Development of a Novel Process for On-Demand Generation of Medical-Grade Gas Microbubbles

By

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Abstract

The use of microbubbles (gas filled bubbles 1-10 microns in diameter) for therapeutic applications is a relatively new field of medicine. There is great potential in using such microbubbles, combined with ultrasound, to improve cell membrane permeability.

In collaboration with Artenga Inc. (Ottawa, ON), a new method for microbubble generation was developed, based on perfluorocarbon bubbles stabilized with a surfactant shell, and a prototype device was built and tested. The resulting microbubbles have been shown to be comparable to existing ultrasound contrast agents in properties such as concentration (1×10^8 microbubbles/ml), and size distribution (mean diameter of 1-3 microns). *In vitro* and *in vivo* tests have also been performed which verify that the microbubbles generated with the new device provide imaging capabilities comparable to those of existing products.

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Nomenclature

Acronyms and abbreviations:

BBB	Blood-brain barrier
HDPE	High density polyethylene
HLB	Hydrophile-lipophile balance
MGD	Microbubble generation device
MI	Mechanical Index
PFC	Perfluorocarbon gas
TBI	Transient bubble infusion (initial device concept)
UCA	Ultrasound contrast agent
US	Ultrasound

Symbols:

γ	Adiabatic ideal gas constant
γ_c	Compressibility term
γ_d	Density term
Δt	Actuation time of single pass
ϵ	Ratio of microbubble radius to initial microbubble radius (r/R_0)
θ	Ultrasound scattering angle
ρ_0	Ambient fluid density
σ	Surface tension at bubble-liquid interface
ν_{water}	Kinematic viscosity of water
C_{MI}	Constant for MI = $1 \frac{MPa}{\sqrt{MHz}}$

D	Diameter of capillary tube
D_w	Diffusivity of air in the surrounding medium
f	Ultrasound frequency
f_0	Resonant frequency of microbubble
f_R	Ratio of actual partial pressure of the gas in the surrounding liquid at saturation
H	Ostwald coefficient
I	Scattered ultrasound intensity
I_0	Incident ultrasound intensity
I_m	Membrane current
k	Wave number
n	Number density of scattering particles
p_0	Ambient fluid pressure
P_a	Ambient pressure
P_{neg}	Peak negative pressure
r	Microbubble radius
R_0	Initial microbubble radius
R_{shell}	Mass transfer resistance of the microbubble shell
Re_w	Water equivalent Reynolds number
V	Ultrasound scattering volume
V_c	Command potential
V_m	Membrane potential
V_s	Volume of syringe

Chapter 1: Introduction

Cancer represents a tremendous burden on societies around the world. The World Health Organization estimates that there were 7.9 million fatalities due to cancer in 2007, and projections indicate that these rates will continue to rise with an estimated 9 million cancer deaths worldwide by 2015.¹ Although cancer fatalities are more prevalent in lower income countries, where resources for prevention, diagnosis, and treatment are limited, more developed countries are affected as well. In Canada, based on current incidence rates, it is estimated that 39% of Canadian women and 44% of Canadian men will develop cancer during their lifetimes. Approximately 1 out of every 4 Canadians will die from cancer.²

The term cancer refers to a group of more than 100 different diseases, characterized by cells which are no longer responding to the body. Various cancers can affect cells in any part of the body. When most normal cells are damaged or mutated to an extent that they are no longer functioning, they are eliminated by the process of apoptosis (programmed cell death). Cancer cells are unable to undergo apoptosis, as a result of having undergone a genetic mutation, leading to abnormalities which result in the altered function. This can manifest itself as uncontrolled growth, invasion of the cells into

surrounding organs and tissues, or metastasis, which is the spread of the cells to other parts of the body via the lymphatic or circulatory systems.

Most cancers are associated with the formation of an abnormal growth known as a malignant tumour. Unlike benign tumours, which are classified as tumours which do not display uncontrolled growth, invasion, or metastasis, malignant tumours are likely to lead to serious health complications. The continued growth and invasion of malignant tumours result in extensive damage to healthy tissue, release of toxic by-products, and compromised organ function. Without treatment, the cancer usually progresses uninhibited, eventually leading to damage severe enough to compromise essential life-sustaining functions.

Approaches to the treatment of cancer are constantly evolving, but the most commonly-used approaches involve targeting the tumours and attempting to destroy and/or remove them while they are still confined to a single location, and have not yet had a chance to metastasize. Once a cancer becomes metastatic, it is much more difficult to treat as the affected cells can travel throughout the entire body, making them difficult to locate and to eliminate. Common techniques for removing or destroying tumours involve surgery, radiation, chemotherapy, or frequently a combination thereof. For many types of cancer, chemotherapy has proven to be a very effective means of destroying cancer cells. Unfortunately, the means by which chemotherapy drugs destroy cancer cells tend to have very serious side-effects to a patient's healthy cells. Typically, the dosage of chemotherapy drugs administered is as high as a patient can withstand, in an effort to maximize the potential destruction of the cancerous cells. Due to the adverse

side effects including severe anaemia, toxicity, and compromised immune function, subsequent doses of the drugs must be reduced as the body's ability to withstand treatment is decreased by the treatment itself. Although this approach can be effective in some cases, it is not always capable of destroying all cancer cells before the side-effects of the treatment deplete the patient to a state of such weakness that further treatment is not feasible.

In some cases, chemotherapy treatments are completely ineffective, due to an intrinsic resistance of the cancer cells to the drug's treatment modality. It is believed that some cancer cells feature membranes which do not physically allow the chemotherapy drug molecules to enter the cell, while others are capable of actively removing the drugs from the cell at a rate which is faster than they are absorbed by the cell. In such cases, reliance on surgery, radiation, or other means are the only option for treatment. Depending on the location and structure of the tumour, such alternatives may not be viable, rendering the cancer essentially untreatable.

1.1 The cell membrane and sonoporation

Improving the ability to transfer chemotherapy drugs across the cell membrane by increasing its permeability could allow for a drug treatment of an otherwise untreatable tumour. In addition to improving cancer therapy, increasing the permeability of a cell membrane can have many other potential uses including targeted drug delivery, gene therapy, and transferring drugs through complex interfaces such as the blood-brain barrier.

Sonoporation is the process by which ultrasound energy is used to create transient perforations in a cell membrane without permanently damaging or destroying the cell. It has also been used to enhance the permeability of capillaries³ and dermal layers.⁴ This process has been shown to benefit a great deal from the addition of cavitation nuclei or microbubbles: a substantial reduction in the acoustic power required to achieve sonoporation has been noted when microbubbles are present.^{3,5,6,7,8,9} During sonoporation treatment, microbubbles are delivered to a location in close proximity to a target cell, and are then exposed to sonic energy using an ultrasound transducer. The ultrasonic pressure waves cause the microbubbles to resonate, the action of which leads to the formation of small tears or pores in the cell membrane. This allows the transfer of active substances, such as large molecules, genes, and particles into the target cell. The specific mechanisms of sonoporation are currently not fully understood, but are believed to involve a possible combination of gas microjets caused by the asymmetric collapse of microbubbles,⁴ the shear forces generated by the liquid flows around the cavitating bubbles,^{10,11} and/or shock waves created by the bubbles as they collapse.^{4,12,13}

Two types of proposed sonoporation therapy procedures exist: reparable sonoporation, and lethal sonoporation.¹⁰ In the case of reparable sonoporation, the cell membrane damage is non-permanent, which can temporarily increase the permeability of the cell membrane, allowing the delivery of drugs, gene therapy agents, or any materials which would normally be unable to traverse the cell membrane without killing the cell. Lethal sonoporation is typically associated with higher-energy disruptions, and leads to

extensive damage to the cell membrane, which is beyond the capability of the cell to repair, ultimately resulting in destruction of the cell.

A clear display of the potential effectiveness of using sonoporation to reparably disrupt a cell membrane has been achieved experimentally.⁵ In the experimental setup (illustrated in Figure 1-1), a *Xenopus* oocyte, the female gametocyte which an egg develops from, was treated and immersed in a solution above an ultrasound transducer as shown.

Figure 1-1 Experimental setup for reparable membrane disruption [5]

The transmembrane current flow was measured under a number of experimental parameters. As shown in Figure 1-2, the ultrasound alone had virtually no effect, but the in the presence of microbubbles the ultrasound pulse resulted in a significant change in

the transmembrane current, most likely the direct result of increased permeability of the cell membrane due to sonoporation.⁵ It is worth noting that the current flow returned to its resting state shortly after the ultrasound pulse was terminated, indicating that the increased permeability was a transient effect, and that the cell membrane was capable of repairing itself after the insonation was terminated.

Figure 1-2 Transmembrane current during sonoporation: effect of ultrasound and microbubbles [5]

Very promising research has indicated that the possibility of using sonoporation to improve chemotherapy drug treatment can lead to dramatic results.¹⁴ Recently, Iwanaga et al. demonstrated improved cytotoxic effects on cancer cells when using sonoporation to deliver Bleomycin (a common chemotherapy agent), leading to almost complete

destruction of gingival squamous carcinoma tumours in mice.¹⁴ Another study by Larina et al demonstrated complete tumour regression by using ultrasound-induced cavitation.¹⁵

Figure 1-3 illustrates the observed effect of treating human colon cancer tumours with sonoporation-enhanced chemotherapy, vs. chemotherapy alone.

Figure 1-3 Complete regression of tumours using sonoporation-enhanced drug delivery [15]

1.2 Therapeutic microbubbles and Artenga Inc.

Artenga Inc. is an Ottawa-based start-up company specializing in the development of a commercially-viable microbubble generation device for improving drug delivery. Although commercially available microbubbles exist in the form of ultrasound contrast

agents (UCAs), the primary design intent for these UCAs has to do with image enhancement and the contrast agents are not necessarily optimized for therapeutic treatment procedures. The intent of the company is to create a commercial device for the on-demand generation of microbubbles which can be customized and optimized for therapeutic applications including sonoporation. Whereas UCA microbubbles make use of microbubbles with properties which are important for imaging, some of the properties which lead to optimal imaging characteristics are not necessarily relevant (or optimal) for therapeutic applications.

Due to prevalence, tumour location, and tendencies towards drug resistance, the most likely candidate cancers to benefit from microbubble-enhanced drug delivery are colorectal, breast, pancreatic, ovarian, and liver. An overview of these target cancers is shown in Table 1-1.

Table 1-1 Drug-resistant cancers suitable for microbubble-enhanced sonoporation treatment [16]

Cancer Type	Drug resistance	Ultrasound-guided intervention	Deaths per year (U.S. & Canada)
Colorectal	Inherent	Yes	56,000
Breast	60%	Near lungs/ribs	48,000
Pancreatic	Inherent	Yes	34,000
Ovarian	85%	Yes	18,000
Liver	high	Yes	14,000
Total			170,000

1.2.1 Original design concept

The Transient Bubble Infusion (TBI) device was the original design concept proposed by James Keenan, the founder of Artenga Inc. The intent of the TBI device was to provide a means of continuous infusion of microbubbles, coupled with the capacity to concurrently inject drugs or other materials directly to the target site. A separate ultrasound transducer was to be used to induce the acoustic cavitation necessary to trigger the sonoporation process. The preliminary concept sketch is shown in Figure 1-4.

Figure 1-4 Concept sketch of proposed Artenga TBI device [16]

The purpose of the current research was to evaluate the feasibility of such a design, and to collaboratively develop a means to achieve the design goals set out by the company.

1.2.2 Microbubble generation

One of the key concepts of a device used for improving drug delivery via the sonoporation process is to develop a means to produce microbubbles which are optimized for therapeutic applications. As research into such mechanisms is currently in its infancy, the optimal microbubble parameters are not yet fully understood. Currently, the majority of microbubble and sonoporation research is limited by the use of commercially available UCAs as the source of the microbubbles. The intent of the Artenga device is to develop a means for generating microbubbles on demand, while maximizing the flexibility to allow

for changes in various microbubble parameters as might be required by research. Allowing control of parameters including mean microbubble size, size distribution, concentration of microbubbles, gas composition, microbubble shell components, and carrier liquid properties, could provide additional options in this field of research which are not currently available.

Additionally, the use of an on-demand device could provide additional flexibility in more complex cases, such as those involving the binding of drugs or targeting ligands to the microbubble surfaces. Depending on the exact requirements, preparing such customized microbubbles with existing technology can involve a complex series of procedures including dilution, mixing, rinsing, and centrifuging of the materials. For consistency, such procedures would need to be carried out by an experienced laboratory technician. Developing a device which could potentially incorporate such series of procedures into a self-contained, consistent, automated process could greatly accelerate both the research, as well as the eventual clinical use of products developed through this research.

Chapter 2: Background and Literature Review

2.1 Microbubbles and ultrasound

Ultrasound imaging is the safest, fastest, least expensive method of scanning for many types of medical diagnoses. Although the quality of images obtained using ultrasound is relatively inferior to other methods of diagnostic imaging, such as magnetic resonance (MRI) and computed tomography (CT), ultrasound is still the most widely-used imaging method worldwide. In the United States, there are approximately 75,000 ultrasound instruments in use, as compared to approximately 7,000 CT units, and 5,000 MRI units.¹⁷ To compensate for the shortcomings of ultrasound imaging, ultrasound contrast enhancement is highly desirable. Microbubble-based ultrasound contrast agents have been established over the past 20-30 years as the most effective means of ultrasound image enhancement.¹⁷ Although there has been a great deal of research conducted with UCA microbubbles in recent years, as well significant advances in microbubble technology, their behaviour is still not yet fully understood.

The use of microbubbles as ultrasound contrast agents was originally discovered in 1968 by Gramiak and Shah, who observed strong, short-lived echoes from agitated saline injected into the ascending aorta during echocardiographic recording.¹⁸ Microbubble UCAs entered widespread clinical application through the 1980s,¹⁹ but it was not until the

turn of the 21st century that major advances in microbubble technology were made. The most significant development was the discovery and production of UCAs filled with heavier-weight perfluorocarbon (PFC) gases, rather than the air-filled microbubbles which had been in use up until that time. The use of PFC gases extended *in vivo* persistence of the microbubbles from a matter of seconds to minutes.²⁰ This allowed for a great deal more flexibility in imaging, as the microbubbles could be injected intravenously and circulated throughout the body. The late 1990s also brought about research into the use of microbubble UCAs in therapeutic applications.

