being most pronounced in the axis of sound wave propagation. The relationship between the amount of virus extravasated and the level of transgene expression suggests that improved *distribution* of virus - rather than absolute quantity - is a key effect of HIFU treatment within the tumor model.

The *in vivo* work involved mice with subcutaneously grown human breast can-

cer tumors. Initial studies established safe operating regimes - including a range of peak pressures, duty cycles, frequencies, and bubble concentrations - which avoided unacceptable side effects, such as skin burns and hemorrhaging.

Next, the effect of HIFU on transgene expression was studied following intratumoral injection of adenovirus and UCAs. IT injection, though less translatable to clinical practice, reaffirmed the hypothesis that HIFU enhances transgene *distribution*. Further histological work would help confirm this effect, by exploring the relationship between cells exhibiting transgene expression and their proximity to functioning vasculature.

Finally, the mouse model was used to explore the effect of HIFU on transgene expression following *intravenous* injection of adenovirus and UCAs. Although visually apparent increases in transgene expression were observed, and *ex vivo* analysis showed an increase in average transgene expression following 1.2 MPa HIFU, the variability in expression from mouse to mouse precluded a statistically rigorous conclusion, given the small sample size (n=12). Analysis of the recorded PCD signal revealed several characteristics, including the absolute rise in broadband emissions upon reinjection, and the persistence of inertial cavitation with time, which may be indicative of the success of injection and treatment.

A discussion of conclusions and visions for the future of this technology is included in the following chapter.



CONCLUSIONS & FUTURE WORK

In this chapter, the original contributions and new insights presented in this thesis are summarized, from concept to implementation of cavitation-enhanced drug transport in small animals.

6.1 CONCLUSIONS

In Chapter 1, hindrance of the progress in cancer treatment by the failure of intravenously injected therapeutics to reach all tumor cells at toxic doses was identified. The failure was attributed to physiologic peculiarities within solid tumors which affect the three stages of drug delivery: vascular transport, extravasation, and interstitial transport. The pathophysiology manifested itself in changes to the tumor's fluid dynamics - areas of low flow rates, unfavorable pressure gradients, low diffusivity - inspiring us to explore the ability of therapeutic ultrasound to locally improve the fluid dynamics.

Therapeutic ultrasound has a broad range of biomedical applications, from thermal ablation to lithotripsy, and equally numerous mechanisms of action. In Chap-

ter 2 parameters were identified which distinguish one treatment regime from another. It emerged that microstreaming, driven by the ultrasonic cavitation of exogenously injected nuclei, was observed under mild sonication regimes, while more violent phenomena such as fluid jetting were observed in more powerful acoustic fields.

Uncertain as to the optimal regime in which HIFU could enhance vascular transport, a tissue-mimicking polymer model was developed in Chapter 3 which simulated the junction between a flowing vessel and an obstructed vessel, to facilitate the *in vitro* analysis of various sonication regimes and their ability to improve the distribution of a dye-labeled macromolecule. In one set of experiments, cavitation was promoted by the introduction of exogenous nuclei.

Cavitation activity was indirectly monitored by detecting, with a PCD, the acoustic emissions generated by microbubble oscillations. A coarse approximation of the type and quantity of cavitation taking place is indicated by the energy spectral density of the acoustic emissions, with broadband energy corresponding to inertial cavitation and ultraharmonic energy corresponding to stable cavitation.

It was shown that 2 seconds of continuous-wave HIFU at 0.5 MHz was indeed capable of improving the distribution of a macromolecule, in some cases propelling the macromolecule at significant concentrations to distances up to 20 mm from the vessel junction. It was also found that the transport was enhanced by up to an order of magnitude by the presence of exogenous nuclei, and that the total quantity of drug delivered into the obstructed vessel was strongly correlated to the energy contained in broadband emissions, which suggested both a mechanism of action of this phenomenon - inertial cavitation - and the potential to develop a non-invasive means of monitoring the course of treatment, which is a second major objective of this work.

Following the success of this proof-of-concept experiment, novel numerical methods were developed in Chapter 4 to identify parameters - such as exogenous nuclei size, acoustic frequency, and peak pressures - which optimize convective transport due to HIFU. First, the experimental work conducted in the previous chapter was reproduced using finite volume (FV) computational fluid dynamics (CFD) techniques, and was found to bear strong agreement to experimental results only at low pressures or in the absence of exogenous nuclei, where the role of cavitation is weak. Incorporating cavitation into the model, however, required significant simplification in order to remain computationally tractable.

