Phospholipid Encapsulation Properties and Effects on Microbubble Stability and Dynamics

James Jing Kwan

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ABSTRACT

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The goal of this doctoral work was to observe and analyze the stability and dynamics of phospholipid-encapsulated microbubbles, and in particular the reaction to sudden submersion in a multi-gas medium. To accomplish this goal, first an experimental technique was developed to observe a microbubble in a single-gas environment suddenly immersed in a multi-gas environment, without perturbing the microbubble position. A modified Epstein-Plesset model was concurrently developed to account for the multiple gas species in the bulk solution. The model was used to analyze previous data for the effect of anesthesia carrier gas on microbubble ultrasound contrast agent in vivo circulation persistence. The focus of the experiments then shifted to microbubbles of different sizes encapsulated with a homologous series of saturated diacetyl-chain lipid surfactants and emulsifiers. Constitutive models for the elastic and gas permeation properties of the lipid encapsulation were developed to elucidate the unique behaviors observed during the experiments.

The experimental techniques employed were: (1) transmission bright field optical microscopy to obtain real-time, digital videos of microbubbles growing and dissolving in response to perturbations in the local gas environment and (2) the Langmuir trough film balance to determine the elasticity of the phospholipid monolayers during compression, expansion, and expansive relaxation. The modeling techniques employed was (1) a forward-wind finite difference method to discretize a series of non-linear differential equations and (2) a Newton-Raphson method to solve the diameter of a microbubble from the mechanical stress balance.
These modeling techniques were used to determine the behavior of a microbubble a priori, whereas the fitting models implemented the iterative methods to solve for parameters without a Newton-Raphson method.

Results showed that microbubbles coated with soluble surfactants and dissolving in a single gas solution could be predicted by the original Epstein-Plesset model. When subjected to a multi-gas medium, the modified Epstein-Plesset model accurately predicted microbubble growth and dissolution. The model was used to analyze the increase in microbubble circulation lifetime observed by others in anesthetized rats inhaling air rather than oxygen as the anesthesia carrier gas. The predictive capabilities of the model broke down, however, if the gas-core was encapsulated with a phospholipid monolayer. A typical, large (>40 µm diameter) lipid-coated microbubble displayed stunted growth, followed by three anomalous dissolution regimes: (1) rapid dissolution back to the initial resting diameter followed by (2) slow, steady dissolution and finally (3) stabilization, where the apparent surface tension approached a near-zero value. The model was modified to allow fitting of the radius-time curve by varying the surface tension. The analysis showed that the surface tension is dynamic, and suggested that a “break up” tension allowed for rapid expansion of the microbubble beyond the initial resting diameter. Lipid jamming was proposed as the mechanism eventually halting dissolution. Further observations of smaller microbubbles (<20 µm diameter) coated with a homologous series of saturated diacyl chain lipids gave significantly different results. Initially the microbubbles grew, but growth was severely subdued, if not eliminated, for more solid encapsulations below a threshold size (~10 µm diameter). Following growth, most microbubbles rapidly dissolved back to their original size. The microbubbles then experienced an anomalous lag time before spontaneously dissolving again. The lag times were highly variable and shown to correlate to the reduced
temperature of the encapsulation, rather than the initial microbubble size. Most of the microbubbles stabilized again at a diameter of 1-2 µm, and this “stable diameter” appeared to be universal and independent of both the initial microbubble size and the rigidness of the encapsulation. Constitutive models were developed to describe these physical phenomena in the early growth and dissolution stages which were verified with independent monolayer relaxation studies.
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Chapter 1: Introduction

Microbubble suspensions are made by emulsification of gas into a liquid. A microbubble, therefore, is a single globule of gas that is suspended in an aqueous environment. Natural microbubbles range in size between 1 – 400 µm, whereas synthetic microbubbles used for medical applications lie between 1 – 10 µm in diameter (Cartmill and Su 1993; Feshitan et al. 2009). Because of their small size, these emulsions are predicted to last for only fractions of a second owing to surface tension effects (Epstein and Plesset 1950). The addition of a stabilizer, which usually is a phospholipid for synthetic microbubbles, mechanically stabilizes the microbubble and limits gas diffusion (Fox and Herzfeld 1954; Yount 1997). As a result of these surfactants, microbubbles can remain stable for days (D'arrigo 1986). This chapter reviews natural and synthetic microbubbles, their acoustic response and their medical and industrial applications.

1.1. Natural Microbubbles

Since Tomlinson’s (Kurzer 2004) impactful work on surface tension and the nucleation of bubbles, these gas particles have been of great interest to the scientific and engineering community. Though bubbles (>1 mm in size) can easily be observed floating to the surface of a liquid, e.g., in soda water, microbubbles (µm in size) were not observed until the age of supersonics. Harvey (Harvey 1930) was one of the first researchers to observe the presence of microbubbles in nature. Upon applying ultrasonic waves, plant cells were lysed by cavitation-generated microbubbles. Harvey questioned how cavitation can occur in a plant cell under 4 – 5 atm turgor pressure when all previous evidence indicated that only 1 – 4 atm prevents cavitation. Harvey (Harvey et al. 1944; Harvey et al. 1944) later demonstrated that cavitation could be
prevented by degassing the cells, implying that microscopic gas bubbles may be present and are necessary to nucleate cavitation events. Fox and Herzfeld (Fox and Herzfeld 1954) proposed that these gas particles under 0.1 mm in diameter (microbubbles) must be stabilized by an organic skin which acts as a mechanical barrier to dissolution. Natural microbubbles have been shown to persist in fresh water (Dean 1944), ocean water (D'arrigo 1986) and in biological media, such as blood and tissue (Harvey et al. 1944; Harvey et al. 1944).

Oceanic microbubbles form from the crashing of waves against the atmosphere-ocean interface and can persist for several days (Cartmill and Su 1993; D'arrigo 1986; Johnson and Cooke 1981). Once formed, organic molecules encapsulate the gas core, providing stability while preventing coalescence (Cartmill and Su 1993; Fox and Herzfeld 1954). Microbubbles have been suggested to account for the discrepancies between theoretical and experimental measurements of the acoustic backscatter at the ocean surface once attributed to Bragg scattering, shown in Figure 1.1 (Dashen et al. 1990; Mcdonald 1991).
Figure 1.1: A comparison of the Bragg theory and data sets of acoustic backscatter of the ocean’s surface at winds greater than 5 m/s, showing a discrepancy of ~10 – 20 dB and demonstrating the need for additional scattering mechanisms. Taken from McDonald (Mcdonald 1991).

Because of their size, microbubbles flow with the currents, forming large clouds of gas. Henyey (Henyey 1991) calculated that the plumes could persist at depths down to 10 m, but recent acoustic measurements have shown scattering at depths down to 25 m (Trevorrow 2003). Microbubble clouds echo sound from SONAR machines, resulting in inaccuracies in avoidance measurements of obstacles during boat navigation (Trevorrow 2003). Johnson and Cooke (Johnson and Cooke 1980) reported that oceanic microbubbles act as “collectors” of organic remains, aggregating debris as the gas core dissolves. Sebba (Sebba 1960) demonstrated that these collectors also transport salts and organics across the atmosphere-ocean interface through the process of “ion floatation”. As a result, microbubbles impact solid and ion transport rates in
the ocean and atmosphere. Recently, Seitz (Seitz 2011) reported that microbubble clouds in the ocean may reflect sunlight and proposed that microbubbles may be a new method for solar radiation management.

1.2. Synthetic Microbubbles and their Applications

1.2.1 Microbubble Production

1.2.1.1. Mechanical Agitation and Size Isolation

Mechanical agitation, through sonication or shaking, is a quick and easy technique to generate a high yield of microbubbles, although with a large size disparity. Feinstein et al. (Feinstein et al. 1984) first described sonication for a gas-liquid interface to generate microbubbles. The principle, however, was previously described Li and Fogler (Li and Fogler 1978; Li and Fogler 1978), who proposed that waves forming on the gas-liquid interface destabilize and pinch off globules of supernatant into the liquid medium. Acoustic waves transmitted from the surface travel throughout the solution, causing inertial cavitation sites in the bulk. These cavitations generate inertia that continuously fragments the emulsion particles to smaller sizes, creating a polydisperse particle size. A cartoon of these events is shown in Figure 1.2. Similar conditions arise when the interface is disturbed by shaking the containment vessel at high frequencies (4 – 4.5 kHz). Both of these methods in mechanically disrupting the interface yield high concentrations of gas-liquid emulsions and at large volumes and short times.
Figure 1.2: Cartoon of the two stage mechanism producing polydisperse droplet size in oil-water emulsions proposed by Li and Fogler. (Li and Fogler 1978; Li and Fogler 1978) First, the gas is entrained by unstable capillary waves at the interface. Second, cavitation sites (filled in circles) fragment the droplets until a stable size is reached. The inset cartoon shows the capillary wavelength ($\lambda$), wave height for instability ($l_{\text{max}}$), and diameter of generated droplet ($d$) at the relevant length scales. Taken from Feshitan et al. (Feshitan et al. 2009).

Production of microbubbles via sonication yields high concentrations at large volumes. Swanson et al. (Swanson et al.) sonicated lipid solutions semi-continuously, creating microbubble suspensions at concentrations up to $10^{10}$ MB/mL at volumes up to 1 L. Large volumes of microbubbles are useful if a continuous supply of microbubbles is needed; however, most clinical studies only require small volumes of microbubbles (1 – 2 mL). For lower volumes
of microbubbles, Unger et al. (Unger et al. 1992) proposed a shaking method of small (2 – 3 mL) serum vials. Pre-microbubble lipid solution can be prepared and stored in serum vials. These serum vials can be shaken with a device similar to a dental amalgamator for immediate generation and single-use injection while maintaining the same polydispersity and concentration as microbubble solutions generated through sonication. The ability to transport and have on-site production is important for clinical applications of microbubbles.

