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ENHANCEMENT AND MAPPING OF
ULTRASONIC DRUG RELEASE FROM
THERMOSENSITIVE LIPOSOMES BY
ACOUSTIC CAVITATION

**A thesis submitted for the degree of
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To my parents.

Abstract

One of the major limiting factors of cancer therapy is the inability to deliver a sufficient dose of drugs to a target tumour without incurring significant side effects. Recent advances in nano-sized liposome formulations have enabled encapsulation of anti-cancer agents that simultaneously reduce off-target toxicity and allow increased accumulation in tumours due to the enhanced permeability and retention effect. Yet, a method for reliably releasing the drug from encapsulation has yet to be found. In this work, low-temperature sensitive liposomes, which release an encapsulated anti-cancer agent (doxorubicin) in the temperature range of 39-45°C, are combined with focused ultrasound-induced mild hyperthermia to deliver a predictable, site-specific, and high dose of anti-cancer agents that has the potential of overcoming the current limitations of targeted drug delivery for cancer.

To reach this aim, acoustic cavitation-enhanced heating was harnessed to produce rapid and efficient temperature rises that could be spatiotemporally monitored and eventually controlled. For this purpose, a reproducible, non-exothermic, cell-embedding tissue-mimicking model was first developed to have an attenuation coefficient, thermal response and cavitation threshold similar to liver. The phantom platform was then utilised to allow for high-throughput optimisation of ultrasound exposure parameters that efficiently and safely produced mild hyperthermia and drug release. Inertial cavitation-enhanced heating was successfully shown to enhance drug release from the liposomes and subsequent cancer cell death due to release of the encapsulated agents, using a 25% Duty Cycle, 1Hz PRF 6 s sonication sequence. The remotely detectable acoustic emissions associated with cavitation were then exploited to enable real-time monitoring and spatial mapping of drug release using a novel passive acoustic mapping method. Finally, these developments and techniques were combined to demonstrate the feasibility of cavitation-enhanced drug release and delivery in a world-unique isolated normothermic liver perfusion model that provides blood-flow rates comparable to those encountered in humans.

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.

Eleonora Mylonopoulou
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ACRONYMS

TMM	Tissue Mimicking Material
HIFU	High Intensity Focused Ultrasound
BSA	Body Surface Area
TTSLs	Traditional Thermally Sensitive Liposomes
LTSLs	Low Temperature Sensitive Liposomes
PAM	Passive Acoustic Map
TEA	Time Exposure Acoustics
PCD	Passive Cavitation Detector
BBB	Blood-Brain Barrier
DC	Duty Cycle
PRF	Pulse Repetition Frequency
PPK	Peak-to-Peak



MOTIVATION AND PHYSIOLOGY

There are more than 200 types of cancer, each with different causes, symptoms and treatments. Liver cancer is the seventh most commonly diagnosed cancer and the fourth most common cause of death from cancer worldwide. In this chapter, existing liver cancer therapies are discussed, along with the advantages and limitations associated with each treatment. An overview of the pathology of cancer is given, with particular attention to the tumour microenvironment, how it proves to be a challenge for current techniques and how it can be utilized to improve treatment. Finally, the potential of a new technique, which employs targeted heat activation of thermally sensitive liposomes with high-intensity focused ultrasound is considered.

1.1 THE BASICS OF CANCER

Cancer is not a single disease, but a group of over 200 diseases that share the ability to invade the body and cause uncontrolled, invasive, and metastatic cellular growth due to damage in the genetic information of cells. Such damage can be caused by inherited factors (up to 10% of all cancers) or can be a result of endogenous factors, such as hormones, or exogenous factors, such as tobacco smoking, alcohol consumption, excessive exposure to UV radiation, infectious agents or occupational exposure to chemicals.

Normal tissues carefully control the production and release of growth-promoting signals that control the progression through the cell growth-and-division cycle, thereby ensuring a homeostasis of cell number and thus maintenance of normal tissue architecture and function. By contrast, cancer cells resist cell death in two ways. They evade growth suppressors and induce and sustain growth-stimulatory signals. These signals promote uncontrolled growth and division of cancer-cells. When cancer cells divide they form new abnormal cells with the same damage in their genetic information. In most cases, apart from haematological cancers, such as leukaemia, this abnormal growth leads to the formation of a mass or tumour (Hanahan and Weinberg, 2011).

Left untreated, tumours that contain only benign cells will stop growing, whilst those that contain malignant cells will continue to grow, divide and possibly metastasise. Metastasis, the migration of cancerous cells away from the initial site of malignancy can cause spread of the cancer through the lymphatic and haematogenous systems and invasion of distant tissues, causing micrometastatic lesions, which later evolve to macrometastatic tumours. Tumours eventually will starve healthy surrounding cells from the nutrients they require and cause organ failure. Figure 1.1 depicts the basic characteristics that define cancer, as described above.

Even though cancer can develop at any age, more than 60% of the cases have been diagnosed in people aged above 65 and especially with the increasing average life expectancy in modern times, cancer has become a leading cause of death worldwide. According to the World Health Organization statistics, it accounted for 7.6 million deaths (13% of all deaths) in 2008 and is projected to continue to rise to over 11 million in 2030. In the UK alone, there were around 300.000 new cases of cancer diagnosed in 2008 (Cancer Research UK).

Early detection of cancer, namely identification of the disease before symptoms

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Figure 1.1: Classical hallmarks of cancer (Hanahan and Weinberg, 2011).

appear, has been shown to reduce mortality from various cancer types, including cancers of the colon and rectum, breast, and uterine cervix (ACS 2011). After a cancerous tumour is identified, there is a variety of methods that can be used for its treatment. The most established therapies that are currently used in the clinic to treat cancer are described in the following section.

1.2 CANCER TREATMENTS

Currently there are many cancer treatments available, with unique advantages and disadvantages, which can be followed either alone or in combination to maximize the therapeutic effect. The most established and commonly used treatments comprise surgery, chemotherapy and radiotherapy.

Surgery is a procedure that involves excising tumours or tumour-containing organs from the patient, and is very effective in cases of well-defined solid tumours. However, being a highly invasive procedure, it has drawbacks such as long recovery time for the patient, possibility of infection or haemorrhaging. Moreover, even in

cases of well-defined tumours, surgery might not be applicable because of the tumour being adjacent to a critical body structure or located in a region offering poor surgical access.

Radiotherapy uses high-energy radiation to kill cancer cells by damaging their DNA through ionizing radiation. Tumour cells are more sensitive to ionizing radiation than normal cells, because they frequently acquire defects in their molecular regulatory mechanisms, and thus a diminished ability to repair damage, during the process of becoming cancer cells (Gudkov and Komarova, 2003). The radiation may be delivered by a device outside the body (external-beam radiation therapy), or it may come from radioactive material placed in the body near cancer cells (internal radiation therapy, also called brachytherapy).

Radiotherapy has acute or chronic side-effects, including tissue damage, hair loss or even secondary cancer in cases of high radiation dosage. In terms of therapeutic efficacy, radiotherapy has a limitation in the cases of solid tumours. As will be discussed later, tumours have hypoxic regions, which are resistant to radiation treatment, because oxygen is a potent radiosensitizer (Gray and Hancock, 1955).

Chemotherapy is a treatment strategy that targets rapidly-dividing cancer cells and involves administration of cytotoxic agents that impair cell division, or cause cell apoptosis. Chemotherapeutic agents are administered either orally or intravenously, alone or in combination, and are successful mostly in highly vascularised tumours. The major drawback of chemotherapy is that it indiscriminately kills healthy and cancerous tissues.

The widespread distribution of a chemotherapeutic agent throughout the patient's body at a dosage adequate to achieve cytotoxicity in tumours inevitably affects healthy tissues, causing undesirable side effects ranging from hair loss, nau-

sea and tiredness (Lerman *et al.*, 1990) to life-threatening heart failure (Swain *et al.*, 2003), thus limiting the drug dosage that can be safely administered (Doroshov *et al.*, 1991; Allen and Cullis, 2004). The balance that must be maintained between minimum concentration of the chemotherapeutic agent in normal tissues and a therapeutically relevant concentration in the target tissue not only results in healthy tissue damage, but also compromises the overall outcome of the chemotherapeutic management of cancer.

In addition to the lack of selectivity of the anticancer agents, other inherent complications of free drug systemic administration include poor solubility and unfavourable pharmacokinetics. These limitations have triggered the investigation of new methods for targeted drug delivery capable of confining the drug action to the tumour site, thus reducing the side-effects and maximizing the therapeutic efficacy (Brown, 2004).

Apart from these commonly used treatments, there are other options currently undergoing clinical trials, such as tumour ablation with microwaves or ultrasound, as well as targeted drug delivery techniques. Ablation techniques are only suitable for discrete solid tumours and can be used as a minimally-invasive replacement for surgery. They are however not applicable to the drug-based treatment of metastatic cancer and are therefore not directly relevant to the present work.

In the next section, targeted drug delivery for cancer is discussed. The characteristics of the tumour microenvironment that distinguish it from normal tissue are addressed and the challenges that it poses to drug delivery are outlined. Additionally, modern methods of addressing these obstacles are presented, including the exploitation of the effects of hyperthermia.

1.3 TARGETED DRUG DELIVERY FOR CANCER

Many of the pharmacological properties of conventional (free) chemotherapeutic drugs, such as poor solubility, tissue damage, poor biodistribution and lack of selectivity, can be improved through the use of drug delivery systems, which include particulate carriers, composed primarily of lipids and/or polymers, and their associated therapeutics. The essence of most targeting strategies is (i) the encapsulation of the therapeutic agent in a biocompatible vesicle, for instance a liposome; (ii) systemic administration of the encapsulated, thus inactive agent; and (iii) its controlled release from the carrying vesicle at the intended treatment site (Cho *et al.*, 2008).

Earlier targeting strategies relied on the natural increased drug uptake from the target tissue (passive targeting), a mechanism known as the Enhanced Permeability and Retention effect (EPR) (Maeda *et al.*, 2001). Drug carriers of the right size range, such as liposomes or macro-molecular drugs tend to accumulate in tumours more than they do in normal tissues. This phenomenon occurs because in certain pathological conditions, such as in a solid tumour, the vascular permeability is increased so that carriers that would normally be excluded from tissue can extravasate and accumulate in the tissue interstitial space in a size-dependent manner.

A step forward from passive targeting is the exploitation of the differentiating characteristics between healthy and cancer cells for active cell recognition from the drug carrying vesicle (Wink, 1999). When the differentiator is a structure on the target cell surface, ligands, namely substances that bind specifically and reversibly to another chemical entity to form a larger complex, or antibodies, namely proteins produced by the immune system, that interact with this specific structure are coupled to the active substance or the drug carrier to serve as a means of active targeting. Targeting ligands are attached to drugs and drug delivery systems act as hom-

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Figure 1.2: Overview of the most clinically relevant drug targeting strategies. (A) Systemic administration of the chemotherapeutic agent. (B) Passive drug targeting by virtue of the enhanced permeability and retention (EPR) effect, which substantially increases the accumulation of the active agent in tumours. (C) Active drug targeting to internalization-prone cell surface receptors (over)expressed by cancer cells. (D) Active drug targeting to receptors expressed by angiogenic endothelial cells aims to reduce blood supply to tumours. (E) Stimuli-sensitive nanomedicines can be activated (i.e., induced to release their contents) by externally applied physical triggers, such as hyperthermia. (F) In cases in which tumours are easily accessible, for example during surgery, sustained-release delivery devices can be implanted or injected directly into the irresectable parts of the tumours. Reproduced from Lammers *et al.* (2008).

ing devices for binding to receptor structures expressed at the target site (Allen *et al.*, 2002; Park *et al.*, 2004).

In the case of potential target structures being at the cell interior, the active substances are manipulated and the drug is only activated when taken up within the cell. As the anti-cancer agent remains inactive while associated with the carrier, drug encapsulation proves to be efficacious in preventing toxic side effects. However, that does not guarantee a therapeutically effective treatment. To be effective,

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Figure 1.3: Distribution of doxorubicin *in vivo*. Fluorescence microscopy image from a section from a mouse mammary tumour showing the distribution of doxorubicin (blue) in relation to tumour blood vessels (red) and regions of hypoxia (green). Note the steep gradient of doxorubicin distribution around tumour blood vessels. Bar = 100 μm . Reproduced from Primeau *et al.* (2005)

anti-cancer drugs must reach all malignant cells that comprise the target population at a therapeutically relevant dose, as the survival of one cell could form the focus of tumour recurrence.

The disadvantage of these methods is that after administration of the targeted drug, there is no control over the treatment. Controlled targeted drug release may be achieved with the establishment of treatment regimens in which tumour-targeted nanomedicines are combined with other clinically relevant treatment modalities. Such modalities are surgery, radiotherapy, localised heating, ultrasound mechanical or thermal effects. (Allen and Cullis, 2004)

Despite the increased vascular permeability of a tumour that enables preferential vesicle localization at the tumour site, the unique interstitial tumour structure and the highly heterogeneous vasculature constitute a very challenging environ-

ment for drug delivery. Figure 1.3 shows distribution of plain doxorubicin (a commonly used chemotherapeutic agent, which intercalates DNA in rapidly growing cancer cells, namely interacts with it inhibiting its replication.) *in vivo* in a mouse mammary tumour. High concentrations of doxorubicin (blue) are localized around blood vessels (red), whereas many regions of the tumours do not contain detectable doxorubicin, thus showing a lack of penetration deep into the interstitial space. Areas of tumour sections without detectable doxorubicin include all of the hypoxic (green) and adjacent (black) regions (Primeau *et al.*, 2005).

1.4 THE TUMOUR MICROENVIRONMENT

The tumour microenvironment is highly heterogeneous and thus presents challenges for targeted chemotherapy. Mostly, these challenges stem from the abnormal and highly heterogeneous tumour vasculature. In Figure 1.4 the difference between normal and tumour vessels can be observed.

To reach all viable cells in the tumour, anti-cancer drugs must be delivered efficiently throughout the tumour vasculature, cross the vessel wall and penetrate the tumour interstitial space. Normal cells in the human body are organized within a close distance ($<100\ \mu\text{m}$) to the blood vessels, so that delivery of nutrients and oxygen from the vessels to the cells is facilitated. On the contrary, tumour cells often have the potential for more rapid proliferation than the cells that form blood capillaries, often compressing blood vessels and forcing them apart (Minchinton and Tannock, 2006). Consequently, vascular density is reduced and a population of cells distant from blood vessels is created. This has a dual effect, as distant cell populations are not only less exposed to potentially lethal concentrations of the cytotoxic agent, but also receive limited supply of nutrients, which results in metabolic changes, causing local hypoxia or acidity (Minchinton and Tannock, 2006; Brown

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Figure 1.4: Microscopic imaging of normal and tumour blood vessels. (a) Scanning electron microscopic (SEM) imaging of polymer cast of normal microvasculature, showing simple, organised arrangement of arterioles, capillaries, and venules. (b) SEM image of cast of tumour microvasculature, showing disorganisation and lack of conventional hierarchy of blood vessels. Arterioles, capillaries, and venules are not identifiable as such. (McDonald *et al.*, 2003)

and Giaccia, 1998; Helmlinger *et al.*, 1997). This influences drug sensitivity, as some drugs might be less active in hypoxic, acidic or nutrient-deprived microenvironments.

Tumour vessels themselves are characterized by abnormalities in form and a poorly organized architecture (Less *et al.*, 1991). They are often dilated and convoluted and their walls may be leaky, since they usually present fenestrations and discontinuous membranes (Trédan *et al.*, 2007). If substances like liposomes (100 - 200 nm in mean diameter) have reached the tumour capillaries, the aforementioned vessel abnormalities present a positive effect to targeted chemotherapy, as they allow the drug to cross the endothelium of blood vessels and extravasate into the interstitial space resulting in increased accumulation at the tumour site with respect to normal tissues (EPR), as illustrated in Figure 1.6. The vascular pore cut-off size for six different types of tumours grown in the dorsal chamber is shown in

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Figure 1.5: (A) Diagrammatic representation of tumour cells and the extracellular matrix (ECM) surrounding a capillary. Cells further from the vessel are subjected to hypoxic and acidic conditions. (B) Schematic representation of oxygen concentration and pH gradient with respect to the nearest blood vessel (reproduced from (Trédan *et al.*, 2007))

Figure 1.7. However, increased extravasation of macromolecular drugs from large vessels at the tumour periphery may lead to drug loss, thus reducing therapeutic effectiveness.

Extravasation is also influenced by gradients in pressure, as the process depends on convection between the vascular and interstitial space. In a solid tumour, the lymphatic network, which is mainly responsible for fluid drainage from tissues, is usually malfunctioning or compressed by rapidly growing cancer cells. Consequently, fluid drainage is inadequate and thus the hydrostatic pressure within the interstitial fluid (interstitial fluid pressure) is elevated compared with normal tissues, approaching micro-vascular pressure (Leu *et al.*, 2000). The resultant diminished pressure gradient inhibits the extravasation of large molecules by convection, even if there are vessel abnormalities, such as fenestrations that would otherwise allow it.

Increased interstitial fluid pressure also leads to vessel blockage thus diverting

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Figure 1.6: Representative electron micrographs of tumour vessels grown in the dorsal chamber (A) Venular-sized vessel with fenestrated endothelium (Bar = 500 nm.) (B) tumour vessel with an open gap measuring 856 nm delineated by the arrows. (Bar = 500 nm.) (C) Open endothelial gap in tumour blood vessel from a long-circulating liposome-injected animal. What appear to be long-circulating liposomes are shown traversing through the open junction (Bar = 300 nm.), adapted from Hobbs *et al.* (1998)

the blood away from the centre of the tumour towards its periphery, which, combined with the composition and structure of the extra-cellular matrix, can slow down the movement of molecules within the tumour causing poor drug penetration.

Finally, high vascular permeability and vessel compression by cancer cells may impair tumour blood flow. In normal vasculature, flow rate is directly proportional to the difference in pressure between arteries and veins and inversely proportional to the viscous and geometric resistance. In tumours, the difference in pressure between arterioles and venules is reduced and viscous and geometric resistance is increased. These abnormalities cause temporally and spatially heterogeneous blood flow. In addition, tumour blood flow rate may change at a fixed location due to tumour growth and necrosis (Trédan *et al.*, 2007; Sevick and Jain, 1989).

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Figure 1.7: Vascular pore cutoff size for six different types of tumours grown in the dorsal chamber (A) and the cranial window (B). The solid circles represent significant extravasation at the indicated liposome size. (Hobbs *et al.*, 1998)

The highly heterogeneous and unfavourable tumour microenvironment underlines the necessity to exploit any of its characteristics that can potentially maximise the therapeutic efficacy of a chemotherapeutic regime. Acidity and hypoxia in tumours increase their resistance to both chemotherapy and radiation. However, these same factors render cancer cells more sensitive to heat (Moyer and Delman, 2008).

In addition, the cytotoxicity of several anticancer agents and the efficiency of radiation are enhanced at conditions of mild hyperthermia. As a result, more sophisticated cancer treatments have been introduced that combine targeted chemotherapy with mild hyperthermia for regional tumour control, due to its selective effect on cancer cells. In the next section the potential of this synergistic action to achieve enhanced therapeutic effects will be discussed and an overview of previous attempts towards that direction will be presented.

1.5 HYPERTHERMIA AND CANCER THERAPY

1.5.1 Mechanism of Action

The rise in temperature in a tissue depends on the amount and duration of heat exposure. In normal tissues it is regulated by mechanisms that control heating and cooling of tissues, so that homeostasis is achieved. Blood flow is one of the major means by which heat is dissipated from tissues and generally the lower the blood flow rate, the easier it is to deliver heat to the tissue. Tumours, being tissues with low and irregular blood flow, are more prone to reaching higher temperatures as heat accumulates (Horsman, 2008).

Upon initial heating (up to 42°C - mild hyperthermia range), blood flow and oxygenation in tumours increases, thus enhancing drug delivery and radiation sensitivity (Moyer and Delman, 2008; Sun *et al.*, 2010). In a study by Song *et al.* (Song *et al.*, 2005) heating rodent tumours at 40–42°C was found to cause an enduring increase in blood flow and oxygenation. Studies with canine soft tissue sarcoma and human tumour clinical studies also demonstrated that mild hyperthermia improves tumour oxygenation, and enhances response of the tumours to radiotherapy or chemoradiotherapy.

In addition, cells in a hypoxic, acidic and nutrient deficient environment, such as a tumour, are more sensitive to the cytotoxic effects of heating than normal cells (Horsman, 2008; Gerweck *et al.*, 1979). Furthermore temperature rises as low as 41°C (Eddy, 1980) have been shown to cause changes in tumour vasculature, such as caliber reduction and dilation.

With further heating that gives a temperature rise above 42°C, due to the abnormal and disorganized tumour vasculature, there is preferential increase in blood cell rigidity, endothelial cell swelling, haemorrhage into capillary lumens and leukocyte

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Figure 1.8: (a) The effect of hyperthermia on perfusion in normal and neoplastic tissues. Both the magnitude and the time of maximum flow appeared to be bimodal functions of temperature, with a maximum of 45.7°C in normal tissue and 43.0°C in tumours. Reproduced from (Dudar and Jain, 1984), (b) Thermal death times for animal and human normal tissues in vivo compared to the thermal death times for tumours. Reproduced from Dickson and Calderwood (1980)

adherence to tumour vessel walls (Song, 1984). This process is first seen in tissues at approximately 42°C and overwhelms all human tissue at 45°C (Horsman, 2006). In addition, the acidic and hypoxic local conditions are aggravated by the additional decrease in oxygen supply that follows heat-induced vascular collapse, so that an otherwise survivable condition becomes incompatible with cell life (Moyer and Delman, 2008).

In Figure 1.8, the effect of hyperthermia on perfusion and the thermal death times for both normal and neoplastic tissues are shown. Both the magnitude and the time of maximum flow appear to be bimodal functions of temperature. They increase with temperature up to a certain critical temperature, and then decrease

at higher temperatures. This critical temperature was found to be approximately 45.7°C in normal tissue and 43.0°C in tumours. Normal tissue requires temperatures greater than 47°C to bring about vascular stasis in less than 1 hr, while stasis occurred in tumours in the same time frame at temperatures greater than 41°C (Dudar and Jain, 1984). Also the thermal death time appears to be shorter for tumours than normal tissue (Dudar and Jain, 1984).

1.5.2 Thermally Sensitive Liposomes

Liposomes are small lipid vesicles with an aqueous interior, which generally range between 50 nm and 200 nm in diameter. Liposomally encapsulated cancer drugs have been developed to increase the efficacy of controversial chemotherapy by site-specific delivery, as previously mentioned, and have demonstrated considerable promise as a carrier for the delivery of anti-cancer agents *in vivo*.

Currently, several anti-cancer agents have been combined with lipid-based systems using a variety of preparation methods. Among them, liposomal formulations of doxorubicin (a commonly used anti-cancer agent), such as Myocet and Doxil are approved for the treatment of metastatic breast cancer and AIDS-related Kaposi sarcoma (Markman, 2006; Rivera, 2003; Rosenthal *et al.*, 2002). Both products contain doxorubicin, but differ particularly in the presence of a stealth coating: the former refers to doxorubicin entrapped in uncoated liposomes, and the latter to surface-modified (or sterically stabilised) liposomes, whereby coating with polyethylene glycol (PEG) is used to inhibit rapid recognition by the reticuloendothelial system, and thereby to prolong circulation time.

These liposomal formulations offer longer circulation times and preferential accumulation to tumours due to the EPR effect in comparison to free doxorubicin. However, their major advantage has been their ability to attenuate drug-related tox-

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Figure 1.9: Schematic representation of mechanisms involved in combination of hyperthermia and thermally sensitive liposomes, as a therapy for solid tumours. (A) Liposomes, shown as blue circles, preferentially extravasate from pores in tumour vessel walls. Hyperthermia increases vessel pore size (B) and triggers drug release (C). Local hyperthermia triggers drug release from liposomes already extravasated in the tumour interstitium (D) and directly causes cytotoxic effects (E), reproduced from (Kong *et al.*, 2000).

icity (e.g., cardiomyopathy, bone marrow depression, alopecia and nausea), rather than to enhance antitumour efficacy (Ranson *et al.*, 2001; Harris *et al.*, 2002).

The combination of the advantages of liposomal formulations of anti-cancer agents with an external modality to trigger localised release is expected to improve accumulation and bioavailability of liposomal drugs in tumours locally. As previously mentioned, localised hyperthermia has a major impact on several tumour pathophysiological parameters, including perfusion and vascular permeability, hence favorable effects on liposome extravasation in tumour areas can be anticipated and have already been demonstrated in some animal tumour models (Kong *et al.*, 2001).

To further exploit the advantages of hyperthermia in tumour treatment, liposomes sensitive to heat have been designed to the principle illustrated in Figure 1.9. Known as thermosensitive liposomes (TSLs), they release their payload preferentially in regions where the local temperature is elevated. Hence, they can be com-

bined with an external source of hyperthermia, which can act not only as a source of additive cytotoxic effects, but also as a release trigger. The therapeutic benefits from liposomes and hyperthermia individually, coupled with the potential advantages seen by their combination, make the synergetic use of the two modalities a very attractive method for targeted chemotherapy.

Work on thermally sensitive liposomes started in the 1970's with the work of Yatvin *et al.* (Yatvin *et al.*, 1978), who introduced the use of phospholipid liposomes that undergo a phase transition from gel to liquid around 44 °C. Since then, there are several temperature sensitive liposomal formulations that have been developed, which in general fall into two categories, namely Traditional Thermally Sensitive Liposomes (TTSLs) and Low Temperature Sensitive Liposomes (LTSLs). TTSLs are triggered in a temperature range between 42°C and 45°C and release their content slowly over 30 minutes of exposure. LTSLs undergo a rapid phase change from gel to liquid when triggered at lower temperatures and release their content in a matter of seconds. (Kong *et al.*, 2000)

The release profiles of LTSLs, TTSLs and non-thermosensitive liposomes (NTSLs) are shown in Figure 1.10. The onset temperature for LTSLs triggered release is a narrow range between 39°C and 40°C. In this range the content release already starts to become significant, even though it is below the main peak of the phase transition (42°C) (Needham *et al.*, 2000).

The advantage of LTSLs with respect to other liposomal formulations has been illustrated in a comparative study by Kong *et al.* (Kong *et al.*, 2000), in which the authors present results on intratumoural drug concentrations, drug distributions and therapeutic efficacies achieved using NTSLs, TTSLs, LTSLs, free doxorubicin and saline in conjunction with hyperthermia. Doxorubicin distribution in tumours is visualized by its inherent ability to fluoresce. The fluorescence intensity of doxoru-

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Figure 1.10: Thermal properties for the three liposomal formulations, LTSLs •, TTSL ■, NTSL ▲ and for pure DPPC (plain phospholipid) ♦. Examples of the rates of DOX release in bovine serum from each liposome at 42°C determined by fluorimetry (a). Examples of the amount of DOX released in bovine serum from each liposome formulation as a function of temperature after 5 min (LTSLs) or 30 min (DPPC, TTSL, and NTSL) (b). Inset: comparison between the heat flow (mW) measured by differential scanning calorimetry for the LTSLs and its release profile from b. Reproduced from (Needham *et al.*, 2000)

bicin is used to quantify the concentration of the anticancer agent at the tumour site and therapeutic efficacy is assessed by tumour growth time. A representative graph of the observed fluorescence intensities is presented in Figure 1.11. As can be seen, the concentration of the anticancer agent is significantly greater in the case of LTSLs.

It should be noted here that the drug used in the present work is ThermoDox® (Celsion Corp., USA). ThermoDox is a lysolipid formulation based on the formulation developed by Needham *et al.* (Needham *et al.*, 2000), that consists of LTSLs containing doxorubicin (DOX). ThermoDox underwent further pharmaceutical development by the biopharmaceutical company Celsion and, in combination with Radio Frequency Ablation (RFA), has reached clinical trial phase III for hepatocellular carcinoma (HCC, or primary liver cancer) and clinical trial phase I for recurrent breast cancer at the chest wall (RCW) (Celsion).

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Figure 1.11: Concentration of doxorubicin in tumours after intravenous injection of the different liposomal formulations and 1 h of hyperthermia at 42°C. Doxorubicin extracted with chloroform only is shown in □ and doxorubicin extracted with chloroform and silver nitrate is shown in ■. Figure adapted from (Kong *et al.*, 2000).

1.5.3 Current Methods of Drug Release from Thermally Sensitive Liposomes

Current methods of promoting thermo-sensitive liposome release mostly employ electromagnetic radiation as an external source of hyperthermia, such as radio frequency (RF) radiation (Aoki *et al.*, 2004) or microwaves (Khoobehi *et al.*, 1989). However, these modalities are only applicable to shallow treatment depths and require insertion of a needle at the tumour site to deliver localised hyperthermia, making them minimally-invasive rather than non-invasive. Only recently has High Intensity Focused Ultrasound (HIFU) - induced hyperthermia been examined as a non-invasive method for drug release from thermally sensitive liposomes.

1.5.4 Ultrasonic Drug Release from Thermally Sensitive Liposomes

High Intensity Focused Ultrasound (HIFU) has recently emerged as a most promising therapeutic modality. Being completely non-invasive, of low-cost, without any known side-effects, capable of penetrating to great depths within the body and giving a more well-defined spatial temperature control than RF, HIFU shows great potential as a heating modality in the context of hyperthermia-triggered drug release. HIFU is discussed further in Chapter 2.

Whilst several studies have been published so far using other modalities for targeted drug release from thermally sensitive liposomes, or using mechanical effects of HIFU for drug release from non-thermally sensitive liposomes, only a small number of studies have been reported that use ultrasound-induced hyperthermia as a potential release mechanism for thermo-sensitive liposomes.

In 2000 De Zwart *et al.* (De Zwart *et al.*, 2000) demonstrated in a preliminary *in vitro* study, the feasibility of localized drug delivery using a combination of thermo-sensitive liposomes and controlled local hyperthermia based on MR-guided focused ultrasound. They showed that the drug release from TTSLs (phase transition temperature 42°C) is possible with ultrasound-induced local hyperthermia.

In another study (McDannold *et al.*, 2004), TSLs were loaded with a paramagnetic contrast agent, where the triggering temperature of the liposomes was set to thermal ablation levels (57 °C). Using an MR-guided HIFU system in combination with the TSLs and imaging for T1-weighted signal intensity enhancement, the authors demonstrated that the temperature elevations required for ablation had indeed been achieved.

In the aforementioned studies, traditional TSLs have been used in combination

with ultrasound as the heating modality. To our knowledge, there is only one study so far that combines HIFU with LTSLs. Dromi *et al.* (2007) used low temperature sensitive liposomes, or LTSLs, with pulsed-HIFU exposures that typically produced temperatures not exceeding 45°C. They showed that combining the exposures with the LTSLs could significantly enhance the delivery of doxorubicin in murine breast cancer tumour xenografts, compared to a commercial nonthermosensitive liposome (i.e. Doxil®). This increase in local drug delivery was subsequently shown to also improve growth inhibition of the tumours. Even though no optimisation of the HIFU exposure conditions was performed, these results demonstrate the potential of combining LTSLs (ThermoDox®) with HIFU.

In a recent study, Staruch *et al.* (2010) evaluated the feasibility of using MRI-controlled focused ultrasound (FUS) and LTSLs (ThermoDox®) to achieve thermally mediated localised drug delivery *in vivo*. A focused ultrasound beam is scanned in a circular trajectory to heat 10–15mm diameter regions in normal rabbit thigh to 43°C for 20-30min to activate intravenously infused lyso-thermosensitive liposomal doxorubicin. This study demonstrated that doxorubicin concentrations were significantly higher in heated compared to unheated regions, showing the potential of focused ultrasound hyperthermia for enhanced local drug delivery with temperature-sensitive drug carriers.

Recently, ultrasound-induced cavitation activity and LTSLs were used in a two-step protocol for enhancement of intracellular drug delivery *in vitro*. As a first step, low-temperature sensitive liposomes were heated at 42°C for release of their content and as a second step ultrasound-induced cavitation in the presence of SonoVue microbubbles led to local permeabilization of the cell membrane and enhancement of ultrasound-mediated intracellular drug delivery. (Yudina *et al.*, 2011). Cavitation activity was used however not for thermal release of the drug from the LTSLs, but

for mechanical enhancement of the cell-membrane permeability and heating was conventionally done. Additionally, microbubbles were used, which in a physiological setting are expected to limit the cavitation activity within the vessels and not near cells as in the *in vitro* model.

1.5.5 Implications for the Present Work

Studies to date have demonstrated that liposomal formulations of potent anti-cancer agents, such as doxorubicin, offer significant advantages in terms of circulation dynamics and tumour accumulation compared to systemically administered free drugs. Furthermore, the use of hyperthermia in the context of cancer therapy is known to offer several advantages, including enhanced permeability of the tumour. The exploitation of localised hyperthermia as a mechanism for drug release from thermosensitive liposomes therefore represents an obvious advancement that can improve both the targeting and uptake of anti-cancer agents by tumours.

Most studies of drug release from thermosensitive liposomes to date have been performed using either minimally-invasive modalities such as RF and microwave, or using non-invasive therapeutic ultrasound in combination with traditional TSLs that require elevated temperatures and longer durations for complete release. Only two studies have been performed that combine LTSLs with ultrasound. Staruch *et al.* (2010) used MR-based thermometry to investigate the feasibility of release using HIFU in non-perfused ex-vivo tissue, but this technique requires the combination of therapeutic ultrasound with a monitoring method that is orders of magnitude more expensive than HIFU itself and took no account of the effect of perfusion. Dromi *et al.* (2007) were first to demonstrate the feasibility and potential therapeutic efficacy of ultrasonic release from LTSLs in a mouse xenograft model, but this was delivered without any intraoperative monitoring and without any optimisation

of the HIFU exposure parameters.

The present work therefore seeks to fill the gap in knowledge in ultrasonic drug delivery from thermosensitive carriers (LTSLs in particular), by optimising the HIFU exposure parameters to achieve maximum release for the smallest possible input acoustic energy, whilst enabling real-time monitoring of the drug delivery process using a low-cost ultrasound-based technique that can help overcome the effects of variable tissue perfusion. This is achieved by exploiting a phenomenon known as acoustic cavitation, described in further detail in Chapter 2, which greatly enhances ultrasound-induced heating.

A reproducible, non-exothermic, cell-embedding tissue-mimicking model is first developed and characterised in Chapter 3, and exploited in Chapter 4 to enable optimisation of the HIFU exposure parameters to maximise release in the absence or presence of acoustic cavitation. The remotely detectable acoustic emissions associated with cavitation are then exploited in Chapter 5 to enable real-time monitoring of drug release using a novel passive acoustic mapping method (Gyongy and Coussios, 2010) that has not been previously applied to drug delivery. Finally, the efficacy of cavitation-enhanced drug delivery in perfused tissue is assessed in Chapter 6, using an isolated normothermic liver perfusion model that provides blood-flow rates comparable to those encountered in humans.



BIOMEDICAL ACOUSTICS

In recent years, High Intensity Focused Ultrasound (HIFU) has evolved into a therapeutic tool with great potential in a wide range of applications, from thrombolysis and lithotripsy to reversible opening of the blood-brain barrier for delivery of therapeutics. In the context of anti-cancer therapy, both mechanical and thermal effects of ultrasound have been utilised in a variety of ways and include ablation of deep-seated tumours and delivery of therapeutic agents to tumour sites. Cavitation activity, which is a phenomenon that may occur under specific ultrasound exposure conditions, has been found to play a key role in these treatments, due to both mechanical and thermal effects. In this Chapter, ultrasound propagation in tissue and the types of cavitation activity that may occur are described and their relevance to the present work is discussed. Moreover, as the purpose of this work is thermal release of chemotherapeutic agents from thermally sensitive liposomes, attention is drawn to the thermal effects of ultrasound and cavitation in tissue. Finally, a method of spatially monitoring and mapping acoustic emissions from cavitation events at the HIFU focal region is described and the potential role this technology may have in mapping drug release is addressed.

Ultrasound has been traditionally perceived as an imaging modality but recent advances have enabled its use for a wide range of applications, ranging from mechanical tissue damage (Roberts *et al.*, 2006) sonoporation (Bao *et al.*, 1997)(Wu *et al.*, 2002) and thrombolysis(Datta *et al.*, 2006a) to rapid and localized tissue heating (ter Haar and Coussios, 2007). The transition from imaging to therapy happens with the use of higher acoustic intensities in the form of a focused beam (High Intensity Focused Ultrasound-HIFU).

In the context of anti-cancer therapy, HIFU-induced heating has been employed for ablation of deep-seated tumors, and only recently in conjunction with Low Temperature Sensitive Liposomes (LTSLs) (Dromi *et al.*, 2007; Staruch *et al.*, 2010; Yudina *et al.*, 2011) as described in Chapter 1. Therapeutic ultrasound has a wide range of advantages over other treatment modalities, including non-invasiveness, low cost and the ability to penetrate deep within the patient's body.

In this Chapter, the principles of ultrasound propagation through tissue and the mechanisms of ultrasound-induced heating are discussed. Moreover, acoustic cavitation, an ultrasound-induced phenomenon, is described and its potential relevance to HIFU-induced heating for hyperthermia-triggered drug release and to treatment monitoring is outlined. Finally, methods for monitoring and spatiotemporally mapping cavitation activity are presented.

2.1 LINEAR PROPAGATION OF ULTRASOUND

Sound is a longitudinal wave of alternating pressure perturbations about an equilibrium pressure, which causes local regions of compression or rarefaction when propagating through an elastic medium. These pressure perturbations are generated at a frequency f and propagate at the speed of sound c .

Ultrasound refers to sound at frequencies higher than the human hearing range (20Hz - 20 kHz). Like all acoustic waves, ultrasound waves obey the wave equation

$$\frac{\partial^2 \cdot p(r, t)}{\partial t^2} = c^2 \nabla p(r, t) \quad (2.1)$$

where p is the pressure, r is the distance from the source, t is time and c represents the speed of sound given by

$$c^2 = \frac{1}{\kappa_0 \cdot \rho_0}, \quad (2.2)$$

κ_0 and ρ_0 being the medium compressibility and density respectively. This wave equation represents linear sound propagation and is valid only for the case when pressure perturbations remain significantly smaller than ambient pressure. For one-dimensional sound propagation in the x -direction, a harmonic plane wave is a solution to the wave equation and takes the form

$$p(x, t) = A \cdot e^{j(kx - \omega t)} \quad (2.3)$$

where A represents the pressure amplitude, ω is the angular frequency and $k = \omega/c$ is the wavenumber.

The acoustic energy density, (W), is:

$$W = \frac{1}{2} \rho u_{\max}^2 \quad (2.4)$$

For a plane wave, the energy of the acoustic wave is dependent upon the pressure amplitude and the properties of the material that the ultrasound beam passes through. The dependence upon the medium properties is described with the *acoustic impedance*, which relates the acoustic pressure p to the particle velocity u :

$$Z = \frac{p}{u} \quad (2.5)$$

Specifically for a plane wave, the acoustic impedance Z is equal to the characteristic impedance of the medium, $\rho_0 c$.

The acoustic intensity (I) is the rate at which energy propagates through a unit area and is given by the acoustic energy density, (W), multiplied by the speed of sound. From Equation (2.4) and Equation (2.5) for a harmonic plane wave this energy density becomes:

$$I = \frac{p^2}{2Z} \quad (2.6)$$

where P is the pressure amplitude. However, this approach is valid under the approximation that the medium response to the mechanical stresses imposed by the sound wave is linear, which applies only for very low pressure amplitudes. For higher pressure amplitudes, non-linear ultrasound propagation has to be considered.

2.2 NON-LINEAR PROPAGATION OF ULTRASOUND

At higher, and more therapeutically-relevant, excitation amplitudes, the pressure perturbations become significant and the particle velocity is not small compared to the speed of wave propagation, thus leading to non-linear effects, such as shock wave formation. This means that particle velocity during the compressional half-cycle is greater than the speed of sound and lower than the speed of sound during the rarefactional half-cycle.

The result is progressive steepening of the wave, an effect more pronounced for higher amplitude waves and for higher frequencies, and cumulative as the wave propagates. After a sufficient propagation distance, a shockwave will form (Muir and Carstensen, 1980; Hamilton and Blackstock, 1998). The distance required for complete distortion of a sine wave into a shockwave is termed the *shock formation distance* and is given by:

$$\bar{x} = \frac{1}{\beta k M} \quad (2.7)$$

where β is the coefficient of nonlinearity of the medium, k is the angular wavenumber of the original sine wave, and M is the Mach number (defined as the ratio of the peak particle speed u to the speed of sound c in the medium (Kirkhorn, 1999)). This shockwave is composed of the higher harmonics of the original periodic signal. Figure 2.1 shows an example of the evolution of a sine wave that is propagating in a

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Figure 2.1: Evolution of a propagating sine wave in a non-linear attenuating medium. (a) Source waveform, $x = 0$. (b) Distortion becoming noticeable. (c) Shock formation, $x = x$. (d) Maximum shock amplitude, $x = (1/2) x$. (e) Full sawtooth shape, $x = 3 x$. (f) High frequency components are attenuated. (g) Shock beginning to disperse. (h) Attenuated sine wave. Adapted from Shooter *et al.* (1974)

non-linear attenuating medium.

In the frequency domain, this results in the appearance of harmonic components of the original fundamental frequency. The degree of non-linearity is primarily a property of the medium, as described by the parameter of non-linearity B/A , but also increases with increasing amplitude, increasing frequency and increasing propagation distance, until the shockwave has completely formed. Then the high frequency components of the shockwave are attenuated resulting in a sine wave of lower amplitude than the initial one. (Hill *et al.*, 2005).

2.3 ULTRASOUND ATTENUATION IN TISSUE

Waves propagating through viscoelastic media lose energy to their environment through attenuation, which depends on absorption and scattering. *Absorption* of acoustic energy results in conversion of mechanical energy into heat due to thermal conductivity, molecular relaxation and viscous losses. Meanwhile, spatial fluctuations of the density and compressibility in the medium may result in *scattering* or reflections, which, along with absorption account for the loss of sound energy in

tissue. Because losses due to absorption usually dominate over losses due to scattering in tissue, absorption is usually used instead of attenuation when estimating the sound wave energy losses.

Both absorption and scattering are frequency-dependent because the scale of tissue structure changes relative to an acoustic wavelength. More specifically, as the frequency increases, the period decreases and thus the time scale for any relaxation and additionally the distance between the regions of compression and rarefaction. Attenuation is thus expected to increase with frequency resulting in greater heat deposition and difficulty in propagation depth.

Attenuation is of particular interest for ultrasound heating applications, such as HIFU ablation or ultrasound-induced hyperthermia for targeted drug release, because it governs the conversion of acoustic energy into heat (Hill *et al.*, 2005; Nyborg, 1981, 1986). The losses in wave energy to the medium can be described by an exponential law with distance. For a plane wave propagating in the positive x direction, a modification can be made to the solution of the wave equation to account for an exponential decrease in the wave amplitude due to attenuation:

$$p(x, t) = Ae^{j(kx - \omega t)} e^{-\alpha x} \quad (2.8)$$

α representing the attenuation coefficient, usually expressed in units of Nepers per meter (Np/m). Numerous tissue data suggest that frequency dependence of attenuation obeys a power law defined as:

$$\alpha(f) = \alpha_0 + \alpha_1 f^y \quad (2.9)$$

The coefficient y depends on the type of tissue each time (≈ 1.1 for liver tissue (Duck, 1990)). Goss *et al.* (Goss *et al.*, 1980a) have published a comprehensive list for the attenuation of a vast range of mammalian tissues. Equation (2.18) also shows

that harmonic components of nonlinear sound are more readily absorbed, resulting in enhanced heating deposition. The liver, which is the main organ of interest in the present work, has attenuation values in the range of 4-7.24 Np/m/MHz (Duck, 1990; Fujii *et al.*, 2002; Goss *et al.*, 1980b; Garra *et al.*, 1987; Kuc and Taylor, 1982; Taylor *et al.*, 1986).

2.4 ULTRASOUND-INDUCED HEATING

Heating of tissue by ultrasound involves an energy balance between sources of heating and mechanisms of cooling by the medium. Sources of heating include metabolic processes and ultrasound power deposition, whilst thermal energy losses can be attributed to mechanisms of heat conduction, convection and perfusion. Heat transfer by convection refers to heat transfer by the bulk motion of a fluid, for instance blood flowing in an artery, whereas heat transfer by perfusion refers to heat dissipation by blood flow in the capillary bed.

Heat transfer due to conduction originates from the second law of thermodynamics, which states that heat spontaneously flows from a region with high temperature to a region with lower temperature. The law of thermal conduction states that the rate of heat transfer through a material is proportional to the negative of the gradient in the temperature. In its derivative form, it deals with the local flux of heat and is expressed as follows:

$$\bar{f}_{cond} = -K_t \nabla T \quad (2.10)$$

where $\bar{f}_{cond}$ is the heat flux, K_t is the material conductivity and ∇T is the temperature gradient.

Convective heat transfer is the transfer of thermal energy through a fluid due to the bulk motion of the fluid and contrasts with conductive heat transfer, which

occurs at a molecular level due to molecular vibrations. When a fluid, like blood, travels from a hot to cold region, it carries away heat. Heat convection by fluid flowing through a pipe, as for instance blood in an artery, has been modeled as follows:

$$\bar{q}_{conv} = \rho_b C_b [\mathbf{u} \cdot \nabla T(r, t)] \quad (2.11)$$

where ρ_b is the blood density, C_b is the blood heat capacity and $\mathbf{u}$ is the blood flow. Finally, a biological process that contributes to heat transfer is perfusion, namely the supply of arterial blood to the capillary bed. It is assumed that blood enters the heated volume at the temperature of the arterial blood (core temperature) (T_∞) and leaves at the local average (and elevated) temperature (T), as expressed in Equation (2.12):

$$q_{perf} = w_b C_b (T - T_\infty) \quad (2.12)$$

where w_b is the average blood perfusion rate. These thermal loss mechanisms in tissue have been incorporated in Pennes bioheat transport equation (BHTE) (Pennes, 1948), which describes the process of heat deposition in biological media:

$$\rho_t C_t \frac{\partial T}{\partial t} = K_t \nabla^2 T - w_b C_b (T - T_\infty) + q_s \quad (2.13)$$

$$\rho_b C_b \frac{\partial T}{\partial t} = K_b \nabla^2 T - \rho_b C_b (\mathbf{u} \cdot \nabla T) + q_s \quad (2.14)$$

$$\mathbf{u} = 2U_0 \left[1 - \left(\frac{r}{r_0} \right)^2 \right] \quad (2.15)$$

Equation (2.13) refers to the tissue domain and Equation (2.14) refers to the blood domain.

Perfusion and conduction depend on fluid flow (Coussios and Roy, 2008). Equation (2.15) is the representation of the flow field (laminar Poiseuille flow) expressed as the linear sum of a fully developed parabolic flow in a pipe of radius r_0 . The average externally driven flow velocity (U_0) is pulsatile, in the sense that it fluctuates with the cardiac cycle.

It should be noted that the primary contribution of large vessels is to cool surrounding tissue (Huang *et al.*, 2004). The effect of blood vessels proximal to the heated region will be discussed in Chapter 6, in which a perfused liver model is exposed to ultrasound-induced heating, in regions close and far away from major blood vessels.

The heat source term is represented by q_s and represents the contribution of HIFU as the acoustic power deposited per unit volume of tissue. Pierce (Pierce, 1989) has computed the absorbed sound power density for linear wave propagation as

$$q_s = 2\zeta I, \quad (2.16)$$

where I is the acoustic intensity and ζ is the absorption. Equation (2.16) is valid only in the case of a traveling plane wave of constant angular frequency. It is thus only strictly applicable to the focal region of a HIFU transducer, where the wave can be assumed to be plane. Furthermore, absorption alone is difficult to measure in tissues, and the attenuation coefficient α is often used instead of the absorption coefficient in Equation (2.16), based on the knowledge that absorption accounts for over 85% of attenuation in tissues (Hill *et al.*, 2005).

Heat deposition by ultrasound and non-linear propagation effects are incorporated in the Westervelt equation (Hamilton and Blackstock, 1998):

$$\left(\nabla^2 - \frac{1}{c^2} \frac{\partial^2}{\partial t^2} \right) p - \frac{1}{\rho} \nabla p \cdot \nabla \rho + \frac{\delta}{c^4} \frac{\partial^3 p}{\partial t^3} + \frac{\beta}{\rho c^4} \frac{\partial^2 p^2}{\partial t^2} = 0 \quad (2.17)$$

where P is the acoustic pressure, c_0 is the speed of sound, β is the local coefficient of non-linearity, ρ_0 the density in the absence of the sound wave and δ the acoustic diffusivity, which is related to the attenuation coefficient by

$$\alpha = \frac{\delta \omega^2}{2c^3} \quad (2.18)$$

where ω is the angular frequency. An adaptation of the Westervelt equation that considers directional sound beams is the Khokhlov-Zabolotskaya-Kuznetsov (KZK) equation (Rozanova-Pierrat, 2006). A limitation of both Equation (2.16) and Equation (2.17) is the assumption that $\alpha \propto f^2$, which implies a purely viscothermal medium, when for propagation through tissue, numerous experimental data suggest that $\alpha \propto f^{1.1}$, as previously mentioned. There is presently no analytical model available capable of reconciling these discrepancies and in the context of simulations the difference in the frequency dependence of the attenuation coefficient is generally handled via an iterative numerical process.

At sufficiently high acoustic pressures tissue heating is dominated by another effect, inertial cavitation activity, which is known to induce rapid and localised heating. In the next section cavitation activity and cavitation-enhanced heating will be discussed.

2.5 ACOUSTIC CAVITATION

When ultrasound propagates through liquid or tissue, it causes alternating cycles of compression and rarefaction in the medium. At sufficiently high amplitudes, and depending on the medium properties, an ultrasonic wave may spontaneously nucleate and excite micron-sized bubbles, a phenomenon known as acoustically induced cavitation inception or nucleation. After these bubbles form, they undergo oscillations due to subsequent ultrasonic excitation, known as acoustic cavitation (Coussios and Roy, 2008).

Acoustic cavitation has been associated with a wide range of bioeffects that include thermal tissue coagulation (ter Haar and Coussios, 2007), enhancement of delivery and efficacy of therapeutics (Rifai *et al.*, 2010; Bazan-Peregrino *et al.*, 2011),

release of encapsulated therapeutic agents (Mylonopoulou *et al.*, 2010; Hussein *et al.*, 2005), reversible opening of the blood-brain barrier (Hynynen *et al.*, 2005), reversible permeabilization of the cell membrane (sonoporation) (Miller *et al.*, 2002; Ohl *et al.*, 2006), mechanical tissue fractionation (histotripsy) (Xu *et al.*, 2004; Roberts *et al.*, 2006; Parsons *et al.*, 2005) or lysis of blood clots (thrombolysis) (Datta *et al.*, 2006a, 2008; Everbach and Francis, 2000). Each of these applications utilises different mechanical or thermal effects of cavitation. The relative strength of each effect is dependent both on the acoustic excitation parameters and the medium, in particular the presence or absence of gas pockets or bubbles i.e. cavitation nuclei. In this section the basic principles and mechanisms of cavitation are explored and the relevance to the present study is discussed.