A flexible and efficient method, general enough to be applied to *any* arbitrary geometry and underlying actuation mechanism, was therefore developed, which computes the steady streaming fields induced by a periodic actuator. After presenting numerical validation examples which demonstrate good agreement with experimental reference data from the literature in cases of both acoustic cavitation and electrowetting-driven bubble oscillations (albeit at larger bubble sizes), the technique was applied to the optimization of convective transport around an acoustically cavitating microbubble.

It was discovered that at low pressures, resonant or near-resonant bubbles were most effective at enhancing the transport of a hypothetical zero-diffusivity scalar. However, the impact of initial bubble size becomes less pronounced as pressure is increased and large expansion ratios are observed. In this case, off-resonance bubbles are nearly equally effective at enhancing convective transport, a finding which had important implications in terms of the exogenous nuclei employed in subsequent experiments. Specifically, if sub-MHz frequencies are employed to reduce cavitation thresholds and minimize heat deposition, the use of clinical ultrasound contrast agents - which generally have a mean radius of $\sim 2 \mu\text{m}$ - may be warranted.

Moreover, it was found that treatment at these higher pressures had the desirable attributes of both reducing treatment time *and* reducing the total acoustic energy necessary to achieve the desired bioeffect.

In Chapter 5, the insight gained from numerical optimization was applied to a more sophisticated *in vitro* model, then a live animal model. To confirm the numerical finding that acoustic energy is more efficiently converted into the desired bioeffect at higher pressures, a constant acoustic energy dose per treatment was maintained by varying the duty cycle with the inverse square of the peak pressure. SonoVue[®] ultrasound contrast agents were used to instigate cavitation, and biologically active adenoviruses were employed as the model therapeutic. Viruses are appealing delivery vectors because of their ability to target cancer cells with high specificity, efficiently transfer genes, and generate robust and exponentially amplifying expression of potentially toxic transgenes.

The *in vitro* work identified sonication parameters under which virus extravasated from a vessel into a cell-embedding agar gel with a porosity similar to that of solid tumors, and the viral genome was expressed in infected cells. It was found that inertial cavitation at 1.2 MPa was most effective in this regard, yielding a 4-fold increase in extravasated viral particles, and a 100-fold increase in transgene expression. The relationship between the quantity of extravasated viral particles and the level of transgene expression suggests that improved *distribution* of virus - rather than absolute quantity - is a key effect of HIFU treatment.

In addition, one parameter which was *not* examined numerically - pulse repetition frequency (PRF) - was found to be critical to sustaining cavitation activity. Specifically, cavitation activity was maximized when the quiescent periods between acoustic pulses were matched to the half-reperfusion time, so that new virus and UCA could enter the vessel and replenish those destroyed by the preceding acoustic

pulse.

These results were supported by *in vivo* work involving mice with subcutaneously grown human breast cancer tumors. After establishing operating regimes which avoided side effects, the effect of HIFU on transgene expression was studied following intratumoral injection of adenovirus and UCAs. IT injection, though less translatable to clinical practice, reaffirmed the hypothesis that HIFU enhances transgene *distribution*, since equal numbers of viral particles were injected into sonicated and control tumors.

Finally, the mouse model was used to explore the effect of HIFU on transgene expression following *intravenous* injection of adenovirus and UCAs. Using diagnostic ultrasound imaging, the half-reperfusion time of the tumor was determined, and guided the choice of a PRF of 0.5 Hz. Visually apparent increases in transgene expression were observed using an *in vivo* imaging system, and luciferin assays and qPCR *ex vivo* showed an increase in average transgene expression following 1.2 MPa HIFU relative to both 360 kPa HIFU and control tumors. However, unanticipated variability in transgene expression from mouse to mouse precluded a statistically rigorous conclusion, given the small sample size.

Analysis of the acoustic emissions during *in vivo* experimentation, though suffering once again from small sample size, revealed adenoviral transgene expression only in the presence of inertial cavitation. Additionally, several characteristics of the power spectra of the PCD signal, including the absolute rise in broadband emissions upon reinjection, and the persistence of inertial cavitation with time, may be indicative of the success of injection and treatment *in vivo*, but further experimentation is necessary to confirm these hypotheses.

6.2 FUTURE WORK

Although the past three years have witnessed the development of this therapeutic modality from concept to pre-clinical promise, there is significant room for advancement, both in the context of the current experimental model and future incarnations of the technology.