Size uniformity is critical in both diagnostic and therapeutic application of microbubbles. Early methods to control microbubble size take advantage of buoyant forces on the microbubbles. Because of their size, microbubbles obey Stoke’s law, which states that the terminal rise velocity will vary with particle diameter. Kvale et al. (Kvale et al. 1996) took advantage of the varying terminal rise velocities to develop a floatation technique to size fractionate a microbubble suspension. Following the same central principles, Feshitan et al. (Feshitan et al. 2009) developed a differential centrifugation technique to efficiently size isolate different size classes from a microbubble suspension with large size disparity. Their results are shown in Figure 1.3. While these techniques require additional steps, they satisfy both the size consistently and adequate volume concerns that are necessary for diagnostic and therapeutic applications of microbubbles.
Figure 1.3: Number weighted (A,C) and volume weighted (B,D) size distributions of the initial polydisperse microbubble suspension and their respective final size isolated disparity, (A,B) 1 – 2 µm and (C,D) 4 – 5 µm. Taken from Feshitan et al. (Feshitan et al. 2009).

1.2.1.2. Flow Focusing

Flow focusing uses a liquid stream to focus a gas stream through a small opening, forming gas microbubbles. As the gas stream is pushed through a small orifice, surrounded by flowing liquid, a gas ligament is created. The liquid surrounding the gas ligament forms capillary waves on the surface, which destabilize and pinch the gas stream into microbubbles. Ganan-Calvo and others (Ganan-Calvo 2004; Ganan-Calvo and Gordillo 2001; Talu et al. 2008) demonstrated that flow focusing can form a continuous stream of monodisperse microbubbles. Recently, Herrada and Ganan-Calvo (Herrada and Ganan-Calvo 2009) proposed an improvement to co-axial flow focusing by adding swirl to the liquid stream. For on-chip generation of
microbubbles, Hettiarachchi et al. (Hettiarachchi et al. 2007) developed the T-junction flow focusing technique, shown in Figure 1.4.

![T-Junction micro-chip showing the direction of flow (arrows) of the gas and liquid streams](image)

**Figure 1.4:** An image of a T-Junction micro-chip showing the direction of flow (arrows) of the gas and liquid streams. The orifice has a 7 µm diameter, generating 3 µm microbubbles. Taken from Hettiarachchi et al. (Hettiarachchi et al. 2007).

They demonstrated that this technique could produce uniform microbubbles of a variety of sizes, down to 5 µm diameter at rates of $6 \times 10^7$ MB/s.

Electrohydrodynamic (EHD) flow focusing is an additional technique to generate microbubbles. Coaxial EHD atomization (CEHDA) uses two concentric stainless steel capillary needles with the inner diameter ranging from tens of micrometers to millimeters (Farook et al. 2009; Loscertales et al. 2002). An electrode is attached to the capillary needles and a ring electrode, maintained at zero potential, is placed a short distance away. The electrodes create an electric potential, which forms a Taylor cone as the gas-liquid stream is passed through. Depending on flow rates and voltage, the diameter of the microbubbles and the production rate
can be adjusted. Because the microbubbles are produced in an open environment, the microbubbles were found to be unstable at the initial diameter. Through gas diffusion, the microbubbles dissolved for an hour before finally stabilizing at 2 µm, and were then stable for up to 40 hours.

1.2.2. Microbubble Characteristics under Ultrasound

Microbubbles have several unique acoustic properties. The gas core is highly compressible and thus responds to pressure changes in the local microenvironment. Microbubbles are small (1 – 10 µm in diameter) compared the wavelength of ultrasound (100 – 1000 µm). Therefore, a microbubble experiences isotropic pressure changes as the ultrasound wave passes through the microenvironment. The microbubble undergoes expansion as the pressure decreases during rarefaction. Upon compression, the pressure increases causing the microbubble to contract. In an ultrasound field, a microbubble goes through radial oscillations, generating spherical waves (echoes) that are detectable by an ultrasound transducer.

Fortunately, microbubbles resonate at MHz frequencies. de Jong and others (de Jong et al. 1992; Medwin 1977; Sirsi et al. 2010) have used the microbubble acoustic scattering cross-section ($\varepsilon_s$) as a metric for echogenicity:

$$\varepsilon_s = \frac{4\pi R^2}{\left(\frac{f_r}{f_d}-1\right)^2 + \delta^2}$$ (1.1)
where \( f_d \) is the driving frequency, \( f_r \) is the resonant frequency, \( R \) is the microbubble radius and \( \delta \) is the overall dampening coefficient. The total dampening in equation 1.1 can be represented as a sum of the re-radiation (\( \delta_r \)), shear viscous losses in the surrounding medium (\( \delta_v \)), thermal transport between the gas and liquid (\( \delta_T \)) and shell frictional damping (\( \delta_F \)).

\[
\delta = \delta_r + \delta_v + \delta_T + \delta_F
\]  

(1.2)

For an unencapsulated gas bubble in an isotropic medium, the resonant frequency is given as (de Jong et al. 1992; Medwin 1977; Sirsi et al. 2010):

\[
f_r = \frac{1}{2\pi R} \sqrt{\frac{3\gamma P_H}{\rho}}
\]  

(1.3)

where \( \gamma \) is the ratio of constant-volume specific heat to the constant-pressure specific heat, \( P_H \) is the ambient bulk pressure and \( \rho \) is the bulk fluid density. Notice that the microbubble resonance frequency depends on the ambient pressure, but not the bulk fluid viscosity. As \( R \) approaches the microbubble size range (1 – 10 \( \mu \)m), the resonant frequency approaches the MHz range (0.3 – 3 MHz). From these equations, it can be shown that driving microbubbles at MHz frequencies provides the strongest backscattered echo. This attribute is important because diagnostic and therapeutic ultrasound applications typically use frequencies on the order of 0.1 – 10 MHz.
In 1905, Lord Rayleigh (Rayleigh 1905) first described the radial motion of a collapsing bubble. Subsequent models by Plesset and others describe the radial oscillations of a microbubble under an acoustic field (Emmer et al. 2009). The Rayleigh-Plesset equation is given as:

\[
R\ddot{R} + \frac{3}{2} \dot{R}^2 = \frac{1}{\rho} \left( P_g - P_H - P(t) - 4\mu \frac{\dot{R}}{R} - \frac{2\sigma}{R} \right)
\]  

(1.4)

where the accents \( \dot{} \) and \( \ddot{} \) represent a first and second derivative with time, respectively, \( P_g \) is the pressure of the microbubble gas core, \( P(t) \) is the external pressure exerted on the microbubble as a function of time (t), and \( \sigma \) is the interfacial surface tension. Figure 1.5 demonstrates the non-linear oscillations of the microbubble, which translates to non-linear scattering of the acoustic signal that can be detected by transducers and separated from the more linear background tissue scatter (Ferrara et al. 2007).
Figure 1.5: A diameter-time streak image of a microbubble oscillating under 2.25 MHz pulse with a peak negative pressure of 360 kPa. The transmitted pulse (white) and predicted radius (grey) are overlaid. Taken from Ferrara et al. (Ferrara et al. 2007).

Though it is sometimes considered axiomatic that microbubbles scatter acoustic signals, research has shown they will also attenuate. Attenuation is the loss of acoustic energy as the ultrasound field propagates in a bubbly medium. In general, the more energy required to oscillate a microbubble, the greater its ability to attenuate the acoustic signal. Medwin and others (de Jong and Hoff 1993; de Jong et al. 1992; Medwin 1977; Sirsi et al. 2010) mathematically described this absorption of energy as the microbubble absorption cross section ($\varepsilon_a$), which is given below:

$$\varepsilon_a = \varepsilon_s \left( \frac{\delta}{\delta r} - 1 \right)$$ (1.5)
where $\delta_r$ is the dampening coefficient for re-radiation, a component of the overall dampening coefficient shown in equation 1.2. The extinction cross-section ($\varepsilon_e$), shown in Figure 1.6, is the total energy of the ultrasound beam lost when passing through a bubble and is defined by the sum of the scattering cross-section (equation 1.1) and absorption cross-section (equation 1.5) of the bubbles (de Jong and Hoff 1993; de Jong et al. 1992; Medwin 1977).

$$
\varepsilon_e = \varepsilon_s + \varepsilon_a
$$

(1.6)

Figure 1.6: (A) Extinction cross-section as a function of microbubble diameter as calculated by equation 1-5. The scattering and absorption cross-sections (equation 1.1 and 1.5, respectively) are shown in grey. (B) Extinction cross-section as the surface tension is changed from 0 mN/m (maximum surface coverage) to 72 mN/m (clean gas-interface). Adapted from Sirsi et al. (Sirsi et al. 2010).
de Jong (de Jong et al. 1992) calculated that at sufficiently small sizes, microbubbles absorb acoustic signals instead of scattering them. Sirsi et al. (Sirsi et al. 2010) recently observed that polydispered microbubbles showed low levels of increased video signal, and proposed that the presence of the small bubbles negated the scattering from larger ones. They demonstrated that larger (>4 µm) size isolated microbubbles scattered signal whereas the smallest size range (1 – 2 µm) actually decreased the signal intensity. This evidence is important because current commercially available and clinically approved microbubbles for ultrasound contrast enhancement all use polydisperse microbubbles with a small mean diameter (~2 µm).

Finally, acoustic radiation force can spatially translate microbubbles (Crum 1975; Dayton et al. 2002; Dyson et al. 1971; Zheng et al. 2007). Dyson (Dyson et al. 1971) observed the presence of an acoustic radiation force when she halted the flow of red blood cells with ultrasound. Several years later, Crum (Crum 1975) reported that large air microbubbles pulsing in a low-frequency vibrating vessel would attract each other due to the acoustic radiation forces. Wantanabe (Watanabe and Kukita 1993) observed that the microbubble translational response was proportional to its size and showed that microbubbles at their resonant size moved to the anti-node in the pressure field. Interestingly, at sufficiently small sizes these microbubbles appeared to have chaotic translational motion. Dayton (Dayton et al. 1999; Dayton et al. 1997) demonstrated that the use of a radiation force could spatially alter the flow patterns of ultrasound contrast agents, which would be a useful tool in localizing microbubbles coated with binding molecules near the site of interest (e.g., a blood vessel wall). More recently, Zheng et al. (Zheng et al. 2007) observed the translational movement of microbubbles pushed by acoustic radiation force with high-speed cameras. The captured images were horizontally stacked to create streak
pictures of the microbubble diameter-time behavior. Figure 1.7 shows the diameter-time behavior of a 2-µm diameter microbubble in an ultrasound wave, with a peak negative pressure (PNP) of 750 kPa.