2.1.1 Ultrasound contrast agents

Current ultrasound contrast agents consist of an exogenous preparation of stabilized microbubbles which is most commonly introduced into the vascular system via intravenous (IV) injection. The microbubbles found in UCAs are typically under 10 microns in diameter, and are coated in either a surfactant or a solid shell (e.g. phospholipid, serum albumin, or polymer). Uncoated free gas bubbles of this size are very unstable and would dissolve too quickly in unsaturated blood to be suitable for use as contrast agents.^{21,22}

The effectiveness of microbubbles as ultrasound contrast agents is the result of their dynamic response to ultrasound. As the gas bubbles are much more compressible than the surrounding fluid or tissue, the rapid fluctuations in pressure resulting from the applied ultrasound waves cause expansion and contraction of the bubbles, which results in the bubbles scattering much more energy than any surrounding tissue. Additionally, the high frequency expansion and contraction cause the bubbles themselves to become

secondary acoustic sources, further improving the detectable acoustic signal. UCA-enhanced ultrasound images prove to be excellent for improving ultrasound imaging in cases where standard ultrasound images provide insufficient detail. This is of particular importance where UCAs are used to image vascular structures, as the sound waves scattered by red blood cells at typical diagnostic ultrasound frequencies are about 1,000-10,000 times weaker than that from solid tissue – rendering them essentially at the level of background noise, and making blood vessels virtually invisible in ultrasound imaging without microbubbles present.^{23,24}

In diagnostic ultrasound, a common expression used to quantify the acoustic power is the mechanical index (MI). This represents the approximate exposure of average tissue to the ultrasound pressure at the focal point of the beam. MI is defined as:

$$MI = \frac{P_{neg} / \sqrt{f}}{C_{MI}} \quad (2-1)$$

In the context of microbubble-ultrasound interactions, MI corresponds to the amount of mechanical work that can be performed on a bubble by the ultrasound beam, with values typically being under 0.1 where linear oscillations are desired, up to 1.0 to promote non-linear oscillation, and values in excess of 1.0 tend to result in disruption of the microbubbles.²⁵

Under ultrasound, the oscillations of UCA microbubbles may be linear or non-linear. The non-linear response of microbubbles typically occurs at higher MI levels

(between 0.1 and 1.0), due to the induced asymmetric expansion and contraction of the bubbles as they are acted upon by the ultrasound pressure waves: as the bubbles are compressed due to the high pressure portion of the ultrasound wave, the increased pressure inside the bubble and decrease in shell elasticity result in increased resistance to further compression. Conversely, as the bubbles undergo expansion during the rarefaction phase of the ultrasound wave, the resistance to expansion decreases, resulting in a significant increase in the bubble diameter above the equilibrium size. These behaviours are highly dependent on the gas contained within the microbubbles, as well as the shell properties.²⁶

For a given UCA microbubble, a number of harmonic resonant frequencies will exist as a result of the non-linear behaviour. These resonant frequencies experienced by microbubbles under ultrasound are incorporated into advanced imaging methods which exploit the non-linear response in order to improve imaging.²⁴ In imaging applications, the harmonic responses can then be isolated from the surrounding tissue, which generally does not exhibit non-linear response to ultrasound, allowing effective UCA-specific imaging methods to be employed.²⁴

UCA microbubbles can be based on either free or shell-encapsulated microbubbles. In encapsulated bubbles, which make up the vast majority of diagnostic imaging microbubbles, the shell serves to help stabilize the bubbles, particularly *in vivo*. While a rigid or semi-rigid shell is often necessary to provide adequate microbubble stability, the drawback is that the shell has a dominant effect on the acoustic response of

the microbubbles, effectively reducing the linear, nonlinear, and transient scattering efficiency.²⁷

2.1.2 Acoustic cavitation

Acoustic cavitation results from the formation and oscillation of gas pockets due to the effect of ultrasound energy being applied to a fluid where cavitation nuclei, such as microbubbles, are present. *In vivo*, the presence and predictability of cavitation nuclei is difficult to assess, and high levels of ultrasound energy are required to induce acoustic cavitation. Due to their uniform size and known composition, injected microbubbles act as excellent cavitation nuclei. They have been shown to significantly reduce the amount of ultrasound energy required to achieve cavitation sufficient to perforate cell membranes.^{5,10,28,29,30,31} Microbubble-enhanced cavitation is achieved as acoustic pressure is increased, and disruption of microbubbles is most easily achieved at lower frequencies than those used in diagnostic imaging.^{32,33}

In the context of ultrasound-microbubble interactions, two types of cavitation may occur: inertial cavitation, and non-inertial cavitation. The former is associated with the violent collapse of the microbubble, whereas the latter generally applies to a stable form of cavitation.

Although the specific modes of action are not fully understood, it is hypothesized that microbubble collapse as a result of inertial cavitation may generate a radial shock wave with enough force to disrupt a cell membrane.¹¹ Oscillations *in vivo* are typically non-spherical, due to irregularities in the surrounding tissue, as well as the presence of nearby oscillating microbubbles.³⁴ In such instances, a microbubble undergoing inertial

cavitation may collapse asymmetrically, resulting in the release of gas or liquid microjets which may perforate a nearby cell membrane.¹¹ Inertial cavitation is typically viewed as an unstable effect, but it has been shown that during an intermediate range of acoustic pressures, fragments of collapsed bubbles may coalesce and reform new bubbles, resulting in a quasi-stable form of inertial cavitation.^{35,36}

Non-inertial cavitation refers to a more stable oscillation of a microbubble around its equilibrium radius. The flow streaming around a non-inertially cavitating microbubble or group of bubbles can provide sufficient shear forces to disrupt cell membranes, as shown in Figure 2-1.

Figure 2-1 Non-inertial cavitation of a microbubble in close proximity to a cell
Frames (captured at 10^7 frames per second) show the magnitude of bubble radius
change relative to the cell of two full oscillation cycles of the microbubble under ultrasound
[37]

The mechanisms of acoustic cavitation which induce sonoporation of a cell membrane can result from a number of different processes, the specifics of which may be dependent on cell membrane properties and the surrounding extra-cellular environment. The mechanics of the sonoporation action are affected by factors including the ultrasound frequency, intensity, and pulse duration, as well as the concentration and mean diameter of the microbubbles present.³⁸ The chemical composition and thickness of the shells surrounding most UCA microbubbles have been shown to have a significant effect on the elastic properties of the shell and on the response of the bubbles to ultrasound.³⁹

2.1.3 Therapeutic applications of microbubbles

A number of unique therapeutic approaches are currently under investigation, which make use of various forms of the ultrasound-microbubble interaction. The methods range from using high intensity ultrasound waves to violently collapse the microbubbles in order to destroy or break up cells or tissues to using sonoporation-based therapy to non-destructively provide targeted delivery of drugs or genes. Although microbubbles are currently in widespread use in imaging and diagnostic applications, the therapeutic use of microbubbles may eventually become their predominant use.⁴⁰

High intensity focus ultrasound tissue ablation (HIFU) is a treatment process which makes use of an array of high intensity ultrasound transducers which can be focused on a specific point within the patient. The focused ultrasound energy provides both a thermal and mechanical (cavitation) effect which can destroy targeted tissue. Although this treatment is non-invasive, procedures involving the destruction of tumours can take

several hours. The addition of microbubbles has been shown to significantly shorten treatment time, while also improving accuracy and decreasing the likelihood of potential complications.⁸

Sonothrombolysis is another ultrasound-based treatment which has been shown to benefit from the addition of microbubbles.^{41,42} The process involves breaking up blood clots using ultrasound probes. Microbubbles injected into the target site have been shown to improve the speed of clot destruction, as well as the effectiveness of thrombolytic drugs. Some results have suggested that the use of therapeutic microbubbles to enhance thrombolysis may negate the need for additional thrombolytic drugs altogether.⁴³

In order to balance the need for an effective drug/gene delivery method without causing excessive cell lysis, the microbubbles and treatment process must be optimized in a different manner than in the case of targeted tumour or tissue destruction, where a high degree of cell lysis may be preferable. Such sonoporation-based applications include targeted drug delivery, gene transfection, and delivery of drugs across the blood brain barrier.

In vivo applications for sonoporation using microbubbles are very promising. Since ultrasound can be focused on almost any part of the body, the targeted delivery of drugs to specific sites using microbubbles and sonoporation can lead to highly selective and efficient localized drug delivery. This form of treatment is non-viral, non-invasive, and inexpensive, offering a number of advantages over other methods of targeted drug delivery currently in use.⁴⁴ Such treatment methods can make it possible to deliver drugs

in such a way that toxic levels are only attained in the desired volumes of tissue, leading to a minimization of associated side effects in healthy tissue.

Microbubble-enhanced gene transfection is a means of using reparable sonoporation to deliver gene therapy treatments to cells. While a great deal of research is currently underway in gene therapy treatments, delivery of the agents is still a major obstacle in future progress towards viable treatment methods. The approach involving microbubbles has been shown to potentially be very effective.^{29,45,46} Combining sonoporation with traditional viral vectors loaded on microbubbles has been shown to significantly improve transfection effectiveness.⁴⁷ Alternate approaches have shown improved gene expression specificity using microbubbles rather than viral vectors in cardiac gene therapy.⁴⁸

Microbubbles have also been shown to have the potential to reversibly open the blood brain barrier (BBB), which would allow drug delivery to the nervous system without damaging neurons.⁴⁹ The BBB is a protective membrane structure which restricts the passage of bacteria, as well as many chemical substances, from being transferred from the bloodstream to the central nervous system. Continued advances in the area of using microbubbles to temporarily and reversibly permeate the BBB could allow for the treatment of brain metastases with chemotherapy drugs, or facilitate the delivery of therapeutic agents for neurodegenerative diseases such as Alzheimer's.³⁸

There is a great deal of potential for future developments in customized therapeutic microbubbles. Current research may lead to the development of bubbles used to encapsulate drugs, potentially featuring targeting ligands bound to the external surface, as shown schematically in Figure 2-2. Treatment using such bubbles would involve

allowing them to chemically bind to a target site via the targeting ligands, at which point the bubbles can be disrupted using ultrasound, increasing the permeability of the membrane of the target cell and simultaneously releasing the drug.^{38,50} Such an approach could substantially improve treatments where the toxic side-effects of the drugs are currently limiting factors.

Figure 2-2 Proposed customized therapeutic microbubble [50]

2.2 Microbubble generation

Generation of gas bubbles within the 1-10 μ m size range used by UCAs is a non-trivial task. Due to the high gas pressures inside the bubbles, and the small total volume of gas contained therein, free gas bubbles in this size range are typically highly unstable and dissolve within fractions of a second. Combining a highly insoluble gas with a rigid or

semi-rigid encapsulating shell allows a significant improvement in stability. Nonetheless, the challenges associated with characterizing such small bubbles and observing their behaviour leads to a limited understanding of the mechanisms involved in creating stable bubbles suitable for use in UCA applications, as well as potential therapeutic uses. In order to develop a method for the generation of acceptable microbubbles, the process of initial bubble formation, as well as development of a means to stabilize the end product must be taken into consideration.

2.2.1 Modelling microbubble stability

Due to the very small size of microbubbles, accurately observing or modelling their behaviour becomes a complex task. Many variables come into play, such as the solubility of the gas in the surrounding liquid, the viscosity of the liquid, the rate of diffusion of the gas through the liquid, the rate of diffusion of the gas through the interface between the liquid and the gas (i.e. the surface of the bubble), and the velocity of the bubble – which may result from upward movement due to buoyancy forces, even if the fluid is essentially quiescent. At present, the stability mechanics of the sub-10 μ m microbubbles used as contrast agents are poorly understood.

In 1950, Epstein and Plesset developed simplified analytic models for bubble growth and dissolution, based on diffusion of gas through the bubble-liquid interface.²¹ The solution was based on the analysis of a free gas bubble in a quiescent liquid. Using the diffusion equation and boundary conditions, an expression for the concentration profile of gas in the surrounding liquid was developed, then used to solve for the mass transfer through the bubble-liquid interface. Three models were created: a full solution which

included the effects of surface tension, a simplified solution which neglected surface tension effects, and a further simplified approximate solution.

The full Epstein-Plesset equation for change in bubble radius with respect to time (dR/dt) is:

$$-\frac{dr}{dt} = H \frac{1 - f_R + 2\sigma / P_a r}{1 + 4\sigma / 3P_a r} \left(\frac{D_w}{r} + \sqrt{\frac{D_w}{\pi}} \right) \quad (2-2)$$

In general, a gas bubble in a liquid-gas solution will either grow or shrink, depending on the level of gas saturation in the solution. The change in volume of the bubble is determined by the mass transfer of gas molecules either into or out of the bubble via the interfacial surface. Furthermore, as the bubble size changes, the bubble boundary is displaced, resulting in a transport term in the diffusion equation, which makes it difficult to obtain a straight analytical solution.²¹

The early work done on modelling bubble stability by Epstein and Plesset neglected this term under the assumption that the concentration of gas dissolved in the solution is significantly smaller than the gas density in the bubble, and the region surrounding the bubble (through which the diffusion takes place) very rapidly becomes much larger than the bubble itself.²¹ Therefore, neglecting the effects of surrounding bubbles, the actual size of the bubble is only important in terms of determining the interfacial area across which the mass transfer takes place.

Effects of buoyancy were also neglected in the model, although as calculated for a bubble 10 microns in diameter, the buoyant force would result in a terminal velocity of approximately 0.3mm/sec. It should be noted that for bubbles of $<10\mu\text{m}$ in diameter, even such a low rate of motion would result in a slightly accelerated diffusion process due to the increased mass transfer by convection.

The work done by Epstein and Plesset on the modelling of bubble behaviour is considered to be a fairly accurate representation of actual free-gas bubble behaviour. However, as is almost invariably the case in UCA and similar microbubbles, addition of surfactants or shells results in significant deviations from the analytical model. Recent research by Borden and Longo has yielded a variation on the original Epstein Plesset equation, which takes into account the effect of gas permeation resistance by an encapsulating shell, which further limits mass transfer of gas molecules across the interfacial surface.³⁹ The steady-state governing equation for the dissolution of a shelled microbubble was determined to be:

$$-\frac{dr}{dt} = \frac{H}{r/D_w + R_{shell}} \left(\frac{(1 + 2\sigma/P_a r) - f_R}{1 + 4\sigma/3P_a r} \right) \quad (2-3)$$

The revised model added a term to account for the mass transfer resistance of the monolayer lipid shell used in the experiments. The analytical model was validated by investigating the effect of microbubble shell resistance as a function of varying the hydrophobic chain length which made up the tail end of the lipid molecules used in the

microbubble shells. The results of the simulation proved to be very consistent with the corresponding experimental work as shown in Figure 2-3.

Figure 2-3 Experimental results (a) comparison to simulation model (b) for various lipid chain lengths [39]

Additional recent research on microbubble stability has been done by Takahira et al.⁵¹ In their experiments, the microbubbles were trapped using a circular laser cone. The laser cone created a stable equilibrium where optical forces from the laser held the bubble stationary, allowing the trapped bubble to be observed with a high-speed camera. The dissolution/expansion data obtained from experiments was compared to analytical models, based on a modified version of the Epstein-Plesset equation. As the bubble was kept stationary by the laser cone, inaccuracies in the model due to the aforementioned buoyancy-induced convective mass transfer were removed. It was discovered that, for the given experimental conditions, there existed a critical radius above which a bubble would continue to grow, and below which the bubble would shrink/dissolve. It was also noted that in a shrinking bubble initially 10 microns in diameter, the rate of change of

bubble size was drastically reduced as the radius approached approximately 4 microns. In this experiment, this was normalized to the coefficient $\varepsilon = 0.4$ (where $\varepsilon = r/R_0$).

The bubbles generated in these experiments were generated from a commercial contrast agent, and were subsequently coated with a thin palmitic surfactant layer. The Epstein-Plesset method was modified to take into account the variation in surface tension, as well as the resistance to diffusion as derived in the approach used by Borden and Longo. The resulting model proved to be very consistent with the experimental data (Figure 2-4). However, in order to ensure this fit, it was necessary to introduce a stepwise variation in the permeation resistance at $\varepsilon = 0.4$. Although the specific nature of the sharp increase in permeation resistance is not fully understood, the data supports the observation that there is a sharp decrease in the gas diffusion out of the bubble, which effectively stabilizes the bubble at $\varepsilon = \sim 0.4$. It was concluded that this increase was a result of increased permeation resistance at the bubble interface due to an equilibrium condition involving the palmitic surfactant layer.

Figure 2-4 Takahira model of surfactant-shelled bubble stability [51]

2.2.2 Microbubble generation methods

Dynamic generation of microbubbles of the sizes useful for the proposed application is a non-trivial task. The means by which typical UCA microbubbles are generated involve sonication of a mixture of surfactants, lipids, or polymers in the presence of the gas to be contained within the bubbles.⁵² The sonication process mechanically agitates the components, causing a large number of inhomogeneous gas bubbles to be formed. The resulting mass of bubbles is then filtered or separated by buoyancy floatation means, sometimes with the aid of a centrifuge, in order to isolate the bubbles in the desired size range. The resulting microbubbles are then typically rinsed in order to remove residual particulate, and sealed in ampoules for delivery to the end users. Alternatively, the

microbubbles are lyophilized (freeze-dried) and provided in powdered form, which must then be combined with saline solution to reconstitute the bubbles.

Typical UCA microbubbles must be extremely stable, as they must be sufficiently robust to be shipped, and must persist in the vials for many months to have a clinically-useful shelf life. This necessitates the use of tougher, potentially stiffer, and more chemically complex shells than those which would be required for bubbles generated on demand, which would need only to be of sufficient stability to persist long enough for the duration of the treatment procedure.

