

**ENGINEERING THE SURFACE PROPERTIES OF
MICROBUBBLES FOR BIOMEDICAL APPLICATIONS**



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Abstract

Surfactant coated microbubbles are widely used as contrast agents (UCA) in medical ultrasound imaging, due to their high echogenicity and non-linear response to acoustic excitation. Controlling the stability of microbubbles *in vivo* represents a considerable challenge. Understanding the characteristics of the bubble surface and how they change with production method, composition and environment is key to addressing this problem. The aim of this thesis is to investigate viscosity, bubble dissolution, and acoustic response as functions of their composition, manufacturing method and environment.

Bubbles were made using combinations of phospholipid and an emulsifier in different molar ratios. Adding the emulsifier decreased both the size and the surface viscosity of the bubbles and caused changes in the scattered pressure amplitude of bubbles under ultrasound.

To increase microbubble stability, solid inorganic nanoparticles were adsorbed on to the microbubble surface. These particles behaved as Pickering stabilisers, and deterred Ostwald ripening. The nanoparticles also enhanced the non-linear behaviour of bubbles at low acoustic pressures.

Three manufacturing methods (sonication, cross-flow and flow focusing) were investigated in order to verify stability differences. Sonication produced bubbles with surface viscosities hundreds of centipoise greater than those produced by

microfluidics. Both pressure amplitude and harmonic content for sonicated bubbles were found to be much larger due to a higher liposomal adhesion rate at the surface.

Solution temperature and bubble age were also investigated. When the solutions were heated above the phospholipid gelling temperature, microfluidic bubbles showed an increased surface viscosity, due to increased liposome adhesion caused by the increased temperature.

Bubble composition, manufacturing method and environment were found to vary the surface characteristics of the microbubbles. Further investigations into the affects of the filling gas, *in vitro* studies, and low temperature TEM characterisation should be conducted to produce a microbubble with the full range of desired characteristics.

Statement of Originality

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

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

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

Graciela Mohamedi

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The FLIM portion of my experimental work was carried out in collaboration with Dr. Neveen Hosny, an inspiring research fellow at Imperial College London. Her involvement made viewing the bubble surface viscosity possible, and her humour and optimism helped see us through difficult and sometimes discouraging times.

The theoretical models used to verify my experimental results, were developed by Mr. Mehrdad Azmin, in collaboration with University College London. His perfectionist attitudes for his models only served to strengthen my experimental work.

The acoustic comparisons were completed with Mr. Paul Rademeyer using his acoustic set-up and analytical code. I thank him for his ever-enduring patience in helping a surface scientist with acoustic measurements.

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Dedication

To Alma and Zorah

Because this song, like all the others I sing is and will always be, for you.

Dissemination

Peer-Reviewed Journal Publications

- M. Azmin, **G. Mohamedi**, Pastoriza-Santos; J. Perez-Juste, L. M. Liz-Marzan, M. Edirisinghe and E. Stride. Dissolution of Coated Microbubbles: The Effect of Nanoparticles and Surfactant Concentration; *Materials Science and Engineering: C*, **2012**, 32(8), 2654–2658
- **G. Mohamedi**, M. Azmin, Pastoriza-Santos I; V. Huang; J. Perez-Juste; L. M. Liz-Marzan; M. Edirisinghe and E. Stride. The Effects of Gold Nanoparticles on the Stability of Microbubbles; *Langmuir*, **2012**, 28 (39), 13808–13815
- N. A. Hosny, **G. Mohamedi**, T. Mengxing, R. J. Eckersley, M. Kuimova and E. Stride. Mapping Microbubble Viscosity Using Fluorescence Lifetime Imaging. *Proceedings of the National Academy of Sciences*, **2013**. doi:10.1073/pnas.1301479110
- **G. Mohamedi**, M. Azmin, P. Rademeyer; I. Pastoriza-Santos; J. Perez-Juste; L. M. Liz-Marzan; M. Edirisinghe and E. Stride. The Effects of Gold nanoparticles on the Non-linear Acoustic response of phospholipid microbubbles. In Preparation.
- **G. Mohamedi**, Y. Park, J. Wong, E. Stride. Changes in phospholipid layer composition and surface properties due to microbubble preparation method. In Preparation.

Conference presentations

- **G. Mohamedi**, N. Hosny, P. Rademeyer, Y. Park, J. Owen, T. Pham, J. Wong, M. Kuimova, E. Stride. Investigating the effect of fabrication method on the stability and acoustic response of microbubble agents. 21st International Congress on Acoustics, and 165th Meeting of the Acoustical Society of America, June 2013, Montreal, Canada.
- M. Azmin, **G. Mohamedi**, P. Rademeyer, L. Liz-Marzan, M. Edirisinghe, E. Stride. Investigating the acoustic response of gold nanoparticle coated microbubbles. 21st International Congress on Acoustics, and 165th Meeting of the Acoustical Society of America, June 2013, Montreal, Canada.
- **G. Mohamedi**, N. A. Hosney, P. Rademeyer, Y. Park, J. Owen, T. Pham, J. Y. Wong, M. Kuimova, E. Stride. Influence of Fabrication Method on Functional Properties of Microbubble Agents for Theranostic Applications. 245th ACS National Meeting, New Orleans, LA

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- **G. Mohamedi**, M. Azmin, P. Rademeyer, M. Edirisinghe, E. Stride. Investigating the acoustic response of gold nanoparticle coated microbubbles. 245th ACS National Meeting, New Orleans, LA
 - **G. Mohamedi**, M. Azmin, P. Rademeyer, M. Edirisinghe, E. Stride. Effects of gold nanoparticles on the stability and non-linear response of phospholipid microbubbles. 2012 International Society for Therapeutic Ultrasound Symposium, Heidelberg, Germany, June 10-13 2012.
 - **G. Mohamedi**, M. Azmin, I. Pastoriza Santos, V. Huang, J. Pérez-Juste, L. Liz-Marzán, M. Edirisinghe, E.P. Stride. The Effects of Gold Nanoparticles on the Stability of Microbubbles. 2011 Materials Research Society Fall Meeting and Exhibition, Boston, MA
 - N. A. Hosny, **G. Mohamedi**, Tang Mengxing, Robert J. Eckersley, Marina Kuimova and Eleanor Stride. Mapping Microbubble Viscosity Using Fluorescence Lifetime Imaging. 17th European Symposium of Ultrasound Contrast Imaging, Rotterdam, the Netherlands, January 19-20, 2012.

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List of Symbols

γ, σ	Surface or interfacial tension	Ca	Capillary number
A	Area	m	Mass
W	Work	K	Nominal Constant
G	Gibbs free energy	t	Time
P, p	Pressure	Φ	Quantum yield
T	Temperature	τ_f	Fluorescent lifetime
H	Enthalpy	χ^2	Goodness of fit
S	Entropy	k_r	Radiative rate
C, c	Concentration	k_{nr}	Non-radiative rate
θ	Contact angle	M	Molar mass
E_B	Desorption energy	D	Coefficient of diffusion
R	Radius	S_A	Effective surface area
B	Universal gas constant	f_{p0}	Initial fractional coverage
V	Volume	d_{s0}	Shell thickness
U	Chemical potential	G_s	Modulus of rigidity
x	Mole fraction	G_{s0}	Stiffness of particle free shell
δ	Notional bubble size	d	Ratio of saturated dissolved gas to gas density
ρ	Density	f	Ratio of initial gas concentration to concentration at saturation
ℓ	Characteristic dimension	ϕ	Particles to shell volume fraction
μ	Dynamic viscosity	I	Initial surfactant concentration
ν	Kinematic viscosity		
Re	Reynolds number		
L	Length		
w	Width		
Q	Flow rate		

List of Abbreviations

UCA	Ultrasound contrast agent
MEM	Microelectromechanical
CT	Computed tomography
MRI	Magnetic resonance imaging
FFT	Fast-Fourier Transform
HIFU	High intensity focused ultrasound
PEG	Polyethylene glycol
LSPR	Localised surface plasmon resonance
DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine
FDA	Federal Drug Administration
o/w	Oil-in-water emulsion
w/o	Water-in-oil emulsion
PDE	Partial differential equation
SD	Standard deviation
RSD	Relative standard deviation
RCF	Relative centrifugal force
PBS	Phosphate buffered saline
CEHDA	Co-axial electrohydrodynamic atomization
SPG	Shirasu porous glass
RMS	Root mean square
PEEK	Polyether ester ketone

HPLC	High pressure liquid chromatography
PDMS	Poly(dimethylsiloxane)
PEG40S	Polyethylene glycol 40 stearate
FLIM	Fluorescence lifetime imaging microscopy
BODIPY-C₁₀	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
EDS	Energy dispersive spectroscopy
BSE	Backscattered electron imaging
SEM	Scanning electron microscopy
L-α PC	L-alpha-Phosphatidylcholine
PFC	Perfluorocarbon
TEM	Transmission electron microscopy
LTTEM	Low temperature transmission electron microscopy

1 Biomedical Microbubbles: Composition, Properties and Applications

This chapter will introduce microbubbles and their physical and chemical properties in relation to their biomedical applications. Microbubbles, gas-filled bubbles with diameters in the 1-100 μm range are currently utilized in a wide range of technological and biomedical applications, such as sources for extreme temperatures and pressures in sonochemical reactions [2] and oxygen delivery vehicles in fermentation processes [3]. Some microelectromechanical (MEM) devices use an oscillating microbubble to drive mixing processes [4]. In medicine, microbubbles of less than 10 μm in diameter are used as ultrasound contrast agents [5-12] and are also being developed for use as drug/gene delivery vehicles [13-17], oxygen carriers [18], and in cancer therapies [17, 19, 20]. The characterisation of medicinal microbubbles and their unique physical and chemical properties will be the focus of this work. Specifically the aims of this work are:

- 1. Understanding the role microbubble lipid shell composition plays on bubble stability and surface properties such as shell viscosity.*
- 2. Investigating how the adsorption of solid nanoparticle particles on the microbubble surface affects the amplitude of excitation needed to produce non-linear oscillations when subjected to an ultrasound pressure wave.*
- 3. Characterising the effects of fabrication method on the physical and acoustic properties of lipid bubbles.*

4. *To study how the dynamics of lipid microbubbles differ with environmental changes.*

By understanding how these variables change the physical properties of phospholipid microbubbles future researchers will be able to produce bubbles with very specific physical properties tuned to their application.

1.1 Biomedical Application of Microbubbles

Microbubbles used for biomedical purposes are typically between 0.5 and 8 μm in diameter, which is the upper limit for passage through the lung capillaries. They consist of a gas core, that is a single chamber and comprises the majority of the total particle volume, surrounded by a shell which may be polymer or surfactant based. The shell acts as a barrier preventing diffusion of the encapsulated gas into the surrounding aqueous medium (Figure 1.1) [10, 21-23]. Their interactions with ultrasound are of great interest in variety of applications within medical diagnostic imaging and therapy. Both the shell and the gas core affect microbubble behaviour and their resulting efficacy in a given clinical application [24].

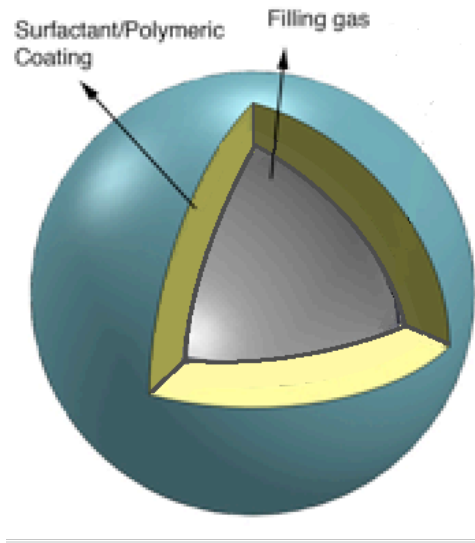


Figure 1.1 Schematic of a coated microbubble

1.1.1 Ultrasound Contrast Agents

Ultrasound is a real-time modality that commonly allows imaging up to 30 frames per second. Compared with computed tomography (CT) and magnetic resonance imaging (MRI), ultrasound offers several advantages: hardware that is relatively inexpensive with the ability to provide real-time images [25]; also since no form of ionizing radiation is used, it is much safer than many other imaging methods, particularly CT and nuclear imaging techniques. Image resolution however is limited, increasingly so with tissue depth, and contrast especially between blood vessels and other tissue can be poor, requiring the use of contrast agents.

Microbubbles function as UCAs by modifying the characteristic signature of the echo from blood [26]. Bubbles oscillate when exposed to an ultrasound beam, rapidly contracting and expanding in response to pressure changes imposed by the

sound field [5]. Microbubbles enhance the backscattered acoustic signal, both due to a large impedance mismatch between the bubbles and the surrounding tissue, and their volumetric oscillations due to their high compressibility [27]. By fortunate coincidence, the range of resonance frequencies for bubbles with diameters between 1 and 10 μm is approximately 2–10 MHz which corresponds to the frequencies typically used in diagnostic ultrasound imaging [5, 7]. Thus microbubbles are several thousand times more reflective or “echogenic” than normal body tissues (Figure 1.2) [5].

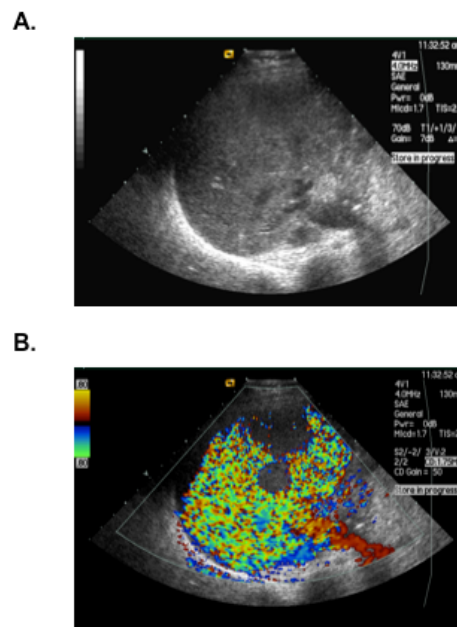


Figure 1.2 Ultrasound Doppler images without (A.) and with (B.) contrast agents. Each pixel indicates the direction and velocity of motion at a particular location. More intense colour indicates higher velocity whilst stationary tissues appear grey.

At low acoustic powers, linear, symmetrical bubble oscillations occur and the frequency spectrum of the scattered signals is the same as that of the transmitted

pulse. At higher acoustic powers, the expansion and contraction phases become unequal because microbubbles resist compression more strongly than expansion. This response is said to be “non-linear”, and the scattered signals contain multiples (and in some cases, fractions) of the insonating frequency (Figure 1.3) [6]. The scattered signals of the microbubbles can thus be easily distinguished from that of the surrounding tissue, and excellent contrast to tissue ratios can be achieved [27, 28]. By taking a fast-fourier transform (FFT) of the acquired scattered signal the frequency spectrum of the insonified bubbles can be produced. This allows for the harmonic behaviour of the bubbles to be investigated.

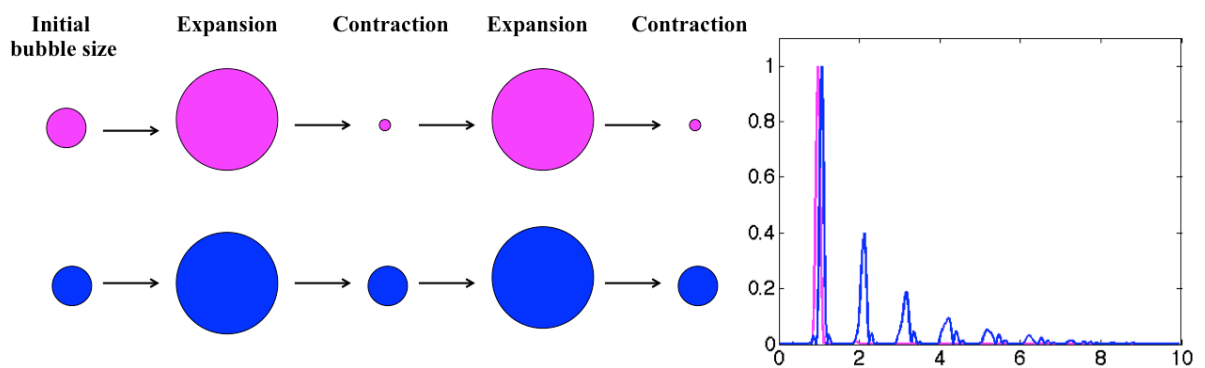


Figure 1.3 Non-dimensionalised comparison of oscillating bubbles and their corresponding FFT. Bubble A (pink) undergoes both expansion and contraction during oscillation resulting in a linear signal characterised by a single peak at the driving frequency, in this case 1. Bubble B (blue) undergoes expansion-only oscillation meaning that the bubble is unable to contract further down than it's original size, which result in a non-linear harmonic signal characterised by several resonance peaks.

1.1.2 Therapeutic Applications

The effects of ultrasound on microbubbles have spurred research into many therapeutic applications including transdermal delivery, delivery to solid tumours, delivery across the blood brain barrier, and enhanced thrombolysis [29]. Ultrasound is also advancing technologies and methods including tumour ablation, transdermal glucose monitors, and systems to enhance the delivery of drug/gene therapies [30]. Ultrasound can produce a wide variety of biological effects *in vitro* and *in vivo* through non-thermal mechanisms, such as acoustic cavitation, radiation force, and acoustic streaming which have been harnessed in developing new therapies (Figure 1.4) [31]. The presence of microbubbles can influence many of these phenomena, [32]. In high intensity focused ultrasound (HIFU) surgery, microbubbles have been used as a means of promoting cavitation in the treatment region [19]. Thus, depending on exposure conditions, the rate of tissue ablation can increase through a combination of mechanical effects, also known as histotripsy [33] and increased heat deposition and hence tissue denaturation [34]. Cavitation (relatively high amplitude bubble oscillations) in an ultrasound field can increase the permeability of the vascular endothelium, allowing small molecules to enter into tissue from the blood stream (Figure 1.5). It can also enable the uptake of molecules by cells a, process known as sonoporation [35].

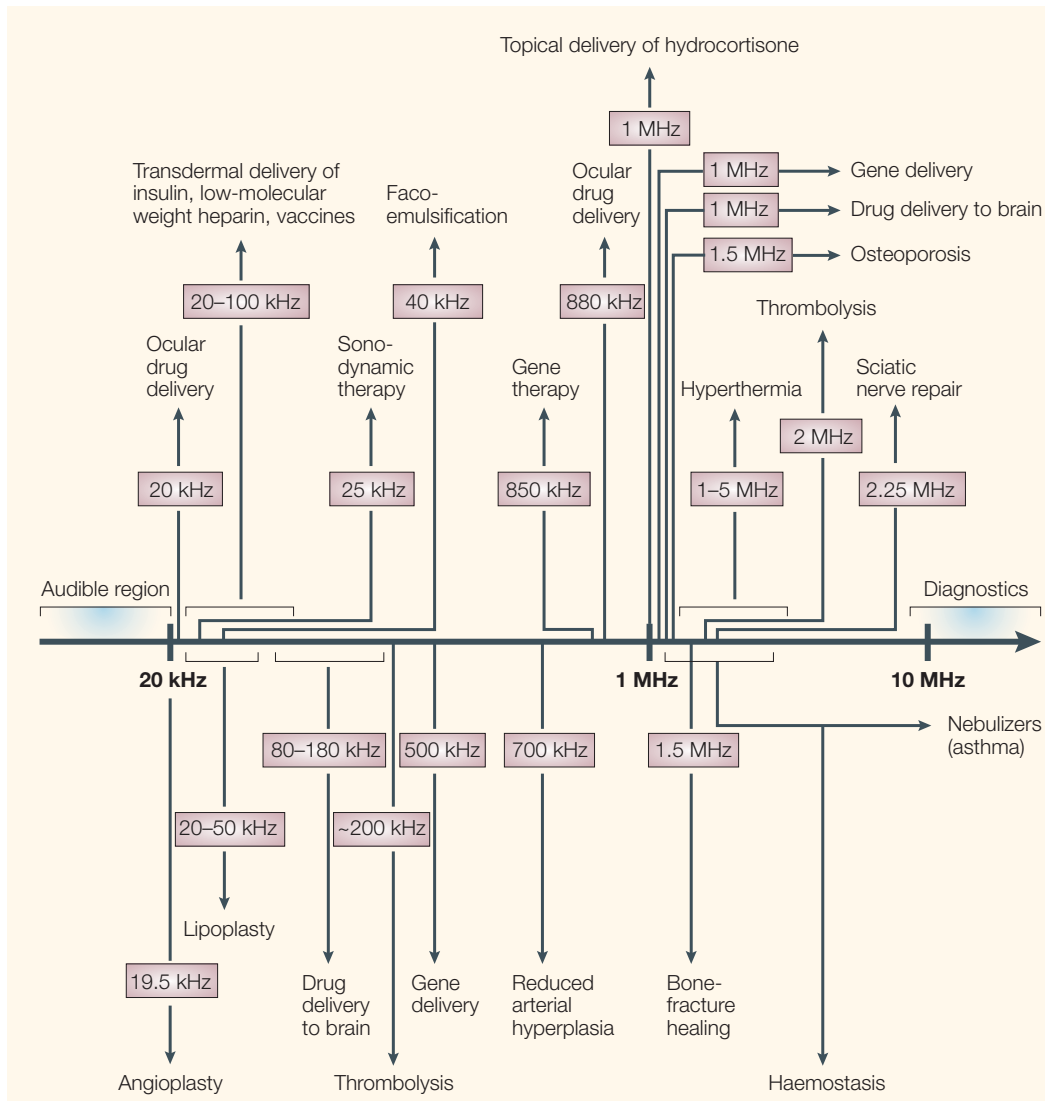


Figure 1.4 Partial summary of ultrasound frequencies used for medical applications. The figure provides an overview of the conditions used for medical ultrasound applications but does not necessarily depict optimal or recommended conditions. For many applications, more than one condition has been used. Only select conditions are represented in the figure for clarity. Ultrasound parameters other than frequency that are also crucial to medical applications, including intensity (i.e. pressure amplitude) and duty cycle (pulse width and repetition frequency), are not listed in the figure, for clarity. A significant number of applications use a frequency of 1 MHz. Reproduced with permission from [30].

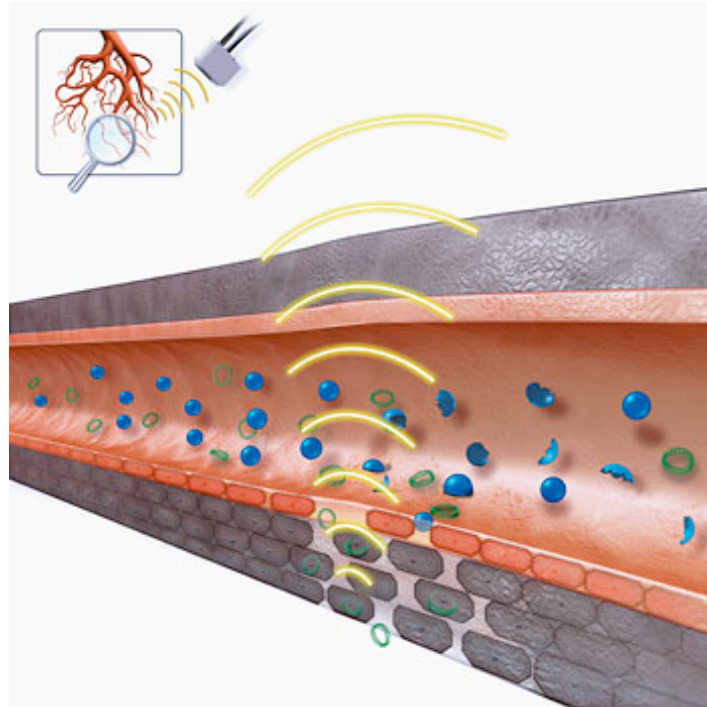


Figure 1.5 Schematic representation of ultrasound mediated delivery. Microbubbles are co-injected into the bloodstream along with therapeutic agents. At the target organ, the microbubbles are subjected to high intensity focused ultrasound (HIFU), causing them to rupture, thus increasing permeability of the blood vessel and cell membranes in the underlying tissue. This facilitates the local uptake of the therapeutic agents. Reproduced with permission from [36].

The exact mechanisms that induce sonoporation are still in question, but there is evidence to suggest that both stimulation of normal membrane transport processes and mechanical pore formation [37, 38] may contribute to changing cell membrane permeability depending on the ultrasound exposure conditions.

In addition to promoting therapeutic uptake, microbubbles can also be used as vehicles for localised drug delivery and gene therapy as they can be loaded with a

range of therapeutic materials during their production [16, 17, 39, 40]. Due to their ability to be clearly seen using conventional ultrasound, the loaded bubbles can be traced to a target site where they can then be destroyed by increasing the ultrasound intensity to release the encapsulated material locally. This can greatly reduce the risk of harmful side effects associated with systemic administration, e.g. of toxic chemotherapy drugs [41].

1.2 Microbubble Composition

When designing a microbubble to generate an optimal acoustic signal, the larger the microbubble the better [10]. However to (a) achieve systemic and pulmonary circulation and (b) be an effective contrast or theranostic agent, microbubbles need to pass through the capillary system intact. Microbubbles for clinical applications are thus designed to be approximately equivalent to the size of a red blood cell, with typical diameters ranging between 1 and 10 μ m. The encapsulating shell may be stiff (e.g., protein, or polymer) or more flexible (phospholipid, surfactant), with a thickness varying from 10 to 200 nm (Figure 1.6). [10, 21-23, 42]. Table 1.1 lists some commercially available microbubbles and their compositions.

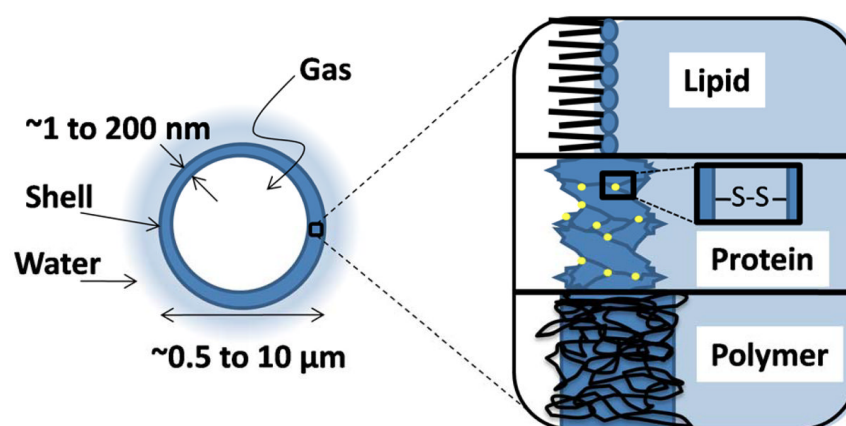


Figure 1.6 Structure of a typical microbubble with different shell compositions. Different shell materials may be used, including lipid (~3 nm thick), protein (15–20 nm) and polymer (100–200 nm). Lipid molecules are held together through physical force fields, such as hydrophobic and van der Waals interactions. Protein is cross-linked by covalent disulphide bonds. Polymer chains are covalently cross-linked and/or entangled to form a bulk-like material. Reproduced with permission from [13].

Table 1.1 Examples of commercial microbubble agents currently available or under development.

Name(s)	Manufacturer	Stabilising coating	Filling gas
SonoVue®	Bracco Diagnostics, Inc.	Phospholipid	Sulphur hexafluoride
Definity®	Bristol-Myers Squibb	Phospholipid	Octafluoropropane
Optison™	Amersham Health PLC	Human serum albumin	Octafluoropropane
Filmix™	Cav-Con Inc.	Phospholipid	Air
Imavist™	Schering A.G.	Surfactant	Perfluorocarbon & air
Albunex®	Molecular Biosystems	Human serum albumin	Air
Sonazoid™	Amersham Health PLC	Phospholipid	Perfluorocarbon
BiSphere™	Point Biomedical Corp.	Polymer/protein bilayer	Air
Quantison™	Quadrant Healthcare PLC.	Spray dried serum albumin	Air
Echovist®	Schering AG.	None	Air
Levovist®	Schering AG.	Palmitic acid	Air
Sonavist™	Schering AG.	Cyanoacrylate	Air
Echogen®	Sonus Pharmaceuticals	Surfactant	Dodecafluoropentane

1.2.1 Protein Shelled Microbubbles

Protein shelled bubbles have been observed by scientists for over a century to possess extraordinary stability due to their solid membrane shells [43]. In the 1980s and 1990s, the potential of microbubbles coated with an adsorbed layer of the protein albumin as UCAs was recognised [44, 45]. As a result, the albumin-coated microbubbles Albunex® and Optison™ (GE Healthcare) were the first commercially available, FDA-approved contrast agents.

The typical production method for albumin-coated microbubbles involves sonication of a heated solution of human or bovine serum albumin in the presence of a gas, either air or a perfluorocarbon [6, 46]. During sonication, microbubbles of the encapsulating gas are formed with a shell of aggregated albumin [13]. Heating is necessary to denature the albumin prior to sonication and facilitate encapsulation; however, the mechanism responsible for forming the microbubbles is a combination of two acoustic phenomena: emulsification and cavitation [46]. Dispersing gas into the protein solution, coupled with chemically cross-linking the protein at the bubble interface results in the formation of a solid shell. More recently, other proteins (e.g. lysozyme and albumin/avadin mixtures), have been used to coat microbubbles [47, 48], and protein-shelled microbubbles have also been functionalized to carry targeting ligands and genetic payloads [14, 49, 50].

1.2.2 Lipid Shelled Microbubbles

Although UCAs can be made of several different materials from proteins to polymers, [51] this thesis will focus specifically on phospholipid-shelled microbubbles made using the naturally occurring phospholipid 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC). Previous studies conducted by Borden and Longo [74] have shown that microbubbles produced using longer chained lipids, specifically DSPC, have a lower rate of gas diffusion than those made from lipids with shorter chain lengths. They also showed that DSPC bubbles did not fragment under an acoustic pressure wave as bubbles produced using lipids with longer chain lengths had a tendency to do [74]. Several commercially available DSPC lipid-coated microbubble formulations are approved for clinical use internationally, with Definity (Lantheus Medical Imaging) and Sonovue® (Bracco Diagnostics) being two of the most commercially successful.

Phospholipid shells have several advantages over their protein or polymer brethren. Phospholipid molecules spontaneously self-assemble into a monolayer at the air-water interface due to their amphiphilic nature [52]. The hydrophobic acyl tails face the gas while the hydrophilic headgroups face the water [7]. Lipid monolayers are extremely cohesive due to attractive hydrophobic and van der Waals interactions between tightly packed acyl chains. Saturated diacyl phosphocholines, which are commonly found in abundance within animal cell membranes, are capable of laterally compressing within the monolayer plane to reach a very low surface tension when the temperature is decreased below their main phase transition

temperature, at which the membrane transforms to a more rigid “gel” phase from a more fluid “liquid phase”. The relative mobility or fluidity of the individual lipid molecules can be directly correlated to the temperature of the phospholipid and to both surface tension and diffusivity. In an uncoated bubble, surface tension at the curved interface induces a Laplace overpressure, which forces the gas core to dissolve [5, 53]. The condensed lipid monolayer, however, stabilises the microbubble and promotes an equilibrium pressure [54]. The high cohesiveness of the lipid molecules gives the lipid shell a solid-like character when below phase transition temperature and can induce expansion only oscillations when a driving frequency is applied [55].

Polymeric microbubbles exhibit quite different acoustic responses to ultrasound when compared to lipid-shelled microbubbles [56, 57]. Lipid shelled bubbles are much more fluid than polymeric, and therefore allow oscillations without shell rupture. At low ultrasound intensity, the stiff polymeric shell will not oscillate actively. Some polymers will allow oscillations but that typically occurs at much lower amplitudes. As pressure increases, shell defects or cracks form, which allow encapsulated gas to escape and eventually cause complete bubble destruction (Figure 1.7).

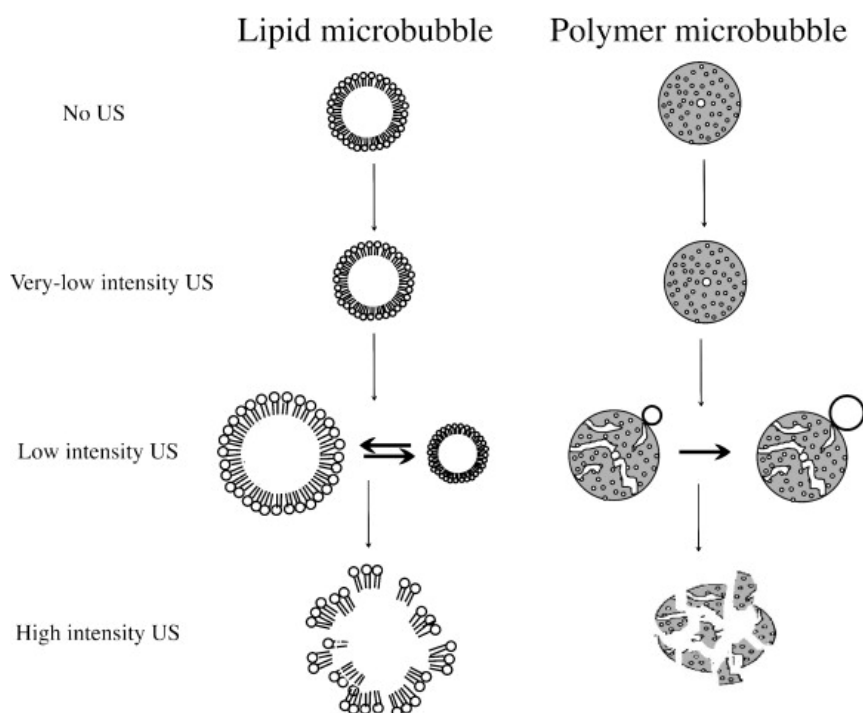


Figure 1.7 Schematic representation of lipid (left) and polymer (right) microbubble interaction with ultrasound of increasing intensity (top to bottom). Reproduced with permission from [56].

Lipid-coated microbubbles can also be functionalized for drug delivery, molecular imaging, or other purposes by incorporating different lipid headgroup species such as polyethylene glycol-biotin (PEG-biotin) linkers (Figure 1.8) or post-production bio-conjugation [58, 59]. Lipid monolayers also provide suitable substrates for inorganic nanoparticles [27].

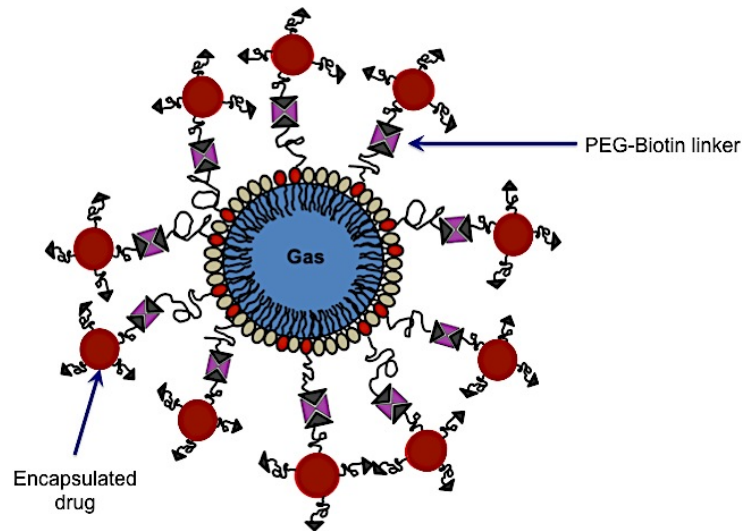


Figure 1.8 Schematic representation of a PEG-biotin linked microbubble for drug delivery

1.2.3 Nanoparticles for Biomedical Applications

Both organic and inorganic nanoparticles have been used in biomedical applications that take advantage of their varied mechanical, chemical, electrical, optical, and acoustic properties, as shown in Table 1.2 [1].

Table 1.2 Imaging and Therapeutic Capabilities of Nanoparticles. Reproduced with permission from [1]

	modality	nanoparticle/agent
imaging	optical scattering/OCT	gold nanoshells, nanorods, nanocages, nanoparticles
	fluorescence	quantum dots, dye-doped silica, carbon nanotubes, organic fluorophores, phosphors
	MRI	manganese-based, iron oxide, gadolinium agents, perfluorocarbon
	PET, SPECT	radioisotopes (^{64}Cu , ^{18}F , ^{124}I , ^{111}In)
	CT	gold nanoparticles, iodine
	ultrasound	polymeric nanoparticles, perfluoropentane
therapeutic actuation	photothermal	gold nanoshells, nanorods, nanocages, nanoparticles
	brachytherapy	^{198}Au , ^{125}I , ^{103}Pd (X-rays)
	photoacoustic	carbon nanotubes
	chemotherapy	anticancer drugs (doxorubicin, paclitaxel, etc.)
	photodynamic	photosensitizer
	gene therapy	siRNA, DNA
	magnetic hyperthermia	iron oxide based nanoparticles
	radiotherapy	^{64}Cu radionuclide
	neutron capture therapy	gadolinium, boron

^aOCT, optical coherence tomography; MRI, magnetic resonance imaging; PET, positron emission tomography; SPECT, single photon emission computed tomography; CT, computed tomography.

Recently there have been several studies conducted on the ability of inorganic nanoparticles, in particular noble metals (Au, and Ag) to target and treat various forms of cancer [60-70]. Colloidal gold is of particular interest due to its varying optical properties, which can be tuned for specific applications by controlling the size and shape of the nanoparticle [60, 69] The interesting optical attributes of metal nanoparticles, are due to their unique interaction with light, as shown in Figure 1.9 [65, 71].

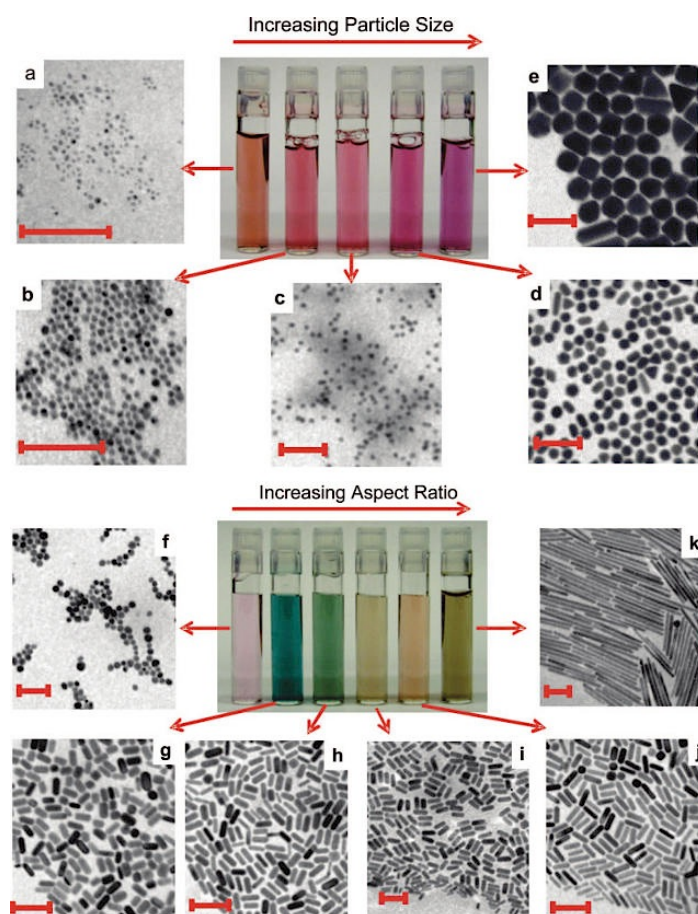


Figure 1.9 Photographs of aqueous solutions of gold nanospheres (upper panels) and gold nanorods (lower panels) as a function of increasing dimensions. Corresponding transmission electron microscopy images of the nanoparticles are shown; all scale bars 100 nm. Reproduced with permission from [65]

In the presence of the oscillating electromagnetic field, free electrons of the metal nanoparticle undergo a collective coherent oscillation with respect to the positive metallic lattice. This process is resonant at a particular frequency of the light and is termed the localized surface plasmon resonance (LSPR) oscillation [60, 63, 65] [72].

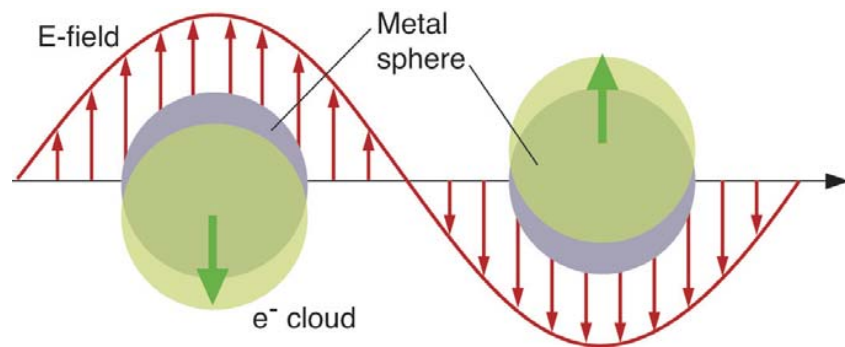


Figure 1.10 Schematic of the interaction of a metal nanosphere with light. The electromagnetic field of the light induces a coherent dipolar oscillation of the metal conduction electron across the nanoparticle. Reproduced with permission from [60].

Due to their tuneable scattering and strong adsorption of light in the near infrared range gold nanoparticles serve as “light-activated nanoscopic heaters” useful for biomedicine, especially the selective laser photothermolysis of cancer cells [65, 72]. LSPR absorption of Au nanoparticles is followed by the rapid conversion (~ 1 ps) of the absorbed light into heat. The ability of Au nanoparticles to convert strongly absorbed light efficiently into localized heat can be exploited for the selective photothermal therapy of cancer [60, 61, 71]. Cancer targeting antibodies, and ligands can be attached to the nanoparticle surface in order to selectively target

cancer cells without significant binding to healthy cells [66, 67, 72]. The cancer cells targeted by the nanoparticles can then be irradiated a laser of whose frequency overlaps the LSPR absorption maximum of the nanoparticles, thus selectively heating and destroying the cancer cells at much lower laser powers than those required to destroy healthy cells making therapy minimally invasive [60, 63, 67].

1.2.4 Nanoparticle Coated Microbubbles

It has been shown that the adsorption of solid nanoparticles on to a bubble surface can potentially improve the efficacy of microbubbles as ultrasound contrast agents by increasing the nonlinear character of the microbubble acoustic response at low excitation amplitudes [73]. The presence of the surface coating both lowers the interfacial tension at the bubble surface and slows gas diffusion, thus preventing rapid dissolution and/or coalescence and disproportionation within the suspension [74, 75]. Typically in order to increase the degree of non-linearity in a bubble, the amplitude of excitation would have to be increased. This can result in undesirable side effects as the bubbles often react violently to the amplitude increase, inducing cavitation or bubble fragmentation as a result [28, 73, 76]. By including inorganic particles on the lipid surface, the non-linear acoustic characteristics of the UCA at low acoustic pressures can be increased while avoiding the undesirable effects [73]. In order to repeat this on a non-particle laden bubble the amplitude of the ultrasound would have to be increased to levels upwards of 10 times the acoustic peak negative pressure [73, 77]. This is due to the “jamming” of particles (Figure 1.3) when the

bubble undergoes compression, leading to an asymmetric volume oscillation and nonlinear acoustic radiation.