![Image](image.png)

Figure 1.7: A diameter-time streak image of a microbubble oscillating under an ultrasound pulse of 2.25 MHz at a PNP of 750 kPa. The microbubble is seen moving vertically (arrow) as the radiation force pushes it. Taken from Zheng et al. (Zheng et al. 2007).

1.2.3. Biomedical Applications

In the United States, contrast-enhanced ultrasonography (CEUS) employing stabilized microbubbles is clinically approved for echocardiograms (Wilson and Burns 2010; Wilson et al. 2009). More than 50 countries worldwide, including those in Europe, Asia and India, use CEUS for routine abdominal imaging with the purpose of, for example, detecting hepatic lesions or masses (Lindner 2004; Lindner 2009; Wilson and Burns 2010). Microbubbles for contrast enhancement have proven to be a valuable asset in the fields of cardiology and radiology, and several clinical trials for imaging cancer and stroke are currently underway in the U.S.
CEUS is safe, portable and cost-effective in comparison to other imaging modalities, such as dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) and x-ray computed tomography (CT).

1.2.3.1. Contrast Enhanced Imaging of the Heart

Echocardiography is a non-invasive and easy-to-perform cardiac imaging technique using ultrasound to generate real-time pictures of the heart. In some cases, the images of the heart are difficult to read and rely on the skill of the radiologist to discern key features, leading to inaccurate diagnostic cardiac measurements. One method to add clarity to echocardiograms is to use microbubble ultrasound contrast agents (UCAs) for contrast-enhanced echocardiography. The increasing use of UCAs clinically is indicative of their importance for echocardiography. Some specific uses of UCAs for echocardiography are illustrated below.

The chambers of the heart are greatly illuminated because microbubble UCAs travel to organs with a high density of RBCs. Hundley et al. (Hundley et al. 1998) demonstrated that intravenous (IV) injections of weight-adjusted doses of perfluorocarbon microbubble UCAs (EchoGen, Sonus Pharmaceuticals) increased visibility of the left ventricular (LV) chamber, clearly outlining the endocardial border, as shown in Figure 1.8.
Figure 1.8: (A) A standard image from an echocardiogram of the left ventricle. (B) An image of an echocardiogram with contrast enhancing microbubbles. Note the clear LV border once the blood pool is illuminated. Taken from Hundley (Hundley et al. 1998).

Their study showed that the LV volumes determined by CEUS matched the MRI results better than standard echocardiography. Kasprzak et al. (Kasprzak et al. 1999) showed similar improvements in LV border measurements when imaging the left ventricle with second harmonic imaging after administering microbubbles. In lieu of the increased visibility of the LV border, CEUS was demonstrated in a recent multi-center study to be the best imaging modality for detection of LV regional wall motion abnormalities (RWMA), a strong indicator of different coronary diseases such as coronary artery disease (CAD) (Hoffmann et al. 2006). More sophisticated CEUS echocardiographic techniques are being developed to characterize RWMA. Takeuchi et al. (Takeuchi et al. 2006) demonstrated the feasibility of using 3-D CEUS echocardiography as an imaging modality for LV RWMA, to diagnose CAD. More recently,
Keator et al. (Keator et al. 2011) showed CEUS capabilities to assess vascular perfusion of the uterus, providing a new tool for monitoring vascular changes in early pregnancies. CEUS showed potential in other studies for improving the detection of heart related dysfunctions such as mural thrombus, LV pseudo-aneurysms and aortic dissection (Lindner 2004).

Microbubbles rheology in circulation is nearly identical to that of the red blood cells (RBC) traveling to the microvasculature of organs (Lindner et al. 2002). Armstrong et al. (Armstrong et al. 1982) was one of the first groups to demonstrate the possibility of utilizing UCAs to assess myocardial perfusion abnormalities, such as acute myocardial infarction. Wu et al. (Wu et al. 1998) demonstrated that CEUS could quantify such obstructions, which was traditionally limited to contrast enhanced MRI (Shan et al. 2004). Though it is unclear which technique is “better”, CEUS demonstrated greater sensitivity to microvessel occlusions. Wei et al. (Wei et al. 1998) reported that the myocardial blood flow (MBF) could be measured independently from other systemic factors. Interestingly, they were able to characterize the MBF at different conditions, effectively simulating symptoms of atherosclerosis, diabetes and neoplasia. Wei (Wei et al. 2001) initiated clinical trials, demonstrating that the myocardial contrast echocardiography (MCE) technique could non-invasively measure coronary blood flow. Such studies opened the door for CEUS echocardiography, ushering in a new era of ultrasound imaging and extending it to the assessment of angiogenesis in tumors (Ellegala et al. 2003; Leong-Poi et al. 2003; Moguillansky et al. 2011; Pysz et al. 2011), locate regions of inflammation (Lindner et al. 2001) and perform diagnostic measurements (Fernandes et al. 2011).
1.2.3.2. Contrast Enhanced Imaging of the Liver

As with echocardiography, ultrasound images of the liver have been shown to be difficult to read and reproduce in the clinical setting (Bolondi et al. 1991; Sabba et al. 1990). For example, patients with obesity, liver cirrhosis, bowel gas, or anatomical variations may not be eligible for sonography of the liver. Fischer et al. (Fischer et al. 1998) demonstrated that injection of microbubbles into the portal and hepatic veins allows imaging of blood flow in the regions that were once acoustically invisible. Similarly, Harvey et al. (Harvey et al. 2000) demonstrated that CEUS of the abdomen improved the planar representation of the liver with resolution less than one centimeter. As a result, defects such as hepatic tumors were highlighted, as shown in Figure 1.9 (Harvey et al. 2000).

![Figure 1.9](image)

Figure 1.9: (A) A B-mode image of the liver on a patient with known hepatocellular carcinoma. The liver appears heterogeneous, but no cancer defects are seen. (B) The same patient’s liver, but enhanced with Levovist®. The cancerous regions are clearly marked as dark spots surrounded by bright rings (arrow). Taken from Harvey et al. (Harvey et al. 2000).
In the following decade more studies have been published demonstrating the efficacy of CEUS in diagnosing masses in the liver (Albrecht et al. 2003; Albrecht et al. 2001; Harvey and Albrecht 2001; Kim et al. 2000; Martin et al. 2010; Ricci et al. 2008; Trojan et al. 2010; Zhou et al. 2010). A multi-center study performed by Albrecht et al. (Albrecht et al. 2003) showed that CEUS improved the sensitivity and specificity of detecting malignant metastases. Macro- and microvascularity of these hepatic tumors can be assessed using CEUS, as demonstrated by Ricci et al. (Ricci et al. 2008), allowing doctors to differentiate malignant tumors from benign ones. More recently, Zhou et al. (Zhou et al. 2010) showed that the hepatic transit time between microbubble appearance in the hepatic artery and the appearance in the hepatic vein could also be used to differentiate malignant tumors from benign focal lesions. Quick and accurate delineation of benign and malignant tumors could substantially improve cost savings and relieve unnecessary procedures on the patient.

1.2.3.3. Targeted Contrast Enhanced Ultrasonography

1.2.3.3.1. Targeting Against Angiogenesis

The current clinical standards use passive (untargeted) microbubbles, but new generations of active (targeted) microbubbles have been formulated for diagnostic molecular imaging. Targeted contrast agents are an emerging technology, bringing ultrasonography diagnostics to a molecular scale. One topic of major scientific interest is the targeting of angiogenesis. Angiogenesis is the process of creating new blood vessels from currently existing ones and is a fundamental biological process that is a critical component in wound healing, embryonic development and growth of tumors (Pysz et al. 2011). Cancers have been observed to display rampant angiogenesis and, as a result, can be characterized by an upregulation of $\alpha_v$-
integrins, for example αVβ3, on the surface of endothelial cells in the blood vessels (Anderson et al. 2001; Pysz et al. 2011). By conjugating monoclonal antibodies, peptides or other targeting ligands, microbubbles can bind specifically to αV-integrins. Leong-Poi et al. (Leong-Poi et al. 2003) conjugated microbubbles with echistatin, which adheres to murine αV-integrins. Ellegalla et al. (Ellegala et al. 2003) and Steiger et al. (Stieger et al. 2008) similarly produced echistatin-coated microbubbles that would bind to αVβ3 receptors exposed on tumor blood vessels. These studies showed an increased retention of targeted microbubbles, suggesting the possibility to characterize the efficacy of angiogenesis inhibiting cancer therapy. More recently, Eisenbrey and Forsberg (Eisenbrey and Forsberg 2010) showed a significant increase in targeted microbubble retention using peptides rather than antibodies as the targeting ligand. The ability to assess the progression of treatment in cancer is paramount for “personalized” cancer therapy.

1.2.3.3.2. Molecular Imaging of Inflammation

There are many cardiovascular diseases that stimulate a major inflammatory response (Lindner 2009). Inflammation is a biological response to harmful stimuli such as pathogens, injury of cells, and irritants and is characterized by the release of cytokines that control leukocyte adhesion and extravasation, increasing the concentration of leukocytes. Christiansen et al. (Christiansen et al. 2002) demonstrated that microbubbles targeted to leukocytes are effective for imaging acute inflammation, where the density of leucocytes is high. Chronic inflammation of blood vessels, often caused by atherosclerosis, has a low density of leukocytes. Consequently, these regions of chronic inflammation often have large concentrations of leukocyte trafficking biomarkers, such as vascular cell adhesion molecule-1 (VCAM-1), on the surfaces of the vasculature. Kaufmann et al. (Kaufmann et al. 2007) demonstrated that microbubbles targeted
for VCAM-1 showed an increase in CEUS signal intensity in mice with atherosclerosis, indicating the presence of VCAM-1 (later confirmed by histology). Because atherosclerosis is a moderate risk for 40% of adults in the United States (Jacobson et al. 2000), it is important to develop non-invasive detection of plaque build-up in major arteries, which targeted microbubble UCAs may provide.