A number of approaches to a more refined process for preparing microbubbles have been investigated. Methods involving the generation of uniform microbubbles by using high speed cross-flowing liquid to shear the bubbles from a gas jet generation orifice have been shown to produce repeatable, controllable microbubbles.⁵³ Another approach known as flow focussing,⁵⁴ which involves forcing a 2-phase liquid and gas flow through a small orifice, has shown potential for equally promising results.

2.2.3 Microbubble shells

The coatings on commercially available contrast agent microbubbles dramatically reduce the rate of gas diffusion, which serves to improve the stability of the bubbles.^{55,56,57} The mechanical characteristics of UCA microbubble shells also have an effect on the optimum acoustic power required to maximize the contrast effect without annihilating the microbubbles.⁵⁸ Although it is possible that microbubble persistence *in vivo* is limited by destruction of the microbubbles by the immune system, it is currently

believed that the eventual method of annihilation of current UCA microbubbles is by dissolution of the bubbles, rather than removal of the bubbles by phagocytic cells.²⁰

The most common UCA microbubble shells typically consist of surfactants, lipids, polymers, or serum albumin. Variation in the properties of the shells, such as stiffness, solubility, gas permeability, and biocompatibility, can have a strong effect on *in vivo* behaviour and interaction with ultrasound.³⁴ It has been found that in polymer-based shells, the length of the polymer chain directly affects shell stiffness.⁵⁸ A stiffer microbubble shell may theoretically lead to more predictable interactions with ultrasound, including a more symmetrical, spherical oscillation pattern. However, non-uniformities in the shell would be accentuated, which may result in complex and unpredictable behaviour.¹⁷

Surfactant-based microbubble shells feature an insoluble surfactant monolayer which functions to increase the resistance of the shell to gas permeation.⁵⁹ In a surfactant monolayer, the interfacial resistance increases with increasing hydrophobic chain length, as well as reducing the mass of the hydrophobic portion of the surfactant molecule.^{39,60}

Microbubbles with solid shells, a feature found on most commercially-available UCA microbubble preparations, tend to have significantly lower scatter efficiency,⁶¹ which has an adverse affect on their capabilities as a contrast agent and may impair the efficiency of a sonoporation-based treatment method. In an attempt to make use of the improved imaging capabilities of free-gas microbubbles, a technique of imaging generally referred to as intermittent imaging has been recently developed.⁶¹ This approach involves transiently increasing the peak ultrasound acoustic pressure beyond a certain threshold in order to

promote the rupturing of the solid encapsulating shells and releasing the gas into the surrounding liquid to form uncoated free-gas microbubbles. Since free gas bubbles provide an optimal non-linear response, and require less ultrasound energy to incite resonance, such techniques would provide obvious advantages for imaging applications, as well as potentially providing additional options for sonoporation or other therapeutic microbubble applications. It should be noted, however, that the disruption of the shells results in a significant decrease in short-term microbubble stability, restricting the effect to a relatively limited time frame (on the order of 1-5ms).

2.2.4 Gases for microbubble use

The persistence of microbubbles *in vivo* has a moderate dependence of the total quantity of microbubbles injected, but most strongly depends on the gas used within the bubbles.

The use of high molecular weight gases with low solubility has been shown to dramatically improve microbubble stability, with *in vivo* persistence increasing from approximately two minutes for a low molecular weight perfluorocarbon (C_2F_6) to in excess of 40 minutes for a significantly heavier molecular weight gas ($C_6F_{14}O_3$).⁶² Apart from the decreased solubility, the larger physical size of the gas molecules impedes their ability to diffuse through the microbubble shell. Furthermore, in UCA-enhanced imaging applications, ultrasound scattering efficiency has been shown to improve with the use of moderate weight gases featuring a high saturated vapour pressure, and comparatively low water solubility.²⁰

One study involving the use of microbubbles for gene transfection found a 10-fold increase in protein expression using perfluorocarbon microbubbles, with no noticeable benefits seen using comparable air-filled microbubbles.⁶³

2.3 Conclusions drawn from literature review

Although there appears to be an abundance of information on the research applications and clinical potential of customized therapeutic microbubbles, a consistent, readily-available source of such bubbles does not currently exist. Any research in the area of therapeutic applications is limited to what can be accomplished with existing commercially-available UCA microbubbles, which are intended and optimized for contrast-enhanced imaging applications only. Development of a system for generating microbubbles with variable parameters such as size, concentration, shell and gas composition, or the availability of binding sites on the microbubble surface would fill a niche for which there is potentially great demand.

The basic layout of the concept proposed by Artenga would lend itself to a device which could produce microbubbles on demand, requiring the development of a means of generating microbubbles of 1-10 μ m in diameter. A number of approaches were investigated, with the emphasis on a capillary tube flow focussing arrangement which had shown potential in early experiments.

From the collected research, there appeared to be sufficient indication that the addition of an encapsulating shell, most likely to be surfactant-based, would improve the persistence of the bubbles sufficiently for the proposed application. Selection of the gas

contained within the microbubbles was to be based on experimental work, though it was expected that a perfluorocarbon gas of some sort would yield the most promising results.

The process of developing an on-demand microbubble generation device involves not only the physical, chemical, and practical aspects of the design, but must also address a means to characterize the end product and verify that the resulting microbubbles met the original design specifications. This was accomplished by characterizing the microbubbles and evaluating their suitability as replacements for UCA microbubbles in sonoporation applications and other areas of therapeutic research.

Chapter 3: Problem Statement

Although microbubbles are currently available in the form of commercial ultrasound contrast agents, these products are optimized for imaging applications and are not necessarily ideal for sonoporation therapeutic techniques. Medical researchers interested in investigating such applications further are limited to either adapting their application to the available products or devising a method of preparing their own microbubbles specific to the application. There are currently no microbubbles approved for therapeutic use in humans.

The goal of Artenga Inc. is to develop a medical device for generating microbubbles on demand. The device would provide a flexible platform for researchers to develop a means to use microbubbles to enhance drug delivery, including chemotherapy treatments, and to potentially provide a means for treating tumours which are currently untreatable.

The current research has included an investigation into a number of new approaches to generating microbubbles, with the eventual end goal of developing an appropriate physical system which could be integrated into a commercial device produced by Artenga Inc. As sonoporation research is currently limited to the use of existing UCA microbubbles, it was decided that the immediate goal would be to create a method of

producing microbubbles which are at least comparable to existing UCAs in terms of mean bubble size, homogeneity, acoustic response, and concentration of microbubbles.

3.1 TBI device design requirements

Details set out in the preliminary design specifications for the Artenga TBI device were used as a guide for general design principles. The key medical requirements for the proposed device¹⁴ were as follows:

1. Enhance treatment efficacy by improving drug uptake at a cellular level.
2. Permit a high dose, localized dose of toxic drugs with minimal negative side effects to the patient.
3. Permit a more flexible combination treatment than is possible with acoustically activated drugs formulated in pharmaceutical settings. Physicians will be able to administer a variety of drugs (enhanced by acoustic activation) at short notice, tailored to an individual patient's indication, reaction to therapy, and disease stage.
4. Enhance the therapeutic efficacy of acoustically activated drugs.
5. Be approved for patient use by the appropriate regulatory agency (US Food and Drug Administration, Health Canada, etc).
6. Enhance the ultrasonic visibility of a needle to permit precise, localized drug delivery, to a depth within a patient, under real-time ultrasound guidance.

In order to accomplish the goals for the eventual medical device, certain design requirements were outlined by the company. The requirements relevant to the undertaken research included:

1. A physician inserts a needle or deploys a catheter to a particular area of interest such as a solid tumour.

2. A drug or combination of drugs is delivered in the form of, or infused with, transient micro or nano bubbles.
3. Ultrasound, preferably at energies above the cavitation limit, activates the bubbles.
Note: microbubbles lower the threshold for cavitation by ultrasound energy and may be used as cavitation nuclei for drug and gene delivery.
4. Acoustically activating micro or nano bubbles, either bubbles comprised of a drug or non-therapeutic bubbles within a drug carrier, will:
 - a. activate the pharmacological activity of a therapeutic agent, such as enhancing drug transport through tissues and across cell membranes, and/or
 - b. create a local hyperthermic condition that can enhance the destruction of diseased tissue such as cancerous tissue, and, or
 - c. further enhance the drug uptake of acoustically activated drug systems by increasing the local cavitation
5. The TBI device may be used to generate gas bubbles within a liquid carrier, liquid bubbles within a liquid carrier, liquid bubbles within a gas carrier, or gas bubbles within a gas carrier. The device may be used with liquefied gas.
6. The device may be used to generate gas or liquid bubbles without a carrier fluid directly within a patient.
7. The bubble size and concentration within a carrier fluid will be adjustable.
8. The bubbles will maintain their desired size and homogeneity for a brief period of time, 2 to 10 seconds minimum, before dissipating or otherwise altering form. If too high a percentage of bubbles dissipate prior to acoustic activation, the therapeutic benefits would not be realized.
9. The carrier fluid:
 - a. must be (reasonably) biologically harmless; possibly medical grade
 - b. may be of a low viscosity to promote the generation of or the stability of bubbles.
 - c. may be of a high viscosity to promote the stability of the generated bubbles.
 - d. may include additives, natural or synthetic, to alter its viscosity
 - e. must have minimal adverse effect on the therapeutic drug

10. Where possible, components will be off the shelf, commercially available. Medical grade components may include: needles, needle adaptors, catheters, syringes, guide wires, infusion pumps, fluid conduits, leak proof fittings, meters, laparoscopes, endoscopes, probes, multiple lumen delivery means and the like. The device may include specialized components with attributes such as MRI compatible materials, coatings to enhance the image guidance of the needle or catheter, and the like.
11. Sterility. All injectate-contacting components along the flow path will be sterile, medical grade. The bubble generating means, i.e. fluid conduit, permeable interface, and piezo sources (if they are positioned in contact with the fluid flow) must be disposable or cleaned/sterilized after use.

The design requirements for the TBI device included specific goals for the microbubble properties, as summarized in table 3-1.

Table 3-1 TBI device microbubble characteristics

Characteristic	Design goal
Nominal Diameter	0.5 – 10 microns (variable)
Homogeneity	80% of bubbles within +/-50% of mean diameter
Concentration	Minimum: 10^6 bubbles/ml
Bubble Stability	Minimum: 10 seconds, minutes preferable

3.2 Objectives

Although the Artenga TBI concept included a complete therapeutic solution, the focus of the current research was to attempt to develop the microbubble generation component of the TBI device. The key design goal of the process by which the microbubbles are

generated is that it could potentially exercise some control over critical microbubble parameters. Bearing in mind that the end goal was to integrate the technology into a commercial product, the process must not only be effective from a technical perspective, but must be designed in a way which will eventually allow for straightforward integration into a final commercial product. This would include considerations such as size, cost, and complexity of operation.

Due to the fact that the desired product involves technology which is currently not in existence to enhance a field of research which is not yet well understood, development of the process included an investigation into the factors controlling key parameters of the generated microbubbles. This would allow future work to refine and optimize the microbubble parameters as deemed necessary by researchers once a system for the generation of customizable microbubbles has been made available to them.

3.2.1 Size and concentration of microbubbles produced

The preliminary approach was to design a microbubble generator which produced stable microbubbles, comparable to commercial UCAs. The size specification for the Artenga TBI device of 0.5-10 μ m in diameter is based on the typical size of microbubbles used for ultrasonic image enhancement.

From the literature review, it was found that the acoustic response of nonresonant gas bubbles is proportional to the sixth power of the radius of the bubble, as per the equation describing ultrasound scattering intensity of small spherical particles.²⁰

$$\frac{I}{I_o} \approx \frac{1}{9} nV \left[k^4 r^6 (\gamma_c + \gamma_d \cos \theta)^2 / d^2 \right] \quad (3-1)$$

This would suggest that larger bubbles would provide improved image enhancement, although the analysis does not take into account the resonant behaviour of gas microbubbles, which would greatly increase the scattering intensity.

Bubbles in excess of approximately 6-8 μ m are trapped by the lung capillaries, effectively filtering them from circulation.^{20,64} As a result, UCA microbubbles, which are intended to be injected intravenously and circulated throughout the bloodstream, tend to have very few microbubbles above this size. For imaging applications which take advantage of the resonant properties of microbubbles, it is preferable to have microbubbles with diameters close to the resonant frequency of diagnostic ultrasound systems. For given conditions, the resonant frequency of a free-gas microbubble as a function of bubble radius can be found:

$$f_o = 1/2\pi \sqrt{3\gamma p_o / \rho_o} \quad (3-2)$$

From this equation, it has been found that microbubble diameters on the order of 5 μ m or less give resonant frequencies in the medical imaging band of 1-10MHz.²² Smaller sized bubbles have also shown to have potential advantages for therapeutic applications, as they are disrupted more easily with ultrasound.³³ One potential drawback to free gas bubbles under 5 μ m in diameter is that they are very unstable, and will dissolve into surrounding unsaturated water or blood within 100ms due to surface tension.²² As a

result, some form of encapsulating shell or other stabilization means is required in order for such microbubbles to be useful *in vivo*.

In situations where microbubble resonance is important, such as harmonic imaging and sonoporation, a population of microbubbles with a narrow size distribution is preferable, as homogeneous microbubbles are more likely to behave in a predictable manner when resonating. The waves which are emitted by individual resonating bubbles can affect surrounding bubbles and tissues. It has been shown that for inhomogeneous bubbles of various sizes, the oscillations of the individual bubbles can lead to a coupling effect, which can reduce the effectiveness of both sizes of bubbles. As much as a 10dB decrease in acoustic response has been shown due to the interactions of a 2.2 μ m bubble with a 10 μ m bubble.⁶⁵ This would obviously affect image enhancement, but also would have the potential to adversely affect sonoporation, requiring additional energy to incite resonance in the microbubble population.

For image enhancement, higher concentrations of microbubbles have been positively correlated with improved image enhancement.⁹ A higher concentration of microbubbles also affords the flexibility to reduce the concentration by dilution, should a lower concentration be preferred. Very high concentrations may be undesirable because when excessive quantities of microbubbles are present in imaging applications, a phenomenon known as acoustic shadowing may be observed: this occurs when the bubbles closest to the ultrasound transducer provide a sufficiently strong response so as to obscure any image enhancement in deeper structures, due to attenuation of the signal as it passes through the densely-populated microbubble regions.²⁰

Due to the lack of a product which allows researchers to produce populations of microbubbles of sizes other than those offered by commercial UCAs, it is currently very difficult to ascertain the optimum microbubble size for sonoporation therapy. As a result, one of the goals of the proposed device was to have some degree of control over the microbubble size, filling a current void in the market. Since the means of treatment using the TBI device involved direct tumour injection, where bubbles would not need to pass through capillary beds, the heart, or lungs, larger bubbles which would normally be filtered by the lungs during intravenous injection could be used. Additionally, it may be desirable to alter the microbubble size for investigating the effects of ultrasound frequencies outside the standard diagnostic range, while still maintaining an appropriate bubble diameter for optimal resonance.

3.2.2 Safety

As the desired end product was to be eventually used in a clinical setting, and substantial *in vivo* testing was to be performed in the interim, the safety of the injected microbubbles was of paramount concern. Ensuring that the constituents of microbubbles and any surrounding carrier liquid were biocompatible and had low toxicity, as well as accounting for a suitable sterilization procedure for any components which were to come in contact with the injected material were important considerations in the development of the microbubble generation device.

The small quantities of materials required to produce the shells of typical UCA microbubbles, as well as the low doses necessary for imaging applications generally lead

to minimal toxic side-effects. For example, the standard imaging dose used by the contrast agent Definity® is a mere 1/1000 of the toxic dose.³⁸

Since the applications of the microbubble generation system are not fully defined, particularly in the area of doses required for therapeutic use, minimizing the use of potentially toxic entities is a significant concern so as not to establish strict dose limitations on the final product. Any gases contained within the microbubbles, chemicals used to suspend, stabilize, or be incorporated into the surface of an encapsulating shell must have been selected in such a way as to minimize the possibility of toxic reactions, microembolism (due to aggregation of shell fragments), or other adverse effects which have been shown to be a concern.⁶⁶

The final concern which was to be addressed is that the microbubbles or shells themselves can be physically harmful *in vivo*, either by creating blockages in capillaries due to excessively large bubbles or the aggregation or coalescence of smaller bubbles. For the eventual clinical use, it is important to note that once the microbubbles are dissolved or ruptured, the remaining fragments or any chemical entities should consist of components which can be readily metabolized or excreted.