Nanoparticles have also been used as stabilisers in a manner akin to the well-known phenomenon of Pickering stabilisation in liquid–liquid emulsions [78, 79], for both bubbles [80] and foams [81]. The underlying physics of this phenomenon will be described in detail in the next chapter. In particular, a number of recent studies have demonstrated that ultra-stable foams can be generated through the use of solid particles [82-85] with applications ranging from food engineering to cosmetics and the biotech and ceramic industries. As described in the above references and the review by Horozov [86], a range of different materials such as polymeric, metallic and ceramic particles and varying, from long rods to spheres have been shown to be highly effective. The sizes of particles used have ranged from a few tens of nanometres to the micrometre scale with cell diameters similarly varying from tens to hundreds of micrometres. Foams have been shown to be stable for periods of several weeks including under harsh ambient conditions. This is of considerable potential interest because the stability of microbubbles in biomedical applications, as well as in other areas, is of great importance. In the case of contrast agents, it determines the period over which diagnostic information may be acquired, and it is equally important in drug delivery to ensure accurate dosing and minimize unwanted side effects. Finally, in addition to tailoring the acoustic response, there are other incentives for coating microbubbles with nanoparticles to achieve enhanced functionality, such as photoacoustic response [27, 87]

1.3 Thesis Structure

This thesis investigates the physical properties, both surface and acoustic; of biomedical microbubbles for use as multi-functional therapeutic and diagnostic agents.

Chapter 1 provides background information about the research field and gives an overview on biomedical microbubbles and their applications for both diagnostic and therapeutic applications. The organisation of this thesis and scope of the research is also outlined.

Since the aim of the research is to investigate the physical properties of microbubbles, **Chapter 2** presents an overview of the interfacial properties and the basic physics and thermodynamic equations which govern them.

Chapter 3 presents a detailed literature review of several different microbubble manufacturing methods and the theory behind the different approaches.

Chapter 4 describes the experimental setup, materials used, experiments and characterisation procedures carried out, and a detailed description of the experimental tools used to achieve the objectives.

An extensive study investigating the effects of composition on the surface properties of microbubbles is detailed in **Chapter 5**. Different molar ratios of DSPC lipid and

PEG 40S were investigated to determine the effect upon bubble size, stability, effective surface viscosity, and acoustic response.

Chapter 6 examines the effects of microbubble surface composition. Gold nanoparticles were adsorbed on the surface of DSPC microbubbles and compared against non-particle coated DSPC bubbles. The non-linearity of the acoustic response and long-term stability of the bubbles were determined and the experimental results compared with those obtained via theoretical modelling.

In **Chapter 7**, the stability, acoustic response and surface viscosity of bubbles manufactured via three different methods are compared.

Chapter 8 details the environmental effects, which can change the behaviour and physical characteristics of bubbles. These include bubble-bubble interactions, temperature of the bubble solution and bubble age.

Chapter 9 presents the conclusions from this work and proposals for future work.

2 Engineering the Interfacial Properties of Microbubbles

Microbubbles pose several interesting engineering challenges due to their dynamic interfacial properties. Those of particle-laden bubbles are even more complex as the addition of a solid particle on the bubble surface adds another interface. In this chapter the different interfacial qualities of typical lipid-coated as well as particle-laden microbubbles are discussed. It begins by describing surface tension and free energy and how they affect the stability of colloidal suspensions. Next it discusses the pressure differences caused by the surface tension (Laplace pressure) and the stabilisation of the microbubble solution through the addition of colloidal particles (Pickering stabilisation), ending with a discussion of thermodynamically driven bubble coarsening (Ostwald ripening) and the differences between large and small bubbles.

2.1 Surface Tension

Surface tension (γ or σ) is a reflection of an unfavourable change to the free energy of a bulk solution as molecules move from the bulk and to the interface between surfaces [88]. As the molecules move a new surface is created. In order to create and expand that new surface area (A_S) a quantity of work (W) is needed.

$$\gamma = \frac{dW}{dA_S} \qquad \text{Equation 2.1}$$

This work is stored as potential energy, which thereby increases the overall Gibbs free energy (G) of the system. Thus, equation 2.1 can be written as equation 2.2 where temperature (T) and pressure (P) are kept constant [89]:

$$\gamma = \left(\frac{\delta G}{\delta A} \right)_{T,P} \quad \text{Equation 2.2}$$

Temperature, however plays a large role in the magnitude of surface tension seen in a system as the viscoelastic properties of membranes tend to change with an increase or decrease in temperature. Therefore a modified equation is needed to account for the effect of temperature. Since surface tension can be directly related to the excess amount of Gibbs free energy at the surface (G^S) the general thermodynamic relationships of G^S can be correlated back to γ [89]:

$$G^S = \gamma = H^S - TS^S \quad \text{Equation 2.3}$$

and

$$\left(\frac{\partial G^S}{\partial T} \right)_P = \left(\frac{\partial \gamma}{\partial T} \right)_P = -S^S \quad \text{Equation 2.4}$$

where H^S is surface enthalpy, T is temperature and S^S is surface entropy. When equations 2.3 and 2.4 are combined a general thermodynamic definition of surface tension is defined:

$$\gamma = H^S + T \left(\frac{\partial \gamma}{\partial T} \right)_P \quad \text{Equation 2.5}$$

In order to lower surface tension between fluids, surfactants can be added into the system. Surfactants are amphiphilic compounds with hydrophilic head groups and hydrophobic tails (Figure 2.1). Because of their amphiphilic nature, these molecules will spontaneously organize into monolayers at the interface between phases (i.e. oil/water, air/water, etc.) [88-90]. This creates a “buffer zone” between phases, which reduces the tension at the interface. While phospholipids are classified as a type of surfactant, their double tail distinguishes them from most other surfactants which have only a single tail. In this thesis the term surfactant will be used when discussing amphiphilic molecules with single tails.

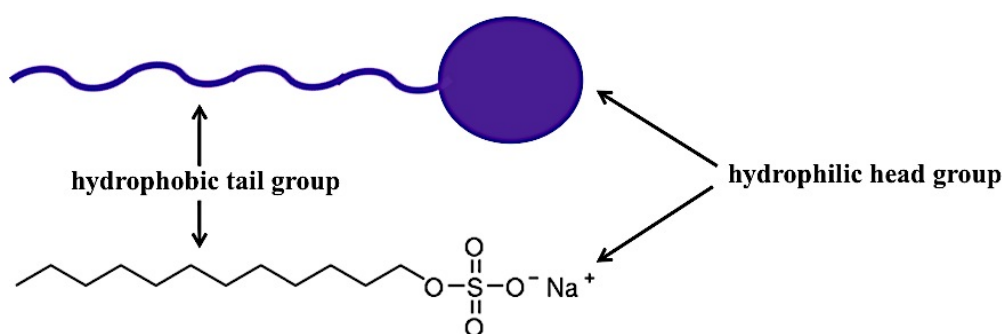


Figure 2.1 View of a typical surfactant structure. Top is a representative cartoon while the bottom is the structure of the common surfactant Sodium Dodecyl Sulphate

2.1.1 Surface Tension In Colloidal Suspensions

In principle, all dispersions of colloidal particles, including ones in which the “particles” are encapsulated gas as in the case of a bubble, are thermodynamically unstable due to the large interfacial area produced upon emulsification, and the corresponding increase in the interfacial Gibbs free energy. In all colloids therefore,

the trend is toward phase separation [91]. The change in Gibbs free energy (ΔG) associated with emulsification, in the absence of any surface-active agents, scales according to:

$$\Delta G \sim \gamma_{pB} \Delta A_{Bp} \quad \text{Equation 2.6}$$

where p designates the particle, B designates the bulk solution, and ΔA is the change area. Therefore, ΔG is positive and the emulsion is thermodynamically unstable. The presence of emulsifiers, such as molecular surfactants or solid particles, affects the change in the Gibbs free energy upon emulsification. Molecular surfactants lower the interfacial tension γ_{pB} of the particle/bulk interface and thereby reduce ΔG .

2.2 Laplace Pressure

Since systems try to find a state of minimum potential energy, a free droplet of liquid or gas naturally assumes a spherical shape, which has the minimum surface area for a given volume. Laplace pressure, P_γ , can most easily be described as the difference in pressure between in the inside and outside of a bubble due to surface tension. For a spherical droplet [92, 93] surface tension can be calculated as shown by the Young-Laplace equation:

$$P_\gamma = \frac{2\gamma}{R} \quad \text{Equation 2.7}$$

As explained by Basford [93], the tension in the walls of a rigid capsule depends on both the pressure of the inside contents and its radius. If the internal pressure increases, the wall tension also begins to increase. Thus, if the pressure contained in a capsule with a fixed radius is doubled, the tension in its walls also doubles. In a non-rigid capsule, like a bubble, the situation becomes more complex. In this case, the walls of a container distend as the pressure increases and change the pressure-volume-wall tension relationships (Figure 2.2 shows a similar example however here a bubble is stuck to a surface).

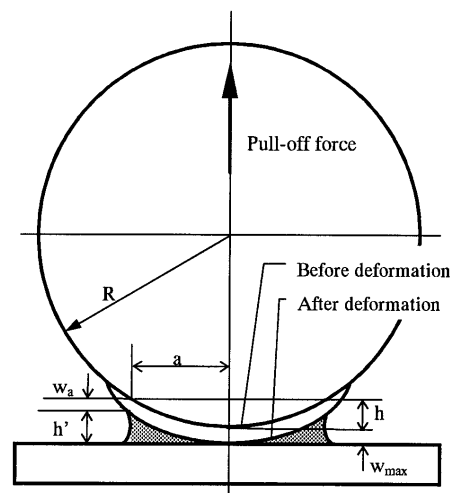


Figure 2.2 Contact geometry between a sphere and a plane surface before and after deformation due to the Laplace pressure. Reproduced with permission from [94]

To further complicate the situation; in a bubble there are several different pressures. There is an internal pressure within the bubble (P_{in}) due to the combination of the gas pressure (P_g) as well as a liquid vapour pressure (P_v) [92]. As a result of surface tension, the pressure within the bubble is greater than the pressure of the liquid

immediately outside (P_{out}) [95], and the internal bubble pressure is equal to the sum of the liquid pressure and the surface tension pressure, Figure 2.3 [92, 95]. It is because of the pressure and gas concentration gradients that bubbles require external stabilisation.

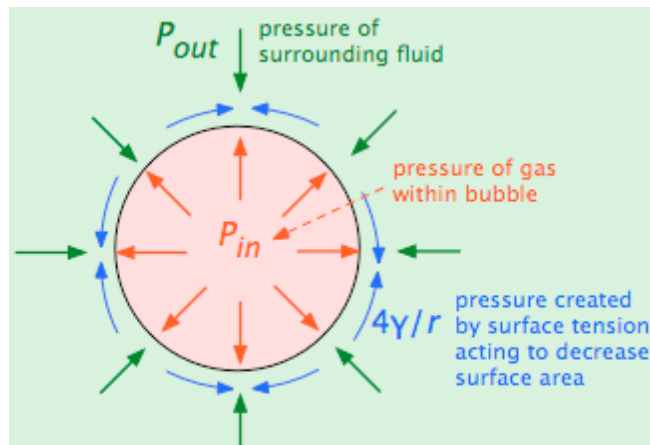


Figure 2.3 Schematic showing internal pressure (P_{in}), surface tension pressure ($4\gamma/r$) across the bubble membrane, and the liquid pressure (P_{out}) surrounding the bubble wall.

2.2.1 Gas Diffusion In Microbubbles

As previously discussed, surface tension forces on the bubble surface create a higher pressure inside the bubble. The equation of state for the gas predicts a higher gas concentration there, which consequently, drives the outward diffusion of gas in the liquid [96, 97]. Therefore, sustained presence of very small bubbles is only possible in presence of a shell which holds the gas in. This was modelled by Epstein and Plesset [53] in 1950 and their work is the basis of most modern bubble dissolution models.

As demonstrated by Katiyar and Sarkar [97]: encapsulation elasticity will slow bubble dissolution. A stable bubble can be obtained when the elasticity overcomes the surface tension (Figure 2.4). When encapsulation is inelastic, stability can be reached when the surface tension is zero. An elastic encapsulation can stabilise the bubble against surface tension based dissolution, but it can only reach a neutrally stable radius in an air-saturated medium. The effective surface tension at these conditions is zero and remains zero, therefore reaching a new equilibrium [96, 97].

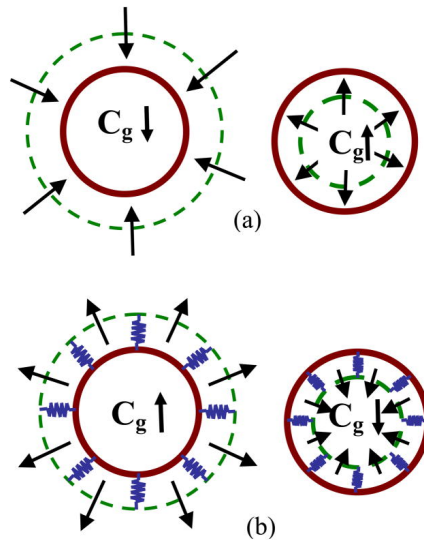


Figure 2.4 Mechanisms for unstable equilibrium for an inelastic encapsulation (a) and stable equilibrium for an elastic encapsulation (b). In (a) increasing or decreasing radius (green dashed line) from the equilibrium (brown solid line) leads to gas movement (radial arrows) to further drive it away from the equilibrium. Corresponding increase or decrease of inside gas concentration C_g causes the gas flux. Opposite happens in (b). Reproduced with permission from [97].

2.3 Pickering Stabilisation

The most common way to stabilise a bubble solution, which is in essence an emulsion, is by adding surfactants to immiscible liquids and providing mechanical energy to break up the mixture into micrometric droplets immersed in a continuous phase [98]. In the early 1900s, however, both Ramsden [79] and Pickering [78] showed that emulsion stability does not necessarily require amphiphilic surfactants reducing the interfacial tension but can also be efficiently promoted by dispersed particles in the colloidal size range. Particles can adsorb strongly to liquid-liquid interfaces, and the energy of attachment depends on the contact angle, θ , the particles make with the oil-water or in the case of bubbles, the gas-liquid interface, Figure 2.5 [99].

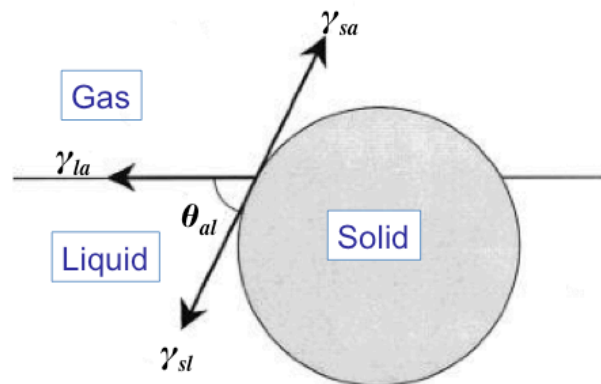


Figure 2.5 Schematic diagram of a spherical solid particle at an air-liquid interface showing the various interfacial energies (γ) and the contact angle (θ) measured into the water phase.

As shown by Binks and Rocher [100], if the stabilising particles are less than a few nm and the fluid–fluid interfacial tension is less than a few mN/m, particles of suitable wettability are adsorbed giving rise to drops and bubbles which are completely stable to coalescence and disproportionation [98, 101]. For solid–liquid–air systems, particle adsorption is thermodynamically favourable if the sum of the solid–air tension γ_{sa} and the solid–liquid tension γ_{sl} is less than the original liquid–air tension γ_{la} [100]. This depends on the contact angle θ of the particle with the interface measured through the liquid phase, as given by the Young equation:

$$\cos\theta = \frac{\gamma_{sa} - \gamma_{sl}}{\gamma_{la}} \quad \text{Equation 2.8}$$

Particle adsorption onto the interface occurs when it is partially wetted by the two fluid phases, i.e. when $0^\circ < \theta < 180^\circ$ as shown in Figure 2.5. The contact angle of the particle to the interface will also determine the type of emulsion that will result [102]. Hydrophilic particles ($\theta < 90^\circ$) protrude more into the aqueous phase and favour the formation of oil-in-water (o/w) emulsions, while hydrophobic particles ($\theta > 90^\circ$) stabilise water-in-oil (w/o) emulsions [103] (Figure 2.6). For the purposes of this work the oil phase corresponds to the filling gas, which result in the ability to model the bubble system as an o/w emulsion.

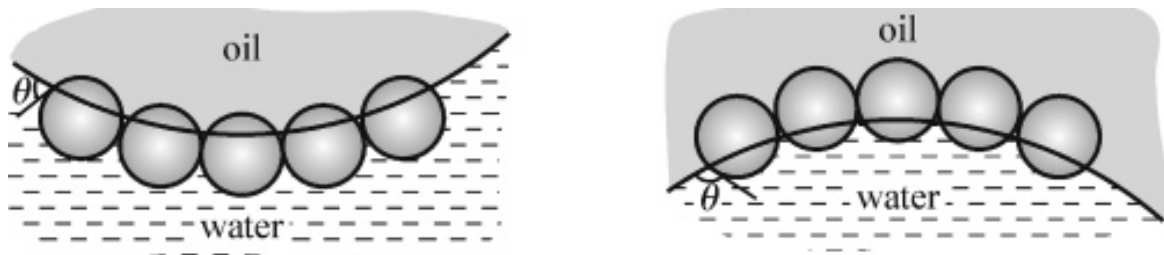


Figure 2.6 Contact angle and emulsion type: The experiment shows that hydrophilic particles ($\theta < 90^\circ$) stabilise oil-in-water emulsions, while hydrophobic particles ($\theta > 90^\circ$) stabilise water-in-oil emulsions. A possible reason for this effect could be the curvature energy of the particle adsorption layer. Reproduced with permission from [103].

The fundamental difference between Pickering and surfactant stabilisation is the comparatively large size of the stabilising agent. However this large size results in very strong binding energies of the particles with the interface [104]:

$$E_B = \pi R^2 \gamma_{ow} (1 - |\cos \theta|)^2 \quad \text{Equation 2.9}$$

Where E_B is the desorption energy of a particle from the liquid interface into the continuous phase of the emulsion, and γ_{ow} is the interfacial tension at the oil/water interface. The key to the stabilisation mechanism is that if the surface energy or contact angle of the particles with the aqueous phase is in the correct range, then the adsorption energy per particle can be very large [105]. Therefore once particles are adsorbed at the interface of a droplet they will not be readily removed either by shrinkage of the droplet or through coalescence between droplets [81, 104].

2.4 Ostwald Ripening

Ostwald ripening or size disproportionation is the process by which large droplets grow at the expense of smaller droplets and is one of the main reasons for emulsion instability. The driving force for Ostwald ripening is the difference in capillary pressure between small and large droplets or, in this particular case, bubbles, as defined by the Young-Laplace equation [106]. Ostwald ripening occurs due to suspended droplets minimizing their surface to volume ratio and thereby minimizing their surface free energy [75, 107]. Smaller droplets gravitate towards equilibrium by shedding some of their soluble surface molecules, which leads to a transport of material from small to larger droplets, with an accompanying increase in the mean droplet size with time, while also lowering the Laplace pressure in the bubble, Figure 2.7 [75, 107-110].

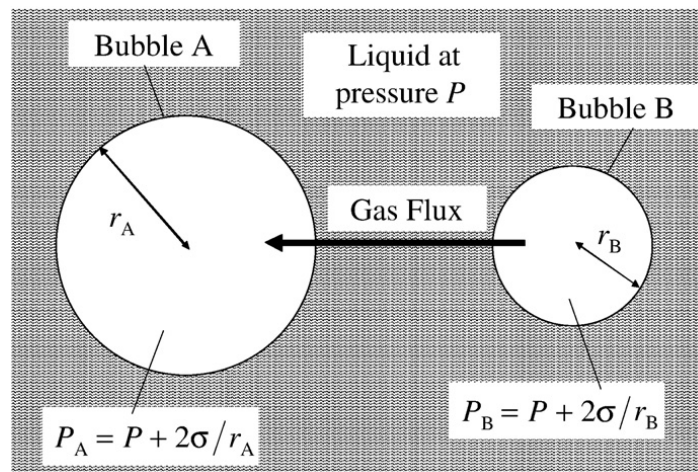


Figure 2.7 Schematic representation of the growth of circular bubble A at the expense of B caused by a pressure driving force. Here surface tension is represented by σ . Reproduced with permission from [109]

While the mean size of the droplets increases, the population density will decrease as will the interfacial energy, which decreases with increasing droplet size (Figure 2.8) [111]. Perfectly monodisperse systems will therefore not exhibit Ostwald ripening [112]. In a suspension of coated bubbles undergoing Ostwald ripening both the shell material and gas can undergo mass transfer. Ostwald ripening requires a finite solubility of the dispersed phase in the continuous phase, such that diffusion from the small to the larger droplets is possible [107].

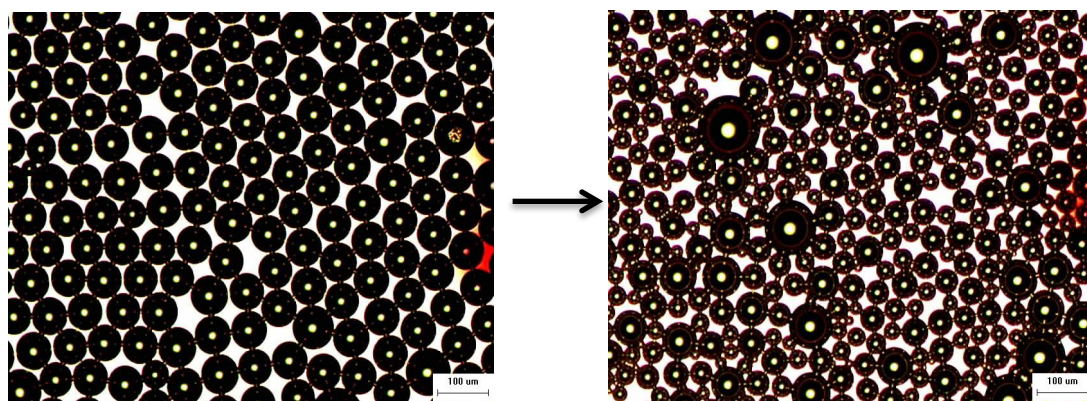


Figure 2.8 Example of Ostwald ripening within a phospholipid bubble population. The two images show the same bubble population at the same location on the slide. The picture on the left was taken at $t=2$ min after bubble production, while the bubble on the right was taken at $t=10$ min.

In theory at least this process continues until one large droplet is left, unless it is stopped or prevented. In order to slow the ripening process, a small amount of a second component with a very low solubility can be incorporated into the system, which results in a difference in composition between large and small droplets. The

concentration of this second component in the droplets increases when Ostwald ripening takes place, creating an osmotic pressure. This may counterbalance the driving force for Ostwald ripening and eventually result in a termination of ripening [107, 108, 111, 113]. For example, high salt concentrations in aqueous emulsion droplets are capable of doing this. Ostwald ripening increases the salt concentration in the small droplets, with respect to the large droplets, which correspondingly increases the osmotic pressure [114, 115]. Solid particles are also able to arrest Ostwald ripening, although through a different mechanism. The particles are irreversibly attached to the droplet due to the extremely high energy of particle detachment. The particle layer is compressed upon shrinking of the droplet. Continued shrinkage of the droplets deforms the menisci (inward bending of the fluid interface between the particles) and results in an opposed capillary pressure, which is capable of arresting Ostwald ripening.

2.4.1 Ostwald ripening in suspensions of large bubbles

If a bubble suspended in an aqueous solution is larger than 50 μm in diameter then it can be assumed to behave as a droplet and Laplace pressure of the bubble is neglected. Laplace pressure is inversely proportional to bubble diameter [106]. So the smaller a bubble the higher the Laplace pressure. The radius dependence of the solubility is obtained from the Kelvin equation [106]:

$$c(r) = c_{\infty} \exp \left(\frac{2\gamma V_m}{RT} \right) \quad \text{Equation 2.10}$$

where R is the droplet radius, γ is the surface tension, V_m is the molar volume of the dispersed compound, c_∞ is its bulk concentration (i.e., the molecular concentration that is in thermal equilibrium with a macroscopic bulk phase), and B is the gas constant. As shown below, the radius dependence of the solubility is related to the radius dependence of the molar chemical potential, U [108, 116]:

$$U(r) = \mu_0 + \frac{2\gamma V_m}{r} \quad \text{Equation 2.11}$$

Assuming that the concentration is in equilibrium with a particle of radius r :

$$U(r) - U_0 = BT \ln \left\{ \frac{c(r)}{c_\infty} \right\} \quad \text{Equation 2.12}$$

Assuming ideal mixing, mixing in a second component results, if the two components are miscible, in a decrease of the chemical potential due to the entropy of mixing [116, 117]. Denoting the major component and the second component as “1” and “2”, respectively, we have for the major component:

$$U_1(r) = U_0 + \frac{2\gamma V_m}{R} + BT \ln(1 - x_2) \quad \text{Equation 2.13}$$

Here x_2 is the mole fraction of the second component in the particle. If the second component is insoluble in the continuous solvent, a ripening process leads to a decrease of x_2 in the growing particles, whereas x_2 increases in particles that shrink. If the distribution of the second component is initially uniform, i.e., the same x_2 value in all of the particles, this may eventually lead to a termination of the ripening

process where a small radius and high x_2 balances a large radius and small x_2 so that the chemical potential and hence the solubility is the same for the different radii.

2.4.2 Ostwald Ripening in suspensions of small bubbles

Small bubbles have different Ostwald ripening conditions due to the fact that the Laplace pressure dramatically increases as the bubble size decreases. Suspensions of small ($< 10 \mu\text{m}$ diameter) bubbles are inherently thermodynamically unstable because their free energy of formation is positive due to their large interfacial area [118]. As shown by Stevenson [109] for a small bubble the pressure difference between any spherical bubble of radius R and a notional bubble size, δ , that is representative of the coarsening behaviour is:

$$\Delta P = 2\gamma \left(\frac{1}{\delta} - \frac{1}{R} \right) \quad \text{Equation 2.14}$$

The Laplace pressure will increase dramatically as bubble radius dips below $10 \mu\text{m}$. At that time the Laplace pressure increases to several hundred kPa depending on the surface tension of the bubble surface [119]. The Laplace pressure of a bubble a few micrometres in diameter for example is approximately 100 kPa. Due to the high-pressure differential, the interfacial tension is unsustainable and thus drives Ostwald ripening [119].

3 Microbubble manufacturing techniques: theory and application

There are currently several different manufacturing methods used to produce phospholipid-coated microbubbles. This chapter discusses several of those methods from theory to application.

3.1 Liposome formation

All production methods used to produce phospholipid microbubbles require a precursor suspension of liposomes. Liposomes are self-assembling particles stabilised by lipid bilayers which are typically spherical and have an aqueous core [33][120, 121]. Most commonly, liposomes are made by dissolving the phospholipid(s) in a solvent, normally chloroform, and then evaporating in order to produce a lipid film [121]. In order to hydrate the film and disperse the lipid either water or an aqueous solution is then added, and the system is subjected to either homogenization or sonication in order to fully disperse the lipid (Figure 3.1) [15].

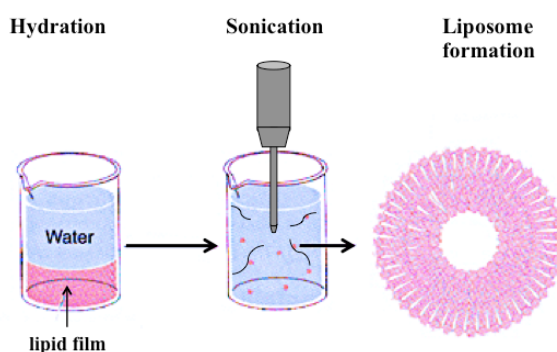


Figure 3.1 Schematic representation of liposome production

In contrast to lipid monolayer structures, liposomes are characterised by extended, two-dimensional, and clearly separated hydrophilic and hydrophobic regions, (Figure 3.2) [120, 122]. The hydrophilic portions of bilayer lipids are directed towards the aqueous phases (external and internal), whereas the hydrophobic portions of both lipid layers are directed towards one another, forming the internal region of the membrane.

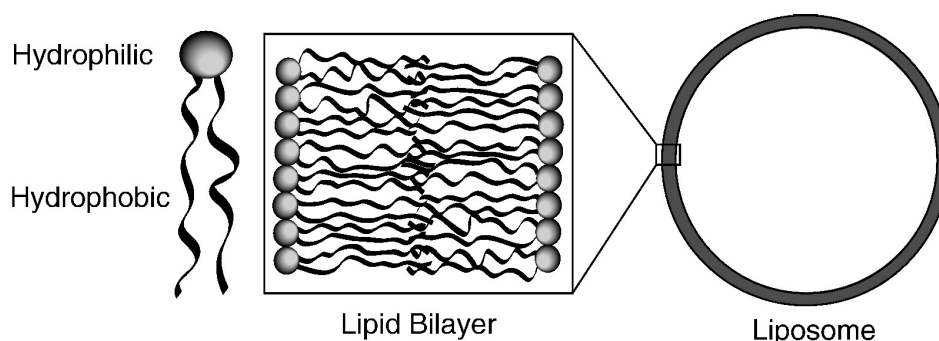


Figure 3.2 Schematic diagram of lipids incorporated into a bilayer membrane to form a liposome. Reproduced with permission from [122].

No matter which fabrication methods are used to create the bubbles, microbubble shells are created by the diffusion of liposomes to the gas/water interface. In order to create a lipid shell, liposomes must adsorb onto the gas/water interface, where they spontaneously re-orient, and laterally organize the lipids into a monolayer film (Figure 3.3). This self-assembled monolayer gives the bubble its inherent stability.

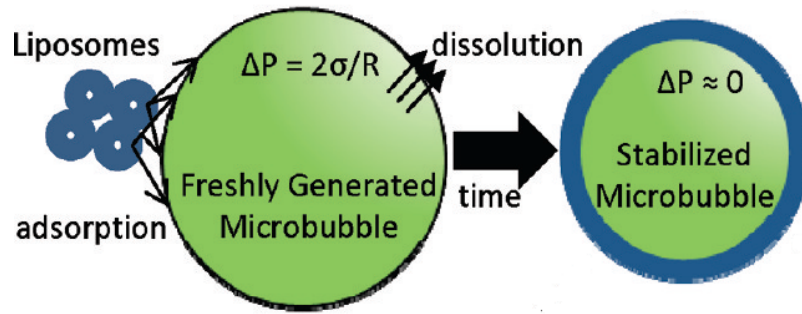


Figure 3.3 Synthesis and stability of microbubbles. Mechanical agitation of liposomes in an aqueous environment results in lipid-coated microbubbles. If the microbubble shell is cooled slowly, domains of lipid components form. Adapted with permission from [15].

3.2 Mechanical Agitation

The most common way to produce large quantities of microbubbles is via mechanical agitation, either high-speed homogenization or acoustic sonication. Both methods are described in more detail in the next two sections.

3.2.1 Homogenization

Homogenization is one of the earliest forms of microbubble production. It has been used for decades in order to produce microbubble contrast agents [123]. It works by emulsifying an aqueous suspension by high shear stirring (rpm > 1000) [124] using another immiscible fluid, such as a gas, and a surfactant to create a sterically stabilised emulsion. More recently homogenization has been used particularly for preparing polymer-coated microbubbles [125]. For phospholipid bubbles, however homogenization has fallen out of use in favour of sonication.

3.2.2 Sonication

The most commonly used method for microbubble preparation is sonication, which involves dispersing gas or liquid in a suspension of a suitable coating material using high intensity ultrasound [125-127]. Typically this is done using a tip sonicator, which can be immersed directly into the solution, similar to the liposome production process previously described. As described by QSonica [128], the makers of the Misonix tip sonicator used throughout this thesis, a conventional tip sonicator works by transforming AC line power to a 20KHz signal that drives a piezoelectric convertor/transducer. This electrical signal is converted by the transducer to a mechanical vibration due to the characteristics of the internal piezoelectric crystals. The vibration is amplified and transmitted down the length of the probe where the tip longitudinally expands and contracts. The distance the tip travels is dependent on the amplitude. In liquid, the rapid vibration of the tip causes cavitation, the formation and violent collapse of microscopic bubbles. The cavitation of thousands of bubbles, releases tremendous energy, both thermal and mechanical in the cavitation field [129].

According to Fogler and Li [130], emulsification occurs in two stages. Initial bubble entrainment occurs as the unstable growth and eventual eruption of interfacial capillary waves produced by sonication. The second stage of acoustic emulsification involves the continual cavitation-induced breakdown of larger particles as a function of sonication time until a stable size is reached [131]. The breakdown mechanism depends on the type of deformation and flow pattern around the bubble. The stable

size results when surface tension forces balance the inertial forces on the bubble [130].

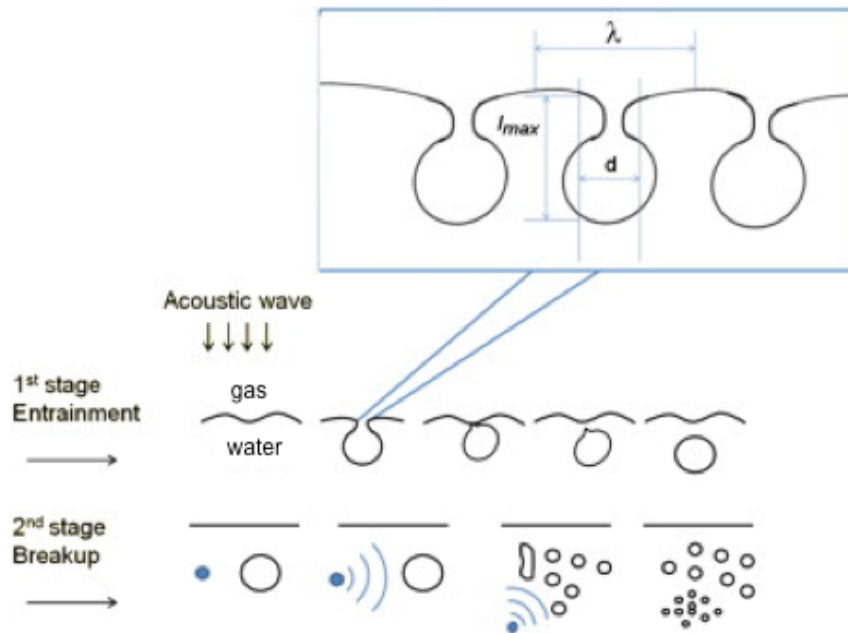
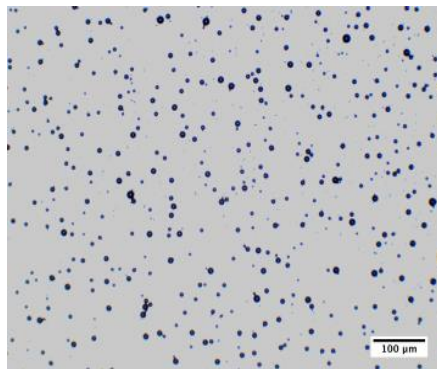


Figure 3.4 Schematic showing origins of polydispersity during acoustic emulsification. Initial bubble entrainment occurs as a capillary instability. Inset shows relevant length scales. Subsequent cavitation in the suspension (shown as filled circle with propagating waves) induces breakup of the larger bubbles to a critical diameter, where surface forces and inertial forces balance. Reproduced with permission from [131].

When the gas and bubble solution is emulsified it forms a suspension of microbubbles [125]. As described in section 3.1, the pre-existing liposomes will then self-assemble onto the microbubble surface and automatically adsorb. Also, because of the high temperatures and pressures generated through cavitation, a chemical modification of the surface layer may occur which improves its stability [125]. As originally discussed by Feinstein [132] the size distribution of the bubbles produced depends upon the frequency, power and pulse regime of the ultrasound,

and there is no simple theoretical relationship between these variables [46]; the size distribution of microbubbles obtained from sonication are inevitably broad, (Figure 3.5) [125, 133, 134]. In order for the bubbles produced to be viable for transport throughout the vasculature they must be further processed to remove large bubbles from the suspension. Without further size separation adverse reactions such as embolism may occur [135].

A.



B.

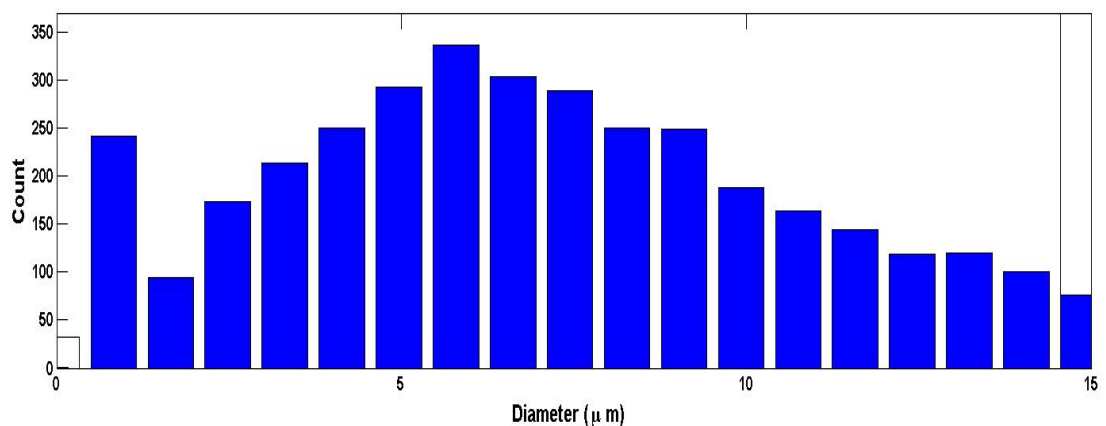


Figure 3.5 A. Sample of sonicated microbubbles, and B. The corresponding size distribution (determined via image processing).

3.2.3 Size separating bubbles

As stated previously, in order to travel through the vasculature without causing any adverse reactions, microbubbles must have a diameter less than 10 μm . Microbubble acoustic behaviour also depends very strongly on size and increasing the microbubble diameter from 1 to 5 μm will change the resonance frequency of an unencapsulated microbubble from 4.7 to 0.72 MHz [28]. Size also affects the biodistribution and pharmacodynamics after intravenous injection, the bioeffects during ultrasound insonification, the gas release profile, and other related behaviours [131]. Since mechanical agitation creates bubbles with large size distributions, size separation is required to produce suspensions that are viable for biomedical purposes. While the bubbles can be filtered through a porous film bubble loss can occur. As a result the most common way of size separating bubbles exploit their natural buoyancy.

3.2.3.1 Flotation separation

Larger microbubbles are more buoyant and therefore rise faster under normal gravitational conditions. This principle allows for separation of microbubbles based on their different migration rates. Kvale et al. described a model for the size fractionation of Infosom, air-filled albumin microbubbles by simple flotation [136]. In their experiments microbubbles were injected at the bottom of a stagnant water column and allowed to rise under normal gravity. Samples were taken of the bubbles at different heights within the column and the experimental data (Table 3.1) were

then compared with a model developed by the group to predict the size distribution of microbubbles at certain distances from the bottom of the column as a function of time. The model used was a second-order PDE that accounted for both the convective motion of the bulk dispersed phase (liquid moved down the column as microbubbles moved up) as well as the Brownian (thermal) diffusive motion of the particles. The crowding effect of the microspheres was accounted for by using a modified version of Einstein's derivation for the effective viscosity in a dilute suspension [137]. The predicted size distributions of the model were shown to match the experimental data collected, thus making it a viable method of controlling the size fractionation process.

Table 3.1 Repeatability of reported Alunex parameters. Pooled standard deviation (SD) and relative standard deviation (RSD) values from triplicate analysis on 30 samples. Reproduced with permission from [136].

Parameter	Mean	SD	RSD (%)
Number concentration, 4.0 to 10.0 μ (10^8 /ml)	2.13	0.03	1.3
Number concentration, ≥ 10.0 μ m (10^8 /ml)	0.29	0.004	1.3
Number concentration, ≥ 25.0 μ m (10^6 /ml)	0.41	0.11	26
Number concentration, 1.0 to 30.0 μ m (10^8 /ml)	7.48	0.09	1.3
Volume concentration, 1.0 to 30.0 μ m (10^8 /ml)	7.5	0.25	3.4
Number mean diameter, 1.0 to 30.0 μ m (10^8 /ml)	3.77	0.02	0.6

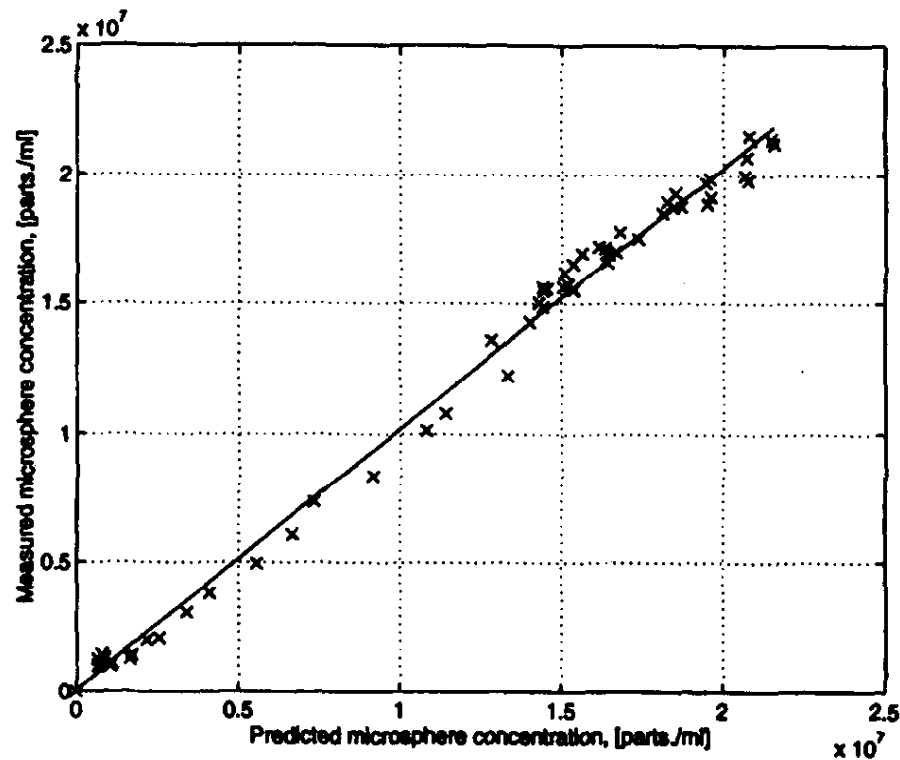


Figure 3.6 Predicted microsphere size distribution versus measured microsphere size distribution in the flotation column. The x-axis represents predicted microsphere concentration (parts/ml) and the y-axis denotes measured microsphere concentration (parts/ml). Reproduced with permission from [136].

3.2.3.2 Separation through centrifugation

The most commonly reported method of size separation is differential centrifugation. Wheatley et al. reported the isolation of submicrometre bubbles using differential centrifugation [138]. Surfactant coated bubbles were centrifuged at 300 RPM for 1, 2 or 3 min. or 500 RPM for 0.5, 1, 2, or 3 min. As a control, samples were also separated by gravity for 5, 10 or 15 min. The use of centrifugation allowed for faster size isolation than buoyancy, however it also led to destabilisation of the surfactant-stabilised microbubbles during subsequent in-vivo

insonification. Microbubbles centrifuged at 500 RCF for 1 min were not stable, whereas those spun at 300 RCF for the same time were relatively stable. Destabilisation was attributed to the extra hydrostatic pressure exerted on the microbubbles, which increased toward the bottom of the column in proportion to the centrifugation speed. Flotation at normal gravity was more time consuming, but less detrimental to microbubble stability.