2.5.1 Regimes of Acoustic Cavitation

Gas bubbles may form spontaneously in tissue and act as cavitation nuclei. In the absence of an acoustic wave or other mechanical stress, such nuclei quickly dissolve and disappear from the medium, but under the influence of an acoustic wave, they may give rise to the following two phenomena (Plesset and Prosperetti, 1977):

- **Stable cavitation**, which refers to the prolonged linear or non-linear oscillation of an acoustically-driven bubble about an equilibrium radius. The response of the bubble is dictated by the compressibility of the gas phase. Effects of oscillating stable bubbles include generation of fluid flows on a scale comparable with the bubble dimensions (cavitation microstreaming (Elder, 1959)), sound scattering or mild localized heating in the viscous boundary layer surrounding the bubble (Prosperetti, 1977), (Holt and Roy, 2001).
- For sufficiently large peak negative pressures of the acoustic field, bubbles

grow to several times their original size and are filled with vapor and small amounts of gas. These bubbles are unstable and may collapse rapidly (supersonic velocities can ensue) due to the inertia of the surrounding medium. This phenomenon is known as **inertial cavitation** and is associated with the re-radiation of part of the incident ultrasonic energy as transient, broadband noise, due to the violent collapse of the bubble wall (Leighton, 1997). These emissions can be detected remotely providing a tool for monitoring cavitation activity noninvasively. Physical effects associated with inertial cavitation include fluid jetting near boundaries, and extreme thermodynamic conditions in the bubble leading to light production (sonoluminescence) and chemical reactions (sonochemistry) (Apfel, 1997). In the context of therapeutic ultrasound, the interaction of inertial cavitation with tissue can cause a range of bioeffects, such as thermal ablation of tissue (Coussios and Roy, 2008), mechanical destruction of the cellular structure of tissue (histotripsy) (Xu *et al.*, 2004) or reversible membrane permeabilization (Ohl *et al.*, 2006). The bioeffects of inertial cavitation will be discussed in more detail in §2.7 and §2.8.

2.5.2 Inertial Cavitation Threshold

Although the concepts and effects associated with stable and inertial cavitation are fundamentally distinct, identifying the transition between the two types of cavitation activity is complex. At low acoustic pressures, the bubble may pulsate nonlinearly, thus producing harmonic emissions at multiples of the fundamental frequency (nf_0 , $n=2,3,4,\dots$).

Further excitation may lead to a transition from periodic to non-periodic pulsations, which may occur in a series of period-doubling *bifurcations* (f_0/m , $m = 2,3,4,\dots$). Eventually, the bubble will reach the *stochastic zone*, which involves inertial cav-

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Figure 2.2: Bubble dynamics. (a) Radius versus time curves of a $7\text{ }\mu\text{m}$ bubble excited at 25kHz across a range of acoustic pressure amplitudes p_a . At low pressures, the bubble undergoes stable cavitation. At a high pressure (dark gray), the bubble radius increase to many times the original size and the bubble collapses violently (inertial cavitation). (b) Photographic series of one cycle of a bubble oscillation driven at 21.4 kHz. The difference from one frame to the next is 500 ns and the frame size $160\text{ }\mu\text{m} \times 160\text{ }\mu\text{m}$. (Lauterborn *et al.*, 2007)

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Figure 2.3: Effects of inertial cavitation. (a) Photo of a jet formation near a solid boundary (Benjamin and Ellis, 1966), (b) Photo of a light-emitting bubble (Lauterborn *et al.*, 2007)

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Figure 2.4: Bubble bifurcation diagrams. (a) Bifurcation diagram of a bubble of $R_0 = 20 \mu\text{m}$ and $f_0 = 36 \text{ kHz}$ with respect to pressure, plotting the non-dimensional maximal (R_+/R_0), and minimal (R_-/R_0) bubble radii during the pulsation period (Ilyichev *et al.*, 1989) (b) Bifurcation diagram of a bubble with $R_0 = 10 \mu\text{m}$ with respect to pressure when $f_0 = 500 \text{ kHz}$ plotting each observed local maximum of the radius-time curve (Behnia *et al.*, 2009).

itation events occurring in the form of chaotic pulsations and the disappearance of the periodic structure altogether. However, further increase of the pressure may lead to the return of periodic bubble pulsations and the process may be repeated (Ilyichev *et al.*, 1989). Figure 2.4(a) shows a theoretical solution for the bifurcation diagram of a bubble of $R_0 = 20 \mu\text{m}$ and $f_0 = 36 \text{ kHz}$ with respect to pressure and Figure 2.4(b) shows the bifurcation diagram of a bubble with $R_0 = 10 \mu\text{m}$ with respect to pressure when $f_0 = 500 \text{ kHz}$ (Behnia *et al.*, 2009).

Even though the transition from stable to inertial cavitation is complex, the concept of a threshold beyond which the bubble behaviour changes drastically has been a very active research area and various threshold criteria have been suggested through the years.

Apfel and Holland (Apfel and Holland, 1991) gave a threshold in terms of the maximum temperature T_{max} of the gas within the bubble during collapse in pure

water, by modeling the adiabatic collapse of bubbles. The resulting thresholds assuming $T_{max} = 5000K$ for three different sonication frequencies are shown in Figure 2.5. According to this numerical model, if bubbles of all sizes exist in the medium, then the threshold pressure at any given frequency is the minimum in the pressure threshold curve. This estimate of the pressure threshold for violent acoustic cavitation became the basis for the Mechanical Index (MI), a scale which is used to judge the likelihood of inertial cavitation occurring during a single cycle of excitation with an intervening tissue path. The mechanical index is currently used to assess the safety of diagnostic ultrasound (Meltzer, 1996):

$$MI = \frac{P_r}{\sqrt{f_c}} \quad (2.19)$$

where P_r is the maximum axial value of rarefactional pressure measured in water, $p_r(z)$ (Szabo, 2004). This study however applies only to exposures consisting of a single pulse, in pure water and assuming that all nuclei sizes pre-exist. In the case of a longer pulse, inertial cavitation can occur in sub-threshold pressures several cycles into the pulse (Flynn and Church, 1988).

Two other measures for inertial cavitation threshold were suggested by Church *et al.*, namely the ratio of the maximum bubble wall speed to the speed of sound (Church, 2002) and the maximum bubble radius being equal to twice the initial radius ($R/R_0 > 2$) (Church, 2005). In the latter study, longer pulses than in Apfel and Holland (Apfel and Holland, 1991) were examined. Note that in Figure 2.4, chaotic behaviour starts at $R/R_0 > 2$.

So far the parameters that have been mentioned as parameters that influence the cavitation threshold are the acoustic pressure, pulse length and bubble radius. In addition, the cavitation threshold depends on the acoustic frequency and the history of the bubble, based on an observed *hysteresis* effect (Neppiras, 1980; Frohly *et al.*, 2000). More specifically, Neppiras (1980) observed that the cavitation thresh-

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Figure 2.5: Cavitation thresholds for numerical models of single cycle excitation at three different sonication frequencies across a range of bubble sizes. The cavitation threshold is determined as the pressure at which adiabatic collapse leads to a temperature rise of the air filled bubble to 5000K from an initial temperature of 300K (Apfel and Holland, 1991).

old is higher when the acoustic power is gradually increased than when it is gradually reduced from the level above the threshold, thus showing that cavitation is influenced by the sample history. Hynynen *et al.* (Hynynen, 1991) observed the hysteresis effect in *in vivo* experiments performed in dog thigh. The hysteresis effect has been exploited in the context of therapy, for instigating cavitation at a high pressure with a short pulse and then using lower pressure pulses for the treatment, which aims to address the undesirable prefocal damage that may occur at very high pressure amplitudes (see §2.8).

2.5.3 Cavitation Nucleation in Tissue

Bubble formation is a stochastic process that depends on the probability of the energy deposited at a specific site to cause a cavity formation of a critical radius. In tis-

sue, the size of the stochastically generated cavities that are typically encountered is on the order of angstroms (Cobbold, 2007), which would require pressures of ~ 10 MPa for nucleation. However, *in vivo* experiments have demonstrated cavitation at lower rarefactional pressures, which suggests that nuclei pre-exist in the tissue (endogenous nuclei) (Coussios *et al.*, 2007b), (Haar and Daniels, 1981).

Some of the mechanisms that have been suggested as being responsible for existence of endogenous nuclei in tissue are the crevice model (Apfel, 1970), (Crum, 1982) the skin-surface model (Yount *et al.*, 1984) and spontaneous nuclear fission (Walder and Evans, 1974). Because the quantity of endogenous nuclei and the environmental conditions vary significantly in physiological conditions, introducing exogenous nuclei has been suggested. Various formulations of exogenous nuclei include ultrasound contrast agents, rigid-shelled microbubbles (Lindner, 2004) or lipid-shelled microbubbles (Lee *et al.*, 2001).

It should be noted here, that in the present work only endogenous cavitation nuclei have been utilised. In Chapter 6 the cavitation threshold of a perfused pig liver model will be identified, supporting the existence of endogenous nuclei, especially in regions with high blood flow.

Regardless of the nature and origin of any pre-existing nuclei, the quality and quantity of cavitation nuclei naturally varies throughout any given medium, as it is a stochastic phenomenon. Additionally, the elasticity of the tissues reduces the bubble oscillation amplitudes and thus could lead to further increases in the cavitation threshold (Yang and Church, 2005). Given the variability of nucleation environments *in vivo*, detection, classification and quantification of cavitation activity is essential for safe and effective treatment delivery. Established and more recent methods of cavitation detection are described in the next section, and are analysed in terms of their respective advantages for the present work.

2.6 CAVITATION DETECTION AND MONITORING

2.6.1 Active Cavitation Detection

Detection of cavitation activity can be done actively or passively. Active cavitation detection (ACD) is based on the emission of a high frequency acoustic pulse and the detection of backscattering from bubbles in pulse-echo mode. However, it has several limitations. The high frequency acoustic signals, which are necessary for adequate spatial resolution, are attenuated through tissue, as previously shown (see §2.3) and may not reach the appropriate depths. Additionally, because the intensity of the therapeutic ultrasound is much higher than the intensity of the diagnostic ultrasound it causes interference on the ultrasound image (Owen *et al.*, 2006) and thus has to be turned off during ACD. Consequently, hyperechogenicity shows only the backscattering cross-section of the bubble post-exposure and not the phenomenon that caused this cross-section. Also, the time delay in generating the B-mode image may be enough for bubbles to have dissolved away (Szabo, 2004), implying that cavitation could have occurred without being detected.

2.6.2 Passive Cavitation Detection

Cavitation activity gives rise to a complex set of acoustic emissions that can be identified by interpreting their frequency content. For the case of ultrasound-induced hyperthermia, inertial cavitation can be a major source of heating, as described in §2.8 and thus monitoring its activity may be an important in enhancing and controlling heat deposition.

Passive cavitation detection (PCD) typically uses broadband ultrasound transducers with a high center frequency to record acoustic emissions from cavitation activity generated by a separate therapeutic transducer. In this manner, a low-

frequency transducer, appropriate for therapeutic applications, can be used to induce cavitation, and a transducer of higher frequency, which will have better spatial resolution and improved frequency separation compared to the main HIFU signal, can be used to listen to the resulting cavitation emissions (Leighton, 1997). This method has already been employed in therapeutic applications, such as monitoring heat deposition non-invasively in cavitation-enhanced thermal ablation (Coussios *et al.*, 2007a), (Kyriakou *et al.*, 2011) and drug delivery (Arvanitis *et al.*, 2011).

In general, the spectrum of any acoustic emissions is a set of discrete components superimposed onto background noise. The discrete components are emissions at a nf_0/m frequency, f_0 being the fundamental. Harmonic, subharmonic or ultraharmonic emissions are distinguished by the different values in m and n . Decomposing the signal into various discrete components is very important when studying cavitation noise spectra, because each of the components is associated with one (or more) different bubble behaviours.

Harmonic emissions are the discrete components that occur at multiples of the fundamental frequency (nf_0 , $n=2,3,4,\dots$ and $m=1$). The occurrence of harmonics of exciting frequency can be attributed to scattering of a non-linear excitation waveform, due to non-linearity of the medium, (§2.2). Additionally, harmonic emissions can be generated from non-linear bubble pulsations (Leighton, 1997).

Subharmonic emissions (nf_0/m , $n=1$ and $m=2,3,4,\dots$) are generally representative of stable cavitation, but can also occur for inertially cavitating bubbles if a bubble continues to grow, skipping the contraction phase, resulting in oscillations at a period multiple to the fundamental (known as period doubling, tripling etc.).

Ultraharmonic emissions (nf_0/m , $n=2,3,4,\dots$ and $m=2,3,4,\dots$) are usually attributed to propagation of subharmonic emissions through a non-linear medium,

even though the exact mechanism that causes ultraharmonic emissions is not fully understood (Ilyichev *et al.*, 1989).

Finally, broadband emissions, (increase in the noise floor of the spectrum) is attributed to inertial cavitation activity. Upon bubble collapse, the bubble wall velocities approach the speed of sound causing shock wave formation and a rapid collapse. Such a signal that is very short in the time domain is very broad in the frequency domain. Hence, broadband noise emissions in the acoustic spectrum are uniquely attributed to inertial cavitation (Ilyichev *et al.*, 1989).

Passive cavitation detection has the great advantage of allowing to record acoustic emissions during the HIFU exposure (real-time), which is something that cannot be done with active acoustic monitoring. Using a single-element PCD transducer, passive detection does not provide any spatial information about the acoustic emissions, because the PCD has a fixed focus. The method described in the following section is a method for real-time acoustic monitoring and spatial distribution mapping.

2.6.3 Passive Acoustic Mapping

As mentioned in §2.6.2 and §2.6.1, bubble activity during HIFU exposure can be traditionally monitored in one of two ways. Single-element high-frequency transducers can be used as passive cavitation detectors (PCDs) that record the acoustic emissions from cavitating bubbles (Farny *et al.*, 2006). However, PCDs have a single focus, thereby providing information in a discrete region only.

Alternatively, hyperechogenic regions in B-mode ultrasound images can enable detection and localization of bubble activity using time-of-flight information (Vaezy *et al.*, 2001). However, B-mode images can only be taken while the HIFU is off to

avoid interference from the therapeutic ultrasound signal and thus only enable detection of cavitation that persists after HIFU excitation has ceased. B-mode monitoring is therefore less sensitive than PCD monitoring, and has been previously shown to be detecting boiling bubbles that are indicative of overtreatment, rather than inertially cavitating bubbles that are indicative of enhanced heat deposition during ablation (Rabkin *et al.*, 2005).

Diagnostic ultrasound systems with access to post-beamformed RF data have been used to provide spatial information about cavitation activity. By turning off pulse transmission, the dynamic receive beamforming algorithm of the ultrasound scanners has been used to resolve cavitation along the transverse direction of the linear array (Farny, 2007), (Farny *et al.*, 2009) and, to a limited extent, axially (Salgaonkar *et al.*, 2009). However, the reliance on sequentially generated RF A-lines precludes these methods from simultaneously detecting emissions from spatially distinct cavitation sources. Moreover, the fixed size of the subapertures used to form the A-lines greatly degrades the axial resolution of the images.

Passive Acoustic Mapping overcomes these limitations, and provides real-time spatial localisation of inertial cavitation activity. Gyongy *et al.* (Gyongy *et al.*, 2008), (Gyongy and Coussios, 2010), developed a system that essentially acts as an array of PCDs that can be electronically focused in order to provide spatial localization of inertial cavitation activity from a single bubble as well as contiguous and disjoint cavitating regions.

In these studies a standard 64-element diagnostic array was aligned coaxially to the HIFU transducer. The acoustic signals that were received passively by each of 64 elements of the array during HIFU exposure were combined to generate maps of inertially cavitating regions of an agar-based TMM, as illustrated in Figure 2.6

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Figure 2.6: Passive mapping of a cavitation region. Left: Estimated cavitation region around HIFU focus. Middle: Passive source intensity map from inertial cavitation recorded during HIFU exposure. Right: Comparison of axial cross section of passive source intensity map with predicted cavitation region. Figure adapted from Gyongy and Coussios (2010)

The algorithm used for the production of these passive acoustic maps, is based on time exposure acoustics (TEA), a passive beamforming technique used in passive seismic imaging. The basic theory for this passive beamforming technique is presented in the following section (§2.6.3.1).

2.6.3.1 Passive Beamforming for Passive Acoustic Mapping

Unlike in pulse-echo imaging, in passive localization and mapping of cavitation produced by a continuous sound wave there is no direct information about the propagation time from a source to a receiver. Therefore, measuring the time at which a cavitation emission is received by a single passive cavitation sensor does not enable the observer to distinguish between a cavitation event that has occurred close to the sensor but several cycles into the HIFU excitation, and a cavitation event that has occurred farther from the sensor but in the early stages of HIFU excitation. Hence, unlike standard pulse-echo imaging techniques, spatial resolution with a single receiver is not possible.

However, a fixed source location will always give the same differences in time of arrival (DToA) for a set of receivers, and changes in the DToA enables resolution of different source locations, even without prior knowledge of which HIFU cycle caused which cavitation event (and hence, the time at which the cavitation events occurred).

Maps of cavitation source intensity can be achieved by beamforming (delay-and-sum) the array element signals and calculating their energy in time. Cavitation sources are broadband and need to be in the near field of the receivers to yield position information. For this reason TEA is the beamforming method used for the generation of the passive acoustic maps.

Assuming that a source field $s(x,z,t)$ with zero temporal mean, causes the pressure field $p(x,z,t)$ to propagate according to the linear wave equation, estimation of the source intensity I (temporal mean squared of s) at a position (x,z) can be done by back-propagating the RF pressure signal $p_i(t)$ for each element i of the array, and taking the following combination of temporal and ensemble moments:

$$I = \frac{1}{T} \int_0^T \left[\left(\sum_i H_i(\tau) \right)^2 - \sum_i H_i(\tau)^2 \right] d\tau \quad (2.20)$$

where $H_i(\tau)$ is the back-propagated signal

$$H_i(\tau) = d_i \cdot p_i \left(t - d_i/c + \tau \right) \quad (2.21)$$

with d_i being the propagation distance from the point (x_s, z_s) to array element i :

$$d_i = [z_s^2 + (x - x_s)^2]^{1/2} \quad (2.22)$$

TEA applied to pre-beamformed RF data has similarities to B-mode images obtained with a conventional US imaging system set to receive only and employing dynamic receive beamforming, but one substantial difference is that TEA effectively

integrates the beamformed signal power over a time T , as defined in Equation (2.20), while the B-mode returns the envelope of the echo it expects to receive at a given time, giving images that are noisy and difficult to interpret. This short integral time suggests that TEA is capable of a high time resolution, comparable to B-mode. Another distinguishing feature of passive beamforming is that axial resolution is largely dependent on the receiving aperture. Limiting the receiving aperture largely degrades the axial resolution of TEA. This is a concern since the initial (and subsequent change in) cavitation distribution during HIFU exposure is mainly along the HIFU transducer axis, which is coaxial with the linear array providing the passive maps.

The main advantages of the passive acoustic mapping method described in this section can be summarised as (i) ability to monitor acoustic emissions in real time during sonications, (ii) quantification and qualification of cavitation activity throughout a treatment plane. Namely, the signal received by the various locations of the PAMs can be decomposed in the frequency domain and the different types of cavitation activity can be classified and their magnitudes quantified. (iii) The use of a linear array gives 2-D PAMs, but this could be implemented to 3-D if a 2-D array is used.

2.7 MECHANICAL EFFECTS OF CAVITATION IN TISSUE.

The effects that cavitation has on tissue can be broadly divided into *mechanical* and *thermal*. Cavitation-enhanced heating and thermal effects will be discussed in detail in §2.8, so this section will focus on the mechanical effects of cavitation and their therapeutic applications.

Tissue erosion may occur either by a shock wave that is emitted upon bubble collapse or by jet formation when a bubble collapses near a rigid boundary. In the

first case, the shock wave is rapidly attenuated and affects only tissue very close to the bubble. In the second case, the presence of the rigid surface causes asymmetrical bubble collapse leading to a high speed fluid jet directed towards the rigid surface. Even though tissue proximal to collapsing bubbles is destroyed, tissue at mid-distance from the cavitating microbubble has shown to exhibit reversible permeabilization, a phenomenon known as sonoporation (Ohl *et al.*, 2006), (Miller *et al.*, 2002). Moreover, in the case of bubble clouds, the mechanical stresses generated during collapse can cause cellular destruction and tissue homogenisation. Another effect is the presence of small-scale acoustic streaming generated within the oscillatory boundary layer surrounding the bubble caused by bubble-scattered sound waves, known as microstreaming. Maximum streaming velocities up to 0.45 mm/s have been reported (Elder, 1959).

The first ultrasound application that was used in the clinic was extracorporeal shockwave lithotripsy, which employs very short pulses at very high amplitudes (~tens of MPa) for destruction of kidney and gall stones. Originally, tissue damage via the shock wave was the intended treatment mechanism, but it has been shown that the shock wave and fluid jet from a collapsing bubble play a role in stone fragmentation. Another application is histotripsy, the mechanical tissue homogenisation (destruction of the cellular and super-cellular level) of tissue, which is caused by the mechanical stresses that occur upon collapse of a bubble cloud.

In the context of drug delivery, inertial cavitation activity has been shown to enhance extravasation and delivery of anti-cancer agents to poorly vascularised areas of tumours (Rifai *et al.*, 2010), (Bazan-Peregrino *et al.*, 2011). Sonoporation has been employed for enhancement of gene transfer into cells (Mehier-Humbert *et al.*, 2005), but not very effectively as it has been coupled with irreversible cell destruction as well. Another area of drug delivery is reversible opening of the blood-brain

barrier (BBB) for transport of therapeutics. It has been shown that cavitation by burst ultrasound in the presence of an ultrasound contrast agent can disrupt the blood-brain barrier with acoustic parameters suitable for completely noninvasive exposure through the skull (Hynynen *et al.*, 2001). In a recent study Choi *et al.* (Choi *et al.*, 2011) investigated the probability of occurrence and spatial distribution of cavitation for optimising the ultrasound pulse design for drug delivery through the BBB. Stable cavitation has been applied for the dissolution of blood clots (thrombolysis) by enhancing the transport of rt-PA a thrombolytic drug to the blood clots. It has been suggested that microstreaming provided a mechanism for the enhanced transport of plasma and rt-PA across the clot surface (Datta *et al.*, 2006b).

2.8 CAVITATION-ENHANCED HEATING

Figure 2.7(b) shows the heating and cooling curves in agar-graphite phantom, exposed to 1 MHz HIFU for 1 s. Even though the pressure difference between 1.1 MPa exposure and 1.2 MPa exposure is 0.1 MPa only, the difference in the observed temperature rise is substantial, of the order of 20°C (Coussios and Roy, 2008).

This gives an example of cavitation-enhanced heating. It has been shown that such a sudden and dramatic change in the thermofluidic response caused by only a small change in the acoustic pressure can be attributed to the onset of inertial cavitation activity (Holt and Roy, 2001). Sometimes, in longer exposures the sudden and dramatic increase in heating can be observed halfway through the exposure.

In Figure 2.7(a) three distinct heating regions can be identified. In the first region, (low pressures) the measured temperature is proportional to (roughly) the square of the acoustic pressure, which corresponds to linear heating. The second region begins with a sudden increase in the measured temperature at a threshold

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Figure 2.7: Temperature rise measured in an agar-graphite phantom exposed to 1 MHz HIFU for 1 s. (a) shows the measured (blue) and predicted (orange) peak temperature rise versus the acoustic peak-rarefaction pressure amplitude. It can be observed that above 1.2 MPa the measured temperature rise is much higher than the prediction of the Westervelt equation, indicating the progression from linear to cavitation-enhanced heating. (b) Measured temperature versus time for increasing peak-rarefaction pressure amplitudes Coussios and Roy (2008)

pressure and the temperature rise that is observed beyond that threshold is much higher than that predicted by linear absorption theory and more erratic. Up to a six-fold increase in rate of heating has been observed (Coussios and Roy, 2008). The third region is characterised by decrease of the observed temperature rise with increase of the acoustic pressure. A possible cause is excess of bubble activity in the focal region, that scatters sound, thus shielding the thermocouple from the HIFU beam (Coussios *et al.*, 2007b).

Figure 2.8 shows temperature measured in an agar-graphite TMM exposed to 1.1 MHz HIFU for 1 s at three pressure amplitudes (Coussios *et al.*, 2007b). This is plotted alongside the corresponding PCD output for each exposure. It can be observed that as the pressure increases, the level of broadband noise emissions increase and coincide with a sudden and dramatic increase in the rate of heating.

As discussed in §2.6.2 ,broadband noise emissions recorded from the focal re-

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Figure 2.8: Measurements of temperature rise (solid line) and PCD output (dash-dot line) as a function of time in an agar-graphite phantom exposed to 1 MHz HIFU for 1 s at three different pressure amplitudes. Broadband noise recorded with the PCD is indicative of inertial cavitation activity. (c) shows low temperature rise and no broadband noise emissions. In (b) sudden temperature rise and broadband noise onset halfway through the exposure. In (a) high levels of temperature rise and broadband noise are observed at the start of exposure. In (a) and (b), the dramatic increase in the observed rate of heating is coincident with the onset of broadband noise, which indicates the onset of inertial cavitation activity. Figure adapted from Coussios *et al.* (2007b).

gion of the HIFU are indicative of inertial cavitation activity. Thus, the sudden 'jump' in temperature can be attributed to inertial cavitation activity. This is especially obvious in Figure 2.8(b), in which both a dramatic temperature increase and broadband noise emissions onset simultaneously half-way through the sonication.

In Figure 2.8 it was shown that there is significant correlation between broadband noise emissions and dramatic heating and in Figure 2.7 it was shown that this rate of heating presents a significant discrepancy from the prediction of the linear

heating model, which applies to ultrasound-induced heating (in the absence of inertial cavitation) and according to which temperature rise is quadratic with pressure (linear to the acoustic intensity). This suggests that temperature rise versus pressure can be used as a more practical alternative than the methods suggested in §2.5.2 for estimating the inertial cavitation threshold. This approach has been followed in the present work for estimating the cavitation threshold in the novel TMM that has been developed, as will be discussed in §3.5.1.2. It should be noted here, that the value of the threshold pressure for the onset of enhanced heating decreases as the insonation time increases (Holt and Roy, 2001).

Two mechanisms have been identified as responsible for the bubbles acting as an additional heat source: (i) they radiate broadband acoustic emissions that are readily absorbed by the medium (Holt and Roy, 2001), (Thomas *et al.*, 2005) and (ii) the shearing stress converts fluid flow to heat in a viscous boundary layer near the bubble wall (Prosperetti, 1984), (Hilgenfeldt *et al.*, 2000).

Cavitation-enhanced heating has been shown to be effective in ablating tissue (WATKIN *et al.*, 1997) (Xu *et al.*, 2004) and in creating larger and deeper lesions (Melodelima *et al.*, 2004). Moreover, it has been demonstrated that cavitation-enhanced heating is also achievable *in vivo*. In their experiments in rabbit thigh, Sokka *et al.* showed that the lesions created by cavitation-enhanced heating have a 2–3 times larger volume, more spherical and closer to the ultrasound transducer than the control lesions (Sokka *et al.*, 2003).

Therapeutic applications of cavitation-enhanced heating for cancer treatment so far include ablation (Coussios and Roy, 2008) and heat-shock (Falk and Issels, 2001) of deep-seated tumours. In these cases, continuous wave (CW) sonications of high intensity focused ultrasound have been used to achieve temperature rises of tens of degrees in focal volumes comparable in size to a grain of rice (Coussios *et al.*,

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Figure 2.9: Comparison of a lesion in *ex vivo* liver caused by 5 s 1.06 MHz HIFU exposure at 6.3 MPa with passive and hyperecho maps. The lesion indicates well-localized activity in both the broadband and harmonic maps.(Jensen *et al.*, 2011)

2007b).

The fact that inertial cavitation is coupled with enhanced heating, could provide a means for non-invasive and inexpensive monitoring of heat deposition by monitoring broadband noise emissions. Several studies have been presented in which the acoustic emissions from the HIFU focus have been used for estimation of cavitation-enhanced heating (Rabkin *et al.*, 2005), (Rivens *et al.*, 2007).

Additionally, the feasibility of using passive acoustic mapping (§2.6.3) for spatiotemporal monitoring of high-intensity focused ultrasound (HIFU) treatment in *ex vivo* bovine tissue has also been demonstrated (Figure 2.9)(Jensen *et al.*, 2011) and it may eventually serve as the basis for a strategy for guiding cavitation-enhanced HIFU therapy in the clinic.

2.9 OBJECTIVES OF THE PRESENT WORK

The objective of the present work is the non-invasive and localised ultrasonic release of anti-cancer agents from low-temperature thermally sensitive liposomes

and the spatial mapping of the release. The theoretical work on ultrasound propagation through tissue and ultrasound-induced heating that has been presented in this Chapter shows that reaching the appropriate levels of hyperthermia in a confined area in tissue with HIFU exposures is feasible.

Moreover, it has been shown that inertial cavitation activity can be employed to enhance heating in a rapid and localised manner, while the acoustic emissions that it causes (broadband noise) can be monitored in real-time, thus providing a means for treatment monitoring. Additionally, in the previous Chapter, it was demonstrated that the LTSLs that are used in the present work release their content in the narrow range of mild hyperthermia.

Therefore, the focus of this work will be to combine (i) the link between monitoring the acoustic emissions of inertial cavitation activity and temperature rise and (ii) the link between mild hyperthermia and drug release from the LTSLs, to achieve monitoring of drug release by monitoring broadband noise emissions at the HIFU focus. There is a series of steps that has to be achieved for the objective of this work to be reached.

Firstly, a tissue mimicking material (TMM) with similar attenuation to liver tissue has to be developed, so that it has a similar acoustic and thermal response. Using a TMM is preferable to using a real liver tumour as a first experimental approach, because it provides a controlled environment, and thus allows for large parametric studies, manipulation of the environment and more accurate determination of thermal response. Such a TMM is developed and characterised in Chapter 3.

The second step to fulfilling the aim of this work, is to identify the HIFU exposure parameters that cause cavitation-enhanced mild hyperthermia. It was shown in §2.8 that cavitation activity has the ability to cause rapid and localised tempera-

ture rise, but it was also shown in §2.5.2 that it is a stochastic and thus unpredictable process. Consequently, the parameters of the sonications have to be identified so that the cavitation-enhanced heating is confined to the mild hyperthermia range. Moreover, cavitation activity may cause mechanical damage to tissues. Even though this has been utilised in a variety of therapeutic applications (§2.5), for the purpose of the present work it is undesirable. It is thus necessary to ensure that any observed therapeutic effect (i.e. death of cancer cells that are embedded in the TMM) is *only* caused by the drug release from the LTSLs due to the thermal effects of ultrasound and not due to any mechanical effects. Optimisation of the HIFU exposure parameters to achieve cavitation-enhanced heating and drug release without direct mechanical damage to cells in the TMM is carried out in Chapter 4

Finally, after the HIFU exposure parameters that cause cavitation-enhanced heating are identified, the passive acoustic mapping method that was discussed in §2.6.3 is used for spatiotemporal mapping of the drug release from the LTSLs by mapping the broadband noise emissions from cavitation activity occurring at the HIFU focus. It is hoped that this will provide a non-invasive, inexpensive and real time method for monitoring and hence controlling targeted chemotherapy for the first time.

Blood perfusion, which according to the Pennes Bioheat Transfer Equation (§2.4) plays a huge role in heat deposition by ultrasound, will have been neglected for simplicity throughout the initial optimisation steps of cavitation-enhanced ultrasonic drug release. However, to ensure clinical relevance, the proposed strategy for cavitation-enhanced drug delivery is tested using an isolated perfused liver model in Chapter 6, enabling validation a blood flow rates similar to those encountered in humans.



PRE-CLINICAL TUMOUR MODEL

In order to optimize ultrasonic exposure conditions for activation of thermally-sensitive liposomes, a pre-clinical liver-tumor model with embedded cells has been developed. Experiments were performed to test the cell and acoustic characteristics of this model and compare them to liver tissue. In addition, as the ultimate objective is the activation of thermally sensitive liposomes by ultrasound-induced hyperthermia, the HIFU excitation parameters that cause clinically relevant temperature rises have been identified both in the novel tissue-mimicking material and in liver tissue.

3.1 PRE-CLINICAL TUMOUR MODEL REQUIREMENTS

The use of real liver tumours as a first experimental approach has several disadvantages, such as inability to do a large parametric study, determine thermal response with accuracy, or manipulate the environment to determine the causes of aberrant temperature rise profiles. For instance, heat rise *in vivo* could be dependent on local concentration of cavitation nuclei, irregular perfusion or local variations in ultrasound properties, such as attenuation and speed of sound. Consequently, as a first experimental approach it is preferable to use an artificial material with acoustic properties similar to liver tissue, so that all the environmental parameters can be

controlled. Such materials are known as tissue-mimicking materials or phantoms. It was deemed necessary for the preclinical tumour model developed for the present work to meet the following requirements:

- **Similar thermal response and acoustic properties to liver tissue** In order to model ultrasound propagation through tissue, the phantom must exhibit acoustic properties similar to liver tissue, so that for the same ultrasound exposure parameters, similar heating levels and heating spatial profiles are achieved. Heating is the most vital parameter to simulate, as it controls the activation of the thermo-sensitive liposomes and thus the chemotherapeutic agent release. As described in Section 2.2, heat transfer into tissue occurs via conduction, convection and perfusion. Convection and perfusion are caused by blood flow in the tissue and thus for liver tissue to be simulated, flow should be incorporated in the phantom. However, as a first approach a tissue mimicking material without flow was manufactured, in order to have better control of the region and more homogeneous distribution of the phantom ingredients (ThermoDox, cancer cells etc.). The absorbed sound power density depends on the attenuation coefficient of the medium and on the local acoustic intensity. It thus becomes obvious that the most important characteristic that the tissue mimicking material should have is a frequency-dependent attenuation coefficient that is similar to liver tissue, as it directly affects ultrasound energy deposition and tissue heating. Comparable temperature profiles are also desirable, to ensure similar heating patterns in both liver tissue and the TMM.
- **Non-exothermic preparation procedure.** ThermoDox is embedded in the phantom while it is still in liquid form, so that a homogeneous drug distribution is achieved throughout the sample. As ThermoDox contains low-

temperature-sensitive liposomes, even a slight temperature elevation during the phantom preparation may cause release of the anticancer agent and thus compromise the experiments.

- **Gel stability.** HIFU exposure causes radiation forces at the focal region due to the mechanical stress that is induced. Consequently, a solid structure is necessary to avoid changes in the phantom composition and reserve its uniformity. Moreover, stability is important for a homogeneous ThermoDox and cancer cell distribution. Usually, during an experimental procedure different regions of the phantom are exposed to different conditions and then compared. As a result, it is vital to have the same conditions throughout the whole phantom for result reliability.
- **Cell viability.** The incorporation of live human hepatic cancer cells is a major step towards achieving a closer simulation of human liver cancer and thus acquiring more clinically relevant results. In order to estimate therapeutic efficacy of the proposed therapy by evaluating cell death due to ThermoDox activation by HIFU-induced hyperthermia, all other potential sources of cell death should be eliminated. As a result, the tissue mimicking material should be cell-compatible and should enable sustained cell-viability for 24 hours or longer, so that the anti-cancer activity of doxorubicin following release can be assessed.

3.2 OVERVIEW OF PREVIOUS WORK

Previously developed generations of materials include agarose gels ((Madsen *et al.*, 1998), (Matsukawa *et al.*, 2002), (Kondo and Fujimoto, 2002)), polyvinylalcohol gels (PVA) ((Kharine *et al.*, 2003), (Manohar *et al.*, 2004), ((Devi *et al.*, 2005)), polyacry-

lamide gels (PAA) ((Takegami *et al.*, 2004), (Lafon *et al.*, 2005), (Howard *et al.*, 2003)) and silicone ((Zell *et al.*, 2007)). Among these, agarose gel was the most promising for experiments with thermally sensitive liposomes, because it has a low gelling temperature (below 37°C), which allows it to be mixed with temperature-sensitive ingredients, without compromising their stability. Also, unlike other materials, agarose is not cytotoxic and permits cell growth ((Chen *et al.*, 2004)).

Because literature values of attenuation in pure agarose gel are much lower than in tissue (Zell *et al.*, 2007), (Cobus *et al.*, 2007)), in several studies particles have been mixed with agarose gels to increase effective attenuation, due to scattering. Agarose-based TMM with dispersed powdered graphite has been widely used, because its acoustic characteristics can be easily controlled in the manufacturing process ((Burlew *et al.*, 1980), (Kondo and Fujimoto, 2002)), but the toxicity of the graphite particles render it ineffective for cell viability evaluation after drug delivery.

Matsukawa et Al. ((Matsukawa *et al.*, 2002)) did a comparative study of the attenuation and speed of sound in particle-compounded agarose gels. The particles were talc powder, glass beads and graphite. Unlike talc particles and graphite, glass beads are cell-compatible. In their study, the used concentrations provided a range of acoustic attenuation coefficients much larger than in tissue, indicating that for the right concentration of glass particles attenuation and thus heating response similar to tissue can be achieved. In the present work, the composition of an agarose-based glass particle-embedding TMM is optimized as a cell-compatible in vitro platform for activation of thermally sensitive liposomes via HIFU-induced hyperthermia.

3.3 MATERIALS

3.3.1 Basic TMM Composition and Preparation

Agar has been identified as a cell compatible TMM from previous work. It is purchased as a powder. Once suspended in PBS and heated it dissolves and then sets as a gel when it cools down. For the purposes of the tissue mimicking material a special type of agarose (Agarose 1000, Invitrogen) with low setting temperature (35°C) was purchased and used.

The low setting temperature allows for mixing agarose with ThermoDox at 37°C, which is below the temperature at which the liposomes release their content. Thus ThermoDox activation prior to ultrasound excitation is avoided. In order to enhance cell viability in the agarose gel, the agarose powder was suspended in one half PBS and one half fetal bovine serum, a commonly used culture medium in cell cultures.

Different concentrations of agarose powder in the solution result in different acoustic properties of the material. The most commonly used agarose concentrations are 0.005 - 0.03 g/ml. In order to choose the concentration that provides acoustic properties most relevant to liver tissue, different concentrations were evaluated. Speed of sound for all phantom formulations was in the range of speed of sound in tissue (1450 m/s - 1640 m/s). However, the acquired attenuation coefficients were significantly lower than the literature values for liver tissue.

In order to increase the attenuation coefficient another ingredient was introduced, namely spherical particles, which were expected to induce losses due to scattering. The particle types that were tried include polystyrene and glass microspheres. Preliminary attenuation measurements that were conducted suggested that polydisperse soda lime glass microspheres (Spherical Glass Materials-Dry 424, Duke Scientific) of diameter 5 μm to 50 μm (Figure 3.1(a)) give higher attenuation than polystyrene microspheres.

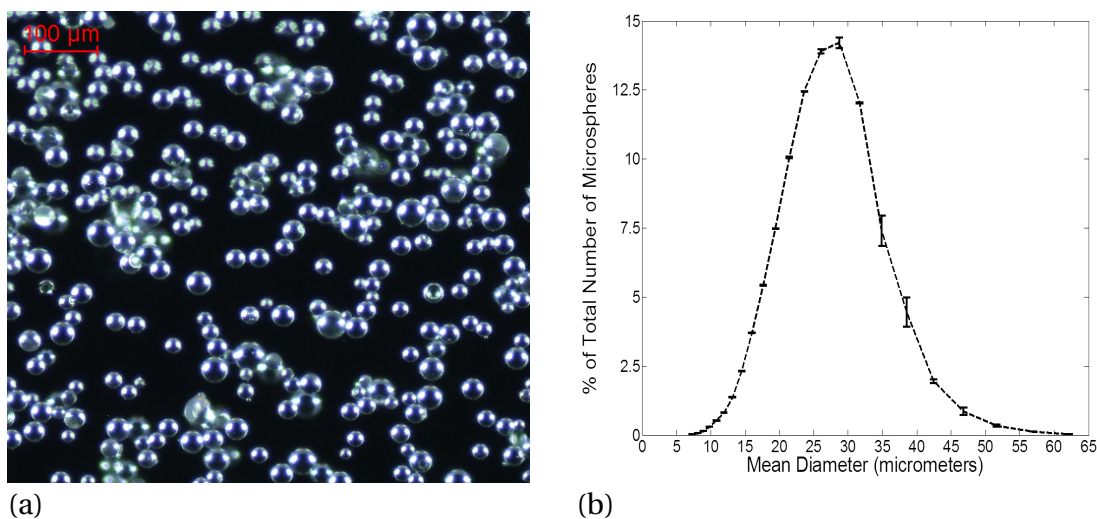


Figure 3.1: Microscopy image of the glass microspheres (a) and their size distribution (b) as measured with a particle size analyzer

The particle size distribution was measured with a particle size analyzer (Master-sizer, Malvern), which relies on the principle that particles passing through a laser beam scatter light at an angle that is directly related to their size. The scattering intensity is also dependent on particle size and decreases in relation to the particle cross-sectional area. Therefore, large particles scatter light at narrow angles with high intensity, whilst small particles scatter at wider angles but with low intensity. The resulting size distribution curve is shown in Figure 3.1(b).

In order to enable adjustment of the attenuation coefficient of the TMM, different concentrations of agarose (0.5% or 1% w/v) and microspheres (0.5%, 1, 2 or 4% v/v) were manufactured and characterised.

3.3.2 Incorporation of Cells and Viability Measurement

Given that the main indication of ThermoDox is primary liver cancer, human hepato-cellular carcinoma cells (HuH-7) were used. The cells were maintained in an incubator at 37°C in appropriate medium and split twice per week (treated with

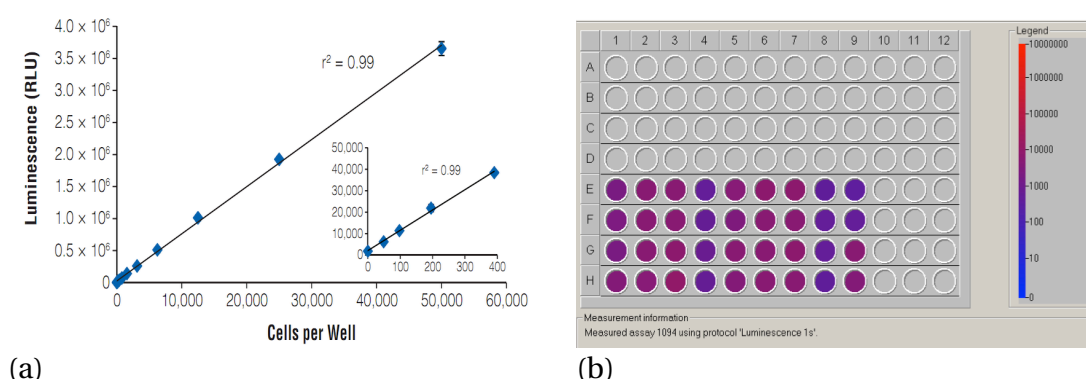


Figure 3.2: Luminescence intensity with respect to cell number per well as calibrated by Promega in Relative Luminescence Units (RLU) (a). Typical display on the luminometer monitor. In this case a 96 well plate has been used, out of which 36 wells were measured. The colour indicates the luminescence intensity as shown at the colour-bar in a log scale (b).

2 ml trypsin and then transported to a new flask at a ratio of 1/10 with 16 ml culture medium) and cell viability in the tissue mimicking material was measured with a luminescent cell viability assay (CellTiter-Glo, Promega), which enables determination of the number of viable cells present in a culture by quantification of the ATP present, an indicator of metabolically active cells. Prior to embedding the cells in the tissue mimicking material and conducting the viability test, the cells were counted using a haemocytometer.

100 μ l samples of all agar and glass microsphere concentrations tested were placed in a 96-well plate, alongside control samples containing no cells or no glass microspheres. Each sample was mixed with 100 μ l of CellTiter-Glo reagent, resulting in the generation of a luminescent signal that is proportional to the amount of ATP and thus indicative of cell viability. Figure 3.2(a) shows the assay calibration, provided by the manufacturer, and Figure 3.2(b) a typical display of the luminometer.



Figure 3.3: ThermoDox bottle as it arrives from the manufacturer and the 1 mL aliquots.

3.3.3 The Liposomal Formulation

Thermodox®(Celsion Corp) is a drug that contains low-temperature-sensitive liposomes at a liquid suspension of 2 mg/ml. When heated to temperatures in the range 39°C to 42°C, the lipid bilayers destabilize and the anticancer agent that is encapsulated is locally released within the heated area. The active drug in ThermoDox is doxorubicin HCl, a well-known and widely used chemotherapeutic agent. Drug maintenance is done at -20°C, and in order to unfreeze the least possible quantity of drug for each experiment, aliquots of 1 mL are made (Figure 3.3).

The therapeutically relevant dose, as provided by the manufacturer, is 50 mg/m². The dose of ThermoDox to be administered to a patient is calculated using Boyd's Formula ((Boyd, 1935)) for Body Surface Area calculations (BSA), because the BSA formula is usually considered a better indicator of metabolic mass than weight, as it is less affected by body fat. Assuming that the average BSA of a patient is 2m² and that the average patient has approximately 5 liters of blood the recommended dose per patient becomes 100 mg/5L, or else 0.02 mg/mL. Doxorubicin release can be measured after HIFU exposures due to its inherent ability to fluoresce at low concentrations (ex/em 487/590 nm). When doxorubicin is encapsulated in the lipo-

somes, it quenches and does not fluoresce. This property allows for measuring the fluorescence of the released drug from the liposomes, and thus evaluating its release, without interference from any doxorubicin that is still encapsulated.

3.3.4 Liver Tissue Preparation

The purpose of the tissue-mimicking material is to simulate liver tissue. It was thus considered advantageous to conduct the same experiments with liver tissue to obtain control values for temperature rises and attenuation under the same experimental conditions. For this purpose freshly excised ox liver was used.

Before each experiment the liver was degassed for 30 minutes at 0.8 atm pressure. Degassing was performed to reduce oxygen concentrations in ex vivo liver to values similar to in vivo liver (even though neither value has been reported in the literature to our knowledge). In addition, inserting liver tissue in its custom designed holder while it is still in the degassing apparatus eliminates the possibility of entrapped bubbles compromising the experiment.

As previously mentioned, HIFU exposure may give rise to cavitation activity that enhances local heating and ultrasound absorption. This possibility is increased with preexisting cavitation nuclei. Consequently, the existence of bubbles in the liver-phantom wall interface may give rise to prefocal cavitation activity.

3.4 EXPERIMENTAL METHODS

As mentioned in §3.1, the acoustic properties that mostly affect ultrasound energy deposition and heating are the attenuation coefficient and the cavitation threshold. These parameters along with the actual heating induced for a range of ultrasound parameters have been measured both in the TMM and degassed ox liver tissue for

comparison. In the present section the experimental procedures for the measurement of *attenuation coefficient*, *cavitation threshold* and *thermal response* are presented.

Due to the importance of accurate determination of the attenuation coefficient, two independent methods were used to measure it. Ultrasound generation by an unfocused transducer, transmission through the tissue-mimicking material and detection of the signal with a similar unfocused transducer is the first method and was used to measure sound attenuation in both liver tissue and the TMM. Detection of the pressure generated from a 1.1 MHz HIFU transducer with a needle hydrophone embedded at various depths in the material is the second method and it was used as a confirmation technique done only with the tissue-mimicking material. With the second method attenuation was measured only at 1.1 MHz (fundamental frequency of the HIFU transducer) and 3.3 MHz (3rd harmonic).

3.4.1 Measurement of the Attenuation Coefficient

3.4.1.1 Attenuation measurement with two unfocused 2.25 MHz transducers

Two unfocused 2.25 MHz transducers (586820 and 586821 V306, Olympus Panametrics NDT) were submerged in degassed water at 37°C in a water tank and the material (liver tissue or TMM) interposed between them. The first 2.25 MHz unfocused transducer was driven by a signal generator (33220A, Agilent Technologies) at a sine wave of 20 cycles, 10 V peak-to-peak in the frequency range of 1 - 4 MHz. The acoustic signal was transmitted through the material and detected by the second 2.25 MHz unfocused transducer. The received signal was analysed with pre-existing custom-made Labview software. The experimental setup is shown in Figure 3.4 (Collin, 2009). This received signal has losses that are caused by sound attenuation in water, attenuation in the tissue mimicking material/liver tissue and reflections at

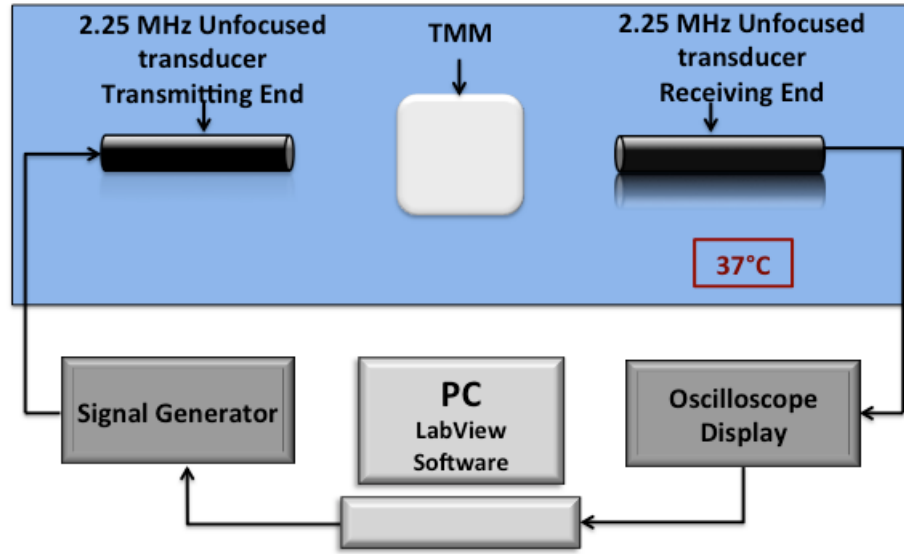


Figure 3.4: Schematic representation of the experimental setup. The ultrasound transducer on the left, driven by the signal generator, emits acoustic pulses that travel through the medium and are detected from the transducer on the right. This transducer is connected to the oscilloscope where the received signal is displayed and forwarded to the PC. The Labview software on the PC closes the loop by storing the data and increments the centre frequency of the signal generator before starting the next loop

the interfaces between water and front wall of the material and water and back wall of the material.