In terms of therapeutic applications, oscillating microbubbles can create a great deal of viscous heating of surrounding fluid, particularly when the microbubbles feature rigid shells. Microbubbles with stiff encapsulating shells require higher acoustic pressures due to the increased damping effects of the shell, which results in additional energy which must then be dissipated by the shell into the surroundings. It has been shown that such effects can lead to localized heating sufficient to produce harmful bioeffects.⁶⁶ Since the

proposed concept is to make use of microbubbles generated on demand, which are not required to maintain stability for many months, it may be possible to generate bubbles with more flexible shells, at the expense of reduced long-term stability. This may help minimize the acoustic energy required to resonate the bubbles, and thus mitigate the potential for harmful side-effects. This is especially important as it has been shown that at high acoustic pressures, ultrasound alone can damage surrounding healthy tissue.⁶⁶

3.2.3 Summary of preferred microbubbles

Microbubbles optimized for therapeutic use should feature a number of key differences from those used in UCAs. Encapsulating shells with improved flexibility, possibly at the expense of increased permeability would be optimal to minimize the ultrasound energy necessary to induce acoustic cavitation. However, the shells should be rigid enough to have the potential to survive *in vivo* for the duration of the treatment to be performed. As there are many unknowns at this point due to lack of research in this area, the short-term goal was to develop bubbles suitable for imaging, while allowing enough flexibility in the device to alter the properties of the encapsulating shell, should the characteristics of the microbubbles require such changes.

Based on the available UCA microbubbles, as well as existing research which is guiding the development of these bubbles,^{20,24,38} the ideal bubble for ultrasound imaging, and therefore the immediate goal of the microbubble generation device should have a few specific characteristics: The diameter of the bubbles should be approximately 1-5 μ m, with as homogeneous a size distribution as possible; the bubbles and carrier liquid should be suitable for intravenous injection, either by bolus or infusion; and the bubbles

must be stable for the duration of the proposed treatment. Stability during cardiac and pulmonary passage would be desired initially, although this may not be a requirement for specific targeted treatments developed in the future. From a safety perspective, the bubbles should not grow or aggregate *in vivo*, and any gases contained in the bubbles, encapsulating shell materials, and contents of the carrier liquid should show some evidence of low toxicity in regards to the quantity administered, and a minimal potential for direct or indirect harmful bioeffects. Additionally, it would be desirable to generate microbubbles using a process which would easily lend itself to potentially incorporating binding or encapsulating drugs for future applications.

3.3 Approach

The device mechanics were developed through attempting a number of different approaches to the generation of microbubbles, before settling on the most promising approach and refining it through a series of iterative design changes. The key parameters which were deemed to be important (and could be readily observed) were the concentration of microbubbles, the relative size distribution of the microbubbles, the persistence over time, and the acoustic response produced by the bubbles under diagnostic ultrasound equipment. These parameters were mostly characterized in diagnostic imaging labs, which have access to expertise in such measurements, as well as the sophisticated equipment necessary to obtain accurate data.

Apart from the investigation of physical means of microbubble generation, a number of experiments were performed to determine the chemical composition of both the gas

contained within the microbubbles, and the chemical components that would make up the encapsulating shell.

A secondary goal of the research was to investigate the possibility of altering certain aspects of the microbubbles produced. Due to the limitations of the existing technology, in that commercially-available microbubbles are currently sold exclusively for imaging applications, there is currently no clear indication as to what parameters are required for therapeutic uses of microbubbles *in vivo*. Allowing for flexibility in the design which would enable customization of the microbubbles for as-yet undefined applications could greatly improve the value of the bubbles for future research.

3.4 Quantifying results

Since it was determined that as a first iteration for the design and characterization of the microbubbles generated the goal was to create bubbles comparable to those of existing UCAs, standard proven methods of characterizing UCA microbubbles were used. This would limit the characterization to basic parameters such as size distribution, concentration of microbubbles, and acoustic response to diagnostic ultrasound equipment, but would be sufficient for a proof-of-concept device which could be subsequently refined as additional resources for characterizing the therapeutic effectiveness of the microbubbles are developed.

Although the eventual goal of the device involves generating microbubbles for therapeutic use, there is no straightforward method of evaluating the therapeutic effectiveness of microbubbles without the use of highly specialized equipment or a complex cell culture analysis which would be well beyond the scope of the current

research. The approach taken was that such detailed analyses would be performed in the future, once the basics of the microbubble generation device had been developed.

3.4.1 Microscope and haemocytometer

As a result of the very small size of the microbubbles, observing the behaviour or characterizing the effects of any experimental changes on the microbubble characteristics was a non-trivial task. During the early stages of the device design, analysis of the microbubbles was conducted at Carleton University with the use of a Meiji ML7000 metallurgical microscope (Figure 3-1). Although it is difficult to quantify purely visual data, certain observations were made, which provided preliminary information on the effectiveness of certain microbubble generation approaches. This provided sufficient information to eliminate or limit certain variables, while indicating approaches which showed promising results. An additional benefit of microscopic analysis was that it allowed direct observation of the microbubbles, which facilitated the characterization of certain dynamic effects such as microbubble coalescence, aggregation, and short-term stability.

Figure 3-1 Microscope setup

An improvement to the basic microscope imaging method was achieved by the use of a haemocytometer. A haemocytometer is a device used for counting cells via a microscope, and has been shown to be an effective means of characterizing microbubble populations.⁵⁸ The haemocytometer provides a grid of known dimensions (Figure 3-2) which is visible across a constant-depth channel. When the channel is filled with the microbubble-containing liquid sample to be analyzed, the microscopic image can be used to provide an estimate of concentration by visually counting the microbubbles in the known volume of liquid.

Figure 3-2 Haemocytometer grid (small squares are $20\mu\text{m}$ X $20\mu\text{m}$ X $100\mu\text{m}$ deep)

Although a haemocytometer is very effective when counting cells, certain limitations exist when gas-filled microbubbles are counted. Due to the buoyancy of the microbubbles, as well as the tendency for a thin film of foam to form on any free surface of the liquid, droplets of the microbubble liquid placed on the haemocytometer tended to form a film on the upper surface, prior to the cover slip being put in place. When the cover slip was added, the film of bubbles on the surface showed a tendency to adhere to the glass cover slip. This behaviour resulted in an over-representation of the concentration of microbubbles, particularly for larger microbubble diameters, to that

which would be expected in the bulk liquid. This phenomenon would not be present when cells are being counted, as they would tend to remain dispersed in a liquid due to much less prevalent buoyant forces. As a result of the difficulties encountered in obtaining reliable results, the microscope and haemocytometer method of microbubble characterization was limited to predominantly qualitative data. Further comparison of the characterization data to more sophisticated equipment led to the conclusion that the microscope approach was adequate only for preliminary testing or analysis of significant trends.

3.4.2 Coulter Multisizer particle analyzer

The Beckman Coulter Multisizer is a particle analyzer typically used for counting and characterizing cells, but has also found widespread use in characterizing microbubbles such as those used in UCAs. It is generally the most popular means of analyzing UCA microbubbles, due to its ability to accurately and consistently measure the concentration and size distribution of a population of microbubbles.

The principle of operation of a Coulter Multisizer is that microbubbles suspended in a liquid electrolyte solution are passed through a small aperture. The electrical impedance across the aperture is measured, and as bubbles or materials pass through the aperture, the electrolytic solution is displaced from the measurement plane, resulting in changes in the electrical impedance across the aperture. By precisely measuring these changes, the Coulter analyzer is capable of providing an accurate estimate of the size of the item passing through the aperture. When a known volume of fluid is passed through the aperture, the absolute quantities of the detected microbubbles can be extrapolated to

provide an estimate of the mean concentration of microbubbles in the sample solution. As well as providing the means to count the microbubbles, the Coulter Multisizer is sensitive enough to provide data on the size of each individual bubble. When sufficient quantities of microbubbles are present in the sample volume, a representative curve of the size distribution can be obtained.

The majority of the quantitative data from the given experiments was obtained using a Coulter Multisizer III at the Sunnybrook Research Institute in Toronto, Canada. The prepared microbubble samples were diluted in an isotonic buffer fluid, and the analyzer was operated in a 50 μ L volumetric mode, with a dilution ratio chosen in such a manner that the final concentration of microbubbles was appropriate for providing clearly discernable data.

3.4.3 Ultrasound and iU22 imaging systems

In order to verify the echogenicity of the microbubbles, as well as to examine their persistence behaviour, a number of ultrasound imaging systems were used.

The methods discussed above did not provide a reliable means to conclusively verify that the spheres observed under the microscope, or particles detected in the Coulter counter were actually gas-filled microbubbles. Similar data could have been observed using such methods on micelles, coalesced surfactant emulsions, or fragmented remnants of dissolved or ruptured unstable microbubbles. As the proposed approach to sonoporation specifically requires bubbles filled with a compressible gas to induce resonance, it was necessary to verify that the materials observed via microscope and detected via the Coulter analyzer were in fact gas-filled microbubbles.

Although the Artenga microbubbles are to be eventually optimized for therapeutic applications, in order to fully verify the present usefulness of the bubbles, it was necessary to compare them to existing UCAs. Treating the generated bubbles as contrast agents allowed the use of existing ultrasound contrast imaging protocols to be used, including the use of non-linear imaging modes where applicable. Such an approach allowed experiments to be performed for both *in vitro* and *in vivo* environments, leading to test data which more closely would approximate the eventual clinical end use.

The diagnostic ultrasound imaging systems were used at various stages in the development to verify that gas-filled microbubbles were produced. As gas-filled microbubbles are so much more efficient at scattering ultrasound energy than any solid or liquid particles, a suitable concentration of microbubbles produced significant enough image enhancement to effectively rule out the possibility that the particles detected with the Coulter Multisizer or observed with the microscope were not gas-filled microbubbles.

Ultrasound imaging of the microbubbles *in vivo* also provides an improved indication of “real-world” acoustic response, persistence, and absorption of the microbubbles into organs.

Three ultrasound imaging systems were used throughout the development of the microbubble generation device: the Philips HDI 5000, the Phillips iU22, and a proprietary ultrasound contrast imaging system developed by a third party.

The Phillips HDI 5000 is currently one of the most commonly used diagnostic ultrasound units in labs as well as clinics. Preliminary experiments were carried out at the Bank St. Ultrasound Clinic in Ottawa, Canada. These tests involved injecting the

microbubbles into animal tissue samples, and observing the imaging effects achieved during the standard clinical B-mode imaging. The goal of these experiments was to verify that microbubbles capable of providing image enhancement comparable to UCAs were being produced.

The Phillips iU22 ultrasound system was used at Sunnybrook Research Institute in Toronto, Canada. This system, combined with an open-loop flow system was used to provide verification of the acoustic response properties of the gas microbubbles, as well as being used as the primary means to quantitatively measure the persistence of the microbubbles in this flow system over time.

In vivo testing was conducted at St. Michael's Hospital in Toronto, Canada, using a Phillips HDI 5000 ultrasound system. The microbubbles produced were injected into an anaesthetised rodent, and the circulation of the bubbles through the left ventricle of the heart was observed.

Additional *in vivo* testing was carried out by a 3rd party which has requested that the data not be published. However, the results obtained were comparable to those observed with the HDI 5000 system at St. Michael's Hospital, and served to provide additional confidence in the accuracy of the data.

Chapter 4: Development of the Microbubble Generation Device

Once the specific design goals were established, development of the microbubble generation device (MGD) began. A number of preliminary experiments was carried out which ruled out certain approaches as not being feasible for the given application. Although a detailed analysis into some of the abandoned approaches was not done, the focus of the research was on the most promising method selected, which led to the development of the final device prototype.

4.1 Chemical composition of microbubbles

The closest comparable products to the microbubbles which are to be produced via the microbubble generation device are the wide array of microbubble-based ultrasound contrast agents currently used in ultrasound/microbubble research. The processes by which these bubbles are produced necessitate the use of specialized equipment, as well as complex chemistry, in order to provide the impermeable microbubble shells needed for long-term stability. Once prepared, many such contrast agents will typically last for up to 6 months before the microbubbles contained within them break down or dissolve. Many manufacturers of contrast agents attempt to improve the stability of the bubbles by using highly insoluble gases in combination with rigid, impermeable shells. Special handling

procedures such as refrigeration must be used in order to preserve the bubbles. Table 4-1 illustrates the composition of common contrast agents

Table 4-1 Composition of commercial ultrasound contrast agents

UCA name	Filling Gas	Encapsulating Shell Material
Aerosomes	Perfluorocarbon	Phospholipid
Albunex	Air	sonicated serum albumin
BG1135	Air	Polymer
Bisphere	Air	Polymer
Cardiosphere	Nitrogen	Albumin
Definity	Octafluoropropane	Lipid
Echogen	Dodecofluoropentane	Surfactant
Echovist	Air	Galactose
Filmix	Air	Phospholipid
Imagent	perfluorohexane/nitrogen	Lipid
Levovist	Air	palmitic acid
MP1950	Decafluorabutane	Phospholipid
Optison	Octafluoropropane	cross-linked serum albumin
Quantison	Air	spray-dried serum albumin
Sonazoid	Perfluorobutane	Phospholipid
Sonogen	Dodecafluoropentane	Surfactant
Sonovist	sulfur hexafluoride	cyanoacrylate (polymer)
Sonovue	sulfur hexafluoride	lipid/surfactant

Using the components of existing contrast agents as a guideline, a number of preliminary experiments on potential microbubble chemicals were performed. The detailed results of the experiments are beyond the scope of the current research, but did yield indications that promising microbubbles were generated using a surfactant shell, and making use of a perfluorocarbon gas. The surfactants used for all experiments undertaken as part of the current microbubble generation research consist of a

combination of the non-ionic surfactants commonly known as SPAN 60 (sorbitan monostearate) and TWEEN 80 (polyoxyethylene (20) sorbitan monooleate) dissolved in deionised water.

The surfactant solution was prepared by a proprietary method developed by Artenga Inc. which appears to yield stable microbubbles, comparable to the results found in literature using similar components.⁵²

The choice of surfactants was based largely on existing research, supported by experiments to confirm compatibility with the generation process. Wheatley et al. generated bubbles by sonication of SPAN 60 and TWEEN 80 in an aqueous solution.⁵² It is believed that this combination of surfactants leads to the formation of a mixed-surfactant monolayer, resulting in microbubbles which consist of essentially gas-filled micelles. The chemical structures of SPAN 60 and TWEEN 80 molecules are very similar, as shown in Figure 4-1. The SPAN 60 molecule contains a hydrophilic sorbitol head group, and a long hydrophobic tail. SPAN 60 is highly insoluble in water and is considered a hydrophobic surfactant, with a hydrophile-lipophile balance (HLB) of 4.7. Conversely, the HLB of TWEEN 80 is 14.9, placing it in the region of hydrophilic surfactants, and allowing it to be soluble in water.

Figure 4-1 SPAN 60 (a) and TWEEN 80 (b) molecular structure (note size of head groups, similarity of tails groups) [52]

The research suggests that the combination of surfactants is essential, as in the proposed arrangement of surfactant molecules in a microbubble shell the SPAN 60 molecules, with their smaller head groups, fill in space between the heads of the TWEEN 80 molecules (Figure 4-2). Due to the size of the TWEEN 80 head group, it is likely that the repulsion forces between neighbouring molecules would be too great to form a stable surfactant shell. Incorporation of the SPAN 60 molecules allows for sufficient distance between the SPAN 60 and TWEEN 80 molecules to create a stable shell.⁵² In the literature, it was determined that the ideal molar ratio for the highest yield of microbubbles using this combination of surfactants was achieved using 1.7 SPAN 60 molecules to each molecule of TWEEN 80.⁶⁷ Replication of this ratio was attempted, although due to significant differences in the preparation process for the surfactant mixture, there was no straightforward means to measure the quantities of components in

the filtered surfactant mixture. Thus the exact molar ratio of SPAN 60 to TWEEN 80 is unknown.