In contrast to surfactant-coated microbubbles, lipid-coated microbubbles have been shown to be stable after centrifugation up to several hundred RCF (relative centrifugal force) due to their much more rigid outer shells [131, 139, 140]. Feshitan et al. more recently developed a protocol for separating bubble populations into very narrow size bands [131]. Initial centrifugation was performed on a newly sonicated bubble population for 10 min, at 300 RCF in order to collect all microbubbles into a cake. The remaining lipid suspension was siphoned off and recycled to produce the next batch of microbubbles. The cakes were then combined and suspended in PBS to improve total yield.

Large microbubbles, those with diameters greater than 10 μm , were removed by performing one centrifugation cycle at 30 RCF for 1 min and discarding the cake. The remaining solution was saved and dispersed in PBS as it contained the microbubbles whose diameters were less than 10 μm . Next, microbubbles of greater than 6 μm diameter were removed by performing a centrifugation cycle at 70 RCF for 1 min. Again the remaining solution was saved and re-dispersed into PBS while the cake was discarded. Finally, microbubbles of less than 4 μm diameter were

separated by centrifuging at 160 RCF for 1 min, discarding the remaining solution and dispersing the cake of small bubbles in PBS. This step was repeated 5-10 times to ensure total bubble separation. Once these initial steps were completed Feshitan et al. isolated two distinct size populations: the 1-2 and 4-5 μm diameter ranges. Microbubbles in the larger diameter range (4-5 μm) were isolated first, while the smaller microbubbles were saved for the subsequent isolation of the 1-2 μm fraction. After repeated centrifugation and re-concentration, both microbubble size populations were successfully isolated from the initial polydisperse suspension (Figure 3.7). The final microbubble suspensions typically had a total volume of 1 ml with concentration on the order of 10^8 to 10^9 ml^{-1} for the 4-5 μm population and 10^9 to 10^{10} ml^{-1} concentration for the 1-2 μm . While this process was shown to be very successful in isolating very specific bubbles populations with a high concentration, a large volume of lipid solution (~30 ml per syringe, minimum two syringes) is needed in order to proceed and a very small yield (~1 ml) is produced.

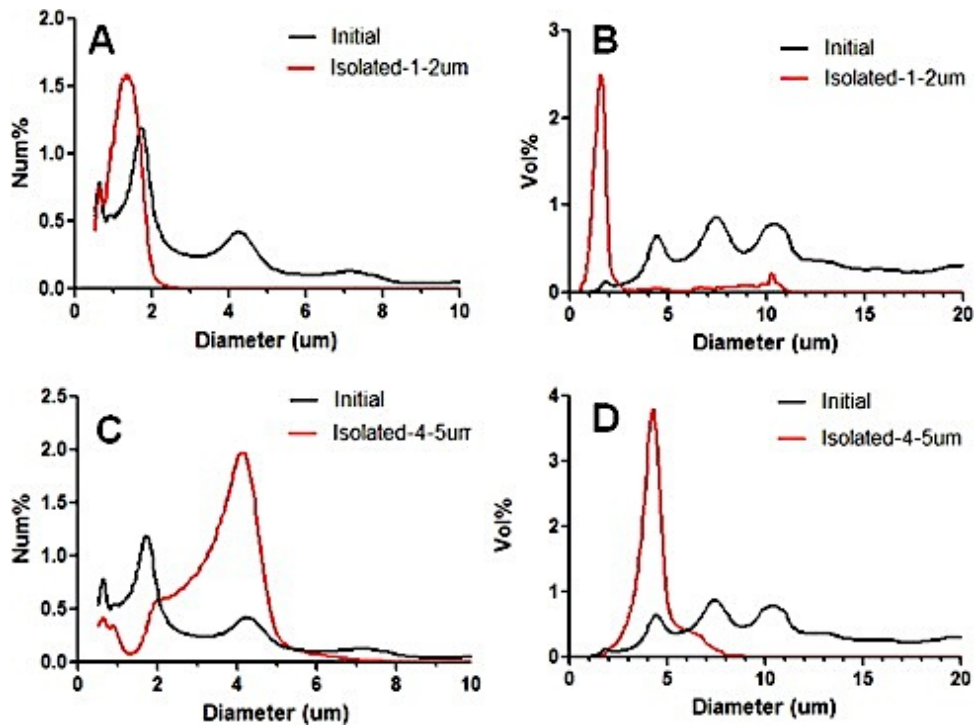


Figure 3.7 Size distributions for initial polydisperse and final size-isolated microbubbles. Shown are isolated subpopulations at the 1–2 μm (A, B) and 4–5 μm (C, D) diameter size ranges. Comparison of number-weighted (A, C) and volume-weighted (B, D) size distributions allows inspection of polydispersity. Reproduced with permission from [131]

3.3 Microfluidics

In order to overcome the issues associated with size separating bubbles, microfluidic devices have been designed to produce bubble populations with low polydispersity. Microfluidic devices facilitate the formation of monodisperse microbubbles as they can be fabricated with internal dimensions on the micrometre scale [141, 142]. There are several designs reported in the recent literature for microfluidic devices to prepare monodisperse microbubbles using gas and liquid

streams and microdroplets with two immiscible liquid streams. These include cross-flow rupturing in a T-junction device [11, 143-145], hydrodynamic flow focusing devices through a small orifice [133, 134, 146-148], and the co-flowing rupturing method [149] among others. Each of these methods utilizes a specific flow field to promote the uniform generation of microbubbles/droplets as illustrated in Figure 3.8.

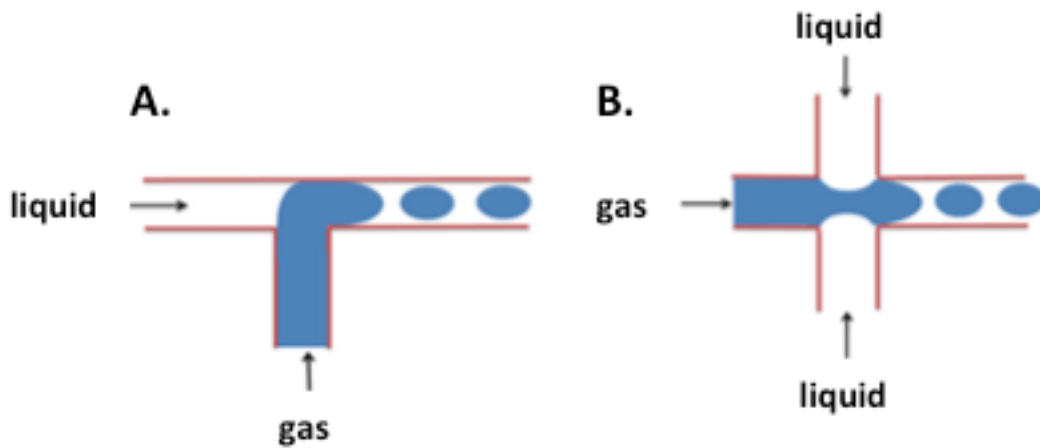


Figure 3.8 Illustrations of the two main microfluidic geometries used for bubble formation. A. cross-flowing streams in a T-shaped junction B. flow focusing geometry. Adapted with permission from [150].

Flow within a microfluidic system is usually characterised by low Reynolds numbers (Re):

$$Re = \frac{\rho u \ell}{\mu} \quad \text{Equation 3.1}$$

where ρ is the fluid density, u is the fluid velocity, ℓ is the characteristic dimension of the system and μ is the dynamic viscosity of the fluid respectively [145]. When

$Re \ll 1$ the fluid flow is dominated by viscous stresses and pressure gradients while inertial effects are negligible. The trajectories of fluidic particles, therefore can be controlled precisely. Continuous-phase viscosity and relative flow rates of the two phases however, have different effects on bubble size. As a result the scaling law describing bubble formation using the cross-flow rupture technique is different from that for hydrodynamic flow focusing [143]. These scaling laws also depend on the capillary number (Ca):

$$Ca = \mu u / \gamma \quad \text{Equation 3.2}$$

where μ is the viscosity, and u is the mean speed of the carrier fluid, and γ is the interfacial tension; as large capillary numbers will have very different flow dynamics than smaller ones [145]. This thesis will focus on two microfluidic manufacturing methods: cross-flow rupturing and flow-focusing through a small orifice.

3.3.1 Cross Flow Rupturing

In the cross flow rupture technique (Figure 3.9), bubble size decreases with increasing continuous-phase rate and continuous-phase viscosity, and is independent of surface tension depending on the liquid used and the length scale [150].

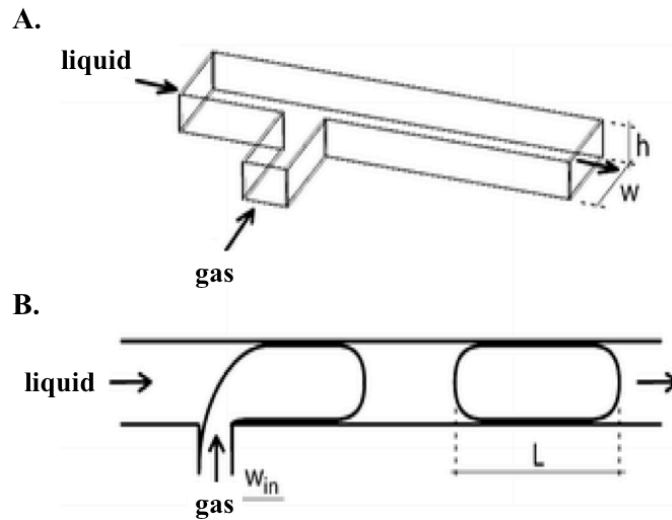


Figure 3.9 (a) A schematic illustration of a microfluidic T-junction composed of rectangular channels. The channels in this schematic are planar and have uniform height h . (b) A top view of the same schematic in a two-dimensional representation. Flow along the main channel proceeds from left to right. The continuous fluid flows along the main channel of width w , and the fluid that will be dispersed flows through the perpendicular inlet of width w_{in} . Adapted with permission from [145].

As described by Garstecki et al. [145] at low values of the capillary number, when the interfacial forces dominate the shear stresses, the dynamics of flow break-up is dominated by the pressure drop across the droplet or bubble as it forms. The size of the droplets or bubbles is determined solely by the ratio of the volumetric rates of flow of the two immiscible fluids. Therefore the size of the discrete fluid segments can be determined using the scaling function:

$$\frac{L}{w} = 1 + \alpha \frac{Q_{in}}{Q_{out}} \quad \text{Equation 3.3}$$

where L is the length of the immiscible slug, w is the width of the channel, $Q_{\text{in/out}}$ are the rates of flow of the dispersed and carrier fluids respectively, and α is a constant depending on the geometry of the T-junction. The scaling relation is independent of the material parameters of the fluids, their viscosities and the interfacial tension between them. Because the capillary numbers are typically small ($Ca < 10^{-2}$), the shear stresses on the interface of the forming bubble are not high enough to distort it significantly. As a result, the bubble blocks almost the whole cross-section of the main channel and confines the flow of the liquid. Upstream of the bubble there is an increase of pressure which leads to 'squeezing' the neck of the immiscible thread. This continues at a rate proportional to the liquid flow rate and determines the amount of time the forming bubble has to grow. The growth of the bubble occurs at a rate proportional to the gas flow rate. In this theses, two geometries of cross-flow devices are used, the common T-junction (described above) and a Y-junction.

3.3.1.1 Y-junction

Like droplet formation at a T-junction, the droplet size produced by a Y-junction decreases with increasing continuous-phase viscosity, and increasing continuous-phase flow rate. Droplet formation in Y-junctions however, can be described more simply than other (shear-driven) microfluidic geometries, as the droplet size is independent of the disperse-phase flow rate [151-154]. Droplets generated at T-junctions are generally larger than at Y-junctions made at corresponding flow rates and have a larger polydispersity (Figure 3.10) [151]. Both the continuous and

disperse phase flow rates determine droplet size produced by T-junctions. In Y-junctions however, droplet size is independent of the disperse-phase flow rate and is described by an one-step model dependent on the capillary number [154].

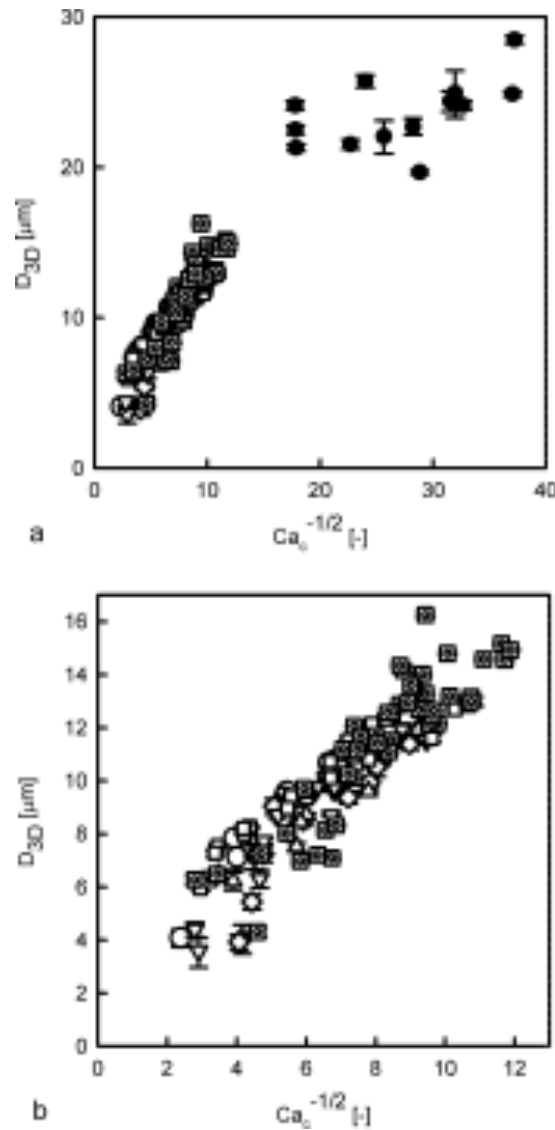


Figure 3.10 Droplet size D_{3D} as function of the inverse square root of the capillary number of the continuous phase Ca_c^{-acs} for Y-junctions a.1 ($\square$), a.2 ($\square$), b ($\circ$), c ($\diamond$), d (∇), and e ($\triangle$), and T-junction f ($\bullet$) at various flow rates and for various ethanol-water phases. Reproduced with permission from [151].

In the dripping and jetting regime, droplet size is determined by the force balance between interfacial tension and the shear force at the position where the neck and the droplet are connected [153]. As a result droplet size is proportional to the diameter of the neck, and can be controlled more precisely than in a T-junction.

3.3.2 Flow focusing

Hydrodynamic flow focusing devices are typically made via soft lithography with very fine flow channels of micrometre (> 20) cross-sectional dimensions. Gas is focused into a micro-jet, which is coated on either side by a premixed bubble suspension and pushed through a very small orifice ($> 10\ \mu\text{m}$) (Figure 3.11) [125, 143, 147].

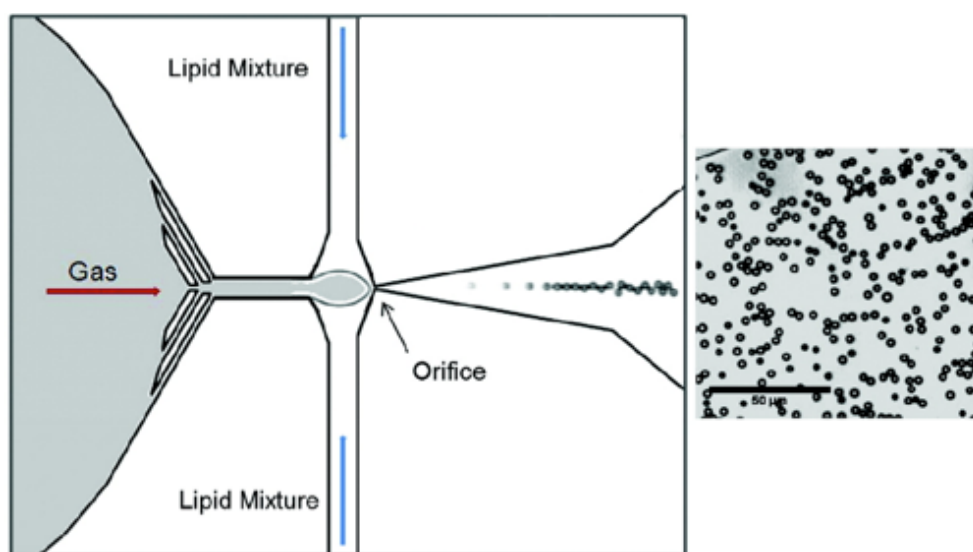


Figure 3.11 Schematic of the microfluidic device. A lipid dispersion and gas stream were focused on the orifice ($6\ \mu\text{m}$) to create lipid-coated/gas-filled microbubbles. To the right is an image of microbubbles created using a device based on this schematic. Adapted with permission from [146].

Due to the high surface energy of this configuration, the micro-jet is not dynamically stable and it breaks to release a bubble into the outlet channel [155]. As described by Garstecki et al. [156] the breakup process of a bubble produced via flow focusing can be described in three stages (Figure 3.12). First the gas enters the orifice, penetrates into the outlet channel, and starts to inflate a bubble. The width of the jet remains constant, and only the portion upstream of the orifice thins (Figure 3.12 A and B). Secondly, a clearly visible neck develops and the thread collapses at a constant rate (Figure 3.12 C-E). In the third stage, the jet collapses rapidly, breaks and retracts upstream of the orifice to begin the process again (Figure 3.12 E and F).

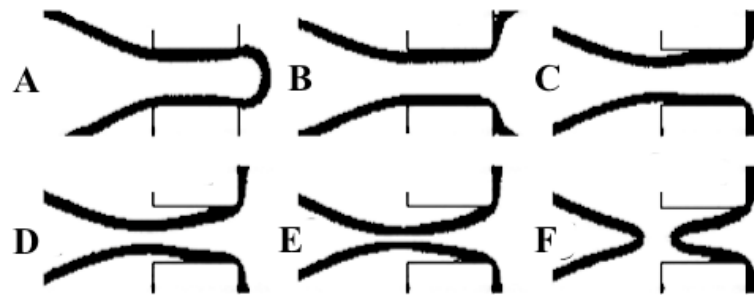


Figure 3.12 Schematic of the gas-liquid interface along the breakup trajectory. Adapted with permission from [156].

3.4 Other methods

While the most common bubble manufacturing methods have been detailed above, there are other methods such as co-axial electrohydrodynamic atomization, ink-jet printing and membrane emulsification which are being used to successfully form microbubble suspensions.

3.4.1 Co-axial Electrohydrodynamic atomization

Co-axial Electrohydrodynamic atomization (CEHDA) or as it is more commonly known electro-spraying, occurs when a liquid or suspension is passed through a pair of co-axial capillary needles at a controlled flow rate under the influence of an electric field. This causes the suspension to undergo various modes of atomization. Because a pair of co-axial needles is used two different liquids or suspensions can be pumped through pumped through two concentrically placed needles (Figure 3.13) [157, 158]. The eventual jet break-up can result in near-monodisperse compound aerosols with the outer material encapsulating the inner one.

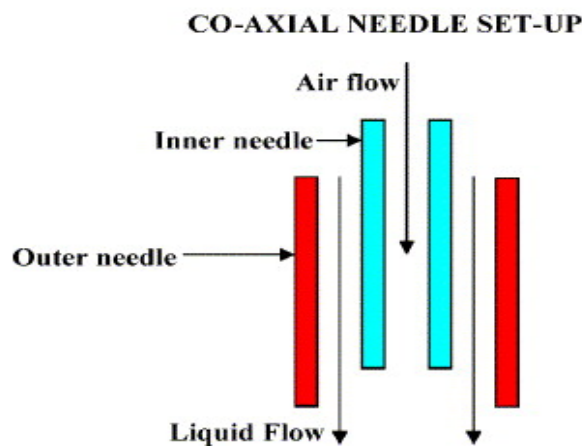


Figure 3.13 Needle arrangement used for co-axial electrohydrodynamic atomization. Adapted with permission from [157].

First shown by *Farook et al.* [157], co-axial electro-spraying can be used to prepare microbubble suspensions with a near monodisperse distribution and a diameter under 10 μm . As proof of concept, glycerol was injected through the outer needle while air flowed through the inner needle at the same time [157, 158].

CEHDA bubbling shows three characteristic spray modes: bubble dripping, coning and continuous bubbling; the latter mode being the most suitable for the preparation of microbubbles for biomedical applications. The process used originally to prepare glycerol bubbles was then adapted to prepare phospholipid-coated microbubbles, however a relatively broad size distribution was seen when the bubble size became optimal for biomedical applications ($>10\text{ }\mu\text{m}$) [159]. The process was again modified and microbubbles were able to be produced with both a narrow size distribution and a high yield ($10^9\text{ bubbles min}^{-1}$) [160]. Suspensions of bubbles with a mean diameter of $6.6\text{ }\mu\text{m}$ and a standard deviation of $2.5\text{ }\mu\text{m}$ (Figure 3.14) were produced and found to be very stable at ambient temperature.

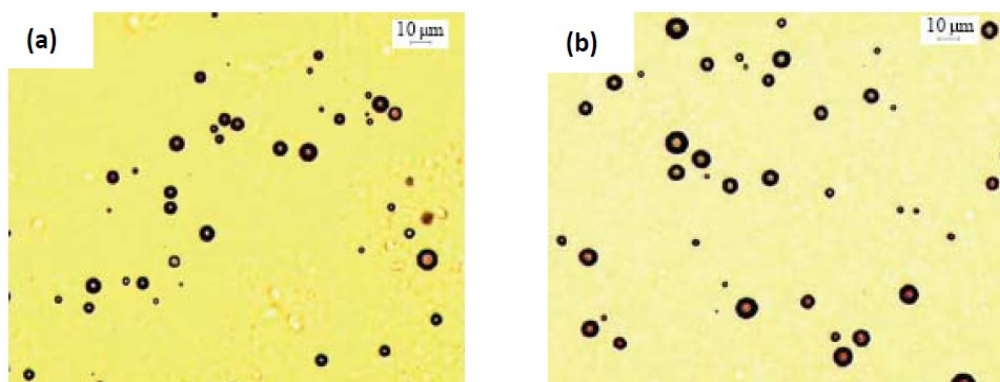


Figure 3.14 Optical micrographs of lipid-coated microbubbles collected at ambient temperature at a flow rate combination of (a) $5\text{ }\mu\text{l s}^{-1} : 5\text{ }\mu\text{l s}^{-1}$ and (B) $10\text{ }\mu\text{l s}^{-1} : 5\text{ }\mu\text{l s}^{-1}$. Adapted with permission from [160].

However at human body temperature (37°C), the mean bubble diameter initially decreased rapidly before stabilising at $1\text{--}2\text{ }\mu\text{m}$ after 20 min [160]. This is an example

of gas diffusing through the gaps between the phospholipid heads until a maximum packing density is reached. More can be read about this phenomenon in Chapter 6.1.

3.4.2 Membrane Emulsification

An alternative to sonication and shear stirring is to produce the emulsion by forcing the components, sometimes repeatedly, through a porous membrane [125, 161, 162]. The most common forms of membrane used for this purpose are Shirasu porous glass (SPG) membranes. In SPG emulsification a dispersed phase (the gas) is permeated into a continuous phase (the bubble solution) through uniform pores [161]. Microbubbles are formed from the inner surface of a porous tubular membrane (Figure 3.15) [161, 163].

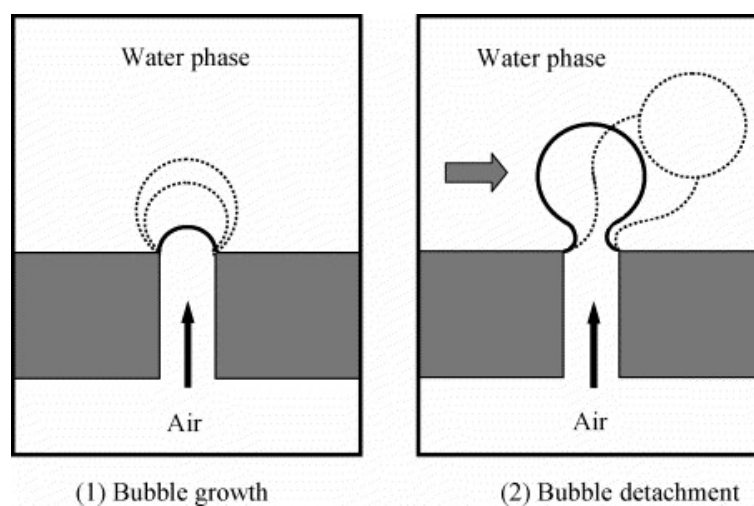


Figure 3.15 Schematic representation of bubble formation process from a pore. Adapted with permission from [162].

As described by Kukizaki and Goto [161], droplet formation using SPG membranes typically involves one of the following two detachment mechanisms, shear-induced droplet formation or spontaneous transformation-based droplet formation by interfacial tension. The drag force caused by the flow of the bubble suspension phase acts on the droplet as a detaching force in shear induced droplet formation. In contrast, spontaneous transformation-based droplet formation occurs where the droplets are formed without a continuous phase flow and the spontaneous detachment of droplets from the membrane surface is strongly dependent on the shape of the pores. As circular pore openings make spontaneous detachment impossible, SPG membranes typically have tortuous pores with irregular cross-sections, which allow the bubbles to be detached from the membrane surface spontaneously. The tortuous pores distort the shape of the gas so that the neck of the bubble at the pore openings is deformed, giving the bubbles a larger interfacial area than spherical bubbles of equal volume. In order to minimize their surface free energy the deformed bubbles spontaneously change shape and detach from the membrane as spherical bubbles.

3.5 Relevance to thesis present work

Within this thesis three different manufacturing techniques will be compared: sonication, microfluidic flow focusing and cross-flow rupturing. Sonication is the most common technique used for producing bubbles and was therefore used as a control when the physical properties of the bubbles were compared. Cross-flow rupturing and microfluidic flow focusing allowed for a tighter size distribution

however the quantity of bubbles produced was much lower. By comparing several techniques a fuller picture of the physical variations between bubbles is achieved.

4 Experimental methods

This chapter describes the materials, apparatus, experimental procedures, post-processing and analytical techniques, used or developed in this study including all relevant hardware and software.

4.1 Bubble manufacturing techniques

Four different bubble manufacturing techniques were used in this study; sonication, T-junction, reverse Y-junction and microfluidic flow focusing.

4.1.1 Sonication

A volume of 80 ml was sonicated at the air/bubble mixture surface using a 3 mm diameter probe, which was held in place by a clamp (Figure 4.1).

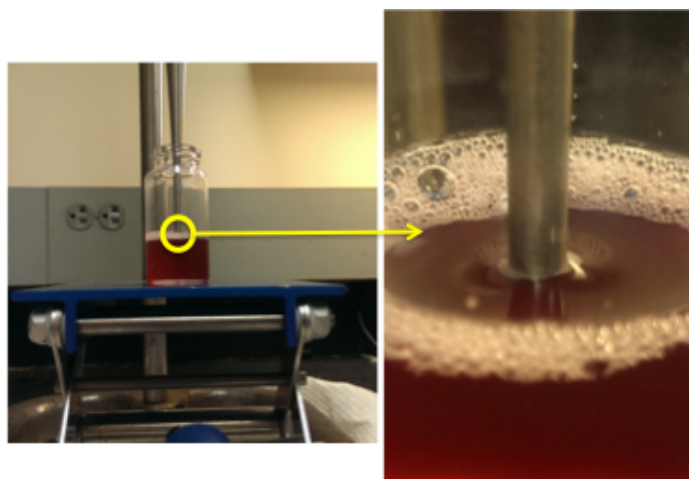


Figure 4.1 Experimental set-up for bubble production through sonication. Note the probe tip only just touches the solution surface.

The probe tip is immersed into the bubble solution at the air/liquid interface just until the solution adheres to the tip. This allows for a greater volume of gas to enter the bubbles solution during sonication, producing a larger number of bubbles. Sonicated bubbles were produced using an ultrasonic cell distributor (Misonix XL200, Labcaire, Dorset, UK) at an output power of 9 W (RMS) and power setting of 4 for 30 seconds.

4.1.2 T-Junction

T-Junction microbubbles were produced using a specially designed T-junction as shown in Figure 4.2.

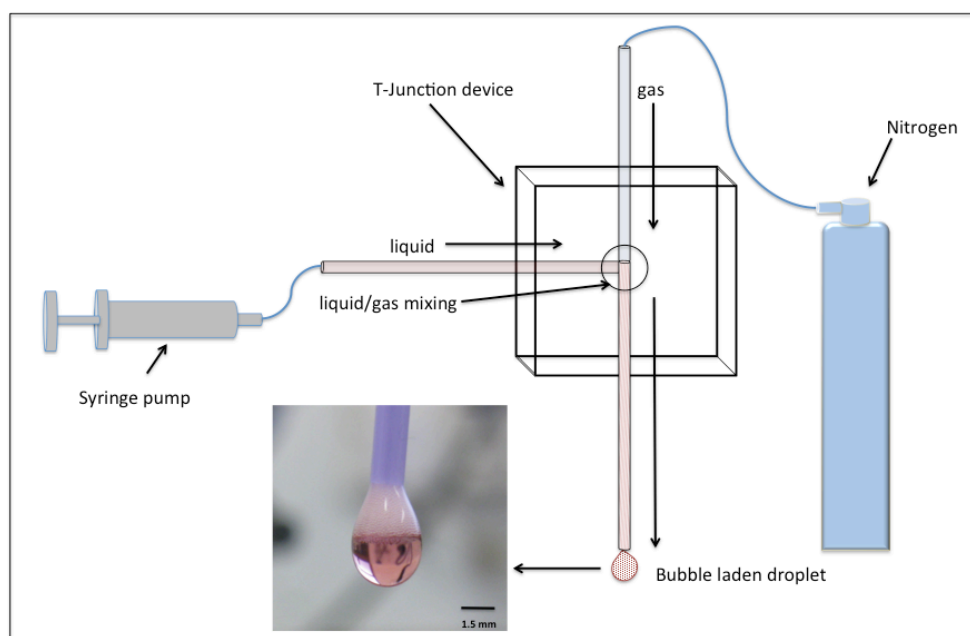


Figure 4.2 Bubble preparation using a T-junction: basic device schematic and image of gold coated microbubbles emerging from the lower capillary prior to collection.

Three polyether ether ketone (PEEK) capillaries with an internal diameter of 150 μm were embedded in a rigid acrylic Perspex block. The ends of the upper and lower (parallel) capillaries were separated in the centre of the device by a distance of approximately 70 μm , with the middle capillary aligned centrally with this gap. The capillaries were held in place using standard high-pressure liquid chromatography (HPLC) connectors and ferrules. All tubing and ferrules were purchased from Gilson Scientific Ltd., Luton, UK. The upper capillary was connected to a nitrogen cylinder, which supplied the gas at a constant pressure of 43.5 ± 2 MPa, as measured by a digital manometer (Digitron 2026P - Digitron Manometer 0-10 BAR). The middle capillary was connected to a digital syringe pump (Harvard Apparatus Standard Infuse/Withdraw PHD 4400 Hpsi Programmable Syringe Pump), which allowed for constant measurable non-pulsatile fluid flow at 50 $\mu\text{l}/\text{min}$. The lower capillary was used to collect the microbubbles after formation.

4.1.3 Reverse Y-Junction

A specially designed Y-junction (Figure 4.3) was also used. Four polyether ether ketone (PEEK) capillaries with an internal diameter of 75 μm were embedded in a rigid acrylic Perspex block. The capillaries were held in place using standard high-pressure liquid chromatography (HPLC) connectors and ferrules. All tubing and ferrules were purchased from Gilson Scientific Ltd., Luton, UK. The upper capillary was connected to a nitrogen cylinder, which supplied the gas at a constant pressure of 210 MPa, as measured by a digital manometer (Digitron 2026P - Digitron

Manometer 0-10 BAR). The two side capillaries were connected to a digital Aladdin syringe pump. The fourth capillary was used to collect the microbubbles after formation [11, 73].

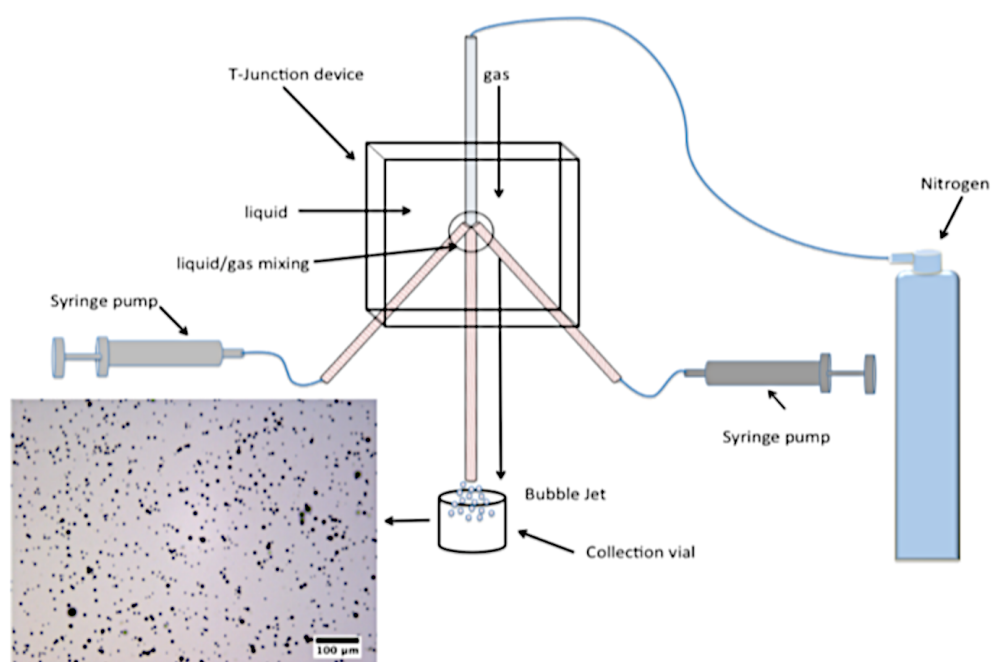


Figure 4.3 Bubble preparation using a reverse Y-junction: basic device schematic and image of a gold-coated microbubble laden droplet prior to collection

4.1.4 Flow-Focusing

A third microfluidic flow focusing device, manufactured in house as described below, was used to make monodisperse bubbles. Gas enters the device through a central 40 μm channel and is focused through an 8 μm orifice by an aqueous lipid mixture dispersion, which flows through two 50 μm flanking channels, as shown in Figure 4.4.

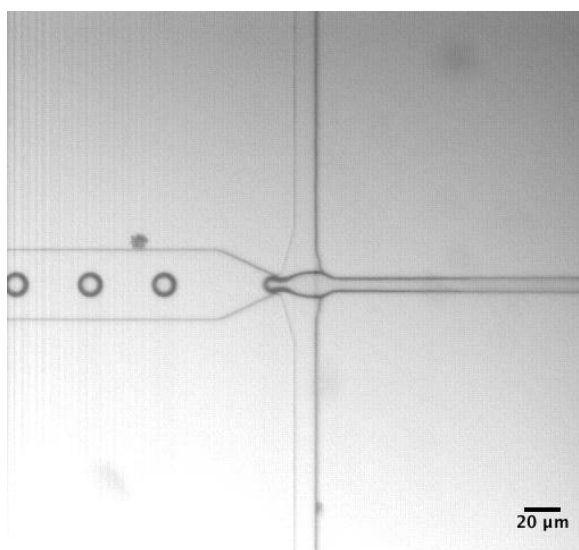


Figure 4.4 Phospholipid bubbles being prepared via microfluidic flow-focusing

All channels were 5 μm high, a dimension controlled by the photoresist layer described below. The focusing of the flow results in a microjet, which periodically pinches off as it exits the orifice resulting in the formation of gas microbubbles with a monolayer of lipids at the gas–water interface. The microfluidic device used here was fabricated from poly(dimethylsiloxane) (PDMS). The mould for the PDMS was made from a silicon wafer (SI-Tech, Inc., Topsfield, MA). The wafer was spin-coated with photoresist (SU8-2005, MicroChem, Newton, MA) to a thickness of 5 μm , and photolithography techniques, using a chromium photomask, were used to create the patterns for the channels. After the photoresist layer was developed, the wafer was used as a mould for the PDMS (Sylgard 184, Dow Corning, Midland, MI). When the PDMS microfluidic devices were separated from the mould, a 0.75 mm diameter needle was inserted through the PDMS into the channels to provide fluid

ports, and the devices were then plasma-treated in a plasma cleaner (model: Atto, Diener Electronic, Ebhausen, Germany) to bond to a glass coverslip. The final devices were plasma-treated again for 5 min in order to render the surfaces hydrophilic to facilitate complete wetting of the interior of the devices.

4.2 Bubble Formulation

Several bubble solutions were mixed and compared (Chapter 5) as part of this study. The compositions are listed in detail in 11.1, but briefly bubbles were produced in 1:1, 1:4, or 1:9 PEG40S:DSPC molar ratios. The final bubble formulation and the one used most often was prepared as follows: A lipid film of 0.13 vol% 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 0.025 vol% 1,2-Distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate-2000 (Na⁺ salt) prepared via solvent evaporation, was added to an aqueous solution containing 0.05 vol% polyethylene glycol 40 stearate (PEG40S), purchased from Sigma-Aldrich Co.

In Chapter 5.3.1, a gold citrate solution was used as the base for the microbubble solution.[73, 164] An aqueous gold citrate suspension (0.51 mM) was used as the base for the microbubble coating. Small monodisperse gold colloids with an average diameter of approximately 15 nm $\pm$ 10% were synthesized according to the sodium citrate reduction method [165] by boiling 95 mL of 5×10^{-4} M tetra-chloroauric acid (HAuCl₄), adding 5 mL of a warm sodium citrate solution (1 wt%) and boiling for 15 min. Suspensions containing only water and PEG40S were also prepared in the same manner. Nitrogen was used as the gas in all cases. For nanoparticle coated bubbles

the lipid/PEG40S mixture was incorporated directly into the aqueous suspension of the Au/citrate particles.

4.3 Characterisation of the solutions

The physical properties of the bubble solutions were measured for comparison to the surface properties of the bubbles obtained subsequently.

4.3.1 Density

The density of the liquid/solution was measured using a 25 ml standard density bottle (VWR International, Lutterworth, UK). The mass of the empty bottle and the mass of the bottle filled with liquid /solution were obtained using an electronic balance (AND HF-1200G A&D Instruments Ltd., Japan). The density (ρ) was calculated as follows:

$$\frac{(m_1 - m_2)}{25} \quad \text{Equation 4.1}$$

where m_1 is the mass of the empty density bottle, and m_2 is the mass of the density bottle filled with liquid /solution.

The mean value of five such consecutive calculations was taken as the density of the liquid /solution and reported in this thesis. Measurements were taken at the ambient temperature and pressure.

4.3.2 Bulk Viscosity

The dynamic viscosities of the solutions were determined using a calibrated micro Ostwald viscometer type 516 10/I with a nominal constant (K) value of 0.01 (SI Analytics GmbH, Mainz, Germany). 2 ml of each sample were pipetted into the viscometer (the larger tube) and allowed to settle for 5 min prior to measurement. The viscometer was suspended in a water bath with a regulated temp of 20°C to ensure temperature control. After the 5 min the solution was siphoned up past the second line on the smaller tube. Timing began when the sample reached the top line and ended when the sample reached the bottom line. Each measurement was made 3 times and the mean value of the time was calculated. Then, the kinematic viscosity (ν) was obtained by multiplying the nominal constant (K) by the time (t):

$$\nu = Kt \quad \text{Equation 4.2}$$

The dynamic viscosity (μ) was then calculated by multiplying the kinematic viscosity by the density (ρ) of the solution:

$$\mu = \nu\rho \quad \text{Equation 4.3}$$

If equations 4.2 and 4.3 are combined then,

$$\mu = Kt\rho \quad \text{Equation 4.4}$$

Ethanol, ACS reagent, $\geq 99.5\%$ (200 proof), was used to calibrate the viscometer. The mean value of five readings was taken as the dynamic viscosity of the sample.

For U-tube viscometers that are not calibrated by manufacturers, use of a calibrating liquid is essential, as the viscosity value cannot be calculated without comparing with another liquid.

4.3.3 Surface tension

The surface tension of the solutions was measured using an Attension Sigma 702 viscometer (Standard Wilhelmy's plate method). The plate was hung from the hook and the beaker containing the sample was placed on the platform. The plate was completely immersed into the sample whose surface tension was to be measured. The plate was then gradually lifted and the surface tension value was directly read when the plate was just about to detach from the liquid surface. In order to minimize errors, the plate was cleaned thoroughly with ethanol and dried before each measurement. The mean values of five readings were taken as the surface tension of the sample.

4.4 *Microbubble Characterisation*

The microbubbles themselves were characterised via several methods.

4.4.1 Bubble Sizing and Stability measurements

The size and stability of the relatively large ($>20\mu\text{m}$) monodisperse T-junction bubbles were characterised using a Nikon Eclipse ME600 optical microscope. The bubble size distribution in each case was measured as a function of time via optical microscopy based on a minimum sample size of 200 bubbles from each image set.

Images were taken every 5 minutes for 2 hours and additionally at 24, 48 and 72 hours. Three images were taken at each time interval. Images were then analysed using ImageJ.

For non-monodisperse bubbles under 15 μm in diameter a slightly different procedure was used to size the bubbles prior to acoustic testing and for stability comparisons. A 20 μl sample of bubbles was taken just prior to acoustic testing and was imaged using a Leica DM500 optical microscope and a QImaging MicroPublisher 3.3 RTV camera. Approximately twenty-five images were taken of each type of bubbles at $\times 100$ magnification. For bubble stability comparisons images were taken every 5 minutes for 2 hours.

The bubble size distribution and concentration were obtained using purpose written image analysis software in MatLab (2010B, The MathWorks, Natick, MA, USA) Full details of the algorithm used and the rationale behind using optical microscopy as opposed to other commonly used bubble sizing and counting methods (coulter counting, and laser diffraction) can be found in [166] and [167]. But briefly, microbubbles are exposed to shear stresses when they are put through either coulter counting or laser diffraction. This can cause the bubbles being sized to deform, which hinders the process and creates a larger error. By using optical microscopy and the algorithm, there is no additional shear stress being put on the bubbles [167]. As described in [166]: the algorithm used by the software treats each image independently and outputs a list of bubbles detected and their diameters. It then accumulates the output across several images and adjusts the final number count to

remove the effects of bubble clustering. The optical system has a resolution of 0.45 μm so bubbles smaller than that are excluded from the counts as they are not diagnostically relevant. All experiments were performed at the ambient temperature in accordance with the conditions under which bubbles were prepared in.

4.4.2 Acoustic Response

The substantial involvement of Mr. Paul Rademeyer in developing and running the acoustic experimental set-up and equipment, and performing the frequency analysis for the gathered acoustic data is gratefully acknowledged.