1.2.3.4. Microbubble Enhanced Therapy

The blood-brain barrier (BBB) is a barrier between the capillaries, which supply the brain with oxygen, and the brain parenchyma. Molecules larger than ~400 Da are excluded by the BBB, and vital large molecules, such as nutrients, must be actively transported. While the BBB restricts the entry of viruses, bacteria and toxins to the brain, it also prevents therapeutic drugs, such as brain-derived neurotrophic factor mimetics, from treating disorders in the brain such as Alzheimer’s, Parkinson’s and Huntington’s disease. High intensity focused ultrasound (HIFU) was shown to generate cavitation near the BBB and locally increase the permeability of the BBB (Mesiwala et al. 2002). Though a promising technique, HIFU operates at conditions (high powers and long pulse lengths) that can cause hemorrhage in brain tissue, leading to permanent neurological damage (Baron et al. 2009).

Microbubbles may provide some assistance in safely opening the BBB using HIFU. Because opening of the BBB requires cavitation, the addition of microbubbles may allow a reduction in the necessary ultrasound intensity and duration. Focused ultrasound (FUS) in conjunction with lipid-encapsulated microbubbles has been shown to transiently and noninvasively open the BBB in mice (Choi et al. 2008). Choi et al. (Choi et al. 2011) injected a
microbubble suspension (Definity) and fluorescently tagged dextran (3 kDa molecular weight) solution into the tail vein of mice to investigate possible FUS operating conditions, shown in Figure 1.10.

![Fluorescence images of a mouse brain](image)

**Figure 1.10:** (A) Fluorescence images of a mouse brain that was recently exposed to microbubble-enhanced FUS to open the BBB. Fluorescent Dextran can be clearly seen diffusing into the left hippocampus, past the BBB. (B) A fluorescence image of the same mouse, but the other side of the brain where microbubble-enhanced FUS was not applied. The fluorescent probe remains in the vasculature and no contrast is seen in the right hippocampus. Taken from Choi et al. (Choi et al. 2011).

They demonstrated that the application of FUS at low pulse lengths could open the BBB to allow passage of dextran. BBB disruption using microbubble-enhanced FUS may deliver drugs like β-secretase inhibitors to treat Alzheimer’s disease, without brain tissue damage.

Not only can microbubbles assist in the delivery of drugs, but they can be part of the therapy. Treatment of acute ischemic stroke, for example, has been shown effective when tissue
plasminogen activator (tPA), a protein used to cleave fibrin clots, is used in conjunction with ultrasound (Alexandrov et al. 2004). Similar to FUS-induced BBB permeability, ultrasound causes cavitation in regions near the clots. Shearing around the clot ruptures fibrin fibers in the clot, exposing them to tPA. Instead of microbubbles produced by inception cavitation, which requires large amounts of ultrasound energy and can possibly damage nearby cells, tPA-loaded microbubbles can be directly injected. Molina et al. (Molina et al. 2006) demonstrated that tPa-microbubbles enhance the breakdown of blood clots. Though ultrasound without microbubbles can accelerate the lysis of clots, the addition of microbubbles increases the safety and efficacy of ultrasound treatment for acute ischemic strokes.

In addition to drug delivery and assistance in therapy, microbubbles are stimulus-response drug vehicles. Effective drug delivery relies on limiting the drug to the region where the dysfunction is. The advantage of restricting drug delivery to the affected areas, especially with chemotherapeutics, is to reduce toxic side effects. Drug loading in the microbubble shell, however, is constrained because the thin monolayer that stabilizes the interface is often tightly packed and easily destabilized with inclusion of macromolecules. Instead of trying to fit drugs into the shell, attaching drugs to the surface of the microbubble may overcome this bottleneck. Molecular linkers like biotin can be attached to polymer-lipids which have polyethylene glycol (PEG) chains that extend into the bulk phase, exposing the biotin molecule. It is possible, therefore, to attach drug-carrying nanoparticles to the shell using avidin-biotin chemistry, which will remain en route until external forces deliver them. Because attaching particles to the interface is less destabilizing to the microbubble, drug loading can be greatly enhanced. As a result, these microbubbles become a stimulus-response drug delivery system. To mimic this system, Lum et al. (Lum et al. 2006) flowed microbubbles covered with avidin-coated
fluorescent nano-beads through a capillary tube coated in biotin. They applied a radiation force, to push the microbubbles, followed by a destruction pulse to deposit the nano-beads. They showed a remarkable increase in bead deposition for bead-coated microbubbles when compared to free flowing beads. The increase in bead deposition suggests that microbubbles loaded with nanoparticles can increase the therapeutic index of drugs. By depositing more drugs at the afflicted regions, the total dosage of drug can be reduced. It is paramount to reduce the toxic effects of chemotherapeutic agents, for example, which may be resolved by lowering the drug dosage through stimulus-response drug vehicles.

1.2.4. Applications in Biotechnology

Micro-electromechanical systems, or MEMS, are mechanical devices driven by electricity with components ranging from 1 – 100 µm in size. More recently, bio-MEMS have become a topic of interest for their applications in biometrics. In these systems, classical volumetric physics and transport phenomena are no longer dominant, and instead surface effects, such as electrostatics and wetting, govern most of the behavior (Ganguly and Puri 2010). Marmottant et al. (Marmottant et al. 2006) took advantage of the microbubble oscillations to construct novel MEMS components. These radial oscillations induced by acoustic waves produce micro-streams that surround the vibrating microbubble, generating local vortexes that mix the surrounding fluid. By modulating the micro-stream flow patterns, Marmottant et al. (Marmottant et al. 2006) demonstrated that the micro-mixers can become pistons, switches, pumps, or micromanipulators in MEMS. Chung (Chung and Cho 2008; Chung and Cho 2009; Chung et al. 2010) has done extensive work on the micromanipulation of objects using microbubbles. Chung et al. (Chung and Cho 2009) demonstrated that a microbubble can
translate, capture and release objects, such as beads, fish eggs and living water fleas, making it evident that microbubbles can be utilized as a soft-touch tool to manipulate living organisms, an important step in bio-MEMS.

Microorganisms submerged in fermentations require nutrients and oxygen, and while water-soluble nutrients are easily supplied, the low solubility of oxygen limits the microorganism’s growth and survival (Weber and Agblevor 2005). Gas spargers and impeller agitation, though traditionally used, form microbubbles that coalesce to form large bubbles with a low surface-to-volume ratio, which are inefficient at transferring gas. Surfactant-coated microbubbles do not coalesce, but instead rise slowly, increasing the amount of oxygen being delivered. Oxygen microbubbles can, therefore, efficiently supply oxygen to microorganisms like Trichoderma reesei (T. reesei), an aerobic fungus capable of producing key enzymes in turning biomass into fuel (Weber and Agblevor 2005). Webber and Agblevor (Weber and Agblevor 2005) compared microbubble dispersion techniques to conventional ones, and showed that oxygen microbubbles increased oxygen mass transfer up to 3 times more than the conventional mixing methods, such as an impeller. Efficient growth of this aerobic fungus is vital in reducing potential bottlenecks in the production of biofuels.

Microbubbles may also aid in generating magnetic metallic nanoparticles, which are currently being tested for applications in magnetic immunoassays (MIA) (Piao et al. 2011) and bio-MEMS (Ganguly and Puri 2010). To generate these particles, extreme amounts of energy or harmful chemicals are usually required. Fortuitously, microbubble cavitation creates local temperatures up to 5200 K, pressures up to 1000 atm, and heating and cooling rates up to $10^{10}$ K/s (Shchukin and Mohwald 2006). Sonochemistry utilizes these extreme settings to perform high temperature chemistry in room temperature conditions. Sonochemical decomposition of
metals, such as gold, palladium, copper, and iron, will yield different nanoparticle physical properties when the surfactant species and concentration are changed (Radziuk et al. 2010; Radziuk et al. 2010; Shchukin and Mohwald 2006). For example, Shchukin and Mohwald (Shchukin and Mohwald 2006) demonstrated that sonochemical decomposition produced iron nanoparticles with almost double the magnetic strength when compared to other deposition methods like metal vapor deposition. Other nanoparticles, such as superparamagnetic colloidal bimetallic particles, single crystal gold nanoprism, and hollow microspheres, can also be produced through sonolysis, providing a vast array of novel nanoparticles. Thus sonolysis is a unique technique in generating nanoparticles with properties ideal in a variety of biotechnologies, such as bio-MEMS.

1.2.5. Applications in the Food Industry

To protect foods and preserve fruits and vegetables before they are harvested, pesticides and other chemicals, which must be removed before reaching the consumer, are used to deter insects and microorganisms. The current technology, however, has trouble completely cleaning the produce, and residual pesticides and mycotoxin produced from microorganisms may be present. For example, chlorine washes are inefficient at killing some spore-forming microbes, leading to a loss in product quality and an increased probability of harming consumers (Karaca and Velioglu 2007). One possible solution is to add ozone gas to the current washing cycles, in order to reduce residual pesticides (Ong et al. 1996).

The addition of ozone is emerging as a powerful sanitizing agent for produce and drinking water. For produce cleaning, ozone has been shown to aid in the removal of residual
pesticides, kill spore producing microbes (Graham 1997) and remove mycotoxins (Cataldo 2008; Karaca et al. 2010; Takahashi et al. 2007). Ozone is a potent sterilizer, it auto-decomposes to oxygen and an oxygen radical. The radical is highly reactive, breaking down molecular components found in microbes and bacterial spores. For example, Khadre et al. (Khadre et al. 2001) showed that oxidation of sulphhydryl groups in microorganisms inhibits their activation, leaving them inert and preventing them from reproduction. The addition of ozone is a safe and effective sanitizer; however, the effects of temperature (Wickramanayake et al. 1984), pH levels (Farooq et al. 1977; Vaughn et al. 1987), ozone-consuming products (Emerson et al. 1982; Khadre et al. 2001), and ozone solubility in solution can lower the efficacy of this potent sanitizer (Khadre et al. 2001).