Figure 4-2 Proposed SPAN-TWEEN molecule arrangement in microbubble shell [52]

From experimental data, it was found that the combination of the SPAN 60 and TWEEN 80 surfactants with a perfluorocarbon gas produced the most stable microbubbles. It is believed that use of the PFC leads to significant improvements in microbubble stability due to the higher molecular weight of PFC, as well as the lower solubility.⁶⁸ Limited experiments with different PFC gases led to the conclusion that Octafluoropropane (C_3F_8) was the most effective gas for producing stable microbubbles in the current application.

Preliminary experiments had been conducted with the various prototype microbubble generation devices to explore the potential of substituting alternate gases, surfactants, or incorporating various additives into the microbubble shell. Although some promising

results have been noted, the specific effects of surfactants and other chemical additives have not been investigated as part of the current research.

4.2 Methods of microbubble generation investigated

Various methods of generation of microbubbles were attempted. Due to the small diameters of bubbles required, many conventional means of bubble generation did not appear to show promise when scaled down to the required size. Early methods attempted included generating the gas microbubbles in a static liquid carrier by forcing pressurized gas through various small submerged orifices. However, the most promising means of generation involved dynamic liquid/gas approaches: (a) forcing pressurized gas through pipette tips, which were immersed in a cross-flowing liquid, and (b) a novel approach involving mechanically mixing the liquid and gas prior to forcing the resulting two-phase flow through a narrow diameter capillary tube.

4.2.1 Stainless steel tubes

A straightforward method of generating gas bubbles in a liquid involves the use of a submerged orifice through which pressurized gas is fed. Preliminary testing of this approach involved having stainless steel tubes manufactured with 5 micron diameter orifices laser-drilled into one end. The process had been shown to have the potential of producing bubbles ranging in diameter from 10 μ m to 1000 μ m.⁶⁹ Pressurized nitrogen gas was forced through the orifice while the tube was submerged in water. Surfactants were added to the water in order to reduce the surface tension force, thus reducing the gas pressure required to create bubbles, as well as reducing the minimum diameter of bubbles

created. As proposed in the TBI concept, the possibility of transversely vibrating the orifice during bubble generation was also investigated: with the aid of a high frequency mechanical transducer, the tube could be vibrated in such a way as to produce high shear forces at the orifice, which would lead to earlier detachment of bubbles, resulting in smaller bubble diameters being produced.

The resulting column of bubbles was observed with a microscope to determine the effectiveness of the method. It was determined that the lack of consistency in manufacturing between the stainless steel tubes led to unpredictable results. The reason for the lack of consistency was postulated to be the result of the microbubble formation being highly dependant on the surface geometry at the location of the orifice. Microscopic inspection of the metal surface surrounding the orifice indicated that the diameter of the hole was significantly smaller than the asperities on the surface finish of the material. This led to the hypothesis that the mechanics of generation were very sensitive to the location of the orifice with respect to irregularities on the surrounding surface. Additionally, due to practical reasons such as concerns about potential sterility problems, as well as the cost of manufacturing the resulting devices, this method of microbubble generation was abandoned in favour of more promising techniques.

4.2.2 Filter screens

In order to overcome the potential problem with the cost of the laser drilled stainless steel tubes, as well as to provide a means to increase the rate of total microbubbles generated, an alternate approach was attempted. Filter screens with pore diameters ranging from

200nm to 5 μ m were tested in a manner similar to the stainless steel tubes: the filters were submerged, and compressed nitrogen was forced through the pores to generate bubbles.

Initial testing indicated that the filter screens were too fragile to handle the pressure required to overcome the surface tension force necessary to generate bubbles. Two filter materials were attempted: a ceramic disk filter, as well as a polymer disk. In both cases, the filter discs would rupture before sufficient pressure was reached to generate bubbles. An improved apparatus was developed for testing, which allowed only a small area of the filter material (a 0.5mm diameter circle) to be exposed, thus reducing the total force on the filter by the pressurized gas. Despite the substantial improvement this offered, the ceramic filter disks still proved to be excessively prone to cracking, and highly inconsistent results were obtained with the polymer filters.

Some experiments with the polymer filters did yield promising results, but further investigation revealed that this was the result of the filter membranes having been deformed by the gas pressure, resulting in irregularly-shaped pores and complex flow geometry which could not be consistently duplicated.

4.2.3 Cross-flow approaches

Early experiments based on published research indicated the feasibility of generating microbubbles by forcing pressurized gas through a pipette tip which was immersed in a cross-flowing liquid channel. Whereas earlier attempts with submerged orifices involved static liquids, the cross-flowing approach was shown to force a more consistent and controllable detachment of the microbubbles, resulting in promising results for customized microbubbles. The transverse shear force on the forming gas bubbles causes

earlier detachment, as well as allowing control of the initial size of the microbubbles. This approach was investigated thoroughly by Genereux.⁷⁰

Although the cross-flowing approach provided a consistent means to generate microbubbles, the rate at which the bubbles could be generated was insufficient for the proposed application.

4.2.4 Two-phase capillary flow

During the testing of the filter screen approach, it was discovered that satisfactory microbubbles could be generated by passing a mixture of liquid and gas through the filter holding apparatus which was used at the time. It was discovered that the filter itself was not required in order to generate the bubbles, leading to the speculation that the bubbles were generated by the agitation of the liquid/gas mixture as it was repeatedly passed through the small diameter capillary tubes present in the filter holder. It was noted that a high flow rate within the capillary tubes was required to generate microbubbles. Further review of the relevant published literature yielded a possible explanation for the observed phenomenon.

A proven means of precisely controllable microbubble generation which has been under recent investigation is the microfluidics phenomenon known as flow focusing.^{54,71} In this application, gas is released into a small orifice, which is concurrently passing liquid in the same direction. The liquid portion of the flow tends to adhere to the outer edges of the orifice, provided that the walls are hydrophilic. The interface between the liquid and gas at the entry to the orifice forces a thin stream of gas to enter the orifice, surrounded by fluid. Due to the high surface energy of this configuration, the gas thread

is not dynamically stable and it breaks up to release bubbles in a periodic fashion. Bubble size at the exit has been shown to be controllable based on the flow rates of liquid and gas and the diameter of the orifice, with experiments showing that uniform bubbles could be produced between 5 and 500 μm in diameter.⁷² With appropriately adjusted parameters, bubbles can be generated which are significantly smaller than the diameter of the orifice.

An excellent illustration of the flow focusing principle was found in an experiment involving a water-in-oil emulsion,⁷³ with surfactant present in order to stabilize to water droplets. Although not identical to the gas bubble generation case, the relevant physical principles are analogous. Figure 4-3 illustrates the effect of various flow regimes. As shown, depending on flow conditions, coalescence of droplets may occur when the downstream droplets are not moving fast enough. At higher flow rates, polydisperse droplet distributions are created. In the present case, it was observed that an orifice 10 μm in diameter could produce droplets in the range of 100s of nanometres in diameter. However, it should be noted that the water-in-oil emulsion would be significantly more stable than a gas bubble due to the lack of tendency of the generated droplets to dissolve in the bulk liquid, as would be the case for gas bubbles.

Figure 4-3 Water in oil emulsion using flow focusing

Q_i = flow rate of water, Q_o = flow rate of oil. Note that bubbles much smaller than orifice were formed in cases (k) and (q) [73]

The concept of forcing a mixture of gas and liquid through a narrow capillary tube at high fluid speed resulted in the design of what was to become the base concept of the microbubble generation device. It was believed that the rapid flow of the two-phase

mixture resulted in a combined mixing effect and a form of dynamic flow focusing which resulted in the generation of a high concentration of microbubbles.

4.3 The microbubble generation device

The final MGD prototype was developed over a series of iterative steps, with improvements incorporated into the design as new observations were noted. All MGD prototypes were built with support from Artenga Inc.

After detailed review of the initial design parameters set out for the original TBI device, it was decided that in order to produce a device for generating microbubbles which would best meet the requirements, such a device would deviate significantly from the proposed concept. During the early stages of the design analysis, it was decided that ensuring sterility of the entire flow path, as required for the TBI concept, would prove to be a complex task. In addition, as the volume of any dose supplied during treatment would ultimately be finite, the need for a device which could produce microbubbles continuously, as achieved by the TBI concept, would not offer any clear advantages.

The revised device concept involved the preparation of a fixed quantity of microbubbles in the hospital pharmacy, after which the bubbles would be placed in a syringe and transported to the physician for injection. As such, the size, shape, and operation of the device had to be such that such an approach was practical.

Due to concerns of spreading infections within hospitals, the current trend in medical devices which contain components which may come in physical contact with patients, particularly in the case of injectables, is to produce single-use disposable products, which are discarded after use. One of the key objectives in the development of the revised

microbubble generation system was to ensure that any components which may come in direct contact with the microbubbles or injectable carrier liquid were contained in a self-contained disposable cartridge, thus facilitating the transition from a proof-of-concept device to a valid commercial product.

It was decided that the new design would feature a separate self-contained cartridge, pre-filled with the necessary combination of gas, carrier liquid, and any additional chemical entities. The filled cartridge could then be sealed in a pouch and terminally sterilized by means of gamma irradiation, thus ensuring the sterility of any components to be injected into the patient. Such a cartridge-based component could be supplied as a single-use disposable component, packaged in a sealed, sterile package.

The general principle of operation of the device was based on what had been observed during the preliminary experiments: microbubbles were generated by means of rapidly forcing a liquid and gas mixture through a small-diameter capillary tube, generating gas microbubbles via a principle similar to the flow focussing method. In the given case, rather than controlling the liquid and gas phases independently, the two phases were mixed and then forced through the capillary tube simultaneously. It was found that this method produced a large quantity of bubbles much smaller than the internal diameter of the capillary tube used.

In order to provide the controlled force necessary to force the two-phase mixture through any intervening geometry, the main component of the MGD concept consists of a reusable actuation system, which contains the controlled mechanical means to consistently generate microbubbles within the disposable cartridge.

4.3.1 MGD1 prototype

The first revision of the MGD was the earliest proof-of-concept prototype, based on the 2-phase mixing and capillary flow focussing principle. The MGD1 was a pneumatically-driven actuation device which forced the liquid and gas mixture through the series of mixing chambers and capillary tubes contained within the disposable cartridge. The result of this was a controlled, repeatable means to generate a 3mL syringe full of microbubbles, with a fairly uniform distribution.

Figure 4-4 MGD1 prototype

(1) Cartridge component (2) Actuation platform (3) pneumatic cylinder (4) solenoid control valve (5) control panel

The cartridge component of the MGD1 consisted of an aluminum housing, which contained a series of acrylic plugs which had been drilled in such a way as to provide a complex flow path which would promote mixing, while simultaneously removing excess foam and large bubbles from the final mixture due to the vertical orientation. The final stage included a 250 μ m diameter capillary tube which effectively converted the amorphous gas and liquid mixture into a relatively uniform dispersion of uniform, small diameter microbubbles.

Figure 4-5 MGD1 cartridge schematic and flow path

(1) capillary tube (2) mixing chambers (which also trap foam due to vertical orientation)

Despite the foam trap arrangements in the mixing chambers, a small amount of foam was still visible in the end product. During testing, this foam was manually purged, prior to injection of the microbubbles. Early results indicated adequate performance, with a substantial quantity of microbubbles produced. At the time, there was no means to quantify the production or characteristics of the microbubbles, other than by visual inspection of the resulting liquid under a microscope.

Based on the observations of microbubbles generated with the MGD1 prototype, an improved variant was built to be used for testing purposes.

4.3.2 MGD2 prototype

The primary improvements of the MGD2 prototype over the previous iteration were in the area of aesthetics and ergonomics. Functionally, the MGD2 was identical to the MGD1 device: both prototypes used a pneumatic actuation system, and both cartridge components contained identical internal geometry. The MGD2 device featured a more robust construction, and was better suited for transport to external research labs which had improved microbubble characterization capabilities, allowing it to be used for quantitative testing of the microbubble generation mechanisms.

Figure 4-6 MGD2 prototype

(1) cartridge component (2) actuation clamp (3) CO₂ cylinder (4) activation button

4.3.3 MGD3 prototype

Although the MGD2 device had shown potential as a commercially-viable microbubble generator, certain aspects of the design were deemed undesirable and in need of improvement. A significant change in the MGD3 device was the use of an automated control system driving an electro-mechanical actuator to replace the pneumatic system in

use in earlier prototypes. This change resulted in more consistent and controllable actuation speed.

Manufacturing of the disposable cartridges used in the MGD1/2 prototypes would have been complex, involving the assembly of a number of small parts, which would have been required to be assembled in a gas-tight fashion, capable of containing the high pressures during actuation. The pneumatic actuation, which relied on disposable carbon dioxide cylinders to provide the compressed gas, was also deemed to be inconvenient for use in a lab or hospital setting.

Figure 4-7 MGD3 prototype

(1) programmable controller and motor driver (2) electro-mechanical actuator (3) cartridge component

The MGD3 was developed primarily as a means to investigate the potential to greatly simplify the disposable cartridge component. It was hypothesized that by using a reciprocating action, it would be possible to provide effective mixing by repeatedly passing the gas and surfactant mixture through the capillary tube, thus eliminating the complex geometry of the multi-stage mixing chambers. The high-speed fluid jet exiting the capillary tube during actuation appeared to be sufficient to encourage the mixing of the liquid and gas components. Repeated passes through the capillary tube in rapid succession would improve the mixture, without the need for separate mixing components, while concurrently breaking up large gas pockets into small microbubbles via the flow focusing process.

The removal of the excess foam was accomplished by means of a small expansion chamber incorporated into the device, which trapped foam and larger bubbles, as illustrated in Figure 4-8. The programmable controller allowed the final actuation stage to be delayed by a set period of time, which was incorporated into the microbubble generation process in order to allow larger bubbles to settle out due to buoyancy, improving the size distribution and homogeneity of the final microbubble preparation.

Figure 4-8 MGD3 cartridge schematic

(1) 3mL syringe (2) capillary tube (3) expansion chamber/foam trap

Although the simplification of the disposable component would come at the expense of increasing the complexity of the actuation device, it was decided that this compromise was worthwhile, as the generation device would potentially be actuating thousands of the disposable cartridges throughout its service life. Simplifying the single-use cartridge resulted in the potential for a substantial cost savings both in materials required, as well as the manufacturing and filling of the cartridges once the system was in production.

As the mixing action was to be achieved by the fluid jet exiting the capillary tube, and the buoyancy traps had been removed, it was anticipated that the vertical orientation of the disposable component during generation was no longer needed. For improved stability, the MG3 device was oriented horizontally.

4.3.4 MGD4 prototype

During testing, it was found that the data obtained with the MGD3 indicated that the mixing process was not always consistent. The design was changed again to orient the cartridge in such a way that any larger gas bubbles were always passed through the capillary tube first. For full automation, this involved repeatedly rotating the cartridge for each subsequent pass. Although this increased the size and complexity of the device substantially, the potential for improved consistency of the microbubbles was deemed to justify the design changes.

As with the MGD3 prototype, the automated control system of the MGD4 incorporated a low-speed purge cycle to remove the foam layer, a procedure which was greatly improved with the vertical orientation. The operation of the foam purge process was similar to that of the MGD3, allowing use of identical cartridges in both units. A delay was incorporated into the final generation cycle, before transferring the microbubbles to the lower syringe. This allowed the larger bubbles to float to the top of the upper syringe and form a foam layer, leaving only the smaller microbubbles dispersed in the bulk liquid. The device would then slowly pass the liquid containing suspended microbubbles through the cartridge. The layer of foam would again be trapped in the expansion chamber inside the cartridge. After the full process was complete, the lower syringe could then be detached, and would contain a negligible amount of foam or excessively large bubbles.