The response of the microbubbles to ultrasound excitation was tested using a specially designed rig (Figure 4.5). Individual microbubbles were hydrodynamically isolated and streamed using a pair of co-axially aligned needles into the focal region of a pair of transducers. The microbubbles were interrogated by exciting the transmitting transducer (1, 2.25, and 4 MHz focussed, Panametrics-NDT) with a Gaussian-windowed 5 cycle pulse train, ranging from 5 MHz to 2MHz sinusoid pulses, generated by an arbitrary function generator (33220A, Agilent). The signal was then amplified (50dB) by an RF power amplifier (325LA, Electric and Innovation) at a pulse repetition rate of 100 Hz. The driving voltage was adjusted to achieve an equal peak negative pressure at each frequency. The scattered pressure was detected at 90 degrees using a 3.5 MHz focussed transducer (V382, Panametrics-NDT) where the signal was amplified (35dB) using a pulser/receiver (DPR300, JSR Ultrasonics) and digitised with an oscilloscope (600 MHz, Xi64-A,

Waverunner, LeCroy). The data were saved to disk and the captured signals were processed in MATLAB using purpose-written code whereby each signal is analysed in the frequency domain, obtained via Fast Fourier Transform (MATLAB function, `fft`). A flow chart of the signal processing steps can be found in 11.2.

Syringe pumps (AL-1000, World Precision Instruments) were used to control the flow rates in the inner and outer needles of the coaxial flow. Deionised water was pumped through the outer needle (910 μm inner diameter) and the microbubble solution was delivered through the inner needle (190 μm inner diameter). A third syringe pump was used to maintain a stable flow by providing suction through a tube (2 mm Inner dia.) located 2 cm away from the centre of the inner needle. The transducers were aligned by maximizing the received backscatter from micrometre sized air bubbles pumped through the inner needle. Precise alignment of the transducers and needles was achieved by separately mounting the components onto linear xyz manually adjustable stages. The transducers and coaxial flow were securely mounted into a degassed and deionized water tank. The incident pressure at the confocal region was measured using a calibrated 75 μm needle hydrophone (Precision Acoustics Ltd.). Microbubble flow was visualized using a high-speed camera (Memrecam GX-8, AC) to confirm that microbubbles were isolated and that the inner flow was confined to a jet approximately 100 μm in diameter travelling with a central line velocity of 0.12 ms^{-1} . All measurements were performed in filtered, deionised water at the ambient temperature, which had been degassed prior to use.

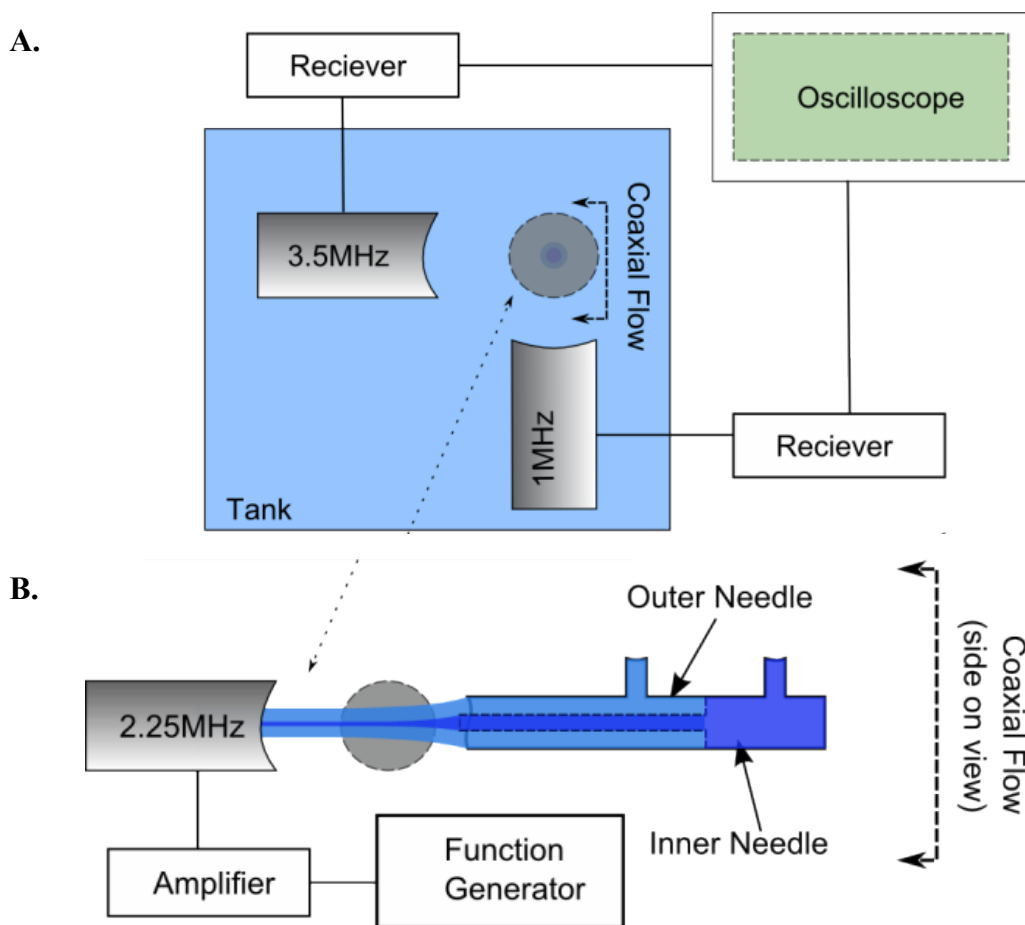


Figure 4.5 Schematic representation of acoustic testing set-up (A) Configuration of the receiving transducers. In a majority of the tests the transducers used were 3.5 MHz and 1 MHz, however in certain tests the transducers were changed. The schematic set up however remained the same. (B) Needle configuration with respect to the transmitting transducer.

4.4.2.1 Acoustic testing for FLIM corroboration

Microbubbles from the different DSPC+PEG ratio mixtures were prepared via sonication and fractionated by allowing them to stand at 4° C for 3 hours.

Microbubbles were hydrodynamically isolated and streamed using a pair of co-axially aligned needles into the focal region of a pair of transducers (Figure 4.4A however with different transducers than shown). The microbubbles were interrogated by exciting the transmitting transducer (2.25 MHz focused, A306S, Panametrics-NDT) with a Gaussian-windowed 5 cycle 2.25 MHz sinusoid pulse, generated by an arbitrary function generator (33220A, Agilent). The signal was then amplified (50dB) by an RF power amplifier (325LA, Electric and Innovation) at a pulse repetition rate of 100 Hz. The scattered pressure was detected at 90 degrees using a 3.5 MHz focused transducer (V382, Panametrics-NDT) where the signal was amplified (42dB) using a pulser/receiver (DPR300, JSR Ultrasonics) and digitized with an oscilloscope (600 MHz, Xi64-A, Waverunner, LeCroy). The peak negative pressure was detected to be 284 kPa, which was comparable with medical ultrasound pressures (Figure 4.6).

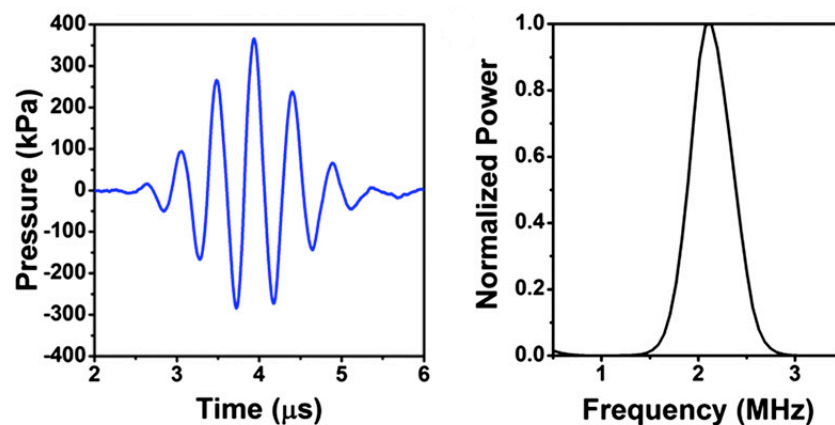


Figure 4.6 (A) The pressure waveform used to excite the bubbles as measured at the focal region with a calibrated needle hydrophone. (B) Corresponding frequency power spectrum of pressure waveform showing narrowband response.

Confirmation and evaluation of scattering events was performed by comparing the frequency spectrum of flow without microbubbles prior to each experiment. The scattered pressure from individual microbubbles was detected by capturing data only when a certain amplitude threshold had been exceeded. The stored data were transferred from the oscilloscope to a PC where they were analysed using Matlab (v.11a, The MathWorks Inc.). The power Fourier transform for each signal was taken and summed within the 6dB frequency bandwidth to obtain an estimate of the scattered power corresponding to the driving frequency.

4.4.3 Surface Viscosity

For the purposes of microbubble imaging we elected to use fluorescence lifetime to detect viscosity. The substantial involvement of Drs. Neveen Hosny and Marina Kuimova in developing and running the Fluorescence Lifetime Imaging Microscopy experiments and analysing the results is gratefully acknowledged.

Fluorescence Lifetime Imaging Microscopy (FLIM) as used in this instance exploits the viscosity sensitive photophysical properties of a ‘molecular rotor’, the meso-substituted 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY-C₁₀) (Fig. 4.7). BODIPY-C₁₀ behaves as a ‘molecular rotor’, due to the rotation of the phenyl ring in and out of the plane of the BODIPY chromophore.

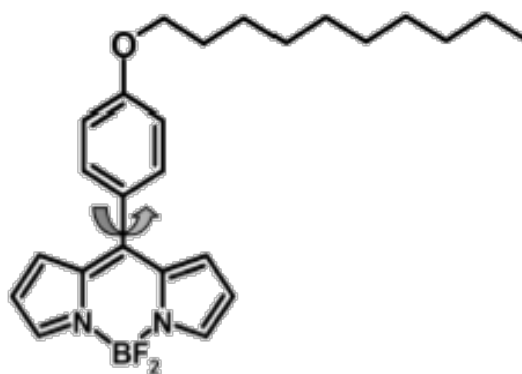


Figure 4.7 BODIPY- C_{10} Chemical Structure

As a result of this rotation, the non-radiative decay pathways are activated, leading to a reduction in the fluorescence quantum yield and the lifetime of the rotor compared with that in a viscous environment [168]. This fluorescent dye can be seamlessly incorporated into structures such as a surfactant coating (Figure 4.8), in this case in the “tail” region of the phospholipid layer surrounding a microbubble. Since it is not a “tag” that is directly bound to the surfactant molecules it does not affect their behaviour [169].

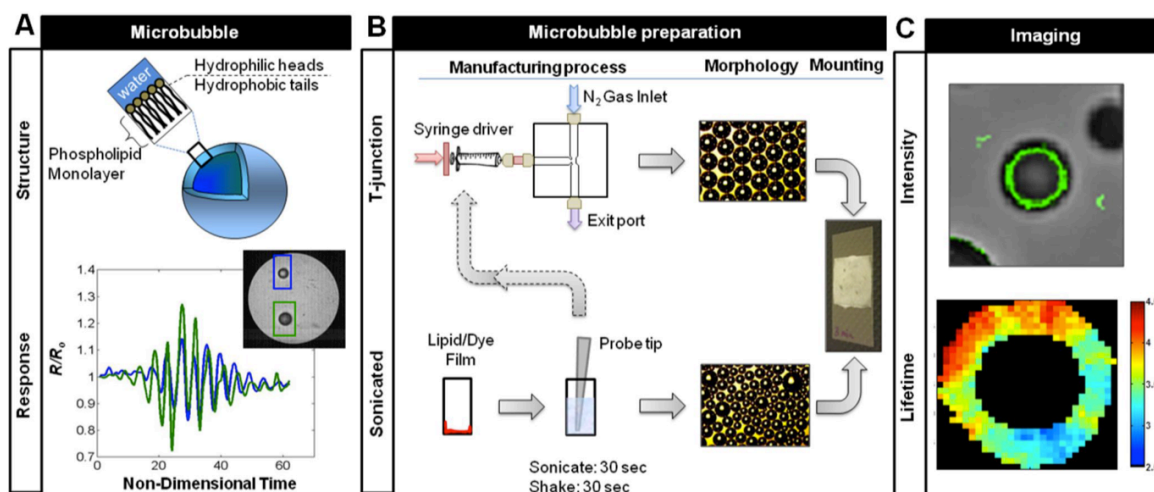


Figure 4.8 Overview of microbubble processing and imaging. (A) Lipid microbubble hypothesized structure and ultrasound response. Shown here are the responses to ultrasound of two bubbles determined using high-speed video microscopy. (B) A diagrammatic representation of the two microbubble preparation processes, T-junction microfluidic preparation and sonication. (C) Brightfield/fluorescence image of a microbubble containing embedded BODIPY-C10 (top) with a lifetime image of BODIPY-C10 showing viscosity distribution in a microbubble (bottom). Previously published in [169].

Unlike fluorescence intensity, which changes as a function of the dye concentration, the fluorescence lifetime can provide a viscosity measure, which is not affected by inhomogeneous distribution of the rotor in a given region of the bubble coating. FLIM can detect individual lifetimes with the spatial resolution of a multi-photon microscope (ca. 200 nm). Thus FLIM of the molecular rotor BODIPY-C10 provides a minimally invasive technique that can directly quantify and image viscosity at a macro- or microscopic resolution from lipid membranes of individual microbubbles. The solution viscosity was correlated to the decay traces using the modified form of the Förster-Hoffmann (Equation 4.5), which describes the

relationship between quantum yield (Φ_f) and viscosity (μ) where z and α represents constants.

$$\Phi_f = z\mu^\alpha \quad \text{Equation 4.5}$$

The quantum yield of a fluorophore can be related to the fluorescence lifetime (τ_f) by the radiative (k_r) and non-radiative rates (k_{nr}), as in:

$$\Phi_f = \frac{k_r}{k_r + k_{nr}} = \tau_f k_r \quad \text{Equation 4.6}$$

Substituting equation 4.6 into 4.5 defines the relationship between the lifetime and viscosity:

$$\tau_f = \frac{z\mu^\alpha}{k_r} \quad \text{Equation 4.7}$$

This can be rewritten as:

$$\log \tau_f = \log \left(\frac{z}{k_r} \right) + \log \mu^\alpha \quad \text{Equation 4.8}$$

A plot of $\log \Phi_f$ or $\log \tau_f$ as a function of $\log \mu$ should yield a straight line with a slope of α , which serves as a calibration graph to convert Φ_f or τ_f into μ , as seen in Figure 4.9 [168-170].

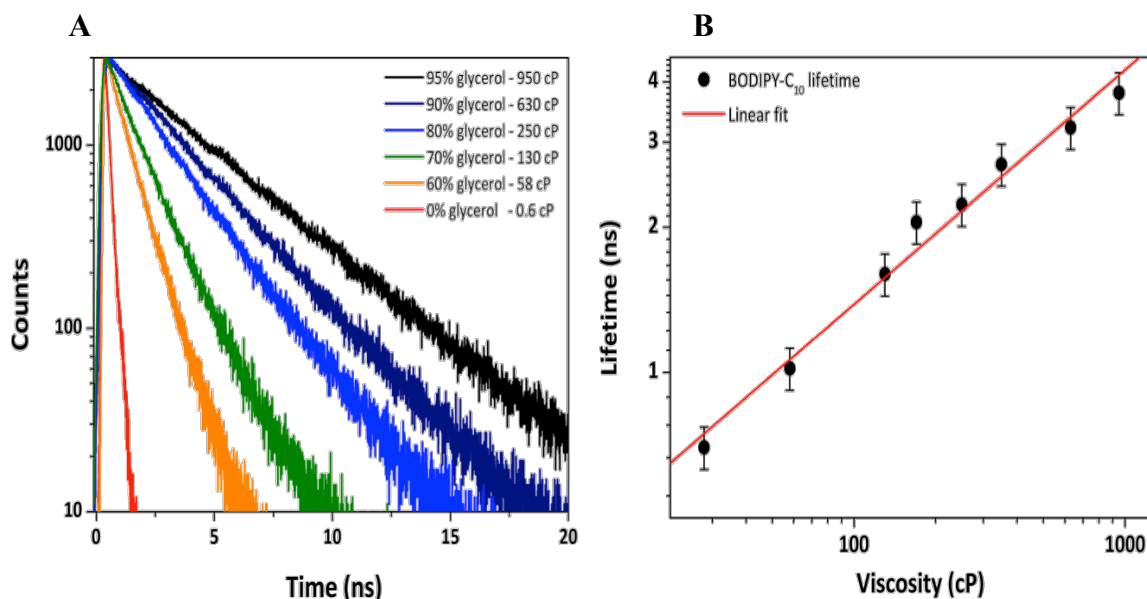


Figure 4.9 BODIPY-C10 fluorescence lifetime calibration vs. viscosity. (A) Selected fluorescence decay traces recorded in methanol/glycerol mixtures (30–95% glycerol) of different viscosity. (B) The logarithmic calibration plot of measured lifetimes vs. viscosity plotted according to the Förster–Hoffmann equation, which was fitted with a linear fit (dash-dot line) and found to be linear between 7.7 and 1,140 cP. Previously published in [169].

4.4.4 Statistical Analysis of FLIM data

All statistical analysis was completed by our collaborator Dr. Neveen Hosny. Microbubble size was determined using Leica LAS software. These measurements were initially verified by importing a brightfield image into ImageJ (4), and a line plot profile was drawn across the image (Figure 4.10A). The graph produced two minima locations associated with the microbubble edge (Figure 4.10B); the distance between these two minima matched exactly the measurements by Leica LAS.

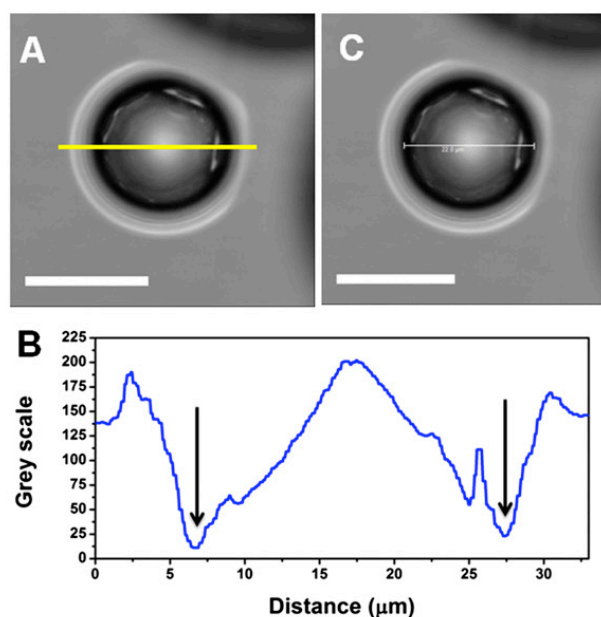


Figure 4.10 Relationship between microbubble diameter and viscoelastic properties. (A–C) Determination of microbubble size. (A) Brightfield image of microbubble with line drawn across it. (Scale bar, 20 μm .) (B) Intensity (grey) scale of line plot across microbubble visualizing the minima points, identified by arrows, across the bubble. (C) Line plot across microbubble obtained in Leica LAS software, using minima points as the reference for the edge of bubble.

All further microbubble diameters were analysed using the minima positioning in the LAS software and values then correlated to viscosity. The relationship between microbubble diameter and viscosity was tested using the Spearman correlation test, which is a nonparametric measure of statistical dependence between two variables and assesses how well the relationship between two variables can be described using a monotonic function. All statistical analysis was completed using Origin scientific software (OriginPro 8.5, OriginLab Corporation).

5 Effect of composition on microbubble physical properties

In this chapter the effects of bubble composition on the stability, effective surface viscosity and the acoustic response of the microbubbles is investigated. The formulation of the bubbles was varied. Two different phospholipids were tested both with and without varying amount of the emulsifier PEG 40S. L- α PC is a naturally occurring molecule with a range of lipid chain lengths. The form used in this study was derived from chicken eggs, and was used in its hydrogenated form in order to lower the presence of double carbon bonds. These double bonds would prevent close packing of the phospholipid heads on the bubble surface and can lower the bubble stability. The molecular structure of DSPC is very similar to the predominant structure found in L- α PC, however the chain lengths of DSPC are much more uniform, (Figure 5.1).

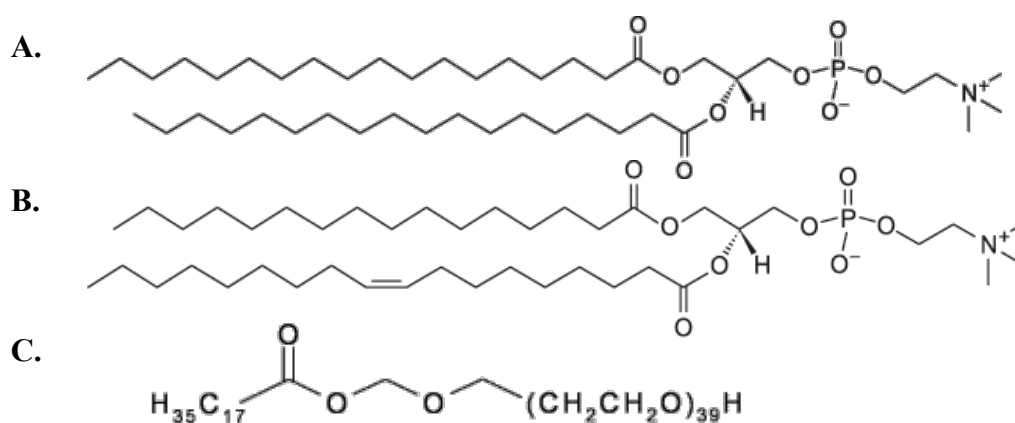


Figure 5.1 The phospholipid structures of A. DSPC, B. L- α PC, and the emulsifier C. PEG 40S

5.1 Bubble Stability

The dissolution rates of DSPC and L- α PC were compared both with and without the addition of PEG40S. When the L- α PC was sonicated without PEG 40S, only liposomes were viewed. The few bubbles that were present behaved like pure DSPC bubbles therefore the stability of the L- α PC can be compared to that of DSPC. Histograms showing the size distribution of a specific bubble population at a given time can be found in 11.4. When PEG 40S was added to the lipid, there was a marked effect upon the size of the bubbles produced. This was expected as the addition of the surfactant PEG 40S facilitated a better emulsification of the bubble solution and the gas. As seen in Figure 5.2 while the DSPC bubbles were fairly stable over a two-hour period their mean size remained around 13 μm . This contrasts greatly with the bubbles made with the addition of PEG 40S as their diameters were between 1 and 2 μm for the first hour.

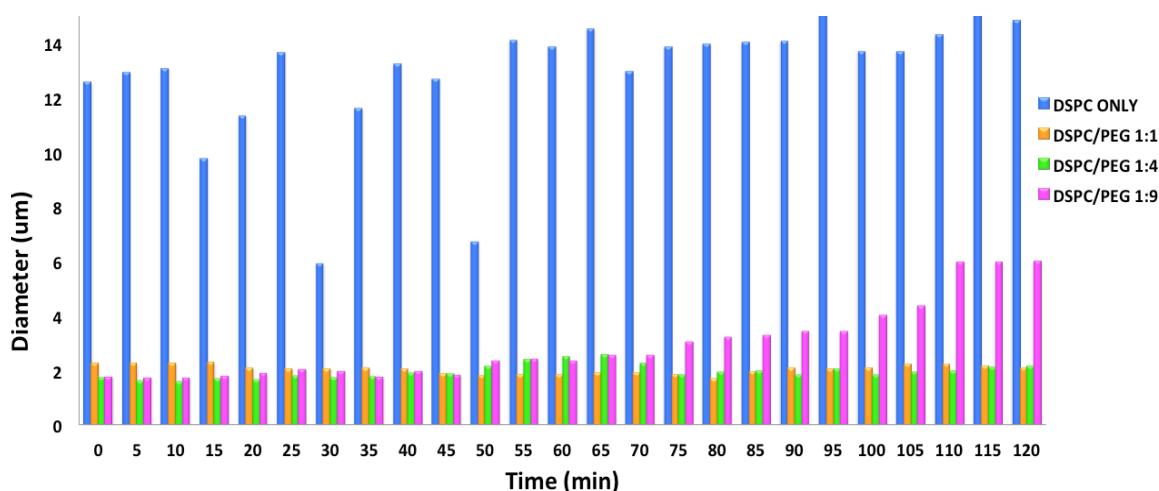


Figure 5.2 Histogram showing the mean size of DSPC and DSPC/PEG 40S bubbles of differing molar concentrations as a function of time. Determined via image processing.

When the changes in size distribution over time of only DSPC/PEG 40S bubbles are compared (Figure 5.3) the concentration of PEG 40S does not seem to change the bubble stability in the short term. The sizes and standard deviations of each molar ratio are fairly well matched. This trend ends at approximately the 75 min mark. The bubbles made using a 1:9 molar ratio begin to exhibit a higher extent of Ostwald ripening, causing the size distribution and standard deviation to increase. While the majority of the bubbles remained within the 1-3 μm limit, bubbles as large as 20 μm were observed. As more PEG 40S was added domains of the surfactant were formed on the bubble surface. These changes in the bubble surface characteristics affected the stability of the produced bubbles. Previous studies have shown that a large number of surface domains indicate that the bubble shell is not at equilibrium [49]. Borden et al [171] showed that the surfactant distributes heterogeneously over the microbubble surface and as a result it will not provide a uniform steric shield against contact by other microbubbles, which can result in Ostwald ripening over time. They also showed that higher concentrations of the surfactant in the bubble solution can result in “squeeze out”, in which the surfactant is pushed out of the surface due to Laplace over pressure, leading to very small rigid microbubbles which may not be optimal for use as UCAs, or targeting agents.

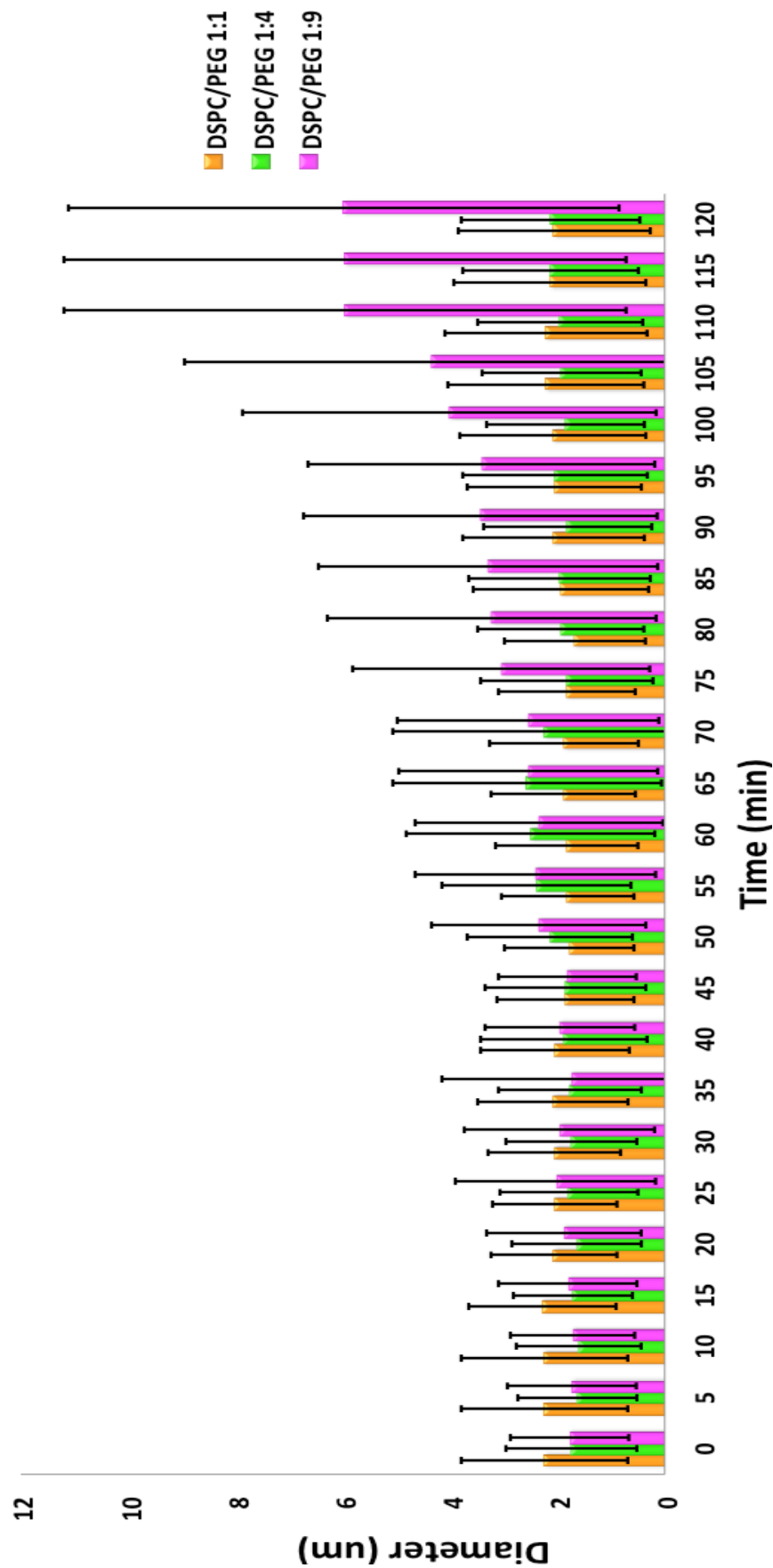


Figure 5.3 Size distributions of DSPC/PEG 40S bubbles of differing molar concentrations over a two hour observation period. Error bars represent $\pm$ one standard deviation. Determined via image processing.

5.2 *Surface Effective Viscosity*

Fluorescence lifetimes detected from microbubbles produced by sonication from lipid-dye films containing DSPC, L-alpha-Phosphatidylcholine (L- α PC) and DSPC+PEG40-stearate in different ratios (11.1) were compared and related back to surface viscosity. This study was published in the Proceedings of the National Academy of Science in 2013 [169].

5.2.1 Bulk Viscosity and Surface Tension Dependence on Dye Concentration

Prior to conducting FLIM the dependence of viscosity and surface tension on the concentration of BODIPY added into the bubble solution needed to be determined. Direct viscosity and surface tension measurements on bulk samples for lipid–dye ratios between 3,600:1 and 28:1, were conducted as previously described (Ch. 4.3.2 & 4.3.3). It was found that the surface tension remained stable at 35.33 (SD 0.9) mN/m, and the viscosity remained constant at 4.17 (SD 0.16) centipoise (cP) across the tested range of lipid–dye ratios (Figure 5.4), confirming the insignificant effect of dye loading on the bulk properties of the microbubble solutions.

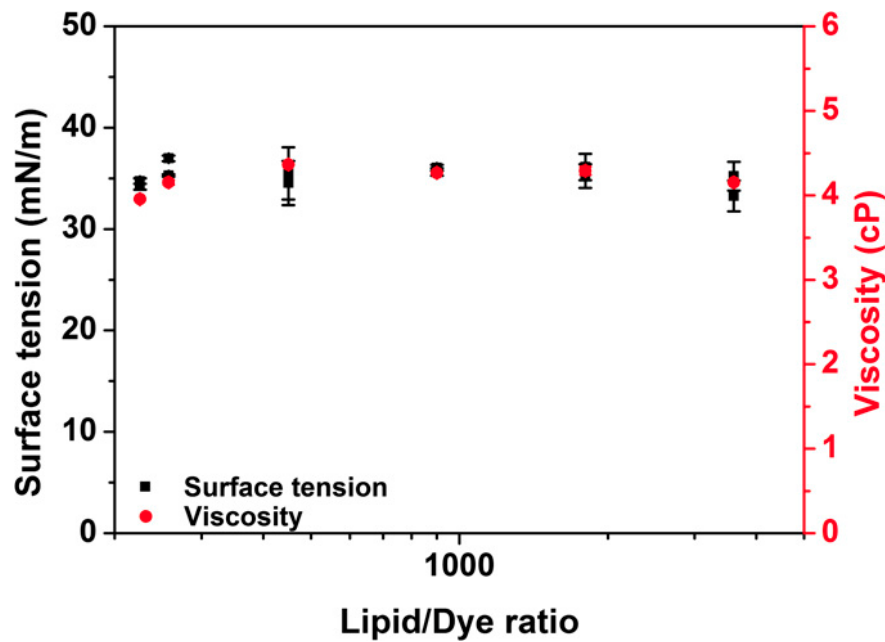


Figure 5.4 Evaluation of the effect of lipid–dye ratio on bulk sample surface tension and viscosity upon increasing the microbubble lipid–dye ratio in bulk samples; ■ surface tension ($n = 2$, repeats = 2) ● viscosity ($n = 3$). Determined via image processing.

5.2.2 Statistical Analysis Of Viscosity And Size Dependence

For all compositions, comparison of the observed lifetime for microbubbles of different size indicated that there was no statistically significant correlation between shell viscosity and microbubble size (Figure 5.5). Previous studies have shown that microbubble ultrasound response is size-dependent [172], and it has been suggested that this is not due merely to the effect upon the resonant frequency of the bubbles but that the coating properties may themselves be size-dependent. Therefore shell viscosity, as measured by molecular rotors, can be correlated to the microbubble size (Ch. 4.4.). The relationship between microbubble diameter and viscosity was tested using the Spearman correlation test, which found that there was no correlation

between viscosity and microbubble size for all composition except DSPC ($P = 0.046$) and PEG 1:1 ($P = 0.007$), and here the level of correlation was low (0.39).

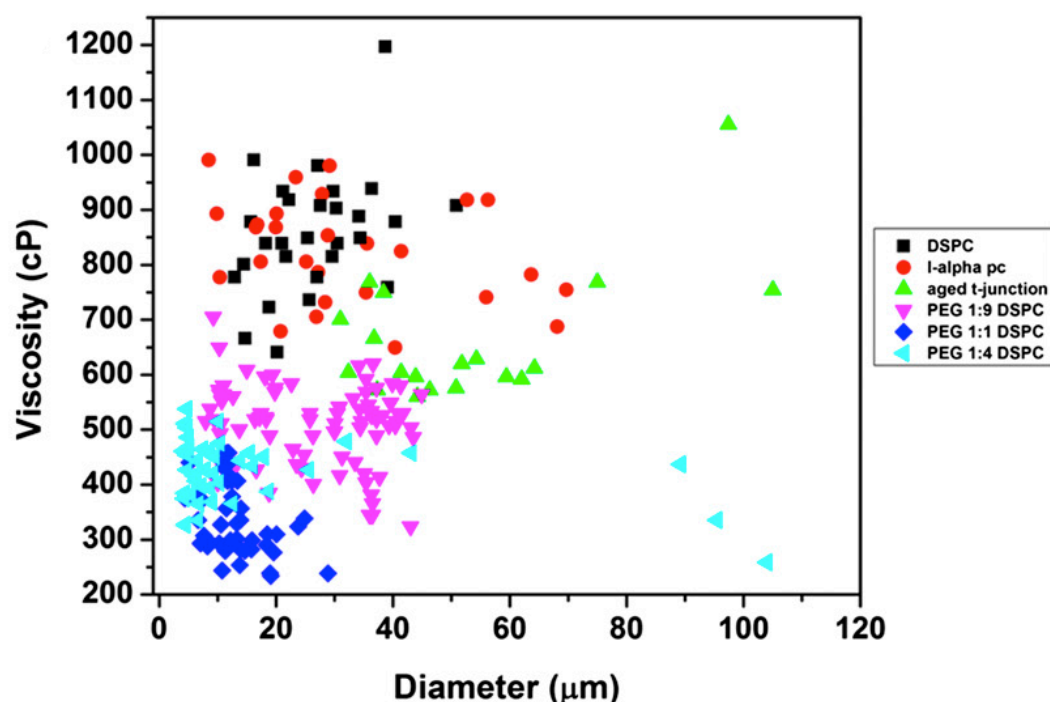


Figure 5.5 Variation of microbubble viscosity with size for different compositions and manufacturing protocols. Determined via image processing.

5.2.3 Surface viscosity measurements via FLIM

The DSPC and L-alpha-PC microbubbles were found to exhibit a similar range of viscosities (Figure 5.6) as might be expected given the similarity in their molecular structure (Figure 5.1, A and B). The addition of PEG 40S to DSPC, however, had a marked effect, with viscosity being significantly reduced with increasing PEG 40S ratio: PEG 1:1 DSPC 337 ± 57 cP (2.49 ± 0.2 ns), PEG 1:4 DSPC 426 ± 57 cP (2.78 ± 0.2 ns), PEG 1:9 DSPC 506 ± 73.9 cP (3.01 ± 0.21 ns) (Figure 5.7). There was also

considerable variation across each population in the measured viscosity for all shell compositions (Figure 5.7). A significant advantage of the molecular rotor-based viscosity determination is the possibility of spatially resolved viscosity imaging, which to the best of our knowledge is currently not possible with any other available technique.

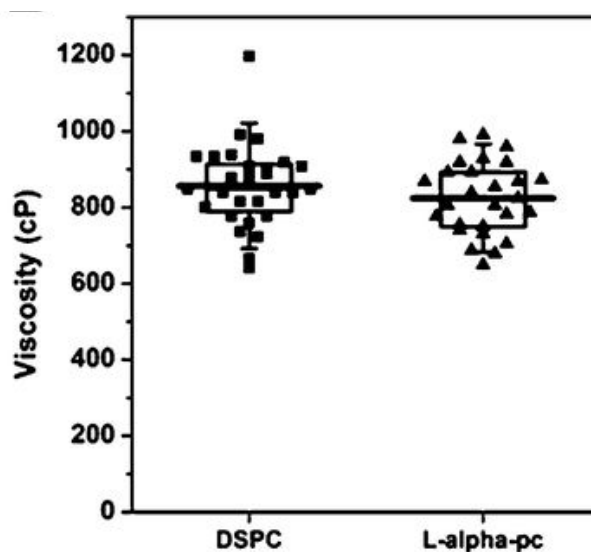


Figure 5.6 Effect of microbubble lipid composition on viscosity for DSPC (■) ($n = 28$), l -alpha-PC (Δ) ($n = 27$). Determined via image processing. Boxes show 25–75% of microbubbles, whiskers are the SD, and thick bar indicates the mean value ($*P < 0.001$). Determined via image processing.

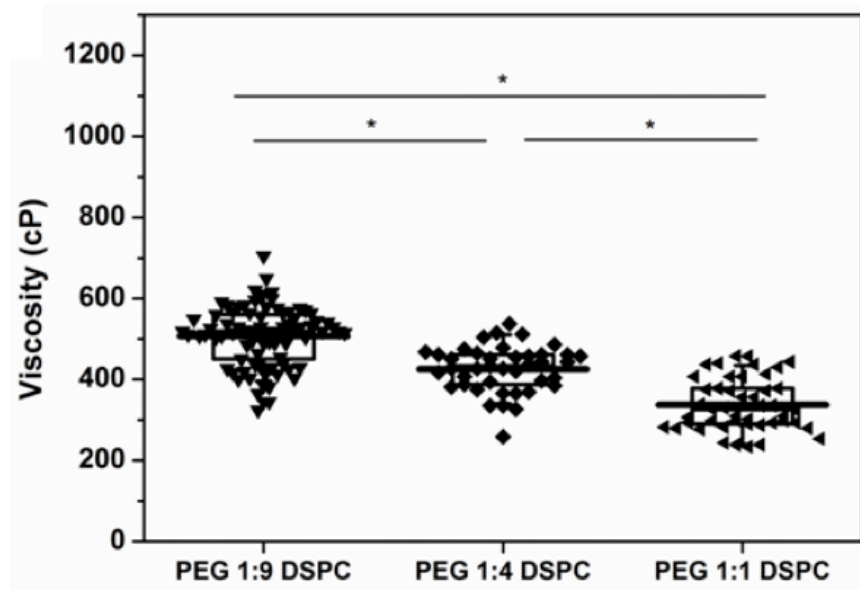


Figure 5.7 Viscosity dependence on microbubble coat composition: PEG 1:9 DSPC (∇) ($n = 82$), PEG 1:4 DSPC ($\blacklozenge$) ($n = 41$), and PEG 1:1 DSPC ($\blacktriangleleft$) ($n = 46$). Boxes show 25–75% of microbubbles, whiskers are the SD, and thick bar indicates the mean value ($*P < 0.001$). Determined via image processing.

5.3 Acoustic response

Three different bubble populations of varying PEG40S to DSPC molar ratios were investigated acoustically. The size distributions of each of the three populations being tested were subsequently determined using purpose written code in Matlab (Ch. 4.4.1). This provided an average Log-normal size distribution of $5.5 \mu\text{m}$ for all compositions ($n > 300$) (Figure 5.8). These data were used in theoretical simulations to determine the expected sensitivity of the scattered pressure to the coating viscosity. This large viscosity variation was reflected in the ultrasound measurements, which also showed a large standard deviation in the scattered

pressure produced by bubbles of the same composition and size distribution (Figure 5.8 and Figure 5.9). The power of the scattered pressure decreased with increasing proportion of PEG40 stearate in the coating, corresponding to a reduction in the measured viscosity (Figure 5.9).

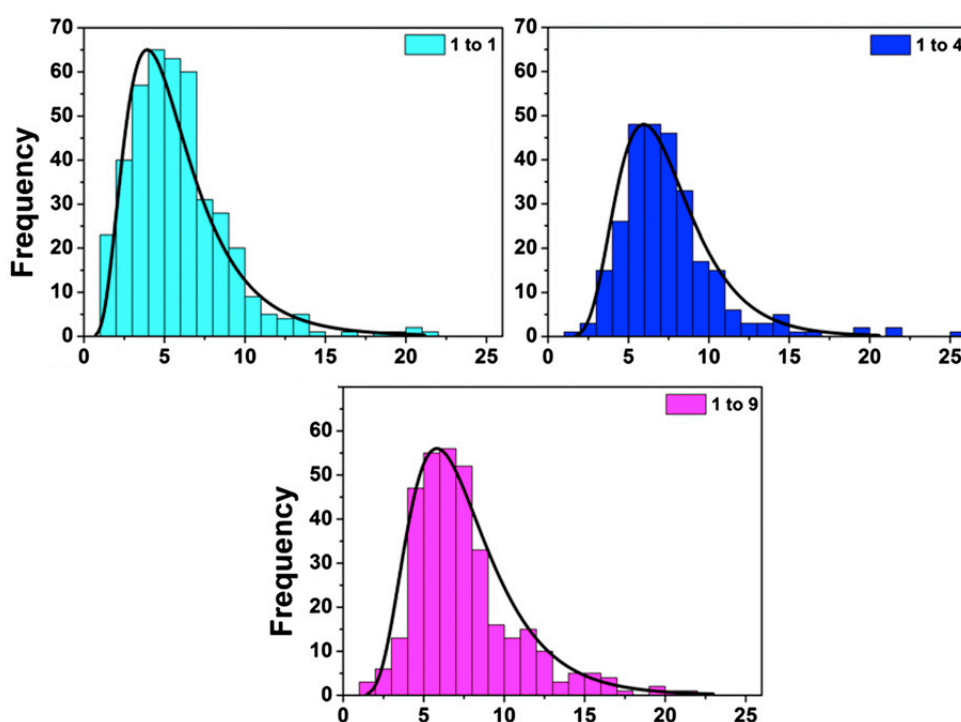


Figure 5.8 Microbubble size distribution fitted to Log-normal distribution curve for solutions PEG 1:1 DSPC ($n = 417$; turquoise), PEG 1:4 DSPC ($n = 276$; blue), and PEG 1:9 DSPC ($n = 342$; pink).