6.2.1 Current Setup

Having identified sonication parameters which enhance transgene expression *in vivo*, further experimentation should be aimed at both refining the treatment protocol and addressing unresolved issues regarding treatment. Primary among these is identifying the mechanism by which cavitation enhances distribution and expression of adenoviral transgenes. Although it was demonstrated that sonoporation was not responsible for the experimentally observed enhanced gene expression *in vitro*, the corresponding experiments should be performed *in vivo* using adenoviral receptor-deficient tumor xenografts.

Next, a series of experiments can be performed to gauge where active transport (e.g. microstreaming, fluid jetting) or passive accumulation (e.g. vessel/cell permeabilization) is responsible for treatment success. One such experiment involves co-injecting adenovirus and UCA prior to HIFU in experimental Group 1, while delaying adenoviral injection until after sonication in Group 2. The relative rise in transgene expression in the two groups should indicate the importance of passive accumulation versus active transfer, since the adenovirus in Group 2 will not be directly subject to the effects of cavitation, but will exhibit variations in transgene expression which arise due to changes in the tumor microenvironment following cavitation activity.

If active transfer emerges as the dominant contributor, then optimal sonication times should also be explored. Acoustic emissions suggest that inertial cavitation activity is only present during the first 30 seconds following UCA injection, so it may be possible to reduce treatment times considerably without compromising efficacy.

If passive transfer emerges as an important contributor, the mechanism can be further explored by identifying the number of acoustic cycles which optimize transgene expression. A finding of enhanced expression with few cycles may indicate that either jetting through the vessel wall, or wall shear stress-induced permeabilization (as in analogous blood-brain barrier extravasation) may be important, and histological analysis could distinguish between the two.

Of course, the ultimate objective of this experimental model is to demonstrate that the combination of HIFU, UCAs, and oncolytic adenovirus can reduce tumor size and/or extend mouse lifespan. Such a study should endeavor to isolate the importance of cavitation activity by both monitoring acoustic emissions as before, and by including an experimental group in which UCAs are not injected before sonication.

The number of mice involved in the proposed experiments can quickly inflate to an unmanageable quantity. Owing to the considerable variability in tumor size, morphology, and vascularity, as well as mouse metabolism, each experimental group often requires a sample size of ~10 to achieve statistical significance given the current assay techniques of viral genome expression and quantification. Reasonable modifications which have been implemented or proposed include normalizing tumor AdDNA against tumor size (though a more fair approach would be to normalize it against viable tumor size or tumoral expression of an appropriate 'housekeeping' gene), normalizing against liver AdDNA (to account for metabolic variability), normalizing against pharmacokinetic data (since this provides a good approxima-

tion of the concentration of virus to which vascularized tumor is exposed), or normalizing against tumor percent vascularity (as measured by Power Doppler ultrasound). Each metric has scientific merit, and though it perhaps naive to attempt to reduce physiologic complexity to a collection of quantities, this level of characterization may be necessary to isolate the impact of HIFU on treatment success, and to do so with a reasonable number of samples.

6.2.2 Improvements to the Experimental Setup

6.2.2.1 HIFU Transducer Design

From a therapeutic perspective, the current experimental setup suffers several limitations which should be resolved in future designs. First, the use of a single HIFU transducer with an f-number of 1 results in poor axial focusing, and high-intensity sound fields in the tumor necessarily extend into the pre-focal skin tissue and the muscle tissue beneath the tumor. Because these adjacent tissues are more well-vascularized than the tumor xenograft, the resulting cavitation field extends to regions where it is not needed - outside the tumor - and where it may cause unwanted side effects or even shield the tumor from high acoustic intensities. Future experiments should employ a single transducer with more powerful geometric focusing, or a pair of transducers, orthogonally oriented, to improve the quality factor of the resulting cavitation field.

6.2.2.2 Treatment Monitoring

Improved HIFU focusing would also improve the quality of extracorporeal treatment monitoring. Currently, the poor axial focusing of the HIFU transducer is exceeded only by the poor axial focusing of the PCD. By improving the focusing of the HIFU field, confidence can be gained that any acoustic emissions detected by the PCD

originated from within the tumor volume, rather than adjacent tissue.

This issue can also be addressed by replacing the single-element PCD with an array of PCDs, such as the linear array of a diagnostic transducer. By passively acquiring the signal detected by a diagnostic transducer array, cavitation activity can be spatially resolved using existing techniques (Gyongy and Coussios, 2010) and possibly even shown to co-locate with histological evidence of treatment efficacy.