Ultrasound attenuation in water is negligible ($26 \cdot 10^{-15}$ Np/m/Hz²), however it was taken into account and corrections for it were made. In order to correct for reflection losses at the surface boundaries, two different material samples of the same composition but of different lengths along the beam path were interposed between the transducers and the received signal was recorded for both of them as illustrated in Figure 3.5. The received signal for each material is

$$p = T_1 T_2 p_0 e^{-\alpha_w x_1} e^{-\alpha_m l_m} e^{-\alpha_w x_2} \quad (3.1)$$

where T_1 is the transmission coefficient of the water-material interface, T_2 is the transmission coefficient of the material-water interface, p_0 is the amplitude of the

incident wave, α_w is the attenuation coefficient of water, α_m is the attenuation coefficient of the material (TMM or liver), x_1 and x_2 is the propagation distance in water before and after the material sample respectively and l_m is the material length. As reflection losses are the same in both samples, any difference in the received signal was caused by the difference in the length of the propagation path within the sample. Division of the received signals provided the attenuation coefficient in Np/m:

$$\alpha = \frac{\ln \frac{p_2}{p_1}}{l_1 - l_2} \quad (3.2)$$

Another parameter that could induce errors in attenuation measurements is diffraction. As a sound beam propagates it spreads. The effect of this diffraction loss was not directly measured or simulated, but it was compensated by adjusting the experimental setup so that the received signal was measured at a distance that corresponds to the same number of wavelengths in both cases of the long and short material being in the propagation path. Sound travels faster in denser media, so when the thicker slice of the tissue-mimicking material comes between the two transducers, the observed time delay is smaller. The physical distance between the transducers was changed so that the acoustic distance remained the same in both cases. For the diffraction correction, the correct distance (x_2) was used for each material in Equation (3.1) (Collin, 2009).

3.4.1.2 Attenuation measurement with the focused 1.1 MHz transducer and hydrophone

A needle hydrophone (HNA-0400, Onda) was coaxially aligned to the focus of a 1.1 MHz HIFU transducer (102E035, Sonic Concepts) and remained fixed at the HIFU focus for the duration of the experiment. The tissue-mimicking material was placed between the HIFU and the needle hydrophone, so that the hydrophone penetrated the material from the far side relative to the HIFU transducer (Figure 3.6).

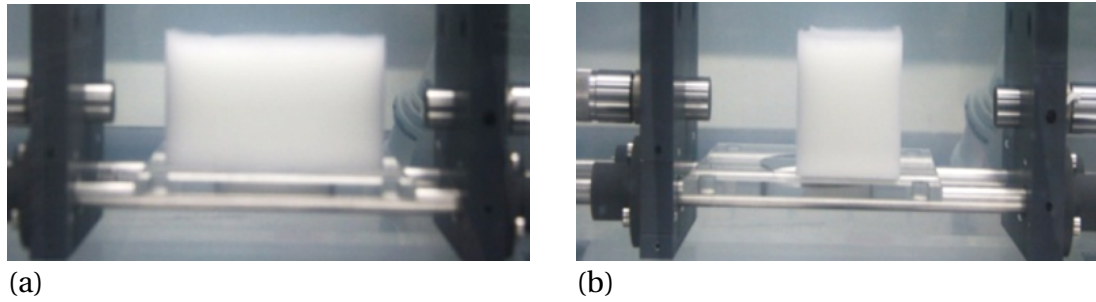


Figure 3.5: The receiving transducer detects the acoustic signal that is passed through two TMMs of different sizes (a) and (b). The attenuation coefficient for a virtual material that has size equal to the difference of the two measured ones is calculated

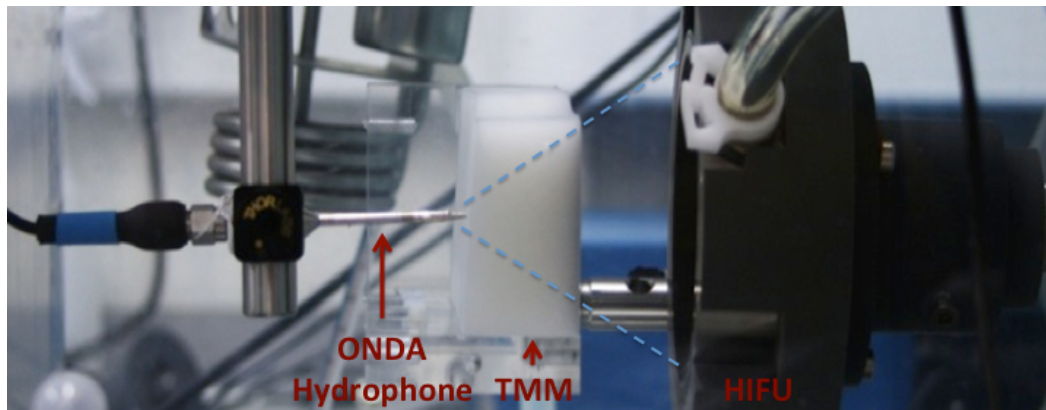


Figure 3.6: Image of the Onda hydrophone (left) receiving the signal from the HIFU transducer during an experiment. The dashed lines indicate the HIFU focus, which is always at the hydrophone tip.

The material was then gradually moved away from the transducer towards the hydrophone in 1 mm steps, until the hydrophone pierced the front wall of the material. At each point the detected acoustic pressure was measured. The attenuation coefficient of the material could be measured, because the only thing changing at each step was the amount of material used between the HIFU transducer and the hydrophone. Acquisition of the acoustic signal right in front and behind of the front wall allowed for direct evaluation of the contribution of the reflection coefficient at the interface as well.

Both the HIFU transducer and the hydrophone were calibrated, so that the relation that governs conversion between acoustic pressure and voltage was known. The experiment was conducted at the HIFU fundamental frequency (1.1 MHz) and 3rd harmonic (3.3 MPa). For the ONDA hydrophone the sensitivity value at the fundamental frequency of 1.1 MHz was 41.43 mV/MPa and at the 3rd harmonic 62.01 mV/MPa.

In order to avoid non-linear effects that would cause attenuation at frequencies other than the driving frequency, thus introducing systematic errors due to attenuation of harmonics, a 0.35 MPa and 50 cycles sine wave was chosen as the input signal. This amplitude was the highest one for which linear propagation was observed (by monitoring the FFT of the detected signal on the oscilloscope). After acquiring the acoustic signal with the embedded hydrophone as a function of distance from the front wall of the tissue-mimicking material, the attenuation coefficient was calculated via the relationship

$$\frac{P_1}{P_2} = e^{-\alpha x} \quad (3.3)$$

where P_1 and P_2 (MPa) are the pressure amplitudes acquired at two different depths in the material, x (m) the distance between them and (Np/m) the attenuation coefficient.

3.4.2 Assessment of Cavitation and Thermal Response

An overview of the experimental setup used for characterisation of the thermal and cavitation response of the TMM is given in Figure 3.7. The key elements of the setup are (i) the HIFU generation loop; (ii) the passive cavitation detection loop and (iii) the temperature measurement loop, which are described in detail in the subsequent sections.

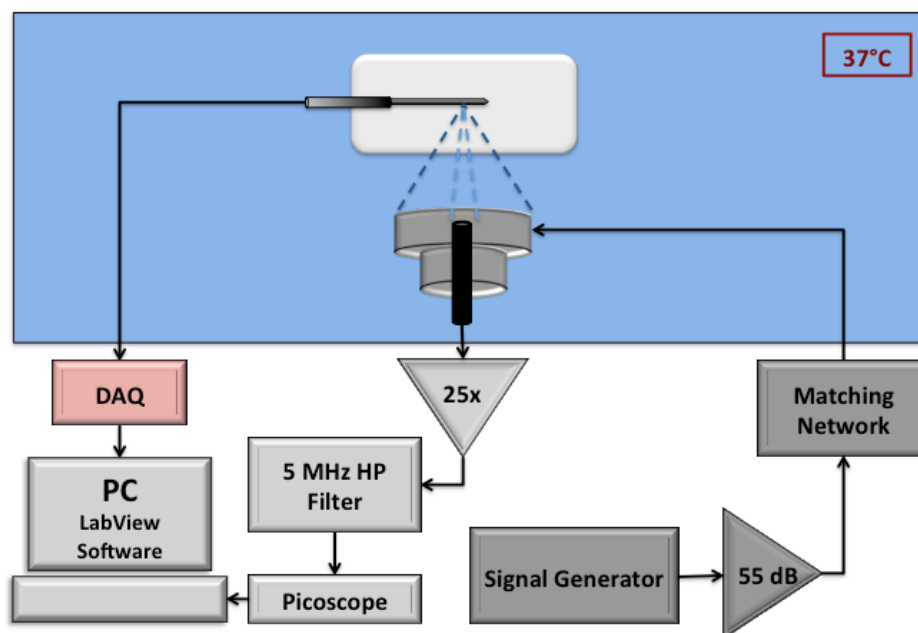


Figure 3.7: Block diagram of the experimental setup for temperature measurements and cavitation detection. The cell and drug-embedding tissue mimicking material was immersed in a water tank maintained at 37°C. A thermocouple was inserted in the material and aligned to the HIFU focus, to enable monitoring of the focal temperature throughout the exposure. Acoustic emissions were monitored with a PCD confocally aligned with the HIFU.

3.4.2.1 Ultrasound Generation

The HIFU transducer (102E035, Sonic Concepts) that was used for TMM characterisation and optimisation of the HIFU exposure conditions in Chapter 3 and Chapter 4 is a single-element piezoelectric spherical shell with 62.64 mm focal length and 1.1 MHz narrowband response. The rationale behind the choice of the 1.1 MHz transducer is that this frequency is high enough to achieve considerable temperature rise and at the same time is low enough to allow for cavitation activity to take place.

The transducer was calibrated using a 0.075 mm-diameter needle hydrophone (1360, Precision Acoustics). The hydrophone sensitivity (mV/MPa) is given by the manufacturer for different frequencies, and calculated with linear interpolation for

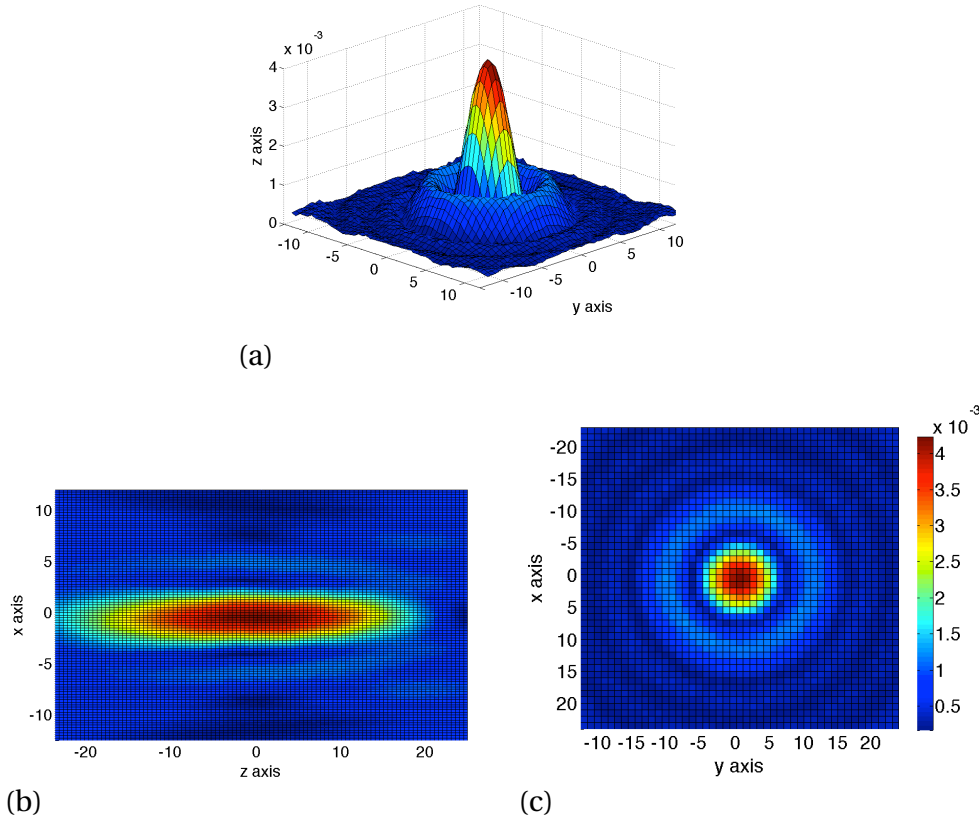


Figure 3.8: 3-D view of the HIFU focus on the transverse plane (a). Longitudinal (b) and transverse (c) pressure profile surfaces of the HIFU transducer, shown on equal scales, with both surfaces intersecting the focal maximum. As a result of the low ratio of element size to focal depth, the focal volume is cigar-shaped, with strong transverse focusing and weak axial focusing.

1.1 MHz, which is the centre frequency of the HIFU transducer. The linear equation that resulted from the calibration and represents the HIFU voltage to pressure relation at the HIFU focus is

$$P = 0.080V + 0.0357 \quad (3.4)$$

where P is the acoustic pressure in MPa peak-to-peak and V is the input voltage after the RF amplifier in V peak-to-peak. The transverse and axial pressure profiles are shown in Figure 3.8.

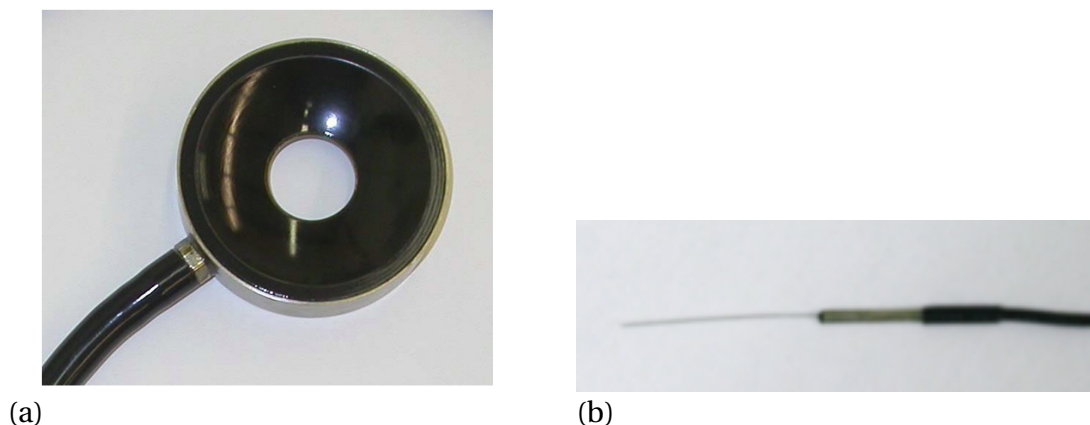


Figure 3.9: Photograph of the HIFU transducer with 20 mm central opening for PCD placement (a) and photograph of the needle thermocouple that is placed in the TMM at the HIFU focus for measurement of the ultrasound-induced local temperature rise (b)

3.4.2.2 Passive Cavitation Detection

The HIFU transducer has a 20 mm central opening, through which a PCD can be mounted Figure 3.9. The PCD is a spherically focused single element transducer with 7.5 MHz center frequency and 75 mm focal length. It is used for listening to the acoustic emissions during each HIFU exposure. Since both the HIFU and the PCD are focused, before each experiment they were coaxially aligned.

The frequency response of the PCD allows for monitoring acoustic emissions over a broad frequency range (5-20 MHz), thus enabling the detection of harmonics (associated with non-linear propagation and stable cavitation) or broadband noise (associated with inertial cavitation). The PCD was used in-line with a 5-MHz high-pass filter (Allen Avionics), as shown in Figure 3.7, which further suppressed emissions at the fundamental frequency, thus making best use of the available dynamic range.

Following filtration, the PCD signal was amplified by a factor of 25 using a low-noise broadband preamplifier (SR445A, Stanford Research Systems) before being

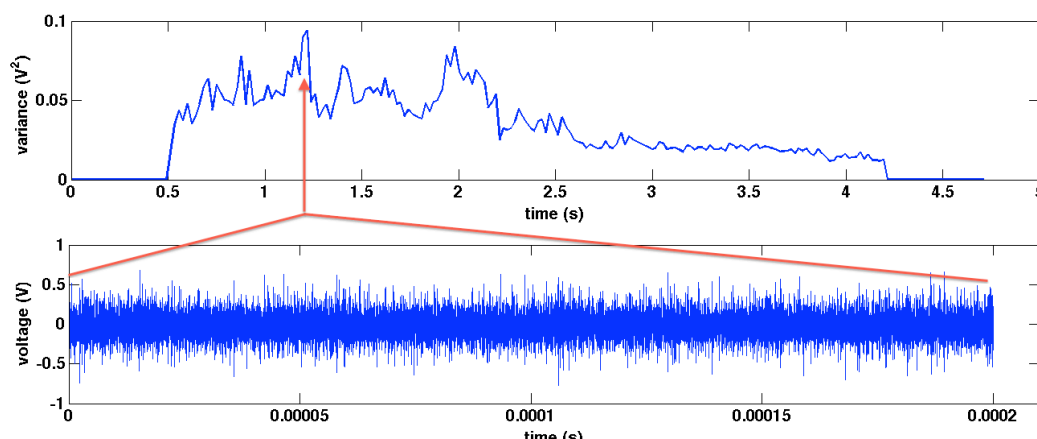


Figure 3.10: The signal variance (a) is a number of points, each one of which is given the value of the variance of the signal received at the PCD at that certain time. The 200 microsecond trace that produces the variance level at each point (b).

connected to an 8-bit Picoscope (PicoScope 3205, Pico Technology) that digitised PCD data with a sampling frequency of 25 MHz.

The digitized PCD traces were initially stored as binary data files, converted into ASCII files off-line and post-processed in Matlab. A typical PCD trace (shown at the bottom of Figure 3.10) is proportional to the broadband acoustic emissions generated at the focal region at that time point. Hence, the trace variance at each time point is proportional to the power of the acoustic emissions at that time point (Kyriakou *et al.*, 2011). Cavitation-enhanced heating has been previously shown to be directly related to the power of acoustic emissions (Coussios and Roy, 2008), therefore the signal variance was used as a metric for thermally-relevant cavitation activity. The signal variance was calculated at each time point of the exposure, as illustrated at the upper part of Figure 3.10, thus giving an indication of the power of acoustic emissions generated at the HIFU focus during the exposure.

Cavitation activity was classified and quantified on the basis of the frequency content of the acoustic emissions, which was calculated by taking Fast Fourier Transforms (FFT) of the PCD time traces. The energy distribution and the FFT of the

fundamental frequency, the harmonics and the broadband noise was calculated. It is assumed that the energy contained within harmonic bands arises from non-linear propagation, and that broadband energy (outside harmonic bands) is indicative of inertial cavitation.

Figure 3.11 shows the decomposition of two sample acoustic signals received. The first (a) has high levels of broadband noise emissions, whereas (b) shows high values only at the harmonics of the fundamental frequency (in this case 1.1 MHz), as shown by the arrows, indicating non-linear propagation of the acoustic wave in the medium. In both parts of the figure, the red dots correspond to the level of broadband emissions at the specific time point, whereas the green dots correspond to the level of harmonic emissions, as calculated by signal decomposition. The contribution of the fundamental frequency is shown by the purple dots, but is in fact zero, as expected, due to the use of a 5 MHz high-pass filter (shown in Figure 3.7).

3.4.2.3 Temperature Measurement

A T-type fine-needle thermocouple (HYP0, Omega Engineering), coated with a stainless steel hypodermic needle of 200 μm outer diameter was embedded in the TMM for focal temperature measurements. The signal from the thermocouple was recorded with a multiplexer with embedded reference temperature compensation (34970A, Agilent Technologies), which sampled and digitised the thermocouple signal 100 times per second before passing the value to a PC (Figure 3.9). The thermocouple was inserted into the TMM via the side face with its longitudinal axis in an approximately horizontal orientation. The flexibility of the fine-needle thermocouple made it difficult to position it in a precisely horizontal location, requiring that the TMM be aligned with respect to the HIFU transducer only after insertion of the thermocouple probe.

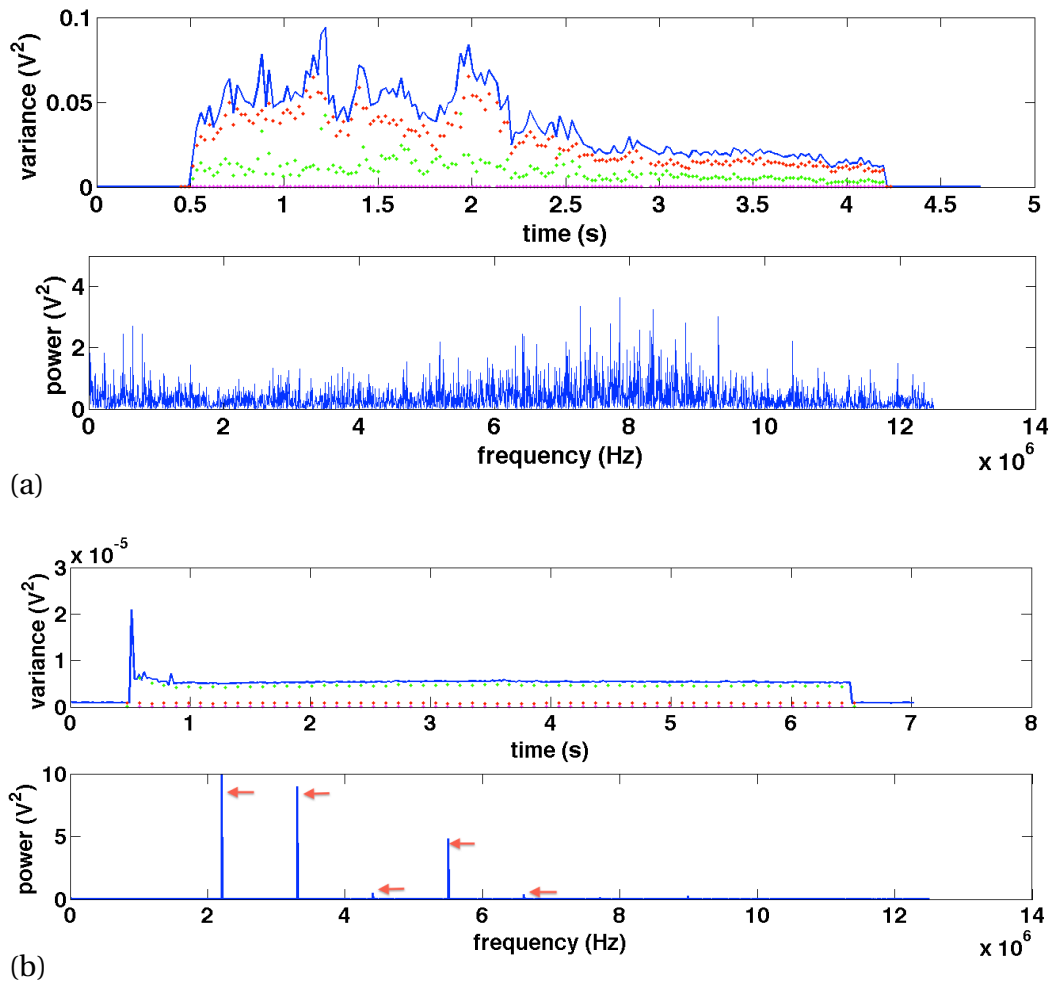


Figure 3.11: Signal variance and spectral decomposition of the signal acquired from a 5.6 MPa peak-to-peak sonication, where cavitation activity is present (a) and from a 4.4 MPa peak-to-peak sonication, where cavitation activity is not present (b) in the TMM. The red dots (·) refer to the level of broadband noise at the specific time-point, whereas the green dots (·) refer to the level of harmonic emissions. High levels of broadband noise can be observed on the spectrum of the signal depicted in (a), whereas dominating harmonic emissions (indicated by the arrows) may be observed on the spectrum of the signal depicted in (b)

The presence of a foreign body, in this case the thermocouple, at the HIFU focus is on its own a source of error, due to the interaction of the HIFU beam with the thermocouple needle. An additional local temperature rise occurs due to the viscous forces that arise from the relative motion between the thermocouple and the surrounding medium as well as from the heat conduction between the thermocouple and the medium (Fry and Fry, 1954b,a). Hynynen *et al.* (1983) have measured that ultrasound sonications at 1 MHz, yielding temperature rises in the range of 5 - 8°C *in vivo*, induce a 0.3 - 1°C additional temperature rise due to thermocouple interference, for thermocouples of diameter up to 300 µm. In all experiments in Chapter 3 and Chapter 4, a 200 µm in diameter thermocouple was used, as described in this section, and the temperature rises of mild hyperthermia that are of interest are below 8°C. Hence, it can be assumed that a thermocouple artefact of 1°C or less is induced in all cases.

In addition to measuring temperature rise only at the HIFU focus with most sensitivity, temperature profiles were acquired for the broader focal region by performing a scan in the transverse and axial planes of the HIFU transducer. For the transverse plane a 0.5 mm step was used and for the axial plane a 1 mm step was used, since the HIFU focal region is broader axially. The assessment of the induced temperature rises, not only at one point but at a broader region is necessary to ensure drug release over the whole treated area .

3.4.3 Experimental Procedure

Before each experiment the HIFU was confocally aligned with the PCD. For this purpose a pulser-receiver (DPR 300, JSR Ultrasonics) was used to create pulses and a small brass sphere was used as a reflective target. The reflected signal was monitored on the oscilloscope screen. The sphere was initially positioned at a distance

corresponding to the HIFU focal length, by using the speed of sound at 37 °C to calculate the approximate time delay at which the reflection from the sphere should appear on the oscilloscope. The sphere was then moved relative to the HIFU transducer both transversely and axially until the amplitude of the reflection was maximized. The procedure was repeated with the PCD transducer, this time moving the PCD transducer relative to the fixed sphere until the amplitude of the reflection was maximised.

The next step was the alignment of the HIFU-PCD assembly with respect to the needle thermocouple, which was embedded in the tissue-mimicking material (or liver tissue), as seen in Figure 3.7. The alignment was done both acoustically and thermally. Acoustic alignment was performed initially to position the thermocouple needle transversely to the HIFU focus and was based on finding the maximum reflection from the thermocouple needle by driving the 1.1 MHz transducer in pulse-echo mode. Even though the PCD has a tighter focus and higher sensitivity than the HIFU transducer, due to the higher centre frequency, the acoustic alignment was performed with the HIFU, because in that case the interference of the glass microspheres in the received signal was less.

Thermal alignment was subsequently performed in order to identify the most sensitive point of the thermocouple needle, which is approximately at a 3–4 mm distance from the needle tip (Figure 3.12). In order to ensure positioning of the HIFU focus on the thermocouple (rather than the needle) tip, the thermocouple was scanned in 0.5mm steps in the plane transverse to the HIFU axis, until the position that yielded the maximum temperature rise was identified. For thermal alignment, low-amplitude (2 MPa pp) HIFU exposures of duration 1s were used, which induced temperature rises not exceeding 2 degrees and did not induce cavitation activity.

During the experiments, the TMM was placed in a custom-made holder with

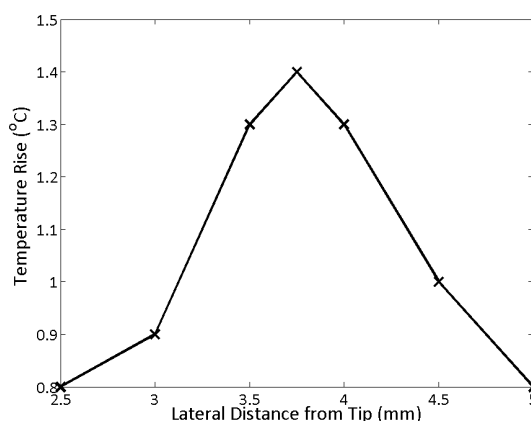


Figure 3.12: Temperature measurements acquired with the hypodermic needle thermocouple acoustically aligned with the HIFU focus, during 2 s low amplitude HIFU exposures. The needle, was moved transversely to the HIFU focus in 0.5 mm steps, until the peak temperature rise corresponding to the location of the thermocouple within the needle shaft was identified.

front and back walls were made of mylar, an acoustically transparent material that minimizes reflections of the incident ultrasound wave.

In order to enable simultaneous acquisition of temperature and acoustic emission data, custom-made LabView software was used to simultaneously trigger the function generator that drove the HIFU transducer, the multiplexer that digitised the thermocouple measurements and the Picoscope used to acquire the PCD measurements. The acoustic emission data was recorded onto the PicoScope buffer in "blocks", then transmitted to the PC. The data acquisition settings that were used were 200 microsecond 'blocks' with 20,000 samples per block. Thus, a 6 second experiment for instance would yield 300 data blocks, each of length 20,000 samples. Recording to and transmission from the buffer did not take place concurrently and data transfer was thus the rate-limiting step.

For each experiment, acoustic emissions and temperature were recorded for 500 ms before and 500 ms after the 6 s sonications to enable comparison of the values of the acoustic emissions before and after the experiment with the emissions dur-

ing the sonication, and also to detect the cooling of the TMM at the focus after the sonication.

Once recording was complete, the thermocouple was repositioned at a new location in the TMM, so that potential effects of cavitation activity or high levels of heating would not affect the next experimental run. This procedure was repeated across peak-to-peak pressures in the range of 2-7 MPa.

3.5 RESULTS

As mentioned in §3.1, for a first experimental approach it was considered advantageous to investigate the effects of HIFU-induced release in a controlled, homogeneous tissue-mimicking material that is capable of replicating the thermal and acoustic properties of liver tissue. Therefore, the focus of the work presented in this Chapter has been mainly the development and characterisation of such a material.

First, degassed ox-liver tissue was characterised in terms of its thermal and acoustic properties. The acquired values were compared to the ones reported in the literature (for attenuation and speed of sound) to ensure that there is agreement and also to the ones obtained in the TMM. After testing a number of candidate materials, the tissue-mimicking phantom best suited to the requirements of the present study and having the closest acoustic and thermal response to the degassed liver tissue was found to be cell-embedding agarose gel containing glass microspheres.

In the present section, the attenuation coefficient, speed of sound and thermal response measured in degassed liver tissue are presented and optimisation of the concentrations of agar and glass microspheres in the TMM is performed to achieve an attenuation coefficient and speed of sound that closely match those of liver tissue. Also, a complete characterisation of this novel tissue-mimicking material in

terms of (i) attenuation coefficient, (ii) thermal response, (iii) frequency response and (iv) cell viability, at various excitation amplitudes is presented.

3.5.1 Comparison of Acoustic Properties in Liver Tissue and the TMM

3.5.1.1 Attenuation Coefficient and Speed of Sound

In order to have control values measured in the same experimental setup (with the two unfocused transducers) for the attenuation coefficient and speed of sound to compare to the TMM, a detailed study of these parameters in degassed liver tissue was performed. The experimental results for attenuation in degassed liver at 37 °C are presented in Figure 3.13 as mean values with errorbars representing double the standard deviation and are compared to the values reported in the literature. The red line represents the linear fit to the experimental data. The two dashed lines represent the upper and lower limit of the reported literature values for liver attenuation in mammals (Goss *et al.*, 1980b; Law *et al.*, 1985; Techavipoo *et al.*, 2004), and $\alpha = 6.67 \cdot f$ Np/m/MHz is a representative linear relationship of attenuation against frequency for ox-liver tissue *in vitro* as reported by (Goss *et al.*, 1980b).

In the present experimental work, the linear fit to experimental measurements in the frequency range 1–4 MHz was found to be $\alpha = 8.3 \cdot f - 2.2$ Np/m/MHz and the speed of sound was found to be $c = 1614 \pm 1.1\%$ m/s. The attenuation coefficient of liver at 1.1 MHz (frequency of interest because it is the central frequency of the therapeutic transducer) was found to be $\alpha = 6.93$ Np/m. The measured mean value of the linear approximation of the attenuation coefficient for liver at 1.1 MHz ($\alpha = 6.93$ Np/m) is in the range of 5.17 - 7.964 Np/m that is found in the literature. The experimentally obtained linear fit to attenuation values, $\alpha = 8.3 \cdot f - 2.2$ Np/m/MHz, is higher than the range of the values reported in the literature, but the power of 1.1

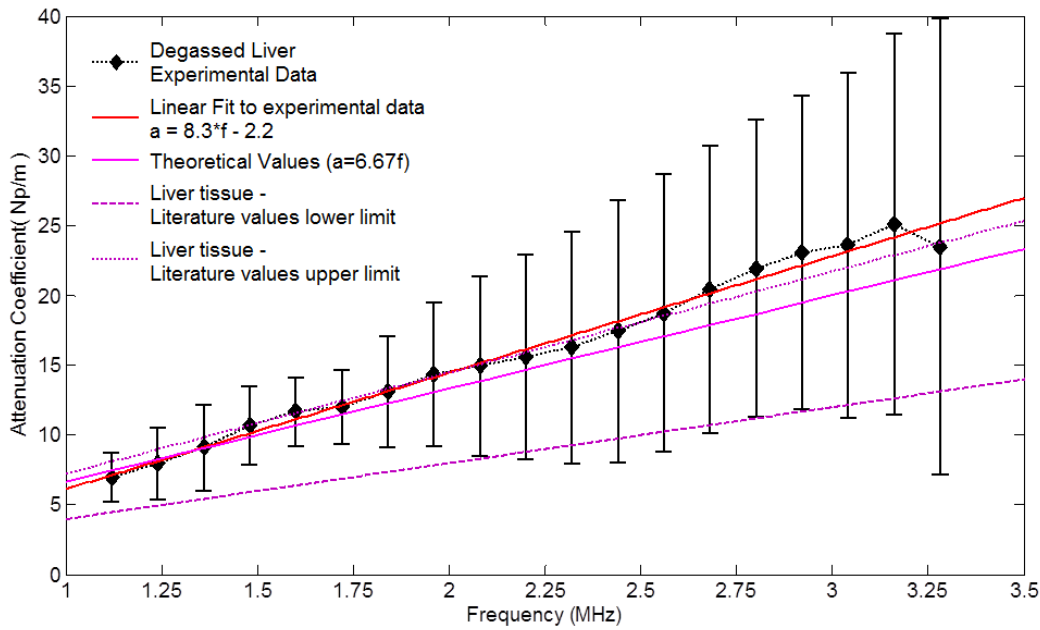
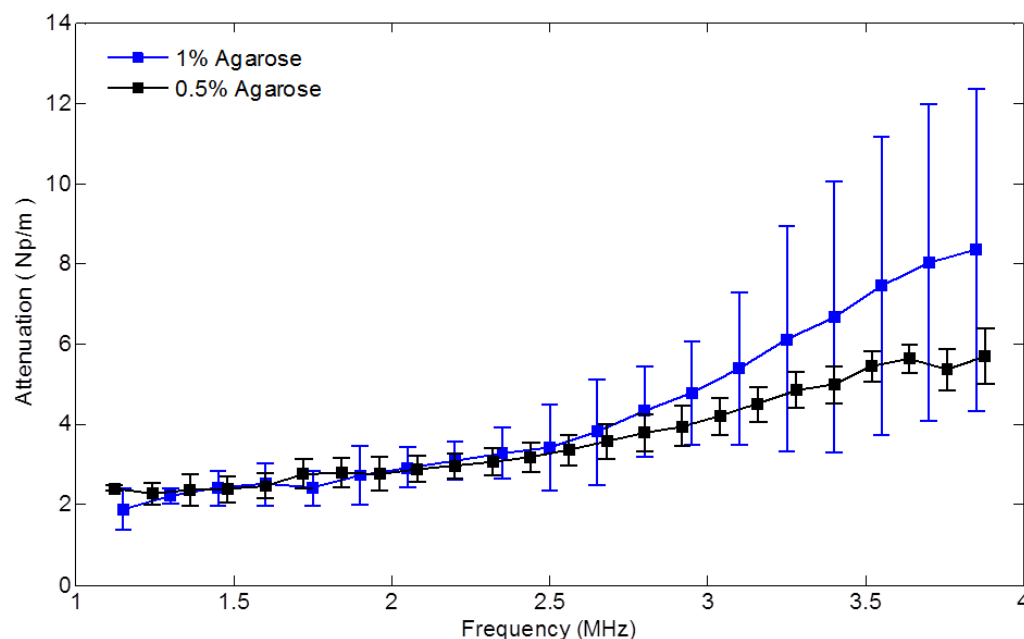


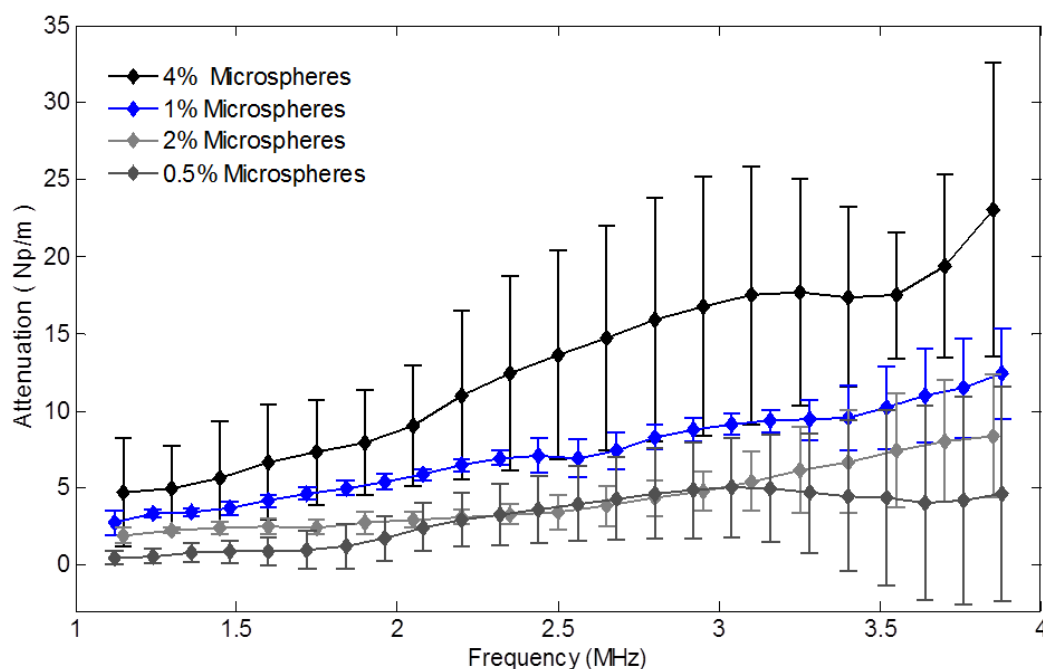
Figure 3.13: Experimental data for degassed bovine liver at 37°C and literature values for liver tissue in mammals ($n = 3$). The experimental results are shown as mean values and errorbars of size double the standard deviation. The red line represents the linear fit to this experimental results. The dashed purple lines represent the upper and lower limits for mammalian liver attenuation as reported in the literature. The continuous purple line shows a representative linear approximation of ox liver attenuation as reported in (Goss *et al.*, 1980b)

approximation, namely $\alpha = 6.04 \cdot f^{1.1}$ lies in the reported range. The speed of sound was measured as $c = 1614 \pm 1.1\%$ m/s, in the range 1570 - 1660 m/s, which is the range of the reported literature values for mammals ((Goss *et al.*, 1980b), (Law *et al.*, 1985), (Techavipoo *et al.*, 2004)).

Having measured the attenuation coefficient and speed of sound in degassed liver and having ensured that it is similar to the reported values, the next step was to optimise the agarose and glass microsphere concentration to achieve similar values in the TMM. Variation in the agarose concentration in the phantom caused a limited change in its attenuation. Figure 3.14(a) shows the attenuation values of 0.005 g/ml and 0.01 g/ml agarose gels with 2% glass particles. At low frequencies the attenuation values were similar and at higher frequencies the 1 % agarose gel



(a)



(b)

Figure 3.14: Measured values of attenuation coefficient with the through-transmission method in a 0.5 % and 1% agarose TMM with 2 % glass microspheres. There is no statistically significant difference between the two TMMs up to 2.5 MHz. Above 2.5 MHz the 1% TMM has a steeper slope of attenuation (a). Attenuation measurements versus frequency for 1 % agarose TMM and 0.5 %, 1 %, 2 % and 4 % glass microspheres (b). The 4% microsphere TMM presents significantly higher attenuation throughout the whole frequency range.

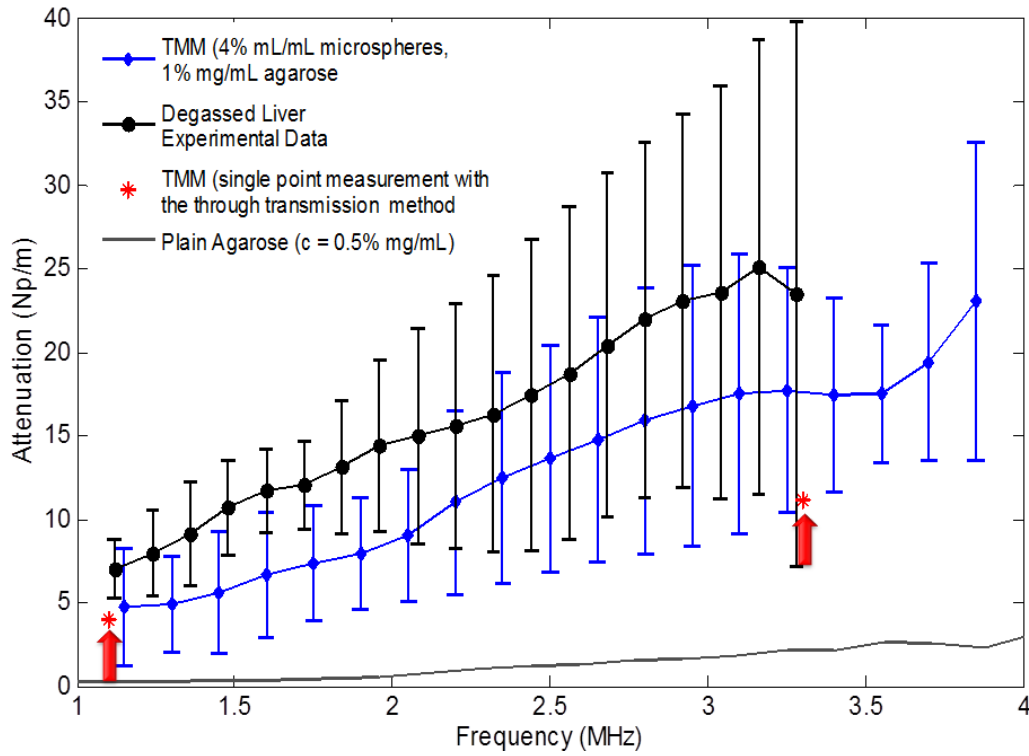


Figure 3.15: Attenuation coefficient of the TMM and the degassed liver

exhibited higher attenuation. It was not possible to go higher in terms of agarose concentration, because up to 1% agarose gel exhibits porosity that is similar to tissue, and thus renders it clinically relevant. Moreover, agarose with concentration 1% or lower is preferable as a cell environment than agarose of higher concentration. For the above reasons agarose of 1% was chosen as the TMM base.

Figure 3.14(b) shows the change in the attenuation with frequency for four different concentrations of glass microspheres; 0.5%, 1%, 2% and 4%. Concentration of 4% microspheres in the TMM gave an attenuation coefficient with statistically insignificant difference to the one of liver tissue, as shown in Figure 3.15. The linear fit to the experimental data for the TMM is $\alpha = 6.5 \cdot f - 3.4$ Np/m/MHz, which is very close to the linear approximation for liver, namely $\alpha = 6.67 \cdot f$ Np/m/MHz, and lies in the range of the attenuation coefficient values found in the literature.

Table 3.1: Acoustic parameters in liver and the novel TMM.

Parameter	TMM	Degassed Liver	Liver (Literature)	Degassed Water	Units
Attenuation Coefficient at 1.1 MHz	3.75, 3.96 ^a	6.93	-	0.0187	Np · m ⁻¹
Attenuation	6.5 · f-3.4	6.04 · f ^{1.1} (linear fit: 8.3 · f-2.2)	4 · f to 7.24 · f	0.017	Np · m ⁻¹ · MHz ⁻¹
Speed of Sound	1545 ± 0.137%	1614 ± 1.1%	1598	1523	m · s ⁻¹
Density	1.06	-	1.05	1	g · cm ⁻³
Characteristic Acoustic Impedance	1.637 · 10 ⁶	-	1.667 · 10 ⁶	1.523 · 10 ⁶	kg · s ⁻¹ · m ⁻²

^ameasured with the ONDA

With the needle hydrophone method the attenuation coefficient was found to be 3.96 Np/m at the fundamental frequency and 11.1 Np/m at the 3rd harmonic (indicated by the arrows). With linear interpolation, the resulting relationship for the attenuation was $\alpha = 3.245 \cdot f + 0.39$ Np/m/MHz. The TMM attenuation values acquired with both methods agree at a low frequency range (3.75 Np/m and 3.96 Np/m measured with method 1 and method 2 respectively at 1.1 MHz) but there is a discrepancy between them at higher frequencies. This discrepancy could be attributed to the fact that at 3.3 MHz the hydrophone size (400 μ m in diameter) was very close to the wavelength (468 μ m) or to alignment issues caused by the narrow focal region at this frequency. For this reason, at higher frequencies the values calculated with the through-transmission method were considered to be more reliable. The calculated values for attenuation and speed of sound in liver tissue and the phantom are also summarized in Table 3.1.

3.5.1.2 Non-Linearity and Cavitation Threshold

Having now established the optimum TMM composition, namely 1% mg/mL agarose and 4% mL/mL glass microspheres, in terms of attenuation, the next step

was to identify the cavitation threshold and confirm a similar heating profile, as it is expected due to the importance of attenuation coefficient in the heating of the material. For this reason all experiments were performed both in degassed liver tissue and the TMM.

Both liver tissue and the TMM were exposed to a range of HIFU pressures at 1.1 MHz, as described in §3.4.3 and as mentioned in §3.4.2.2, during each sonication a PCD detected passively acoustic data from the HIFU focus. Application of FFT to the data-sets gave the frequency content of the signal and signal decomposition gave the contribution of each frequency component (harmonic emissions, broadband noise and fundamental frequency) to the signal power at each time-point.

Figure 3.16 shows representative results for the frequency content of the recorded emissions from liver tissue and the TMM for 2.2, 4.4 and 8.8 MPa peak-to-peak acoustic pressures. The fundamental frequency is cut-off with a 2 MHz high-pass filter. The red circles indicate the three first harmonic emissions. For both materials the n_{th} harmonic is $f_n = 1.1 \cdot n$, $n = 2, 3, 4, \dots$, because the fundamental frequency of the HIFU is 1.1 MHz. Degassed liver (left-hand side) presented linear response at very low sonications (2.2 MPa peak to peak), whereas at higher exposures non-linear behaviour was observed (4.4 and 8.8 MPa peak-to-peak). Non-linear response is indicated by the presence of harmonic emissions and was expected, because liver tissue is known to be a highly non-linear medium (B/A ranging from 6.2 to 8.9) (Law *et al.*, 1985).

The TMM on the other hand expressed non-linear behaviour even at low exposure conditions (2.2 MPa peak to peak), which was also present at higher exposure conditions. At 4.4 MPa peak-to-peak, harmonic emissions were dominating, but broadband noise was also observed, indicating onset of inertial cavitation activity. From 4.4 to 8.8 MPa peak to peak a fifty-fold increase in the broadband noise was

observed. It should be noted here that the acoustic emissions acquired from the TMM are 2 orders of magnitude higher than the emissions acquired from liver tissue. Different axes were used in the figures for the two materials to show the lower energy signals.

In order to have a graphical representation of the frequency content of both the TMM and liver, the spectrograms of 6 s sonications at 4.4 MPa peak to peak are shown in Figure 3.17. Colour intensity represents the power spectral density at each frequency. The colour scaling is the same for both cases. Discrete horizontal lines indicate that signal at that frequency was emitted throughout the 6 sec duration of the exposure, suggesting harmonic emissions and thus non-linearity of the media. They are present in both the TMM and liver. As it may be observed by the color-bar on the right, harmonic content in liver tissue is significantly lower than in the TMM, in accordance to the FFTs shown in Figure 3.16

The vertical lines at a specific time-point, indicate that the frequency content of the signal was broadband which is indicative of inertial cavitation emissions. In this case, their intensity is very low (also supported by Figure 3.16). When the spectrogram of the 6 s, 4.4 MPa sonication is compared to the equivalent sonication at 5.6 MPa, in which inertial cavitation activity dominates, the difference becomes more pronounced (Figure 3.18).

The threshold for cavitation-enhanced heating of the TMM was evaluated from the temperature data that were acquired (and will be shown in the following section: §3.5.2). In Chapter 2, it was shown that in the absence of inertial cavitation activity, temperature elevation at the HIFU focus due to sonications follows a linear profile with respect to the acoustic intensity of the sonication. When inertial cavitation is instigated, cavitation enhanced heating causes irregularities in the temperature profile and thus deviation from the linear profile.

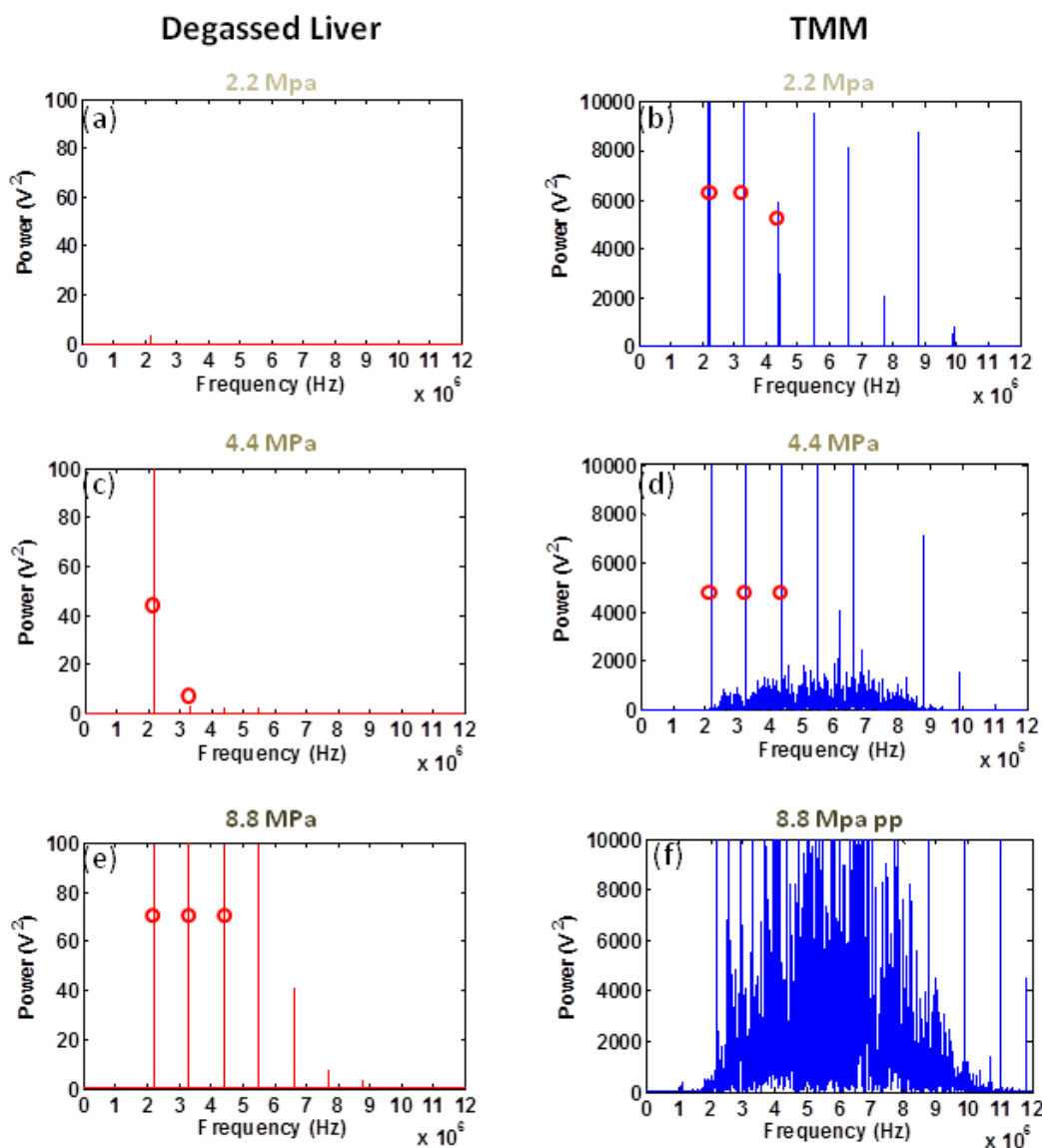


Figure 3.16: Representative FFT of the signal received after exposure of degassed liver and the novel TMM to HIFU. The red circles indicate the first 3 harmonics (2.2, 3.3 and 4.4 MHz). (a),(c) and (e) correspond to 2.2, 4.4 and 8.8 MPa peak to peak HIFU exposures on liver tissue respectively. At 2.2 MPa no harmonic or broadband emissions are observed, indicating linear sound propagation, whereas at 4.4 and 8.8 MPa there are harmonic emissions indicating non-linear sound propagation. No inertial cavitation activity is observed at any frequency. (b), (d) and (f) correspond to the same acoustic pressures applied to the TMM. Non-linearity of the medium can be seen by the high levels of harmonic emissions even at the low pressure of 2.2 MPa peak to peak. At 4.4 MPa, there are both harmonic and broadband emissions, with harmonic emissions dominating, whereas broadband emissions dominate at 8.8 MPa. Note that the axes for liver and the TMM are different.