All of the current ozone sanitizing applications use bubbles dispersed into the cleaning solution because of the low solubility in aqueous solution. Bubbles on the order of one millimeter in size rise quickly, and as a result, ozone bubbles have a lower efficacy as a sanitizing agent. Ozone microbubbles (OMB) (<50 µm in diameter) have a low rise velocity, increasing the residence time in solution and therefore raising the potency as a disinfectant. It has been shown that direct injection of OMBs through a microbubble-sparger increases the concentration of ozone and free radicals, and thus removes residual amounts of pesticides (Ikeura et al. 2011). The use of OMBs can be an immediate alteration to the already emerging use of ozone as a safe and effective produce and potable water sanitizer.
1.3. Experimental Techniques for Observing Microbubbles

1.3.1. Acoustic Quantification of Microbubble Properties

Since the pioneering work of Harvey (Harvey 1930; Harvey et al. 1945; Harvey et al. 1944; Harvey et al. 1944), who first observed the presence of “gas cavitation nuclei” in biological fluids under a supersonic field, ultrasound has been the primary technique to observe these gaseous microparticles. This encounter with the invisible echoing particles was also observed by Dr. Joyner (1960), who noticed a transient increase in ultrasound signal when saline solution was injected into patients undergoing M-mode echocardiography. This chance encounter initiated the field of CEUS, and ushered an era of non-invasive diagnostic measurement techniques (Lindner 2009; Wilson and Burns 2010) and scientific interest in microbubbles (Ferrara et al. 2007; Sirsi and Borden 2009).

After the early discovery of natural microbubbles, more deliberate acoustic techniques were developed to acoustically characterize microbubbles. Kabalnov et al. (Kabalnov et al. 1998) varied the microbubble gas core composition and demonstrated in vivo that large per-fluorocarbon osmotic agents persisted for longer periods of time than nitrogen. Mullin et al. (Mullin et al. 2011) controlled the gas that was inhaled by the rats and concluded that the blood gas have a strong influence on microbubble persistence. Sirsi et al. (Sirsi et al. 2010) showed that the contrast enhancement in mice was affected by microbubble size, verifying the theoretical predictions made by de Jong (de Jong and Hoff 1993; de Jong et al. 1992).
1.3.2. Optical Observations of Microbubble Behavior

The simplest form of optically verifying the presence of microbubbles is by observing the opacity of a solution change from clear to a milky white (Seitz 2011; Takahashi et al. 2007). More sophisticated techniques have been developed to characterize these suspensions by exploiting the colloidal properties. For example, techniques such as light-obscuration particle counting (Feshitan et al. 2009; Takahashi et al. 2007) and flow cytometry (Feshitan et al. 2009) utilize dilution to pass single particles past a laser, where detectors sense the scattering of light to quantify the size of and concentration of the microbubble suspensions.

To provide a deeper understanding of microbubble related phenomena, optical microscopy techniques have been employed to directly observe microbubbles. For example, Johnson and Cooke (Johnson and Cooke 1980) showed that photo-microscopy could be used to observe dirt aggregate formation from dissolving oceanic microbubbles. Experimental work from Borden et al. (Borden and Longo 2002) used video-microscopy to demonstrate the effect of the encapsulating shell on microbubble dissolution behavior. Duncan and Needham (Duncan and Needham 2004) expanded on this study, and reported diameter-time behaviors of different microbubbles under a variety of gas-saturation conditions. Using bright-field microscopy, they were able to observe the wrinkled surface structures that form on insoluble-lipid coated microbubbles as they dissolved. Klein et al. (Klein et al. 2007) used bright-field microscopy to image large (>100 µm in diameter) lipid-encapsulated microbubbles dissolve and oscillate, or “click,” proposing that the “clicks” were a result of different equilibrium states.

Though quantification of surface characteristics of monomolecular films have traditionally been restricted to large scale Langmuir trough studies, microscopy studies of microbubbles have also been used to determine important properties such as elasticity, viscosity
and yield shear of lipid monolayers. Micropipette aspiration microscopy studies conducted by Kim et al. (Kim et al. 2003) demonstrated that the monolayer required a yield stress before any shear strain could occur, shown in Figure 1.11.

![Figure 1.11: Videomicrographs of a microbubble being manipulated with micropipettes.](image)

(A) A captured microbubble situated in a saturated aqueous medium. (B) The microbubble is moved to a degassed medium. As the microbubble dissolves, some lipid is projected into the micropipette. (C) Transferring the microbubble to a saturated medium prevents further dissolution. The microbubble stabilizes and is ready for surface measurements. (D) A measuring micropipette captures the microbubble. (E) Suction pressure is applied to remove the lipid projection. In-line pressure transducers measure the pressure required for strain. (F) Further suction removes the projection and lipid flows into the micropipette. The surface viscosity is determined from the rate at which the projection is moved into the micropipette. (Kim et al., 2006)(Kim et al. 2003)
Marmottant et al. (Marmottant et al. 2005) fitted theoretical models to the measured radial-time curves to back out physical parameters of the encapsulating shell such as shell elasticity and friction. In addition to the shell elasticity, dilatational surface viscosity has also been quantified from more sophisticated models analyzing the observed radial-time behavior of microbubbles induced by ultrasound (Paul et al. 2010; Sarkar et al. 2005). Characterizing the physical properties that govern the harmonic behavior of microbubble UCAs used in diagnostic CEUS is critical to developing newer and more powerful contrast enhancing agents and pulse sequences (de Jong et al. 2009).

Non-linear oscillations have long been observed using sophisticated video microscopy. Marmottant et al. (Marmottant et al. 2005) used high speed video-microscopy to observe asymmetric behavior when microbubbles are insonified at low acoustic pressures, attributing it to a yield stress similar to Kim’s results (Kim et al. 2003), shown in Figure 1.12. This asymmetric behavior, exhibited a strong compression with negligible growth. de Jong et al. (de Jong et al. 2007) demonstrated that smaller microbubbles exhibited “compression-only” behavior more consistently at lower frequencies. Interestingly, they demonstrated that regardless of the acoustic parameters, all of their microbubbles under 4 µm in diameter exhibited “compression-only” behavior.
1.4. Microbubble Dissolution

1.4.1. Microbubble Single-Gas Dissolution

In 1950, Epstein and Plesset (Epstein and Plesset 1950) reported a model that predicted that microbubbles dissolve in fractions of a second, which was later verified by Duncan and Needham (Duncan and Needham 2004). The model accounts for surface tension and dissolved gas content for a single-gas system. The Epstein-Plesset model couples Fickian diffusion of a single gas into and out of a bubble (situated in an infinite medium) to the molar balance at the gas-liquid interface. As demonstrated by Duncan and Needham (Duncan and Needham 2004), the Epstein-Plesset theory can demonstrate stabilization using a zero surface tension term,
removing the effects of Laplace pressure. The model, however, was not designed account for the other effects of an organic skin, which acts as an elastic mechanical barrier to strain and gas diffusion (Fox and Herzfeld 1954; Yount 1997). As a result, Borden and Longo (Borden and Longo 2002) accounted for the organic shell by appending a shell gas resistance term to the gas diffusion resistance in the bulk medium. They were able to show that the addition of finite shell permeability would indeed increase the observed microbubble dissolution time. The modified Epstein-Plesset model proposed by Borden and Longo (Borden and Longo 2002), however, did not account for the elastic properties of the monolayer. Katiyar et al. (Katiyar et al. 2009) later applied a dilatational surface elasticity term in addition to shell permeability to account for long-term stability that is observed in nature.

1.4.2. Microbubble Multi-Gas Dissolution

There is much interest in using microbubbles in biological systems as gas carriers, drug delivery vehicles and ultrasound contrast agents. This interest fuels the need for a deep understanding of what happens to a microbubble when surrounded by a complex medium, such as blood. There are several analytical and numerical models which predict the effects of a multi-component system on the dissolution of a microbubble (Kabalnov et al. 1998; Karichev et al. 1998; Yung et al. 1989). Yung et al. (Yung et al. 1989) applied a momentum balance, analogous to the Rayleigh-Plesset equation, to construct a numerical model. The model was applied to glass melts, absorption processes, and other non-biological applications. Although Yung et al. (Yung et al. 1989) shows an initial influx of foreign gas, the radius of the bubble was not predicted to increase because the system they modeled was undersaturated.
In 1998, Karichev et al. (Karichev et al. 1998) introduced a model for oxygen microbubbles incorporating the effects of blood gases. The model neglected surface tension and momentum at the gas-liquid interface, but instead varied the oxygen concentration in blood and the effects of hemoglobin. They investigated four different cases: bubble gas pressure is (1) equal to, (2) greater than gas pressure far away from the bubble, (3) the gases have equal permeability, and (4) the bulk fluid changes with the changes in dissolved gas.

Kabalnov et al. (Kabalnov et al. 1998) worked from similar assumptions made by Epstein and Plesset. The model was applied to intravenously administered ultrasound contrast agents. UCA’s usually consist of a low solubility filler gas. Similar to Van Liew and Burkard’s (Burkard and Vanliew 1994) work, Kabalnov et al. (Kabalnov et al. 1998) reported an initial growth period, owing to the influx of oxygen and nitrogen. The growth stage was followed by dissolution and a phase change, induced from the Laplace pressure (~3 atm for n-perfluorobutane at 37 °C) forcing the filler gases to condense.

1.5. Role of the Lipid Monolayer

1.5.1. Microbubble Shell Rheology

As molecules adsorb onto an interface they exert a surface pressure (Π). They resist the close packing of the molecules, resulting in a decrease in interfacial tension. This relationship is shown below in equation 1.7 and can be measured using a technique developed by Agnes Pockel (Pockels 1902), later improved upon by Langmuir (Langmuir 1917).
\[ \Pi = \sigma_0 - \sigma \]  

(1.7)

where \( \sigma_0 \) is the surface tension of an interface with no surfactants. As the density of surfactant is increased on the surface, through compression or increased adsorption from the bulk, the surface pressure will increase. At sufficiently large surface pressures, lipid monolayers begin to exhibit rheological Newtonian and non-Newtonian properties (i.e., monolayers can display elastic, viscous, strain-hardening, and strain-softening properties). A parallel can be drawn between these two-dimensional interfaces with bulk three-dimensional volumetric materials (Edwards 1961).

Before the 1960’s, the notion of an interface having rheological properties was fairly new. Boussineq (Boussinesq 1913) applied the concept of a surface viscosity to describe the settling velocities of fluid droplets. Later, Scriven (Scriven 1960) provided one of the first descriptive models for the rheology of Newtonian interfaces. When applied to spherical systems, such as the lipid encapsulation around a microbubble, tensile and viscous forces become dominant. The tensile forces, which are determined by surface tension, are elastic by nature and have been proposed many times as the dominant stabilizing force allowing microbubbles to persist much longer than was predicted by Epstein and Plesset (Epstein and Plesset 1950; Fox and Herzfeld 1954; Katiyar et al. 2009; Vandente.M et al. 1965; Yount 1997). The dilatational viscous forces have also been studied to predict the behavior of microbubbles under an acoustic beam (de Jong et al. 2009; de Jong et al. 1992; Doinikov and Dayton 2007; Doinikov et al. 2009; Paul et al. 2010; Sarkar et al. 2005). Understanding the nature of how elasticity and viscosity
affect the stability and dynamics of microbubbles is paramount in the engineering of novel microbubbles.