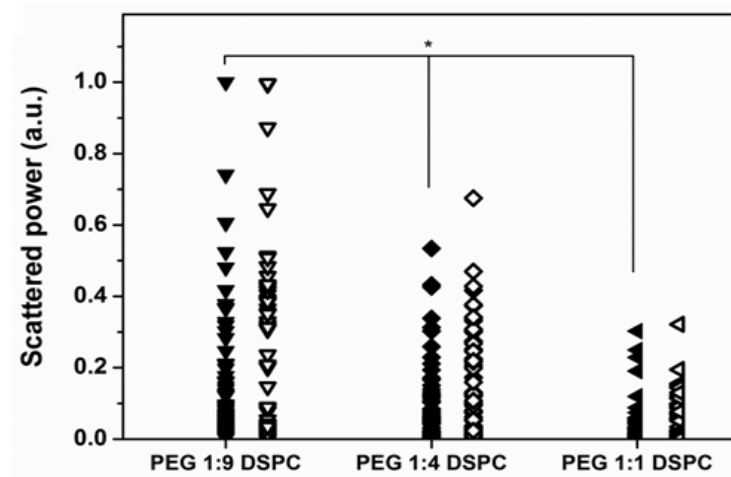


Figure 5.9 Normalized scattered power at the fundamental frequency (2.25 MHz) for ratios of PEG 1:9 DSPC, PEG 1:4 DSPC, and PEG 1:1 DSPC ($n = 150, 80, \text{ and } 69$) ($*\chi^2 < 0.05$) are compared between experiment (closed symbol) and simulations (open symbol) using a modified Rayleigh–Plesset equation. The simulations use an elastic modulus and viscosity, respectively, of 70 MPa and 507 cP (1:9), 40 MPa and 426 cP (1:4), and 10 MPa and 337 cP (1:1). Previously published in [169]

Theoretical simulations (not part of the work presented in this thesis) indicate that the scattered pressure is sensitive to variations in both the viscosity and effective elasticity of the microbubble coating, both of which would be expected to vary with composition.

5.3.1 Surface Composition Variations

To further examine the differences between bubbles within a single population, we determined the fluorescence lifetimes in each pixel of individual DSPC microbubble images (Figure 5.10).

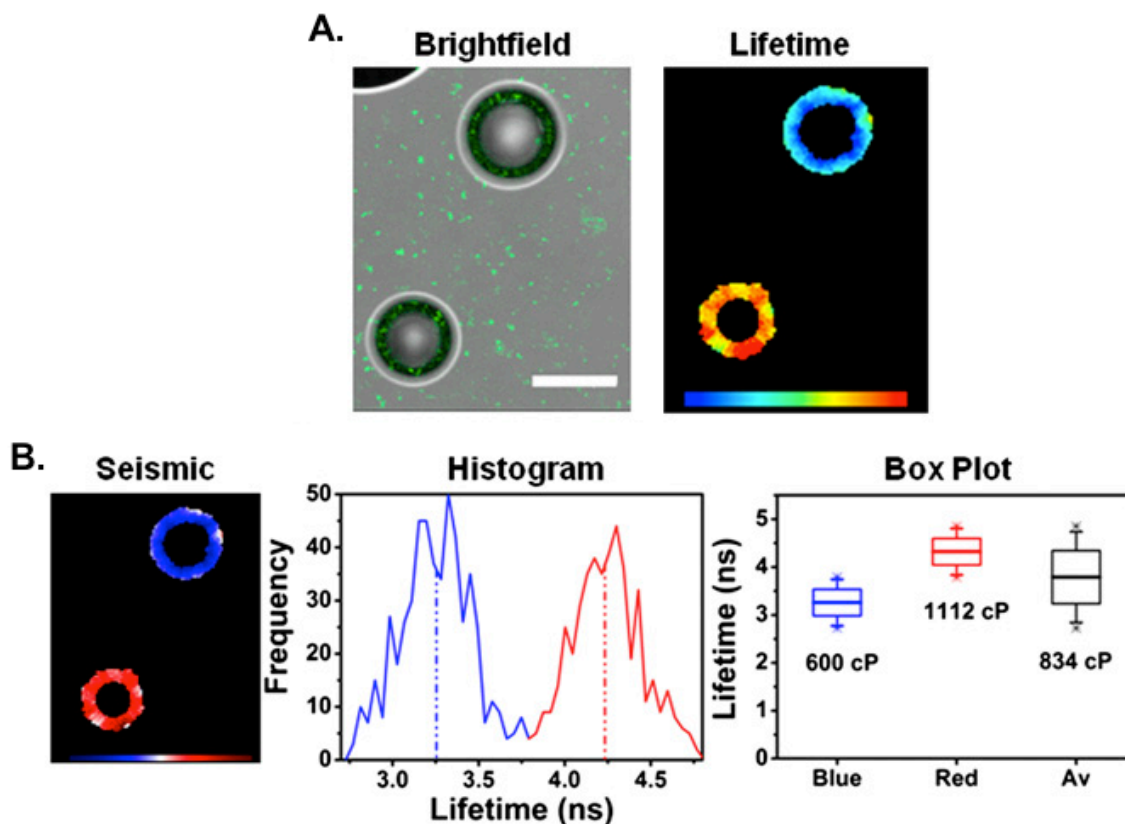


Figure 5.10 Brightfield image of two individual DSPC bubbles of a similar size containing BODIPY- C_{10} , overlaid with confocal fluorescence images of the two. (Scale bar, 20 μm .) Also shown are the results of FLIM lifetime distribution ($\tau = 2.5\text{--}5\text{ ns}$). (B) Analysing lifetime histogram from bubbles in A. shows two distinct peaks with maxima at 3.25 ns and 4.2 ns. Applying a seismic colour hue midpoint at 3.75 ns identifies that short (blue) and long (red) lifetimes are originating from the two different microbubbles. The lifetime distributions and viscosities for each bubble and the whole image are shown on a box plot.

Figure 5.10A shows two bubbles of a similar size, but with different lifetime distributions, as identified by two separate Gaussian peaks in the lifetime histogram (Figure 5.10B). We applied a seismic colour palette to the lifetime histogram (assigned a single colour to each lifetime peak), which clearly differentiated the two

Gaussian distributions. This operation confirmed that the lifetimes in each peak originated from the two separate bubbles, which could be assigned viscosities of 600 ± 140 cP (3.26 ± 0.33 ns) and $1,112 \pm 191$ cP (4.32 ± 0.33 ns) according to the calibration graph (Figure 4.8). In addition, we produced lifetime maps over the surface of single bubbles. Even though the lifetime decay was found to be monoexponential in every pixel, significantly different viscosities were observed across the same bubble (Figure 5.11A). For example, in Figure 5.11B, we resolved the average lifetimes in two separate regions of the same bubble, $A = 3.43 \pm 0.11$ ns and $B = 3.63 \pm 0.11$ ns, corresponding to viscosity differences of 670 ± 50 cP and 761 ± 54 cP, respectively. These data clearly demonstrate that even within a single bubble made up of a pure DSPC lipid, a vastly heterogeneous viscosity distribution could be observed. Our experiments were conducted at an ambient temperature that was much lower than the gel transition temperature of DSPC (55°C), and lack of membrane fluidity may be partly responsible for this inhomogeneity. In addition, the regions of higher apparent viscosity may correspond to regions of lamellae within the coating. These observations confirm that microbubbles should not be modelled as having a continuous shell, even for single lipid component systems.

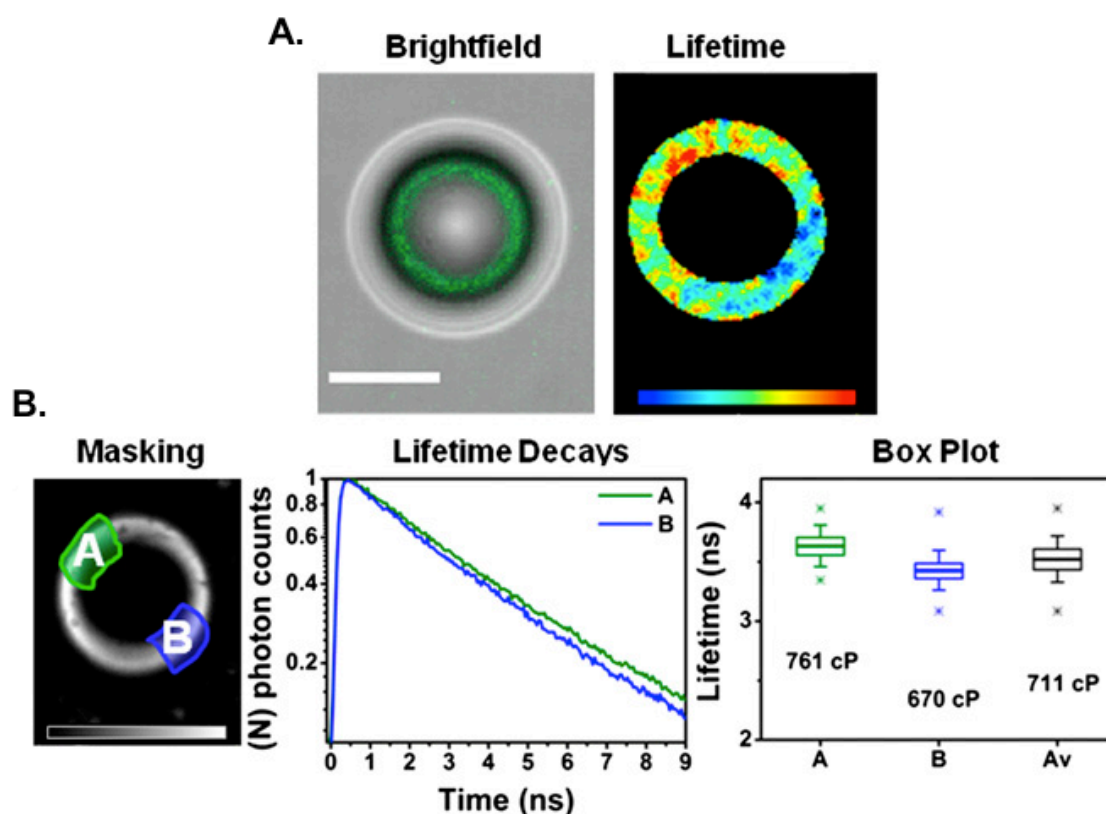


Figure 5.11 A. Individual microbubble with heterogeneous lifetime morphology across the shell, $\tau = 3\text{--}4$ ns. (Scale bar, $20\text{ }\mu\text{m}$.) (B) Masking of high lifetime variation zones A (green) and B (blue) from the lifetime image allows lifetime/viscosity determination in each area. Separately binning all pixels in zones A and B identifies two clearly distinct monoexponential decays, as shown. The analysis of the decay within each bubble as well as within the image as a whole is shown on the box plot.

6 Bubble Surface Structure Effects

In this chapter the effects of surface structure, namely the addition of nanoparticles onto the bubble surface, on the stability and the acoustic response of the microbubbles is investigated. Gold nanoparticles of 15nm diameter were added into the formulation to act as Pickering stabilisers, and increase the “jamming” effect which causes phospholipid bubbles to undergo expansion-only oscillations under acoustic excitation.

6.1 Increasing stability through the addition of Au nanoparticles

These experiments compared the stability of lipid bubbles with and without nanoparticles incorporated into the shell. The results demonstrate the ability of gold nanoparticles to increase the stability of phospholipid coated microbubbles. Comparison with results from a theoretical model developed by collaborators at University College London will also be presented. As well as a comparison between the stability of bubbles made by varying methods. This study was published in Langmuir in 2012 [80].

6.1.1 Confirmation of Nanoparticle Deposition

Gold deposition was confirmed using two methods during this study. The first being the examination of a dried bubble sample viewed and analysed using a Hitachi S-3400N SEM attached with an Oxford Instruments attachment for performing energy dispersive spectroscopy (EDS). Backscattered electron imaging (BSE) was

used to detect differences in particle composition. A bubble sample was dried for twenty-four hours then carbon coated. EDS was run several times at random intervals on the sample in order to conclusively determine the presence of gold at the surface of the bubbles. The EDS spectrogram clearly shows a gold peak, which was verified through several scans at randomly selected points at the bubble interface, Figure 6.1. The gold was only found on the interface and not in the centre of the bubble.

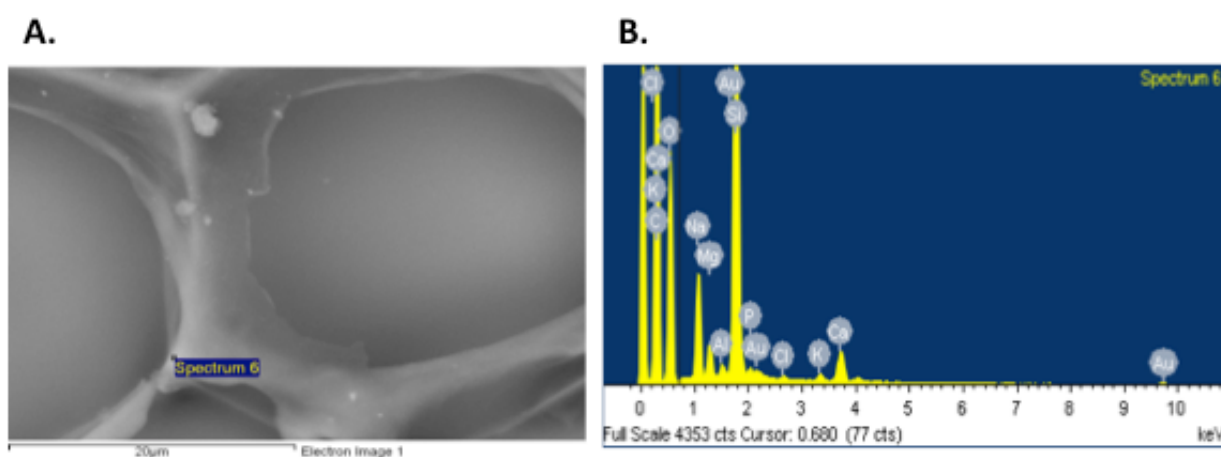


Figure 6.1 (A) SEM of dried bubble residue and (B) backscatter analysis confirming the presence of gold at the bubble surface. Bubbles are assumed to be particle loaded but the absolute gold concentration on the surface cannot be determined.

The second method was performed using optical and hyperspectral images along with hyperspectral data captured in collaboration with CytoViva® using their advanced darkfield illumination system and integrated hyperspectral imaging system (CytoViva Inc., Auburn, AI). This method was used because no vacuum was needed

in order to image the nanoparticles on the bubble surface, and therefore undeformed bubbles could be imaged in suspension. Using CytoViva's hyperspectral image analysis software, the nanoparticles were located and mapped based on the unique spectral response of the gold. This was accomplished without any special staining or tagging. In addition, changes in the sample's surface chemistry can be spectrally detected.

In this device the advanced darkfield condenser illuminates the sample with fixed Kohler illumination and adjustable critical illumination. The light is incident on the sample at an oblique angle, which ensures that only the photons reflecting off the samples are collected by the microscope objective. The integrated hyperspectral imaging camera collects the light from the sample one line at a time. By moving of the automated stage and capturing the released data the series of lines to be analysed are created. This allows for both spatial and spectral data to be collected from each pixel in the line. Compiling the pixelated lines into a single image then creates the data cube.

Once an image has been created, analysis is performed to spectrally determine the location of material of interest. The Particle Filter algorithm is used to collect spectral information in a consistent, repeatable pattern based on the material of interest. Comparing the particle filter results to a negative control sample creates a reference spectral library, which removes any false positive spectral data and ensures a valid result. The spectral library is then compared to an unknown sample. The software algorithm compares every spectrum in the reference library to every

spectrum in the unknown sample and then pseudo colours the matching pixels red (Figure 6.2). The final result gives spatial and quantitative results based on the matching spectral signatures in the unknown sample.

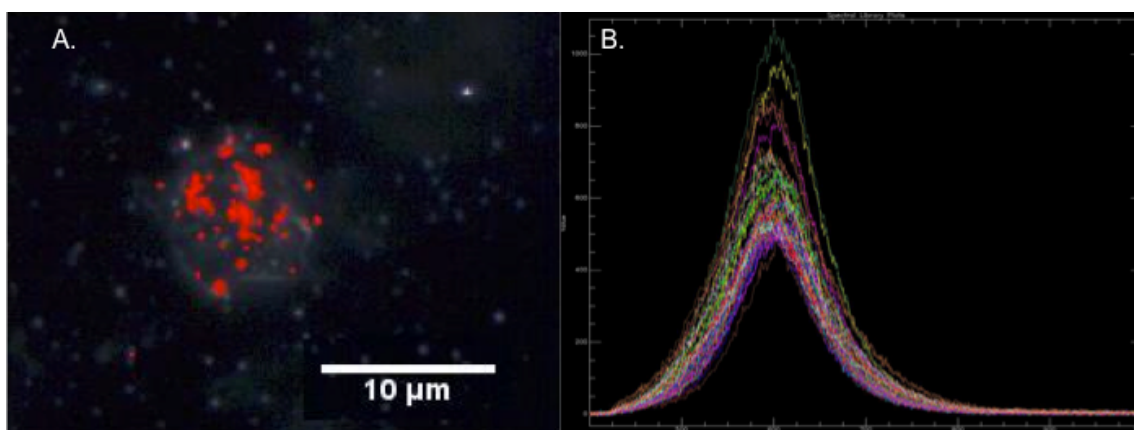


Figure 6.2 (A) Zoomed hyperspectral image of Au nanoparticle coated phospholipid microbubbles. Gold nanoparticles have been pseudo coloured in red. (B) The spectral library of citrate coated Au nanoparticles.

6.1.2 Experimental Results of Au microbubble stability tests

Five different bubble suspensions of 9:1 molar ratio DSPC and PEG40S were tested, containing different concentrations of gold nanoparticles were made using the T-junction device (Ch. 4.1.2). It is important to mention that the purpose of this experiment was to compare experimental results against the theoretical predictions. Larger bubbles than those used for ultrasound contrast enhancement were therefore studied in order to be able to obtain measurements of bubble size to an acceptable precision under optical microscopy. Typical contrast agent microbubbles are of the order of 1–3 μm in diameter and will shrink to sizes below the optical resolution

limit over the time scales involved in this study. In its current form, the model is equally valid for bubbles with diameters between hundreds of nanometres and hundreds of micrometres, and the latter were therefore investigated.

6.1.2.1 Optical Comparisons

Immediately upon emerging from the T-junction each of the bubble populations was near monodisperse and had a diameter of approximately 150 μm . In the time taken to transfer the bubbles from the device to the adjacent microscope, however, the bubbles coated with surfactant only had undergone a considerable reduction in mean size, unlike the bubbles prepared from the gold solution (Figure 6.3A and D). Over the course of an hour at the ambient temperature and pressure, significant Ostwald ripening was observed in the surfactant only suspension, causing the size distribution of the bubbles to broaden greatly (Figure 6.3B). By the end of 24 hours, both the concentration and size of the bubbles were considerably reduced and after 72 hours only a negligible quantity were still detectable under the microscope. The bubbles prepared from the gold solution however remained near monodisperse and underwent a much smaller reduction in mean size over 72 hours (Figure 6.3E and F). Over a much longer measurement period of 30 days, a reduction in bubble concentration and increase in polydispersivity was observed in the suspension (Figure 6.4).

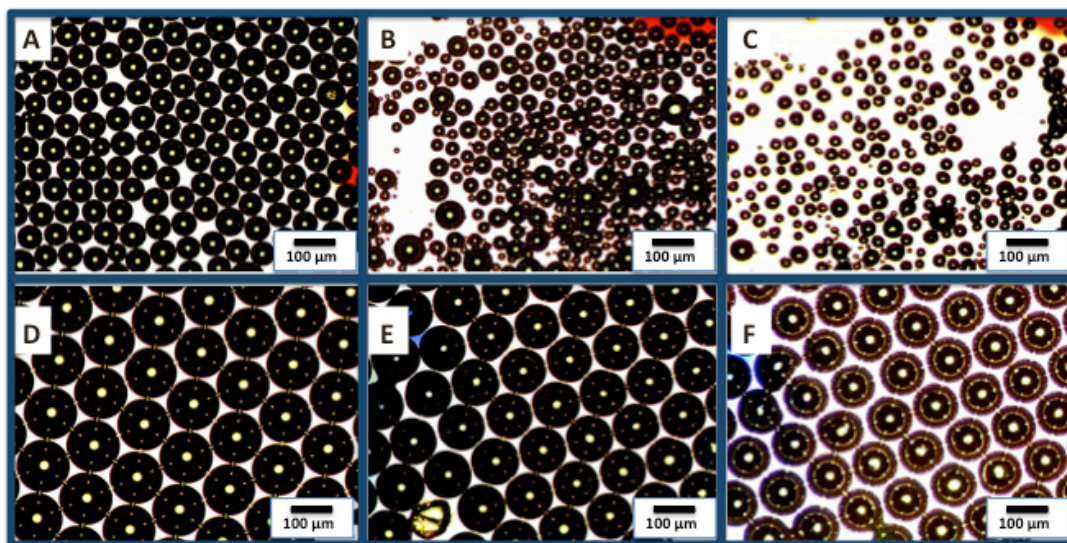


Figure 6.3. Comparison of the stability of surfactant coated microbubbles without (A-C) and with the addition of gold nanoparticles (D-F). Examples of bubble suspensions at: A. $t=0$, B. $t=60$ min, C. $t=24$ hours, D. $t=0$, E. $t=24$ hours, F. $t=72$ hours.

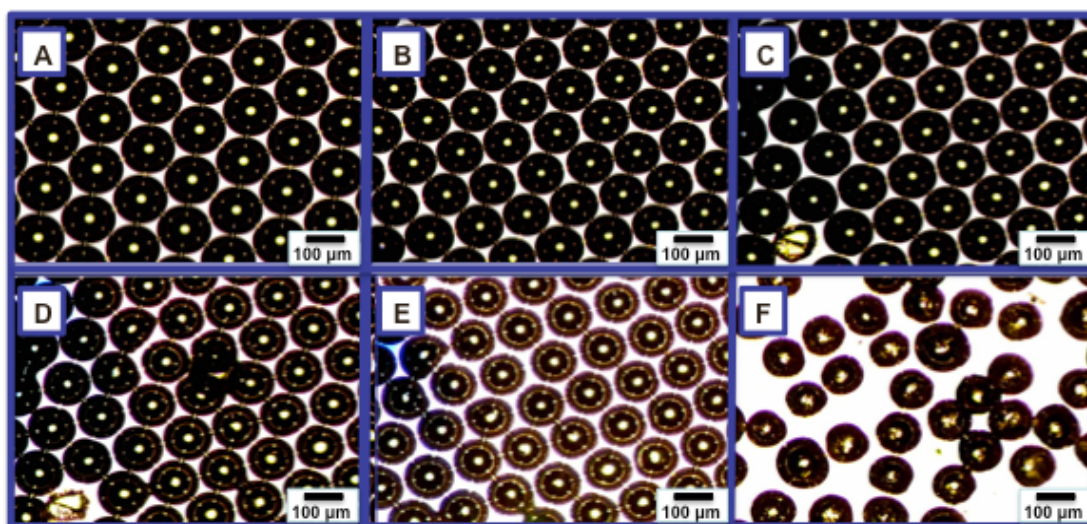


Figure 6.4 Time study of bubbles: (a) Time = 0 min. (b) Time = 60 min. (c) Time = 24 hrs. (d) Time = 48 hrs. (e) Time = 72 hrs. (f) Time = 30 days. (a), (c), and (e) repeated from Figure 6.3.

The changes were still much less significant than those seen in the surfactant only bubble suspension in just 24 hours however. As seen in Figure 6.3, after 30 days the bubbles are no longer spherical. At this time both air diffusion and Ostwald ripening have been effectively terminated and the gold particles have reached their packing density. Interfacial tension is minimized and the gold particles counter the mass-transfer effects due to diffusion and Ostwald ripening [106, 173].

6.1.2.2 Analytical Comparisons

Once the initial stability studies were completed, additional experiments were conducted to determine if the concentration of gold nanoparticles in the original suspension would affect the bubble stability. Figure 6.5 shows how the mean diameter and standard deviation varied with time for samples of 200 bubbles prepared using gold nanoparticle suspensions with varying concentrations. As may be seen, the bubbles undergo a reduction in radius under the influence of interfacial tension and the gas concentration gradient with respect to the surroundings. The surfactant only bubbles, however, undergo more rapid shrinkage due to the higher interfacial diffusivity and larger surface area available for diffusion. In the case of the gold nanoparticle coated bubbles, after a certain period of time (~60 mins in this study) the rate at which the bubble size decreases becomes negligibly small.

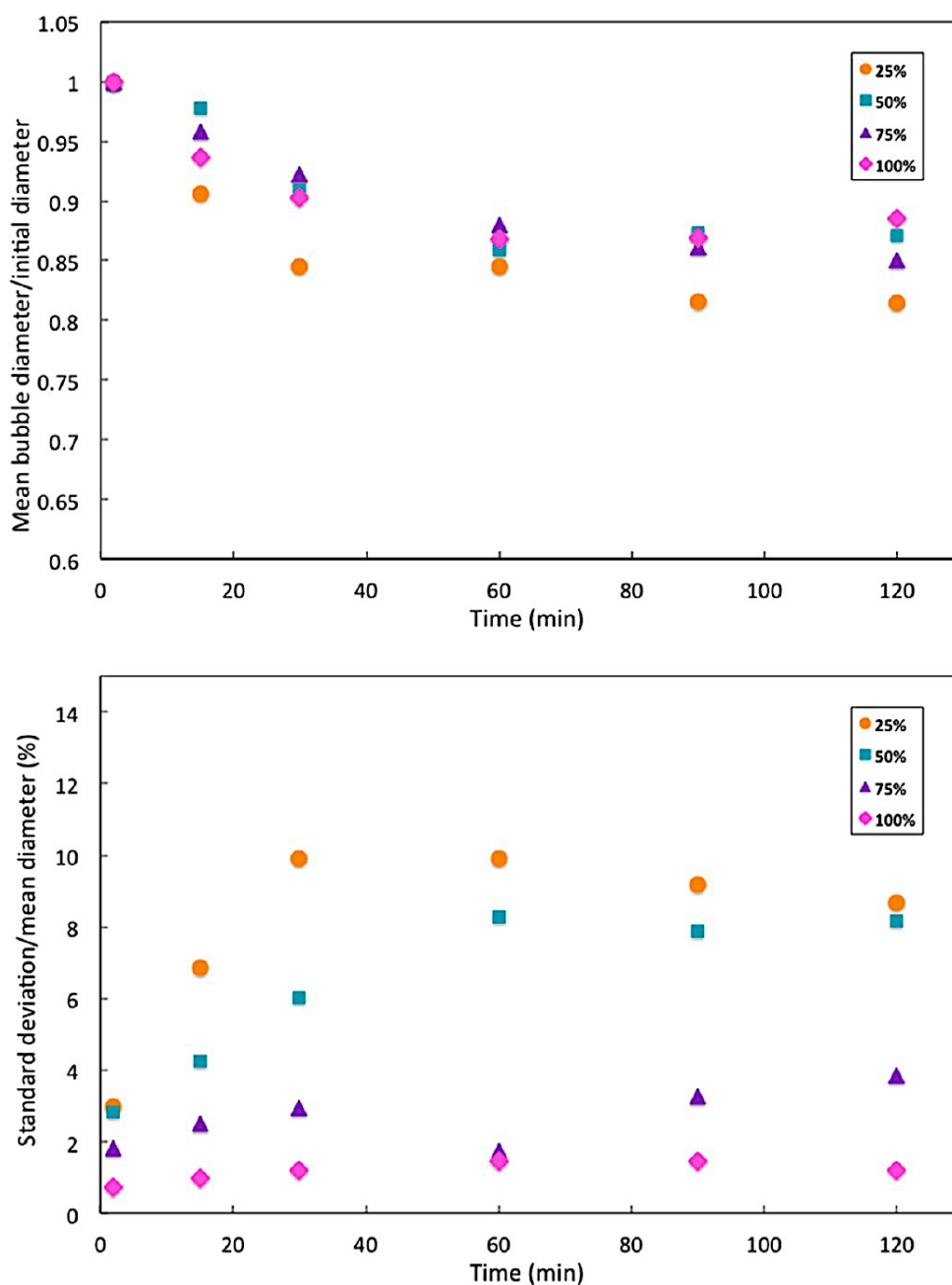


Figure 6.5 (a) Change in mean bubble size over time for bubbles prepared from suspensions containing different concentrations of gold nanoparticles ($n = 200$ bubbles). (b) Ratio of the standard deviation to mean diameter as a function of time for different nanoparticle concentrations. 100% refers to the 0.51 mM gold suspension used for the earlier experiments (Figure 6.5 and Figure 6.6) and 25%, 50%, and 75% to volume dilutions of this. Determined via image processing.

It is hypothesized that this corresponds to the point at which the gold particles reach their packing density on the bubble surface, thereby both minimizing the surface area available for further gas diffusion and significantly reducing the effective interfacial tension due to a decrease in the surface to volume ratio [174]. The rate of shrinkage of the surfactant only coated bubbles also reduces considerably after approximately 20 minutes and this is thought to be due to an analogous increase in the concentration of surfactant molecules on the bubble surface and hence reduction in diffusivity and interfacial tension, as discussed in Chapter 5.2.3. The change is less significant than that affected by solid nanoparticles, however, and the bubbles will continue to shrink with time until they reach equilibrium and full dissolution occurs.

6.1.2.3 Theoretical Model

A mathematical model¹ was developed in order to account for the experimental observations. Full details of the model derivation may be found in Azmin, M, *et al.* [174] but briefly: microbubbles are inherently unstable in liquids due to the effects of capillary pressure acting on the bubble surface as given by the Laplace equation. For an uncoated bubble suspended in a liquid at a constant pressure and temperature, the rate of dissolution depends upon the magnitude of the interfacial tension, the size of the bubble, and the concentration and diffusivity of the gas in the liquid. Assuming

¹ This model was derived by Mr. Mehrdad Azmin (Mechanical Engineering Department, UCL) who studies the theory behind microbubble dissolution as part of a larger collaborative project.

the effect of convection to be negligible, Epstein & Plesset [53] derived the following equation for the rate of change of bubble size under constant interfacial tension (σ) and coefficient of diffusion (D):

$$\frac{dR}{dt} = \frac{D(C_i - C_{sat})}{\rho(\infty) + \frac{2M}{3BT} \frac{2\sigma}{R}} \left[\frac{1}{R} + \frac{1}{(\pi Dt)^{\frac{1}{2}}} \right] \quad \text{Equation 6.1}$$

Where C_i and C_{sat} are the initial and saturation concentrations of the dissolved gas in the liquid respectively, M is the molar mass, B the universal gas constant, T the gas temperature and $\rho(\infty)$ the gas density at zero curvature interface. The addition of a surfactant layer on the bubble surface together with the embedded nanoparticles will affect the rate of dissolution in several ways:

The surfactant film affects the rate of diffusion by creating a barrier to the mass transfer of the gas in and out of the bubble as well as reducing the interfacial tension. Both of these phenomena are dependent on the concentration of the surfactant on the bubble surface. The effective concentration dependent surface tension can be written as:

$$\sigma(R) = \int -2\Gamma_0 \frac{\partial \sigma}{\partial \Gamma} \frac{R_0^2}{R^3} dR = \sigma_0 + \frac{K\Gamma_0^{x+1}}{x+1} \left(1 - \left(\frac{R_0}{R} \right)^{2(x+1)} \right) \quad \text{Equation 6.2}$$

where Γ_0 is the initial surfactant concentration on the bubble surface at which $\sigma = \sigma_0$, K and x are constants characterising a given surfactant and R_0 is the initial bubble radius. The dependence of the coefficient of diffusion on the surface concentration

can be written as an exponential function using a similar treatment to that of Cable and Frade [175], where a and b are constants for the individual surfactant.

$$D(R) = aD_0 \exp \left(b \left(1 - \left(\frac{R_0}{R} \right)^2 \right) \right) \quad \text{Equation 6.3}$$

The presence of the nanoparticles on the bubble surface will reduce the surface area available for gas particles to pass through. This effective surface area (S_A) can be therefore modelled as:

$$S_A(R) = \frac{4}{3} \pi R^2 \left(1 - f_{p0} \left(\frac{R_0}{R} \right)^2 \right) \quad \text{Equation 6.4}$$

where f_{p0} is the initial fractional coverage of particles on the bubble surface. Furthermore, as the bubble contracts, there will come a point that the nanoparticles will reach their packing density. At this point the particles will form a solid shell, which prevents the bubble from further dissolution. This is assuming that the forces present between the particles are not large enough to overcome the attraction force between the particles and the interface and therefore dislodge the particles from the bubble surface. This assumption has been previously supported in theory [173, 176].

Using terms described in 6.2, 6.3 and 6.4 and re-deriving equation 6.1:

$$\frac{dR}{dt} = \frac{(C_i - C_{sat})}{\rho(\infty) + \frac{4M}{3BTR}\sigma(R)} \left[\frac{D(R)}{R} + \frac{\sqrt{D(R)}}{(\pi t)^{\frac{1}{2}}} \right] \left(1 - f_{p0} \left(\frac{R_0}{R} \right)^2 \right) \quad \text{Equation 6.5}$$

Following Epstein and Plesset [53] the following relations can be defined:

$$f = \frac{c_i}{c_s}$$

$$d = \frac{c_s}{\rho(R)} = \frac{c_s}{\rho(\infty) + \frac{\tau\sigma(R)}{R}} \Rightarrow c_s = d \left(\rho(\infty) + \frac{\tau\sigma(R)}{R} \right)$$

$$\frac{c_i}{\rho(\infty)} = fd$$

$$b' = \sqrt{\left(\frac{d}{2\pi} \right)}$$

Here f is the ratio of the initial gas concentration to the concentration at saturation and d is the ratio of saturated dissolved gas to gas density. It is assumed that the value of d is not affected by change in surface tension. After some mathematical simplification:

$$\frac{dR}{dt} = - \frac{f - 1 - \frac{\tau\sigma(R)}{\rho(\infty)R}}{1 + \frac{\tau}{3\rho(\infty)} \frac{2\sigma(R)}{R}} \left[\frac{D(R)}{R} + \sqrt{\frac{D(R)}{\pi t}} \right] \left[1 - f_{P0} \left(\frac{R_0}{R} \right)^2 \right] \quad \text{Equation 6.6}$$

Equation 6.6 can be recast in terms of new variables: $x'^2 = 2dt / R_0^2$ and $\varepsilon = R / R_0$ and solved numerically using a 4th order Runge-Kutta method. This was achieved via the function ODE45 in MATLAB (R2010b, MathWorks Inc., USA).

6.1.3 Comparison between experimental and theoretical results

Utilizing the developed theoretical model a comparison was made between the experimental data and the predictions from the theoretical model. Figure 6.6 and

Figure 6.7 show how the mean size and standard deviation varied with time for samples of 200+ bubbles prepared from the surfactant only and surfactant with gold nanoparticle solutions respectively.

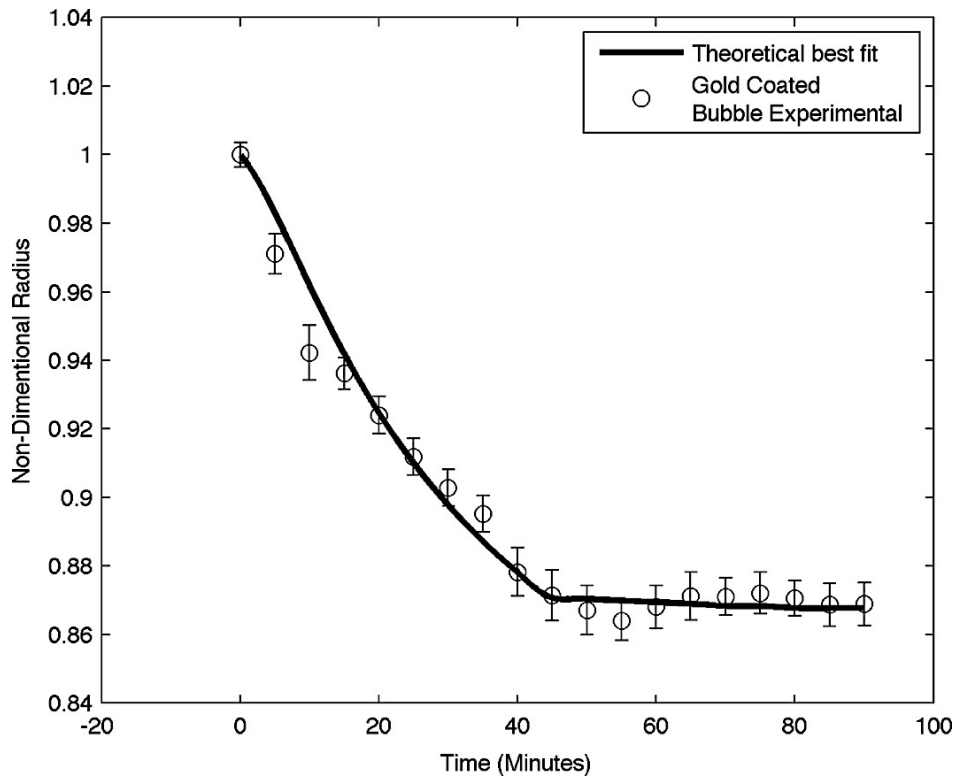


Figure 6.6 Experimental results fitted by the diffusion eq 6.2 for microbubbles coated with surfactant and gold nanoparticles (fitting parameters were as follows: $f p_0 = 0.52$, $a = 0.8$, $b = 9$, $f = 0.85$). Average error for experimental measurements $\pm 1.34\%$ deviation of the diameter within the measured population. Radii are non-dimensionalised with respect to the mean initial value.

The rate of shrinkage of the surfactant-only bubbles also reduced considerably after ~ 20 min (Figure 6.7), and this is thought to be due to an analogous increase in the concentration of surfactant molecules on the bubble surface and hence reduction in diffusivity and interfacial tension. The change is less significant than that effected

by solid nanoparticles, however, and the bubbles continued to shrink with time until complete dissolution occurs.

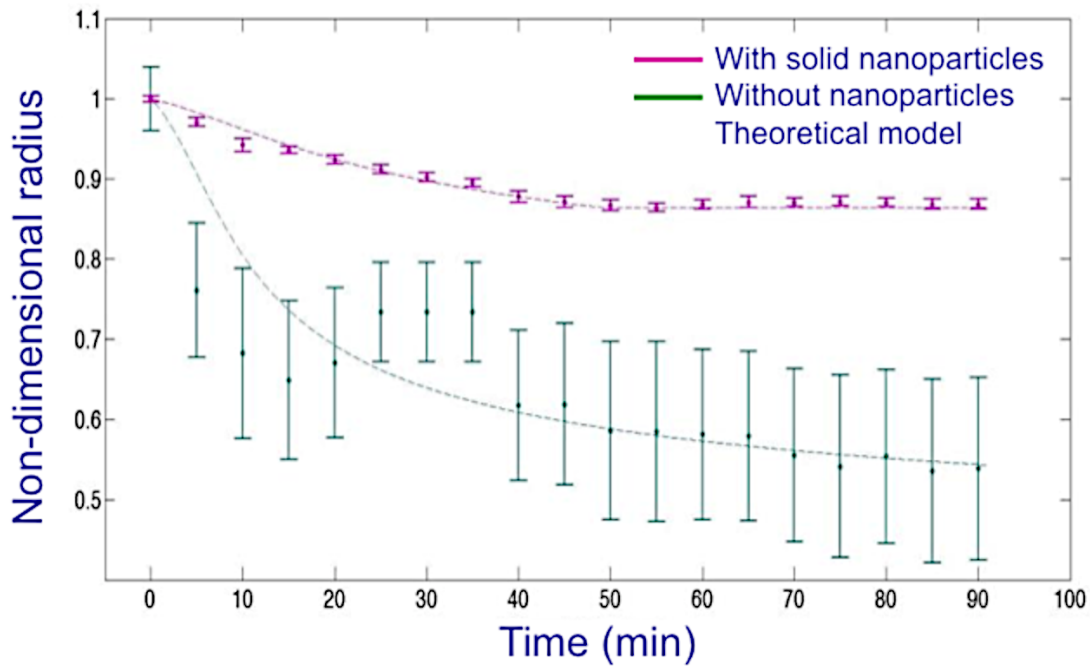


Figure 6.7 Experimental results fitted by the diffusion equation 6.2 for microbubbles coated only with surfactant (green) compared with the results of bubbles coated with Au nanoparticles (pink). Fitting parameters for surfactant-only bubbles were as follows: $f_{p0} = 0.0$, $a = 0.8$, $b = 4$, $f = 0.5$. Average error for experimental measurements of surfactant only bubbles: $\pm 33.7\%$. Radii are non-dimensionalised with respect to the mean initial value.

As mentioned above, the parameters characterising the bubble (a , b , f_{p0} , and f) that provided the best fit to the experimental data were found to be different for the bubbles with and without the gold nanoparticles (see captions to Figure 6.6 and Figure 6.7). This is despite the fact that the bubble preparation and measurement protocols were identical in both cases. It is perhaps less surprising however when

the ideal system considered in deriving equation 6.2 is compared with the actual experimental conditions. The equation describes a spherical single bubble stationary in an infinite volume of liquid. The experimental measurements, however, were conducted on multiple bubbles closely packed and near the surface of a finite volume of liquid. Although the microbubbles remained largely spherical, their close proximity inevitably will result in inconsistencies with the model. For example there will be a constant flux of gas from the bubbles to the liquid and from the liquid to the surrounding air. Therefore, f will be time dependent. Controlling or even measuring f in such small volumes was not found to be practical. As a result, it is plausible for the effective value of f to have differed between the experiments. In addition, the diffusion coefficient will be strongly influenced by the amount of solution around the microbubble as well as the extent to which it has mixed with the glycerol during collection. This would be manifest in some variation in parameter b . In fitting the data for the different particle concentrations it was found that the least mean-square error was obtained by keeping b constant and varying f_{p0} from 0.48 for 25% to 0.59 for 50% and 75%. The corresponding values for f were 0.8, 0.91, and 0.91 for 25%, 50%, and 75%, respectively. The increase in initial particle surface concentration is commensurate with the higher concentration in the surrounding liquid if it is assumed that some time is required to achieve a close-packed structure (as the diffusion curves indicate).

A further significant factor is the absence of any bubble interactions in the theoretical model. In the surfactant-only bubble suspensions, however, the increase

in the standard deviation in bubble size can be attributed at least in part to size disproportionation or Ostwald ripening. This phenomenon occurs as a result of suspended droplets, in this case bubbles, minimizing their surface to volume ratio and hence their surface free energy through transport of material from small to larger droplets, with an accompanying increase in the mean droplet size with time [75, 107]. While the mean size of the droplets increases, the population density will decrease as will the interfacial energy, which decreases with increasing droplet size. In order to slow the ripening process, a small amount of a second component with a very low solubility can be incorporated into the system, which results in a difference in composition between large and small droplets. This difference may counterbalance the driving force for Ostwald ripening and eventually result in a termination [113, 177].

As seen in Figure 6.4E, after 30 days the bubbles are no longer spherical, with some bubbles adopting highly irregular shapes even before this. At this time the rates of both gas diffusion and Ostwald ripening have become negligible, and the gold particles are assumed to have reached their packing density and thus significantly inhibit further mass transfer [73, 175]. The loss of sphericity also indicates a significant reduction in interfacial tension, which may be due to the reduction in curvature of the gas/water interface between the close packed particles. As discussed in Azmin et al. [174], it is implicitly assumed in the theoretical treatment that the bubble remains spherical and that the nanoparticles are rigid. Thus, the predicted radius of the bubble cannot undergo further change. The amount

of gas inside the bubble could, however, continue to decrease until either: the curvature of the bubble surface between the particles becomes convex and the Laplace pressure becomes sufficiently negative that the direction of gas diffusion is reversed or the close-packed particle structure buckles. Other studies, albeit with much larger particle to bubble diameter ratios, have also shown that nonspherical bubble shapes can be stably maintained over long periods of time [178].