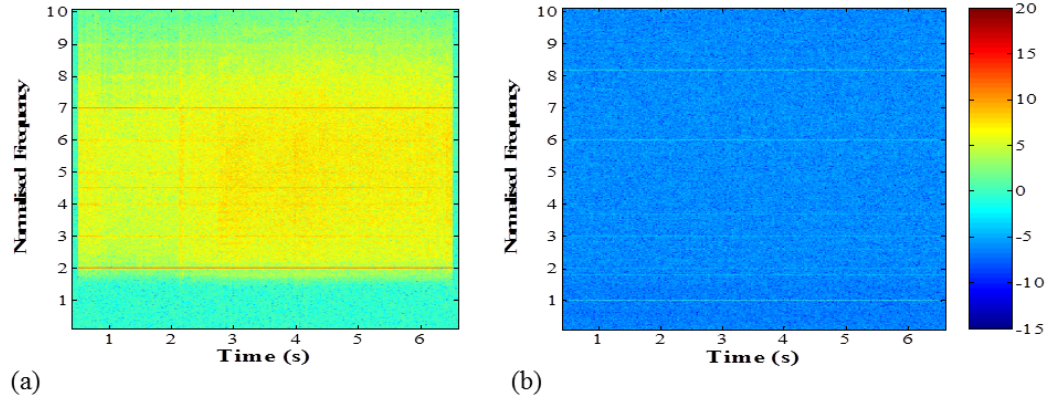


Figure 3.17: Spectrogram for 6 s exposure at 4.4 MPa peak to peak of the TMM (a) and liver (b). Horizontal lines indicate harmonic emissions, whereas vertical lines indicate broadband noise emissions.

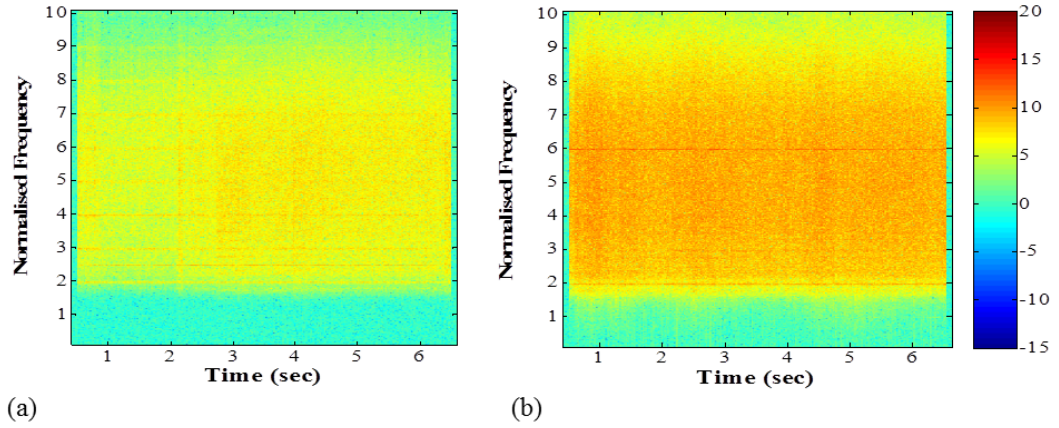


Figure 3.18: Spectrogram for 6 s exposure at 4.4 MPa peak to peak (a) and 5.6 MPa peak to peak (b) of the TMM. Vertical lines, indicating presence of inertial cavitation activity, have significantly higher intensity in (b).

In Figure 3.19, the maximum temperature rise that was recorded at the HIFU focus during 6 s CW sonications in the range of 2.5 - 5.6 MPa peak to peak HIFU excitation, with 0.5 MPa step, is plotted against the respective acoustic intensity for representative samples of both liver tissue and the TMM.

Temperature rise in liver tissue increases linearly with intensity across the whole pressure range investigated. This was expected, as the spectral decompositions of the acoustic data acquired during sonications in liver tissue demonstrated no

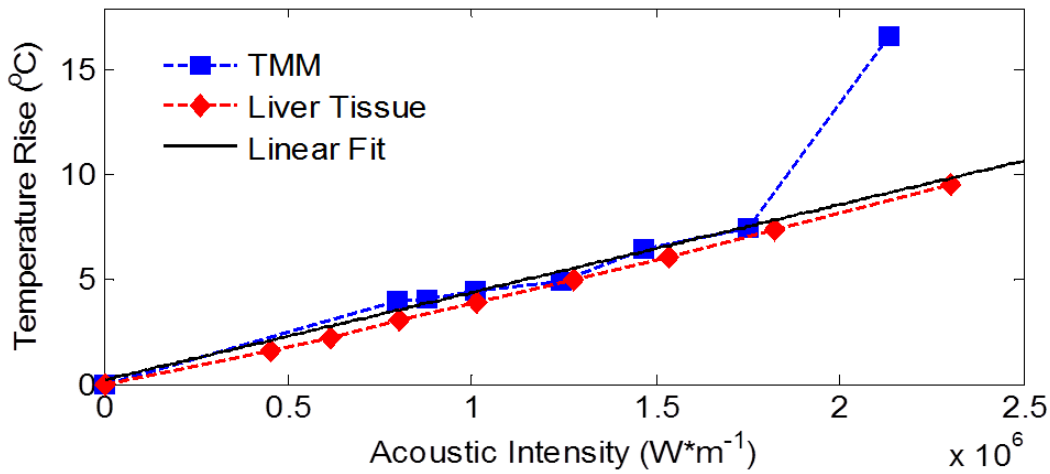


Figure 3.19: Cavitation Threshold of the TMM. At 4.8 MPa peak to peak (corresponding to $I = 1.8 \cdot 10^6 W \cdot m^{-2}$, the maximum temperature rise caused to the TMM was almost 10° higher than the one predicted by the linear model. This implies that there is an additional source of heating apart from sound attenuation. This additional source of heating is inertial cavitation activity.

broadband noise, indicative of absence of inertial cavitation. On the contrary, the temperature rise in the TMM demonstrates a sudden increase (at 4.8 MPa peak to peak). The recorded temperature rise at 4.8 MPa is much higher than the expected value of the linear fit. This indicates the onset of another heating mechanism, namely inertial cavitation activity.

3.5.2 Comparison of Thermal Response to Liver Tissue

In this section, the results of the comparison of the thermal response of the two materials is presented. All experiments were performed in the setup shown in Figure 3.7, with degassed and deionized water being at a base temperature of $36.6^{\circ}C$, to mimic normal body temperature.

For acoustic pressures up to 4.4 MPa peak-to-peak, the induced levels of hyperthermia in tissue are in good agreement with those in the TMM, as shown in Figure 3.20(a), and do not show any statistically significant difference. Mild hyperther-

mia (up to 6°C) was achieved in both materials for the same exposure conditions. The maximum difference in the observed temperature rise was 2°C, and that was only in a small amount of samples from the whole sample lot.

Figure 3.20(b) shows cases of three different pressures in which the acquired absolute temperature rises in the TMM and the liver tissue have less than 0.2°C difference. Activation of the liposomes starts at 39°C and maximum release of its content occurred from 39°C to 42°C (with a maximum at 42°C). All the experiments were conducted at 37°C to mimic physiological body temperature, so the acquired 6°C-6.5°C temperature rise that was induced with a 4.4 MPa sonication leads to a final temperature of 43°C- 44°C (Figure 3.20(a)). Thus, the range in which the liver and phantom thermal behaviour is the same coincides with the therapeutically relevant range of mild hyperthermia.

Reaching 42°C at the HIFU focus is very important, as it suggests that at that point there is adequate hyperthermia for liposomal activation. However, in a therapeutically relevant situation liposomal activation has to be induced in the broader focal region during each exposure, so that an amount of drug adequate for causing the desired cytotoxic effects is released.

The acquired temperature profiles in the transverse plane of the HIFU for liver tissue and the TMM, shown in Figure 3.21, strongly suggest similar heat diffusivity in both materials, as the temperature profiles recorded are similar both on-axis and off-axis. Each line represents the temperature rise with time for a point close to the HIFU focus, when the focus is being heated with HIFU. The temperature rise at the HIFU focus is the highest, and it decreases symmetrically at both sides, as distance from it increases. They also indicate that with each exposure which causes a hyperthermia of 42°C - 43°C at the focus, the broader area is heated in the mild hyperthermia range.

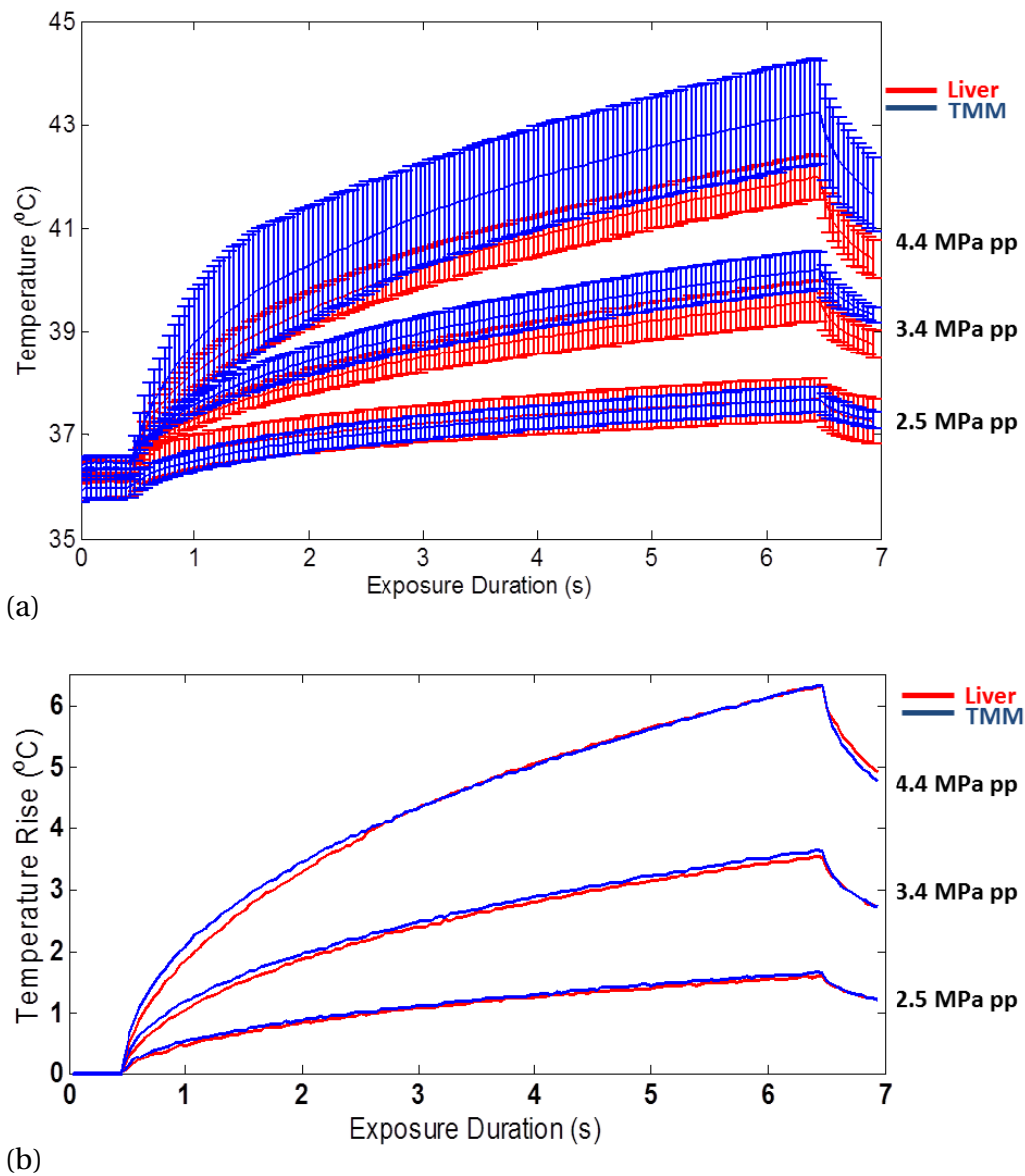


Figure 3.20: Temperature measurements at the HIFU focus in the TMM ($n = 4$) and liver ($n = 3$) below the cavitation threshold. (a) shows the temperatures acquired for three different pressures, 2.5, 3.4 and 4.4 MPa peak to peak. (b) shows 3 cases in which the temperature rise in the TMM and the liver present less than 0.1°C difference. The measurement at 4.4 MPa has additional significance, because the produced hyperthermia falls in the range in which low-temperature sensitive liposomes are activated.

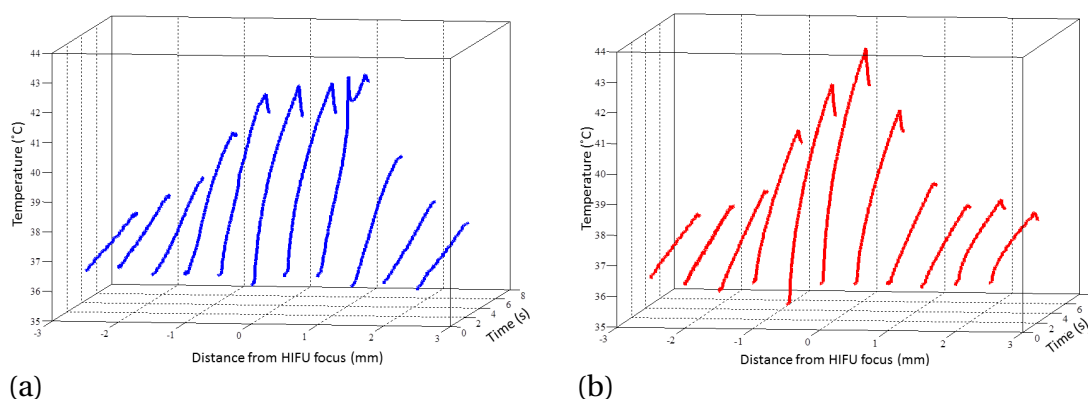


Figure 3.21: Acquired temperature profiles in the transverse plane of the 1.1 MHz HIFU focus during a 6 s exposure at 4.4 MPa in the TMM (a) and liver tissue (b)

In Figure 3.19 it was assumed that above 4.4 MPa inertial cavitation activity is instigated, because there was a discrepancy of the observed temperature rise from the linear profile that is suggested by linear ultrasonic heating theory. The presence of inertial cavitation activity is further supported from the thermal results at 6 s exposures at 4.8, 5.6, 6.6 MPa peak to peak that are shown in Figure 3.22. The temperature rises caused are significantly higher in the TMM than in liver for the same acoustic pressures. For reasons of completeness, the respective FFTs for these exposures are included in Figure 3.23. As expected, the TMM demonstrates high levels of broadband noise, indicative of inertial cavitation activity, whereas the FFTs of the exposures done on the liver tissue, show only harmonic emissions.

3.5.3 Cell Compatibility

The results of the cell viability studies are presented in Figure 3.24, in luminescence intensity units, which can be directly correlated to the number of live cells present per well. Luminescence intensity of all samples containing cells appears significantly higher than the background value, suggesting cell-compatibility of the TMM. It is also stronger in the cases of higher content of glass microspheres. This could be

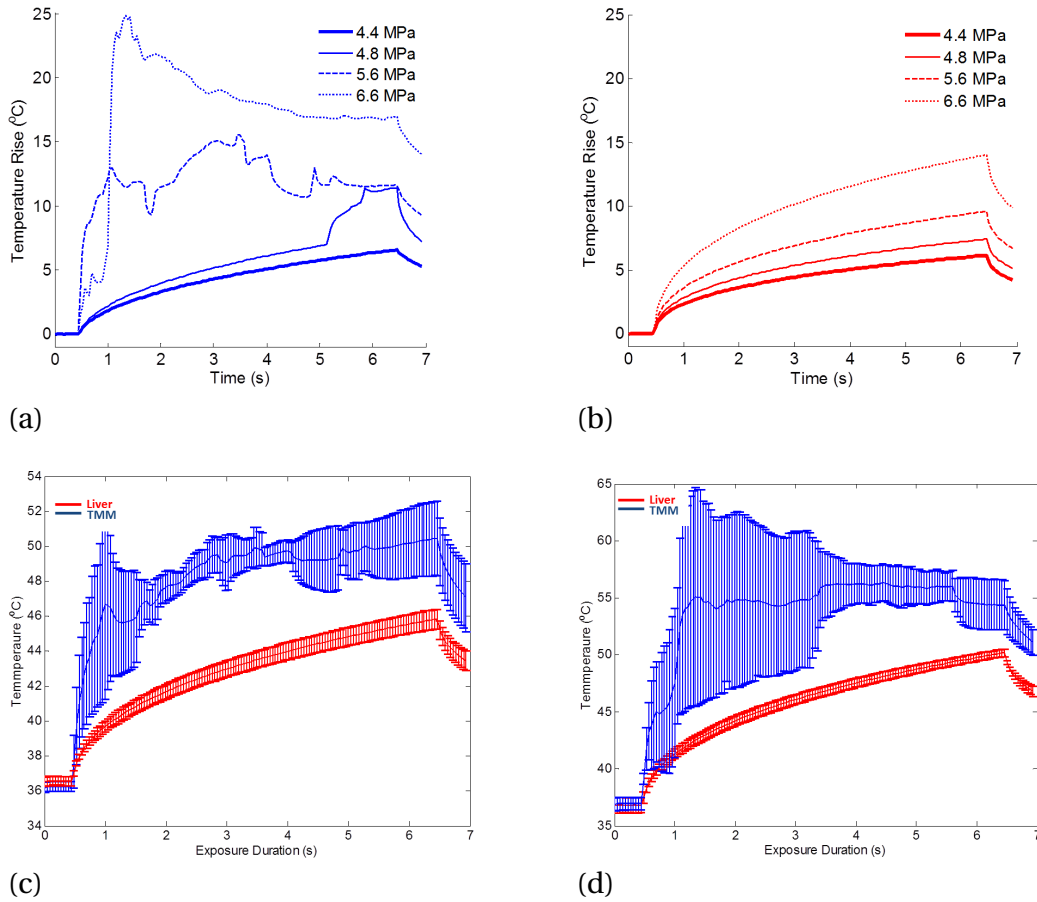


Figure 3.22: Acquired temperature rises in the TMM ($n = 3$) and liver ($n = 3$) above the cavitation threshold. (a) and (b) are a comparison of the temperature rises acquired in the TMM and liver respectively, for 6 s HIFU exposures at 4.8, 5.6 and 6.6 MPa peak to peak, all above the cavitation threshold. The temperature rises in the TMM show great discrepancy from the temperature rises in the liver tissue and are very irregular. This irregularity is a clear indication of the presence of inertial cavitation activity. They are compared to the temperature rise acquired at 4.4 MPa peak to peak, which is similar in both materials. (c) and (d) show representative absolute temperatures that were reached in the TMM and liver for 5.6 MPa and 6.6 MPa respectively.

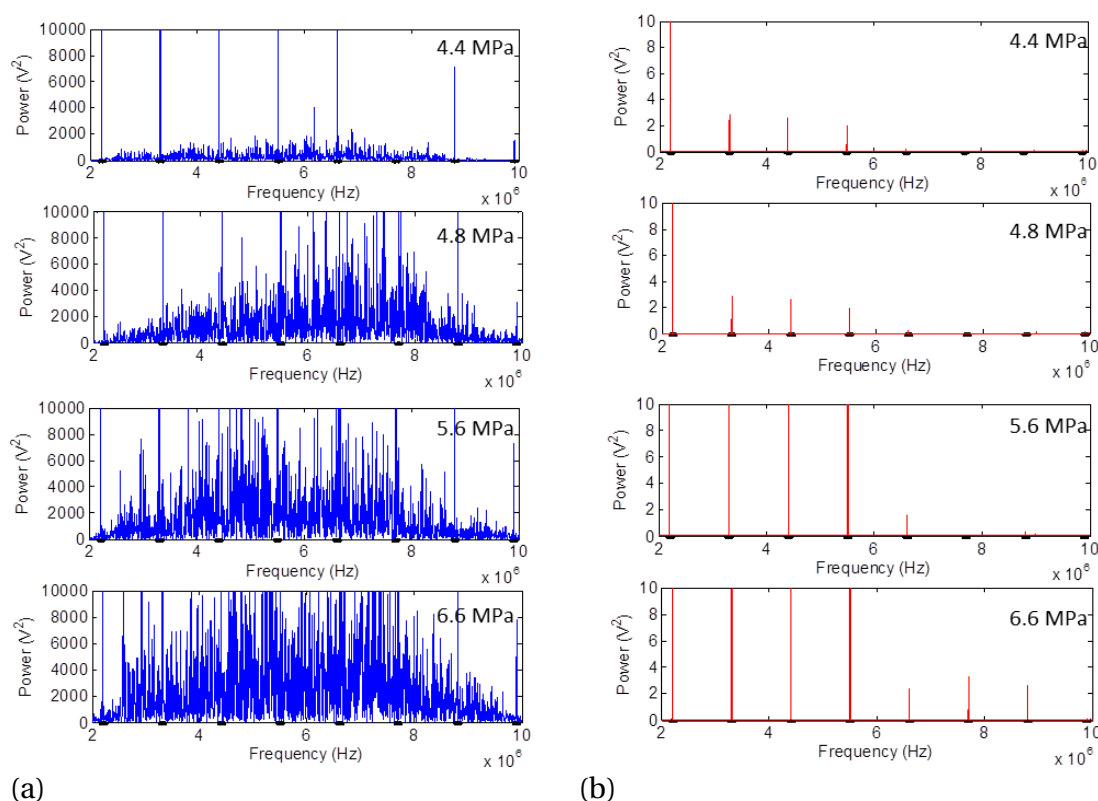


Figure 3.23: FFT of sonications above the cavitation threshold in the TMM (a) and liver (b). The FFTs correspond to the exposures that caused the temperature rises shown in *Figure 3.22(a)* and (b) respectively. The black dots at each FFT indicate the frequency of the harmonic emissions.

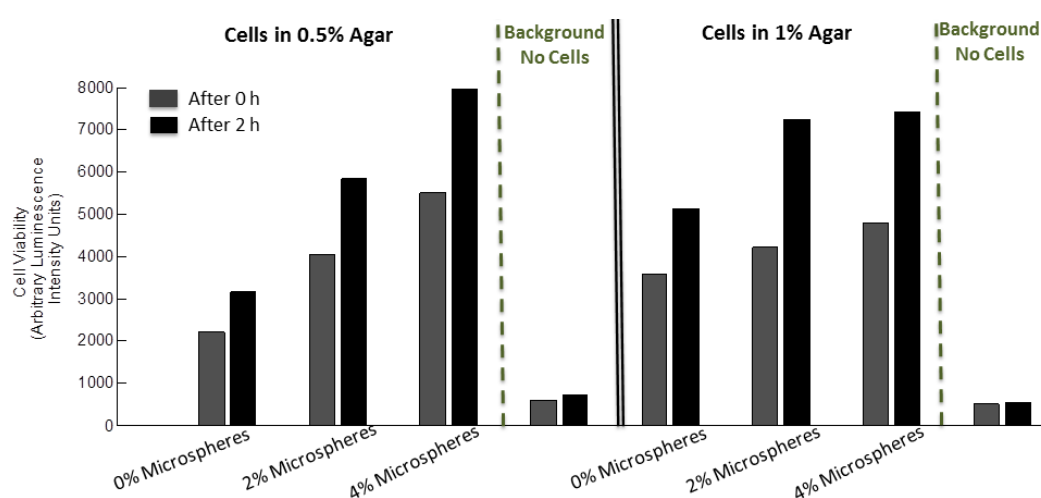


Figure 3.24: Cell viability in various TMM compositions.

attributed to the initial cell number in each well. This experiment is very sensitive to the number of cells, and even slight variations may compromise its outcome. However, for the purposes of the present study, extremely thorough precision in the cell content is not necessary, as more qualitative than quantitative results are sought.

3.5.4 Activation of the Thermally Sensitive Liposomes

The results presented in this chapter have demonstrated that the TMM that was developed has similar acoustic properties and thermal response to liver tissue. It has also been demonstrated that it is cell compatible. This means that the novel TMM can be used for a wide range of applications that involve experimentation in liver tissue. However, because the purpose of the present project is the thermal activation of LTSs, and more specifically ThermoDox, an initial study to ensure that release occurs indeed in the TMM was performed. The in-depth study of the optimal exposure conditions for liposomal activation will be explored in the following chapter.

Figure 3.25 shows the fluorescence intensity results in ThermoDox embedding TMM after HIFU exposure. Higher values at the exposed areas with respect to the non-exposed clearly indicates liposomal activation at 42°C due to HIFU excitation, providing us with a proof of concept that the novel TMM is appropriate for studies of HIFU mediated drug-delivery by mild hyperthermia, while justifying the choice of the specific HIFU excitation parameters. In Figure 3.20 it was shown that this exposure provides heating in the range that activates the thermosensitive liposomes. The fact that the fluorescence intensity of the exposed samples is higher than the fluorescence intensity of the non-exposed samples indicates successful drug-release in the novel TMM with the selected exposure conditions.

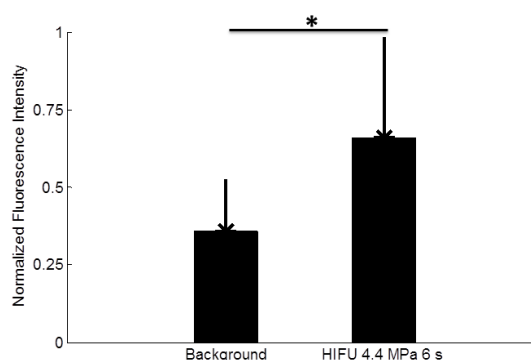


Figure 3.25: Assessment of drug release from thermosensitive liposomes by measurement of the fluorescence intensity of the samples taken from the areas of the TMM that were exposed to 4.4 Mpa pp HIFU for 6 s ($n = 4$). The (*) indicates statistically significant difference (p value < 0.05)

3.6 DISCUSSION

The aim of this work was to produce a TMM with acoustic and thermal properties similar to liver tissue that can be utilized as a platform for ultrasound-induced hyperthermia and drug-delivery experiments. For this reason, it should exhibit similar acoustic properties to liver tissue and, most importantly, similar heating behavior under HIFU excitation, as this was the principal goal. The attenuation coefficient was the most interesting acoustic property due to its direct link to heating. As shown in Figure 3.15, the experimental data for liver and TMM attenuation coefficient are in agreement. Most importantly, they both fall in the range of the liver attenuation values as reported in the literature Figure 3.13.

The size of the error bars in the measurements for liver attenuation could be attributed to the fact that liver is a highly heterogeneous tissue and thus there are structural differences within different samples, as well as within the same sample. Apart from that, before each experiment the tissue was cut into square pieces of the size of the holder in which they were mounted. As this procedure was done by hand, there could be slight variations in the thickness of the samples, or the curvature of

their sides that were exposed to ultrasound.

The size of the error bars in the TMM attenuation results, could be attributed either to entrapped bubbles, or small variations in the local density of the glass microspheres. The existence of $5\text{ }\mu\text{m}$ to $50\text{ }\mu\text{m}$ microspheres renders the gel inhomogeneous. A small variation of the local concentration (or size) of the glass microspheres could cause a variation in the measured attenuation values. As it can be observed in Figure 3, even a small change in the concentration of the glass particles in the TMM (for example from 0.44% to 1%) can cause significant difference in the measured attenuation value. Moreover, the phantom was not degassed before the experiments, as that would be unfavorable for the viability of the embedded cells. Care was taken not to induce bubbles in the gel or the gel/mylar interface, which would compromise the experiment.

However, the similar heating curves (Figure 3.20) between liver and TMM are not in agreement with the wide range of attenuations that has been measured in the two materials, suggesting that the observed discrepancies in attenuation do not have a detrimental effect on the overall heating behaviour of the materials. Attenuation depends on absorption and scattering and governs the wave propagation in the medium, but it is absorption that governs the conversion of acoustic power to heat (see Equation (2.16)). This, in conjunction with the temperature measurement results (see Figure 3.20), suggests that the method for measuring attenuation may not be as accurate as the method of measuring temperature rise, from which the absorption can be derived. Given the present application, which has as an end goal ultrasonic and cavitation-enhanced heating for the release of drug from thermally sensitive liposomes, absorption is more relevant than attenuation.

It is notable that the TMM and liver tissue exhibit a remarkable similarity in HIFU-induced heating up to 4.4 MPa of peak-to-peak applied pressure, whereas at

higher amplitude exposure regimes there is divergence Figure 3.22. As Figure 3.19 indicates, this relates to the onset of inertial cavitation activity in the phantom at acoustic pressures higher than 4.4 MPa. This similarity in acoustic properties and thermal response along with the cell-compatible environment that the novel TMM provides strongly suggest that it is a successful platform for studies of ultrasound-induced hyperthermia and ultrasound-induced drug release. This is further supported by the results presented in Figure 3.25, which shows that thermally-sensitive liposomes can be successfully activated and release the encapsulated drug.

3.7 CONCLUSIONS

In this work a cell-embedding tissue-mimicking material, which has close acoustic and thermal properties to liver tissue was developed and tested. The TMM was manufactured in a non-exothermic process, which renders it appropriate for use in temperature-sensitive studies. The values of its speed of sound and acoustic attenuation coefficient over a range of frequencies (1 MHz - 4 MHz) are similar to those of liver tissue. Most importantly, the TMM exhibits almost identical temperature profiles to liver tissue upon exposure to 1.1 MHz up to 4 MPa peak-to-peak, making it an ideal test medium for studies of HIFU-induced hyperthermia and drug release.



OPTIMISATION OF ULTRASONIC RELEASE FROM THERMOSENSITIVE LIPOSOMES

In the previous Chapter it was shown that ultrasound induced hyperthermia can cause targeted heating for activation of the liposomes in the TMM. The work presented in this Chapter is the optimisation of the ultrasound exposure parameters, to introduce cavitation-enhanced mild hyperthermia for drug activation. The treatment safety and efficacy is also assessed and it is shown that with cavitation-enhanced mild hyperthermia drug activation is achieved in the region of interest in a safe and efficient manner. As it will be demonstrated in this Chapter, cavitation-enhanced heating has the substantial advantages of being rapid and requiring less input acoustic energy. Most importantly though, the presence of cavitation activity can be monitored, thus opening the way for spatial mapping of the treatment, as will be shown in the following Chapter.

4.1 OBJECTIVE

The objective of any clinically relevant pharmacotherapeutic strategy is the efficient and safe drug release at targeted sites of pathology, so that the drug is concentrated at the site of interest without affecting healthy cells. Especially in the case of cancer, the widespread distribution of a chemotherapeutic agent throughout the patient's

body at a dosage adequate to achieve cytotoxicity in a tumor inevitably results in systemic toxicity and causes severe side-effects. These limitations of current therapeutic approaches both in terms of efficacy and of patients' quality of life have motivated the investigation of targeted drug delivery techniques to restrict the action of chemotherapeutic drugs to the tumor region.

In Chapter 1 it was shown that, amongst these techniques, a very promising approach is the use of thermosensitive liposomes that encapsulate an anticancer agent and release it only when the appropriate temperature is reached, thus enabling spatiotemporal control of the chemotherapeutic action. It was also discussed that although the liposomes will respond to any kind of heat stimulus, ultrasound-induced hyperthermia was only recently investigated as a potential release mechanism from thermo-sensitive liposomes, greatly supporting the combined use of thermosensitive liposomes with ultrasound.

These studies however do not exploit inertial cavitation activity. In Chapter 2 it was demonstrated that the violent bubble collapse results in conversion of part of the incident acoustic energy into broadband noise emissions, which are readily absorbed by the surrounding medium causing rapid and local heat deposition.

Apart from enhanced heating, these broadband noise emissions have an additional advantage. They can be detected passively and remotely using conventional ultrasound equipment, as described in §2.6.3., which in a clinical setting could enable real-time non-invasive monitoring of heat deposition and consequently control of the spatial distribution of drug release.

The objectives of the *in vitro* work presented in this chapter are (i) the identification of the ultrasound parameters that cause inertial cavitation in a liposome-embedding clinically relevant platform (the novel TMM presented in Chapter 3),

such that liposomal activation happens because of cavitation-enhanced mild hyperthermia, (ii) ensure that the identified exposure regime is safe in the absence of the liposomes and (iii) assess the effectiveness and the reproducibility of cavitation-enhanced heating as a method of drug release from thermosensitive liposomes.

For this purpose, low-temperature sensitive liposomes containing doxorubicin (lyso-thermosensitive liposomal doxorubicin - ThermoDox®, Celsion Corp.) were mixed in the novel TMM along with hepatocellular carcinoma cells. The cell- and liposome-embedding TMM was then exposed to high acoustic intensities in the form of a focused beam for investigation of the parameters that induce cavitation-enhanced mild-hyperthermia. The TMM samples were imaged with fluorescence microscopy for evaluation of the effects of the HIFU exposures. Finally, throughout this process, cell viability studies were performed on the HIFU-exposed samples, to ensure safety of the exposure conditions.

4.2 EXPERIMENTAL DESIGN AND SETUP

4.2.1 Cell and Liposome-Embedding Liver Model

The agarose-based, cell-compatible TMM that was described in Chapter 3 was used in the present study. As previously mentioned, human hepato-cellular carcinoma cells (HuH-7) were embedded in the TMM at a concentration of 80,000 cells/mL to assess the response of liver cancer cells to the treatment. The TMM was prepared with a non-exothermic procedure, to avoid liposomal activation during preparation, so that any doxorubicin release and cell death that is observed can be safely considered to be a result of the heating induced by the ultrasound exposures.

The therapeutically relevant concentration of ThermoDox for intravenous administration was provided by the manufacturer as 0.02 mg/mL, but when the

drug travels in the vasculature it reaches the desired tumor site with extravasation at lower concentration than the 0.02 mg/mL intravenously administered. To our knowledge, there are no literature data available to provide a specific number for the ThermoDox concentration that reaches the tumour site, because this greatly depends on the environment each time. For this reason, a cell viability and doxorubicin-release experiment was performed in samples of the TMM to define the optimal ThermoDox concentration.

The samples (N=4) were heated in a water bath in the temperature range 24–45°C for 2 minutes and this cell viability study showed that even at normal body temperature (37°C) significant cell death was caused when the clinical concentration of 0.02 mg/mL, recommended by the manufacturer, was used, rendering it unsuitable for the ultrasound experiments. This can be attributed to leakage from the liposomes, which in light of the high concentration and the static environment results in elevated doxorubicin concentrations. A concentration of 0.005 mg/mL was used as a trade-off between a low-enough concentration of ThermoDox for the cells to survive its static presence in their immediate vicinity and a high-enough concentration for measurable amounts of drug to be released.

4.2.2 Experimental Procedure

The experimental setup, ultrasound generation, cavitation detection and temperature measurement technique are the same as described in §3.4.2. The HIFU transducer (102E035, Sonic Concepts) is a single-element piezoelectric spherical shell with 62.64 mm focal length and 1.1 MHz narrowband response and is the same used for the experiments in Chapter 3. The rationale behind the choice of the 1.1 MHz transducer is that this frequency is high enough to achieve considerable temperature rise and at the same time is low enough to allow for cavitation activity to take

place. A 7.5 MHz PCD was mounted through the 20 mm central opening of the HIFU to detect acoustic emissions during the sonications.

4.2.2.1 Sonication Protocol

Two different sonication protocols were followed.

Single-shot sonications for identification of the exposure conditions that induce cavitation-enhanced heating. In this regime, a T-type needle thermocouple was aligned transversely to the HIFU focus (see §3.4.2.3) to record the temperature profile acquired for each sonication. Single-shot HIFU exposures were performed at 4.4 and 5.6 MPa pp with a varying duty cycle in the range 5-25% with 5% step at 1 Hz PRF for appropriate durations so that the total energy delivered was the same in all cases. The acoustic energy delivered to the focus can be estimated as

$$E = \frac{P^2}{2\rho_0 c} \cdot A \cdot t \quad (4.1)$$

where P (Pa) is the acoustic pressure amplitude, ρ (kg/m^3) is the medium density, c (m/s) is the speed of sound, t is the duration that the HIFU was ON per exposure and A (m^2) is the -6dB cross-sectional area of the beam. Since ρ , c and A are the same in all cases, by changing the acoustic pressure and DC, the delivered energy was kept constant by modifying the exposure duration. Before each sonication, the thermocouple and sonication location was changed, so that previous sonications would not cause interference.

Sonication scans for cell viability and liposomal activation experiments. In these cases a larger area had to be sonicated so that the difference in luminescence and fluorescence intensity, indicative of cell viability and liposomal activation respectively, between the treated and non-treated areas was more pronounced. The HIFU exposure regimes that were tested are summarised in Table 4.1. For each ultrasound

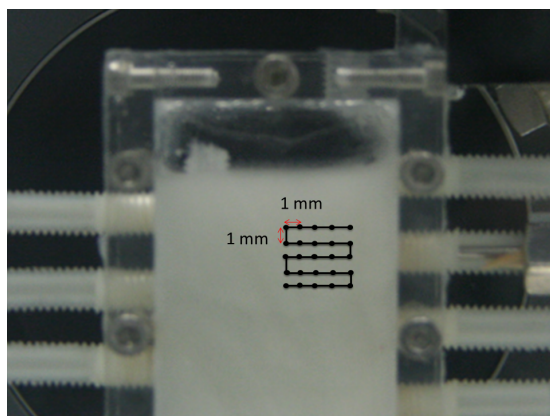


Figure 4.1: Close-up photograph of the TMM in front of the HIFU transducer during an experiment. The white bars on both sides are thermocouple holders. A thermocouple inserted in the TMM can be seen on the second holder from the top on the right side. 25 sonications were performed per sample, and $N = 4$ samples were taken per condition.

exposure regime, scans ($N = 4$) of 5×5 adjacent sonications with 1 mm step were performed (Figure 4.1).

The -6dB ultrasound beam-width at the focus on the transverse plane is 1.5mm and the step between the points was chosen to be 1 mm for the focal pressures of the adjacent exposures to partially overlap at the transverse plane of the transducer, so that the whole area is treated. Our previous thermal results (§3.5.2) have shown that an area of radius 1mm in the transverse plane is being heated at least 3°C below the heating at the focus, due to heat diffusivity of the material. Since the temperature range for drug activation is 39°C to 45°C , it was assumed that the whole exposed volume was therefore adequately heated.

4.2.2.2 Sampling Method

After the insonation scans for all exposure regimes were performed, the TMM was sliced and samples of 125 mm^3 ($5 \text{ mm} \times 5 \text{ mm} \times 5 \text{ mm}$) were extracted from the treated volumes. The extraction took place with custom-made tubes and the sam-

Table 4.1: Exposure conditions tested for cell viability and liposomal activation

Peak-to-Peak Pressure (MPa)	Duty Cycle (%)	PRF (Hz)	Exposure Duration (s)
5.6	100	-	3.7
5.6	25	1	15
5.6	25	1	10
5.6	25	1	15
5.6	5	1	74
4.4	100	-	6
4.4	25	1	24
4.4	25	1	5
2.7	100	1	6

ples were placed in 96-well plates (Greiner Sensoplate glass bottom multiwell plates, Sigma - Aldrich). Microplates with optical glass bottom for optical clarity and black walls for low autofluorescence were used.

4.2.3 Cell Viability Measurement

Cell viability was measured with a Luminescent Cell Viability Assay (CellTiter-Glo, Promega). After the treated samples were placed in the 96-well plate, they were maintained in an incubator at 37°C for 24 h and then mixed with 125 μ l of CellTiter-Glo reagent. CellTiter-Glo assay is a method of determining the number of viable cells present in a culture by quantization of the ATP present, an indicator of metabolically active cells. The reagent caused cell lysis and generation of luminescent signal proportional to the amount of ATP present. Prior to embedding the cells in the TMM and conducting the ultrasound experiments and viability test, the cells were counted using a haemocytometer. The number of live cells after each experiment was calculated as a percentage of the number of live cells in the untreated areas.

For the liposomal activation experiments, the cells were stained with 10 μ g/mL Calcein AM (Calcein AM, Sigma- Aldrich) before they were embedded in the TMM.

Calcein AM is a fluorescent dye with excitation and emission wavelengths of 495/515 nm, respectively, which binds to live cells. Cell fluorescence was then measured immediately after the sonications with fluorescence microscopy (Ti-Eclipse Inverted Microscope System, Nikon) with a FITC fluorescent filter block (ex 465-495 nm, em 515-555 nm) and 4x objective at the center of each well.

4.2.4 Drug Release Measurement

Doxorubicin release was measured with fluorescence microscopy due to its inherent ability to fluoresce at low concentrations. When doxorubicin is encapsulated in the liposomes, it quenches and does not fluoresce. This property allows for distinguishing encapsulated from free doxorubicin, thus evaluating release from the liposomes.

Samples from the sonicated and non-sonicated areas of the TMM were placed in a 96-well plate (Greiner Sensoplate glass bottom multiwell plates, Sigma - Aldrich) immediately after the acoustic exposures and fluorescence intensity readings were taken with the microscope (Ti-Eclipse Inverted Microscope System, Nikon) with a 4x objective at the center of each well. Doxorubicin fluoresces at the red range so TRITC filter (ex:545/30 nm, em:620/60 nm) was used. In each experiment the intensity range was kept constant. For each sample in the well plates a scan was performed throughout the whole sample depth and fluorescence intensity was evaluated as the total intensity of the sample volume (Figure 4.2(a)). Representative images of the samples are shown in Figure 4.2(b).

The control values for fluorescence intensity were obtained by heating ThermoDox-embedding TMM samples (N=4), with $C_{dox} = 0.005$ mg/mL (similarly to the one used at the HIFU experiments) in a water-bath at a 36 - 45 °C temperature range for 6 minutes and measuring their fluorescence intensity. As shown in

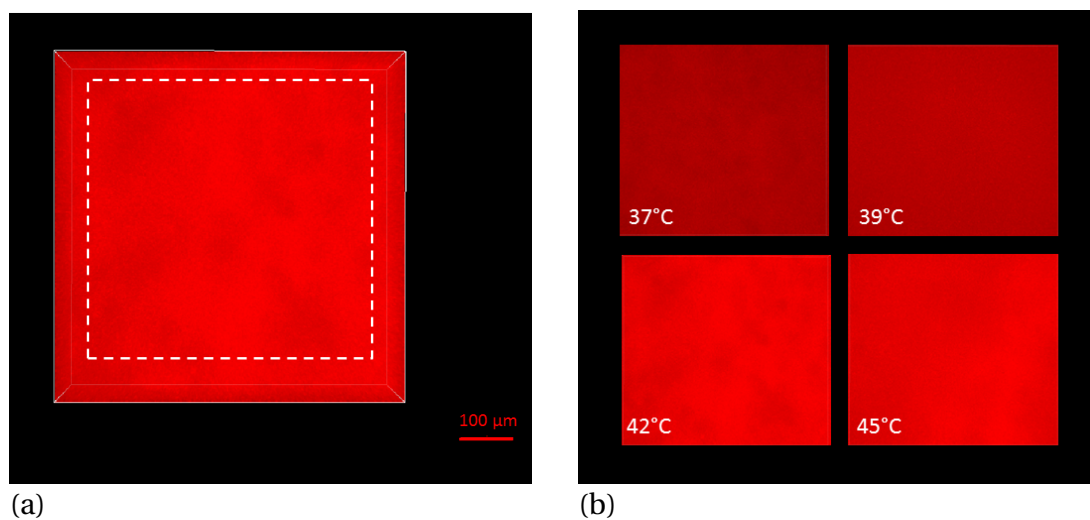


Figure 4.2: (a) The volume projection of the sample. The white rectangle represents the area that is shown in the microscopy images in (b). (b) Microscopy images of the liposome-embedding TMM samples with the TRITC fluorescence filter. The red colour is pseudocolour representing the pixel intensity.

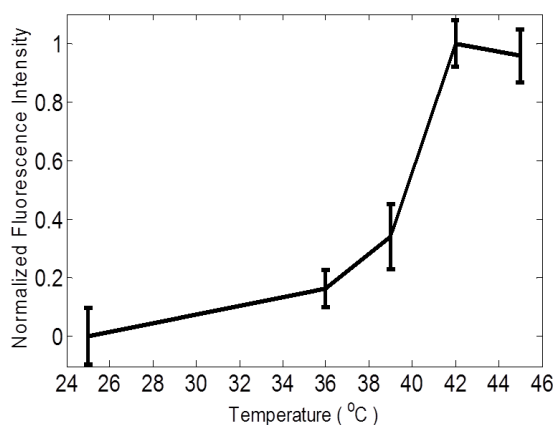


Figure 4.3: Fluorescence intensity of the liposome-embedding TMM samples after they have been heated in a water-bath for 6 min ($n = 4$). Release of the anti-cancer agent occurs in the 39 $^{\circ}\text{C}$ to 45 $^{\circ}\text{C}$ temperature range, reaching a plateau at 42 $^{\circ}\text{C}$.

Figure 4.3, release of the anti-cancer agent was observed in the 39 $^{\circ}\text{C}$ to 45 $^{\circ}\text{C}$ temperature range, reaching a plateau at 42 $^{\circ}\text{C}$, in agreement to the data provided by the manufacturer (Appendix A)

4.2.5 Statistical Analysis

Data are presented in the form of their mean value with error bars representing double the standard deviation. A Student's t-test was performed to determine statistically significant difference among datasets. Datasets with p-value smaller than 0.05 were regarded as significantly different.

4.3 RESULTS

4.3.1 Optimisation of Exposure Conditions

Passive cavitation detection of the tissue-mimicking material, shown in Figure 3.23, demonstrated that, for 4.4 MPa peak-to-peak acoustic pressure, the acoustic spectrum was dominated by harmonic emissions attributable to non-linear propagation, whilst at 5.6 MPa the spectrum was dominated by broadband emissions due to inertial cavitation.

As shown in Figure 3.22, at 4.4 MPa peak-to-peak, the temperature profiles were regular and showed temperature rises (6°C) within range to that required for drug release ($3\text{--}8^{\circ}\text{C}$). At higher acoustic pressures, the temperature profiles became much more erratic, and resulted in considerably larger temperature rises than those needed for liposomal release.

This unpredictability in heating levels is undesirable, because in order to serve the purpose of this work, which is to employ cavitation-enhanced heating for liposomal activation, the induced temperature rise has to be *controlled* and *confined* in the desired 3°C to 8°C range. On the other hand, maintaining cavitation activity during the heating process is desirable as this could provide a process that can be remotely monitored to provide evidence of drug release.

A way of keeping the high pressure amplitude but lowering the temperature rise was to shorten the duty cycle of the exposure. This way, inertial cavitation activity was still present, while the level of induced hyperthermia is decreased (Figure 4.4).

For the exposures shown in Figure 4.4, the total delivered energy was kept constant, by varying the exposure durations, as shown in Table 4.2. More specifically, the exposure durations were 74 s for the 5% Duty Cycle 5.6 MPa exposure, 37 s for the 10% Duty Cycle 5.6 MPa exposure, 15 s for the 25% Duty Cycle 5.6 MPa exposure, 3.7 s for the 100% Duty Cycle exposure (above the cavitation threshold) and 6 s for a 100% Duty Cycle 4.4 MPa exposure (below the cavitation threshold). Maintaining the same total energy levels was necessary in order to ensure that any changes observed in liposomal activation or cell viability (which will be discussed later) were only caused by changes in the heating pattern. Continuous-wave exposures at 4.4 MPa were found to produce adequate levels of hyperthermia for drug release, whilst of the pulsed exposures above the cavitation threshold at 5.6 MPa, only a 25% duty cycle produced sufficient heating for drug release.

In order to enable better understanding of the hyperthermic conditions developed in regions of the sound field that are above and below the cavitation threshold when the focus is exposed to 5.6 MPa peak-to-peak, the temperature rises and associated acoustic emissions at 4.4 MPa and 5.6 MPa under otherwise identical exposure conditions (25% duty cycle, 5 s duration, 1 Hz PRF) are shown in Figure 4.5. Figure 4.5. The 4.4 MPa exposure causes minimal heating, whereas the 5.6 MPa exposure induces the desired levels of hyperthermia for drug release. The respective broadband emissions are presented in Figure 4.5 (b) and (d). The temperature profile in the transverse plane of the HIFU focus for a 5.6 MPa 25% DC exposure is shown in Figure 4.6. It confirms that an area with 1 mm radius around the HIFU focus reaches a mild hyperthermia range as well.