Experimental studies of microbubble rheology are generally conducted by indirect numerical fitting methods to observed microbubble geometries. Kim et al. (Kim et al. 2003) observed a monotonic increase in yield shear and shear viscosity of the encapsulating shell with the reduced temperature of a lipid and concluded it was due to the increase in the van der Waals interactions between the acyl chains. Chatterjee and Sarkar (Chatterjee and Sarkar 2003) found that protein coated microbubbles have a dilatational viscosity around 0.065 Ns/m and an elasticity of approximately 850 mN/m. Findings from Marmottant et al. (Marmottant et al. 2005) showed that a lipid interface behaves as a Bingham fluid, and requires that a yield stress for symmetric oscillations. Most recently, Paul et al. (Paul et al. 2010) utilized a strain softening model to estimate the dilatational viscosity (1.2 × 10⁻⁸ kg/s), unstrained surface tension (19 mN/m) and elasticity (550 mN/m) of the lipid monolayer encapsulating a microbubble. These findings suggest that microbubbles are resistant to external forces, and add to the explanation for their inherent stability.

1.5.2. Monolayer Resistance to Gas Permeation

The thin molecular shell on the microbubble not only resists changes in area, but also is a barrier for gas diffusion. Langmuir trough studies performed by Zhang and Unwin (Zhang and Unwin 2003) used an ultra-micro electrode to measure bromine gas transfer across a fatty acid monolayer. They determined that the monolayer resistance increased exponentially with increasing surface pressure, and concluded, along with others (Archer and Lamer 1955; Barnes and Hunter 1990; Cunnane et al. 1988; Langmuir and Schaefer 1943), that for a gas to diffuse
past the monolayer wall a pore the size of the collision area of the gas molecule must open. This energy barrier for gas diffusion was also observed in microbubble systems. Borden and Longo (Borden and Longo 2002) observed that lipid-encapsulated microbubbles dissolved longer than Epstein-Plesset theory predicted which was attributed to the resistance in gas permeation through the shell and increased with increasing lipid insolubility. Borden and Longo (Borden et al. 2003) also observed a drop in permeation in with the more insoluble lipids. Pu et al. (Pu et al. 2005) demonstrated that for bi-phase lipid encapsulations, one could increase the shells permeability by increasing the interstitial domain boundary density, adding another dimensional space for microbubble stability.

1.6. Specific Aims

Synthetic microbubbles are being developed for use in the food sciences and cosmetics to add volume and texture, as well as in the biomedical sciences for drug delivery, medical imaging and systemic gas transport. Recent data shows that rats breathing medical air, as opposed to pure oxygen, will more than double the systemic blood pool persistence time of lipid stabilized microbubbles (Mullin et al. 2011). We hypothesized that this phenomenon may be explained with a generalized dissolution model for a microbubble in a multi-component medium. Preliminary models of multi-component dissolution have been developed, but generally neglect the effects of the lipid shell (Kabalnov et al. 1998; Sarkar et al. 2009; Yung et al. 1989). Ultimately, one must account for the influence of the organic shell (or skin) which, as will be shown below, is necessary to stabilize the microbubble against surface-tension induced dissolution. We hypothesize that lipid monolayer’s rheology greatly affects dissolution owing to mechanical and permeation properties. We further hypothesize that these properties can be
elucidated by careful experimentation and analysis using theoretical models. Three hypotheses were then made:

1. A single-gas microbubble will grow and then dissolve when suddenly surrounded by a multi-gas medium where the dissolved gases are faster diffusing than the microbubble filling gas. For soluble-surfactant coated microbubbles, this phenomenon can be explained by a multi-gas diffusion model analogous to the Epstein-Plesset model.

2. A single-gas microbubble coated with a lipid surfactant will show non-Epstein-Plesset growth and dissolution upon sudden exposure to a multi-gas solution. The physical properties of the insoluble monolayer will restrict the amount of growth, hinder gas diffusion in and out of the microbubble, and stabilize the microbubble from completely dissolving.

3. The physical and rheological properties of the monolayer, such as the gas permeation resistance, domain dissolution, and surface elasticity are the governing factors in regulating the stability and dynamics of lipid-coated microbubbles.

The research project was broken down into three specific aims to test these hypotheses:

1. Directly observe the behavior of an SF$_6$-filled microbubble submerged in an air-saturated medium and analyze its behavior with a multi-gas diffusion model.

2. Observe the effects of the lipid shell on the stability and dynamics of an SF$_6$-filled microbubble by modulating the encapsulating lipid composition and microbubble size.
3. Add the properties of the lipid shell to the multi-gas diffusion model and analyze the experimental results to develop a constitutive model for the mechanics and gas transfer properties.
Chapter 2. Multi-gas Dissolution: Soluble Versus Insoluble Monolayer Encapsulations

2.1. Introduction

In Chapter 1.1, microbubbles found in natural bodies of waters were discussed to form from the breaking waves and were shown to have a profound impact on acoustic properties, solid sedimentation rates, and mass transport rates across the atmosphere-ocean interface (D'arrigo 1986; Edwards 1961; Johnson and Cooke 1981; Leighton 1994; Lozano et al. 2007; Vagle and Burch 2005). As mentioned in Chapter 1.2., synthetic microbubbles less than 10 µm in diameter are currently being used as UCAs (Wilson and Burns 2010; Wilson et al. 2009) and are also being developed for use as molecular imaging contrast agents (Bloch et al. 2004; Liang and Blomley 2003), drug or gene delivery vehicles (Chen et al. 2006; Choi et al. 2008; Kabalnov et al. 1998; Kabalnov et al. 1998; Mayer and Bekeredjian 2008), and systemic gas transport (Burkard and Vanliew 1994; Karichev et al. 1998; Swanson et al. 2010). For all of these applications, microbubble size and lifespan are critically important.

2.1.1. Single-Gas Microbubble Systems

The classic Epstein-Plesset theory predicts the rate of microbubble dissolution in a quiescent medium for a single gas component (Epstein and Plesset 1950). The Epstein-Plesset model includes effects of constant surface tension (\( \sigma \)) to generate a Laplace pressure in the gas core. Duncan and Needham (Duncan and Needham 2004) used a micropipette technique to show that microbubbles around 15 µm in radius and stabilized with soluble surfactant, such as sodium dodecyl sulfate (SDS), dissolve according to the Epstein-Plesset model. Insoluble
surfactant mixtures, such as lipids, deviate from the predictions of Epstein and Plesset. Borden and Longo (Borden and Longo 2002) accounted for the increase in microbubble dissolution time by including a finite shell permeability term to the Epstein-Plesset model. Katiyar et al. (Katiyar et al. 2009) applied a dilatational surface elasticity term in addition to shell permeability to account for long-term stability under saturated conditions.

Situations often arise, however, when multiple gases are present in the bulk medium surrounding the microbubble. In such cases, the initial microbubble may comprise a single gas, but the surrounding fluid may have several dissolved gases. For example, lipid-coated microbubbles used in medical applications are exposed to multi-gas environments, such as blood which has oxygen, nitrogen, carbon dioxide and possibly anesthetic gases as well. Naturally occurring microbubbles formed in the ocean and synthetic microbubbles used in bioreactors and microfloatation remediation may experience multi-gas environments.

2.1.2. Multi-Gas Microbubble Systems

Several models have been developed to predict the dissolution rate of a microbubble in a multi-gas medium (Burkard and Vanliew 1994; Kabalnov et al. 1998; Karichev et al. 1998; Katiyar et al. 2009; Sarkar et al. 2009; Yung et al. 1989). Kabalnov et al. (Kabalnov et al. 1998) developed a model to explain the in vivo behavior of microbubbles injected into rabbits and pigs. They adapted the Epstein-Plesset model to consider two-gas dissolution influenced by a pressure schedule due to cardiopulmonary circulation. Dissolution was predicted to have occurred over three stages: 1) an initial growth period, 2) steady dissolution and 3) a vapor-to-liquid phase change of the low vapor pressure gas acting under Laplace pressure. Sarkar et al. (Sarkar et al.
2009) derived a modified Epstein-Plesset model for a two-gas system for a lipid-coated microbubble accounting for the effect of shell permeability on the characteristic growth step and dissolution rate. Katiyar et al. (Katiyar et al. 2009) later applied a dilatational surface elasticity to explain longer dissolution times. Air was treated as a pseudo-single gas in these studies.

Here, a theoretical and experimental study was undertaken to determine the fate of a microbubble suddenly suspended in a medium with several gas species as in, for example, the injection of an ultrasound contrast agent into the bloodstream. The model expands on Epstein and Plesset’s analysis to include any number of gases. An experimental system was developed which isolates the microbubble in a permeable hollow fiber submerged in a perfusion chamber, allowing rapid exchange of the external aqueous medium. Experimental verification of the model was performed with individual SF$_6$ microbubbles coated with the soluble surfactant, sodium dodecyl sulfate (SDS). First, SDS-coated microbubbles were placed in a saturated SF$_6$ environment, where they dissolved under Laplace pressure. Second, SDS-coated microbubbles were placed in an air-saturated medium, where they initially grew with the influx of O$_2$ and N$_2$ and then dissolved under Laplace pressure. Finally, SF$_6$-filled microbubbles coated with the highly insoluble lipid dibehenoyl phosphatidylcholine were placed in an air-saturated medium. The lipid-coated microbubble gave significantly different results owing to variable surface tension. The initial growth phase was diminished, possibly owing to a shell “break up” tension that exceeds the pure gas/liquid surface tension. Three dissolution regimes were observed: an initial rapid dissolution followed by slower, steadier dissolution and finally stabilization, where the apparent surface tension approached zero. Our results demonstrate the importance of considering both the effects of multiple gas species and a variable surface tension in predicting the lifetime of lipid-coated microbubbles.
2.2. Theoretical Aspects of Microbubble Dissolution

A numerical method is presented which is not limited to the number of gases in the system. Air is treated as two independent gases. The Epstein-Plesset equation was separated into two components, the mass balance coupled with diffusion and the Laplace pressure equation, thus allowing the Epstein-Plesset form to be used for a system of any $N$ number of gases. The numerical model allows estimation of the apparent surface tension when the diameter of the microbubble is known as a function of time. The fit to experimental data gives insight into the dynamics of the microbubble shell during growth and dissolution.