6.2 Acoustic response of nanoparticle and phospholipid coated bubbles

These experiments examined the acoustic response of nanoparticle-coated bubbles. Bubbles with and without nanoparticle coatings were made using two different methods, sonication and reverse Y-junction. The size distribution of each population was measured to ensure that the populations could be compared. The results demonstrate the ability of gold nanoparticles to increase the non-linearity of phospholipid-coated microbubbles. Comparison with results from a second theoretical model also developed by collaborators at University College London will also be presented.

6.2.1 Theory

A mathematical model² was developed in order to account for the experimental observations. The oscillation of an uncoated microbubble in response to acoustic excitation at a given frequency and pressure, is dependent on its initial radius, liquid viscosity and density, and interfacial tension. The addition of a coating will result in additional resistance to oscillation due the elasticity and viscosity of the coating. The presence of nanoparticles would increase the effective stiffness and viscosity of the shell at the point of reaching their packing density. This effect will therefore be radius dependent and is modelled as such. Full details are provided in 11.3. The results are illustrated in and where the microbubble was excited using a pulse similar to that used in the experiments with the peak negative amplitude of 500 kPa. It is clear that the inclusion of nanoparticles result in a distinct subharmonic peak as well as moderate increase of power at harmonic frequencies.

² This model was derived by Mr. Mehrdad Azmin (Mechanical Engineering Department, UCL) who studies the theory behind microbubble dissolution and acoustic excitation as part of a larger collaborative project.

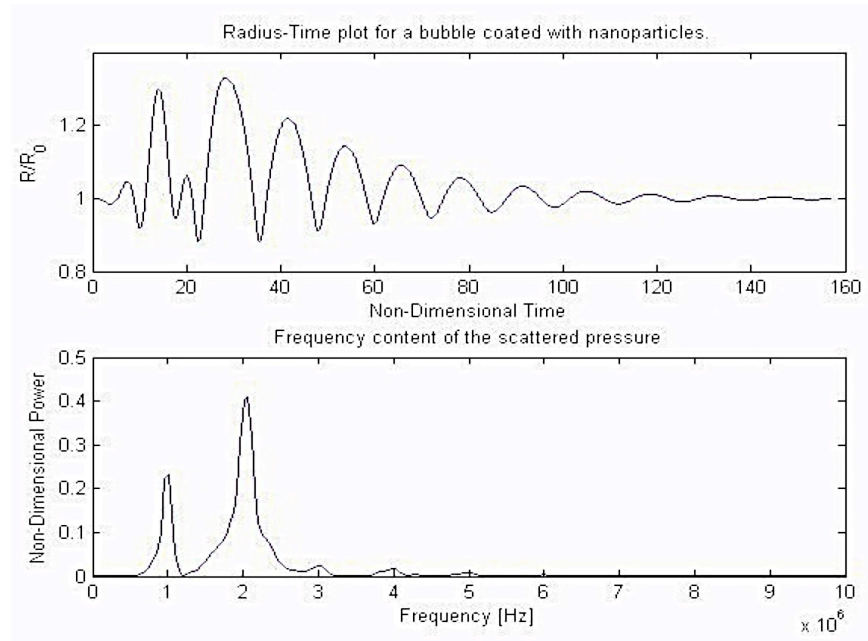


Figure 6.8 Radial response and the frequency content of the resulting scattered pressure between microbubbles with nanoparticle coating.

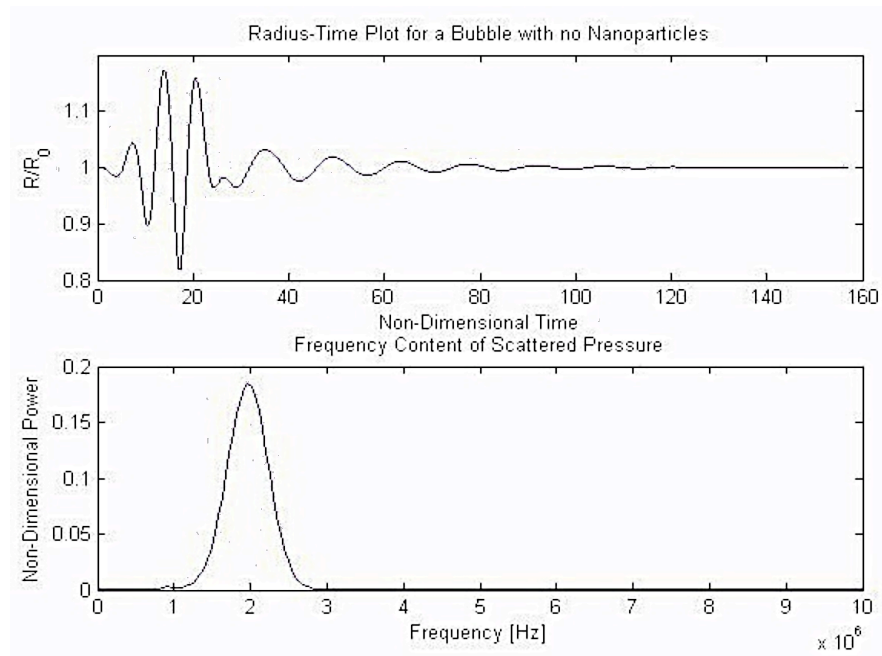


Figure 6.9 Radial response and the frequency content of the resulting scattered pressure between microbubbles without nanoparticle coating.

6.2.2 Experimental Acoustic Comparisons

Prior to testing the acoustic response, the mean size distribution of the 4 different microbubble populations was measured, to ensure that the results could be compared between the different formulations and manufacturing methods. These results can be seen in Figure 6.10.

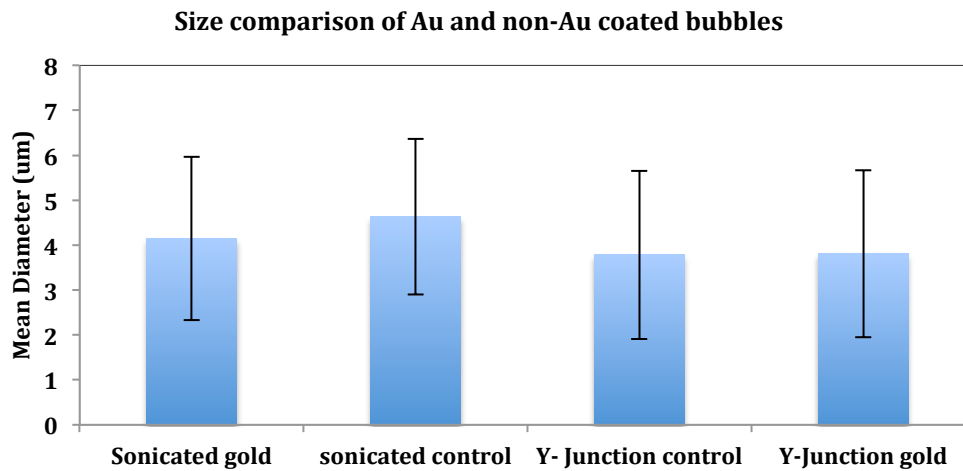


Figure 6.10 Comparison of bubble population mean diameter for all types of bubbles tested acoustically. The error bars represent one standard deviation of population diameter. Statistically there is very little difference between bubble populations' diameters therefore a valid comparison can be made through acoustic testing. Determined via image processing.

The experimental results are shown in Figure 6.11 and Figure 6.12. As may be seen, the amplitude of both the fundamental and subharmonic components increased with increasing driving frequency. This was as expected given that the mean diameter of the microbubbles was approximately 4 μ m and the linear resonance frequency would therefore be expected to be below the centre frequency of the transducer (2.25 MHz).

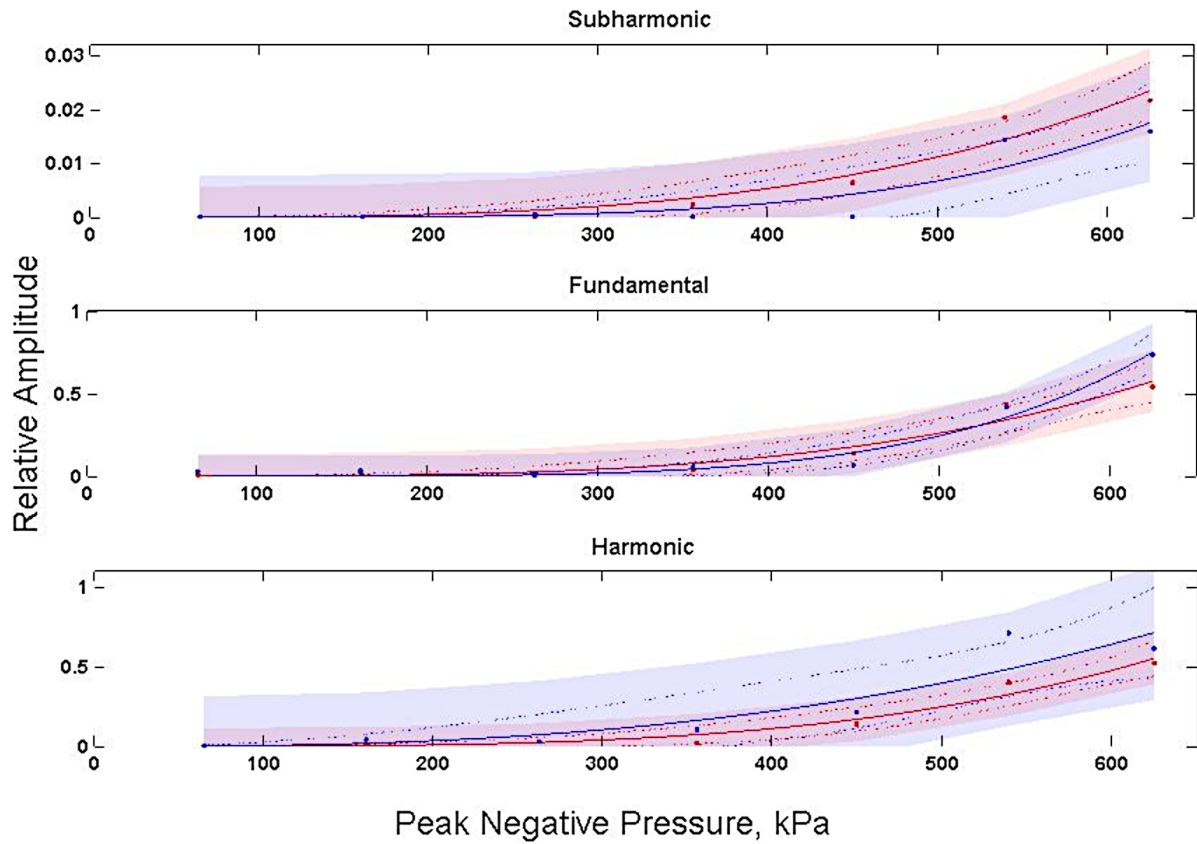


Figure 6.11 Relative amplitude of the subharmonic, fundamental and harmonic components of the scattered acoustic pressure from single microbubbles produced via sonication with (red) or without (blue) a coating of gold nanoparticles excited at different peak negative pressures. The solid line represents the best fit between the mean squared means for each scattering distribution, and “clouds” represent the confidence interval of the data plotted.

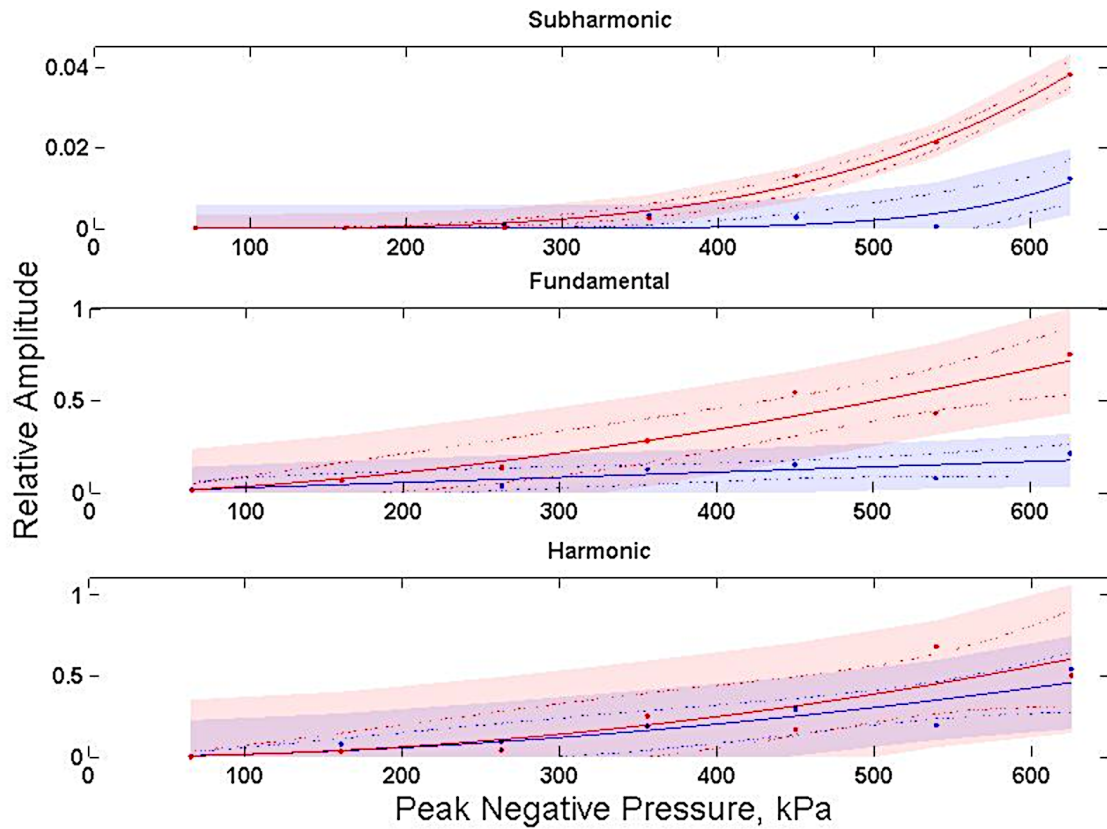


Figure 6.12 Relative amplitude of the subharmonic, fundamental and harmonic components of the scattered acoustic pressure from single microbubbles produced using the reverse Y-junction device with (red) or without (blue) a coating of gold nanoparticles excited at different peak negative pressures. The solid line represents the best fit between the mean squared means for each scattering distribution, and “clouds” represent the confidence interval of the data plotted.

The gold nanoparticle coated bubbles consistently showed a stronger subharmonic response at each frequency in accordance with a more pronounced nonlinear character. In order to confirm the increased non-linear behaviour of the nanoparticle coated bubbles, a second test using a 1 MHz driving frequency was also conducted. It was believed that the increased subharmonic in the previous results (Figure 6.11

and Figure 6.12) was due to the bubbles being hit off resonance. Bubbles with a similar size distribution as those previously tested (Chapter 11.6) were tested using two 1 MHz focused transducers, one to pulse and the other to receive. As seen in Figure 6.13, Figure 6.14, and Figure 6.15 the trend originally seen using the 2.25 MHz centre frequency transducer was continued.

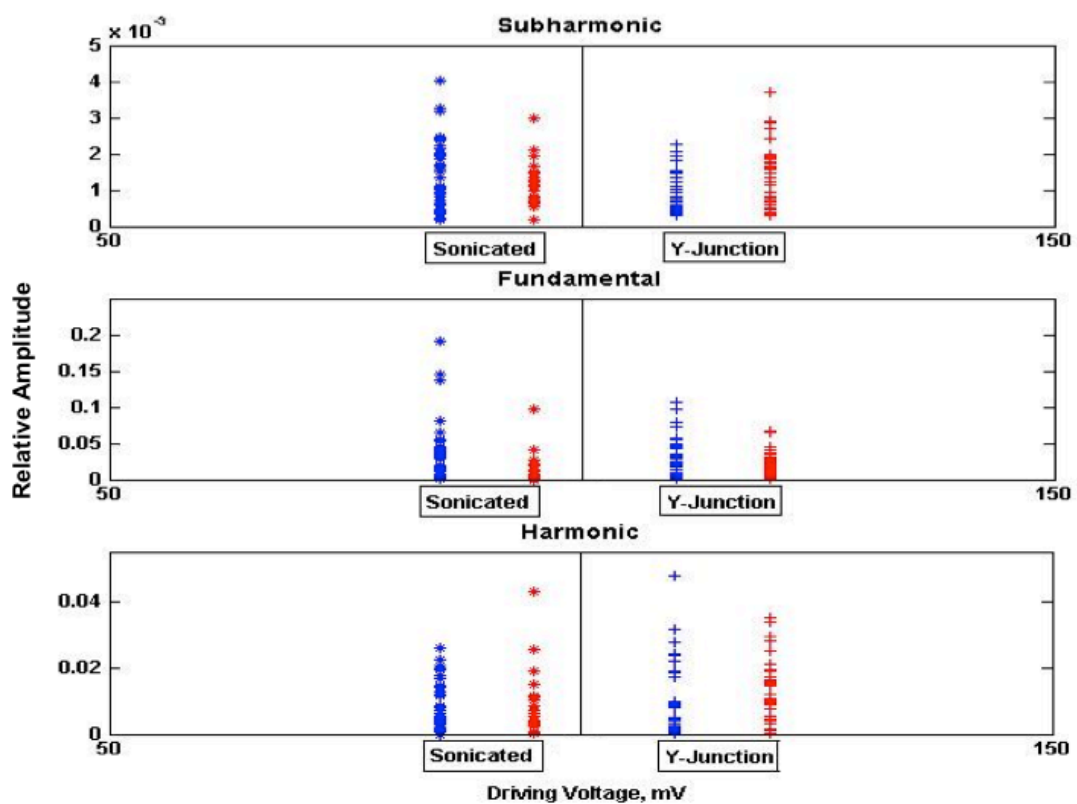


Figure 6.13 Acoustic backscatter results using 1 MHz transducer at a driving voltage of 100 mV. The subharmonic, fundamental and harmonic responses are each shown for sonicated, and Y-junction bubbles both with (red) and without (blue) gold nanoparticles. No confidence intervals are shown as only three different pressures were examined which was not enough to produce reasonable error bars across the range of pressures.

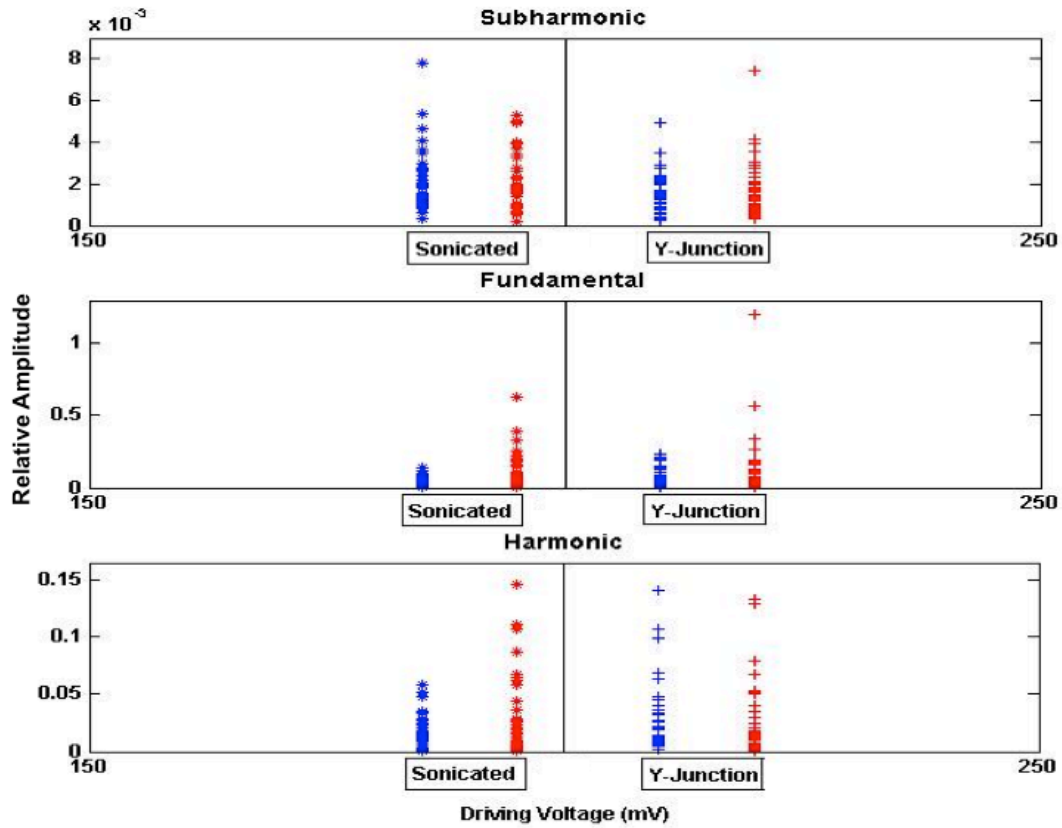


Figure 6.14 Acoustic backscatter results using 1 MHz transducer at a driving voltage of 200 mV. The subharmonic, fundamental and harmonic responses are each shown for sonicated, and Y-junction bubbles both with (red) and without (blue) gold nanoparticles. No confidence intervals are shown as only three different pressures were examined which was not enough to produce reasonable error bars across the range of pressures.

As the driving frequency was increased the non-linearity of the bubble oscillations also increased. Unlike the previous results, however it was the harmonic and not sub-harmonic responses, which showed the largest increase, thereby verifying that the bubbles had been hit off resonance originally.

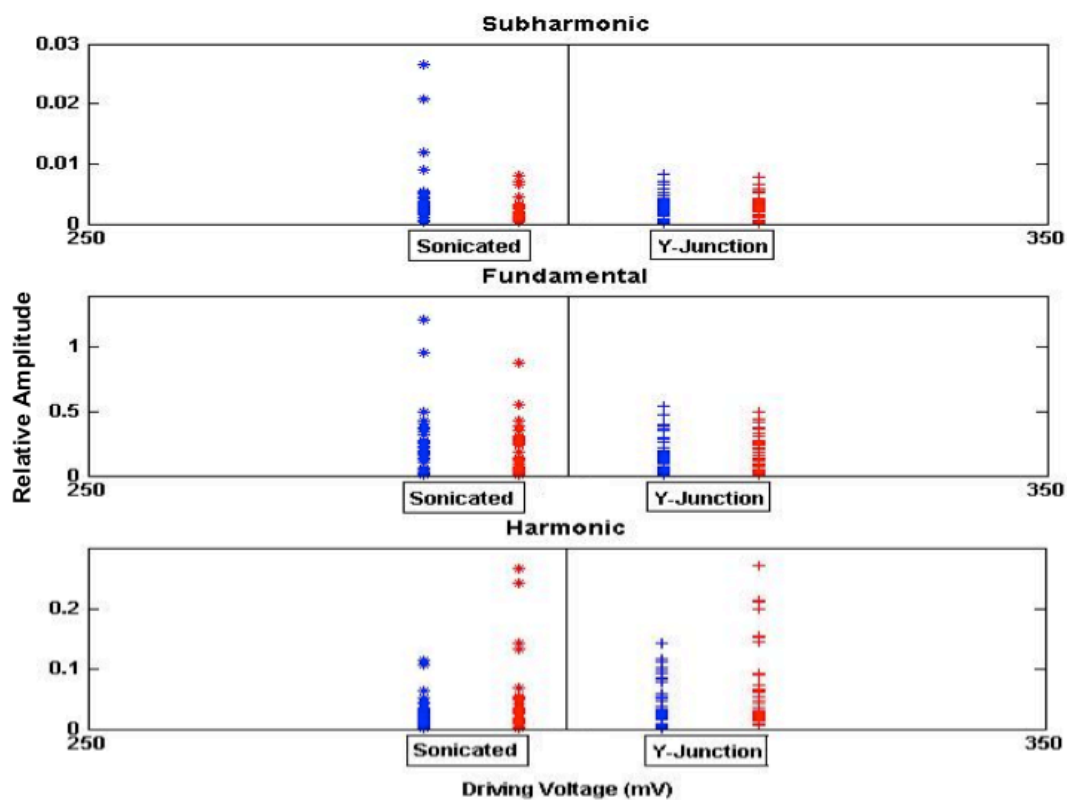


Figure 6.15 Acoustic backscatter results using 1 MHz transducer at a driving voltage of 300 mV. The subharmonic, fundamental and harmonic responses are each shown for sonicated, and Y-junction bubbles both with (red) and without (blue) gold nanoparticles.

7 The effects of manufacturing method on bubble properties

This chapter details the effects of manufacturing method on the surface properties of phospholipid microbubbles. To better understand the surface properties causing both large stability and acoustic response disparities between microfluidic and sonicated bubbles, full dissolution, FLIM, and acoustic studies were performed.

7.1 Time study

Prior to conducting any FLIM or acoustic tests, the dissolution rates of bubbles manufactured via sonication, reverse Y-junction and microfluidic flow focusing were compared (Figure 7.1). As previously discussed in Chapter 3.2.2, when bubbles are produced using sonication the liposomes are broken up not only through high mechanical shear but also as a result of the increased temperatures. This allows for a more complete break up of liposomes, which seems to increase the overall stability of the bubbles when compared to gentler manufacturing methods. As bubbles are produced much more slowly via both the cross-flow rupturing technique used in the reverse Y-junction and the flow focusing technique collecting a bubble population large enough for testing takes several minutes. The bubbles produced initially must sit in the excess solution for several minutes before they are analysed, allowing for both gas diffusion and lipid shedding to begin at different times. When originally produced, the bubbles formed are either monodisperse or have a very low polydispersity, however as seen in the histogram by the time analysis takes place the

size distribution varies wildly and the standard deviation of the mean diameter only grows with time.

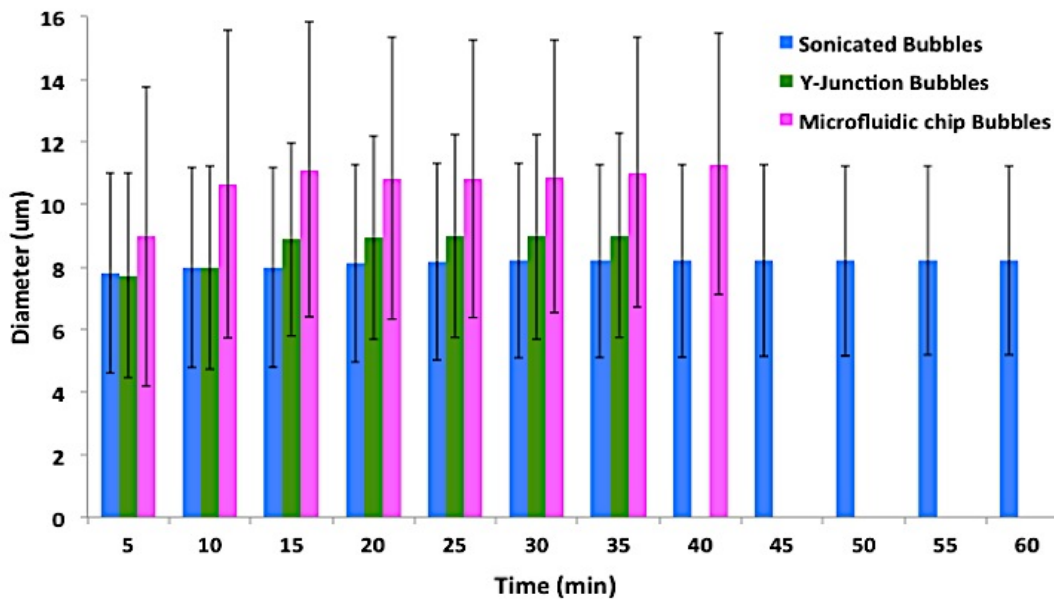


Figure 7.1 Population size distribution in relation to time. Error bars represent $\pm$ one standard deviation of the population diameter at that time. Determined via image processing. Y-junction and microfluidic bubbles either dissolved or aggregated significantly after approximately 35 min and therefore were not able to be quantified after that time point.

7.2 FLIM surface viscosity testing

For the initial test, a single phospholipid (DSPC), was used to prepare microbubbles using both a conventional sonication method (Chapter 4.1.1) and a microfluidic T-junction device (Chapter 4.1.2). The latter enables the preparation of bubbles with a high degree of control over their size and uniformity, while the former produces bubbles with a broad size distribution. The sonication method has

the advantage of producing bubbles in high yield and also with superior stability. However, the underlying reasons for the difference in stability are not fully understood. By applying the molecular rotors approach, we were able to determine the mean viscosity of T-junction microbubbles as 663 ± 119 cP (3.40 ± 0.26 ns), which was found to be significantly lower than for the sonicated bubble population at 857 ± 110 cP (3.83 ± 0.22 ns) (Figure 7.2). This is consistent with lower diffusivity and hence the greater stability of the sonicated microbubbles. This is an instance of the direct physical characterisation of the effect of preparation method upon microbubble structure, which could explain the differences in their behaviour.

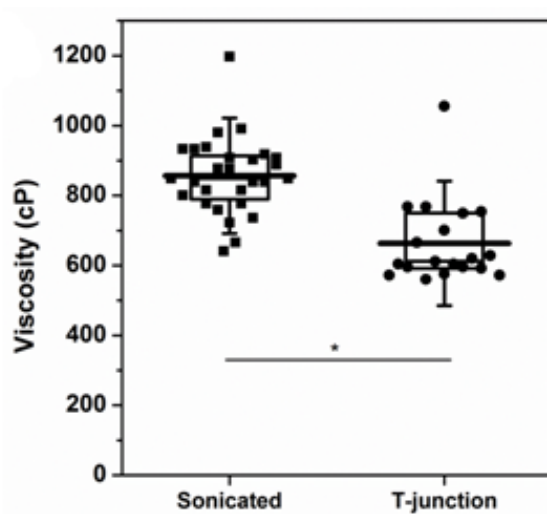


Figure 7.2 Quantification of microbubble shell viscosities. (A) Viscosity dependence on microbubble manufacturing method as measured by BODIPY- C_{10} lifetime, comparing sonication (■) ($n=28$) to T-junction preparation (●) ($n=19$) ($p < 0.001$). Determined via image processing.*

Figure 7.2 showed that manufacturing method also has a marked affect on the surface viscosity of bubbles made using the same recipe. As seen in 5.2.3, the addition of PEG had a marked affect on the effective surface viscosity of the tested microbubbles. Additional FLIM testing was completed using DSPC/PEG40S in a 9:1 molar ratio in order to see if the addition of PEG changed this effect.

When the fluorescent lifetimes and chi squared (goodness of fit) values of each of the three manufacturing processes were compared (Table 7.1), it was highly apparent that the addition of PEG exacerbated the viscosity differences seen in bubble populations. As all of the bubbles were made using the same solution, which had been premade in bulk then split, the viscosity differences cannot be attributed to slight differences in the bubble solution.

Table 7.1 Comparison of mean lifetime and goodness of fit (χ^2) for 1:9 DSPC/PEG 40S bubbles produced via sonication, cross-flow rupturing (Y-junction) and microfluidic flow focusing.

n $\approx$ 20	Sonicated	Y-Junction	Flow focusing
Mean lifetime (ns)	2.56 $\pm$ 0.015	1.697 $\pm$ 0.051	1.47 $\pm$ 0.063
χ^2	0.7577-1.277	0.6874-1.319	0.7461-1.277

The difference in lifetime found between the sonicated bubbles and the two microfluidic methods was considerable. As lifetime is correlated to effective surface viscosity on a logarithmic scale, a lifetime difference of one ns corresponds to a viscosity change of a few hundred centipoise. When the bubbles were viewed more closely there was a distinct difference in the lipid coverage of the shells (Figure 7.3).

The sonicated bubbles were seen to have a much thicker lipid coating, which explains the increased viscosity and increased stability.

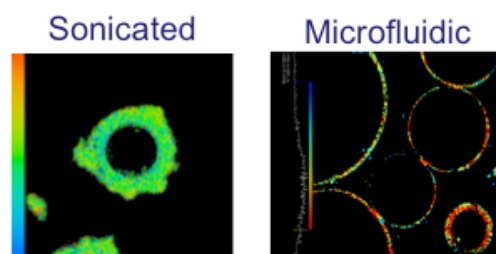


Figure 7.3 FLIM surface comparison of sonicated vs. microfluidically produced microbubbles. Note intensity scales and bubble sizes vary. This image is included only to show a distinct difference in surface character between the different bubbles.

7.3 Acoustics

Acoustic testing was conducted on bubbles prepared using sonication and reverse Y-junction. Testing was also attempted on bubbles produced using microfluidic flow-focusing but they did not survive long enough for measurements to be obtained. SonoVue® (Bracco Diagnostics, Milan, Italy) is one of the most widely used commercial UCA and therefore was chosen as a control for comparisons. The mean size of each bubble population was approximately 4 μm (11.6) so an increased subharmonic response was expected.

When the data were compared (Figure 7.4) an increased acoustic response was viewed for both sonicated and Y-junction bubbles as the incident pressure was increased. Unsurprisingly, sonicated bubbles showed a much stronger response than Y-junction bubbles.

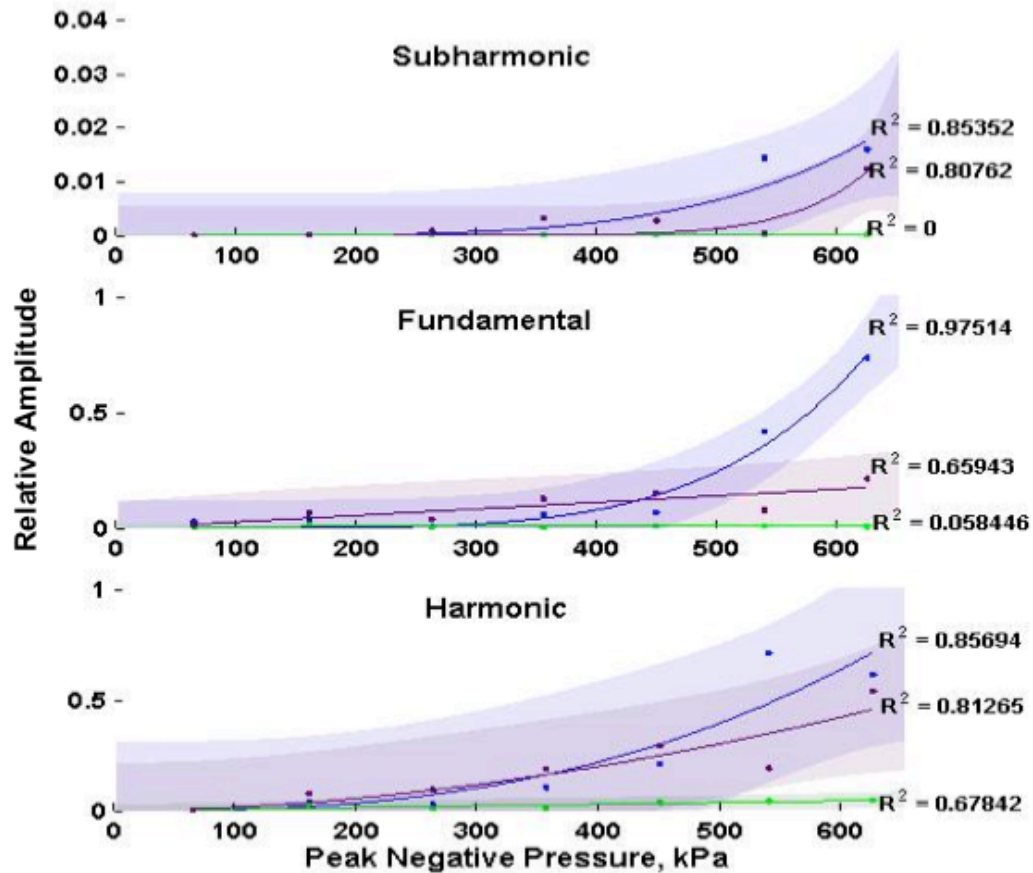


Figure 7.4 Relative amplitude of the subharmonic, fundamental and harmonic components of the scattered acoustic pressure from single microbubbles produced using both sonication (dark blue) and the reverse Y-junction device (purple) compared with SonoVue® (green) excited at different peak negative pressures at a driving frequency of 2.25 MHz.

As shown in 7.1 and 7.2 sonicated bubbles are more stable than those produced microfluidically and have a more viscous shell. The more stable a bubble, the longer it will be able to oscillate before dissolution occurs. Therefore a bubble with a more viscous shell, such as a bubble produced via sonication will demonstrate a stronger acoustic response.

SonoVue, which is the most commonly used ultrasound contrast agent in Europe, however showed very little acoustic response across than either of the other bubble types. It had been assumed that SonoVue would have the greatest response within the fundamental frequency response, however the response was quite low when compared to the strong response of the sonicated bubbles. This could be due to the abundance of submicron bubbles, which were prevalent within the SonoVue sample. The sample of SonoVue showed large Brownian motion of very small particles ($> 0.5 \mu\text{m}$), which the code used to analyse the mean size distribution disregards as too small. So it is very possible that the mean size of the SonoVue sample was actually closer to $1\text{-}2 \mu\text{m}$ as opposed to $4 \mu\text{m}$.

8 Nature vs. Nurture: How the bubble environment affects its surface properties

The environment in which a bubble is both made and maintained, contributes greatly to its behaviour. This chapter investigates the role of bubble interaction, temperature and age on viscosity and acoustic response.

8.1 Heating viscosity effects

In order to fully incorporate the lipid into the bubble solution it is typically heated above its gelling temperature. In the case of DSPC that temperature is approximately 57° C. Sonication releases a large amount of thermal energy into the surrounding media (Chapter 4.1.1) enabling the liposomes to adhere to the gas interface while in their fluid state. Microfluidically produced bubbles however typically use a bubble solution which is at room temperature. This could be a reason for the differences in viscosity between microfluidic and sonicated bubbles (Chapter 7.2). In a multi-component system, bubble partitioning and domain formation in the shell occurs. These domains govern the surface microstructure and affect the viscoelastic properties of the microbubble shell and hence their functional behavior [51, 74, 179]. By knowing the viscosity distributions of individual bubbles, they can be tuned for specific applications.

A series of tests were performed in order to look at the differences in effective surface viscosity according to not only manufacturing method, but also solution temperature.

A 20 ml bulk bubble solution of 9:1 DSPC/PEG40S was used and split into 10 vials containing 2 ml each. Using a bulk solution and splitting it evenly eliminated any variables produced by the mixing of the solution. The bubble solutions were heated to one of four temperatures (23.5, 50, 60, and 100°C) for a minimum of one hour before bubble production and subsequent FLIM testing. This was done to ensure that any liposomal changes within the solution are allowed to go to completion before testing occurred. Both sonicated and Y-Junction bubbles were tested.

The hypothesis upon beginning these tests was clear: bubble made using liposomes above their gelling point would be more viscous as more liposomes would be able to self-assemble into the bubble monolayer. Bubbles made using cross-flow rupturing showed results in keeping with the proposed hypothesis (Figure 8.1). Bubbles heated well above the gelling temperature showed the highest lifetime and therefore the highest viscosity. The lifetime range between the lowest temperature bubbles and the highest was also quite large, almost 0.7 ns. This corresponds to a very large jump in viscosity, analogous to several hundred cP. Also the solutions heated above gelling seemed to relax (reach a steady viscosity) much more quickly than those below. It is believed that by heating the solution prior to use the liposomes are allowed to more fully incorporate into the solution and self-assemble at the gas interface more efficiently.

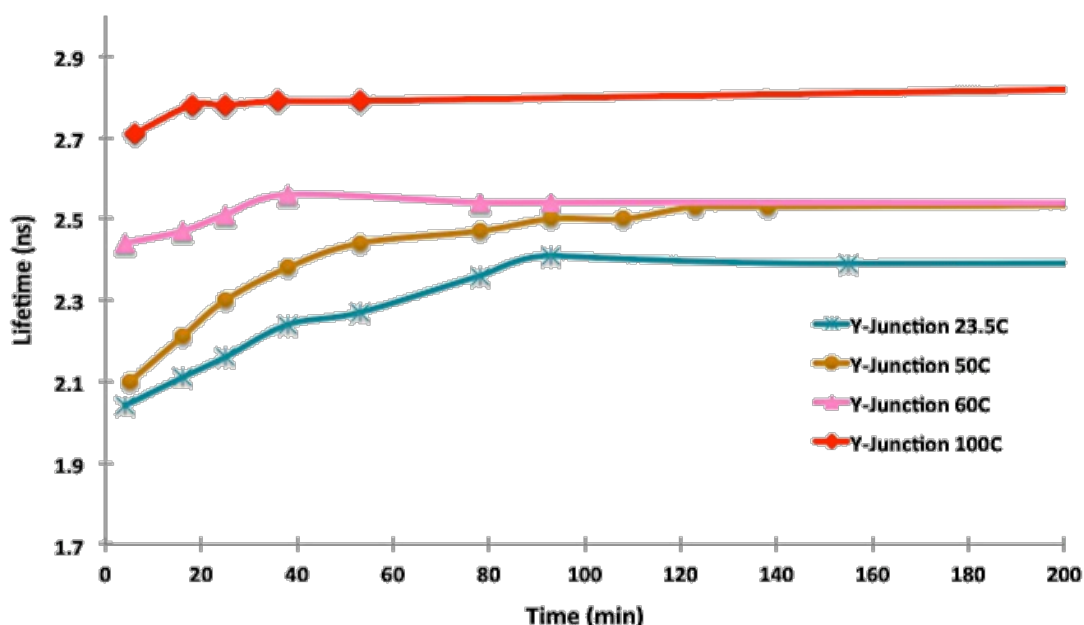


Figure 8.1 The mean lifetime plotted as a function of time for bubbles produced using a reverse Y-junction device. Each colour represents a different initial temperature (blue: 23.5 °C, orange: 50 °C, pink: 60 °C, red: 100 °C)

For bubbles made via sonication however the results were reversed. The bubbles made with solution at room temperature (23.5 °C) actually showed the highest mean lifetime and therefore the highest viscosity (Figure 8.2). The mechanisms for bubble stabilisation is different in sonication and heating will also affect gas content and hence bubble formation, which can result in a lower viscosity with increasing temperature. Within the results there seem to be two distinct viscosity regions: one for bubbles heated above the gelling temperature and one for bubbles heated below. This shows that heating either near (60 °C) or well above (100 °C) the gelling temperature makes little difference when sonication is being used.