Figure 4-9 MGD 4 prototype

(1) cartridge component (2) rotating disk (3) control panel (4) linear actuator

The MGD4 was the final prototype phase from the perspective of the microbubble generation mechanics. The subsequent MGD5 prototype design developed by Artenga Inc. was purely an improvement for the purposes of production, as the MGD5 devices were suitable for commercial research use. The microbubble generation mechanics of the MGD5 are identical to the MGD4 process. At the time of publication, a number of Artenga MGD5 devices are in use by researchers around the world.

Figure 4-10 Artenga MGD5 microbubble generation device

Chapter 5: Experimental Results and Discussion

5.1 Validation of characterization means

5.1.1 Microscope and haemocytometer

Use of the microscope made it possible to observe microbubble behaviour, particularly dynamic effects such as the stability or collapse of individual microbubbles. While it was possible to provide some quantitative data on microbubble concentration using the haemocytometer, it was found that the measurements were not consistent enough to provide reliable conclusions.

Figure 5-1 Microbubbles in haemocytometer 40x magnification, each square contains 250 μ L of microbubble suspension

All microscopy methods suffered from complications due to the tendency of larger bubbles to adhere to the slide cover slip. As a result, for a given sample, the larger sizes were overrepresented. This also created problems in some cases wherein the larger bubbles would obstruct the view of smaller bubbles, resulting in the inability to obtain accurate counts of the bubbles in a given area on the haemocytometer.

5.1.2 Coulter Multisizer

Unless indicated otherwise, the majority of the quantitative data on microbubble parameters was obtained by use of a Coulter Multisizer. This proven method for

microbubble characterization provided analysis of size distribution of the microbubbles, as well as a measure of the overall concentration.

Due to the widespread use of the Coulter Multisizer in microbubble characterization, the data obtained were accepted as providing an accurate measurement of the key microbubble parameters. It should be noted, however, that certain factors may suggest that the Coulter Multisizer alone may not be a sufficient means to fully characterize the microbubbles. The Coulter detected particles well in excess of the normal background levels when the original surfactant preparation was analyzed. This may be due to small fragments of undissolved SPAN 60. Although the surfactant preparation procedure attempts to create an extremely fine dispersion of SPAN 60, it is possible that some large residual fragments may have survived the surfactant preparation, or alternately formed by subsequent coalescence of sub-micron SPAN 60 particles.

Furthermore, as the Coulter Multisizer uses impedance changes to detect microbubbles, it does not have a means to differentiate gas bubbles from, for example, fragments of surfactant which were formed by the collapse of former gas bubbles.

In order to address the relatively high particle counts detected in the initial surfactant preparation, a refined process was developed for preparation which featured filtration through a 0.2 μ m filter as a final step. This was shown to substantially reduce the number of particles detected in the surfactant preparation alone with the Coulter analyzer, without having any noticeable adverse effects on microbubble generation.

5.1.3 Ultrasound imaging

In order to verify that the particles which were detected by the Coulter Multisizer were indeed gas-filled microbubbles, *in vitro* ultrasound imaging was used. During the preliminary analysis, this was verified by simply immersing the ultrasound transducer in a beaker of water to which microbubbles were added. As shown in Figure 5-2, it is clear that the microbubbles provided a clear acoustic response, both with standard B-mode imaging, as well as the microbubble-specific pulse inversion (PI) imaging mode. The clear PI imaging response strongly reinforces the presence of gas-filled microbubbles, as the images generated with the PI method rely specifically on the non-linear response of UCA microbubbles.

Figure 5-2 Microbubble ultrasound images

left image is standard B-mode imaging mode, image on right is non-linear (PI) imaging mode

As shown in Figure 5-3, verification of the surfactant with the *in vitro* ultrasound setup suggests that the particles detected in the original surfactant preparation with the

Coulter Multisizer are not echogenic, and are very likely not the result of gas-filled microbubbles.

Figure 5-3 Surfactant preparation (before microbubble generation) ultrasound image

Note: grey area represents background noise, no microbubble signals are visible

5.2 Preliminary testing of gases and surfactants

Although a number of combinations of surfactants and gases had been attempted, the only means which were shown to produce stable microbubbles, as viewed under the microscope, were PFC gases combined with the SPAN-TWEEN surfactant shelled microbubbles. A limited number of alternate formulations was tested during the preliminary proof-of-concept phase. The two gases of highest interest were Octafluorocyclobutane (C_4F_8) and Octafluoropropane (C_3F_8), both of which produced stable bubbles as viewed under the microscope. Although the C_4F_8 is a larger molecule, the lower solubility of the C_3F_8 proved to be a more significant factor for maximizing

microbubble production. The Coulter Multisizer analysis (see table 5-1) clearly indicated that the quantity of bubbles produced was much lower with the C_4F_8 than with the C_3F_8 .

Table 5-1 Coulter data: comparison of perfluorocarbon gases tested

Sample	Microbubble Concentration (/ml)
Isotonic buffer fluid	$<0.1 \times 10^7$
Surfactant preparation alone	0.2×10^7
Octafluoropropane microbubbles (C_3F_8)	1.7×10^7
Octafluorocyclobutane microbubbles (C_4F_8)	0.4×10^7

A detailed analysis of surfactant types was considered to be beyond the scope of the current research. However, the effects of certain highly relevant additives were investigated for future consideration. As the surfactant preparation uses distilled water as a base, the resulting mixture produces a hypotonic liquid for injection. For animal testing, it is desired to provide an isotonic solution, in order to minimize potential harmful effects to tissues at the injection site.

Sodium Chloride (NaCl) was added to the prepared surfactant in order to bring the concentration of NaCl to 0.9% (w/v), as found in isotonic saline. Haemocytometer analysis did not indicate a significant change in microbubble concentration. However, after a few minutes, it was observed that many of the individual microbubbles were aggregating into large clusters of bubbles.

Figure 5-4 NaCl-induced microbubble aggregation

In comparison, the standard microbubbles, shown in Figure 5-1, generally appear to disperse in a random manner and have no tendencies to aggregate. The addition of NaCl in quantities above 0.1% (w/v) appeared to cause aggregation of the microbubbles, as shown in Figure 5-4. A possible explanation for this behaviour is that the microbubble shells acquire a slight electrostatic charge during generation and develop repulsive forces as a result of the standard generation cycle. The addition of the free Na^+ and Cl^- ions allows the charges on the microbubble surfaces to be neutralized, which negates the repulsive force. Thus, microbubbles which come into direct contact with each other as a

result of random motion are free to adhere to one another, forming the observed aggregate clusters. Further observation concluded that the aggregation force was weak, and was easily disrupted by vigorous fluid movement, such as that which could be expected to occur during injection.

The majority of the testing was done with the standard hypotonic surfactant preparation, which did not include NaCl. It is believed that the negligible effects of the addition of NaCl suggest that data obtained for the hypotonic microbubbles would be applicable to an equivalent isotonic preparation.

5.3 Comparison of MGD prototypes

5.3.1 MGD1 vs. MGD2

The MGD1 prototype provided a preliminary proof-of-concept that the chosen approach was a feasible means to produce microbubbles. The MGD1 was used only for qualitative observation of the microbubbles using the microscope. Having established the validity of the process, the MGD2 prototype was built in collaboration with Artenga Inc. The construction of the MGD2 unit was substantially sturdier and more practical for transportation and use off-site, which allowed it to be used for the detailed characterization experiments conducted at various institutions.

A detailed comparison of the MGD1 and MGD2 prototypes was not carried out, as such an analysis was not necessary. The two devices were identical from a performance standpoint, differing only in the improved construction of the MGD2, as well as cosmetic improvements to the aesthetics and ergonomics. The internal workings, pneumatic

actuation systems, and cartridge geometries were identical, leading them to be treated as equivalent from a technical standpoint.

5.3.2 MGD2 vs. MGD3

Although the MGD2 prototype provided satisfactory results, the multi-stage foam trap system did not prove to be very effective, and the resulting consistency of microbubbles produced was difficult to ensure. The MGD3 device was developed in order to incorporate an electro-mechanical actuation system to replace the less practical pneumatic means, to investigate improvements in the microbubble generation process, and to simplify the single-use cartridge component.

As described earlier, the MGD3 prototype differed significantly from the MGD2. Due to the horizontal orientation, combined with the simplified cartridge geometry, it was necessary to repeatedly actuate the cartridge in order to encourage mixing of the liquid and gas components which would maximize the quantity of microbubbles produced.

Preliminary measurements were done with a modified MGD2 cartridge, in order to measure the effect of multiple passes through a capillary tube. The results are summarized in table 5-2. The data seemed to indicate that the concentration reaches a plateau and does not benefit from actuation beyond approximately four passes. For testing purposes with the MGD3 device, a six-pass cycle was selected as a conservative starting point.

Table 5-2 Effects of multiple capillary tube passes on microbubble concentration

Sample	Microbubble concentration
single pass through capillary tube	0.3×10^7
4 passes through capillary tube	1.8×10^7
8 passes through capillary tube	1.5×10^7

Testing of the MGD3 prototype supported the effectiveness of the reciprocating design feature. It was found that six passes through the cartridge significantly improved the concentration of microbubbles. The concentration for the multiple pass samples increased to 3.4×10^7 microbubbles/mL, from the 1.2×10^7 microbubbles/mL observed with the single pass samples. As illustrated by the increase in total concentration, as well as the plot of size distribution shown in Figure 5-5, the use of multiple passes clearly improved the number of microbubbles generated. Apart from the increase in concentration, increasing the number of passes through the device did not appear to have a significant effect on the size distribution of the microbubbles. The apparent lack of microbubbles above approximately $2\mu\text{m}$ is most likely due to the improved foam purging method of the MGD3 device. The buoyancy-trap purge cycle appeared to effectively limit the presence of microbubbles above approximately $1.5\mu\text{m}$ in diameter in the end product.

Figure 5-5 Size distribution comparison of single-pass vs. multiple passes (MGD3 prototype)

For comparison, the MGD2 device was actuated multiple times to determine if similar effects might be present. The concentration values for the single and multiple pass samples tested on the MGD2 until were 1.7×10^7 and 1.6×10^7 microbubbles/mL, respectively. The size distribution is shown in Figure 5-6.

Figure 5-6 Size distribution comparison of single-pass vs. multiple passes (MGD2 prototype)

It is apparent that significant differences between the single and multiple-pass readings do not exist in the case of the MGD2 prototype. This result was not unexpected, as the MGD2 microbubble cartridge was optimized to generate microbubbles in a single pass, at the expense of much more complicated flow geometry.

Direct comparison of microbubbles generated by the MGD2 and MGD3 prototypes illustrated significant differences between the two devices (Figure 5-7), particularly in terms of the size distribution. Although the two-fold increase in microbubble concentration with the MGD3 over the MGD2 was significant, the size distribution of the resulting bubbles emphasizes the extent of the improvement. While the MGD2 appeared

to exhibit a slightly higher concentration of microbubbles in the 1.7-4 μ m range, there was a significant difference in the size distribution below approximately 1.7 μ m. Although this may have been the result of variation in samples (a similar trend was observed between the single and multiple pass analysis of the MGD2 prototype), the most likely explanation in the context of comparison to the MGD2 is the improved foam purging system in the MGD3 prototype.

The foam purging system in the MGD3 functions by inserting a delay of approximately 5 minutes prior to the final actuation stroke. The final stroke is carried out at a very low rate, which minimizes any mixing in the cartridge, and prevents any of the foam from being passed through the cartridge. The expansion chamber bored out inside the cartridge traps the foam once the cycle is complete. The steeper curve of the MGD3 size distribution indicates a sharper transition in the size distribution of the microbubble population, suggesting that the foam trap system effectively resulted in microbubbles which were generally more uniform in diameter.

Figure 5-7 Size distribution comparison of MGD2 vs. MGD3

It should be noted that the MGD3 device is capable of a slightly higher and more uniform flow rate, due to the direct electro-mechanical actuation replacing the pneumatic system used in the MGD2 prototype. Also, the highly simplified cartridge of the MGD3 configuration was particularly appealing from a manufacturing perspective, as it requires less material and leads to a significant reduction in complexity of the parts to be produced. Such considerations are of particular practical importance since the cartridges are intended to be discarded after a single use.

Figures 5-8 and 5-9 illustrate differences between microbubbles generated with the MGD2 and MGD3 prototypes, as captured with the Richardson RTM-3 microscope. The images are representative of the observed microbubbles, and support the Coulter counter

data indicating a substantial increase in concentration of microbubbles with the MGD3 microbubble generator over the MGD2 variant. It should be emphasized that, particularly in the case of the MGD3 microbubble sample, larger microbubbles are overrepresented due to their tendency to adhere to the cover slip on the microscope slide.

Figure 5-8 MGD2 microbubbles, Richardson RTM-3 microscope (40X)

Figure 5-9 MGD3 microbubbles, Richardson RTM-3 microscope (40X)

5.3.3 MGD4

During a series of sterility experiments (discussed in section 5.4) conducted with the MGD3 prototype, it was noted that visual observation of the microbubbles during generation could be used to predict which samples would yield substantially higher concentrations of microbubbles according to the Coulter counter measurements. The quality of the mixing effectiveness was inferred based on the appearance of the surfactant/gas mixture after the first pass through the cartridge. In cases where the first pass of the generation cycle appeared to be most effective in mixing the liquid and gas components, the concentration of microbubbles produced was highest. It was observed

that the effectiveness of the mixing was impeded by the horizontal orientation in that the gas component within the cartridge would not necessarily be passed through the cartridge in a consistent manner.

Further experimentation showed that improved mixing mechanics could be achieved when the cartridge was in a vertical orientation, similar to the earlier MGD2 prototype. The vertical orientation would ensure that the gas would be forced through the cartridge first, which would lead to improved mixing of the liquid and gas during actuation of the cartridge.

This trend was apparent when qualitative data taken at the time of the experiments were compared to the plots of experimental data as shown in Figure 5-10. As all samples appeared to consistently produce microbubbles, this secondary effect became the predominant observation. In cases where mixing appeared to be particularly poor (denoted by red curves), the actual concentration of microbubbles generated was towards the lower end of the measurements. More significantly, in cases where mixing appeared to be particularly effective (denoted by the green curves), there was a very strong correlation with the Coulter data. The yellow curve, representing the sample used for the persistence analysis, was obtained by manually orienting the cartridge vertically to promote improved mixing. After a single pass, the well-mixed sample was placed in the generator for the subsequent passes. As expected, the forced mixing appeared to yield the highest curve in the data set, and resulted in the highest concentration of microbubbles (1.3×10^8 microbubbles/mL).

Figure 5-10 MGD3 Coulter size distribution comparison: sterility samples

The observations on the importance of orientation led to the development of the MGD4 prototype, which incorporated a rotation mechanism to reorient the cartridge between actuation cycles to maintain a orientation prior to each actuation cycle.

As the MGD4 prototype functioned identically to the MGD3, with the exception of the orientation of the cartridge, the size distributions for the two devices were comparable. The consistent mixing of the MGD4 prototype effectively narrowed down the variability in microbubble concentration between samples, while consistently producing higher concentrations than were observed with the MGD3 device. The results are summarized in table 5-3

Table 5-3 Comparison of microbubble concentration for MGD prototype versions

Prototype version	Mean size	Microbubble concentration (/ml)
MGD1/MGD2	1-4 μ m	$1.2-1.7 \times 10^7$
MGD3	1-2 μ m	$0.3-1.3 \times 10^8$
MGD4	1-2 μ m	$1.0-1.5 \times 10^8$

5.4 Microbubble persistence: iU22 ultrasound data

The iU22 ultrasound experiments illustrated the acoustic response of the microbubbles as a function of time over a period of approximately 40 minutes. The experimental setup consisted of an open-loop water flow apparatus, connected to a syringe pump, which injected microbubbles at a controlled rate into the water stream.