For a microbubble instantaneously submerged in a quiescent medium with $N$ number of gases, the concentration profile around the microbubble is assumed to develop orders of magnitude faster than the movement of the bubble wall (Duncan and Needham 2004). The random walk diffusive velocity of the slowest gas is several orders of magnitude faster than the microbubble shell velocity (verified in results below), which confirms that a fully developed diffusion profile approximation is appropriate. We assume that no convection occurred due to efflux of gas or bulk flow surrounding the microbubble. The gas components are assumed to behave ideally. The molar flux is written as:

$$J_i = -D_i \left( \frac{\partial c_i}{\partial r} \right)_{r=R} = -D_i \left( \frac{c_{i,0} - c_{i,s}}{R} \right)$$  \hspace{1cm} (2.1)$$

where the diffusion zone is equal to the microbubble radius (i.e., Sherwood number = 2 for pure diffusion from a sphere) (Deen 1998). The component balance is:
\[
\frac{dn_i}{dt} = -4\pi R^2 J_i
\]  \hfill (2.2)

where, \(n_i\) is the moles of component \(i\), \(t\) is time, \(R\) is the microbubble radius, \(J\) is molar flux, \(D\) is diffusivity of component \(i\), \(C\) is molar concentration (subscripts “0” and “s” represent the bulk and surface, respectively). The microbubble gas pressure \((P_g)\) given by the Laplace equation (Edwards 1961):

\[
P_g = \frac{2\sigma}{R} + P_H
\]  \hfill (2.3)

where \(\sigma\) is the effective surface tension and \(P_H\) is hydrostatic pressure. The total pressure is equal to the sum of partial pressures, which are related to the moles of gas through the ideal gas law.

\[
P_{\text{g}} = \frac{BT}{3\pi R^3} \sum_{i=1}^{N} n_i
\]  \hfill (2.4)

\[
\frac{BT}{3\pi R^3} \sum_{i=1}^{N} n_i = \frac{2\sigma}{R} + P_H
\]  \hfill (2.5)
where $B$ is the ideal gas constant and $T$ is temperature. The diffusion of mass and heat inside the microbubble is sufficiently fast such that the gas core has uniform concentration and temperature. Assuming the interface is at local thermodynamic equilibrium, Henry’s law relates the gas partial pressure to the concentration on the fluid side of the boundary. The interfacial concentration of species $i$ is given by:

$$C_{s,i} = K_{H,i} \left( \frac{2\sigma}{R} + P_H - \frac{3BT}{4\pi R^3} \sum_{j=1}^{N} n_j \right) \text{ where } j \neq i$$  \hspace{1cm} (2.6)

where $K_{H,i}$ is the molar Henry’s constant. The saturation fraction, $f$, relates the bulk partial pressure with the saturation pressure ($P_{\infty,i}$).

$$C_{0,i} = K_{H,i} P_{\infty,i} f_i$$  \hspace{1cm} (2.7)

Substituting equations 2.6 and 2.7 to the molar balance 2.2 gives:

$$\frac{dn_i}{dt} = -4\pi D_i K_{H,i} \left( \frac{2\sigma}{R} - P_{\infty,i} f_i + P_H - \frac{3BT}{4\pi R^3} \sum_{j=1}^{N} n_j \right)$$  \hspace{1cm} (2.8)

Rearranging equation 2.3 and applying the ideal gas law gives:
Equations 2.8 and 2.9 are used to construct the numerical model, which may be solved for $N$ number of gases. In the single-gas case, the model reduces to the classic Epstein-Plesset model.

\begin{equation}
0 = 2\sigma R^2 + P_w R^2 - \frac{3BT}{4\pi} \sum_{i=1}^{N} n_i
\end{equation}

### 2.3. Materials and Methods

#### 2.3.1. Langmuir Isotherms

Filtered phosphate buffer saline (PBS) (Sigma-Aldrich, St. Louis, MO) solution was used as the sub-phase on a Langmuir trough (KSV, Monroe, CT). Stock solution of 1,2-dibehenoyl-sn-glycero-3-phosphocholine (DBPC) (Avanti Polar Lipids, Alabaster, AL) and polyethylene glycol 40 (PEG40) stearate (Sigma-Aldrich, St. Louis, MO) were dissolved into chloroform at a molar ratio of 9:1 and a concentration of 1 mg/ml. The stock solution was then deposited onto the PBS sub-phase. After the chloroform evaporated (10-15 min), the deposited monolayer was compressed at a rate of 382.5 mm$^2$/min. The monolayer was compressed from 8925 mm$^2$ to 7548 mm$^2$ and then expanded back to 8925 mm$^2$. The monolayer elasticity was determined using the equation below (Gaines 1966).

\begin{equation}
\chi = -A \frac{d\pi}{dA}
\end{equation}
where $\chi$ is the elastic modulus, $\pi$ is the surface pressure and $A$ is the surface area.

2.3.2. Preparation of SDS Microbubbles

SDS (Sigma-Aldrich, St. Louis, MO) was dissolved to 10 mM in purified water (18 MΩcm, Milipore, Billerica, MA) filtered through a 0.2 micron filter (Whatman, Maidstone, England). The solution was placed into 3 ml serum vials and put under vacuum for 5 min. SF$_6$ (Airgas, Radnor, PA) was flowed into the vials at a gauge pressure of 40 kPa for 5 min. After SF$_6$ was introduced, six cycles of vacuum and SF$_6$ (40 seconds per cycle) were applied to ensure there was no remaining air in the vial. After the last cycle, the serum vial was left under 40 kPa gauge pressure of SF$_6$ for 8 min. Before use, each vial was vented for 4-6 seconds. A dental amalgamator (Vialmix, ImaRx) was used to shake the solution to entrain gas and create SDS microbubbles.

2.3.3. Preparation of Lipid Microbubbles

A lipid formulation was used as an insoluble surfactant system. DBPC and PEG40 stearate were dissolved into chloroform. The two solutions were mixed such that the molar ratio of lipid to emulsifier was 9 to 1. The chloroform was allowed to evaporate in a vacuum chamber overnight. The lipid/emulsifier appeared as a white powder film at the bottom of the vial. PBS solution was used to re-suspend the lipid/emulsifier powder to a concentration of 3 mg/ml. The lipid/emulsifier was suspended by heated bath sonication. The gas exchange method described above was used to replace air with SF$_6$. Before shaking, the serum vial was brought up to 80 °C,
which is above the main phase transition temperature of DBPC. Once shaken, the vial was immediately cooled under flowing tap water back to room temperature.

2.3.4. Observation and Measurement of Microbubble Growth and Dissolution

Figure 2.4 shows the experimental setup. A porous micro-dialysis hollow fiber (18 kDa molecular weight cut off, Spectra/Por, Rancho Dominguez, CA) was threaded through a modified Warner Instruments RC-20 perfusion chamber to trap the microbubbles and rapidly exchange the gas concentration around the microbubble.

![Perfusion chamber diagram](image)

**Figure 2.1:** Perfusion chamber diagram. A) Flow chamber setup showing the injection sites of the microbubbles and bulk fluid. The microfiber was threaded through the center of the flow chamber to allow rapid exchange of the medium surrounding the microbubble.
B) A side view of the apparatus showing arrangement of the optics and the location of the microbubble in the hollow fiber.

The perfusion chamber had two inlet ports (Fig. 2.1A). One inlet was used as a microbubble injection site, and the other was used to inject the indicated bulk fluid. The microbubbles were trapped in the fiber due to buoyancy (Fig. 2.1B). The perfusion chamber (48 μL volume; 1 mm height) was designed to allow flow around the microfiber without disturbing the microbubble inside. A threaded, 500-μL gas-tight syringe (Hamilton, Reno, NV) was used to manipulate the microbubble in the microfiber. A syringe pump was used to inject the bulk fluid into the flow chamber at a rate of 5 ml/min for 10 seconds. Thus, a volume of 83 μL was applied to ensure the previous solution was fully purged. The flow chamber setup was placed on an Olympus IX71 inverted microscope with a 20x or 50x objective lens (and an additional 1.6x zoom). Time lapse images were acquired using a digital CCD camera (Pixelink, Ottawa, ON).

First, SDS-coated SF₆ microbubbles were placed in SF₆-saturated 10 mM SDS solution. The microbubble was allowed to settle within the hollow fiber, and time lapse images of microbubble dissolution were obtained. Second, microbubbles were injected into an SF₆-saturated solution. Once a single microbubble was found and trapped with no flow observed within the microfiber, the syringe pump was activated to purge the system of the SF₆-saturated solution and replace it with air-saturated solution.
2.3.5. Image Processing

ImageJ (NIH) was used to determine the diameter of each microbubble and in some cases the aspect ratio (ratio of the major and minor axis of a fitted ellipse). From a single microbubble image, ImageJ processed four different diameter measurements: 1) diameter based on area, 2) diameter based on perimeter, 3) maximum distance between 2 points within the microbubble and 4) minimum distance between two points within the microbubble. Differences between these four different measurements were used to obtain error bars.

2.3.6. Simulation

Using the initial microbubble radius and gas composition as an initial condition, a forward-wind finite-difference method (Farlow 1982) was employed to estimate future molar compositions of the gas microbubble interior, as given below:

\[
\sum_{i=1}^{N} n_i^{\tau+1} = -4\pi h R D_i K_{H,i} \left[ \frac{2\sigma}{R} - P_{\tau,i} f_i + P_H - \frac{3BT}{4\pi R^3} \sum_{j=1}^{N} n_j^\tau \right] + \sum_{i=1}^{N} n_i^\tau 
\]  

(2.11)

where \( \tau \) is the numerical time step and \( h \) is the difference in time between \( n_i^\tau \) and \( n_i^{\tau+1} \). Using equation 2.11 for a single-gas system, the prediction is identical to that of the Epstein-Plesset model. After determining the new composition of the microbubble, a new radius was calculated using the Newton-Raphson method (Stewart 1999):
To avoid instability in the modeling, the time step was made variable with respect to the microbubble radius. The time step was set equal to the time for a molecule of the slowest dissolving gas (diffusivity = $D_{min}$) to diffuse a distance equal to the microbubbles radius.

$$h = \frac{R^2}{4D_{min}}$$ (2.13)

Table 2.1 gives the parameters of solubility and diffusion coefficients for oxygen, nitrogen and sulfur hexafluoride in water used in the model.