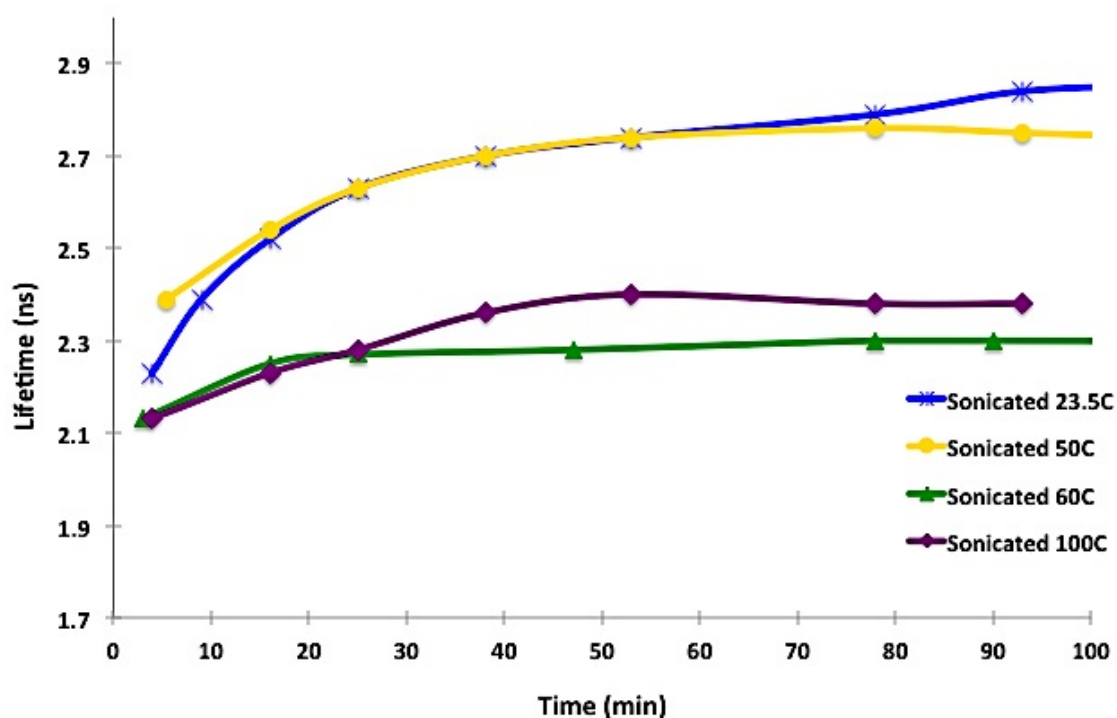


Figure 8.2 The mean lifetime plotted as a function of time for bubbles produced using sonication. Each colour represents a different initial temperature (blue: 23.5 °C, yellow: 50 °C, green: 60 °C, purple: 100 °C)

When the graphs of the two manufacturing methods are plotted together it is clear that groupings appear. This trend was seen through various iterations of the experiment. Curiously, while the overall lifetime trends were reproducible, each batch or lipid mixed seemed to have its own distinct viscosity distribution. This leads us to believe that there are other factors, such as solution/phospholipid age, temperature during the solvent evaporation process to produce the lipid film, whether the film is aspirated which also affects the overall bubble viscosity.

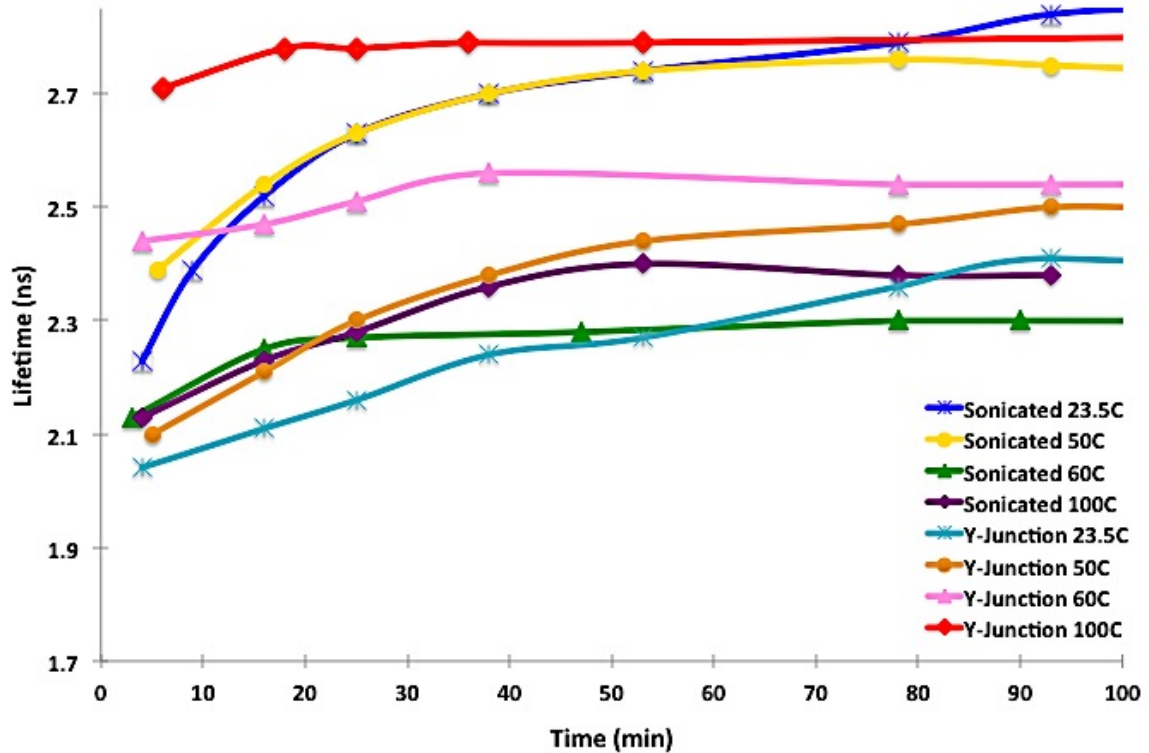


Figure 8.3 The mean lifetime plotted as a function of time for bubbles produced using a reverse Y-junction device and sonication. Each colour represents a different initial temperature. The Y-Junction is represented by light blue: 23.5 °C, orange: 50 °C, pink: 60 °C, red: 100 °C; sonication is represented by dark blue: 23.5 °C, yellow: 50 °C, green: 60 °C, purple: 100 °C.

8.2 Aging effects

Over time bubbles reach a packing density in which they reach their most stable point. However where that lies is difficult to define. Because of diffusion, Ostwald ripening, and lipid shedding the bubble shell continues to change until total dissolution. Here we look at viscosity and acoustic response of bubbles made using cross-flow rupturing and allowed to age for up to one week.

8.2.1 FLIM

In the last section we showed that bubbles made from solutions heated to different temperatures seem to have different points of relaxation (where the bubble's viscosity becomes steady). However the effect of gas diffusion over long periods (<24 hrs) was neglected. No matter how stable, bubbles will undergo gas diffusion. As the gas diffuses and the bubbles become smaller, the Laplace pressure becomes higher and so the phospholipids at the gas interface will rearrange themselves in an attempt to lower their free energy. If lipid shedding is ignored, this process should theoretically create a “thicker” or more viscous bubble shell with time. Here the Y-junction bubbles from the prior experiment (Figure 8.1) were aged for 72 hours and then reimaged (Figure 8.4, Figure 8.5).

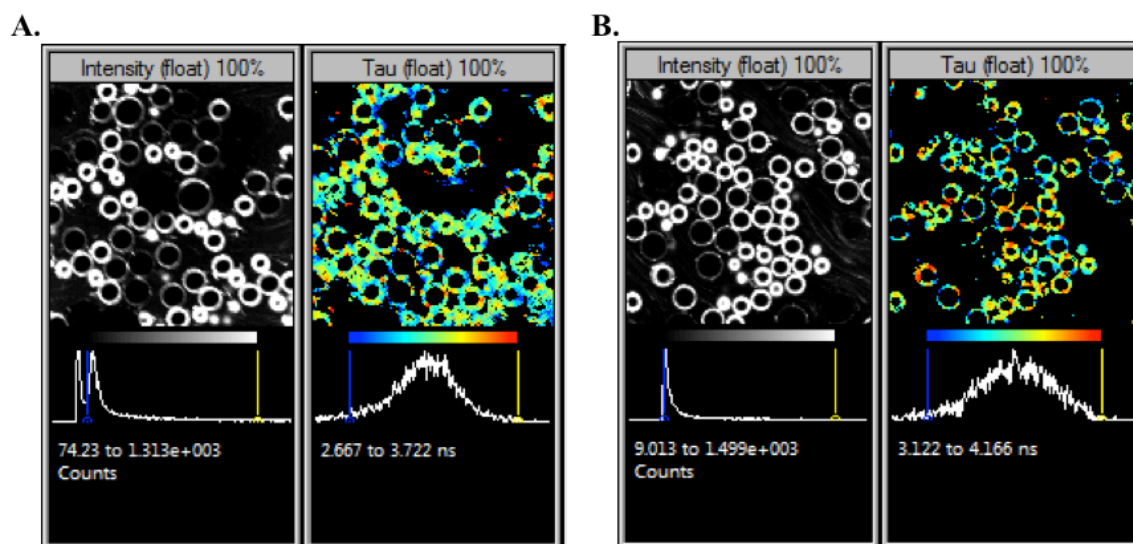


Figure 8.4 Y-junction bubbles heated to 100 °C viewed with FLIM at A. 18 min after manufacture and B. 72 hours after manufacture.

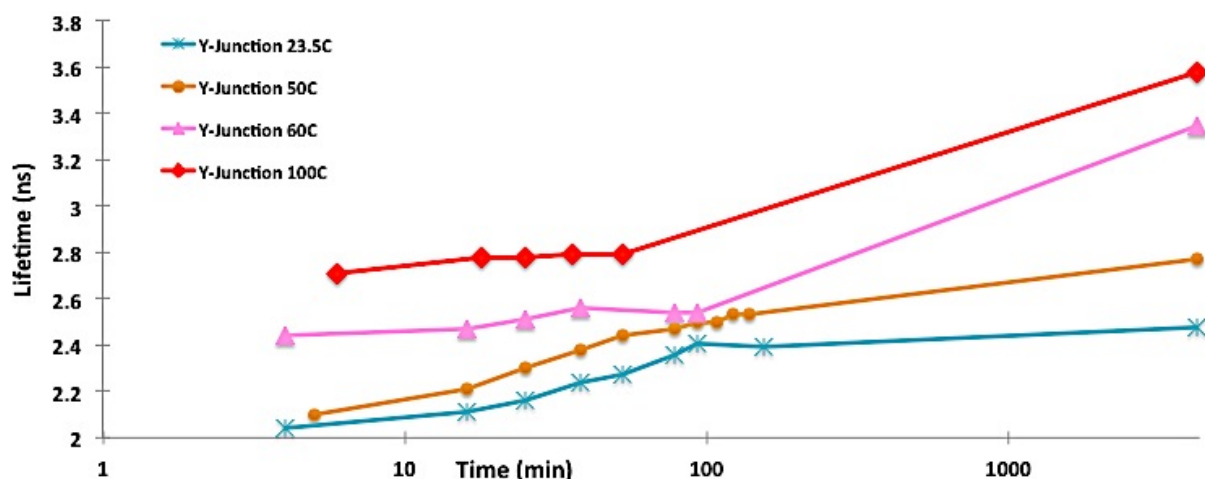


Figure 8.5 The mean lifetime plotted as a function of time for bubbles produced using a reverse Y-junction device. Each colour represents a different initial temperature (blue: 23.5 °C, orange: 50 °C, pink: 60 °C, red: 100 °C). Time has been plotted on a logarithmic scale so that the overall trend can be more easily viewed.

Table 8.1 Change in bubble lifetime as it corresponds to both time and temperature of the initial solution

n ≈ 100	T= 23.5 °C	T= 50 °C	T= 60 °C	T= 100 °C
Initial lifetime (ns)	2.04	2.1	2.44	2.71
72 hour lifetime (ns)	2.48	2.77	3.35	3.58
Change (ns)	0.44	0.67	0.91	0.87
%	21.57	31.90	37.29	32.10

The fact that all of the bubbles showed an increasing viscosity over time was not surprising, however the percentage increase was higher than expected. Final bubble viscosity increased by up to 37%, indicating a large agglomeration of lipid on the bubble surface. More testing is needed to confirm that this is the reason for the drastic viscosity changes observed. Also it must be noted that while mean viscosity is

plotted there were large variations in viscosity within each bubble as can be seen in Figure 8.4. Therefore while the standard deviation of the mean lifetimes calculated were approximately ± 1 ns, the compounded error cannot be determined without significant further analysis by Dr. Hosny.

8.2.2 Acoustics

Like viscosity, as the bubbles age, their non-linear acoustic response also increases. When the responses of freshly made Y-junction bubbles, both control and nanoparticle coated, (Figure 6.10) are compared with those of bubbles aged one day (Figure 8.6) the amplitude of the subharmonic seems to decrease while the fundamental increases. The harmonic response of both gold coated and lipid only coated bubbles shows the most pronounced change as the amplitude more than doubles. The response for gold-coated bubbles is especially strong as the peak negative pressure increases; at 650 kPa the response is approximately five times higher than that of fresh gold-coated bubbles. This indicated that the gold particles are “jamming” on the bubble surface, thereby not allowing the bubble to contract during oscillations. As the bubbles have also begun to undergo gas diffusion, jamming is increased. While the diameter of the bubbles has decreased the concentration of gold on the surface does not change. This “jamming” effect also occurs on non-particle coated bubbles, but the effects are not as pronounced.

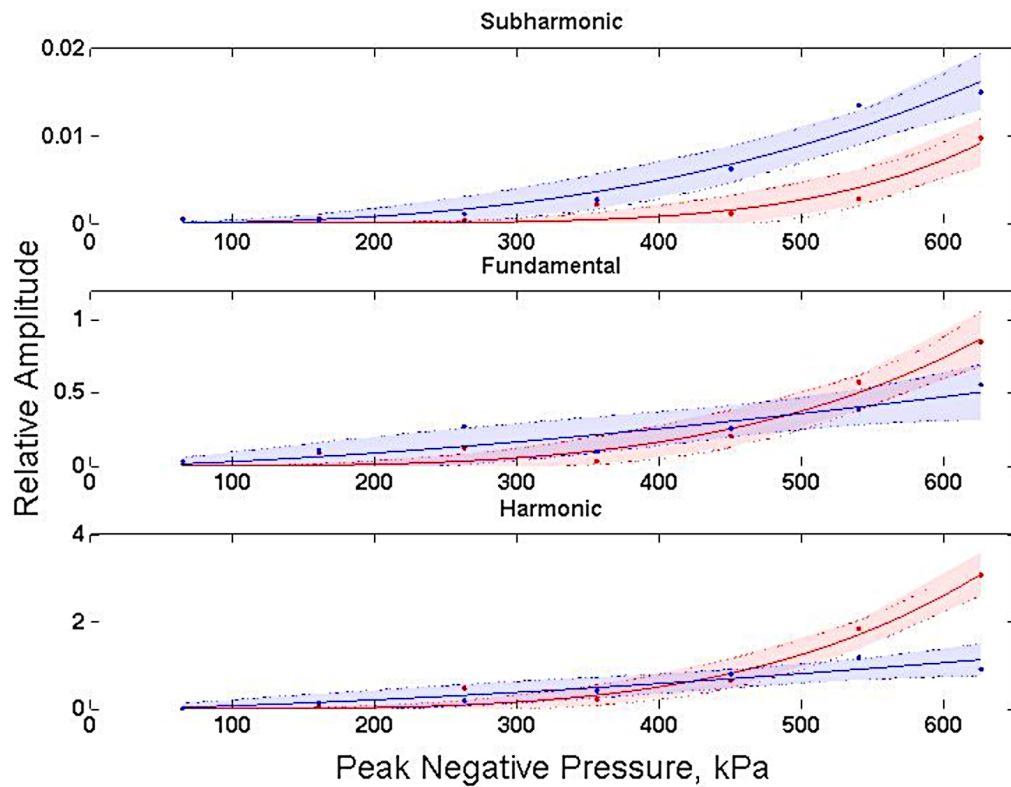


Figure 8.6 Relative amplitude of the subharmonic, fundamental and harmonic components of the scattered acoustic pressure from one-day-old single microbubbles produced using the reverse Y-junction device with (red) or without (blue) a coating of gold nanoparticles excited at different peak negative pressures.

9 Conclusions and future work

This chapter details the conclusions drawn from the studies described in the previous chapters and proposes areas for future work.

Most models used to predict microbubble dynamics view the bubbles as having a cohesive monolayer shell with uniform thickness and viscosity. The work shown throughout this manuscript demonstrates that this is not the case, and bubbles therefore should not be modelled as having homogeneous shells. This work also shows that it is possible to tune bubbles for specific applications simply by changing its method of manufacture and thereby changing the physical characteristics of the microbubbles. Further characterisation of microbubble behaviour both in-vitro and in-vivo however is necessary prior to the application of these bubbles in clinical applications.

9.1 Conclusions

The overall contribution of this work is the demonstration that the physical properties of bubbles depend not only upon surface composition, but also the method and environmental conditions under which they are manufactured and suspended.

Specifically:

9.1.1 Surface composition effects

Experimental results showed that the lipid to emulsifier molar ratio had a large effect on the stability, surface viscosity and acoustic response of the bubbles. The

addition of the emulsifier PEG 40S decreased both the size and surface viscosity of the bubbles. The amplitude and frequency content of the scattered pressure of bubbles under ultrasound exposure also varied with composition. The distribution of molecules on the bubble surface itself was shown to be inhomogeneous possibly due to a lack of fluidity. Most importantly, the experiments showed that a bubble shell is not continuous, but instead made up of domains of varying viscosities, and therefore cannot be modelled as a continuous shell as is the current standard.

9.1.2 Au and non-Au coated bubble comparisons

Both the theoretical and experimental results demonstrate that the modification of the microbubble surface through the addition of solid nanoparticles not only significantly influences the bubbles' stability but also their acoustic response. This is thought to arise from the increased asymmetry in the microbubble oscillation produced by the presence of the particles in the coating, which leads to increased resistance to compression. This phenomenon is potentially beneficial for improving contrast to tissue ratio in microbubble imaging and for tracking of microbubbles used for drug delivery or other therapeutic applications, in particular since a pronounced enhancement in the subharmonic response was observed experimentally. As expected, with increasing incident pressure bubble response became more non-linear, but this effect was more pronounced for the gold-coated bubble, especially when aged for 24 hours. Increased non-linearity was more pronounced for the sonicated bubbles, possibly due to differences in the nature of the coating.

9.1.3 Manufacturing comparisons and viscosity

The method of manufacturing made a large impact on the acoustic and physical properties of the corresponding bubble. A surface viscosity difference was clearly seen when sonicated bubbles were compared with those produce using microfluidics. Sonicated bubbles were seen to have a “fuzzy” shell layer with a higher average viscosity. Microfluidics produced bubbles with a lower effective viscosity than sonication, most likely due to a lack of liposome adhesion at the gas water interface. Heating the bubble solution was seen to overcome this lack of adhesion in microfluidics as the surface viscosity increased when the solution was heated above gelling temperature. Acoustically, sonicated bubbles showed a much higher fundamental and incrementally higher subharmonic and harmonic responses, most likely due to their increased stability which was in turn due to an increase in lipid deposition during the manufacturing process. By choosing one method over the other bubbles can be tuned to have the specific properties desired, whether that be a small polydispersity, ease of manufacture or increased stability.

9.2 Future work

While the results presented in the previous sections suggest that the overall objectives of the project have been achieved substantial work remains to produce a microbubble with the full range of desired characteristics.

9.2.1 Stabilising effects of different gasses i.e. Ar and perfluorobutane

The most common type of gas used to produce commercially available microbubbles is a perfluorocarbon (PFC). Typically PFCs have a high density, up to over twice that of water, due to their high molecular weight when compared to liquids of similar boiling points. They also have low viscosities and surface tension and are not miscible with most organic solvents. Lastly they have very low solubility in water, meaning that diffusion of the gas out of the bubble while it is suspended in an aqueous solution will be extremely low. However in vivo, gas exchange with Nitrogen (N_2) and CO_2 may occur as the bubble flows through the blood stream, so the stability benefits of the PFC may be limited to the long-term storage of microbubbles.

Argon, while much lighter than PFCs is over twice as heavy as Nitrogen: the gas currently used in bubble production. Ar has a high solubility in water, but is also inert. If the bubble solution is saturated with Ar and the created bubbles are stored in an Ar rich environment, diffusion may be limited. As a result bubbles filled with N_2 , Ar and PFC should be compared in order to determine the stability benefits of differing the filling gas.

9.2.2 Viscosity comparisons

Acoustic attenuation measurements in contrast agent suspensions have been used in order to calculate the surface dilatational viscosity, surface tension and surface dilatational elasticity of the bubble shell [180]. This poses a problem, however, since

bubble size and surface properties vary across a population and change over time. The method described in this thesis based on FLIM viscosity measurements to be made on the surfaces of individual bubbles. It may be possible to link surface effective viscosity to the attenuation coefficient of an individual bubble by comparing measured or known attenuations with FLIM surface viscosity measurements. Thus allowing models for acoustic response, dilatational viscosity and surface elasticity to be parameterised based upon a simple FLIM measurement.

9.2.3 Characterisation of the bubble surface using TEM

Previously optical microscopy and SEM have been used in order to characterise the bubble and its surface. Conventional optical microscopy can image the overall size and shape of the bubbles, but it is limited in spatial resolution and to surface features. The thickness of the bubble coating as well as the configuration of the nanoparticles on the bubble surface cannot be viewed. SEM also has large limitations due to the need for a complete vacuum within the chamber, thus only collapsed bubbles can be imaged. Therefore in order to fully characterise the microbubble surface low temperature transmission electron microscopy (LTTEM) should be used.

In traditional transmission electron microscopy (TEM), a beam of electrons is transmitted through an ultra thin specimen, interacting with it as the beam passes through, and an image is formed from the interaction of the electrons. TEMs are capable of imaging at a significantly higher resolution than light microscopes, owing

to the small de Broglie wavelength of electrons. This enables the instrument's user to examine fine detail, which is tens of thousands times smaller than the smallest resolvable object in a light microscope. Traditional TEM however still requires the specimen to be held in vacuum.

LTTEM allows the observation of specimens that have not been stained or fixed in any way, therefore showing them in their native environment. This occurs simply because the TEM observation of the sample is being conducted at sub-zero temperatures. The samples are imbedded in a vitrified layer of water, thus hydrated samples can be viewed without the risk of collapse or dehydration. By viewing the bubbles under LTTEM the surface conditions of the microbubbles can be observed, including the packing density of the nanoparticles, the thickness of the bubble shell, and the inherent microstructure of the surface.

9.2.4 Imaging bubbles in a tissue mimicking phantom

In vitro studies to assess the effectiveness of nanoparticle coated microbubbles for ultrasound contrast enhancement should be completed in a suitable tissue phantom. A gelatine/agar mix can be used to match tissue properties, and a clinical ultrasound scanner to look at contrast specific imaging. A schematic for a proposed experiment is shown in Figure 9.1.

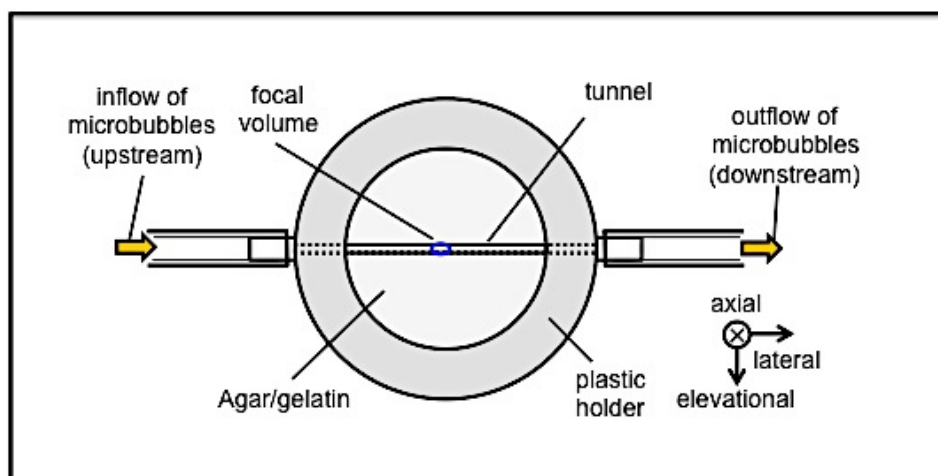


Figure 9.1 Schematic representation of flow phantom imaging tests set-up

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11 Appendices

11.1 Bubble formulations

Concentration		
Lipid Stock	3.80E-03	(Chloroform 3.33ml + DSPC 200mg)
Dye Stock	4.22E-06	
Dye Range (ul)	Concentration	Lipid/dye ratio
0.5	1.05E-06	3600.00
1	2.11E-06	1800.00
2	4.22E-06	900.00
4	8.44E-06	450.00
7	1.48E-05	257.14
8	1.69E-05	225.00
16	3.37E-05	112.50
32	6.75E-05	56.25
64	1.35E-04	28.13

Concentration effect

- 1) Add dye range concentrations to 100 µl of lipid stock (glass bijou)
- 2) Vortex to ensure good mixing
- 3) Evaporate under N₂ until dry film forms
- 4) Add 2ml of de-ionized water to dried film
- 5) Vortex, bath sonicator for 1 min no temp, if still attached to wall use spatula to scrap off
- 6) Transfer solution to plastic bijou tube
- 7) Sonicate for 30 sec (keep in bottom of solution for 25 sec and raise to top of solution for 5 sec) and shake for 30 sec
- 8) Immediately remove microbubbles from interface of water and bubble and transfer to slide with glycerol drop.

PEG:DSPC ratio

- 1) Add 2 μ l dye stock to 100 μ l of lipid stock (glass bijou)
- 2) Vortex to ensure good mixing
- 3) Evaporate under N₂ until dry film forms

PEG:DSPC	Stock PEG solution (ul)	Add dH ₂ O (ul)
1:4	77.64	1922.36
1:1	310.57	1689.43

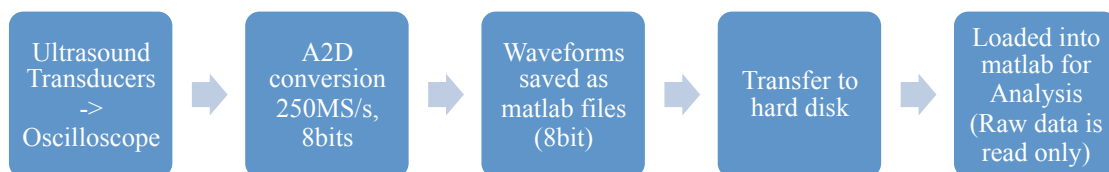
- 4) Mix stock solution PEG quantities with distilled water to reach final volume of 2ml
- 5) Add the 2ml PEG+dH₂O solution to the lipid dried films
- 6) Vortex, bath sonicator for 1 min no temp, if still attached to wall use spatula to scrap off
- 7) Transfer solution to plastic bijou tube
- 8) Sonicate for 30 sec (keep in bottom of solution for 25 sec and raise to top of solution for 5 sec) and shake for 30 sec
- 9) Immediately remove microbubbles from interface of water and bubble and transfer to slide with glycerol drop.

11.2 *Signal processing flow chart for single bubble characterisation*

The signal processing can be split into 4 different processes:

1. Data acquisition
2. Processing
3. Frequency analysis
4. Weighted averaging / Regression analysis

11.2.1 Data Acquisition



11.2.2 Processing



*Erroneous signals include too much noise to distinguish bubble scatter, multiple bubble scatters at different time points, air bubbles (normally noted in log book during experiments) and periodic noise from unknown source (possible to do with receiving amp)

11.2.3 Frequency Analysis

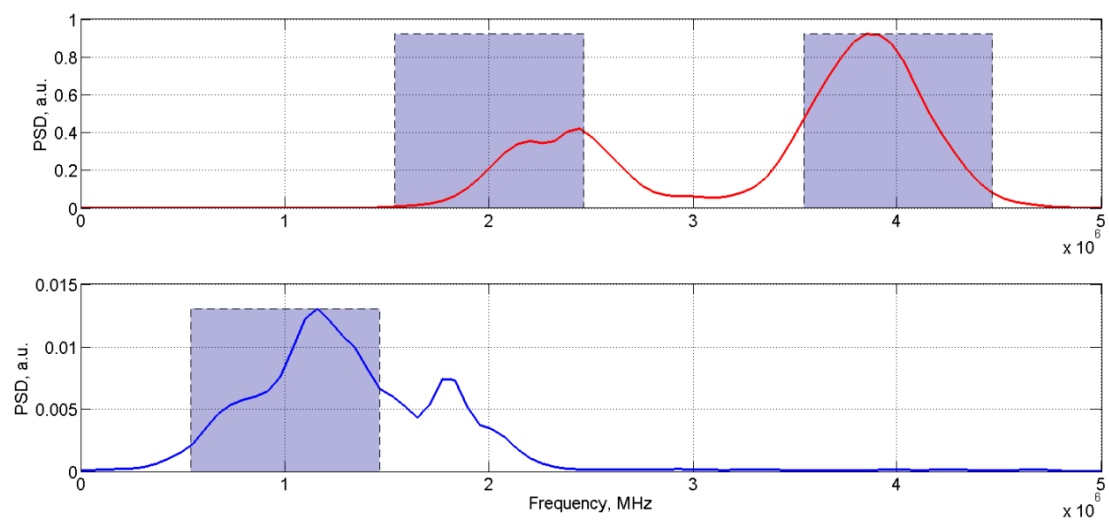
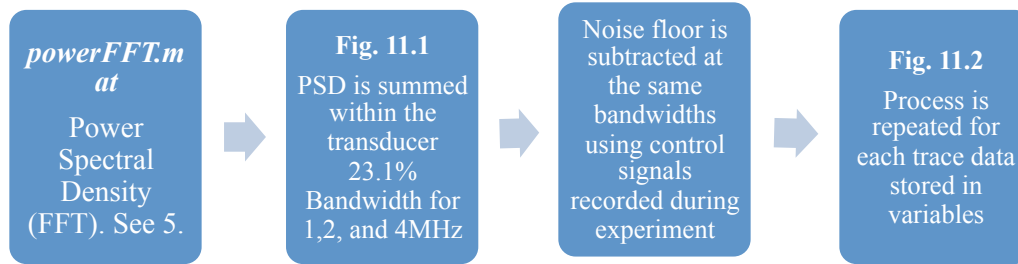


Figure 11.1 Average PSD of single microbubbles at 2MHz and 200kPa. The two graphs are the two receiving transducers. Shaded areas show the 23.1% bandwidth (corresponding to the driving waveform) for the subharmonic, fundamental and 2nd harmonic components. Note, not corrected for transfer functions of transducers.

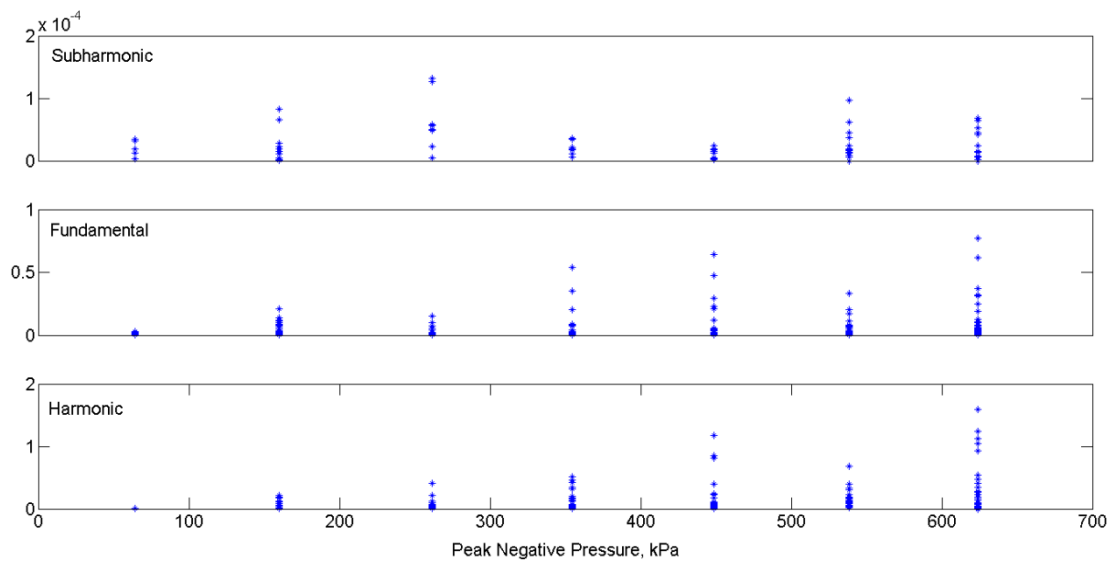
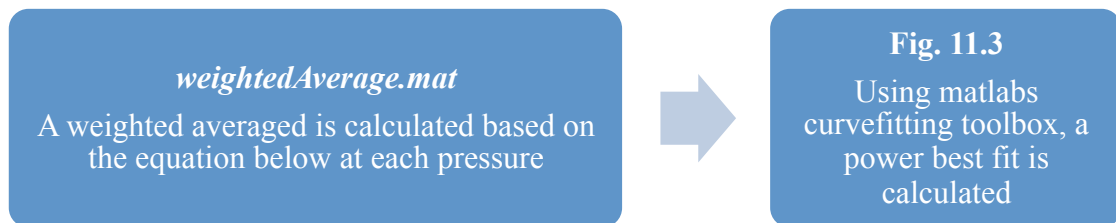


Figure 11.2 Summed power of single microbubbles at subharmonic, fundamental and harmonic. Each blue cross represents a single microbubble trace.

11.2.4 Weighted Averaging / Regression analysis



$$\overline{M}_{weighted} = \sqrt{\frac{\sum_{n=1}^N X_n^2}{N}}$$

$$y = ax^b$$

$$\text{Where } X_n = \sum P SD|_{F(-6DB)}$$

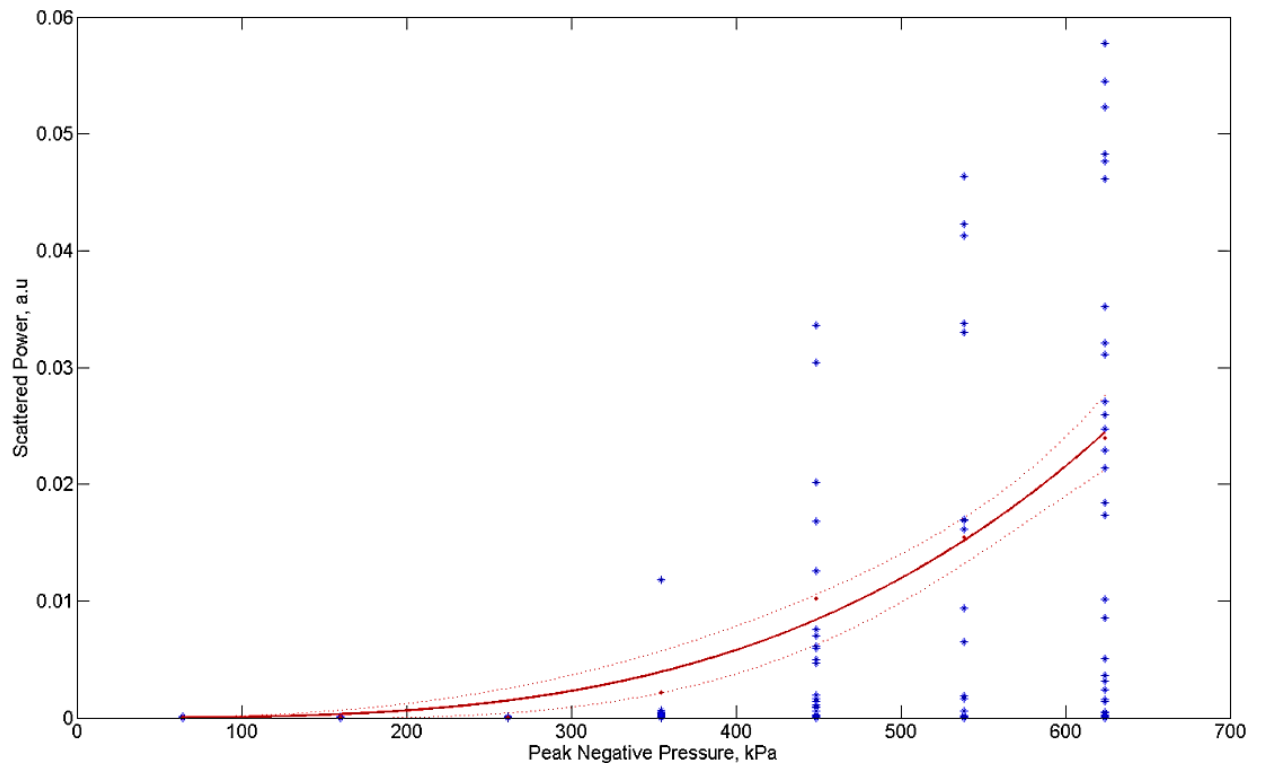
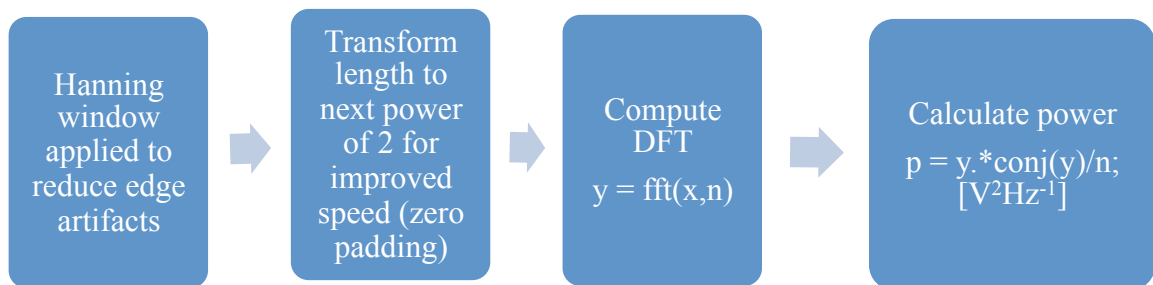


Figure 11.3 Exponential fit (red solid line) on acoustic data based on the weighted mean average at each pressure (red dots). Dashed lines represent the observational confidence intervals at 95%, described in appendix.

11.2.5 Spectral Power flow chart



11.2.6 Matlab code

11.2.6.1 TidyUp.mat

```
function [data, Fs] = TidyUp(data_in)

    data(:,2) = data_in(:,2) - mean(data_in(:,2)); % Remove mean
    Fs = (1/(data_in(2,1)-data_in(1,1))); % Calculate
    Sampling frequency
    Ts = 1/Fs; % Interval size
    % Change timing, so samples start at 0 seconds.
    T = 0:Ts:length(data_in)*Ts-Ts
    data(:,1) = T';

end
```

11.2.6.2 powerFFT.mat

```
function [X,freq]=powerFFT(x,fs)

    % Apply hanning window
    x = x.*hanning(length(x));
    m = length(x); % Window length
    n = pow2(nextpow2(m)); % Transform length
    y = fft(x,n); % DFT of signal
    f = (0:n-1)*(fs/n); % Frequency range
    p = y.*conj(y)/n; % Power of the DFT

    freq = f(1:floor(n/2));
    X = p(1:floor(n/2));

end
```

11.2.6.3 weightedAverage.mat

```
function [fitresult,meanW] = weightedAverage(data,peakNegative,bubName)

    for k = 1:length(bubName) % Loop through each pressure
        meanW(k) = sqrt(mean(data(data(:,k)>0,k).^2));
    end

    % Regression fitting:
    [xData, yData] = prepareCurveData( peakNegative, meanW );

    % Set up fittype and options.
    ft = fittype( 'power1' );
```



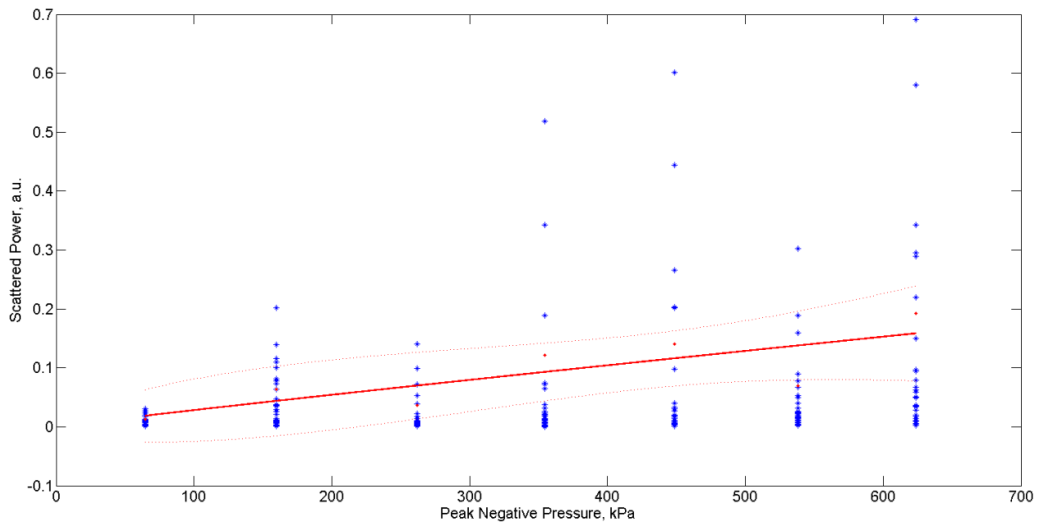
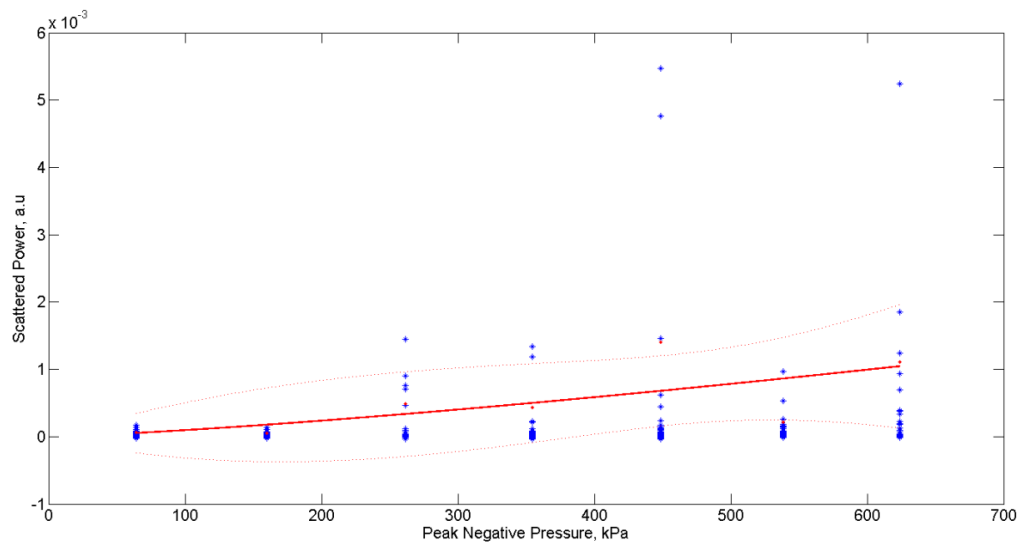
```

opts = fitoptions( ft );opts.Display = 'Off';
opts.Lower = [-inf -inf];
opts.Upper = [Inf Inf];
opts.StartPoint = [0 0];
% Fit model to data.
[fitresult] = fit( xData, yData, ft, opts );

```

```
end
```

11.2.7 Example Fits



The exponential fit appears to be robust to noise and outliers as in the above examples, the confidence intervals ($p < 0.05$) are much larger for these cases with potential outliers/noise

11.2.8 Confidence intervals

$$\mathbf{P}_{n,o} = \mathbf{y} + t\sqrt{s^2 + \mathbf{xSx}^T}$$

Where s^2 is the mean squared error, t is calculated using the inverse cumulative distribution function and S is a covariance matrix of the fitting coefficient estimates

<http://www.mathworks.co.uk/help/curvefit/confidence-and-prediction-bounds.html>

11.3 Theoretical models describing the bubble coating

The model presented here was developed by Mr. Mehrdad Azmin and corresponds to Chapter 6.2.1. Here we make use of we make use of two different methods of describing the bubble coating, as a viscoelastic layer of finite thickness and a surfactant monolayer. The effect of the particles is included by defining stiffness and viscosity terms that are functions of the radius.