At a sufficient distance downstream of the syringe pump, an ultrasound transducer observed a cross-section of the water-filled tubing, as shown in Figure 5-11. Images were captured at regular time intervals, where a region of interest was specified at the location of the microbubble/water stream to compare the successive ultrasound images. A software algorithm was used to quantify the acoustic signal intensity in the region of interest (ROI), which generated a numeric value for the mean acoustic backscatter intensity for each captured image. The data was then converted to a plot of average acoustic response in the ROI vs. time.

Figure 5-11 Typical iU22 ultrasound image

The first persistence tests conducted with the iU22 setup were performed on microbubbles generated with the MGD3 device. The iU22 data, shown in Figure 5-12, indicated that these microbubbles provided their optimal response within approximately 13 minutes of generation, after which the acoustic response dropped to an amplitude roughly 1/3-1/2 of the initial intensity. Between 20 and 40 minutes, the decay rate of the acoustic response appeared to decrease significantly, possibly indicating a stage of secondary stability beyond which acoustic response stabilized over the course of the given time scale.

Figure 5-12 MGD3 iU22 persistence data

During the iU22 persistence testing, a portion of the microbubble sample was tested concurrently in the Coulter Multisizer. Normalizing both sets of data to compare the shapes of the persistence curves over time indicates that the acoustic response of the microbubbles appears to be very closely correlated to the concentration. Figure 5-13 illustrates the strong correlation between the concentration and the iU22 data. Although the concentration data contains only three points of data within the range of the iU22 experimental data, all three points appear to match the acoustic response curve very well. This was not unexpected, as a higher concentration of microbubbles is typically associated with an improved acoustic response.

Figure 5-13 Normalized comparison of iU22 and Coulter persistence data (MGD3)

The sharp drop in concentration which occurred between the initial time of generation and approximately 20 minutes was not typical of normal microbubble behaviour. It was hypothesized that the initial sharp drop off was due, at least in part, to the collapse of some of the smaller microbubbles.

A more detailed analysis was carried out with the MGD2 device for comparison, which yielded a more gradual drop-off in acoustic response. However, since the MGD3 produced a higher concentration of microbubbles, the data from the two curves had to be normalized in order to compare the shape of the curves. The data was superimposed and normalized with the MGD3 data, the results of which are shown in Figure 5-14.

It is likely that the increased presence of slightly larger bubbles in the MGD2 sample may have formed a dominant component in the acoustic response. Since larger bubbles are generally more stable, it would be reasonable to expect the improved relative persistence which was observed. Also, owing to the higher concentration of microbubbles present in the MGD3 samples, it is very likely that the acoustic response was initially much higher for the microbubbles generated with the MGD3 prototype, which may have emphasized any subtle time-dependent effects.

Figure 5-14 Acoustic response persistence: comparison of MGD2 to MGD3

The MGD4 persistence data appeared to be more similar to the trend observed with the MGD2 microbubbles than what was observed with the MGD3 microbubbles. As

shown in Figure 5-15, the decay rate was low and fairly consistent. Again, the data was normalized and compared to the Coulter Multisizer curve, which appeared to follow a similar trend.

Figure 5-15 Normalized comparison of iU22 and Coulter data (MGD4)

5.5 *In vivo* testing

Although the *in vitro* testing performed with the Coulter Multisizer, ultrasound imaging systems, and microscopic analysis provided a great deal of useful data, the most effective test of the effectiveness of the microbubbles as they compare to UCAs was obtained by means of *in vivo* testing.

A number of significant environmental differences are present *in vivo*, which are not accounted for during *in vitro* testing of the microbubbles. When microbubbles pass through the circulatory system of a living organism, it is possible that the bubbles may be filtered by the lungs or destroyed by macrophages. In order for microbubbles to be clinically useful, it is also important to verify that they do not dissolve due to interactions with blood chemistry or dissolved gases. Furthermore, an *in vivo* environment subjects the microbubbles to movement and shear stresses due to the flow of blood through the vessels, as well as fluctuations in ambient pressure and temperature which are present in various stages of circulation.

The *in vivo* testing was conducted with the generated microbubbles on a rodent model, the layout of which is shown in Figure 5-16. A Sprague Dawley rat was injected with microbubbles administered using a syringe pump through the jugular vein, and the ultrasound transducer was oriented for imaging of the left ventricle. Initial concentration of the microbubbles was measured at 1.2×10^8 microbubbles/mL, but the sample was diluted with saline to a concentration of 1.0×10^8 microbubbles/mL to remain consistent with experiments performed with UCA microbubbles.

Figure 5-16 Experimental setup for *in-vivo* rodent imaging

The bubbles were infused at rates ranging from $50\mu\text{L}/\text{min}$ to $300\mu\text{L}/\text{min}$. It was noted that at rates in excess of approximately $150\mu\text{L}/\text{min}$, microbubble perfusion into the myocardium tissue was visible. Such behaviour is desirable for cardiac imaging, and is a positive indicator of the *in vivo* stability of the microbubbles. Image enhancement due to the microbubbles is shown in Figure 5-17.

Figure 5-17 Rodent left ventricle image enhancement with microbubbles

The image enhancement by the microbubbles observed on the ultrasound was described as being consistent with previous observations of UCA microbubbles. The image enhancement by the generated microbubbles was deemed comparable to commercial UCA microbubbles, with both adequate microbubble replenishment and persistence.

5.6 Sterility testing

In order to minimize the chances of sepsis or other infections, medical devices which feature components which come into contact with injectable materials must be sterilized. Depending on the specific design and characteristics, a number of sterilization methods are available for the sterilization of medical devices. The most commonly used means are heat sterilization (autoclaving), chemical sterilization (ethylene oxide or equivalent), sterile filtration, and irradiation.

As the microbubble generation unit does not come into contact with the injectable components, there is no need to ensure sterility of the generation device itself. However, the cartridges which contain the gas and surfactant mixture from which the microbubbles are formed must be free of any pathogens prior to use. Although this is an absolute requirement for medical devices from a regulatory perspective, many of the experimental procedures performed during the current research did not necessarily require the use of sterilized materials. Nonetheless, for the majority of testing, it was preferred to provide sterile samples, in order to reduce the risk of interference due to the presence of foreign organisms and materials, as well as to attempt to mimic the final product as closely as possible.

Once the basic characterization had been completed, and the device and cartridges had been refined to a point by which it was deemed suitable for *in vivo* testing, sterilization procedures were put into use. Initially, the sterilization process consisted of filtering the surfactant with a sterile 0.2 μ m pore filtration system prior to filling the cartridges, in order to remove any microorganisms which may be present in the liquid. As more stringent requirements for sterilization became necessary, suitable methods were investigated for terminally sterilizing the assembled and filled cartridges.

Since the cartridges contained a number of plastic components which would likely be affected by excessive heating, autoclaving was ruled out as a potential means of sterilization. Avoiding a heating method also provided flexibility for additional considerations such as packaging and labelling of the final product.

Although chemical sterilization could have been suitable for sterilizing the unassembled components, once the cartridges are filled and assembled, the interior containing the liquid and gas is sealed from the external environment. Exposing the cartridges to ethylene oxide (EtO), or other such sterilizing chemicals, would not ensure sterility of the contents of the cartridge, which is of greatest interest as they are the components to be injected into the patient.

As a result of essentially a process of elimination, the most feasible approach proved to be gamma irradiation of the filled, assembled, and packaged cartridges. A sufficient dose of radiation would effectively sterilize the cartridge and contents. Sealing the filled cartridges in packages prior to sterilization yielded the end product after irradiation in the form of a sealed, sterile package suitable for use where the presence of pathogens would be unacceptable. Such a method could yield a convenient, repeatable means of ensuring sterility which is compatible with the current composition and configuration of the cartridges.

The radiation dose was selected based on what was believed to be appropriate for the size and materials used in the cartridges.⁷⁴ Optimal radiation doses are typically determined by a series of experiments on a finalized product. However, for the current research, it was decided that selecting a conservative dose in the range of 30-35kGy would be adequate to ensure sterility, possibly at the expense of compromised material properties. Once the cartridges had been sufficiently refined, a batch of packaged samples was sent for formal sterility testing (Nucro Technics, Scarborough Ontario). The

cartridges successfully met the criteria for sterility, providing evidence that the selected sterilization dose was suitable for preliminary work.

As very high doses of radiation were required in order to ensure sterility, it was important to verify that the radiation did not exhibit adverse effects on the materials used in the cartridges or chemical components contained therein. Once sufficient baseline characterization data had been obtained on the microbubbles produced, a series of experiments were conducted in order to determine what effects the addition of an irradiation step would have. A number of different combinations of components was chosen to facilitate the investigation of the effects of irradiating various materials on the microbubble parameters. The sterilization testing was performed using the MGD3 prototype to generate the microbubbles; however, the cartridge components used in the MGD3 prototype are identical to those used in the final MGD4 version.

Table 5-4 Sterility testing summary

Sample Designation	Gamma sterilized	Cartridge housing material	Needle mount material	Adhesive type	Syringe material	Microbubble Count (/mL)
SPPM	Y	HDPE	PP	MG	PP	3.3×10^7
SPPG*	Y	HDPE	PP	GP	PP	
SPMM	Y	HDPE	SS	MG	PP	4.1×10^7
SPMG	Y	HDPE	SS	GP	PE	4.4×10^7
SNPM	Y	Nylon	PP	MG	PP	4.2×10^7
SNMG	Y	Nylon	SS	GP	PP	6.7×10^7
SNPG	Y	Nylon	PP	GP	PP	6.0×10^7
SNMM	Y	Nylon	SS	MG	PP	3.8×10^7
PPM*	N	HDPE	PP	MG	PP	
PPG*	N	HDPE	PP	GP	PP	
PMM	N	HDPE	SS	MG	PE	1.0×10^8
PMG	N	HDPE	SS	GP	PP	6.4×10^7
NPM	N	Nylon	PP	MG	PP	9.0×10^7
NMG	N	Nylon	SS	GP	PP	3.8×10^7
NPG	N	Nylon	PP	GP	PP	3.5×10^7
NMM	N	Nylon	SS	MP	PP	3.4×10^7

PP: Polypropylene

SS: Stainless Steel

MG: Medical grade cyanoacrylate adhesive (Loctite® 4013)

GP: General-purpose cyanoacrylate adhesive

* Concentration data is not available for these samples: Samples PPM and PPG were rendered unusable due to adhesive clogging the needle orifice. During testing, the SPPG sample failed at the needle/syringe connection.

Figure 5-18 Size distributions of sterility test samples

The variation on the results was substantial, due in part to the use of the MGD3 version of the actuation device for the tests in question. However, there was sufficient evidence to conclude that the microbubbles generated were not significantly affected by the sterilization procedure and the quantity of bubbles produced was within the typical range for the MGD3 prototype. Within the resolution of the experimental data, there did not appear to be a drastic reduction in concentration of microbubbles produced, or significant variation in the mean size of the microbubbles. Visual inspection of the components of the sterilized cartridges showed no signs of degradation of the materials, other than some minor discolouration. During normal handling procedures, there were no

noticeable changes to the mechanical properties of the materials, such as embrittlement, deformation, compromised function, or leaks in the cartridges.

It was noted that the medical grade adhesive produced a noticeably stronger bond to the plastic housings than the general purpose adhesive did. The samples bonded with general purpose adhesive were significantly more prone to failure at the glue joint between the cartridge housing and the needle. Overall, there were no detectable signs of increased fragility as a result of the irradiation procedure, although further investigation would be required in order to ensure that such behaviour does not occur in the samples over an extended period of time.

Analysis of the data showed no clear trends in microbubble concentration or size distribution, attributed specifically to the various materials or experimental parameters. The primary goal of the sterilization experiments was to verify that the irradiation process did not have an adverse effect on the materials used in the cartridges, or the microbubbles which could be produced. Since all samples produced an adequate quantity of microbubbles, this verification was complete. The observed variation in these experiments led to the development of the MGD4 device, as described in section 5-3.

5.6.1 Outgassing in plastics

Although the sterilization procedure did not appear to have an adverse effect on the microbubbles produced, an effect which was observed as a direct result of the sterilization process was that the volume of gas inside the cartridges appeared to increase. Further testing on the materials involved indicated that both the Nylon and HDPE materials used exhibited some form of outgassing when exposed to the 30-35kGy dose of

radiation. A detailed investigation was performed by isolating various materials and irradiating them individually. The results are summarized in table 5-5.

Table 5-5 Outgassing effects of sterilization on materials

Material	% increase in gas volume
Distilled water	0
Stainless steel	0
Polypropylene	0
Polycarbonate	2
Nylon (sample 1)	4
Brass	4
Nylon (sample 2)	7
Nylon (sample 3)	16
HDPE (pre-sterilized)	16
HDPE (sample 1)	16
HDPE (sample 2)	18
HDPE (sample 3)	22

Based on the experimental data, the design of the cartridges was modified to incorporate polycarbonate housings in order to mitigate the problem. Black polycarbonate was chosen for aesthetic purposes, as it did not display any effects of discolouration due to the irradiation procedure.

All subsequent testing was performed with the MGD4 prototype and made use of black polycarbonate housings.

5.7 Safety

As mentioned previously, in order to ensure sterility in the end product, the microbubble generation system features a cartridge-based design, which isolates and sterilizes all components which come into direct or indirect contact with any injectable materials. Although the use of gamma irradiation was shown to address the issues of biological contamination due to pathogens, additional safety factors were considered.

Both of the surfactant chemicals used in the microbubble preparation are food-grade, and are commonly used as food additives. As would be expected, these chemicals have very low toxicity. A preliminary estimate of safety was achieved by obtaining the MSDS data for lethal doses. For SPAN 60, the LD₅₀ (lethal dose for 50% of the subjects) for an oral dose administered to rats is 31g/kg.⁷⁵ Translated to the concentration used in the microbubble formulation, this would equate to a dose of approximately 3.4L/kg, which is many orders of magnitude above any feasible injection dose. Similarly, in the case of TWEEN 80, the LD₅₀ for an oral dose administered to mice is 1790mg/kg,⁷⁶ which translates to a dose of prepared microbubbles of 48mL/kg. Although significantly more toxic than SPAN 60, the quantity of TWEEN 80 used is likely to be very safe, as the typical proposed *in vivo* doses of the prepared microbubbles are well under 3mL/kg.

These estimates suggest a wide safety margin, although it must be noted that the LD₅₀ dose data is based on oral administration, rather than the intravenous injection route which is used for microbubbles.

In order to minimize the toxicity caused by components of the cartridge, all experiments were performed with medical-grade adhesives, fittings, and materials

whenever possible. It is expected that in future work, the cartridges can be manufactured entirely from suitable approved medical-grade materials.

5.8 Sensitivity analysis of microbubble generation geometry

With the completion of the MGD4 prototype, the design had been refined to a point where batches of microbubbles could be consistently produced, with an acceptable degree of variation between samples.

The mean size of microbubbles produced with the current procedure seemed to be independent of the cartridge geometry, with no clearly discernible trends in changes to the size distribution. The stable size of the microbubbles generated with the MGD4 prototype is likely governed by a combination of the gas and surfactant interactions, coupled with the buoyancy trap system which essentially eliminates larger bubbles from the microbubble population. As the variation in size distribution was minimal, and provided no indications of being affected by cartridge geometry or flow rate, the size data is not presented. The concentration of microbubbles, on the other hand, did show signs of being highly variable and dependent on the microbubble generation conditions.

In order to compare the effects of various geometry parameters, a standardized means to isolate each parameter was developed. It was noted that changes in the flow rate through the capillary tubes was greatly affected by the diameters of capillary tubes tested. The range of capillary tube diameters tested varied between an inside diameter of approximately 0.004" to 0.069". At similar actuation speeds, this would result in drastically different flow conditions in the capillary tubes, which would likely render a comparison of results to be largely inconclusive. It was decided to measure, and where

practical to alter the flow rate through the capillary tubes to minimize variation between samples. Such data could then be used to compare results.