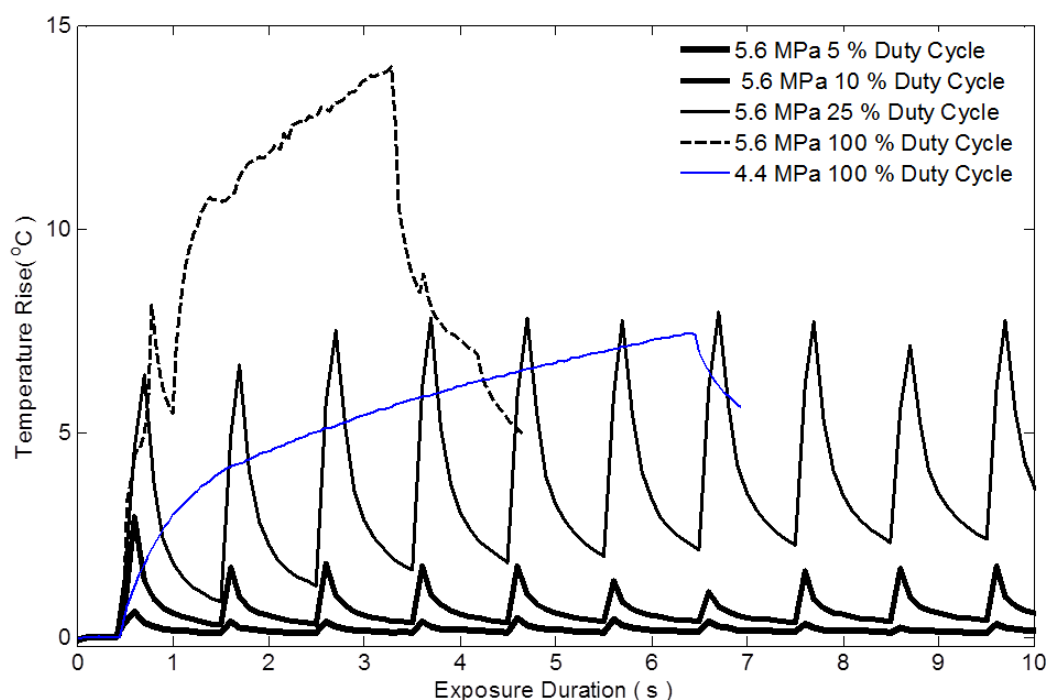


Figure 4.4: Optimisation of the duty cycle of the sonication. The temperature rise in the TMM induced by 5.6 MPa HIFU exposures with varying duty cycle is shown and compared to a CW sonication at 4.4 MPa. The total acoustic energy delivered was kept constant between sonications by altering the exposure duration each time. The respective durations were: 74 s for the 5% Duty Cycle 5.6 MPa exposure, 37 s for the 10% Duty Cycle 5.6 MPa exposure, 15 s for the 25% Duty Cycle 5.6 MPa exposure, 3.7 s for the 100% Duty Cycle exposure (above the cavitation threshold) and 6 s for a 100% Duty Cycle 4.4 MPa exposure (below the cavitation threshold). Only the first 10 s of the sonications are shown in order to have a closer view of the temperature rises caused by the sonications that had the smallest duration. The 25% DC exposure produces adequate temperature rise for drug release.

The significance of this result should not be underestimated. It suggests for the first time that a set of exposure conditions can be identified (1 MHz, 25% Duty Cycle, 1s PRF), for which adequate hyperthermia for drug release is achieved only when cavitation occurs, which coincides with the remote detection of acoustic emissions. In regions of the field where the pressure does not exceed the cavitation threshold, release is unlikely. The implications of this finding are discussed further in §4.3.1.1

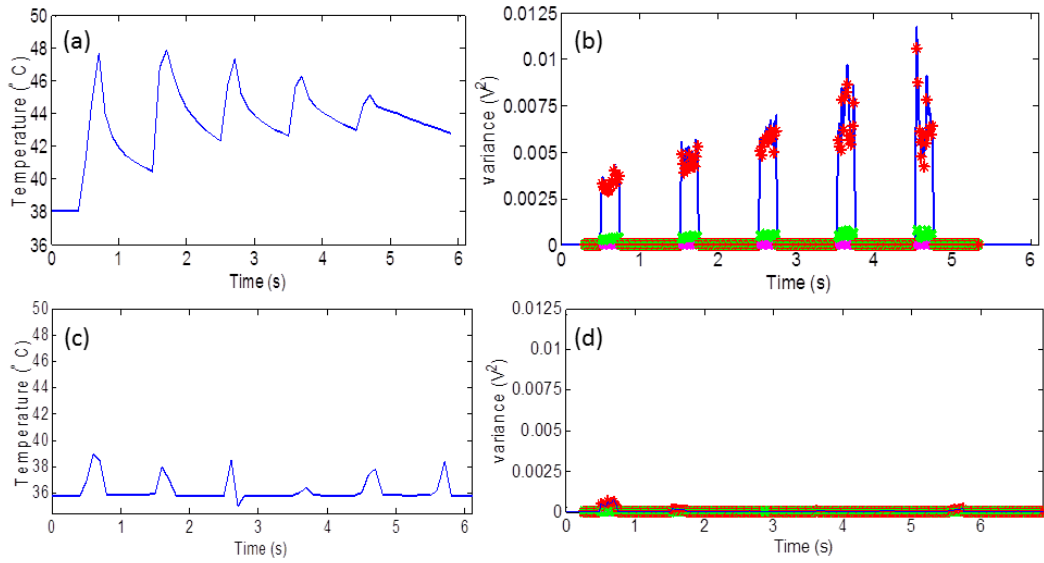


Figure 4.5: Signal variance and induced temperature for the same conditions below and above the cavitation threshold. Acoustic signal variance and temperature for 5.6 MPa 25% duty cycle 5 s exposure (b) and (a) and 4.4 MPa 25% duty cycle 5 s exposure (d) and (c). High levels of signal variance at 5.6 MPa as shown in (b) indicate presence of cavitation activity (denoted with the red asterisks (*)) and produce high temperature rise (a). On the contrary, the 4.4 MPa exposure causes minimal temperature rise (c) and has minimal signal variance, indicating lack of inertial cavitation activity (d). The green asterisks (*) correspond to the levels of harmonic emissions.

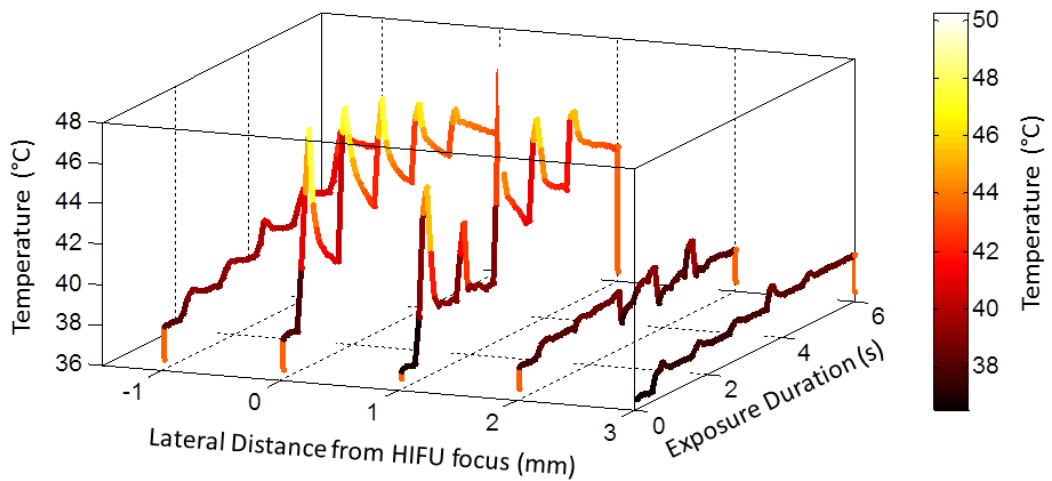


Figure 4.6: Lateral temperature profile for 5.6 MPa 25% DC 5 s sonications.

4.3.1.1 Exploiting cavitation-enhanced heating.

One of the great advantages of cavitation-enhanced heating is that it is much faster than heating in the absence of bubble activity. This difference in the rate of heating is demonstrated in Figure 4.7(a), in which the heating curve of the first second of two continuous wave sonications, is shown. The sonication above the cavitation threshold (5.6 MPa peak to peak) has significantly faster heating than the sonication below (4.4 MPa peak to peak), which is the one that produces the desired temperature rise in the range 3–8°C.

In Figure 4.4 it was demonstrated that lowering the duty cycle of the 5.6 MPa exposure to 25% duty cycle, with 1 Hz PRF, the desirable mild hyperthermia is achieved. Now, in Figure 4.7(b), it is shown that even for the 250 ms that the pulse is 'ON' the rate of heating is significantly greater than the rate of heating caused by the 4.4 MPa exposure, and the peak temperature reached more than 3 times higher.

In the first 250 ms of the pulse, the 5.6 MPa exposure (above the cavitation threshold) has already caused 6.4°C, whereas the 4.4 MPa sonication (below the cavitation threshold) has only caused a 1.8°C temperature rise. After the first 250 ms of the exposures, the 'OFF' time of the pulsed sonication begins and a cooling curve is observed, whereas the continuous wave still heats the focal region. At 1 s the maximum heating caused by the pulsed sonication (6.4°C) is still more than the maximum heating caused by the continuous sonication below the cavitation threshold.

As is already known ((Holt and Roy, 2001)) and has been shown in a numerous instances in this work (§3.5.2, Figure 4.4), inertial cavitation activity produces highly irregular heating. This is a severe disadvantage of cavitation-enhanced heating for the purpose of activation of low-temperature sensitive liposomes, because in this

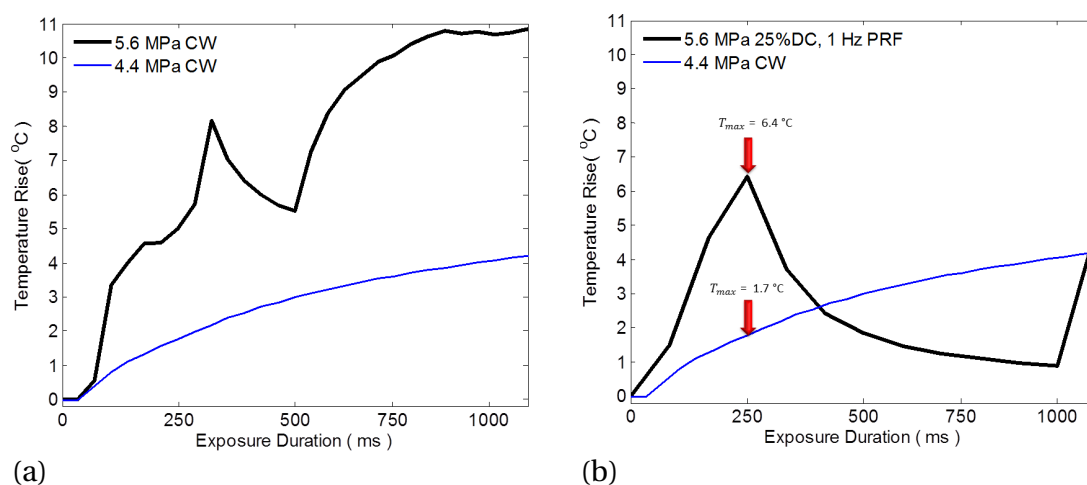


Figure 4.7: Rapid rate of cavitation-enhanced heating seen even at the first 250 ms of the exposures. The first second of two continuous wave sonications above (5.6 MPa) and below (4.4 MPa) the cavitation threshold is shown (a). By lowering the duty cycle of the 5.6 MPa exposure to 25% with 1 Hz PRF (thus 1 sec PRP), the rapid rate of heating causes a temperature of 6.4°C to be reached, on the contrary of the 1.7°C reached in the absence of inertial cavitation activity.

specific case the temperature rise that is induced has to be in the narrow range of 3°C - 8°C.

By lowering the duty cycle of the 5.6 MPa exposure to 25%, it can be shown that this disadvantage can be circumvented. Figure 4.8 displays the variability of the temperature rises observed over 6 identical exposures at a peak-to-peak pressure of 5.6 MPa and a duty cycle of 25%, with error bars showing the standard deviation. The maximum deviation from the mean is never found to be more than 3°C, suggesting that short HIFU exposures resulting in cavitation-enhanced heating can be highly reproducible. Although the reason for this is not certain, it may be due to the progressive heating in the case of the continuous wave effectively changing the medium. Also, heating promotes cavitation and finally cavitation depends on the sample history (hysteresis effect described in §2.5.2)

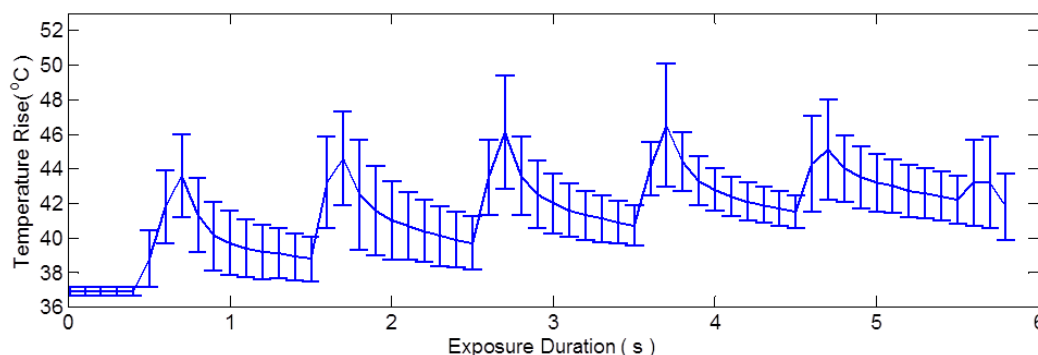


Figure 4.8: Small variation among the induced temperature rise during 5 s, 25% duty cycle, 5.6 MPa sonications (n=6).

4.3.2 Treatment Safety

Another important aspect of this optimisation study was to determine that the ultrasound parameters that are being used for liposomal activation are safe for the patient and more specifically, to ensure that the action of ultrasound or the drug alone, in the absence of the liposomes, was non cytotoxic, and only the combined use of ultrasound and liposomes could cause localised cell damage.

The reason for this is that this treatment relies on the preferential accumulation of the liposomes in tumours due to the EPR effect, so that local concentration of the liposomes is higher in tumours than in healthy tissues and so that exposure to ultrasound-induced hyperthermia causes local drug release. If ultrasound alone was cytotoxic, sonication of healthy tissues surrounding the tumour with little or no liposomal accumulation would result in healthy tissue damage. In a clinical setting it is possible to expose to ultrasound a region of healthy tissue adjacent to the desired treatment site, due to patient movement or lack of precision in imaging guidance.

For this reason, cell viability studies were performed in the exposed areas of the cell-embedding TMM, for a range of sonication parameters. In all of these stud-

ies the TMM contained *no* liposomes. The purpose was to identify the effect of the HIFU exposures *alone* on the TMM. Consequently, a 100% cell viability would be optimum. The presence or absence of liposomes in the TMM has been found to have no influence on the cavitation levels and threshold of the TMM. This was expected because the liposomes consist of a lipid bilayer and only contain doxorubicin, a macromolecular drug. The absence of any gas content or crevices that could introduce gas pockets makes it hence improbable that they have a nucleating effect.

The exposure conditions that were investigated are summarised in Table 4.2 and the results of their effect on cell viability in the absence of the liposomes are summarized in Figure 4.9. In all exposures the total energy delivered was kept constant, in order to ensure that any observed changes in cell viability were attributable to differences in cavitation activity or heating caused by the different exposure conditions, rather than to the total acoustic energy to which cells were being exposed. This was achieved by varying the exposure durations as shown in Table 4.2. Treatment safety was assessed by measuring cell viability with a luminescence intensity assay, as described in §4.2.3.

Table 4.2: Exposure conditions investigated for treatment safety.

Peak-to-Peak Pressure (MPa)	Duty Cycle (%)	PRF (Hz)	Exposure Duration (s)
5.6	100	-	3.7
5.6	25	1	15
5.6	5	1	74
4.4	100	-	6
4.4	25	1	24
4.4	5	1	120

Low duty cycle exposures caused no cell death, indicating that mechanical effects of ultrasound both in the presence and absence of cavitation activity do not affect the cells. The observed cell death in the continuous wave exposures in both cases indicates thermal damage to the cells. For high (100%) and low (5%) duty cy-

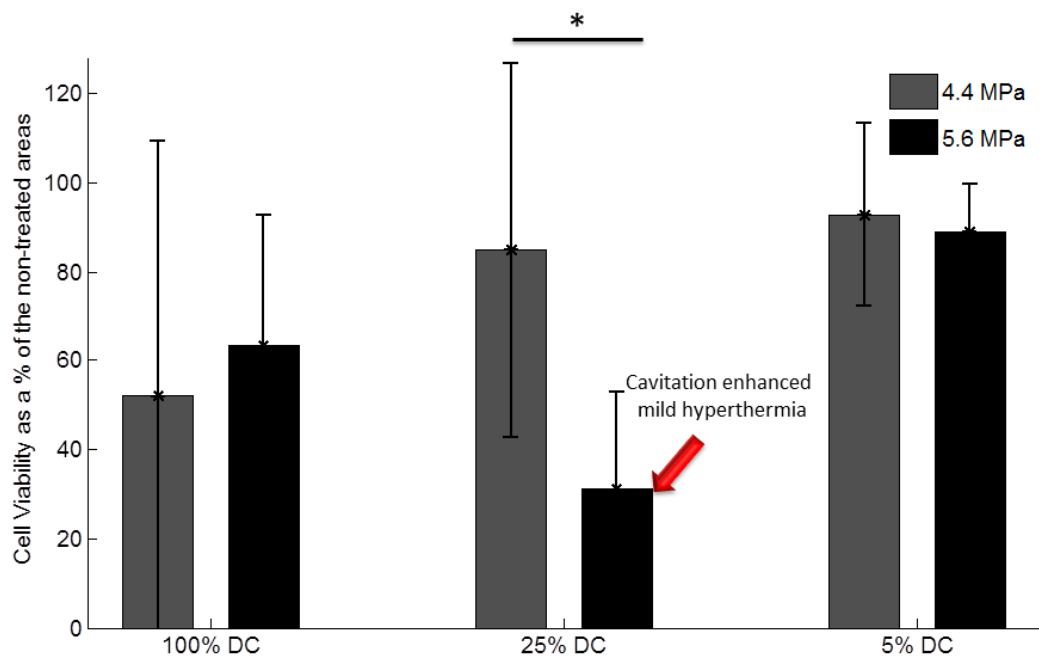


Figure 4.9: Assessment of treatment safety, by measuring cell viability in samples of the TMM without liposomes, 24h after HIFU exposure ($n = 4$). 5% DC exposures cause no heating, thus showing that mechanical effects associated with cavitation activity or acoustic radiation force cause no cell death. The 25% at 5.6 MPa exposure (15 s), which produces the desired levels of hyperthermia with cavitation activity is shown to cause undesirable cell death. The asterisk (*) shows statistical significance ($p < 0.05$)

cles the cell viability results for exposures below and above the cavitation threshold have no statistically significant difference.

As indicated by the arrow in Figure 4.9, the 5.6 MPa, 25%, 15 s exposure caused significant cell death. It is also the only exposure that showed statistically significant difference ($p < 0.05$) in cell viability below and above the cavitation threshold, as indicated by the asterisk (*). It was hypothesized that this loss of cell viability was due to the extensive duration of HIFU exposure and associated heating (15 s). The effect of maintaining a 25% duty cycle whilst reducing the overall exposure duration was thus investigated.

By lowering the total energy delivered by one third and two thirds, complete

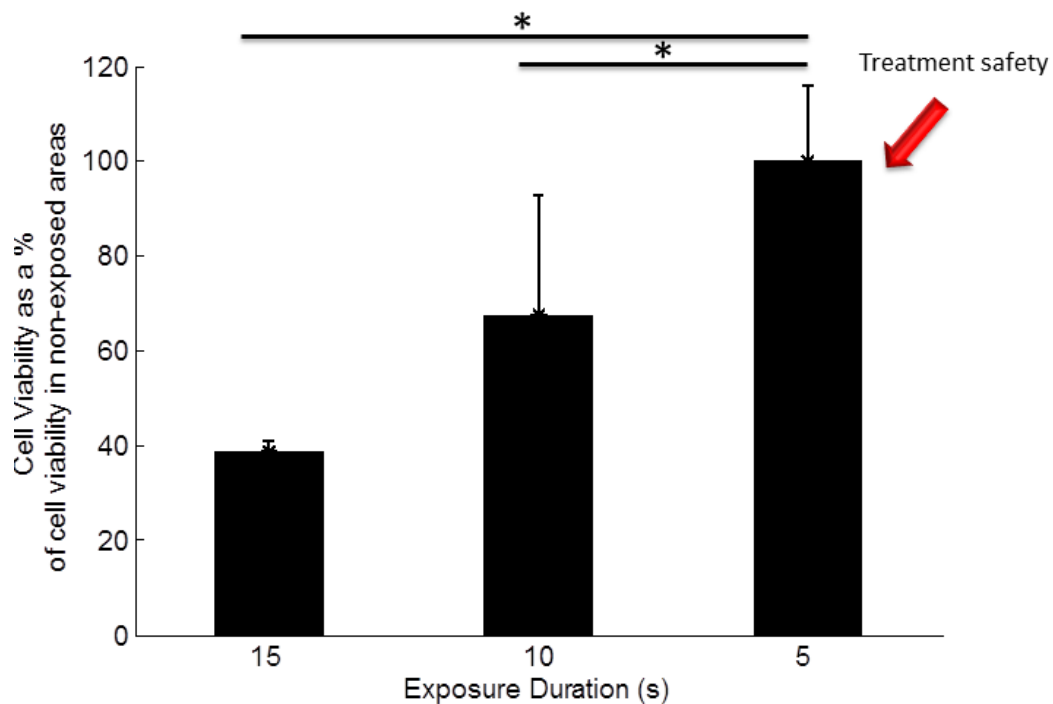


Figure 4.10: Effect of exposure duration on safety of cavitation-enhanced hyperthermia at a 25% duty cycle ($n = 4$). Decrease of the total energy of the exposure by two thirds (by decreasing the duration to 5 s from 15 s) gives 100% cell viability, thus safe cavitation-enhanced mild hyperthermia. The asterisk (*) indicates statistically significant difference (p value < 0.05) in the cell viability achieved.

cell viability was achieved for the desired pressure and duty cycle, as shown in Figure 4.10, in the absence of the liposomes. The asterisks (*) show statistical significance ($p < 0.05$) between the exposure durations indicated by the horizontal lines. Total cell viability in the absence of the liposomes is maintained with the 5 s duration of the 5.6 MPa 25% duty cycle exposure, and exhibits statistically significant difference to both 10 s and 15 s exposures.

4.3.3 Safe Activation of Low-Temperature Sensitive Liposomes with Cavitation-Enhanced Heating

In the previous sections of this chapter the HIFU exposure parameters (5.6 MPa, 25% duty cycle, 1 Hz PRF, 5 s) that induce safe cavitation-enhanced mild hyperther-

mia were identified. In this section, the effect of these exposure conditions on drug release from thermosensitive liposomes is investigated. The liposomal activation caused by cavitation-enhanced mild hyperthermia is compared to the liposomal activation caused by a series of control HIFU exposure conditions. These conditions and the rationale behind their choice are summarised in Table 4.3

Table 4.3: HIFU exposure conditions for assessment of liposomal activation.

	Acoustic Pressure (MPa ^a)	Duty Cycle(%)	PRF (Hz)	Exposure Duration (s)	Acoustic Energy (J)	Desirable Effect
A	2.7	100	-	6	10.94	Mild exposure, mild heating (39°C), no inertial cavitation
B	4.4	25	1	5	6.05	Same exposure regime as (C), but below the cavitation threshold
C	5.6	25	1	5	29.04	Cavitation induced mild hyperthermia
D	4.4	100	-	6	9.8	Ultrasound induced mild hyperthermia

^apeak to peak values

Figure 4.11 shows the qualitative and quantitative data for doxorubicin release from the low-temperature sensitive liposomes that were obtained with fluorescent microscopy for representative samples of the exposure conditions A,B,C and D described in Table 4.3. The control samples that were used are the following: (i) negative control - Huh-7 cells stained with Calcein AM for background fluorescence, (ii) negative control - TMM with Huh-7 cells and liposomes at 37°C for minimum release, and (iii) positive control - high ultrasound exposure (5.6 MPa 15 s continuous wave) for maximum release. As previously mentioned in §4.2.3, live cells stained with calcein appear in green and the inherently fluorescent doxorubicin, which is the active chemotherapeutic agent, appears in red pseudocolour.

When stained cells are in culture medium, background fluorescence is not observed. However, this is not the case when they are embedded in the TMM. The cell and ThermoDox embedding TMM has elevated levels of background fluorescence,

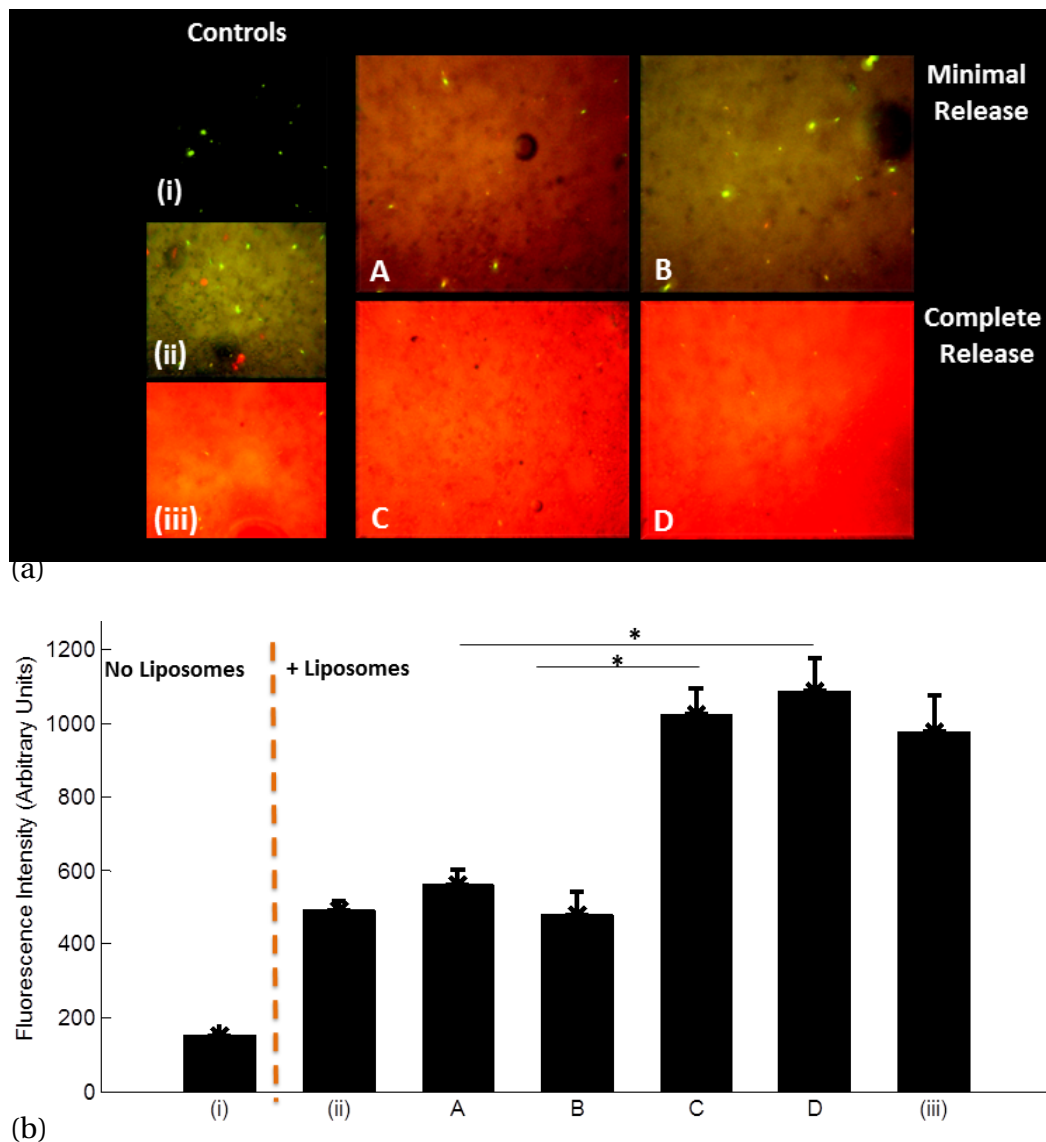


Figure 4.11: (a): Images of Huh-7 cells and ThermoDox-embedding TMM samples after exposure to HIFU taken with fluorescence microscopy.(b): Quantitative data of fluorescence intensity of the samples, for the same exposures ($n = 4$). In both (a) and (b) the control samples are: (i) Huh-7 cells stained with calcein AM (ii) TMM with cells and liposomes at 37°C. (iii) TMM with cells and liposomes after exposure to 5.6 MPa for 15 s. In both (a) and (b) the exposure conditions are; A: 2.7 MPa pp CW 6 s, B: 4.4 MPa pp 25% DC 1 Hz 5 s, C: 5.6 MPa pp 25% DC 1 Hz 5 s, D: 4.4 MPa CW 6 s. The red colour is representative of the activation of the liposomes. It can be observed that for the exposure conditions that cause mild hyperthermia, namely C and D, complete drug release is achieved throughout the whole volume of the samples. In (b) the asterisk (*) indicates statistically significant difference (p value < 0.05) in the fluorescence intensity.

due to autofluorescence of the material, the presence of the calcein AM fluorescent dye and the presence of the liposomes. Even though ThermoDox has been designed so that it does not release its content below 39°C, and doxorubicin is thought not to fluoresce at high concentrations, such as while encapsulated, fluorescence of the liposomes has been observed even at 37°C, both in the TMM or in liquid suspension. Whether this is due to liposomal fluorescence or leakage of the active agent is yet to be investigated.

Condition (A) causes a mild temperature rise of $\sim 2^{\circ}\text{C}$, thus causing minimal increase in fluorescence intensity, without statistically significant difference to the background fluorescence values, as shown in Figure 4.11 (b). Similarly, condition (B), which does not induce temperature rise, does not induce drug release. Maximum drug release is achieved by ultrasound-induced mild hyperthermia (see Figure 4.4). Increasing the applied acoustic pressure and reducing the duty cycle (D), results in cavitation-enhanced mild hyperthermia (see Figure 4.4) and subsequent maximum drug release in the heated volume. The drug release achieved by ultrasound-induced heating does not have statistically significant difference to the drug release achieved by cavitation-enhanced heating and they are both statistically similar to the maximum release control (control iii).

It is suggested that heating is the mechanism that causes drug release from the liposomes and no other mechanical effect of inertial cavitation or ultrasound, because it has been demonstrated in our previous work (Mylonopoulou *et al.*, 2010). The results from these experiments are also presented in Appendix A because they have been conducted in a TMM, with similar composition to the one presented in Chapter 3, but not identical.

The key advantages of cavitation-enhanced drug release are that it requires only one third of the total energy delivered for conventional ultrasound mediated drug

release (see Table 4.3) and that it allows for spatiotemporal monitoring of release at the target region by passive acoustic mapping of cavitation emissions (see §2.6.3). Last but not least, a comparison between drug release caused by exposure regimes (B) and (D) shows that for the same exposure conditions (25% DC, 1 Hz PRF, 5 s duration) below and above the cavitation threshold, no and maximum drug release is induced respectively. If drug release can be passively mapped by mapping cavitation emissions (as will be discussed in Chapter 5), this result implies that absence or presence of cavitation emissions in the target region could be correlated to absence or presence of treatment, even if the exact pressure that reaches the target area is unknown.

4.3.4 Treatment Efficacy

Treatment efficacy is defined as the level of cancer cell death occurring as a result of doxorubicin release by ultrasound. Figure 4.12 shows the level of cell death induced by each of the four ultrasound exposure regimes described in Table 4.3 in the liposome-embedding tissue mimicking material. Low-amplitude ultrasound exposures that do not cause mild hyperthermia (2.7 MPa CW and 4.4 MPa 25% DC) are found to have no effect on cell viability, which was expected on the basis that drug release does not occur for either set of conditions. However, both sets of conditions that are known to induce hypethermic release (4.4 MPa CW and 5.6 MPa 25% DC) result in a 75–80% decrease in cell luminescence intensity.

It should also be noted that exposure regimes that use the same duty cycle (25%), PRF (1 Hz) and exposure duration (5s) but deploy pressure amplitudes above (5.6 MPa) and below (4.4 MPa) the cavitation threshold result in vastly different cell viabilities, with great statistical significance. This is an important finding. In a clinical setting, where the pressure amplitude reaching the target region may not be known,

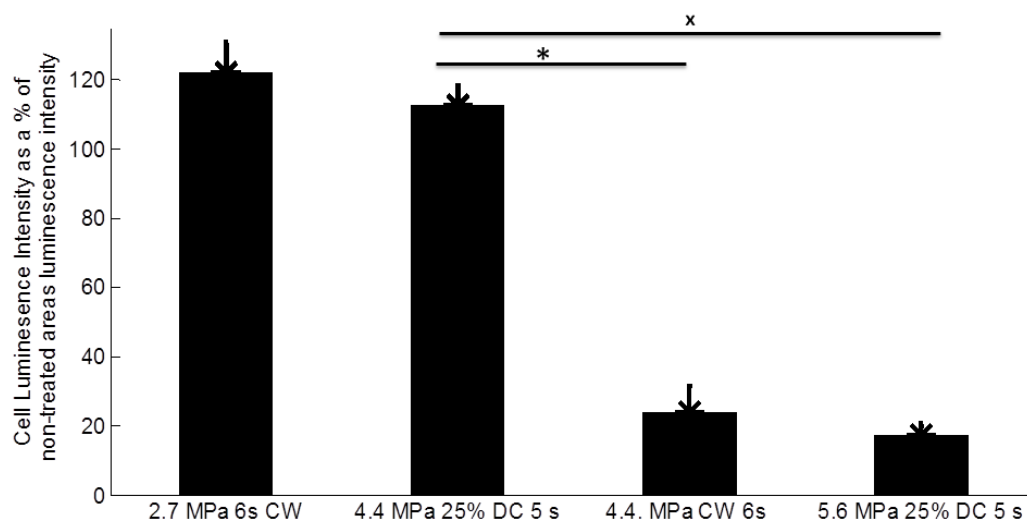


Figure 4.12: Cell Viability in samples of TMM with liposomes, 24h after HIFU exposure with the regimes discussed in Table 4.3 ($n = 4$). The first three exposures are below the cavitation threshold. From them, only the third causes heating at 42°C . The 2.7 MPa exposure produces mild heating (up to 39°C). The last exposure causes cavitation-enhanced mild hyperthermia, and subsequent cell death, due to drug activation. The asterisk (*) indicates statistically significant difference between the 4.4 MPa 25% DC 5 s exposure and the 4.4 MPa 100% DC 6 s exposure. The 'x' indicates statistically significant difference between the 4.4 MPa 25% DC 5 s and the 5.6 MPa 25% DC 5 s exposure. Note how the only difference in these two exposure regimes is the acoustic pressure. 4.4 MPa is below and 5.6 MPa is above the cavitation threshold.

mapping cavitation activity could provide a means of monitoring the location and extent of liposomal activation and subsequent cell death.

4.4 DISCUSSION

It has been shown in this chapter that for 5.6 MPa 5 s HIFU exposure with 25% duty cycle at 1.1 MHz the desired levels of hyperthermia for activation of thermally sensitive liposomes were achieved (Figure 4.4), thus allowing for exploitation of the unique advantages of inertial cavitation; rapid and localized enhanced heating and spatial mapping of the temperature rise by mapping the cavitation activity.

It could be argued that inertial cavitation activity is unsuitable for the purpose of this work, because it is an unpredictable phenomenon, which causes levels of heating that cannot be controlled. However, this issue has been overcome by using exposures with a relatively low duty cycle (25%). In this manner, the major disadvantage of cavitation-enhanced heating, namely unpredictable levels of hyperthermia, was successfully circumvented. As seen in Figure 4.8, the standard deviation of the temperature rise induced at each time-point of the exposure is less than 3°C, falling in the range of mild hyperthermia. This makes the results reproducible.

A comparison of the effect of the suggested HIFU exposure (5.6 MPa 25% duty cycle 5 s) on cell viability in the absence (Figure 4.10) and presence (Figure 4.12) of liposomes in the TMM, shows that the mechanical or thermal effects of inertial cavitation alone did not damage the cells. The combined use of liposomes and adequate levels of ultrasound-induced hyperthermia was in fact a necessary and sufficient condition to induce more than 80% cell death. as seen in Table 4.3.

Figure 4.11 and Figure 4.12 show the liposomal activation and cell death respectively caused by a 4.4 MPa 25% duty cycle, 5 s exposure. When compared to the liposomal activation and cell death caused by the 5.6 MPa 25% duty cycle, 5 s exposure, it can be observed that for exactly the same exposure conditions, with the only difference being that the 4.4 MPa is below and the 5.6 MPa is above the cavitation threshold the effects differ dramatically. This is perhaps the most significant finding of the present study: in the presence of cavitation-enhanced heating, drug release (Figure 4.11) and associated cell death (Figure 4.12) are achieved with just one third of the acoustic energy required for release in the absence of cavitation. In a clinical context, this suggests that release can be achieved successfully and with the possibility of remote monitoring at energy levels that are less likely to cause undesirable side-effects.

A limitation of the present study was that the experiments were conducted in a static environment. The tissue mimicking material did not incorporate vessels to account for the effects of flow, as the case would be in clinical situations. Thus, the effects of heat loss due to perfusion and conduction were not considered (Pennes, 1948) and will be studied further in Chapter 6 using a perfused liver model.

Another issue was the amount of drug release from the liposomes at 37°C, as indicated by the background fluorescence values shown in Figure 4.11. Various factors could affect this release, such as the phantom pH (not measured), leakage from the liposomes or the static environment. However, this does not have a limiting effect in the present study, as the cell viability values were plotted as a% of the cell viability at 37°C.

4.5 CONCLUSIONS

The objective of the work presented in this chapter was the identification and testing of a HIFU exposure regime that induces mild hyperthermia, in the presence of inertial cavitation activity, for thermal activation of the low-temperature sensitive liposomes and total release of their content. Moreover, the objective was to ensure safety and efficacy of this exposure regime for drug release in a clinical setting.

The ultrasound exposure regime that caused cavitation-enhanced heating and thermal release of doxorubicin from the low-temperature sensitive liposomes in a safe and efficient manner was identified as 5.6 Mpa peak to peak 5 s 25% duty cycle at 1 Hz PRF at 1.1 MHz.

With these exposure conditions efficient and safe liposomal activation throughout the treated area has been demonstrated and tissue damage has only been observed due to liposomal activation and not due to any other ultrasound induced

effects alone.



ULTRASOUND-BASED MAPPING OF DRUG RELEASE

In Chapter 4 it was shown that cavitation-enhanced heating can cause safe targeted drug release with only 1/3 of the input acoustic energy needed without cavitation. In this Chapter, passive acoustic mapping of emissions generated *during* sonication are shown to enable spatiotemporal monitoring of drug release. Furthermore, the concentration of released drug is found to be proportional to the acoustic source energy predicted by passive mapping. A potentially clinically applicable method for low-cost real-time monitoring of drug release in a clinical setting is thus developed.

In the previous chapter, cavitation-enhanced heating was shown to have several key advantages over ultrasonic heating: increased heating rates, reduced treatment duration, and a lower input acoustic energy required to achieve temperature rises relevant to drug release. The results thus demonstrated an efficient, safe, and localised drug release method. However, one of the concerns with both conventional ultrasound and cavitation-enhanced heating is the ability to monitor the therapy in real-time, so that under- and overtreatment can be avoided. In cavitation-enhanced heating, unknown temperature rises may be due to the unpredictability of the location, type, and magnitude of cavitation activity, which may be due to two rea-

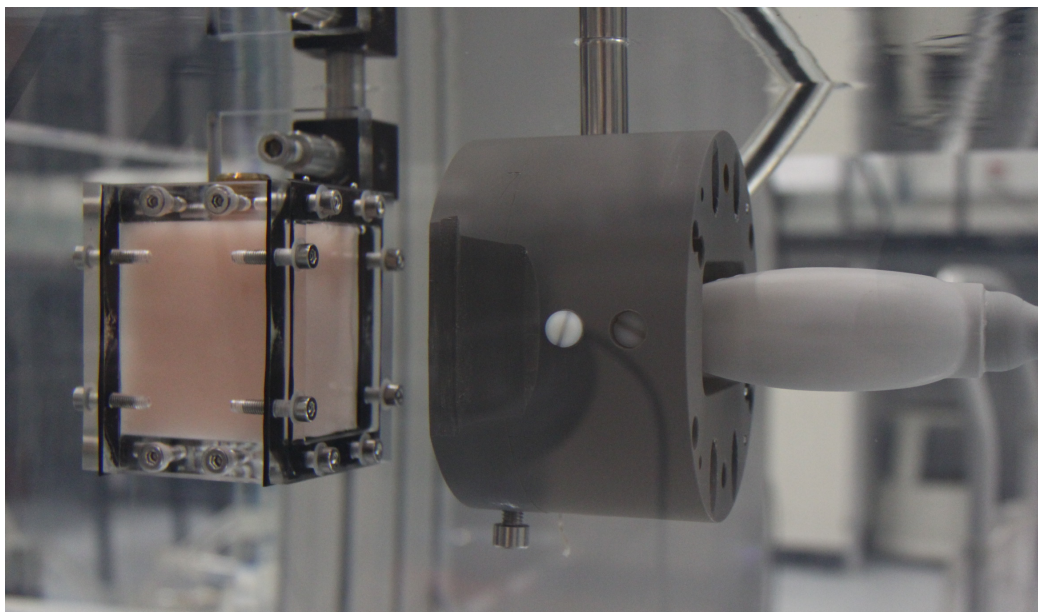


Figure 5.1: Photograph of the HIFU/linear array and TMM submerged in deionized water for a cavitation and drug release mapping experiment. The imaging array is placed in a rectangular hole in the center of the HIFU transducer. The novel TMM is placed in a phantom holder with an acoustically transparent front surface (Mylar). Note that the subtle pink colour is due to the thermally sensitive liposomes (ThermoDox, Celsion Corporation) that are incorporated in the novel TMM.

sons. First, ultrasound may propagate through tissue inhomogeneities and bubbles, thus generating an unknown HIFU pressure field. Second, the pressure cavitation threshold at which inertial cavitation occurs at any given position within the HIFU field may be dependent on local variations in the temperature and cavitation nuclei size distribution and concentration.

In this Chapter, passive acoustic mapping is investigated as a method for spatially and temporally monitoring drug release during treatment. The study was performed using the previously described TMM while acoustic emissions were acquired using a multi-element diagnostic linear array. Passive acoustic maps of cavitation activity generated during therapeutic ultrasound exposure were overlaid to post-exposure fluorescence images of the same TMM slice, thus enabling a direct comparison between the maps and the region over which successful doxorubicin

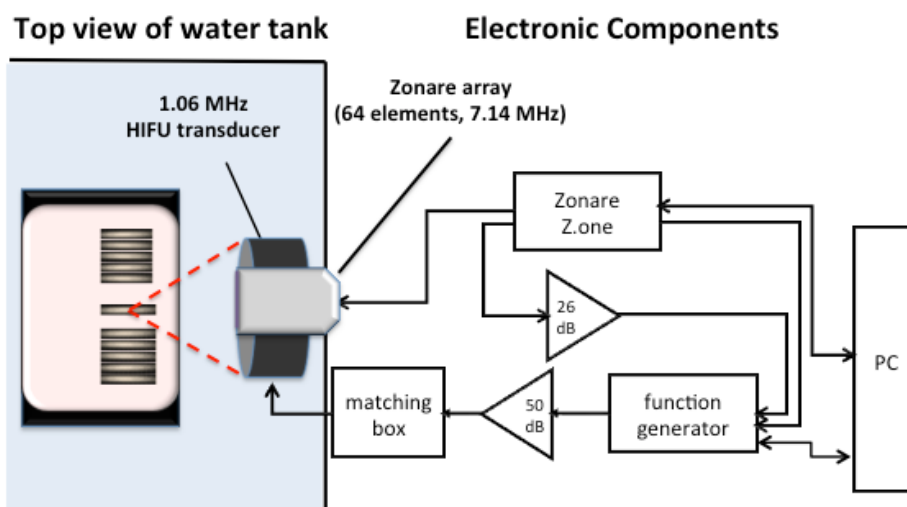


Figure 5.2: Block diagram of the acoustic energy and drug release mapping experimental setup used in this work. The 1.06 MHz HIFU transducer is driven by the signal generator in a range of acoustic pressures that cause temperature rise in the TMM, which is located in front of the HIFU. The acoustic emissions from the HIFU focus are detected with a multi-element linear array positioned in a rectangular hole in the middle of the HIFU transducer. The data was recorded with the Zonare Z.One ultrasound imaging engine and then passed to the PC for further processing. The HIFU was moved with a high-precision 3-D motor system and all experiments were performed in degassed and deionized water at 37°C to mimic normal body temperature.

release from thermosensitive liposomes had occurred.

5.1 EXPERIMENTAL METHODS

An overview of the experimental setup, including the TMM holder, exciting HIFU transducer and coaxial diagnostic array used for passive acoustic mapping is shown in Figure 5.1. A schematic representation of the experimental setup is further presented in Figure 5.2. MATLAB was used to trigger the signal generator that drove the HIFU, the imaging linear array for passive acoustic data acquisition and the high precision 3-D positioning system that controlled the HIFU movement. All experiments were performed in deionised and degassed water at 37°C.

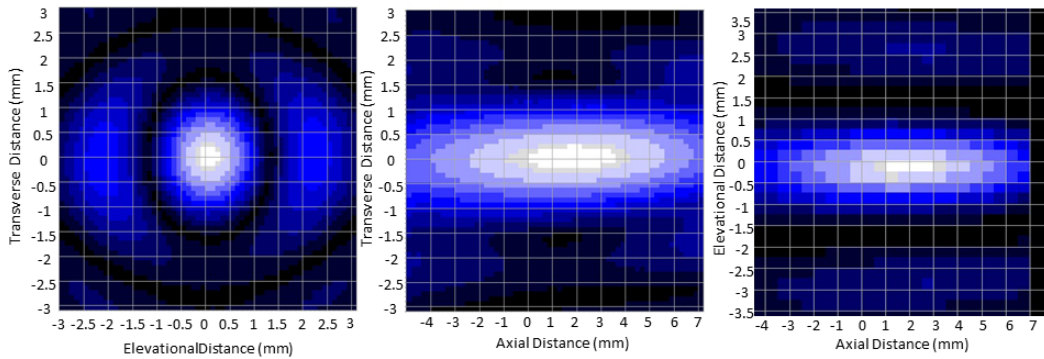


Figure 5.3: Calibration of the 1.06 MHz HIFU transducer. Due to the rectangular hole in the middle, the HIFU focus is not rotationally symmetric and has axial, transverse, and elevational -6 dB focal widths of 11-, 1.4-, and 1.1 mm, respectively.

5.1.1 Ultrasound Generation

Ultrasound was generated with a spherically focused HIFU transducer (H-102B SN22, Sonic Concepts) with a 64.0-mm active aperture diameter and a 60.6-mm focal length. The HIFU transducer used was essentially equivalent to that used in previous chapters, but with a rectangular rather than circular central opening to enable insertion of the diagnostic array. Due to the rectangular opening, the field generated by this HIFU transducer was not axisymmetric about the axis of sound propagation. It was thus fully characterized in water using a calibrated needle hydrophone (SN1060, Onda), with transverse, axial and elevational profiles shown in Figure 5.3. The -6 dB beamwidth was found to be 11 mm axially, 1.4 mm transversely and 1.1 mm in the elevational direction.

Because of the rectangular hole, the transducer had a smaller surface area than the 1.1 MHz transducer that was used in the previous experiments. As a result, the efficiency of the 1.1 MHz transducer was higher than that of the 1.06 MHz transducer. For this reason, the 1.06 MHz transducer was recalibrated, to identify the driving voltages that provided the same acoustic pressures at the HIFU focus as the

previously used transducer. The voltage/pressure relationship was measured as

$$P = 0.031V + 0.0099, \quad (5.1)$$

where P is the acoustic pressure in MPa peak-to-peak and V is the voltage after the signal generator in mV peak-to-peak.

5.1.2 Source Energy Mapping with Linear Array

Throughout each experiment, a 38-mm aperture, 128-element, diagnostic linear array (L10–5, Zonare Medical Systems) was used to acquire both B-mode images and passive acoustic emissions. The linear array was connected to an ultrasound imaging engine (Z.one with Research Package, Zonare Medical Systems) capable of recording 64 channels of IQ data (Kirkhorn, 1999) (7.14 MHz center frequency, 5 MHz bandwidth) simultaneously at a user-specified frame rate. (Mo *et al.*, 2008).

Data acquisition with the Zonare system was designed to capture emissions throughout the entire sonication duration Figure 5.4. Each exposure had $94.6\mu\text{s}$ of data captured at an 80-Hz frame rate and for a total of 438 frames. Meanwhile, each frame consisted of 64 channels of data with a 50-Hz sampling frequency. For exposures consisting of 5 pulses with 25% DC and 1-Hz PRF, the acquisition settings implied the capturing of 20 frames per 250-ms of pulsed sonication ON time. The IQ data were transferred after each exposure to a USB memory device and converted to a Matlab data file for further processing.

Because the axial and lateral resolution of the passive acoustic mapping improves with increasing aperture (§2.6.3.1), the channels selected for data acquisition spanned the entire aperture. However, increasing the distance between any two consecutive recording elements creates grating lobes closer to the main lobe. Thus, elements 2, 4,..., 64, 65, 67,..., 127 were selected as recording channels to en-

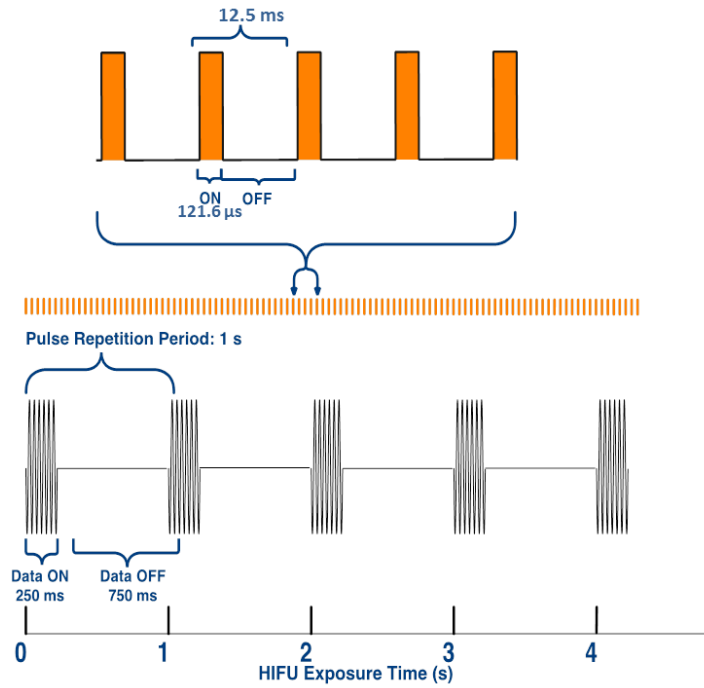


Figure 5.4: Timing of Data Acquisition with the Linear Array. The frame rate is 80 Hz, thus per 1000 ms, which correspond to 1 pulse of the 5-pulse therapeutic exposure, the system acquires 20 frames with acoustic data, and 80 frames without. The frame rate is a trade-off between having the maximum amount of frames in the 250 ms that the pulse is 'ON' and not reaching the limits of the Zonare buffer.

sure that the entire aperture was spanned while any two consecutive recording elements were not spaced further than two elements apart ((Gyongy and Coussios, 2010), (Gyongy *et al.*, 2008)).