The incorporation of a diagnostic array would also offer advantages in treatment planning and execution. As previously discussed, vascularity - and therefore reperfusion rates - vary significantly from tumor to tumor, and even within a single tumor. By interleaving passive cavitation detection with B-mode imaging, a diagnostic array can monitor UCA reperfusion in real time by hyperecho, and treatment parameters such as PRF and re-injection can be tailored to the unique vascularization of each tumor. Even in the absence of a diagnostic array, it may be possible to arrive at a rough estimate of reperfusion by operating a single-element PCD in pulse-echo mode and quantifying nonlinear echogenicity using pulse-inversion imaging or other suitable technique.

Regarding the measurement of cavitation activity, algorithms need not be limited to quantifying broadband and ultraharmonic emissions as indicators of inertial and stable cavitation, respectively. The combination of time- and frequency-domain analysis, which has shown promise in gauging the progress of lithotripsy ((Leighton et al., 2008)) (though admittedly, this involves more pronounced and discrete cavitation events), may prove equally predictive of treatment success in the context of drug delivery.

6.2.2.3 Cavitation Nucleation Agents

In light of the numerical and experimental evidence that sub-resonant micro-bubbles, when driven to inertial cavitation, can elicit the desired bioeffect, further work should consider the potential for hydrophobic nanoparticles as cavitation nuclei. Their size, which can be precisely tuned by adjusting synthesis conditions, makes them an ideal candidate for localizing cavitation within cancerous tissue, since sub- μm particles passively accumulate in tumor interstitium via the EPR effect. By functionalizing and tagging these particles, it may be possible to significantly augment the focusing ability of the HIFU transducer with efficient localization of nuclei within the tumor. Analogous studies have employed gold nanoparticles, which selectively accumulate in tumor tissue, to absorb electromagnetic radiation and thereby localize heat deposition in ablative therapy.

6.2.2.4 Therapeutic Agents

The use of adenoviruses in the current experimental setup represents a major step towards clinical relevance, since transfection inefficiency during systemic administration is one of two major challenges to this otherwise promising branch of therapy (anti-viral immune response is the other). However, characterization of the mechanisms by which cavitation promotes drug delivery is still at an early stage, and the use of viruses introduces an additional level of complexity which, though potentially ‘enhancing’ results through targeting and signal amplification, may nevertheless confound the phenomena at play. Advancement of the technology may therefore benefit from performing experiments with simple tracer particles, such as fluorescently labeled dextran of various molecular weights, so that the relationship between particle size and drug delivery can be explored (to see if there is, for example a cut-off size beyond which extravasation does not occur). Histological analysis

could also be used to quantify the interstitial transport length scales of various sized dextrans and test the hypothesis that diffusion is not an important contributor to delivery of macromolecules.

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PHANTOM PREPARATION

For all experiments described herein, the tumor phantom was prepared according to established protocols Khokhlova (2006). However, for our purposes, the addition of 7% Bovine Serum Albumin (BSA) to increase attenuation was omitted from the original recipe.

Note that this protocol involves hazardous substances, including carcinogens, radical initiators, and strong oxidizing agents. Accordingly, appropriate protective clothing should be worn, and all reactions should be performed in a fume hood.

Briefly, add 45mL Tris (trishydroxymethylaminomethane) to 232mL water in a conical flask containing a magnetic stirrer for mixing. Bring to pH 8.0. Add 168.75mL of a 40% acrylamide solution. Add 378mg Ammonium Persulfate (an oxidizing agent used to catalyze the polymerization reaction). De-gas at -0.9 bar (relative to atmospheric pressure) for 20 minutes to reduce dissolved gas concentration in the phantom and suppress cavitation in the phantom material during experimentation.

Add 225 μ L TEMED (Tetramethylethylenediamine, the polymerizing agent) to the solution and immediately pour into the perspex cast (Figure 3.2). Using a spatula or other suitable device, remove all bubbles from the phantom walls and mandrels, particularly the junction between the mandrels, as this will be the focus of our experimentation. Seal the back wall of the cast. The polymerization will generally be complete within 45 minutes, though for our experiments, phantoms were

allowed to set overnight. If leaving overnight, allow 45 minutes polymerization, then immerse the phantom cast in an isotonic solution to prevent dehydration.

Before removing the mandrels, immerse the cast in an isotonic solution, remove one wall, and let sit for 10 minutes to ensure complete hydration of the gel. Then, slowly remove the mandrels, one at a time, by rotating and withdrawing.

Note that the polymerization reaction, unlike agar gelification, is exothermic. Therefore, if including BSA for denaturation experiments, it may be necessary to reduce the levels of radical reactant, slowing the reaction and allowing heat to dissipate, to prevent the premature denaturation of BSA. Also note that this radical reaction is under kinetic - not thermodynamic - control, i.e. refrigeration does not measurably affect reaction rate.