As the void fraction of gas in the mixture was low (approximately 10%), it was hypothesized that the mean Reynolds number of the flow could be used to standardize the comparison. Due to complexities involved in measuring and calculating actual Reynolds numbers for the surfactant mixture, while attempting to account for the effects of the gas dynamics, it was decided that a more appropriate solution would be to use an equivalent Reynolds number based on water (Re_w). Each cartridge was completely filled with deionised water, and the time of actuation was measured. Where possible, the actuation rate was adjusted in the MGD4 control program in order to attempt to yield comparable Re_w values across different samples. In the extreme cases, which were beyond the capabilities of the actuator, the cartridges were actuated manually, and approximate Re_w values were calculated, based on average flow rate determined by the actuation time.

$$Re_w = \frac{4(V_s / \Delta t)}{\pi v_{water} D} \quad (5-1)$$

Although the Re_w was not a direct measurement of the actual test conditions, it is expected that the results based on the Re_w would be comparable to one another. The Re_w values do not take into account the effect of the gas, which may be compensated for by using a void fraction term. It would also be possible to empirically measure the viscosity of the surfactant solution, providing the necessary information for calculating the actual Reynolds number. However, due to the short actuation time (typically under 2 seconds) and the compressibility and non-uniform distribution of the gas, it would be difficult to

accurately model the actual flow through the capillary tube. For the purposes of the current test, it was assumed that since both the surfactant solution used and the void fraction were constant for all samples, the results based on Re_w were comparable to one another, and were sufficient to determine any significant trends in the data.

It should be noted that, in order to prevent leaks in the syringes, as well as to accommodate the capabilities of the actuation device, the actuation speed at the design point was reduced in order to match Re_w the with the greatest number of samples. This resulted in a slight reduction in the concentration of microbubbles produced, but was considered to be an appropriate representation to evaluate any trends as parameters were altered.

5.8.1 Water equivalent Reynolds number

As shown in Figure 5-19, the combined data of all experiments indicated a clear relationship between the Re_w and the resulting concentration of microbubbles produced. The data is based on an amalgamation of experimental data, including a series of samples which were specifically prepared to provide data on various flow rates.

Figure 5-19 Effect of water equivalent Reynolds number on microbubble concentration

It is worth noting that the data observed yielded some unexpected results well outside of the current design point. While there appeared to be a gradual increase in concentration with Re_w between Re_w values of approximately 2000 to 13000, beyond roughly 13,000 there appeared to be a stronger effect of increasing Re_w . Although this range is well beyond the actuation capabilities of the current device, it is interesting to note for future development. As the quality of data in the range beyond Re_w values of 13,000 is limited in this case, future device development should include a more detailed analysis of this apparent transition point, as well as the possibility of achieving significant gains in concentration by greatly increasing the Re_w values.

In the range of the design point of the current device, when taking into account the limitations of the actuation speeds which could be achieved, there does not appear to be potential for a significant increase in concentration of microbubbles. Although it might be possible to increase the flow rate by reducing the capillary diameter, such a change would result in extremely high pressures required to drive such a flow. The cartridges are currently limited in sealing ability by the plunger interface in the syringes used. Further increases in pressure had been shown to lead to failure at the interface between the rubber syringe plunger and the barrel of the syringe, leading to leakage of the contents.

5.8.2 Capillary tube length

The length of capillary tubes tested did not appear to provide conclusive evidence of any definite trends. As shown in Figure 5-20, there did not appear to be a significant change in microbubble concentration with increasing capillary tube length alone. There is some indication of a mild tendency for the larger diameters tested to exhibit a slight decrease in concentration with increasing length. It should be noted that, although the Re_w values are similar for each diameter of capillary tube tested, the absolute Re_w values differ significantly between each set of data. The minor differences in Re_w within the same capillary diameters are due to a slight reduction in the flow velocity which resulted from the increased frictional resistance of the longer capillary tubes. The effect was very minor, and compensating for this by correcting the actuation speed of the MGD4 was not possible due to limitations on the resolution of actuator speed control.

Figure 5-20 Effect of capillary tube length on microbubble concentration

In order to elucidate the results, the concentration values were normalized with respect to the water equivalent Reynolds number, and scaled appropriately (Figure 5-21). As shown, the trend of decreasing concentration with increasing capillary length seemed to be slightly more obvious when the data is presented in this manner. Nonetheless, the small sample size, coupled with the presence of values which appear to contradict the trend, makes it difficult to draw definite conclusions.

Figure 5-21 Normalized microbubble concentration vs. capillary length

It is important to note that the 1.5" length capillary sample was the longest which could be incorporated into the existing device and cartridge geometry. Increasing the length beyond this value would not be feasible without making significant changes to the overall design of the cartridge and the microbubble generation unit. The data obtained suggests that there are no benefits to increasing the length of the capillary tube beyond the current 0.5" in use. There is some indication that there may be minor gains in concentration of microbubbles produced made by reducing the length of the capillary tube to 0.25" or lower. As noted in the literature review, the flow focussing methods made use of an orifice, rather than a capillary tube, suggesting that even very short sections of capillary tube may be sufficient.

Due to lack of strong trends suggesting gains in concentration were possible by making use of longer capillary tubes, it was recommended that from a practical and manufacturing standpoint, the shortest size of capillary tube available (0.25") be used. This would slightly simplify the manufacturing of the cartridges by reducing the need for deep, small-diameter holes to be created. Shorter capillary tubes may also help with assembly of the components of the cartridge.

Although it may be interesting to more thoroughly investigate the shorter capillary tube lengths, such work would have limited practical applications for the Artenga device, as (a) a source for needles under 0.25" in length may be difficult to locate, and (b) there do not appear to be significant gains to be made in the concentration of the microbubbles produced, especially when compared to other potential means such as increasing the Re_w , as discussed above, or possibly through manipulation of the surfactant combinations used.

5.8.3 Capillary tube diameter

Varying the diameter of capillary tubes used did not appear to directly yield any clear trends. As shown in Figure 5-22, the highest concentrations tended to be obtained with diameters above 0.03", although the trends do not appear to be consistent. Increasing the diameter of the capillary tube for shorter lengths seemed to lead to increased microbubble concentration. However, for longer capillary tube lengths, there appeared to be an optimal diameter, beyond which the concentration would drop. This may be attributed to the shear forces in longer capillary tube breaking up microbubbles. The substantial gains observed with larger diameters of the shorter lengths of capillary tubes can be explained

by the fact that the larger diameters allowed higher flow rates to be achieved for a given applied force. As mentioned, the maximum applied force is governed by the maximum pressure the syringes could sustain without failure.

Figure 5-22 Effect of capillary diameter on microbubble concentration

Normalizing the concentration data with respect to Re_w yielded an even less clear situation, as illustrated in Figure 5-23. Although it is interesting to note the peak values for the 0.033\" diameter capillary tubes in the longer lengths, there does not appear to be sufficient consistent data to make any generalizations on the effect of altering capillary tube diameter. As with the length of capillary tubes, it is unlikely that changes in the capillary diameter would result in substantial gains in the concentration of microbubbles.

Based on the experiments, changes in the capillary diameter appeared to have minimal effects on the microbubble concentration within the design range.

Figure 5-23 Normalized microbubble concentration vs. capillary diameter

5.8.4 Current design point analysis

Overall, the sensitivity study has shown that there do not appear to be substantial gains to be made by altering the design of the capillary geometry of the cartridge within the current design envelope. While it was interesting to note the potential increases in concentration, making use of these observations would require an extensive redesign of the device.

In theory, the current design could be optimized such that the smallest capillary tube diameter is used which does not lead to leaking at the syringe plunger when the device is

operating at its full actuation speed. However, as the current design operates reliably at very close to the maximum speed of the actuation system, additional improvements are not likely to be significant.

Furthermore, it is not clear whether such modifications would result in clinically useful improvements to the characteristics of the microbubbles, as a clear concentration goal has not yet been set, and the initial goal concentration has been exceeded by a wide margin. As future research is conducted, it may be found that a higher concentration of microbubbles is desired for the proposed therapeutic work. In such a case, the design can be revisited and a more detailed analysis into the feasibility of a high Re_w alternative can be conducted. Based on the available data, all indications are that the microbubble concentration is presently adequate for the proposed applications. Although the concentration is still lower than many commercially-available UCAs, sufficient therapeutic work has not been conducted to ascertain any benefits of extremely high concentrations.

It is also worth noting that the concentration itself is not the critical factor for in vivo applications. It is of higher importance to maximize the ratio of concentration to toxicity. Since the preliminary data on toxicity indicate a wide safety margin, it is possible that increases in total microbubble volume may be achieved simply by increasing the dose.

Chapter 6: Conclusions and Recommendations

The development of a method and prototype device to generate consistent microbubbles was successful. The technology developed over the course of the given research was directly incorporated into the latest Artenga MGD5 prototype devices which are currently in use by researchers around the world. As shown in table 6-1, with the exception of control of microbubble diameter, the key design goals of the original concept were all met or exceeded, and a number of preliminary experiments have verified that the microbubbles generated are at least comparable to commercially available UCA microbubbles.

The homogeneity, as laid out in the initial design goals proved to be difficult to quantify, as the minimum size of microbubbles extends beyond the range of any measuring equipment which was available. The homogeneity can be quantified as 99% of the microbubbles being below 2.5 μ m in diameter, which is comparable to the homogeneity of UCAs.

Table 6-1 Comparison of design goals to actual parameters achieved

Characteristic	Design goal	Actual result
Nominal Diameter	0.5 – 10 microns (variable)	0-2 microns
Homogeneity	80% of bubbles within +/-50% of mean diameter	Note: see text
Concentration	Minimum: 10^6 bubbles/ml	$1-1.5 \times 10^8$ microbubbles/mL
Bubble Stability	Minimum: 10 seconds, minutes preferable	In excess of 40 minutes

The potential advantage of the Artenga approach is that changes in the microbubble parameters may be achieved with minimal additional complexity. A simple substitution of the cartridge or an adjustment to the control program could potentially yield significant changes to the microbubbles produced. The flexibility of the design allows for the incorporation of various additives into the microbubble shell, which may prove to be of great significance to research involving binding drugs or other molecules to the microbubble surface at the time of generation.

It was discovered that the current device can potentially provide control over the concentration of microbubbles produced by varying the water equivalent Reynolds number (Re_w) of the fluid in the system. An increase in Re_w was positively correlated to a corresponding increase in microbubble concentration. Such results can be achieved by

the use of a different cartridge, possibly featuring an alternate needle diameter or changes to the control program to vary the flow rate through the cartridge. It should be noted, however, that in order to achieve substantial changes in concentration, significant changes would need to be made to the mechanical design of the prototype device as the existing geometry is not conducive to achieving Re_w significantly in excess of 13,000.

The experiments conducted to this point have shown that there is little potential for control over the mean stable size of microbubbles by physical means alone. No clear correlations were found in the mean size by altering the flow geometry, rate, or microbubble preparation procedure with the prototype device. Nonetheless, the size of microbubbles obtained is well within the range of requirements for any current applications, and has shown promise as an adequate replacement for the UCA microbubbles currently in use by researchers.

A preliminary analysis was completed on potential safety of the device, and the cartridge components have been shown to be suitable for terminal sterilization by a 30-35kGy dose of gamma radiation.

The development of the MGD prototype will enable Artenga Inc. to provide researchers with a valuable tool for future sonoporation studies. This will, in turn, expedite the understanding and optimization of therapeutic use of microbubbles, which may eventually lead to a means to treat tumours which are currently considered untreatable. It is likely that additional applications for the technology will be found in the future which may lead to as-yet undiscovered therapeutic uses for customized microbubbles.

6.1 Recommendations

Despite the functionality of the current design showing indications of being adequate for certain imaging applications, as well as *in vitro* and *in vivo* sonoporation research, a number of potential areas of improvement or continued research have been noted which could yield additional advantages.

6.1.1 Error analysis

The majority of the experimental data collected lacked a thorough analysis of error within the data. This was due to the fact that any changes made to the properties of the bubbles required a lengthy process of construction, filling, and sterilizing of additional cartridges, followed by characterization tests, which required the use of unique equipment and facilities which were not readily available.

Although the microbubbles produced have been shown to be well within the acceptable range for their current use, in the future it may be advantageous to perform a more thorough investigation into the parameters studied, particularly in the area of the capillary tube geometry. The collected data do not appear to indicate strong correlations in the mean size, concentration, or homogeneity of the microbubbles produced by adjusting the given variables. However, a more comprehensive analysis may illustrate some weak trends which could be further exploited for future refinements to the Artenga device.

6.1.2 Potential for higher microbubble concentration

While the concentration of $1.0\text{--}1.5 \times 10^8$ microbubbles/mL which the current prototype is capable of producing is adequate for preliminary research, commercially available UCAs are available in significantly higher concentrations, sometimes exceeding 10^9 microbubbles/mL. For the purposes of research, most microbubble applications require dilution to a lower concentration. However, it would be advantageous if the maximum concentration achievable by the Artenga device were as high as possible, allowing dilution (either manually, or incorporated into the device/control program) to provide researchers with a wider range of options.

As shown in the results, the sensitivity study experiments yielded some unexpected results. Data at the extreme range of the measurements suggested that significant increases in concentration may be possible. Additional characterization studies in that region could enhance understanding of the potential for increasing concentration. Practical use of such data would require a dramatic redesign of the device, as the current actuation system is optimized for relatively high pressures and low flow rates, rather than the high pressures and lower flow rates which would be optimal for the larger capillary diameters and the resulting potential to increase the values of Re_w .

6.1.3 Surfactants and chemical properties

While beyond the scope of the current research, a more thorough understanding of the chemical mechanisms involved in the interaction of the surfactants, and the formation and stabilization of the microbubbles post-generation could provide a chemical means to control parameters such as the stable size of the microbubbles, as well as more long-term

considerations such as shelf life, and stability *in vitro* or *in vivo*. Additionally, the possibility of substituting alternate surfactants with similar structures should be investigated. This could potentially prove to be particularly useful for the binding of additional material to the microbubble shell during the microbubble generation process, or allow variations in the properties of the microbubble shells.

It is also very likely that the stable size of the microbubbles produced can be altered by chemical means. This could involve changes to the properties of the bulk liquid, changes to the surfactants which form the shell of the microbubbles, and/or changes to the gas (or combination of gases) contained within the microbubbles. In the literature, evidence exists that microbubbles with different surfactant shells have shown significant differences in concentration, mean size, and persistence behaviour when surfactants were varied.^{55,77,78}

6.1.4 Chemical binding to the microbubble surface

Currently, a great deal of research is being done in the area of binding targeting ligands, drugs, proteins, or any number of other molecules to the surfaces of microbubbles.³⁸ This has tremendous potential in both diagnostic and therapeutic applications for microbubbles binding. The possibility of incorporating linking molecules into the shells of the microbubbles could allow the microbubbles to be substituted for common applications where binding therapeutic agents to various targeted delivery systems are being investigated.

6.1.5 Safety and toxicology

Although preliminary experiments have been done in an attempt to minimize future complications relating to toxicity, it should be noted that in-depth toxicity analysis of the cartridges and microbubbles has not yet been undertaken. There was no investigation of leaching from the plastic components used, which may be particularly relevant in terms of shelf-life of the cartridges. Also, although the sterilization process was shown to address the issues of biological contamination due to pathogens, this does not preclude the presence of endotoxins or exotoxins produced by bacteria which may have been present prior to irradiation. Presumably, the use of approved clean room and certified pyrogen-free chemicals can mitigate such factors.

6.1.6 Future device improvements

One of the drawbacks of the current configuration is the size required for the device. The understanding of the mechanics of the chosen bubble generation method indicates that there is no strict need for a rotating device. However, given the current technology, it is possible that a much smaller device could be constructed, which relies on an improved cartridge layout and a slightly more complex multiple-actuator system to achieve the same mixing and foam purge effects without the need for a rotating assembly. As well as reducing the overall size and portability of the device, this would provide the additional benefit of enabling further investigations to be performed, as independent control of the syringe plungers can lead to the possibility of exercising a great deal of control over the pressures inside the syringes during various stages of the microbubble generation cycle.

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