During passive acquisition mode, transmission pulses were switched off so that only source emissions generated from the therapy transducer were recorded. The recording itself was in the form of 5-MHz-bandwidth In-phase/Quadrature (IQ) data (Mo *et al.*, 2008), whose central frequency was variable. Among frequency bands available within the US system, 4.64–9.64 MHz was closest to 5–10 MHz and was chosen for the present study.

5.1.3 Experimental Procedure

The objective of this set of experiments was the *spatial* mapping of liposomal activation by mapping the inertial cavitation activity occurring at the HIFU focus. As a result, spatial alignment of the HIFU/Linear Array assembly with the TMM and knowledge of the exact location where each exposure took place in the TMM were of vital importance. In order to ensure the best possible alignment, the following procedure took place before each exposure.

First, the focal point of the HIFU transducer was recorded relative to B-mode images captured with the linear array. In this setup, the HIFU was driven at a low pressure amplitude (DPR 300, JSR) and a hydrophone (HNA 0400, Onda) connected to an oscilloscope (44MXi, UK Lecroy) was used to find the HIFU focal point. At the focal point a B-mode image of the hydrophone was captured and its position relative to the array was recorded. This would be later used as a guide to position the TMM appropriately and cross-reference with the passive acoustic maps (Figure 5.5).

Elevational, axial and lateral alignment of the TMM to the HIFU and linear array complex was then performed. For each exposure condition, a 5x5 sonication raster scan with 1-mm step sizes was performed in the same manner as described in Chapter 4. The TMM holder (dimensions: 50 mm lateral, 50 mm axial, 60 mm elevational with respect to the HIFU), similarly to Chapter 4, had removable thin metal rods fitted to the front wall, which were used for vertical alignment with the HIFU in pulse-echo mode. The central scan row of the 5 sonication scan columns was at the level of the metal rod (which during the experiments was removed), so that 2 scan rows of sonication were above and 2 below.

The HIFU focal point was axially positioned 10 mm into the TMM (as shown

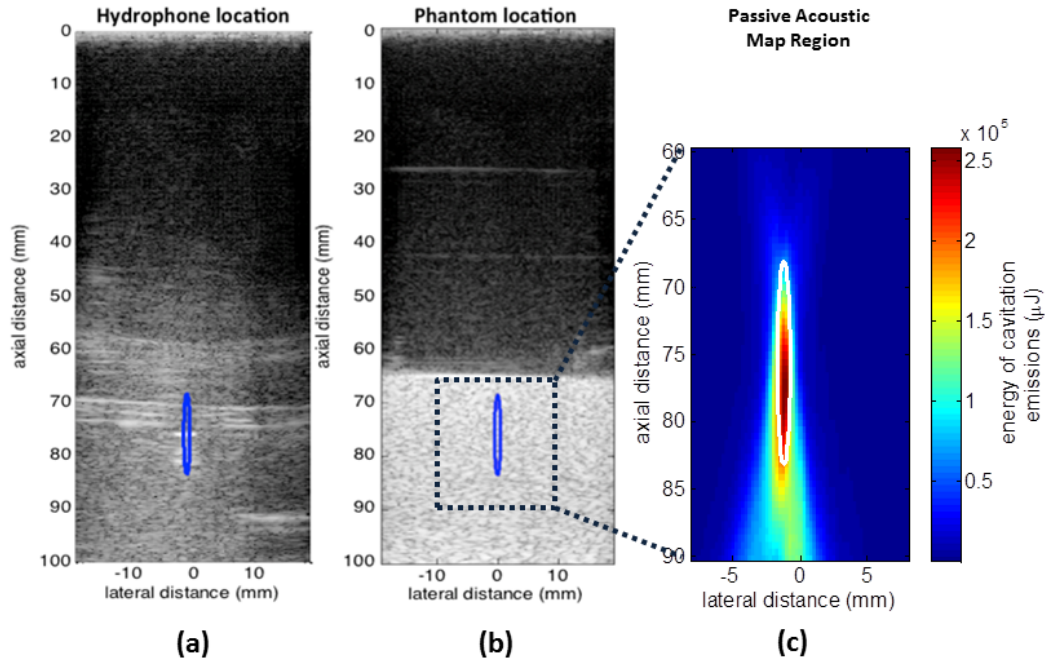


Figure 5.5: B-mode images of the HIFU focal region. In all images the axial distance from the linear array is shown (imaging depth). The image on the left is the B-mode image captured with the hydrophone positioned at the HIFU focus. The hydrophone tip can be distinguished as a bright spot at 75 mm from the surface of the array. The oval shape that is overlapping, is a mathematical representation of the HIFU focus (-6dB focal dimensions), according to the HIFU calibration. The position of the HIFU focal region with respect to the imaging array remains constant, and thus provides guidance as to where the TMM should be placed with respect to the HIFU/array complex. In the B-mode image in the middle, the hydrophone has been replaced by the TMM (bright rectangular region). On the right, a representative passive acoustic map acquired over the region demarcated by the dotted line in the centre pane is shown. The dimensions of this image are the dimensions of the passive acoustic maps (PAMs). By being able to cross-reference the location of the exposure to a PAM, useful information on the spatial distribution of the emitted acoustic energy can be acquired.

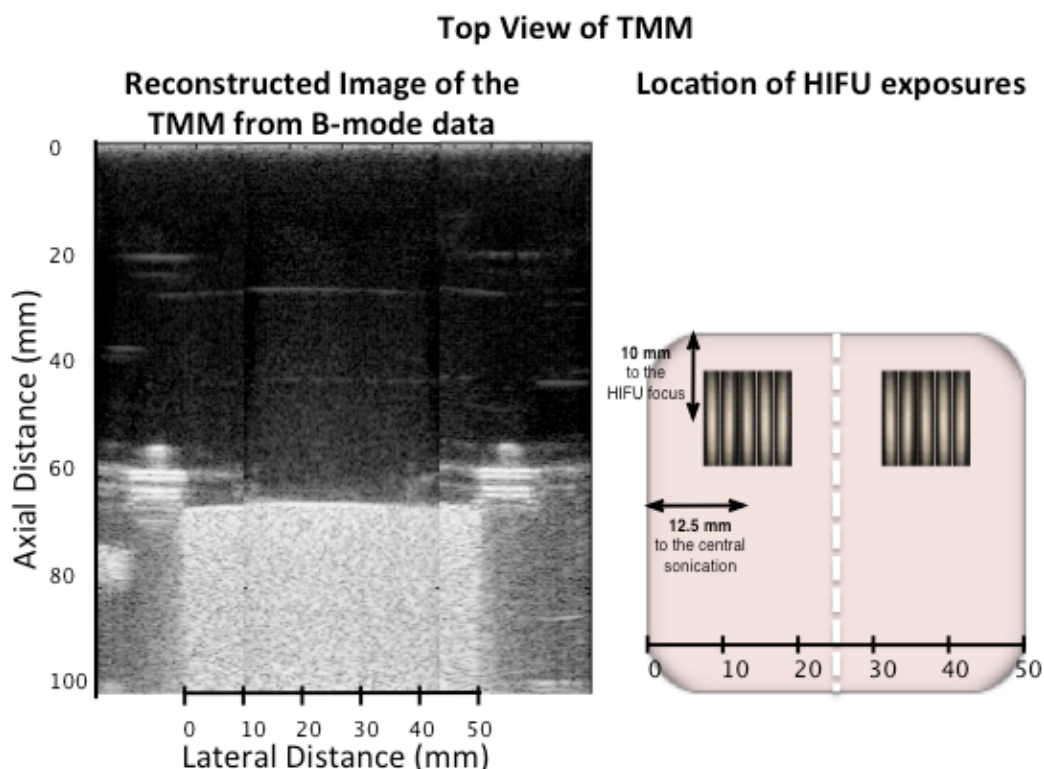


Figure 5.6: Reconstructed B-mode image of the TMM, as seen by the linear array and illustration of the exposure protocol. One 5x5 sonication scan was performed in each half of the TMM. The central HIFU exposure in each 5x5 scan was positioned so that the HIFU focus lay 12.5 mm from the side wall, and 10 mm from the front wall of the TMM.

in Figure 5.5-middle) using B-mode imaging. Lateral alignment was achieved by identifying the sides and the middle of the TMM with B-mode imaging. Two 5x5 sonication raster scans (5 sonications in the lateral and 5 sonications in the elevational plane) were then performed with select exposure conditions. The orientation and dimensions of the exposure regimes are presented in Figure 5.6.

The HIFU transducer insonated the TMM continuously at 1.06 MHz for 6 s at 4.4 MPa pp and in pulsed sequences of 25 % DC for 5 s at 4.4 MPa and 5.6 MPa pp. Also exposures at lower pressures were used to identify any drug release below the cavitation threshold.

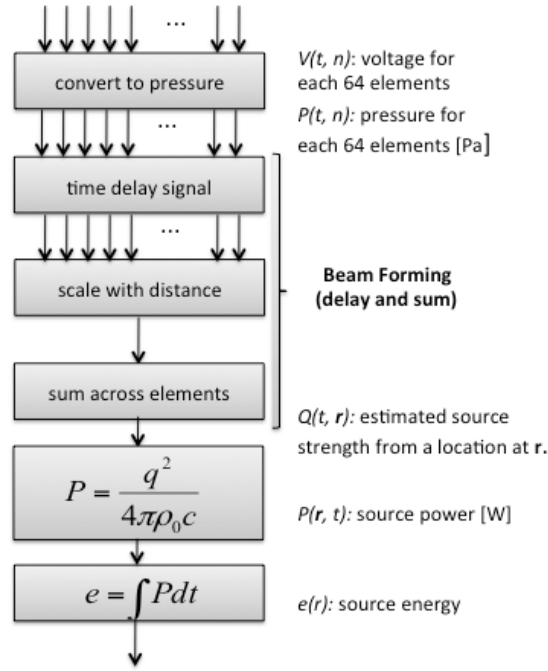


Figure 5.7: Passive acoustic mapping algorithm.

For the scans, Matlab code was used that performed the following steps in series for each one of the 25 sonication points: i) triggered the Zonare system for data acquisition to start, ii) triggered the signal generator to start the exposure, iii) stopped the exposure, iv) stopped the data recording, v) saved the recorded data and vi) moved the HIFU to the next position.

5.1.4 Processing of Acoustic Data

Upon recording, data was transferred to a PC for offline post-processing. The IQ data were converted to RF data upsampled to 200 MHz. The passive mapping algorithm was implemented in MATLAB (Figure 5.7). Passive acoustic maps of 16 mm width and 60 to 90 mm distance from the surface of the linear array were generated for each frame for all the exposures (438 frames per exposure). The 438 maps were then summed into one cumulative map for each exposure set, leading to 25 maps per scan that formed a grid. The 25 maps were overlaid with elevational and

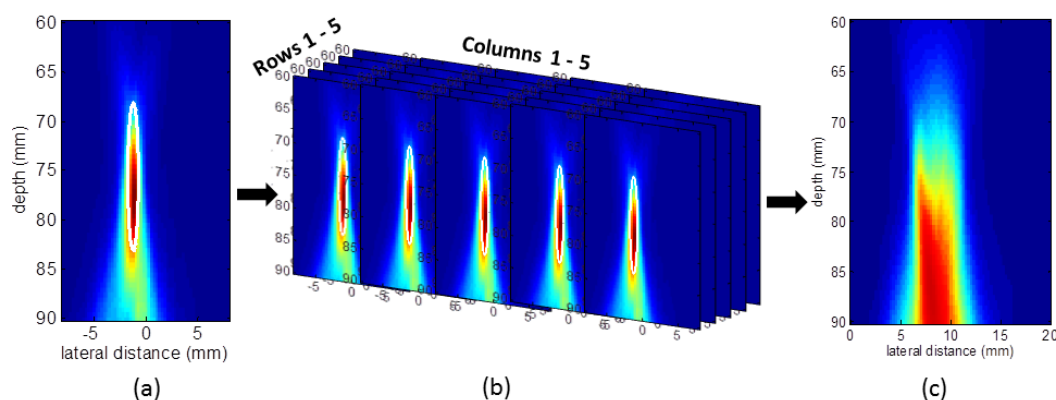


Figure 5.8: Generation of cumulative passive acoustic maps for the 25 sonications that formed each scan. (a) shows the PAM of a single sonication. The white oval shape indicates the location of the HIFU focal region for this exposure. For each exposure condition, a scan of 25 exposures (five rows and five columns) was performed. (b) shows the way the 25 PAMs were overlaid to form a cumulative PAM for the whole scan. The PAMs for each one of the five sonications that formed a column were summed into a cumulative PAM for the column and then were overlaid laterally with the other four columns to form the cumulative map for the scan. Laterally they were overlapped by 1 mm apart, which corresponds to the step by which each sonication was performed. (c) shows an example of the total PAM that is generated for all the sonications.

horizontal (after shifting by 1 to 4 mm according to their position) summation, to generate a map of the total acoustic energy generated from the 5x5 sonication scan. This total acoustic energy map was then compared to the liposomal activation map. (Figure 5.8).

5.1.5 Imaging of Drug Release from the Liposomes

Fluorescence microscopy was utilized to image doxorubicin release from liposomal encapsulation. As previously described, free doxorubicin can be excited at 487 nm to induce emissions at 590 nm. In the present study, the sonicated TMM sections were imaged using fluorescence microscopy (Ti - Eclipse Inverted Microscope, Nikon) and a filter block sensitive to free doxorubicin (C-FL Epi-FL Filter Block N B-1A, Nikon) was used. The filter block was selected to have a narrow passband excita-

tion range (470-490 nm) in order to minimize autofluorescence and photobleaching and increase image contrast. Meanwhile, a highpass filter in the emissions range (520 nm longpass) allowed for high sensitivity to free doxorubicin.

The TMM was sectioned horizontally at the centre of the 25-exposure scans, which formed an axial-lateral plane. Two 25 cm², 2-mm thick sections were acquired, above and below the central level for each exposure condition. Each 25 cm² sample was scanned with a 1X objective and a two-dimensional motorized stage with auto-focus capability. Captured fluorescence data were stitched into a single large image. The two images corresponding to each scan were then overlaid in Matlab to give an image of the total fluorescence intensity produced per ultrasound scan. Although the objective was calibrated before each experiment and 25 pixel overlap was used, the stitched large image had an artifact of lower fluorescence intensity along the edges of the individual images that were stitched, which was taken into account. The same fluorescence intensity settings were used for all the images acquired and post-processing of the images was done with Nikon proprietary software (NIS Elements Software, Nikon) and Matlab.

5.2 RESULTS AND DISCUSSION

5.2.1 Imaging of Drug Release from the Liposomes

Samples of fluorescence images of two TMM sections are shown in Figure 5.9. The images correspond to 2-mm-thick slices and are orientated such that the HIFU beam is coming from the top, as illustrated in Figure 5.6. The increase in fluorescence intensity corresponds to doxorubicin released from encapsulation. In (A) and (D), the TMM was exposed to 4.4 MPa peak-to-peak with a 25% DC for 5 s and a 100% DC for 6 s, respectively. In (A), no fluorescence increase was observed,

whereas in (D), fluorescence increase was observed throughout a large region. This is in good agreement to results shown in Chapter 4 for the same exposures. In (B) and (C), the exposure conditions were the same, namely 5.6-MPa peak-to-peak pressure, 25%-DC, 1-Hz PRF and 5-s duration. Although the exposure parameters were the same, the amount and distribution of fluorescence increase differed.

The square shapes with dark contours observed throughout the large image are imaging artifacts. These artifacts are from individual images acquired with a 1X lens that were stitched together to form the large image of the entire TMM slice. Using a different objective (4X) or more than 25 pixel overlap resulted in the same issue and is speculated that it is caused by a nonuniform fluorescence illumination. However, this artifact was taken into account during post-processing. Both images are depicted using the same absolute colourbar range to allow for interslice comparative analysis. In addition, an interesting observation was that in the portion of the focal volume proximal to the transducer, a dark pattern on the TMM was observed, which appeared to be erosion of the agar. At 4X magnification, brightfield images were captured and depicted in Figure 5.10. Although the cause for this pattern is unknown, it could be attributed to displacement of the glass microspheres in the TMM due to radiation force.

The images in Figure 5.11 were processed in Matlab (see §5.1.5) and depicted in arbitrary fluorescence intensity units. Each image corresponded to a magnified view of the sonicated region in Figure 5.9 and corresponded to the orientation and dimensions of the generated PAMs. These images were generated by summing the fluorescence intensity of slices above and below the sectioned level of the TMM. Hence, the total drug release from the liposomes caused by each raster scan could be directly compared to the total energy of acoustic emissions.

The axial distance on the images is the distance from the front wall of the TMM

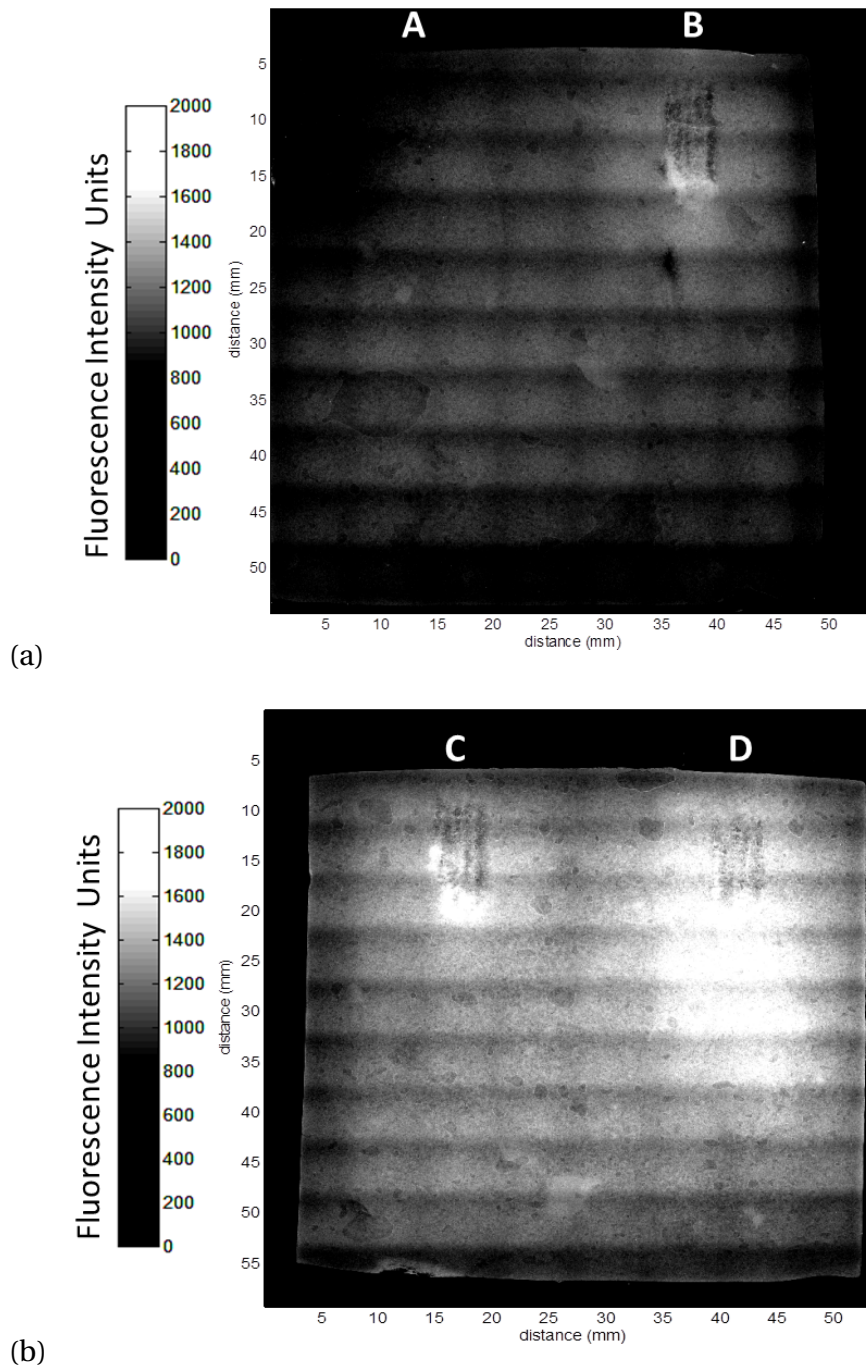


Figure 5.9: Images of drug release in samples of TMM slices after HIFU scans. Increase in fluorescence intensity indicates drug release from the liposomes. In this orientation the HIFU beam is coming from the top with the HIFU focus 10 mm inside the TMM and the scans are centered 12.5 mm from the side walls of the TMM. (a) shows drug release caused by two HIFU scans at 4.4 MPa peak-to-peak (A) and 5.6 MPa peak-to-peak (B) with 25% DC, 1 Hz PRF and 5 s exposure duration. (b) shows drug release caused by a HIFU scan at 5.6 MPa peak-to-peak, 25 DC, 1 Hz PRF and 5 s duration (C) and 4.4 MPa 6 s continuous wave (D).

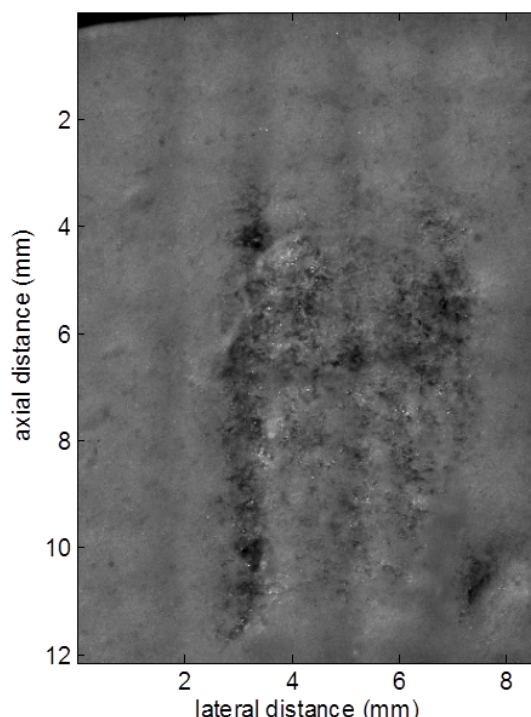


Figure 5.10: Brightfield image with 4x magnification of the region in the TMM where the raster scan was performed. In this orientation, the top of the image corresponds to the front wall of the TMM (closest to the HIFU) and the axial distance refers to the distance from the front wall. In the middle of the image a distortion of the TMM can be observed and five lines of displacement can be distinguished, 1 mm apart, at locations that overlap with the five adjacent HIFU sonication locations. this indicates that there may have been prefocal damage to the TMM

and the lateral distance is the width of the PAMs. Similarly to the PAMs, the fluorescence images have been centered so that the five adjacent sonications are in the middle. The position of the axial HIFU profile is shown on the right for convenience. The exposure conditions in (A) are 4.4 MPa peak-to-peak, 25% DC, 1 Hz PRF and 5 s duration. It is in agreement with the results of Chapter 4, where it was shown that for these sonication parameters temperature rise (Figure 4.5) and drug release (Figure 4.11) was minimal. (B) and (C) have the same exposure conditions, namely 5.6 MPa peak-to-peak, 25% DC, 1 Hz PRF and 5 s duration. In the previous Chapter it was shown that with these exposure conditions cavitation-enhanced mild hyperthermia was achieved. It can be observed that in both (B) and (C) drug release was

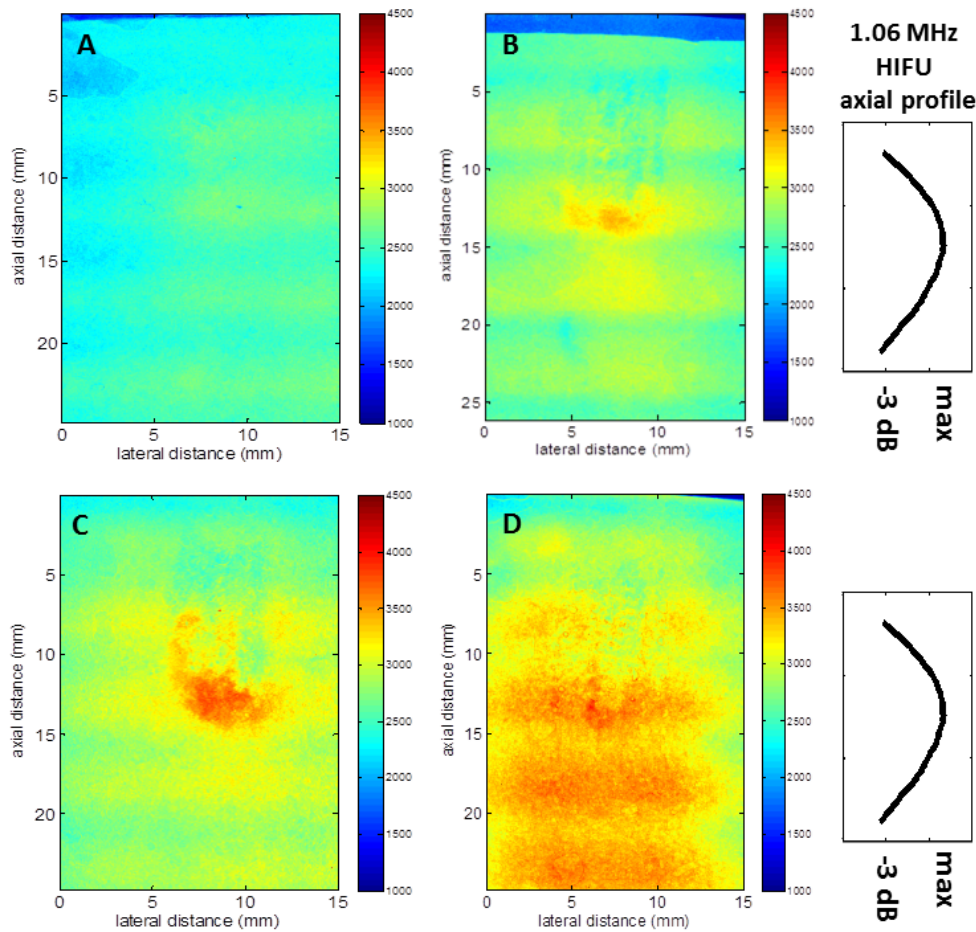


Figure 5.11: Fluorescence intensity of TMM slices after the 25- HIFU sonication scans. The colormap represents arbitrary fluorescence intensity units as measured with the microscope. The dimensions and orientation of each image is the same as in the PAMs. (A) is 4.4 MPa peak-to-peak, 25% DC, 1 Hz PRF and 5 s duration (B) and (C) are 5.6 MPa peak-to-peak, 25% DC, 1 Hz PRF and 5 s duration, and (D) is 4.4 MPa peak-to-peak, 100 % DC and 6 s duration.

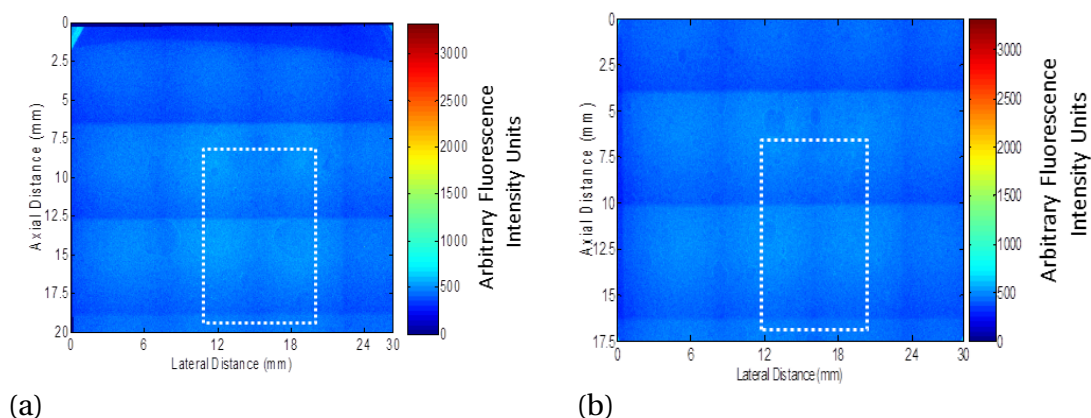


Figure 5.12: Fluorescence intensity of TMM slices after 25-sonication scans at 2.9 MPa continuous wave (a) and 4.12 MPa 25% DC, 1 Hz PRF, 5 s (b). The rectangles indicate the orientation of the five sonications at the level of the TMM dissection.

achieved, but that different free doxorubicin concentrations were achieved. Maximum fluorescence was observed at the peak of the HIFU beam in both cases. (D) shows the release for 4.4 MPa peak-to-peak 6 s continuous wave exposure.

Exposures at lower amplitudes did not cause any drug release as seen in Figure 5.12. A 25-sonication raster scan was performed in this case as well, at 2.9 MPa peak-to-peak pressure and 6 s continuous wave (a) and 4.12 MPa peak-to-peak 25%-DC, 1-Hz PRF, 5-s (b). The background fluorescence is lower than in Figure 5.9, because a different slicing method was used in this case and the sample thickness was 1.7 mm instead of 2 mm.

5.2.2 Passive Acoustic Mapping

As described in §5.1.4, total passive acoustic maps were acquired for the 25 sonications that were performed for each exposure condition. The results are shown in Figure 5.13. Exposure (A) showed the lowest amount of acoustic emissions and exposure (D) the maximum. Even though in both cases the acoustic pressure used was the same, namely 4.4 MPa peak-to-peak, the total energy delivered in (D) is five

times more than in (A) (116.16J and 24.2J respectively), because of the different exposure duration.

The exposure conditions of (B) and (C) were the same (total energy delivered is 39.2J), but the energy of the acoustic emissions generated at the target area was different. Being above the cavitation threshold of the TMM (Chapter 3), 5.6 MPa can be expected to cause inertial cavitation activity events. Inertial cavitation is a stochastic phenomenon, hence difference in level and distribution of the acoustic emissions that are produced is expected. This further emphasises the importance of real-time monitoring of acoustic emissions as a true predictor of cavitation-enhanced heating.

A limitation of this method is that it does not account for heat and doxorubicin diffusion. When a bubble collapses at a specific location, generating the desired temperature rise, the surrounding region is heated as well, due to heat diffusion. As shown in Figure 3.21, a peak temperature of 45°C in the TMM causes temperature elevation above 40°C in a region of 1 mm radius, hence causing drug release in that region as well. In addition any drug that has been released diffuses in the adjacent region.

At the PAMs for the 5.6 MPa peak-to-peak exposures (slices (B) and (C)) a spatial bias of the acoustic energy received can be observed. There is higher amount of acoustic energy generated from the sonications on the left. As previously mentioned, the 25-sonication raster scan was performed so that 5 sonications were made 1 mm apart in each plane, from left to right. Thus the sonication on the left (as shown in the PAM) was performed first. Hence, the shift of the acoustic energy on the left-hand side implies higher amount of acoustic emissions from the first exposure in each plane.

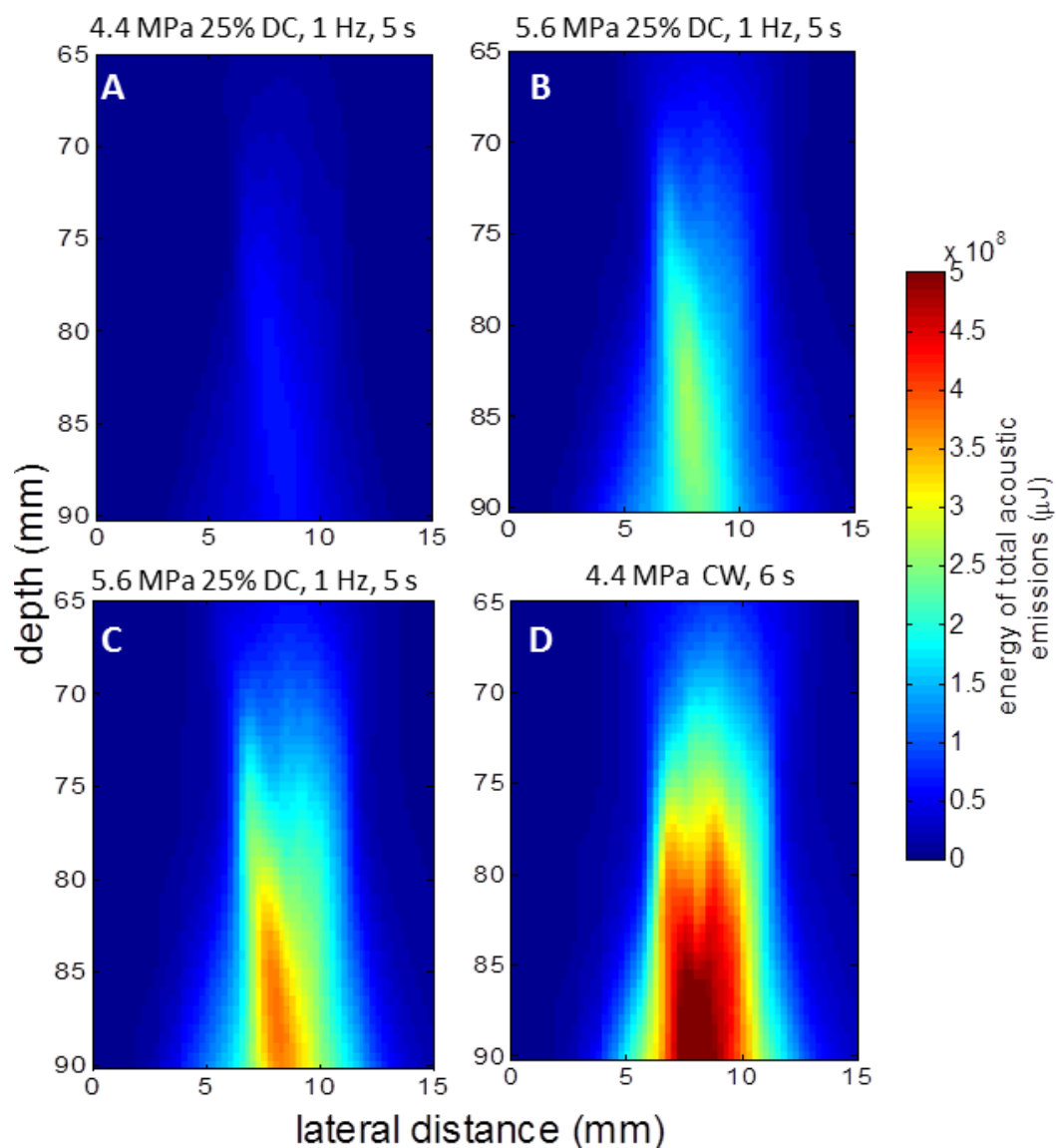


Figure 5.13: Cumulative passive acoustic maps for the scanned regions. For all three sets of exposure conditions investigated: 4.4 MPa 25% duty cycle for 5s, 5.6 MPa 25% DC for 5 s (two repeats), 4.4 MPa CW for 6 s.

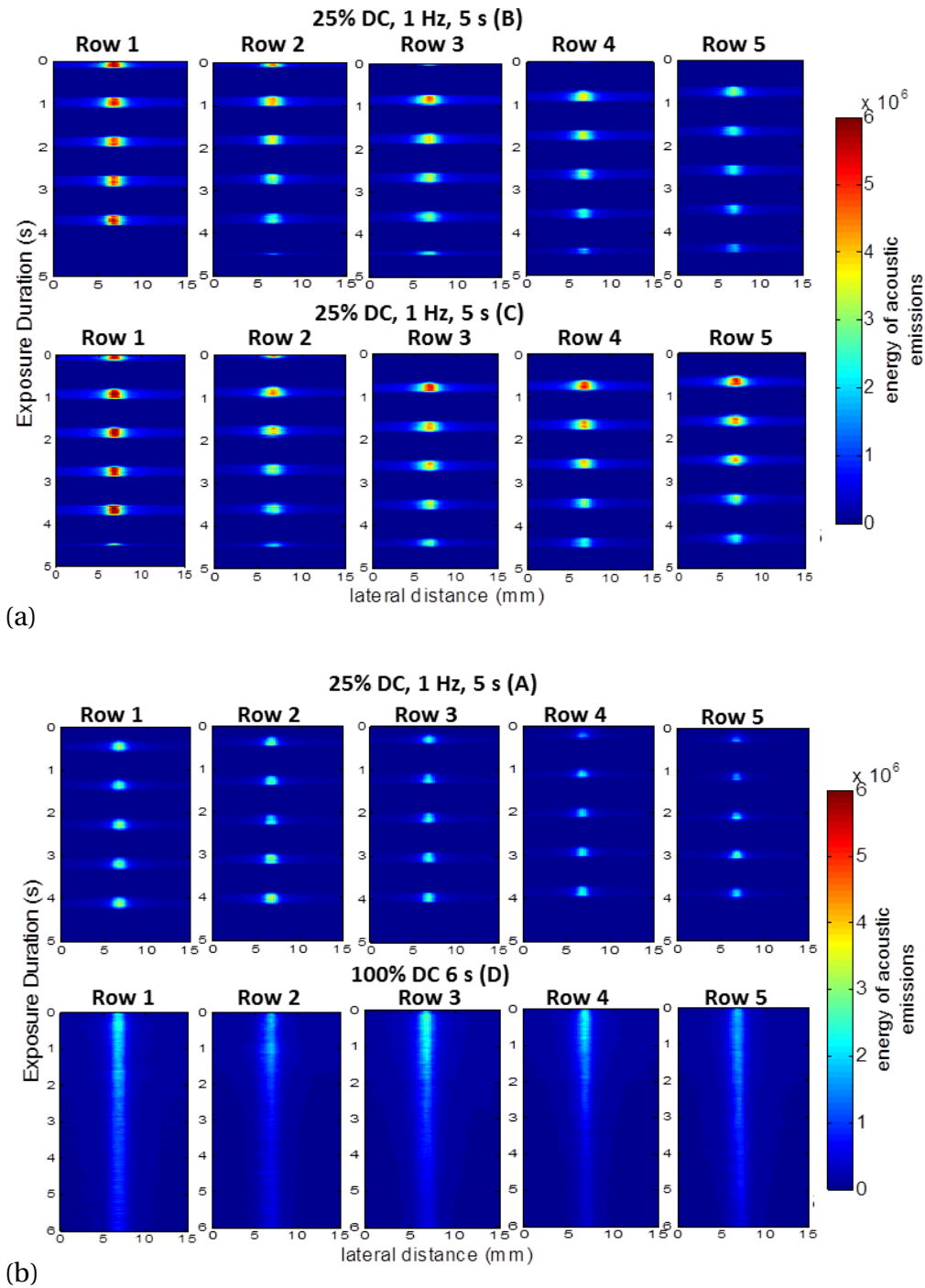


Figure 5.14: Evolution of the total acoustic energy emitted from the distance in the TMM where the maximum of the HIFU focus is, in time, summed for all the five sonications that constitute one row of the 25-sonication scan. The acoustic pressure in (a) is 5.6 MPa peak-to-peak and the acoustic pressure in (b) is 4.4 MPa peak to peak. The acoustic emissions generated in row 1 are higher than in the other four rows at 5.6 MPa, resulting in spatial bias of the emissions seen in the passive acoustic maps.

In order to have a better understanding of this effect, the temporal evolution of the cumulative acoustic emissions generated at the HIFU focus for each column (five sonications in the elevational plane with respect to the HIFU transducer) of sonications is presented in Figure 5.14. The (A), (B), (C) and (D) notation corresponds to Figure 5.11 and Figure 5.13. The data that is presented is the evolution of the total acoustic energy generated at the HIFU focal point summed for all the five sonications that constitute one column of the 25-sonication scan. The acoustic pressure in (a) is 5.6 MPa peak-to-peak and the acoustic pressure in (b) is 4.4 MPa peak to peak.

In both cases in (a), where the acoustic pressure is 5.6 MPa peak-to-peak, the energy of the emissions generated in the first row is higher than in the other four rows, in agreement with the spatial bias observed in the respective PAMs. On the contrary, as seen in (b), for the lower pressure (4.4 MPa peak-to-peak), there is no such bias both for the pulsed (A) and the continuous wave (D) exposure. In all cases the acoustic emissions have higher energy during the first pulse (or first seconds in the case of the continuous wave), but then gradually decrease.

The frequency content of these exposures is shown in Figure 5.15. It can be observed that even at 4.4 MPa pp the level of harmonics is minimal compared to broadband emissions. Therefore, filtering out the harmonics is unnecessary, and may even degrade the signal, since stable cavitation also has a harmonic component. With the 1.06 MHz HIFU transducer that was used for the experiments presented in this Chapter, the cavitation threshold of the TMM appears to be lower than what was found in Chapter 3, as there is broadband noise present in all cases, even at 4.4 MPa ppk indicating events of inertial cavitation activity. As expected, at 5.6 MPa ((b) and (c)) the level of broadband noise is higher than at 4.4 MPa ((a) and (d)). The fact that the level of broadband noise emissions in (b) is higher than (c), is

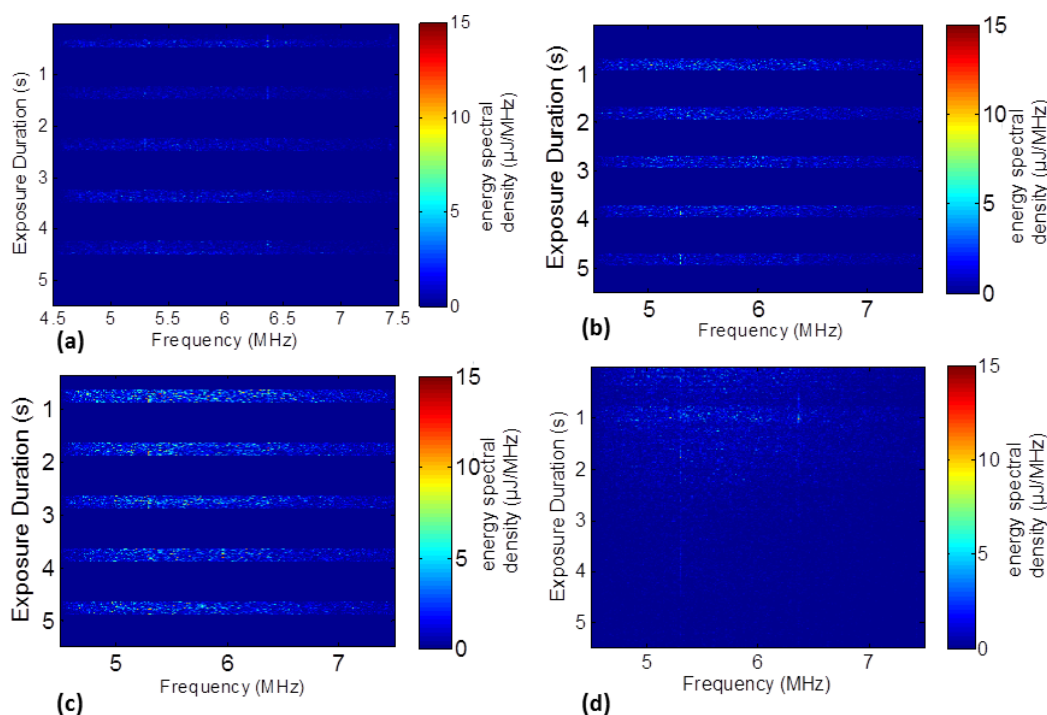


Figure 5.15: Representative spectrograms of sonications from each one of the four scans. The exposure parameters in (a) are 4.4 MPa peak-to-peak 25% DC, 1 Hz PRF and 5 s duration, the exposure in (b) and (c) is 5.6 MPa peak-to-peak 25% DC, 1 Hz PRF and 5 s duration and the exposure in (d) is 4.4 MPa 100% DC for 6 s. Broadband noise emissions that are indicative of inertial cavitation activity are present in all cases, but more pronounced in (b) and (c).

in agreement with the results shown in Figure 5.13 and Figure 5.14.

5.2.3 Comparison of the Spatial Distribution of Drug Release with the Passive Acoustic Maps.

In Figure 5.16 the spatial maps of drug release from the liposomes and of energy of acoustic emissions that were shown in Figure 5.11 and Figure 5.13 respectively are shown together for reasons of comparison. Also, in Table 5.1 the major characteristics of the exposures under discussion are presented, to facilitate the reader. Several important observations can be made.

Table 5.1: HIFU exposure characteristics.

	Acoustic Pressure (MPa ^a)	Duty Cycle(%)	PRF (Hz)	Exposure Duration (s)	Acoustic Energy (J)	Temperature Rise
A	4.4	25	1	5	6.05	Minimal heating (39°C), same exposure as B and C but at a pressure close to the cavitation threshold
B	5.6	25	1	5	9.8	Cavitation-enhanced mild hyperthermia
C	5.6	25	1	5	9.8	Cavitation-enhanced mild hyperthermia
D	4.4	100	-	6	29.04	Ultrasound induced mild hyperthermia

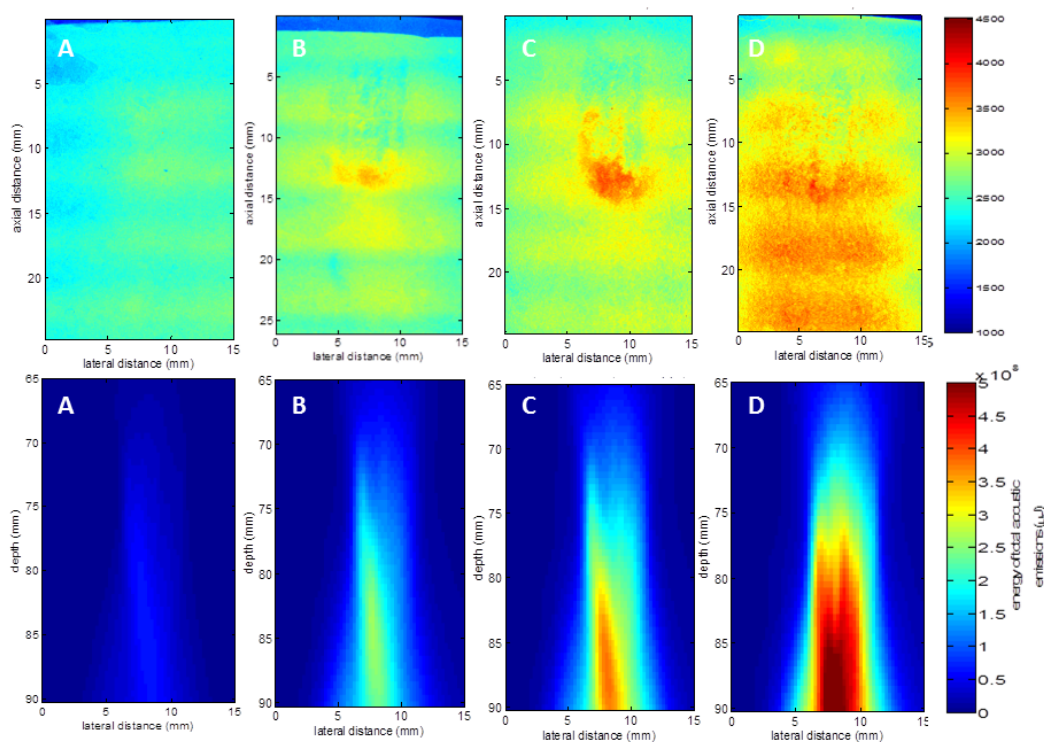
^apeak to peak values

Figure 5.16: Spatial comparison of PAMs and fluorescence maps for the four cases described in Table 5.1. The dimensions and orientation is the same in all maps.

Correlation of doxorubicin release from the liposomes with temperature rise. As expected, there is correlation of doxorubicin release from the liposomes with temperature rise. Exposure A causes minimal heating, and thus no drug release, whereas exposures B, C and D that cause heating in the mild hyperthermia range, either

cavitation-enhanced (B,C) or ultrasound-induced (D), cause drug release from the liposomes as well.

Correlation of doxorubicin release with acoustic emissions. A strong correlation is observed between the acoustic emissions depicted in the PAMs and the fluorescence intensity of the TMM slices. From slice A to D the energy of the acoustic emissions is increasing, and so is the drug release. Interestingly, slices B and C present different magnitude of acoustic emissions *and* also different amount of drug release, even though they were exposed to exactly the same HIFU conditions. As previously mentioned, inertial cavitation is a stochastic process, so the same exposure conditions may result in different levels of cavitation, or in cavitation occurring at different locations in the HIFU focus. As a result, the exact same ultrasound exposure may result in a different amount and in a different spatial pattern of heating and consequently drug release. Having a method for obtaining information on the energy levels of each emission and their location and correlating this information to the observed drug release, instead of relying only on information on the exposure conditions and the HIFU focus location, essentially circumvents the unpredictability of cavitation activity, thus addressing one of the major drawbacks of this phenomenon.

In this case it is shown for the first time that with the passive acoustic mapping method, the amount of acoustic emissions caused by inertial cavitation activity at the HIFU focal region can be directly linked to the amount of localised drug release. The fact that passive acoustic mapping can be carried out in real time during HIFU exposure could help address two of the major challenges in current clinical drug delivery, namely (i) knowing when the drug has been successfully delivered and (ii) knowing where the drug has been successfully delivered. PAM could thus provide a clinically useful tool to avoid both under- and over treatment in the context of drug

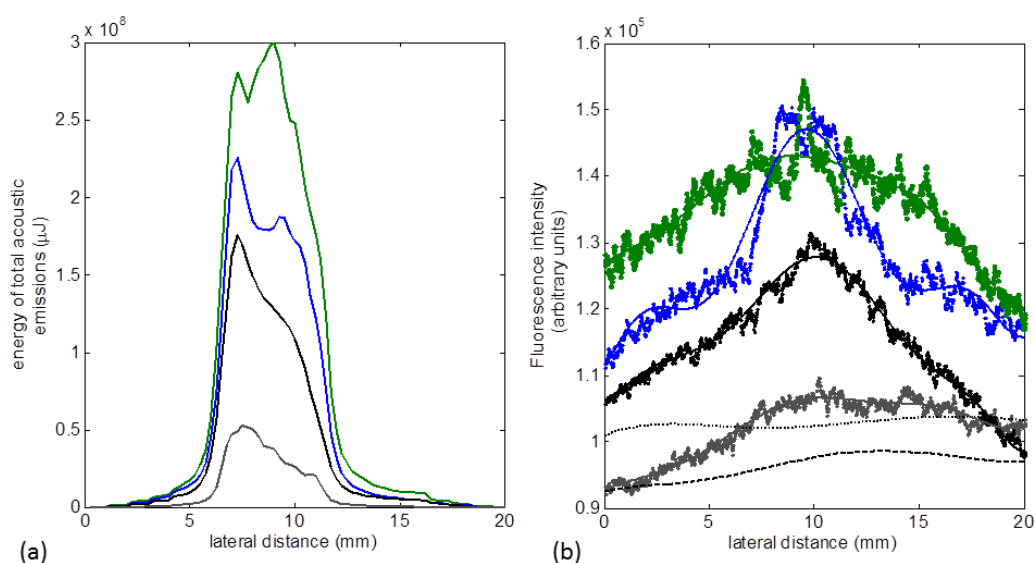


Figure 5.17: Comparison of energy of acoustic emissions and fluorescence intensity at the HIFU focus for exposures A (grey), B (black), C (blue) and D (green). The energy of acoustic emissions generated at the HIFU focus is shown in (a) and the fluorescence intensity is shown in (b). The dashed line represents the background fluorescence for slices A and B and the dotted line represents the background fluorescence level for slices C and D.

delivery by ultrasound.