11.3.1 Dynamic Models

The dynamic model proposed by Church [181], originally developed to model albumin coated Albunex®, was used for a microbubble with a finite thickness shell.

$$\rho_S R_1 \ddot{R}_1 \left[1 + \left(\frac{\rho_L - \rho_S}{\rho_S} \right) \left(\frac{R_1}{R_2} \right) \right] + \rho_S \dot{R}_1^2 \left[\frac{3}{2} + \left(\frac{\rho_L - \rho_S}{\rho_S} \right) \left(\frac{4R_2^3 - R_1^3}{2R_2^3} \right) \left(\frac{R_1}{R_2} \right) \right] =$$

$$p_0 \left(\frac{R_{01}}{R_1} \right)^{3\kappa} - \frac{2\sigma_1}{R_1} - \frac{2\sigma_2}{R_2} - p_0 - p_A - \frac{4\dot{R}_1}{R_1} \left(\frac{V_S \mu_S(\phi) + R_1^3 \mu_L}{R_2^3} \right) - \frac{4V_S G_S(R)}{R_2^3} \left(1 - \frac{R_{e1}}{R_1} \right) \quad \text{Equation 11.1}$$

Subscripts 1 and 2 denote values at their respective interfaces. Subscripts S and L denote the properties at shell and the surrounding liquid. R is the instantaneous radius and the dots indicate its derivatives with respect to time. R_{01} is the radius of interface 1 at $t=0$. Density is denoted by ρ ; p_A and p_0 refer to acoustic and atmospheric pressures; κ the polytropic exponent, σ is the interfacial tension; μ is viscosity, ϕ denotes particles to shell volume fraction and G_S is the modulus of rigidity. Expressions for V_S and R_{e1} (the unstrained radius) are given by:

$$V_S = R_{02}^3 - R_{01}^3 \quad \text{Equation 11.2}$$

$$R_{el} = R_{01} \left[1 + \left[\frac{2\sigma_1}{R_{01}} + \frac{2\sigma_2}{R_{02}} \right] \left[\frac{R_{02}^3}{V_s} \right] \frac{1}{4G_{s0}} \right]$$

Equation 11.3

was applied to the limit of a thin shell by Hoff [57] . The resulting equation was used in modelling monolayer shelled microbubbles.

$$\rho_L \left(R\ddot{R} + \frac{3}{2} \dot{R}^2 \right) =$$

$$p_0 \left(\frac{R_0}{R} \right)^{3\kappa} - p_0 - p_A - \frac{4\mu_L \dot{R}}{R} - 12\mu_S \left(\frac{d_{s0} R_0^2}{R^3} \right) \left(\frac{\dot{R}}{R} \right) - 12G_S(R) \frac{d_{s0} R_0^2}{R^3} \left(1 - \frac{R_0}{R} \right)$$

Equation 11.4

Here d_{s0} is the thickness of the shell, $R_0 = R_{01}$, and all other variables have their usual meaning. Note that in this scenario, the shell viscosity is assumed not to be affected by the presence of particles.

11.3.1.1 Radius Dependent Stiffness

It is assumed that the nanoparticles are adsorbed at the gas/liquid interface inside of the microbubble and that the area between the particles at the interface is occupied by an initial concentration of lipid molecules. As the interfacial radius decreases, the particles and lipid molecules will require more energy to move due to their decreasing proximity. This effectively increases the stiffness of that interface. This continues until the particles reach their packing density at which point no further shrinkage can occur (Figure 11.4). This effect can be modelled by an exponential function of the form:

$$G_S(R) = G_{S0} a \left[\frac{A_{Fi}}{A_{Fmax}} \right]^b \quad \text{Equation 11.5}$$

$$A_{Fi} = A_{F0} \left(\frac{R_0}{R_i} \right)^2 \quad \text{Equation 11.6}$$

where a and b are determined by the properties of the lipid and G_{S0} is the stiffness of the particle free shell and A_{F0} and A_{Fi} are, respectively, the initial and instantaneous area fractions covered by particles. A_{Fmax} is the maximum square packing density and R_0 and R_i are the initial and instantaneous radii of the microbubble.

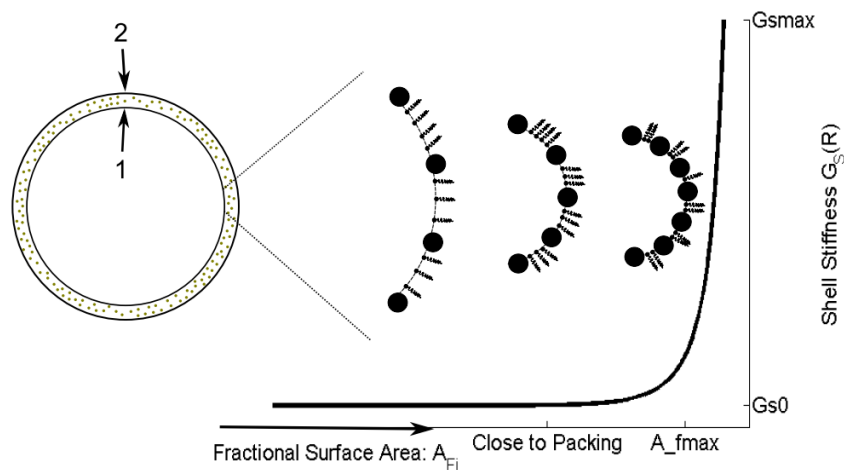


Figure 11.4 Schematic of the effect of nanoparticles upon shell stiffness. As the internal face contracts, the particles become ever closer to each other, eventually reaching their packing density and thus dramatically increasing the shell stiffness.

11.3.2 Effective Viscosity

It is well known that dispersion of colloids in a liquid, such as nanoparticles in a shell of finite thickness, will increase its effective viscosity. This phenomenon, in the case of dilute suspensions of hard spherical particles, was considered by Einstein [182] and was later extended to higher volume fractions by Beenakker up to the second order [129]:

$$\mu(\phi) = \mu_0 \left(1 + 2.5\phi + 4.84\phi^2 + o(\phi^3) \right) \quad \text{Equation 11.7}$$

where μ_0 and μ are the pure fluid and effective viscosities. It is evident that the effect of such particles upon viscosity depends on the volume they occupy rather than their size.

11.3.3 Modelling

A script containing Equations 11.5 and 11.6 was written in MATLAB 2012a 64-bit (The MathWorks, Inc., USA) and solved using the 4th order Runge-Kutta function 'ode45.m'. In order to ensure the accuracy of the solution, the optional tolerance parameter 'RelTol' in 'odeset.m' was set to $o(10^{-14})$. In the absence of consistent experimental data for shell parameters, the values listed below were used for the purpose of conducting a qualitative analysis. These are $p_0 = 0.1 \text{ MPa}$, $\kappa = 1.4$, $\rho_L = 1.0 \text{ g/cm}^3$, $\mu_L = 1 \text{ mPa.s}$, $\rho_S = 1.1 \text{ g/cm}^3$, $\mu_S = 8 \text{ Pa.s}$, $G_{S0} = 150 \text{ MPa}$, $\sigma_1 = \sigma_2 = 0$. $R_0 = R_{01} = 4 \mu\text{m}$. Where nanoparticles were present, $A_{F\text{max}} = 0.72$, $a = 1.4$ and $b = 20$. It was assumed that the initial particle concentration was near maximum packing

density at $A_{Fi} = 0.69$. It was also assumed that the microbubble had a monolayer shell of 1nm thickness and therefore equation 4 was used to simulate its oscillations.

11.4 Microbubble size distribution used during acoustic testing of nanoparticle coated microbubbles

Size distributions for Chapter 6.2.2

11.4.1 2.25 MHz test

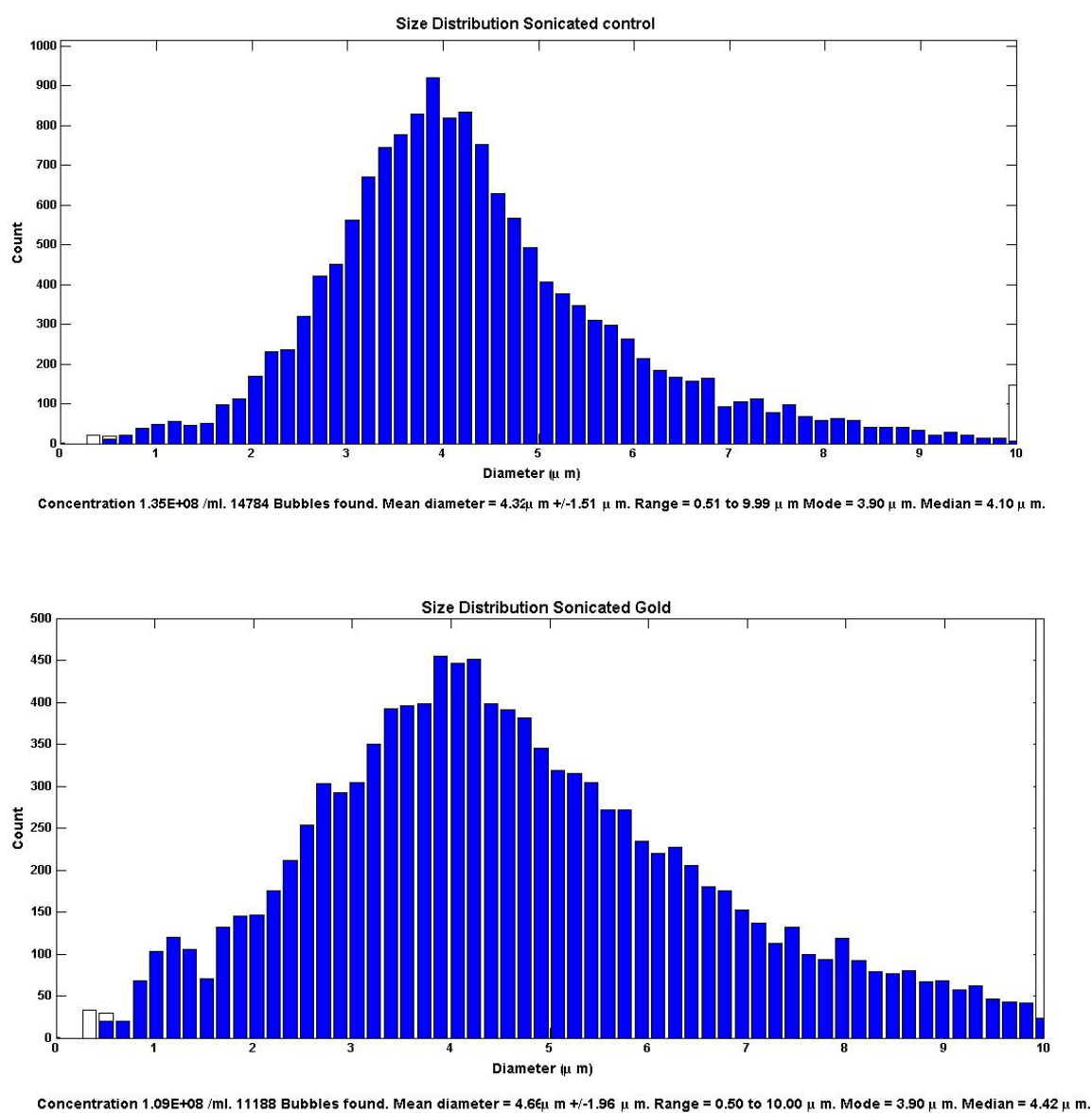


Figure 11.5 Size Distribution of microbubbles produced via sonication both without (top) and with (bottom) the addition of gold nanoparticles.

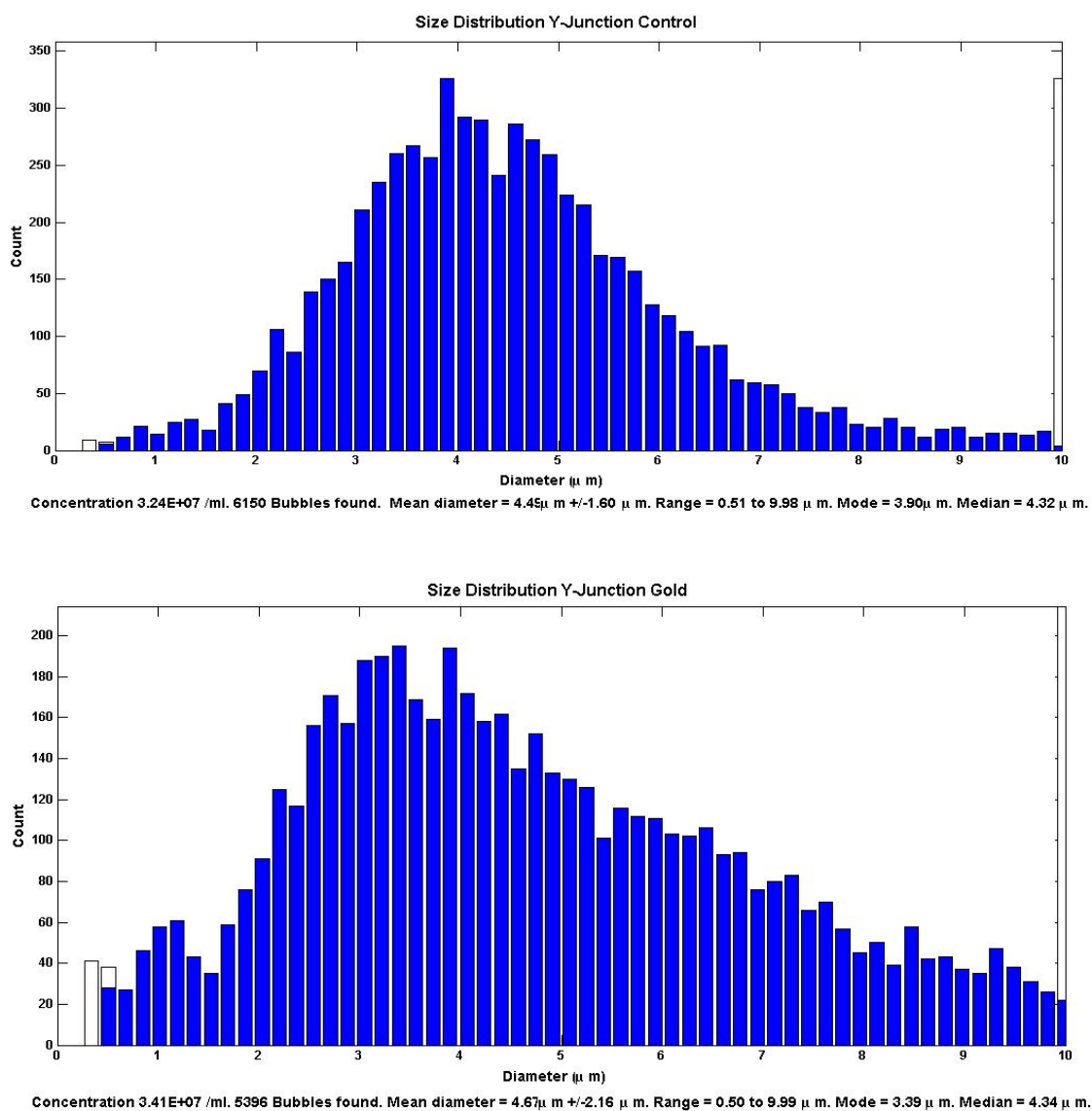
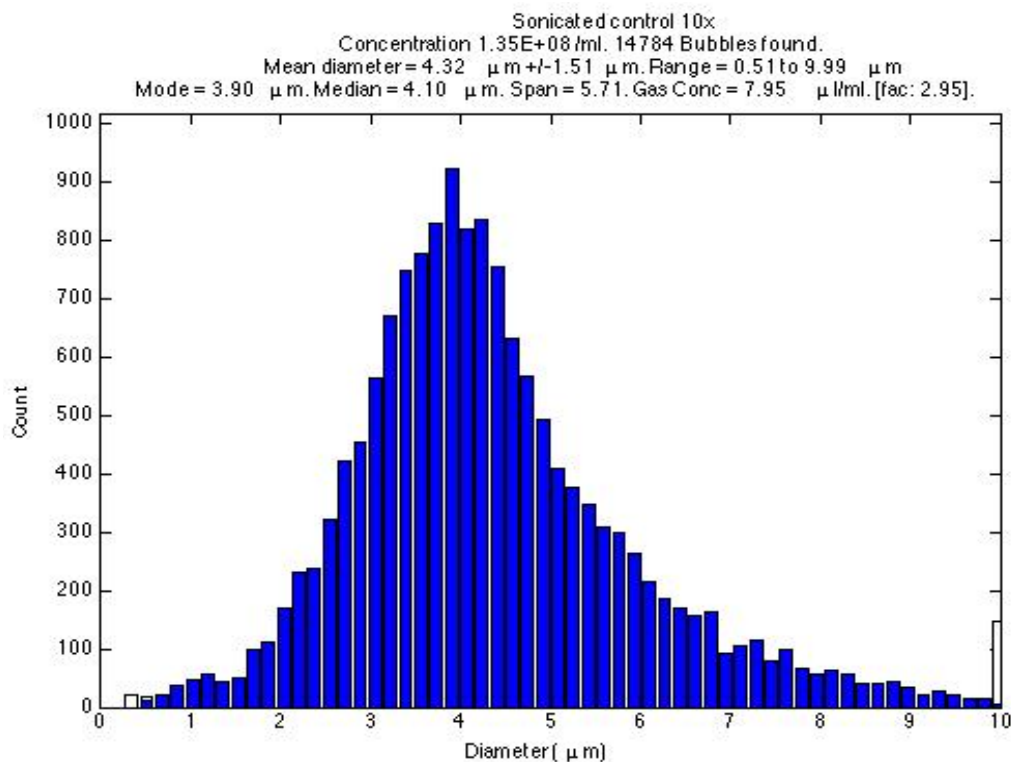


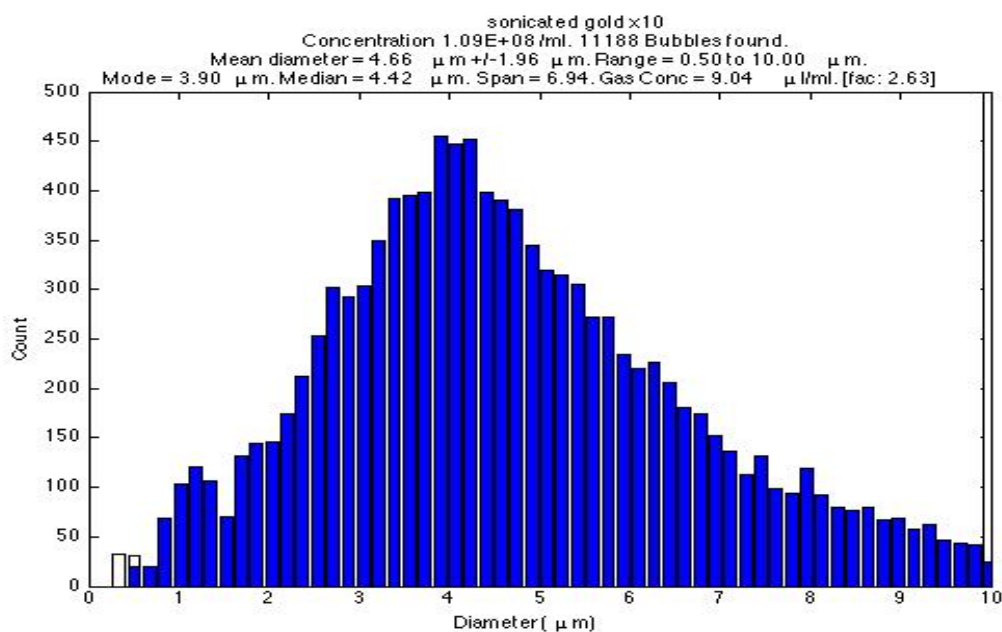
Figure 11.6 Size Distribution of microbubbles produced via reversed Y-Junction both with (B) and without (A) the addition of gold nanoparticles

11.4.2 1 MHz TEST

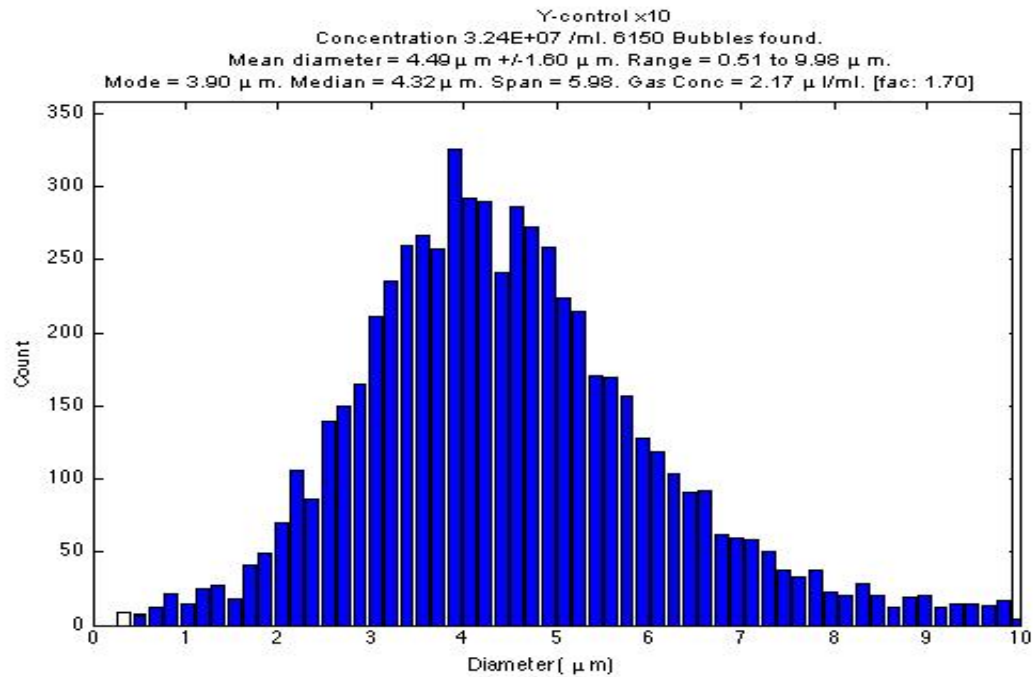
Sonicated control



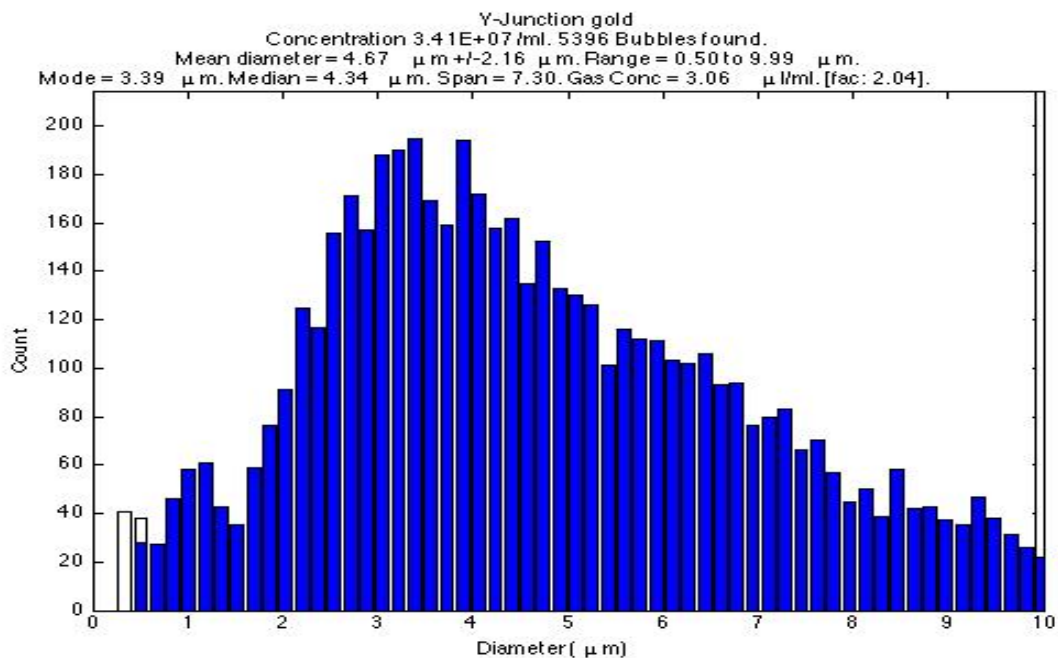
Sonicated Gold



Y-Junction Control



Y-Junction Gold

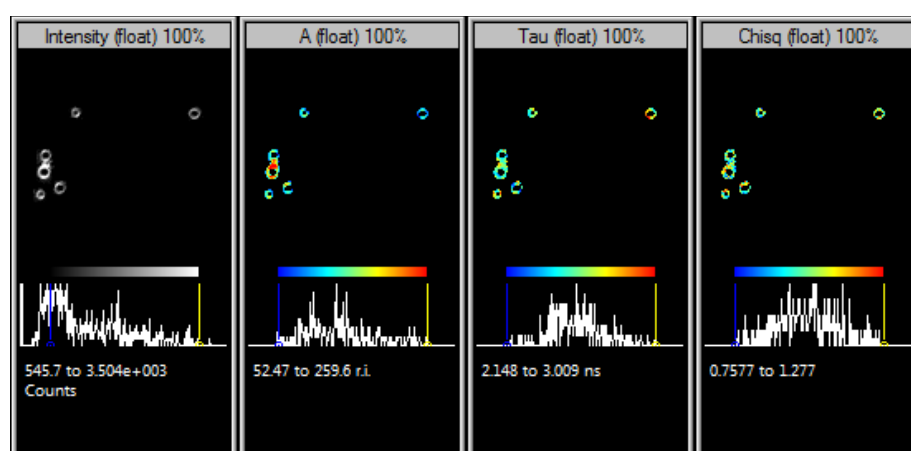


11.5 FLIM testing results

The results are in reference to chapter 7.2. The image labels are as follows: *Intensity* refers to the number of photons, *A* refers to the amplitude, *Tau* shows the lifetime distribution, *Chisq* refers to the goodness of fit.

Sonicated

Test 2 mono-exponential fit 2.56 ± 0.015 ns (s.d.)

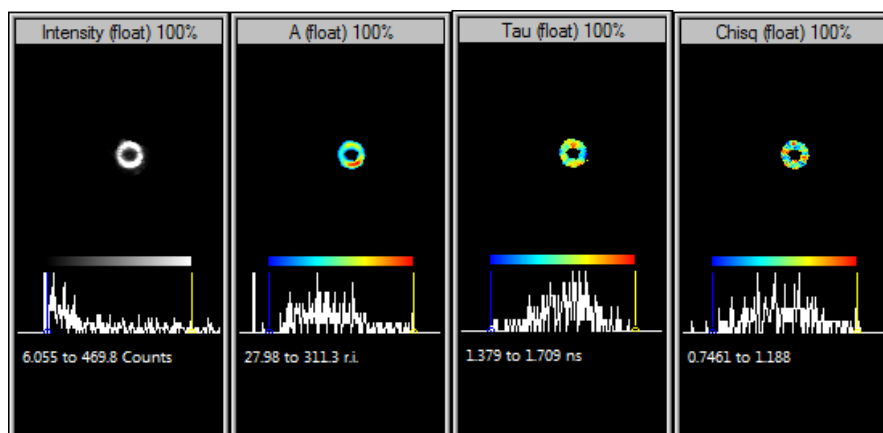


Individual averages of bubbles above:

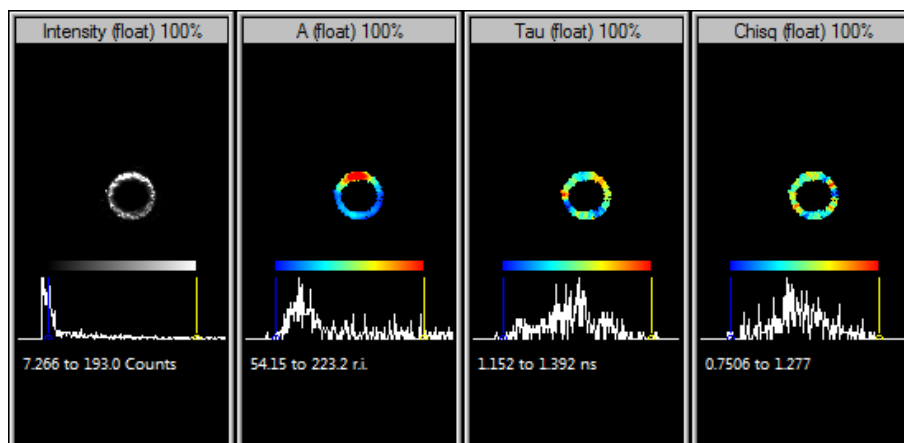
1. 2.51 ± 0.043 ns
2. 2.5 ± 0.03 ns
3. 2.51 ± 0.026 ns
4. 2.44 ± 0.03 ns
5. 2.62 ± 0.04 ns
6. 2.64 ± 0.045 ns

Flow focusing microfluidic device

Test 6 mono-exponential fit average histogram lifetime 1.55 ± 0.061 ns (s.d.)



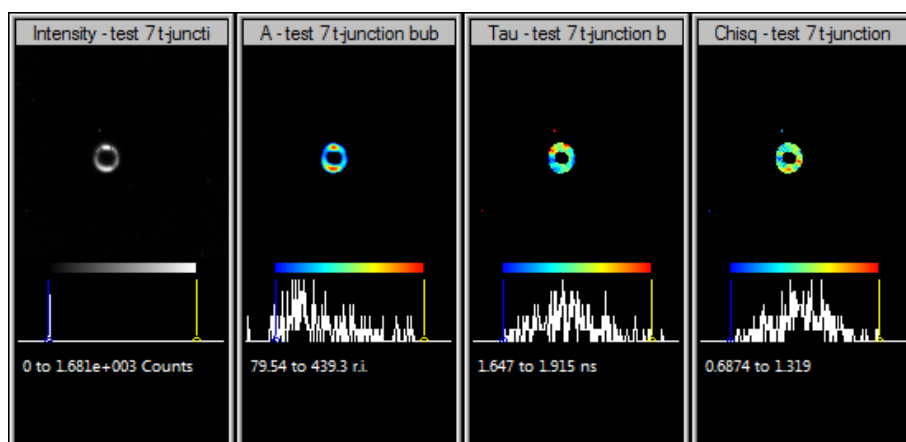
Test 5 Mono-exponential fit 1.26 ± 0.047 ns (s.d)



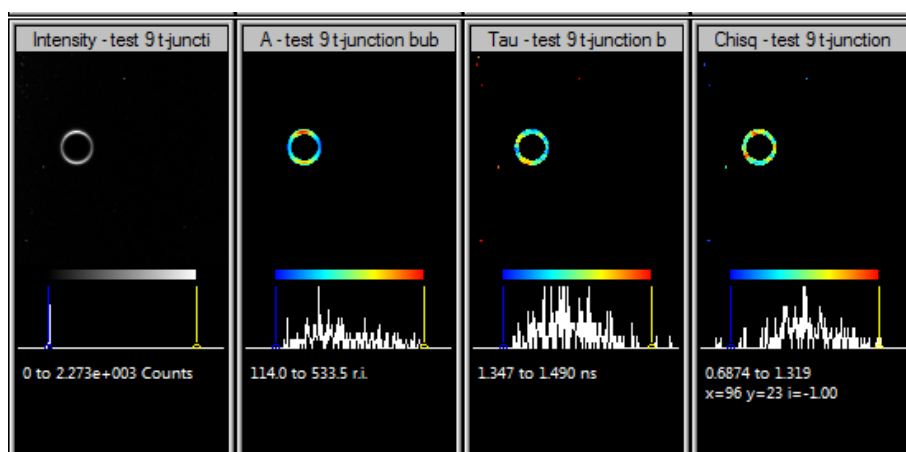
Test 4 Mono-exponential fit 1.62 ± 0.08 ns (s.d.)

Cross flow rupturing reverse Y-junction device

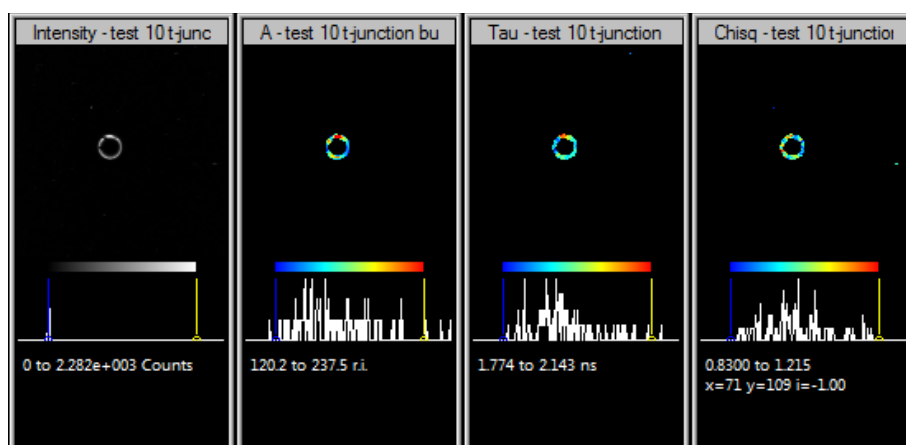
Test 7 Mono-exponential fit 1.76 ± 0.055 ns (s.d.)

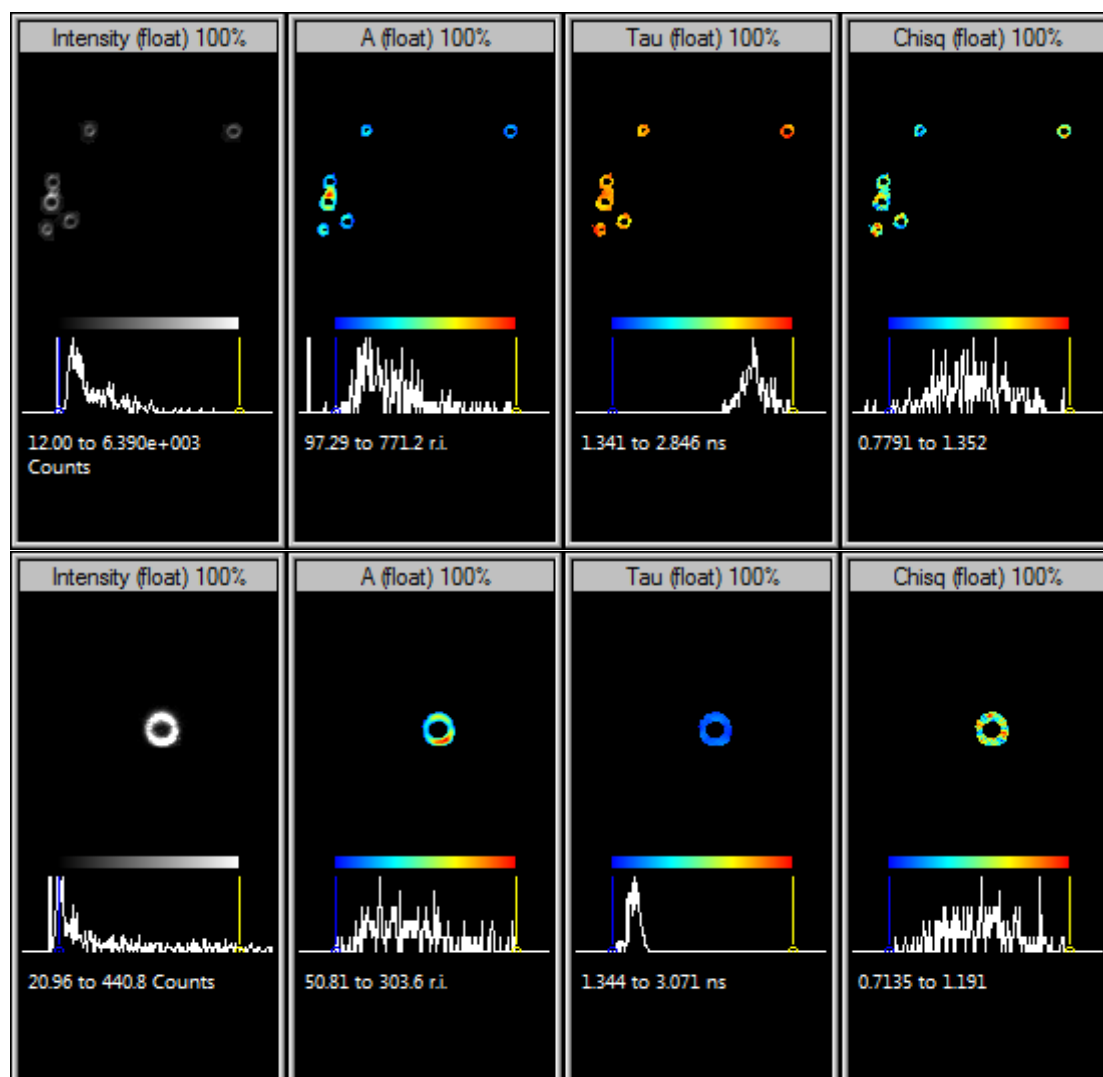


Test 9 Mono-exponential fit 1.41 ± 0.026 ns (s.d.)



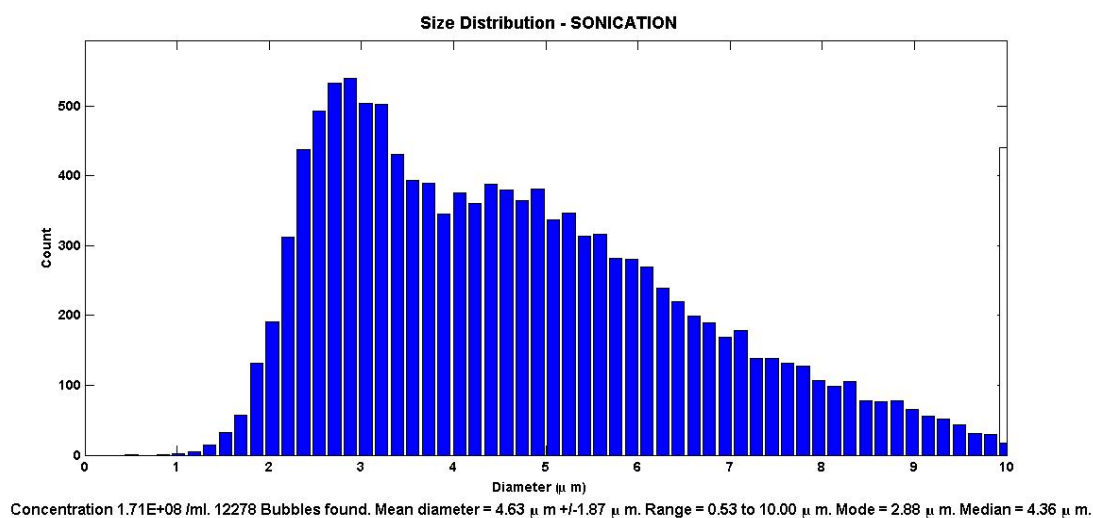
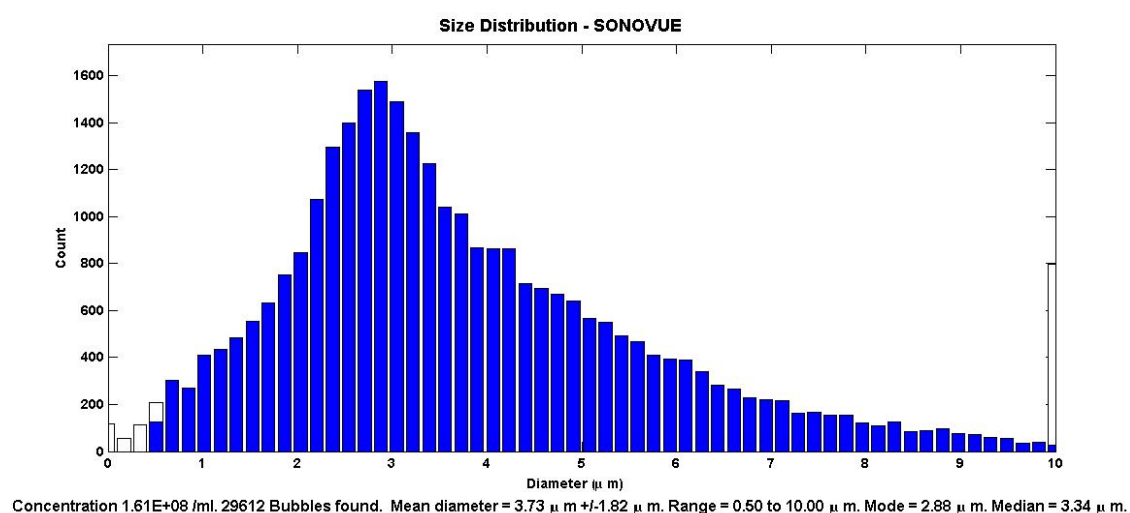
Test 10 mono-exponential fit 1.92 ± 0.071 ns (s.d.)

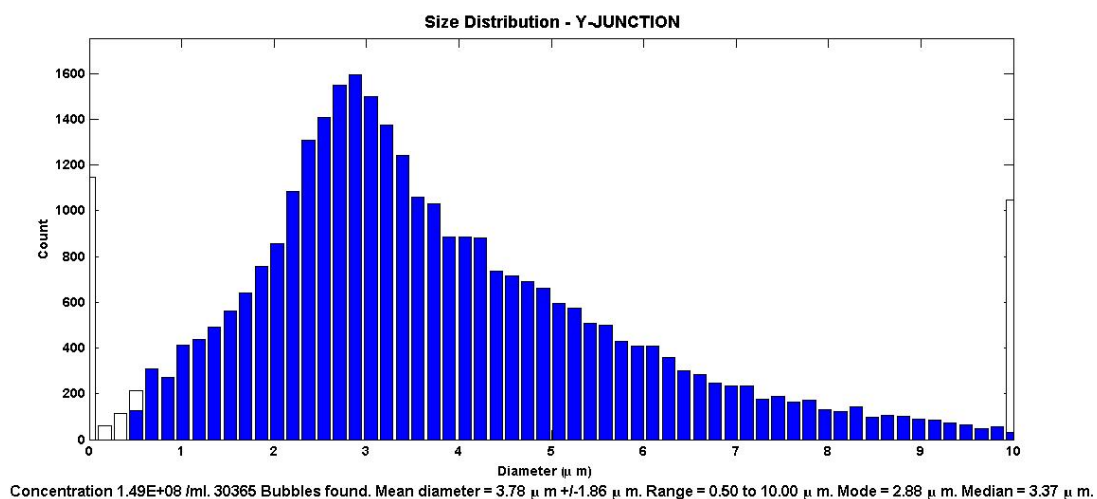


Comparison of test 2 (sonicated) and test 6 (microfluidic)

11.6 Size distributions for Manufacturing process Acoustic Comparison

Size distributions of the bubbles acoustically tested produced through each manufacturing method (Chapter 7.3). A. Sonovue B. Sonication, and C. microfluidic Y-Junction.





1. Bardhan, R., et al., *Theranostic Nanoshells: From Probe Design to Imaging and Treatment of Cancer*. Accounts of Chemical Research, 2011. **44**(10): p. 936-946.