In Figure 5.17 the energy of acoustic emissions and fluorescence intensity at the HIFU focus is shown, further supporting this hypothesis. Because the axial resolution of the PAM is $500\ \mu\text{m}$ and the axial resolution of the microscope images is $12.87\ \mu\text{m}/\text{pixel}$, the fluorescence intensity of 39 pixels was summed in order to enable a direct comparison.

The magnitude of fluorescence intensity corresponds well to the magnitude of acoustic emissions. Increase in the total input acoustic energy does not result in increased therapeutic effects, as the total input acoustic energy for B and C is one third of the input acoustic energy of D (39.2 J and 116.16 J respectively).

The fluorescence intensity profiles are broader than the acoustic emission profiles in all cases, which is expected for two reasons. First, when the focal volume

is heated with each sonication, the surrounding area is heated as well due to heat diffusion, which can result in minor drug release from the liposomes in the broader region, leading to increase in fluorescence intensity. It was shown that for exposures that cause mild hyperthermia at the HIFU focus, the temperature exceeds 39°C for a volume of 2 mm in diameter laterally with respect to the HIFU focus. Additionally, doxorubicin, being a macromolecule (molecular weight: 580 Daltons), can be expected to diffuse to the TMM portion surrounding the focal regions, where it was initially released.

Spatial mapping of drug release with passive acoustic emissions. Good agreement between the passive acoustic maps and the fluorescence increase maps is observed. For exposure A, there are minor acoustic emissions and minor fluorescence increase as seen in Figure 5.16. Higher levels of acoustic emissions are observed in exposures B and C, which is expected due to the higher acoustic pressure, and higher levels of fluorescence intensity are observed as well.

In both B and C, the maximum of the fluorescence intensity is observed at the axial distance from the HIFU where the acoustic emission energy presents a sudden increase. As previously discussed, the acoustic emissions generated from exposure conditions B and C present a spatial bias towards the side of the first column of sonications of the raster scan. The same spatial bias can be observed in the fluorescence intensity maps as well. In both cases drug release is mostly contained in the region of the focal volumes of the 25 sonications.

Fluorescence increase is present laterally, due to heat and drug diffusivity, but at a lower level than in the region of the sonications. Even though the lateral drug distribution is broader than the distribution of the acoustic emissions, the fact that intensity and spatial features of the PAMs match to intensity and spatial features of the

fluorescence maps, indicates that monitoring of drug release from low-temperature sensitive liposomes caused by cavitation-enhanced mild hyperthermia by monitoring the respective acoustic emissions is feasible. In Figure 5.18, the PAM for slice B is overlaid on the respective fluorescence map, showing agreement between the two.

It should be noted here, that what appears to be high energy of acoustic emissions on the PAMs at a distance of 85 mm from the focus and further, is caused by an artifact in the PAM algorithm, and does not correspond to actual high levels of acoustic emissions. This artifact appears as a 'tail' of the emission energy generated at the HIFU focus at every sonication. Summation of the energy from all the sonications generated at the focus that gives the cumulative acoustic maps causes amplification of the artefact. More accurate passive beamforming algorithms, such as the robust capon beamformer (Coviello *et al.*, 2010) are currently under development to enable reduction of this artefact.

Slice D however, that shows drug release for ultrasound-induced hyperthermia at a much lower level of inertial cavitation activity presents a different case. The ultrasound exposures that are employed here require triple the input acoustic energy than cases B and C to achieve drug release, because it is necessary to achieve the desired levels of hyperthermia (Chapter 4). The fluorescence map that corresponds to these exposures, implies an almost homogeneous distribution of the drug that cannot be mapped to the region of the acoustic emissions. This indicates that drug-release in the absence, or more accurately in the mild presence of inertial-cavitation activity, is not as accurately mapped to the acoustic emissions generated at the HIFU focus. A comparison of slices B and C to D shows that in the present case better spatial mapping of drug release to acoustic emissions may be achieved at a lower input acoustic energy when the pressure amplitude is higher.

In this chapter, monitoring of acoustic emissions has been used as a method

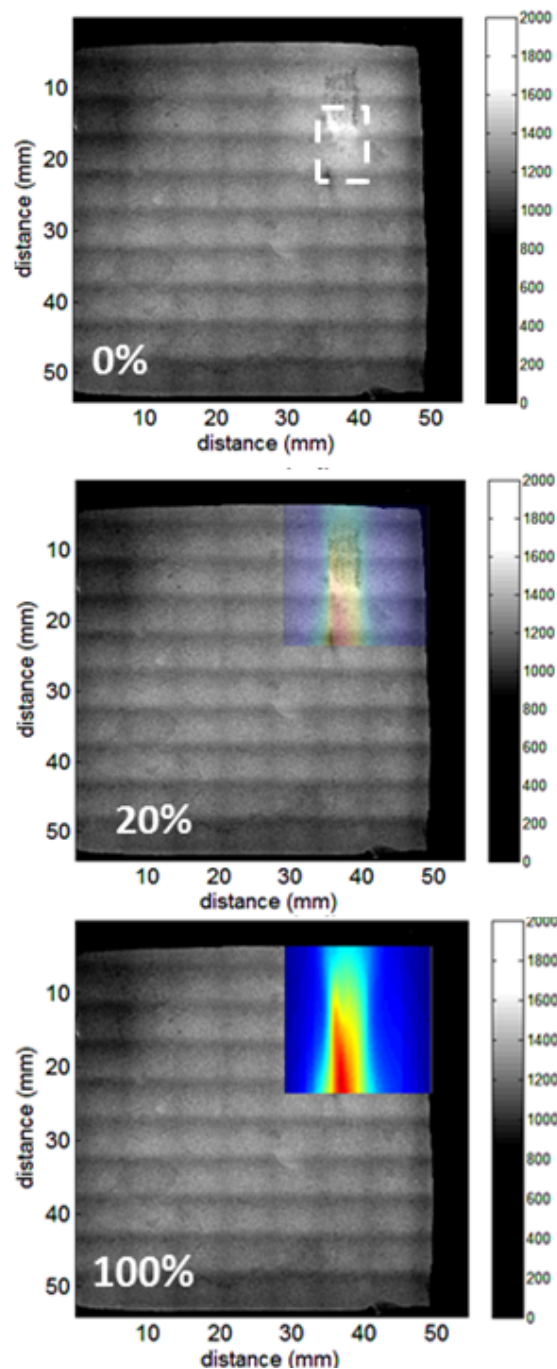


Figure 5.18: Overlap of the PAM of the 25-sonication raster scan at 5.6 MPa 25% DC 1 Hz PRF 5 s (slice B) to the respective fluorescence map. Higher levels of fluorescence intensity indicate higher levels of drug release. Top figure: Fluorescence image of the TMM. The region of the TMM where drug has been released from the liposomes can be observed on the top right (indicated by the dashed rectangle). Middle figure: the respective cumulative PAM for this raster scan has been overlaid to the fluorescence image with 20% opacity, so the similarities in the spatial distribution of the release and the acoustic energy can be observed. Bottom figure: The cumulative map is overlaid to the fluorescence image at 100% opacity.

for real-time non-invasive monitoring of drug release in the TMM and it has been claimed that this is a technique that can be used in the clinic for ultrasonic drug delivery applications. However, mild hyperthermia, which is vital for drug release from the liposomes, has not been spatially mapped. Spatial mapping of temperature rise would provide a direct method of assessing drug release instead of an indirect method such as PAM.

Currently, there are various applications for non-invasive temperature monitoring in tissue. Magnetic resonance (MR) imaging is still the golden standard for determining temperature change in a clinical setting, but it has several drawbacks such as high cost and low accessibility. In addition, the spatial resolution of temperature is lower than what would be necessary for reliable measurement of HIFU-induced mild hyperthermia. Several ultrasound techniques have also been developed for the imaging of temperature (Simon *et al.*, 1998; Miller *et al.*, 2004; Farny and Clement, 2009) that are based on the changes of speed of sound with temperature rise. In the context of the present application, namely monitoring of mild hyperthermia (and subsequently drug release), the speed of sound technique ((Simon *et al.*, 1998)) could be used as it provides a reliable temperature estimation for temperature rises up to 10°C. This technique is based on the detection of shifts in echo location of backscattered ultrasound from a region of tissue undergoing ultrasonic heating. These shifts are caused by the combination of the local temperature dependence of speed of sound and thermal expansion in the heated region and are measured with an imaging array similarly to PAM.

However this method has several characteristics that render it unsuitable for temperature estimations in a clinical setting. Because it is based on an echo tracking approach it is sensitive to body motion and deformation, and thus not preferable as an imaging technique for *in vivo* experiments or in a clinical setting. In addition,

it is affected by thermo-acoustic inhomogeneities, which are the norm when tissue is involved. On the contrary, the PAM technique is not sensitive to the medium; if there is a cavitation event at the HIFU focus during the exposure there will be emitted signal that subsequently will be mapped, whereas if there is no cavitation event, there will be no signal on the passive map. The fact that it is only sensitive to the broadband emissions emanating from the HIFU focus, it gives real time feedback and as has been shown in this Chapter gives good correlation of acoustic emissions to drug release, makes it a very promising method for clinical use.

5.3 CONCLUSIONS

The objective of the work presented in this Chapter was to investigate whether the acoustic emissions at the HIFU focus and the doxorubicin release from thermally sensitive liposomes caused by cavitation-enhanced mild hyperthermia could be spatially correlated. The results presented show that spatial mapping of drug release with acoustic cavitation emissions is feasible. It was shown that drug release due to cavitation-enhanced heating can be more accurately mapped to acoustic emissions than drug release due to ultrasound-induced heating. This finding opens the way for real-time monitoring of cavitation-enhanced heating and drug release, as the PAMs can be acquired real time. Thus one of the major clinical challenges of targeted drug release could be addressed. Moreover, it was shown that apart from spatial distribution, the level of drug release may be correlated to the energy level of the acoustic emissions generated.

This study was performed on a solid TMM, that had controlled conditions and no effects of flow. A study on a more clinically relevant platform, would provide more comprehensive results. In the next Chapter, initial experimentation on a perfused liver model is shown, taking the work that has been done so far one step closer

to clinical conditions.



THERMAL DRUG RELEASE IN A PERFUSED LIVER MODEL

In preceding chapters, the HIFU exposure conditions that create cavitation-enhanced heating were identified, and passive mapping of the acoustic emissions associated with cavitation were exploited for spatiotemporal monitoring of drug release. However, all work up to this point was carried out without taking the heat sink effect due to perfusion into account, which is likely to significantly affect the level of ultrasound-induced hyperthermia *in vivo* and in materials with a different cavitation threshold. A normothermic isolated liver perfusion model is exploited in this chapter to assess the feasibility of ultrasound-induced and cavitation-enhanced hyperthermia for drug release in regions that are proximal or distal from major blood vessels.

6.1 INTRODUCTION

After having optimised and tested the ultrasound parameters for cavitation-enhanced mild hyperthermia and spatially mapped drug release in a TMM, the next step is to assess the feasibility of cavitation-enhanced heating for drug release from the liposomes in a more clinically relevant platform. The experiments discussed in this chapter were performed on surgically excised pig livers that were connected to a normothermic liver perfusion device.

This device was developed to improve organ transplantation, by maintaining the liver in physiological temperature with provision of oxygen and nutrition. Unlike conventional cold preservation technology, in which progressive deterioration of the organ is inevitable and has a time limit, normothermic perfusion has shown the potential to keep the liver stable for more than 24 h (Brockmann *et al.*, 2009). In the present work, porcine liver was kept connected to the normothermic perfusion machine for experiments to be performed.

The normothermic perfusion model provides conditions almost identical to treating patients, as the organ maintains normal hemodynamic, metabolic and synthetic characteristics throughout the 24 hours of perfusion. The use of a porcine liver model is in many ways ideal for the present work, as it has a similar mass and exhibits similar flow rates to those of humans (0-2 L/min). Furthermore, compared to small animal models, the length scales encountered by the HIFU are much more representative of their clinical equivalent. The only limitations of this model are the absence of a tumour microenvironment, as a porcine carcinoma model is not presently available, and the fact that HIFU exposures have been performed without the presence of a ribcage and overlying muscle and fat tissue layers which may affect the incident sound field.

6.2 MATERIALS AND METHODS

6.2.1 Perfused Liver Model

For all experiments white Landrace pigs weighing 70-85 kg were used, in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. Livers were procured using a standard technique described by (Brockmann *et al.*, 2009) and (Jamieson *et al.*, 2011). Blood was collected from the same pig via the aortic can-

nula and was stored in dextrose supplemented blood transfusion bags (CPDA Single Blood Collection Systems, Baxter) for subsequent use. The liver was cold perfused with hyperosmolar citrate solution (Soltran, Baxter) via the aortic cannula. Following venesection, the thoracic aorta was cross-clamped and the suprahepatic IVC was divided. After 2L of cold perfusate had been administered, the hepatic vessels and infrahepatic IVC were divided; the liver was removed by the surgeon and placed in a bowl of saline ice slush. The liver was placed in sterile intestinal bags (Vi-Drape, MCD) and was packed with ice in a cool box for transportation.

Normothermic perfusion was performed, using a perfusion circuit that incorporated a hollow-fibre membrane oxygenator (EOS ECMO PHISIO M, Sorin), a centrifugal pump (BPX80, Medtronic) and a soft-shell reservoir (Capiiox 1500, Terumo) (Brockmann *et al.*, 2009). Two heat exchangers maintained the blood at 37-39°C. Pump speed, portal and arterial flow were automatically controlled in order to maintain arterial and portal pressures within pre-defined limits.

The circuit was primed with ~ 1500mL of autologous whole blood, heparin anticoagulation (10000 Units, CP Pharmaceuticals), calcium gluconate (10mL of 10%; Braun, UK) and cefuroxime (750mg, GlaxoSmithKline). Blood was circulated through the perfusion circuit before the liver was attached so that a blood temperature of 36°C was achieved prior to attachment of the liver. Immediately before perfusion of the liver, continuous infusions of insulin (6.7IU/hr, Novo Nordisk), heparin (750IU/hr), prostacyclin (epoprostenol sodium; 8µg/hr, GlaxoSmithKline) and bile acid (sodium taurocholate 2%; 140mg/hr, NZP) were commenced.

Prostacyclins increase portal flow and increase total blood flow and oxygen transport following reperfusion after warm ischaemia, whilst bile acid supplementation is necessary to replace the enterohepatic recirculation. The liver, suspended in a silicone sling over a stainless steel bowl, was connected to the perfusion cir-

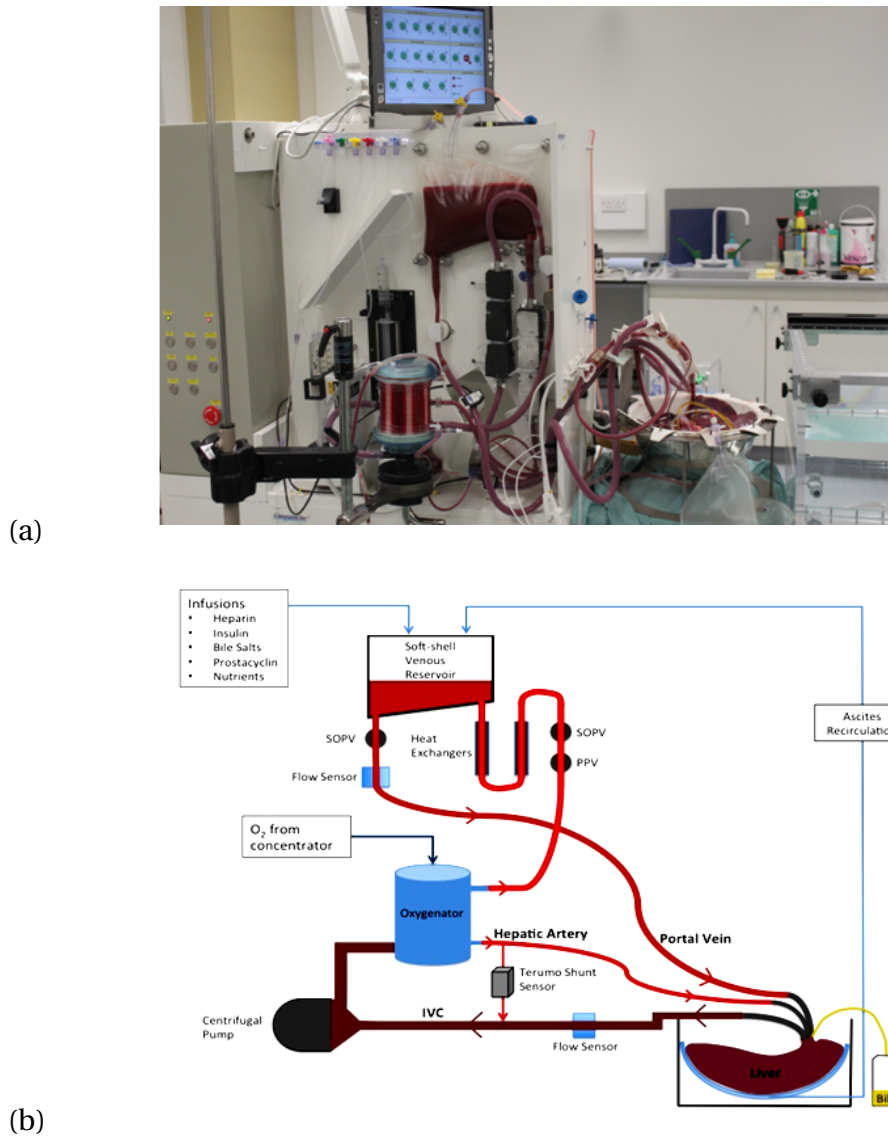


Figure 6.1: Normothermic liver perfusion circuit. (a) Photograph of the normothermic perfusion setup. The liver is placed in the metal bowl on the right side. (b) Schematic representation of the normothermic perfusion setup

cuit and perfusion was initiated with a progressively increasing pump speed over a few minutes until an IVC pressure of 0-5mmHg was achieved. Ascites, serous fluid physiologically produced by the liver and resorbed by the peritoneum *in vivo*, was collected in the stainless steel bowl and recirculated automatically via a peristaltic pump (Watson Marlow).

Blood gas analysis and acid-base status were monitored continuously by a Terumo in-line sensor (CDI510H, Terumo) connected to a Terumo CDI 500 system. PO₂ and PCO₂ were controlled automatically so that they remained in the range 12-20 kPa and 3.5-6 kPa respectively. Blood glucose analysis was performed hourly (Contour blood glucose test strips and Contour machine, Bayer) and once blood glucose was below 12mmol/L, a nutrient infusion (Nutriflex Plus, Braun) was commenced at 15ml/hr in order to maintain perfusate glucose levels in the range 4 to 8mmol/L. Organ connection to the circuit was performed by Prof. Constantin Cousios, whilst Dr. David Holroyd performed the retrieval and was present throughout the 24-hour perfusion to ensure that the organ was maintained in a physiological state.

6.2.2 Experimental Setup

Following approximately 30 minutes of normothermic machine perfusion, the ascites recirculation was stopped, the stainless steel bowl was removed from under the liver to avoid reflections and the liver, supported by a custom-built silicone sling, was placed in a isotonic saline bath (0.9% NaCl, Sigma) which had been degassed and pre-heated to 37°C for experimentation with ultrasound. The bottom of the tank was lined with an acoustic absorber (Aptflex F28, Precision Acoustics) to minimize reflections and prevent standing wave formation.

The ultrasound therapeutic transducer was coaxially aligned to the imaging

multi-element linear array (see §5.1) and positioned in a horizontal orientation on top of the liver, so that the HIFU focus was approximately 10 mm into the tissue for comparison with the previous experiments. A 3-D positioning system was mounted on the side wall of the water tank and was used for positioning the ultrasound transducer/imaging array assembly.

The experimental setup is shown in Figure 6.2. The signal generator (33250A, Agilent Technologies) that drove the 1.06 MHz therapeutic transducer (H102B-22, Sonic Concepts) and the Zonare system were triggered simultaneously by the PC. When pulsed exposures were performed, a second signal generator was used to control the timing of the pulses with respect to the timing of the data acquisition from the Zonare system. At each experiment, the Zonare Z.one system was initially set on B-mode to image the HIFU focus location and surroundings (see §5.1.3) and to achieve thermocouple alignment. It was subsequently used in passive mode (no pulse emitted by the array) during HIFU exposure for passive acquisition of emissions from the HIFU focal region. Data acquisition and capture was done with the L10-5 probe with the same method and settings described in §5.1.

Thermal measurements were performed with a thermocouple that was positioned in the liver tissue (further details are provided in §6.2.3) and the signal from the thermocouple was recorded with a multiplexer with embedded reference temperature compensation (DAQ 34970A, Agilent Technologies) and passed to the computer by serial port. The DAQ was triggered by the PC and set to gather temperature data for 10 s.

Initially, fine needle (0.2 mm) T-type thermocouples (HYP0, Omega) were used, similarly to the work described in previous Chapters, but because their alignment with the therapeutic transducer proved to be very time-consuming (of the order of 4 h at times), large needle (0.7 mm) T-type thermocouples (HYP2, Omega) were used

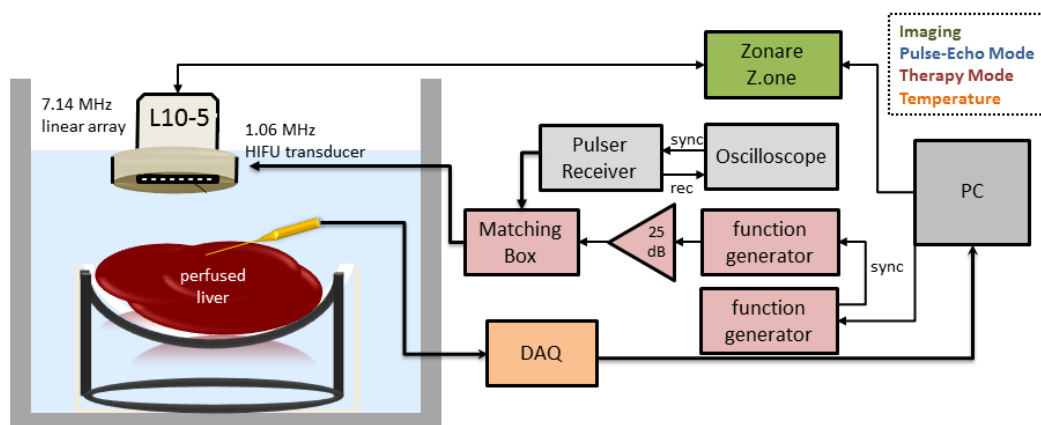


Figure 6.2: Experimental setup for acoustic and temperature measurements in perfused liver. The 1.06 MHz therapeutic transducer was coaxially aligned to the L10-5 imaging probe and driven by a signal generator. The signal generator and the Z.One imaging system were simultaneously triggered by the PC. A needle thermocouple was inserted in the liver and temperature readings were recorded by a multiplexer and passed to the PC for further processing. When pulsed exposures were performed, a second signal generator was used to control the timing of the pulses with respect of the timing of the data acquisition from the Zonare system. Thermocouple alignment to the HIFU was done by pulse-echo and B-mode. The experiments were conducted in isotonic saline bath (0.9% NaCl), degassed and pre-heated to 37°C.

in most experiments. In order to avoid or minimize hemorrhage of the liver due to the insertion of the needle thermocouples, they were not removed after measurements were conducted at each location. Regarding the thermocouple artifact, temperature measurements conducted with both thermocouples during the same exposure conditions at similar locations in different livers each, yielded similar results, indicating that in the mild hyperthermia range the size of the large needle thermocouple does not induce a significant added thermocouple artifact.

6.2.3 Thermocouple Positioning

Thermocouple positioning proved to be a challenging step of the experimental procedure because it was not clearly visible on the B-mode images and because after the first ~ 6 h of the liver being in the saline bath there was slight movement of

the liver that compromised the alignment. Due to the morphology of the liver, the thermocouple was placed at an angle approximately 45° perpendicular to the HIFU axis. Alignment of the thermocouple to the HIFU focus was done by both B-mode imaging and pulse-echo. Dr. Daniel Chung who is a radiologist identified regions of microvasculature or large vessels via B-mode imaging and placed the thermocouple there. Doppler images were acquired at each location in order to assess the presence or absence of large vessels proximal to the tip before the therapeutic sonications. Care was taken not to move the thermocouple and the therapeutic transducer, so that the most sensitive point of the thermocouple needle was at the location of the HIFU focus.

Rough registration of the most sensitive point of the thermocouple to the HIFU focus was done by B-mode imaging and pulse-echo of the HIFU transducer. Precise positioning of the thermocouple was subsequently done thermally by recording the temperature before and after short (2 s) HIFU exposures at low amplitudes (below 3.7 MPa ppk in the microvasculature region and 6 MPa ppk close to large vessels), so that cavitation activity and temperature rises above 3°C were avoided. In some cases this alignment exposures caused cavitation activity, but in these cases the location of the thermocouple was changed and a lower pressure was subsequently used. The position at which the thermocouple recorded the maximum temperature for the same exposure corresponded to the most sensitive point being at the HIFU focus.

6.2.4 Experimental Procedure

Before the series of experiments in perfused livers, the HIFU transducer was recalibrated to acquire the driving voltage - acoustic pressure curve. The calibration curve that was used is: $P = 0.0366V + 0.0521$, where P (MPa) is the peak-to-peak pressure measured with the hydrophone (HNA-0400, Onda) and V (mV) is the peak-to-peak

voltage recorded after the signal generator. The spatial position of the HIFU focus was identified before each experiment with the use of the hydrophone and recorded for subsequent use for the thermocouple alignment. The regions of interest were then identified with B-mode (microvasculature, large vessels) and the thermocouple was aligned.

The imaging array was then put on passive mode, the therapeutic ultrasound sonications were performed over a range of pressures and the acoustic and thermal data from the exposed region were recorded. At each thermocouple location a Doppler image was acquired before and after the sonications.

6.2.5 Exposure Protocol for Acoustic and Thermal Characterisation of the Perfused Liver Model

Experiments were performed in four perfused livers. The liver was sonicated with gradually increasing pressure from 0.95 to 14.7 MPa ppk with a 0.95 MPa ppk step under two sets of exposure conditions, dictated by prior work in Chapters 3-5: continuous wave with 6 s duration and pulsed wave 25% DC with 1 Hz PRF and 5 s duration. During each exposure the acoustic emissions from the HIFU focus were recorded with the Z.one imaging array and the induced temperature rise with the T-type needle thermocouple. Exposures were performed both at locations proximal to large vessels and at locations of microvasculature. Large vessels were considered vessels of mean diameter of ~ 5 mm or above, as measured by Doppler imaging. For the temperature readings in regions near a large vessel, the thermocouple was positioned so that the thermocouple tip was adjacent to the vessel. Between sonications a 1 minute time interval was applied so that the liver tissue would return to normal base temperature (37 °C).

Additionally, a study on the effect of perfusion on acoustic properties and thermal response was performed in the microvasculature region. Each region was sonicated first with the blood perfusion 'ON' and then with blood perfusion 'OFF'. Before the sonications were applied without perfusion, a temperature measurement was taken to ensure that the thermocouple reading for the base temperature of the liver was the same as before turning the perfusion off. The liver was kept non-perfused for less than 10 min in total over the course of the 24 hour experiment, which is the maximum time that it can stay without blood and nutrient flow and keep physiological functions.

6.2.6 Drug Release Study

A feasibility study for drug release in the perfused model was performed using ThermoDox® as the liposomal formulation. ThermoDox® was introduced in a 1/4 IV, 3/4 filtered water and 5% Dextran solution and the solution was administered as a bulk injection into the hepatic artery (Figure 6.3). The composition of the solution for the injection was provided by the manufacturer and its purpose is to keep the liposomes stable when not heated in a clinical setting. In total, 0.02 mg/mL of ThermoDox® was injected per 2 L of blood volume, which is the concentration suggested by the manufacturer for intravenous injection. Before the drug was mixed with the solution, Dextran was filtered with a 20 μm filter for removal of any bacteria.

To study the pharmacokinetics of the liposomal formulation, the hepatic vein blood (blood exiting the liver) was sampled every 1 min for the first 10 minutes post-injection and then every 5 min up to 2 h post-injection. The samples were sealed in vials and stored in the -80°C freezer, along with control samples (pre-injection). According to confidential information from the manufacturer, plasma and tissue

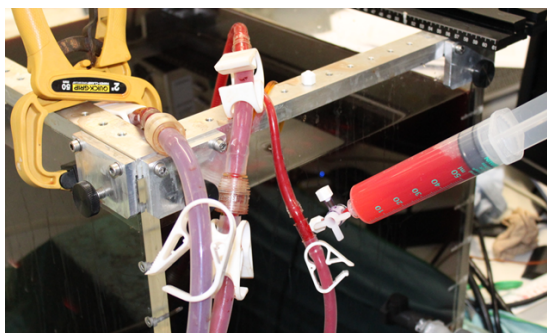


Figure 6.3: ThermoDox® bulk injection in the hepatic artery.

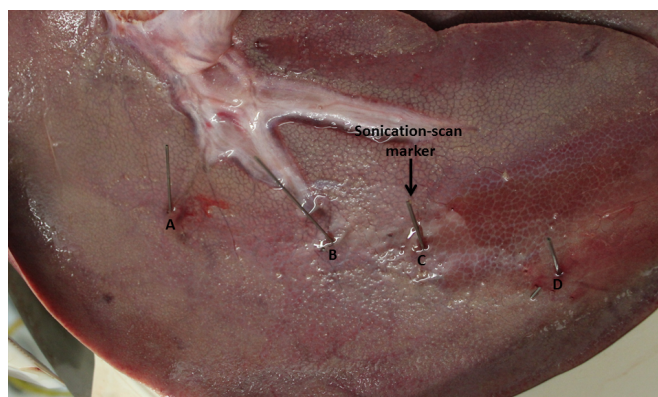
half-life of ThermoDox® is 5 min and 20–48 h respectively.

To study the drug release, three raster scans of 3x3 sonications were performed in different regions of the liver, with the conditions described in Table 6.1.

Table 6.1: Scan configuration for assessment of feasibility of drug release.

Scan Index	Exposure Conditions	Region
A	Pressure: 11 MPa ppk, DC:25%, PRF: 1Hz duration: 5s	near vessel
B	Pressure: 11 MPa ppk, DC:25%, PRF: 1Hz duration: 5s	near vessel
C	Pressure: 7.4 MPa ppk, DC:25%, PRF: 1Hz duration: 5s	near vessel

As a point of reference for the location of each scan, fiducial markers were used. The markers were placed at the focus of the middle sonication immediately after each raster scan was finished. Thin metal rods were positioned on top of the markers, to identify their location during sampling. After the end of the experiment, the liver was sliced in ~ 2 mm thin slices, and rectangular samples of surface $\sim 1\text{cm}^2$ were extracted, as shown in Figure 6.4. Subsequently, the samples were imaged with fluorescence microscopy as described in Chapter 5. For each sample, a surface image of fluorescence intensity was acquired. The conditions used during fluorescence microscopy were the same for all scans. The samples were then stored in -80°C freezer, along with control samples (liver parenchyma from different lobe).



(a)



(b)



(c)

Figure 6.4: Sampling of liver tissue for assessment of drug release. (a) The thin metal rods protruding from the liver indicate the location of the fiducial markers. (b) A slice of liver tissue (corresponding to scan C). The oval shape indicates the location of the fiducial marker. (c) A sliced rectangular sample for fluorescence imaging.

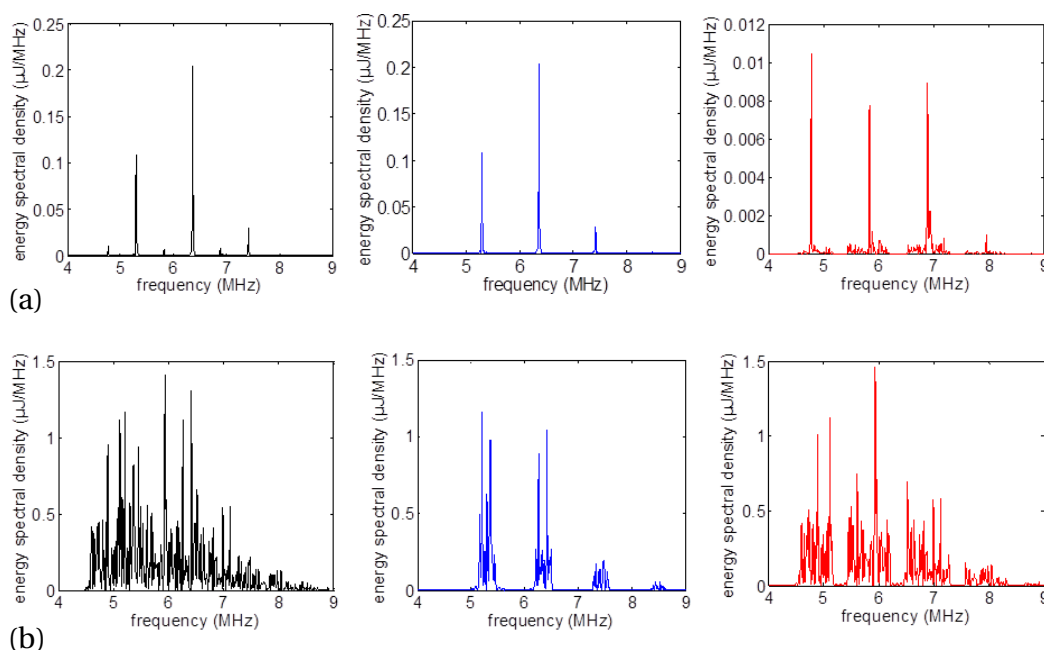


Figure 6.5: Example of the filter used for separation of harmonic and non-harmonic emissions. The total energy of the signal is shown in black, harmonic emissions in blue and non-harmonic emissions in red. (a) shows an example in which harmonic emissions dominate. Note that the y axis of the non-harmonic emissions figure is not the same as the axis of the harmonic and total emissions. (b) shows an example in which non-harmonic emissions dominate.

6.2.7 Passive Acoustic Mapping

Passive acoustic emissions captured with the linear array were post-processed in MATLAB. The energy of emissions was estimated at the focal point for each sonication and harmonic and non-harmonic contributions were extracted using a comb filter. Harmonic emissions were acquired by filtering the channel data at multiples of the center frequency of 1.06 MHz at a 0.3 MHz bandwidth while non-harmonic contributions were the result of energy generated between harmonics and at a 0.706 MHz bandwidth.

6.3 RESULTS

In this section, the acquired results from four experiments with the perfused liver model are presented. The livers were sonicated in regions near a large vessel and in regions of the peripheral liver, namely in the microvasculature region, in order to identify the effect that blood flow conditions have on the thermal response and acoustic properties of the liver. Moreover, the perfusion was temporarily stopped and acoustic and temperature measurements were acquired before and after stopping it, in order to assess the effect that it has on a region.

Even though a rigorous statistical analysis cannot be performed on the acquired results due to the small sample-size in some conditions, an indication of the behaviour of different regions of the perfused liver model under a large range of acoustic pressures in both continuous and pulsed mode is obtained for the first time. Later in this section, results that show the feasibility of drug release in the perfused liver model for the first time are also presented.

6.3.1 Cavitation Threshold in Perfused Liver

The cavitation threshold was estimated in two types of locations: regions proximal to a large vessel and peripheral regions of microvasculature (far away of a vessel) in the presence of perfusion. It was also estimated in regions of microvasculature after perfusion was temporarily ceased. At each one of these three conditions (region proximal to a large vessel with perfusion, region away from large vessel with perfusion and regions away from large vessel without perfusion) two types of HIFU exposures were performed at a range of acoustic pressures, namely CW for 6 s and pulsed sequence of 25% DC, 1 Hz PRF for 5 s.

Figure 6.6 shows the total energy of the non-harmonic emissions that was ac-

quired per exposure at each acoustic pressure. In each one of Figure 6.6(a), (b) and (c) the thick lines correspond to the continuous wave exposures and the thin lines to the pulsed exposures. Additionally, the same line-type corresponds to the same liver in each figure.

Figure 6.6(a) shows the non-harmonic energy emitted near a large vessel. In the case of the CW exposures a sudden increase in the non-harmonic energy is observed at ~ 7.4 MPa ppk. The energy emitted at 6.45 MPa ppk is $21 \mu\text{J}$, whereas the energy emitted at 7.4 MPa ppk is $2400 \mu\text{J}$ and is further increasing to $17000 \mu\text{J}$ at 8.3 MPa ppk. This is indicative of the onset of inertial cavitation activity, as it is associated with the generation of broadband emissions.

In the case of the pulsed exposures, the sudden increase occurs at a lower acoustic pressure (4–4.4 MPa ppk). A sudden decrease is also observed (7.4 MPa ppk) in the energy emitted during one of the pulsed sonications (dashed line). One explanation could be depletion of the cavitation nuclei that were present in the region. In all cases inertial cavitation was instigated at an acoustic pressure below 7.4 MPa ppk (more than $10^3 \mu\text{J}$ non-harmonic emission energy) in regions near large vessels.

Figure 6.6(b) shows the non-harmonic energy emitted in the peripheral microvasculature region. Again in all cases a sudden increase in the non-harmonic emissions is observed, indicative of the onset of inertial cavitation. In three out of the seven cases presented in this figure, there is a very sudden increase at 4.4 MPa ppk (two orders of magnitude increase with only 0.5 MPa ppk increase in the applied pressure), and in two cases there is a sudden increase at 3.7 MPa ppk (two orders of magnitude increase with 0.8 MPa increase in the applied pressure). In one case of pulsed exposure a more gradual increase may be observed (from $1353 \mu\text{J}$ to $10000 \mu\text{J}$ at 3.7 MPa ppk and 4.8 MPa ppk respectively). In all cases however, a high amount of non-harmonic emissions (above $10^3 \mu\text{J}$) has been generated below 4.6 MPa ppk.

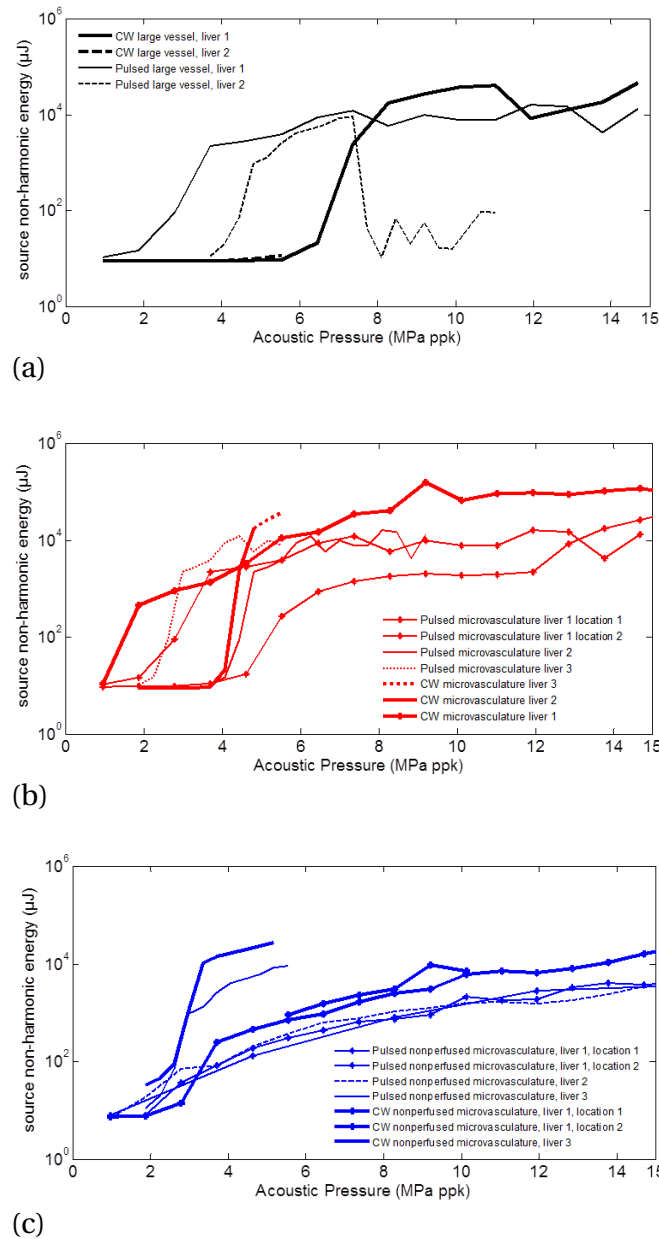


Figure 6.6: Non-harmonic emission energy in a perfused liver model in (a) region proximal to a large vessel, (b) microvasculature and (c) microvasculature after blood perfusion has temporarily ceased. At each of these three conditions two types of HIFU exposures were performed over a range of acoustic pressures, namely CW for 6 s and pulsed sequence of 25% DC, 1 Hz PRF for 5 s. The thick lines correspond to continuous wave exposures and the thin lines to pulsed exposures. In each figure the same line type in corresponds to the same liver.

Additionally, it may be observed that in both (a) and (b) the magnitude of the non-harmonic emission energy generated at the HIFU focus reaches a plateau at pressures ~ 9 MPa ppk and does not increase substantially above that. In the microperfusion area the magnitude of the acoustic emissions generated at the HIFU focus is generally higher than in the area proximal to a large vessel. The emissions generated in the microvasculature region during the CW exposures present even a one order of magnitude difference to the emissions generated in the region close to a vessel at such high pressures ($10^5 \mu\text{J}$ and $10^4 \mu\text{J}$ respectively).

When the HIFU exposures shown in Figure 6.6 (a) and (b), were performed, blood perfusion was ON. The acoustic response of the microvasculature region after perfusion has temporarily been stopped is shown in Figure 6.6(c). It may be observed that the magnitude of the energy generated is in most cases lower than in (a) and (b). Non-harmonic emissions of the order of $10^3 \mu\text{J}$ are observed even at low acoustic pressures (3.4 MPa ppk). Additionally, the results obtained in the absence of perfusion appear to have more consistency across livers than in the presence of perfusion.

As shown in Figure 6.6, the energy of the non-harmonic component of the emissions generated at each exposure increased rapidly by one order of magnitude in almost all cases, indicating the onset of inertial cavitation activity. Figure 6.7 shows the energy of the harmonic emissions that was observed during these exposures. By contrast to the non-harmonic emissions, harmonic emissions present a smooth increase of energy with acoustic pressure, instead of a sudden 'jump' (apart from one case near a large vessel).

It is also observed that the magnitude of the harmonic energy is comparable to the magnitude of the non-harmonic energy. If it is taken into account that the filter that was used to separate the harmonic to the non-harmonic components al-

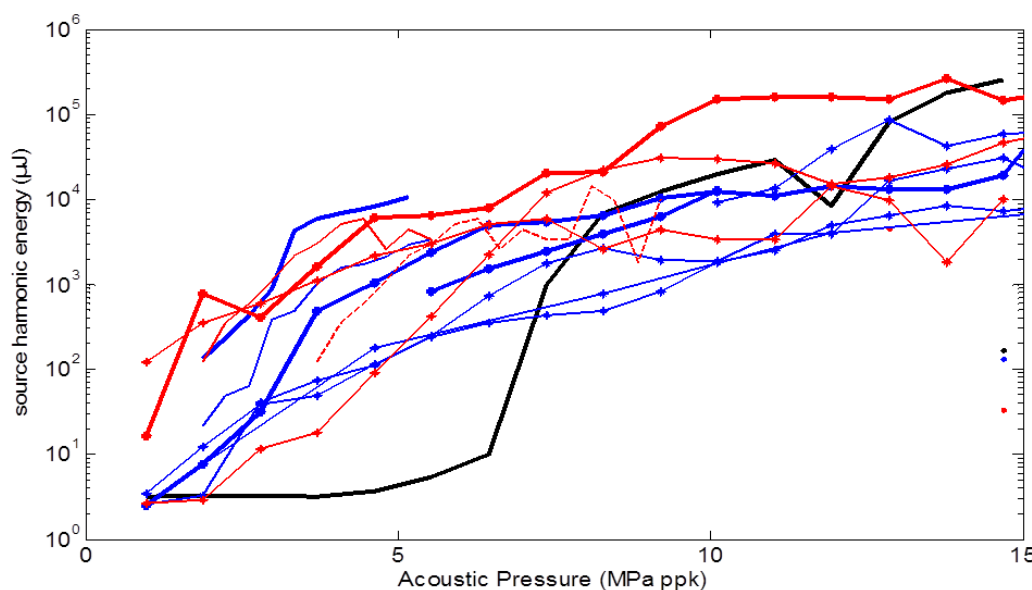


Figure 6.7: Harmonic emission energy in a perfused liver model. The black lines correspond to exposures performed in regions proximal to a large vessel, the red lines correspond to exposures performed in regions away from a large vessel and the blue lines correspond to exposures performed in regions away from a large vessel after perfusion has stopped. Thick lines correspond to 6 s CW exposures and thin lines correspond to 5 s pulsed exposures with 25% DC and 1 Hz PRF. The same type of line indicates exposures performed on the same liver.

lowed only a 0.15 MHz bandwidth for the harmonic emissions and that the energy which is plotted in these figures is the cumulative energy of each exposure, it can be concluded that the amount of harmonic emissions is very high in all cases. This is expected, as liver is a highly non-linear medium (see §2.3).

6.3.2 Ultrasound-Induced Hyperthermia in Perfused Liver

In Chapter 4 it was shown that inertial cavitation can cause mild hyperthermia with only one third of the energy that would be required for conventional ultrasound heating. It is thus very interesting to observe the thermal response of the different regions of the perfused liver model for different exposure conditions that instigate inertial cavitation activity, in the presence of the heat sink effect of blood flow.

Figure 6.8(a) shows the maximum temperature rise from ambient temperature at the HIFU focus over a range of acoustic pressures (MPa peak-to-peak) for 6 s CW HIFU exposures. Figure 6.8(b) shows the respective non-harmonic emissions at the HIFU focus of the exposures that caused the temperature rise shown in (a). In both figures, the line colour and style indicate the same sonications. Black colour corresponds to exposures in regions close to a large vessel, red colour represents exposures in regions of microvasculature and blue colour represents exposures in regions of microvasculature when perfusion has been stopped.

The temperature rises caused in regions of peripheral liver with or without perfusion do not present significant differences, and the temperature rises caused in the absence of perfusion are slightly lower. This indicates that the effect of flow in the microvasculature region is negligible. The effect of flow is much more pronounced in the case of the exposures near a large vessel. The temperature rises acquired in these cases are significantly lower than the ones acquired by exposing the microvasculature areas to HIFU, suggesting strong heat dissipation due to blood perfusion.

In Chapter 2 it was mentioned that during linear ultrasound heating the induced temperature rise follows a parabolic profile with respect to acoustic pressure, and once cavitation-enhanced heating is instigated, the temperature increase becomes becomes greater than parabolic.

Such behaviour is clearly observable in Figure 6.8. The effect of cavitation-enhanced heating is better observed in the microvascular region. In both cases of perfused and non-perfused conditions, a sudden increase in the non-harmonic emissions between 3 and 4 MPa peak-to-peak (indicating the onset of inertial cavitation activity) coincides with a dramatic increase in the observed temperature rise. This is most pronounced in the case of perfused microvasculature heating indicated

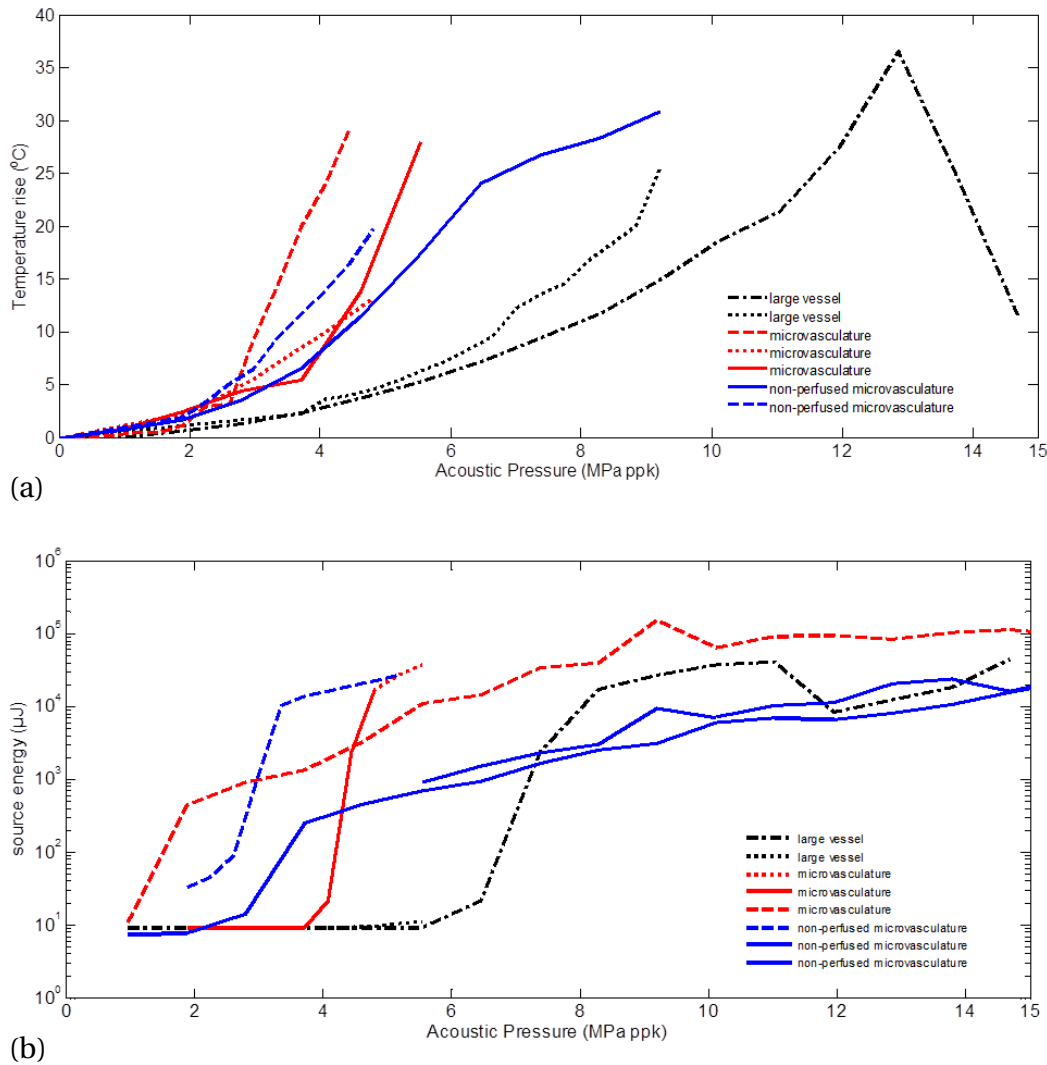


Figure 6.8: (a) shows the maximum temperature rise above 37 °C at the HIFU focus over a range of acoustic pressures (MPa ppk) for 6s CW HIFU exposures. (b) shows the corresponding non-harmonic emissions at the HIFU focus of the exposures that caused the temperature rise shown in (a). The line colour and style indicate the same sonications. Black colour corresponds to exposures in regions close to a large vessel, red colour represents exposures in regions of microvasculature and blue colour represents exposures in regions of microvasculature when perfusion has been stopped.

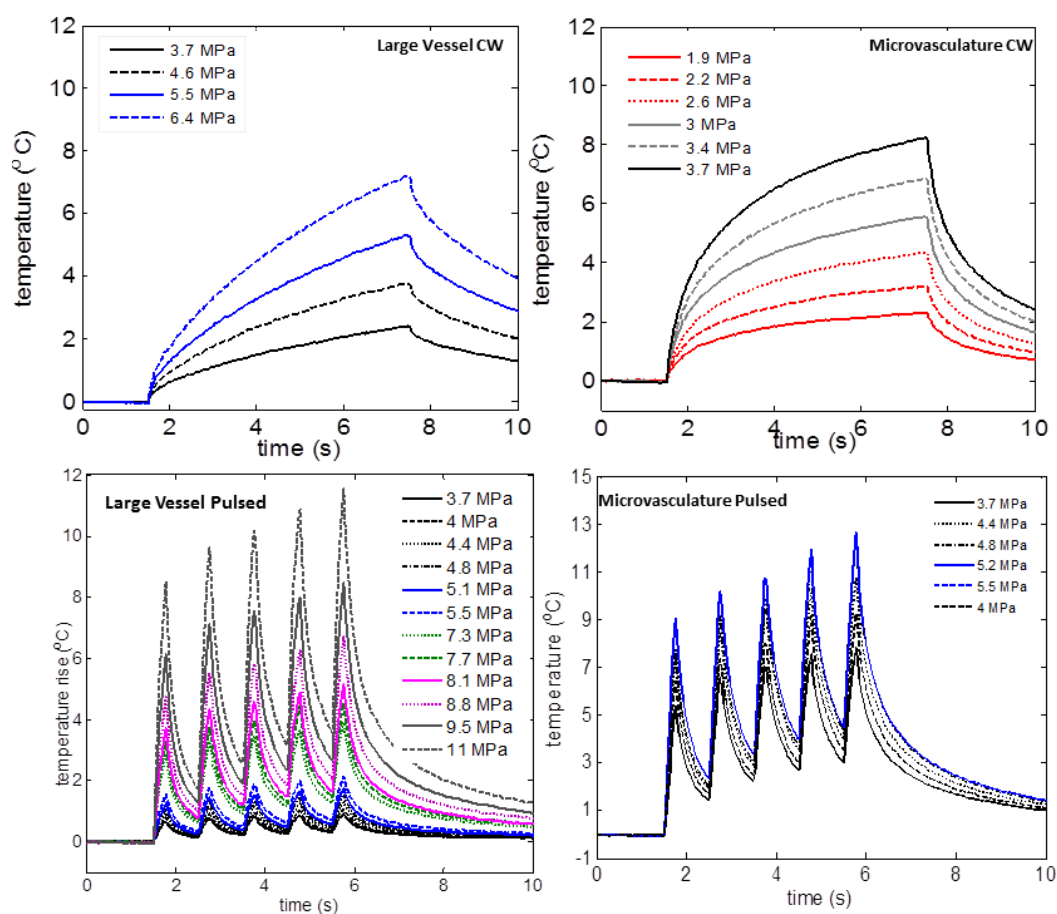


Figure 6.9: Representative acoustic pressures (MPa ppk) that give temperature profiles in the mild hyperthermia range in perfused regions near and away from large vessels.

by the solid red line.

Representative acoustic pressures (MPa ppk) that give temperature profiles in the mild hyperthermia range in perfused regions near and away from large vessels are shown in Figure 6.9. In regions proximal to large vessels acoustic pressures of 5.5–6.4 MPa ppk CW were necessary to achieve mild hyperthermia, whereas in regions away from large vessels, lower pressures, of the order of 3 to 3.7 MPa ppk CW were necessary. When pulsed sequences were applied, mild hyperthermia was achieved with 7.3–9.5 MPa ppk near large vessels and 3.7–5 MPa ppk away from large vessels. The dramatic increase in the pressures needed to induce mild hyper-

thermia in regions proximal to large vessels is indicative of the cooling effect that blood flow has.

A comparison of these results to those of the TMM (see Figure 4.4), shows that mild hyperthermia was achieved in the TMM for pressures (4.4 MPa ppk for continuous wave and 5.6 MPa for 25% DC exposures) similar to those that yielded mild hyperthermia in the microvasculature region. The pressures that caused mild hyperthermia in the large vessel region were 2 MPa ppk and 3 MPa ppk higher than the pressures that caused mild hyperthermia in the TMM, for CW and pulsed exposures respectively, and the pressures that caused mild hyperthermia in the microvasculature region were 1 MPa ppk lower than the pressures which caused mild hyperthermia in the TMM. Thus, the ideal exposure conditions for mild hyperthermia derived in the TMM appear to hold in the microperfused regions, but fail to produce the required hyperthermia in regions of blood flow.

6.3.3 Feasibility of Drug Release

After having characterised the acoustic and thermal response of the perfused liver model, a feasibility study of drug release from LTSLs (ThermoDox) was performed. As an initial step, a pharmacokinetics study was done (Figure 6.10) to assess the concentration of the liposomes in blood plasma after administration. Instructions from the manufacturer suggest that ThermoDox clearance from human blood plasma happens in 5 min and from the tissue in 24–48 h. The experimental results showed however that, in the perfused liver experimental model, 50% of the drug is cleared from porcine blood after 9 min and 90% after 20 min.

Three scans were performed for the assessment of drug release, far from major vessels at the following conditions: 11 MPa ppk 25% DC, 1Hz PRF, 5 s duration (both A and B) and 7.4 MPa ppk 25% DC, 1Hz PRF, 5 s duration (C) (also described

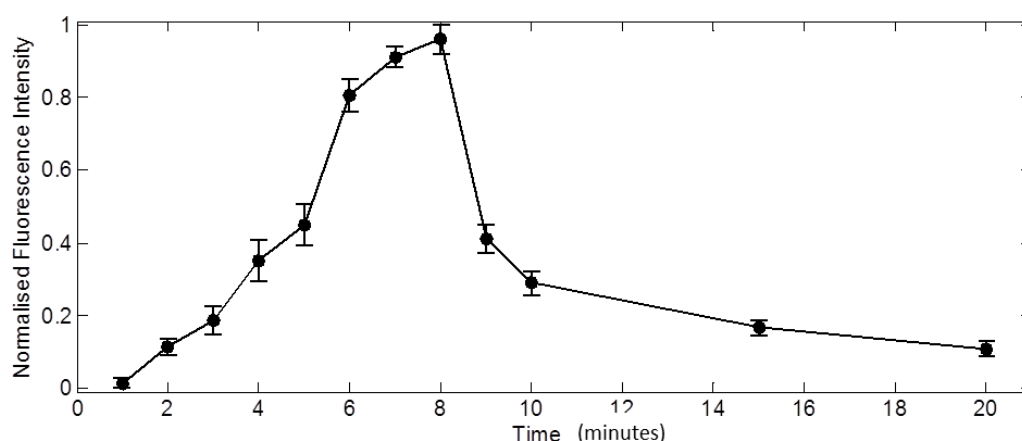


Figure 6.10: Pharmacokinetics study of ThermoDox in a perfused liver model after bulk injection of 0.02 mg/mL ThermoDox into the hepatic artery. Doxorubicin concentration is represented by the normalised fluorescence intensity in time.

in §6.2.6). The acoustic pressures were chosen according to the results shown in Figure 6.9, because they cover the ends of the pressure range over which mild hyperthermia is caused. Even though the 11 MPa ppk is shown to cause temperature rise up to 11°C, it was chosen to account for the thermocouple artefact. Representative PAMs for sonications from each scan are shown in Figure 6.11. In all cases near a large vessel cavitation activity is observed, in accordance to the results shown in Figure 6.6 for the cavitation threshold. An interesting observation is the spatial distribution of cavitation. In all A, B and C scans, cavitation activity was observed both inside and outside of the HIFU focal region. On the contrary, in the PAMs shown in Figure 5.13, when sonications were performed in the TMM in the absence of flow, all activity occurred inside the HIFU focal region. The lateral distribution of source emission energy at the line of the focal point in time is shown in Figure 6.12. In cases A and B emissions are caused by every pulse of the sequence, whereas in C emissions are mostly caused by the first pulse. The respective spectrograms of emissions emanating from the HIFU focal point for these sonications are presented in Figure 6.13. Broadband noise emissions, indicative of inertial cavitation activity

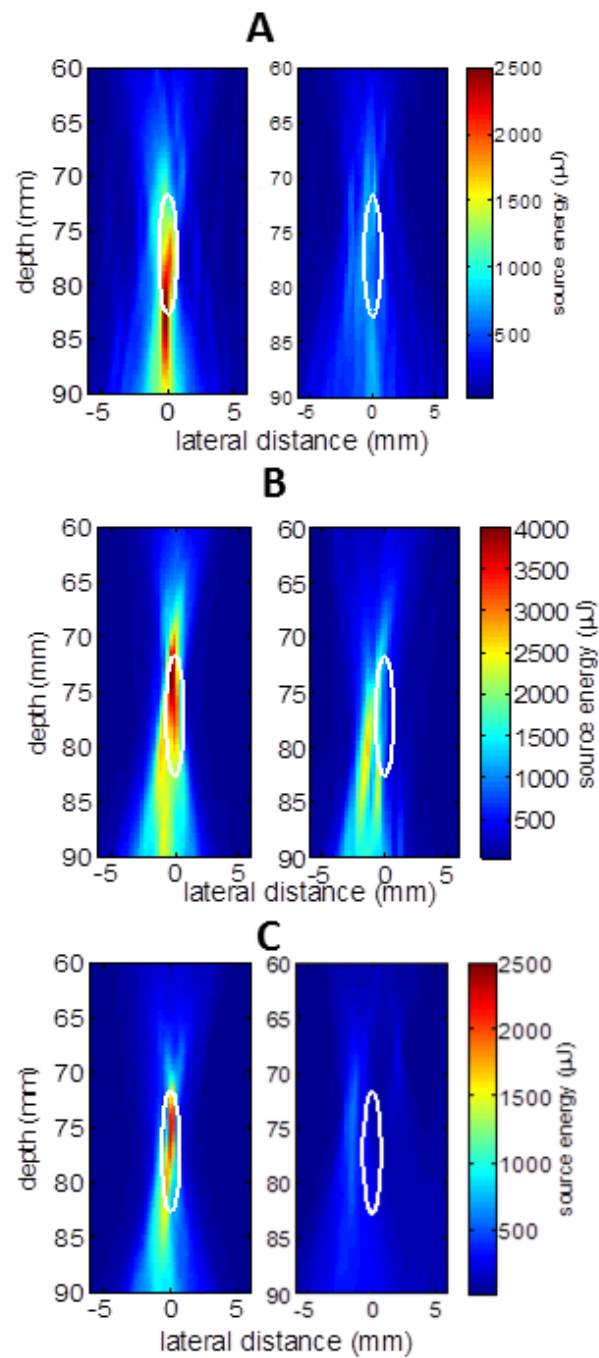


Figure 6.11: Representative passive acoustic maps from sonications done in the perfused liver model. Exposure conditions: 11 MPa ppk 25% DC, 1Hz PRF, 5 s duration (both A and B) and 7.4 MPa ppk 25% DC, 1Hz PRF, 5 s duration (C). All sonications were performed near major vessels.

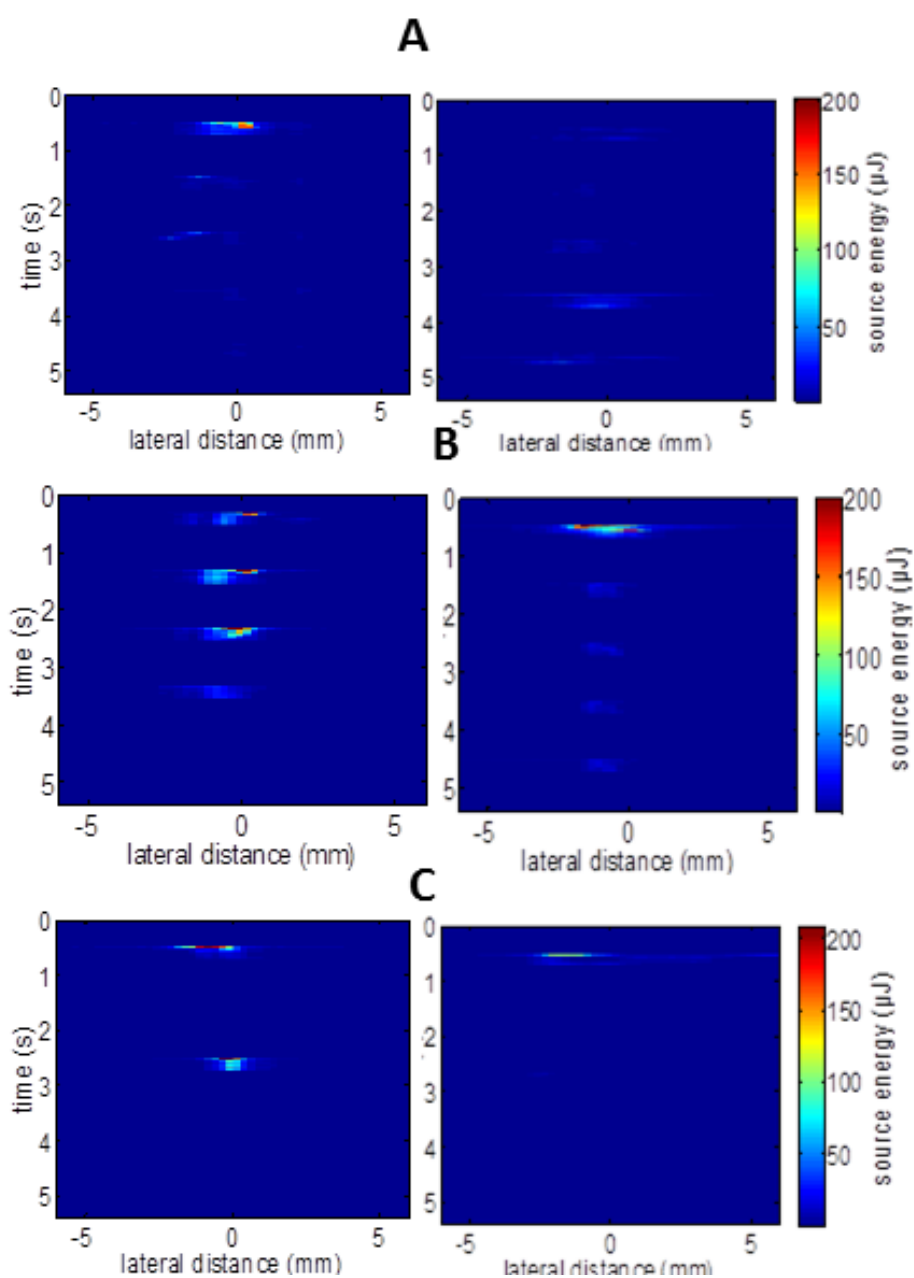


Figure 6.12: Evolution of the total acoustic energy emitted from the distance where the maximum of the HIFU focus is, in time, for representative single sonications from each scan. The acoustic pressure in (A) and (B) is 11 MPa peak-to-peak and the acoustic pressure in (C) is 7.4 MPa peak-to-peak. These scans were performed near large vessels. The acoustic emissions in A and B are generated in all pulses, whereas the acoustic emissions in C are generated mostly in the first pulse.

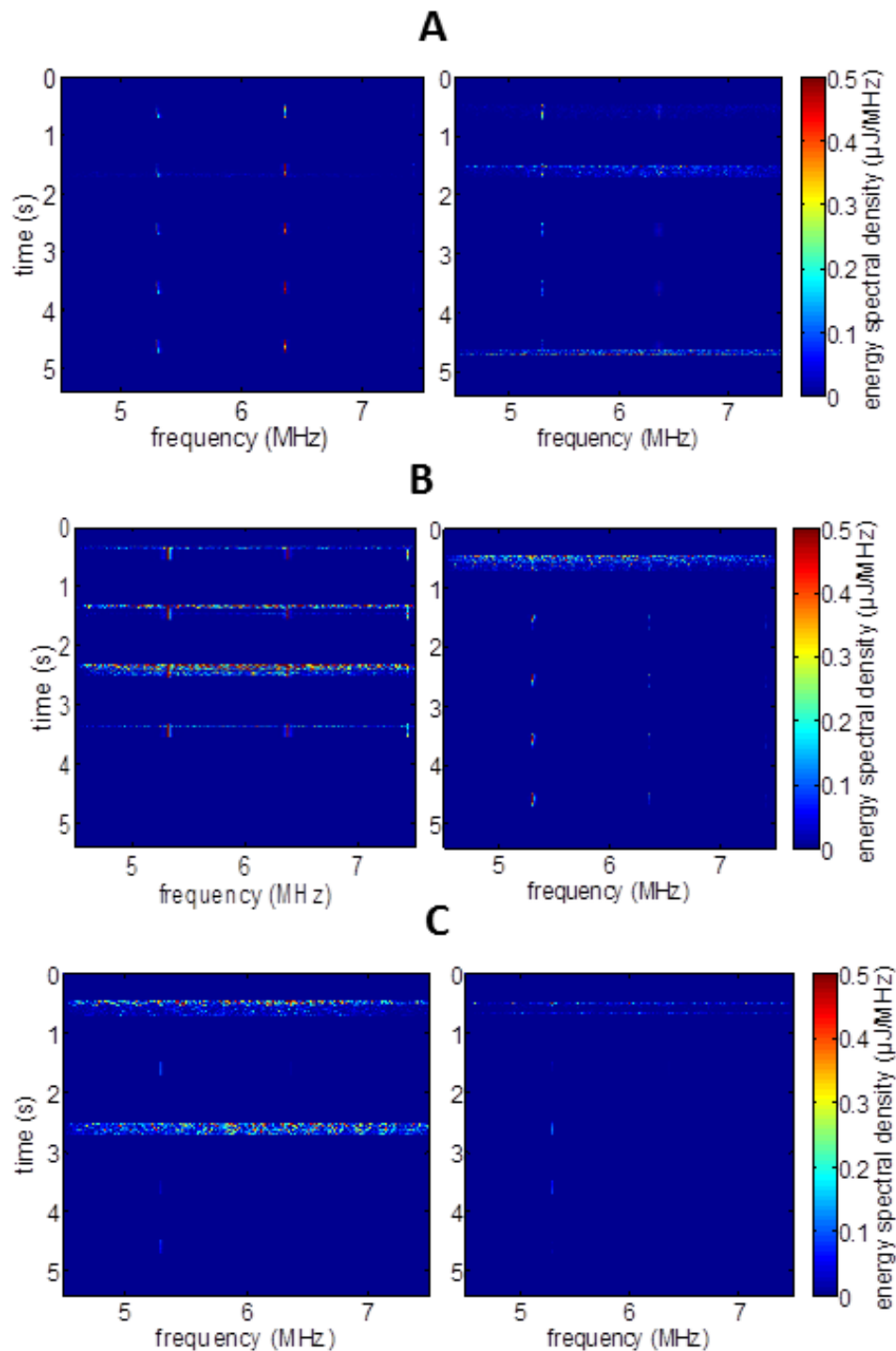


Figure 6.13: Representative spectrograms of sonications from each one of the three scans. Exposure conditions: 11 MPa ppk 25% DC, 1Hz PRF, 5 s duration (both A and B) and 7.4 MPa ppk 25% DC, 1Hz PRF, 5 s duration (C). All sonications were performed near major vessels. Broadband noise emissions that are indicative of inertial cavitation activity are present in cases A, B and C.

are observed in all A, B and C cases.

It should be stressed again that Figure 6.11 shows emissions from a rectangular *area* around the HIFU focus (PAM), whereas Figure 6.12 shows the evolution in time of the emissions from a *line* of the PAM at the distance of the HIFU focus and Figure 6.13 shows the spectrograms from emissions from a *single point* coinciding with the HIFU focus. Especially in the perfused liver experiments, the value of the passive acoustic maps is amplified, because cavitation activity occurs outside of the focal region in many cases, due to blood flow. If a PCD was used instead of a linear array, this information would be lost. Figure 6.14 shows the drug release at the location of each of the three scans. The drug release was measured by fluorescence microscopy, and drug release can be observed in the case of the 7.4 MPa ppk exposure in the region near a large vessel. This is the first time that low temperature sensitive liposomes have been 'activated' and have released their content due to cavitation-enhanced heating in a perfused liver model.

In order to assess with certainty why the drug was not released in the other cases, more experiments need to be performed. Possible reasons include higher or lower temperature rise than that predicted, movement of the liver tissue that could have compromised the alignment of the scans alignment and the registration of the histology slices with the passive maps, or very low or no extravasation of the liposomal formulations, due to their 100 nm diameter. On this last point, it should be noted that porcine endothelium is surrounded by a layer of muscle that is not present in humans, and the effect of this layer on extravasation of nanoparticles remains unknown and should form the basis of further investigation.

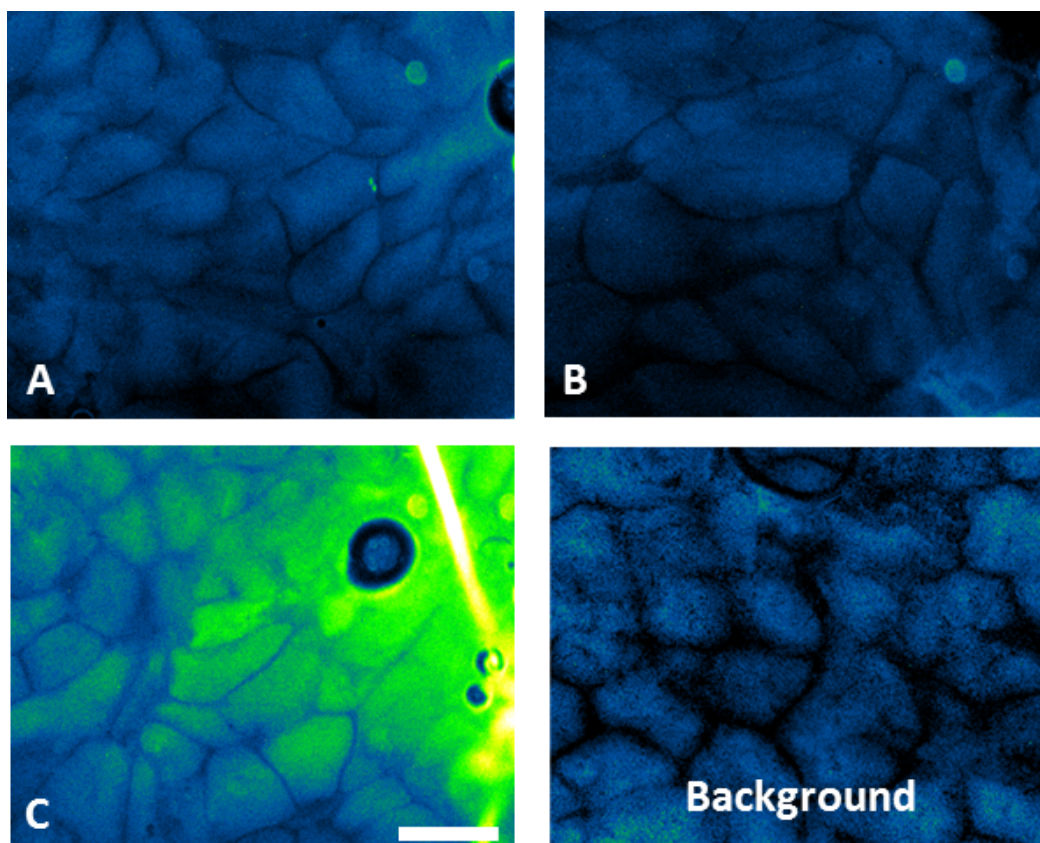


Figure 6.14: Drug Release in the perfused liver model. (A) 11 MPa ppk 25% DC, 1 Hz PRF, 5 s duration, (B) 11 MPa ppk 25% DC, 1 Hz PRF, 5 s duration and (C) 7.4 MPa ppk 25% DC, 1 Hz PRF, 5 s duration. Background control: liver parenchyma from different lobe. All samples were imaged with the same conditions. Fluorescence intensity range : 735 to 1775 arbitrary units, Bar: 1.287 mm. Increase in fluorescence intensity, indicative of drug release is observed in (C).

6.4 DISCUSSION

The purpose of the work presented in this chapter was to characterise the acoustic and thermal properties of perfused liver, so that the likely clinical relevance and feasibility of drug release from thermally sensitive liposomes by cavitation-enhanced mild hyperthermia could be assessed under flow conditions representative of human livers. The effect of vessel proximity on the cavitation threshold was estimated (vessels of diameter ~ 5 mm), and the thermal response of the various regions of the liver to a wide range of acoustic pressures was identified.

Additionally, the effect of perfusion on temperature rise and cavitation threshold was assessed, by ceasing the perfusion temporarily. Finally, a feasibility study of drug release from LTSLs by cavitation-enhanced heating was performed. In this section the effect of perfusion, and blood flow on the cavitation threshold and thermal response will be discussed and the acquired results will be compared to the experiments that were performed on characterisation of degassed liver and the novel TMM in Chapter 3.

6.4.1 Characterisation of Cavitation Threshold and Thermal Response

In Figure 6.6 the effect of vessel proximity on the cavitation threshold is shown. Higher acoustic pressures are required in regions closer to vessels for instigation of inertial cavitation activity, than in regions far from large vessels. More specifically, a difference of 3 MPa ppk has been observed as the pressure difference that is required to have a substantial amount of cavitation activity (on the order of $10^3 \mu\text{J}$) in both cases. It has also been observed that the reproducibility of cavitation activity is greater in microperfused regions. Blood flow proximal to the sonications induces higher levels of uncertainty. At the moment the reason for this is unclear, because there is no clear indication of where the cavitation nuclei originate, i.e. in the microvasculature or the veins and more experiments need to be conducted to identify that.

In Figure 6.6 a sudden decrease in the energy level of cavitation is also observed at 7 MPa ppk. That could be attributed to depletion of endogenous nuclei or to shielding due to pre-focal cavitation activity that is outside the field of view of the passive maps. From Figure 6.6 it can also be observed that the magnitude of the energy of the acoustic emissions at the HIFU focus (non-harmonic emissions) is

higher in the case of the microvasculature than in the case of the large vessel being close to the exposures. In both cases however, the magnitude of the non-harmonic energy generated reaches a plateau at ~ 9 MPa ppk.

It is expected that perfused liver, being a non-homogeneous tissue will have local variations in the content of endogenous nuclei, and hence there will be variations in the cavitation threshold both across livers and within the same liver. This was consistently observed across all perfused liver experiments, both close and far away from large vessels. However, it has been observed that the maximum magnitude of acoustic emission energy that was generated was similar across livers and in different regions in the same liver. Interestingly, there were two cases (solid red line and dotted red line) for exposures at the microperfusion region, in which the acoustic emissions generated were almost identical (the two lines overlap and the dotted line is visible underneath the solid line at pressures above 4.4 MPa ppk.).

In terms of the temperature rises observed, if there is a large vessel present in the vicinity of the HIFU exposure, the acoustic pressure that is required in order to reach a certain level of hyperthermia is much higher than what is required if there is not a vessel present. That was anticipated, because blood perfusion creates a heat sink effect, as described by the BHTE equation (Equation (2.13)). An interesting observation is that the perfusion in the capillary bed does not appear to have a significant effect on the induced temperature rise. A possible explanation could be that the blood flow in the capillary bed is low and there is not significant heat-sink effect to affect the HIFU-induced temperature rise, thus similar temperature rises are observed both with perfusion and when perfusion is ceased. Cavitation-enhanced heating especially is rapid and thus less affected by perfusion.

6.4.2 Comparison with the novel TMM

If the observed cavitation threshold and temperature rises in the perfused liver model are compared to those of the novel TMM, described in Chapter 3, it can be concluded that the TMM is more similar to liver tissue than what was thought when it was first developed. One of the major differences that the TMM had from the degassed liver tissue that it was compared to, was the cavitation threshold. In the TMM the cavitation threshold was identified to be around 4.4 MPa ppk, whereas the cavitation threshold of the degassed liver was much higher, and, to be precise, throughout the whole range of acoustic pressures that was investigated, no inertial cavitation activity was detected.

On the contrary, the cavitation threshold of the microvasculature regions of the perfused liver model is very close to 4.4 MPa ppk, as described in §6.3.1, with cavitation events being observed at even lower pressures. Better agreement is also seen between hyperthermia levels observed in the TMM and in perfused liver. In the TMM 4.4 MPa ppk were required for 6 s CW exposures and 5.6 MPa ppk for 25% DC 5 s exposures. In the perfused microvasculature region of the liver, mild hyperthermia was observed at 3.7 MPa ppk for the CW exposure and 4.8–5.6 MPa ppk for the pulsed exposure. Even though these results are very promising, this is work in progress, and many parameters have yet to be tested. Table 6.2 gives an overview of the exposure conditions that caused mild hyperthermia for drug release in the degassed liver, perfused liver and TMM, for comparison

6.4.3 Drug Release from Low-temperature Thermally Sensitive Liposomes

The drug release feasibility study showed for the first time drug release by cavitation-enhanced heating in a perfused liver model. Even if mild hyperthermia

Table 6.2: Comparison of the effect of various exposure conditions in the TMM, degassed liver and perfused liver.

Material	Exposure conditions	Effect
TMM	4.4 MPa ppk, CW, 6s	mild hyperthermia and drug release
TMM	5.6 MPa ppk, 25% DC, 1 Hz PRF, 5 s	cavitation-enhanced mild hyperthermia and drug release
Degassed liver	4.4 MPa ppk, CW, 6s	cavitation-enhanced mild hyperthermia, drug release not tested
Perfused liver - mv ^a	3– 3.7 MPa ppk, CW, 6s	mild hyperthermia, drug release not tested
Perfused liver - mv	4.4– 5.5 MPa ppk, 25% DC, 1 Hz PRF, 5 s	cavitation-enhanced mild hyperthermia, drug release not tested
Perfused liver - lv ^b	5.5–7.5 MPa ppk, CW, 6s	cavitation-enhanced drug release not tested (cavitation above 6.5 MPa)
Perfused liver - lv	7.3– 11 MPa ppk, 25% DC, 1 Hz PRF, 5 s	cavitation-enhanced mild hyperthermia, drug release at 7.7 MPa

^amicrovasculature^blarge vessel

was reached in the target region of the perfused liver, it was not necessarily expected that drug release would be achieved, because the amount of liposomes that can accumulate in the liver parenchyma is unknown. As described in Chapter 1, the liposomes have a diameter of ~ 100 nm, in order to extravasate through the leaky tumour vasculature and *selectively* accumulate in tumours. Since the porcine liver that was used was healthy, it was not necessarily expected that liposomes would accumulate in the tissue. This accumulation that facilitated liposomal 'activation' with the appropriate HIFU exposure parameters, can be attributed to the inherently leaky vasculature of liver tissue.

In future work, release over a wider range of parameters has to be tested. Another challenge of the drug release experiment was timing of the sampling. The sonication scans for the assessment of drug release were performed immediately after the drug injection. As each sonication scan requires a considerable amount of time to be performed, all the scans were not performed simultaneously.

Additionally, the samples were not collected at the time of the sonication, because that would compromise the condition of the perfused liver, but after all scans were finished. Thus, a different amount of time had passed between the time of the sonication and the time of the sampling for each case. These limitations could be overcome in future through the use of biopsy techniques that do not compromise the overall integrity of the perfused organ.

6.5 CONCLUSIONS

In this chapter, the cavitation threshold and thermal response of a perfused liver model is characterised for the first time. The effects of vessel proximity and blood perfusion on the cavitation threshold and temperature rise were assessed. It has been found that in regions proximal to large blood vessels (mean diameters ~ 5 mm) inertial cavitation becomes substantial at acoustic pressures around 7.4 MPa ppk, and that in regions away from blood vessels inertial cavitation becomes substantial at acoustic pressures around 4.4 MPa ppk. Moreover, it has been shown that vessel proximity influences temperature rise, rendering tissue heating more difficult, as predicted by the BHTE equation, which states that blood flow is a mechanism of heat loss.

The effect of perfusion has been assessed as well, by stopping the blood perfusion temporarily and assessing the cavitation threshold and temperature rise induced at microvasculature regions. Interestingly it has been found that blood perfusion in the microvasculature region does not have a significant effect on temperature rise induced by HIFU excitation. It does however reduce the amplitude of the acoustic pressure required for instigation of inertial cavitation and the magnitude of the acoustic emissions generated at the HIFU focus.

The comparison of the perfused liver to the novel TMM described in Chapter 3, showed that the acoustic properties and thermal response of the TMM are significantly closer to those of perfused liver (particularly microvascular regions) than they are to ex vivo degassed tissue.

Last but not least, cavitation-enhanced heating has been successfully employed for the first time to achieve localised drug release from low-temperature sensitive liposomes



CONCLUSIONS AND FUTURE WORK

In this Chapter, a summary of the novel contributions of this work and the insight gained throughout the course of this research project is given. Also, methods to improve the understanding and control of this technology are suggested.

7.1 CONCLUSIONS

Liposomal formulations of anti-cancer agents that are approximately 100nm in size have long been known to benefit from improved accumulation in tumours due to the enhanced permeability and retention effect. The formulation of the shell to yield low-temperature-sensitive liposomes, such as ThermoDox® (Celsion Corporation), further enables the use of an external heat source to trigger complete release of the encapsulated therapeutic agent in the temperature range 39-42 °C, resulting in a potent combination of mild hyperthermia and the anti-cancer drug. Prior to the present work, initial studies had demonstrated that High Intensity Focused Ultrasound could be successfully used to release anti-cancer agents from thermosensitive liposomes in mice (Dromi *et al.*, 2007), providing evidence of the great potential

of therapeutic ultrasound to act as an effective remote trigger for release.

The novelty of the present work lies in the exploitation of cavitation-enhanced heating to optimise the ultrasound exposure conditions required to achieve maximal doxorubicin release for the lowest possible input acoustic energy, and in the use of passive cavitation mapping to enable low-cost, ultrasound-based spatio-temporal monitoring of drug release during therapeutic ultrasound exposure. The concept of cavitation-enhanced ultrasonic release from thermosensitive liposomes is ultimately tested in a world-unique isolated perfused porcine liver model, which enables the investigation of the heat-sink effect of blood flows similar to those encountered in humans.

In order to enable optimisation of the ultrasound exposure conditions, a novel cell-embedding tissue-mimicking material was developed in Chapter 3 to exhibit a similar thermal response to liver tissue upon ultrasound exposure whilst enabling the assessment of cell viability pre- and post- ultrasound exposure. Furthermore, it was essential that the phantom preparation procedure be non-exothermic, to enable embedding of low-temperature sensitive liposomes without release prior to ultrasound exposure.

In order to provide an environment in which cells could survive for several days using a non-exothermic procedure, an agar-based tissue phantom was chosen. Its acoustic attenuation over the frequency range 1-4 MHz that is most relevant to hyperthermic drug release by ultrasound had to be adjusted to mimic that of liver tissue. This was achieved through the addition of glass microspheres. Incorporation of several mean glass sphere diameters and concentrations and subsequent assessment of the attenuation and thermal response of the phantom yielded a composition which behaved nearly identically to liver tissue: 1% (mg/mL) agar, 4% (mL/mL) spheres of mean diameter 35 μm (5-50 μm).

The resulting phantom displayed a frequency-dependent attenuation that is well approximated by $\alpha=6.5f-3.4$ Np/m/MHz and was found to be well within the range of that measured experimentally and reported in the literature for liver tissue. Its speed of sound was found to be 1614 m/s which compared favourably to the 1570-1660 m/s range reported for liver tissue. Furthermore, based on the heating-cooling curves obtained the thermal conductivity was also found to be comparable between the two media. The only notable discrepancy between the novel tissue phantom and liver tissue was found to be the cavitation threshold, which was measured as 4.8 MPa pp at 1.1 MHz for the tissue-mimicking material and closer to 8 MPa for degassed liver tissue: this enables successful comparison of ultrasound induced heating below 4.4 MPa at 1.1 MHz, but above that pressure, the contribution of cavitation-enhanced heating is expected to be significantly greater in the tissue phantom.

The newly developed cell-embedding tissue-mimicking material was then used to optimise ultrasound exposure conditions in Chapter 4. Appropriate temperature rises to achieve complete ThermoDox release were achieved beyond peak-to-peak pressures of 4.4 MPa, for 6 s continuous wave exposures. However, as 4.4 MPa ppk was measured to be the cavitation threshold of the medium, a higher pressure was necessary to ensure that cavitation activity would occur for every exposure. 5.6 MPa ppk was chosen as optimal because it is the lowest pressure above the cavitation threshold at which inertial cavitation emissions were observed during all exposures.

Optimisation of ultrasound parameters at 5.6 MPa ppk for cavitation-enhanced heating involved a balance of rapid temperature rise with sufficient cooldown period to allow for mild hyperthermia levels. In order to achieve this, a range of duty cycles and exposure durations were tested, with 25% DC at 1 Hz PRF 15 s providing the desired temperature rises, at the same total input energy as the 4.4 MPa CW ex-

posure. Reduced total acoustic energy delivered was achieved by reducing the total exposure duration to 5 s.

The optimal exposure parameters: 5.6 MPa ppk, 25% DC, 1 Hz PRF, 5 s duration were evaluated for treatment safety. Huh-7 hepatocellular carcinoma cells were embedded in a TMM without liposomes, the TMM was sonicated and the cell viability was measured 24 h after the exposures. The aforementioned exposure conditions caused no loss in cell viability in the absence of ThermoDox. Apart from treatment safety, this also ensured that any observed effect on cancer cell death in the TMM would be a direct consequence of drug release from the liposomes, rather than a result of ultrasound-induced mechanical and thermal effects.

The efficacy and efficiency of drug release with the newly developed ultrasound exposure conditions were evaluated by measuring drug release and cell viability in the novel liposome- and cell-embedding TMM after it was exposed to HIFU. The optimised exposure conditions (5.6 MPa ppk, 25% DC, 1 Hz PRF, 5 s) resulted in 100% drug release in the focal volume and more than 80% cell death at 24 hours. Neither cavitation nor drug release were observed for a pressure amplitude of 4.4 MPa ppk, suggesting that at a 25% duty cycle cavitation activity is a necessary and sufficient condition for drug release.

In Chapter 5 passive acoustic mapping was employed for spatial mapping of doxorubicin release from thermally sensitive liposomes. The spatial distribution of drug release was compared to the spatial distribution of the passively acquired acoustic emission, demonstrating a correlation for the first time. Maximum drug release was observed at the locations where cavitation activity was maximal, and it dropped rapidly by 60% around them, to almost the background values. Around the sonication locations there was an up to 40% release observed, which was expected both due to drug diffusion (the molecular weight of doxorubicin is 544 Da)

and heat diffusion which causes low temperature rise at the exposure surroundings (see Chapter 4). The extent of release was also found to correlate with the energy of the generated emissions. Passive acoustic mapping could thus provide a novel method for assessing both when and where the drug has been successfully released in a clinical context.

Having now optimised the HIFU exposure parameters for safe and efficient cavitation-enhanced mild hyperthermia and having demonstrated feasibility of spatially monitoring the treatment by passive acoustic mapping in a novel tissue-mimicking material, the final endeavour was to translate all these findings in a world-unique isolated perfused porcine liver model in Chapter 6. The perfused liver model, being very similar to human liver, enables further optimisation of ultrasound exposure parameters to account for the effect of blood flow, which was not considered during experimentation in the solid TMM.

The liver model was characterised in terms of cavitation activity and thermal response for a wide range of acoustic pressures. The cavitation threshold was identified as 7.4 MPa ppk, 4.6 MPa ppk and 3.4 MPa ppk in perfused regions adjacent to large vessels, perfused regions distant from large vessels and non-perfused regions distant to large vessels respectively. It was also observed that near large vessels the cavitation threshold was lower by approximately 2 MPa ppk when a pulsed exposure was performed than when a continuous wave exposure was performed. The cavitation threshold of microperfused regions was in much closer agreement with the cavitation threshold of the TMM than that in degassed liver, hence highlighting the clinical relevance of the TMM.

Vessel proximity and thus bulk blood flow was identified as a major cause of heat loss, whereas microperfusion did not present any significant effect on tissue heating. Acoustic pressures lower than 4 MPa ppk were necessary to accomplish mild

hyperthermia in perfused and non-perfused regions of microvasculature, whereas more than 5.5 MPa ppk were required to achieve the same level of heating in regions proximal to a large vessel, for continuous wave exposures. For the 25% DC, 5 s pulsed sequence, the lowest acoustic pressures at which 45°C was reached were 8.8 MPa ppk, 3.7 MPa ppk and 5.6 MPa ppk for perfused regions proximal to a large vessel and perfused and non-perfused peripheral regions respectively.

In the final part of this work, a feasibility study for drug release in the perfused-liver model was performed. Raster scans of pulsed sequences (25%DC, 1 Hz PRE, 5 s) at 7.3 MPa pk and 11 MPa ppk were performed in regions proximal to large vessels and a raster scan at 5.5 MPa ppk 6 s continuous wave was performed in perfused peripheral (microperfusion) region. Drug release was observed at the 7.3 MPa ppk sonication scan near a large vessel, thus showing for the first time that cavitation-enhanced heating can be used for localised drug release from thermally sensitive liposomes in a perfused liver model.

7.2 FUTURE WORK

Although the feasibility of cavitation-enhanced drug release in a perfused liver model was demonstrated, a comprehensive optimisation of acoustic parameters remains to be performed. In the experiments done in the present work, temperature measurements were acquired close to large vessels, but only at one location at a time. It would be very interesting to position thermocouples at a range of locations at an increasing distance from the vessel and measure the temperature rise during sonication, to gain insight into the spatial distribution of temperature rise. Furthermore, a comprehensive study should be done for a wide range of vessel diameters and blood flow rates, which would provide a more direct relationship between blood flow/vessel diameter and cavitation threshold/thermal response. The cavi-

tion activity and thermal response of the liver requires also further characterisation across several livers.

This more detailed study will give more confidence for identifying the parameters for cavitation-enhanced drug release in a clinical environment. In the current study the feasibility of drug release in the perfused liver model was demonstrated, which is an accomplishment on its own as healthy liver tissue does not exhibit the large vessel fenestrations and gaps that aid liposomal accumulation in the tumour parenchyma (EPR effect). A more comprehensive study can be performed to optimise the conditions for maximum drug release from LTSs due to cavitation-enhanced mild hyperthermia in regions both adjacent and distant to blood vessels. In this manner, if this treatment reaches the clinic, the clinician will be able to identify the parameters to be used by acquiring a B-mode or Doppler image of the target area.

One major challenge that was faced in this work and has to be addressed in the future is quantification of drug release from the liposomes in the perfused-liver model. Because the sonication-scans were not performed simultaneously in all regions, the liposomal accumulation in the liver tissue during each scan was different and because the samples were not acquired immediately after the scans, a control value of the liposomal accumulation at a non-treated region could not be acquired at the exact time that each scan was performed.

This caused a dual complication: First, a different amount of liposomal accumulation causes a different amount of fluorescence intensity, even at normal body temperature (as shown in Chapter 4 and in the pharmacokinetics study in Chapter 5, there is fluorescence even at 37°C, and it is unknown whether it is caused by fluorescence coming from the liposomes themselves or from minor doxorubicin leakage). This compromises the qualitative comparison of drug release among regions

sonicated at a different time, because the background fluorescence may be different in each case. Furthermore, not having the absolute fluorescence value at each different time point of a non-treated area as a control makes the assessment of the absolute amount of drug release impossible.

According to the instructions provided by the manufacturer the half-life of the liposomes in tissue is 24-48 h, which implies that if all scans are performed after all the injected drug has already circulated through the perfused-liver vasculature (approximately 10 min as measured in the pharmacokinetics study) the amount of liposomes in the tissue should remain relatively constant, making comparisons easier. However, this is something that has yet to be tested.

Finally, mapping of the drug release by mapping cavitation activity can be done in the future. In the experiments presented in this work, the necessary setup was already used (multi-element linear imaging array aligned to the HIFU transducer) and passive acoustic maps were acquired. However, in order to be able to map the release with the acoustic emissions and correlate the emission energy magnitude to the amount of drug release, more work needs to be done, and a large sample size has to be collected from areas both near and away from vessels.

As a longer term plan, if mapping of drug release and acoustic emissions proves successful in 2-D, which is attempted here, it can be translated in 3-D by applying the same principles but using a 2-D multi-element imaging array instead of a linear one.

Longer-term, it is clear that the desired outcome of the present work is the clinical implementation and adoption of ultrasound-guided targeted drug release from thermosensitive liposomes. The clinical HIFU device in Oxford is already equipped with an array that enables passive acoustic mapping during exposure, and it is

hoped that in due course a clinical trial of thermosensitive drug release can be conducted that utilises the exposure conditions and release mapping methodologies developed in the present work.

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PRELIMINARY RESULTS ON LIPOSOMAL ACTIVATION

Preliminary results on drug release from LTSs in a TMM that is a precursor to the optimised TMM presented in Chapter 3

In order to investigate the feasibility of releasing doxorubicin from ThermoDox with HIFU-induced hypethermia and the effects of (i) ultrasound, (ii) inertial cavitation and (iii) heating on the release, an initial study was carried out in a non-optimised TMM which consisted of 0.5% g/ml agarose and 0.2 g/ml sucrose (Mylonopoulou *et al.*, 2010). A HIFU transducer of 1.15 MHz frequency was used and the experimental setup was similar to the setup shown in Figure 3.7 in Chapter 3.

Four different exposure conditions were used: (i) 10.5 MPa ppk CW, 6 seconds for mild hyperthermia in the absence of cavitation, (ii) 10.19 MPa ppk, 10% DC, 1 Hz PRF, 60 seconds, for similar acoustic effects and same total energy delivered as (i) without mild hyperthermia, (iii) 12 MPa ppk, 10% DC, 1 Hz PRF, 43 seconds, for no heating in the presence of inertial cavitation activity and (iv) 1 second CW

exposure at 11.702 MPa ppk, which results in the same temperature rise in 1 second (7.2 °C), as is achieved over a 6 second exposure at 10.5 MPa ppk, to dissociate the effect of absolute temperature rise from rate of heating. Drug release was measured as described in §3.3.3.

The results (Figure A.1) suggest that the 6-second exposure (exposure i) presents significant drug release. At the 60 seconds exposure (exposure ii), there is some fluorescence increase, indicating partial doxorubicin release. This release is not necessarily because of the ultrasound radiation force or mechanical effects, since it is possible that the temperature even though it was well below 42°C, could have reached a level close to 39°C. At the 43 seconds exposure (exposure iii) there is no rise of the fluorescence intensity, indicating that cavitation activity alone does not affect ThermoDox and does not cause release. Finally, the absence of fluorescence rise at the last exposure type (exposure iv) illustrates the significance of the rate of heating. The very similar temperature rises that are caused by exposure (i) and exposure (iv) have been directly compared and only the former results in drug release, suggesting that liposomal activation is not instant.

Figure A.2 shows confidential information provided by the NIH proving drug release as a function of temperature in a water bath. Release is seen to start at temperatures as low as 38°C, but only becomes significant and almost instantaneous for temperatures in excess of 40°C.

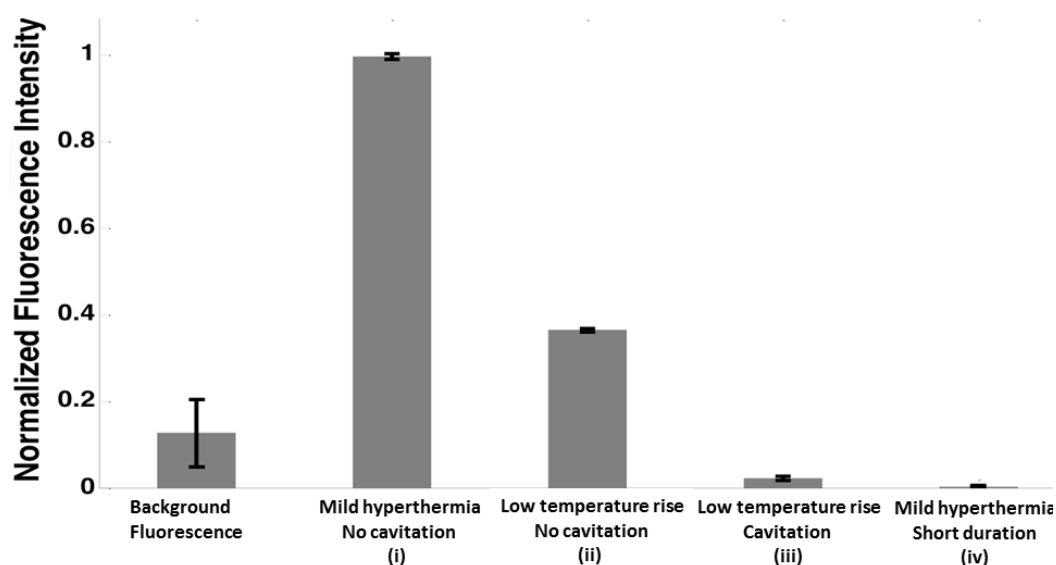


Figure A.1: Fluorescence intensity indicative of doxorubicin release from low-temperature sensitive liposomes (LTSLs), measured after the LTSL-embedding tissue-mimicking material (TMM) was exposed to the following exposure conditions: (i) 10.5 MPa ppk CW, 6 seconds for mild hyperthermia in the absence of cavitation, (ii) 10.19 MPa ppk, 10% DC, 1 Hz PRF, 60 seconds, for similar acoustic effects and same total energy delivered as (i) without mild hyperthermia, (iii) 12 MPa ppk, 10% DC, 1 Hz PRF, 43 seconds, for no heating in the presence of inertial cavitation activity and (iv) 1 second CW exposure at 11.702 MPa ppk, which results in the same temperature rise in 1 second (7.2°C), as is achieved over a 6 second exposure at 10.5 MPa ppk, to dissociate the effect of absolute temperature rise from rate of heating.

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Figure A.2: Doxorubicin release from ThermoDox at a temperature range from 37°C to 45°C, as provided by the manufacturer. Release starts at temperatures as low as 38°C, but only becomes significant and almost instantaneous for temperatures in excess of 40°C. Maximum release is reached during the first seconds at temperatures above 42°C.