<table>
<thead>
<tr>
<th>Gas Type</th>
<th>Henry’s Constant ([g/m^3]/Pa) x 10^4</th>
<th>Diffusivity ([m^2/s]) x 10^9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen ([O_2])</td>
<td>3.948(^a)</td>
<td>2.42(^{d,e})</td>
</tr>
<tr>
<td>Nitrogen ([N_2])</td>
<td>1.713(^a)</td>
<td>2(^{d,e})</td>
</tr>
<tr>
<td>Sulfur Hexafluoride ([SF_6])</td>
<td>3.4596(^{a,b,c})</td>
<td>1.2(^f)</td>
</tr>
</tbody>
</table>

Table 2.1: Key Parameters for the Different Gases

Table 2.1: \(^a\) – (Wilhelm et al. 1977), \(^b\) – (Bullister et al. 2002), \(^c\) – (Mroczek 1997), \(^d\) – (Himmelblau 1964), \(^e\) – (Lide 2009), \(^f\) – (King and Saltzman 1995)
Another algorithm was developed that solved for an effective surface tension. Equation 2.11 was solved to determine a possible microbubble composition under an initial surface tension. Equation 2.12 was then used to solve a new surface tension value. The process was iterated until the two surface tension values were identical.

2.4. Results and Discussion

We report on the effects of dissolved gas content and surfactant solubility on microbubble dissolution. First, we observed microbubbles coated with a soluble surfactant in a single-gas environment. We then observed the same microbubbles submerged in a multi-gas environment. Finally, we observed the dissolution of microbubbles coated with an insoluble surfactant (lipid) in a multi-gas medium.

2.4.1. SDS/SF$_6$ Microbubble in an SF$_6$-Saturated Medium

We used 10 mM SDS as the soluble surfactant because it maintains a constant surface tension (40 mN/m) above the critical micelle concentration (CMC) (Duncan and Needham 2004). Solutions inside and outside the fiber were at the same SDS concentration in order to maintain a constant chemical potential for the surfactant in the system. SDS-encapsulated SF$_6$-filled microbubbles were introduced into the porous hollow microfiber, which was submerged in saturated SF$_6$ solution. The solution surrounding the microfiber was not changed in order to allow surface tension driven dissolution in a single-gas environment. Figure 2.2A shows the dissolution times of SDS microbubbles of varying diameter. A representative diameter-time curve is shown Figure 2.2B. Figure 2.2A also shows the theoretical dissolution time calculated
for a saturated solution, \( f = 1 \) (Duncan and Needham 2004). To fit the data, a correction factor of 1.5 was applied to the solubility (or, equivalently, the diffusivity) of SF\(_6\). The enhanced solubility (or diffusivity) may be due to SDS micelles present in solution which may act as hydrophobic reservoirs for facilitated diffusion (Dungan et al. 2003; Huang and Lee 2001).

**Figure 2.2:** Dissolution times of SF\(_6\)-filled SDS-coated microbubbles dissolving in an SF\(_6\)-saturated aqueous solution. A) Experimental dissolution times (scatter), numerical model predicted values (open squares) and the analytical prediction from Duncan and Needham (Duncan and Needham 2004) (line). A correction factor of 1.5 was applied to the Henry’s constant (solubility) of SF\(_6\) in water. B) A typical microbubble dissolution curve.

2.4.2. SDS/SF\(_6\) Microbubble in an Air-Saturated Medium

We next tested the effect of sudden exposure to an air-saturated environment (i.e., multi-gas). This experiment simulates the injection of a microbubble into blood. Figure 2.3A shows the observed microbubble diameter changing with time. The microbubbles had an initial growth phase followed by dissolution. The model predicted a growth phase as a result of the influx of
oxygen and nitrogen, as shown in Figure 2.3B. Nitrogen and oxygen diluted the SF₆ and reduced the concentration gradient and therefore the dissolution rate.

**Figure 2.3:** Growth and dissolution of a typical SF₆-filled SDS-coated microbubble suddenly exposed to an air-saturated environment. A) Microscopy images of the microbubble. Spots on the bubble are artifacts of the CCD camera used to obtain the images. B) Experimental and theoretical diameter-time curves. C) Predicted gas content inside the microbubble.

The growth also caused a reduction in the Laplace pressure, which is inversely proportional to the microbubbles radius. These combined effects caused the efflux rate of SF₆ gas to decrease.
with time. Once SF$_6$ effectively exited the microbubble, the diameter-time curve followed the Epstein-Plesset model for a quasi-single component. The SDS-encapsulated microbubble ceased to be an SF$_6$ microbubble and instead became an air-filled microbubble, as observed by Sarkar et al (Sarkar et al. 2009). These results illustrate that microbubbles may exchange their gas core within seconds of immersion. Furthermore, they indicate that the concept of an osmotic gas for microbubbles used as oxygen substitutes may only be appropriate when the microbubble is initially submerged (Unger et al. 2004; Vanliew and Burkard 1995; Vanliew and Burkard 1995).

We assumed that the diffusion profile surrounding the microbubble was fully developed during microbubble growth and dissolution. The observed SDS-encapsulated SF$_6$-microbubble in Figure 2.3B had a maximum radial velocity of 0.42 μm/s. Theoretically, the initial growth phase peaked at a maximum velocity of 1.7 μm/s. A single SF$_6$ gas molecule diffuses at a velocity of 560 μm/s assuming random walk dynamics, which are several orders of magnitude faster than the microbubble surface velocity. Thus, the quasi-static assumption was valid.

We compared the observed microbubble dissolution times to the predicted dissolution times to verify the model (Fig. 2.4A). Statistical software (Prism, Graphpad Software, CA) was used to determine the correlation between the observed and predicted values. The coefficient of determination, R$^2$, for the model (with the independently determined correction factor) was 0.9473. The value was near unity and suggests that the model has strong predictive value. The residual scatter plot shows that although the residual tends to be positive, there was no trend associated to it (Fig. 2.4B). Note that although there is good agreement between the theoretical and experimental dissolution times, not all of the simulations traced the diameter-time curves as accurately as Figure 2.3B.
Figure 2.4: Dissolution times of SF\(_6\)-filled SDS-coated microbubbles in air-saturated solution. A) Experimental and theoretical values for the dissolution time as a function of the initial diameter. The numerical model assumed that the microbubble had a constant surface tension of 40 mN/m in the 10-mM SDS solution. B) Observed versus predicted dissolution times. Residuals are shown in the inset.

2.4.3. Lipid/SF\(_6\) Microbubble in an Air-Saturated Medium

We repeated the air-saturated experiment using the insoluble surfactant mixture, DBPC and PEG40S. This experiment most closely simulated an ultrasound contrast agent being injected into blood. Figures 2.5 and 2.6 show selected microscopy images and the diameter-time curve for a typical lipid-coated microbubble.
Figure 2.5: Growth and dissolution of a typical lipid-coated SF$_6$-filled microbubble suddenly exposed to an air-saturated environment. Microscopy images show: A) Initial size, aspect ratio (AR) = 1.021. B) Spherical growth, AR = 1.013. C) Non-spherical dissolution, AR = 1.141. D) Elliptical microbubble, AR = 1.165. E) Stable microbubble, AR = 1.041. Spots on the bubble are artifacts of the CCD camera used to obtain the images.
Figure 2.6: Experimental diameter-time curve corresponding to the microbubble shown in Figure 2.5. The aspect ratio (AR) is also plotted. The labeled growth and dissolution regimes are discussed in the text.

When subjected to the air-saturated environment, an initial expansion phase was observed for the lipid-coated microbubble. However, expansion was much less pronounced than for the SDS microbubbles. The shorter growth period observed for the lipid-coated microbubbles indicated a large dynamic surface tension that resisted expansion. We therefore made surface tension a fitting parameter in the model. The fit required an increase in surface tension during the growth period, with a peak at 520 mN/m (Fig. 2.7). The effective surface tension was seven-fold larger than that of the surface tension for an air-water interface (73 mN/m). This large tension may be explained by a “break-up” tension, i.e., a tension that resists lipid monolayer expansion from its fully condensed state.
Figure 2.7: Effective surface tension of the lipid-coated microbubble shown in Figure 2.5. Also shown is the fitted diameter-time curve. The cartoons show the suggested behavior of the lipid monolayer shell, as discussed in the text.

The concept was originally put forward by Marmottant et al. (Marmottant et al. 2005) for lipid-encapsulated microbubbles sonicated by 2-MHz pulses over 60-ms intervals, which exhibited “compression only” behavior below a threshold acoustic pressure. For a 1.6-µm diameter BR14 microbubble (Bracco Diagnostics), the break-up tension was estimated to be approximately 130 mN/m (Marmottant et al. 2005). The higher break-up tension in our system may have arisen due to the larger microbubble size and lower expansion rate. Lipid shell composition and microstructure also may explain the higher break-up tension. DBPC is well below the main phase transition temperature (75 °C), and DBPC microbubbles are known to exhibit high yield shear and surface viscosity (Kim et al. 2003).
The resting microbubble may be assumed to be in the fully condensed state (Borden and Longo 2004; Kim et al. 2003). In this state, the lipid molecules are maximally compressed and the intermolecular van der Waals forces are maximized. The strength of these van der Waal forces is also evident by the rigidity (Kim et al. 2003) and low permeability (Borden and Longo 2004) of the monolayer. The large break-up tension for the DBPC microbubble may have arisen due to the force required to suddenly overcome the short range intermolecular forces between the highly compressed lipids. In addition to short range van der Waals forces, hydrophobic interactions also may resist monolayer break-up.

Lipid monolayers undergo transitions from liquid to solid phases when compressed (Gaines 1966). During compression, domain formation must overcome electrostatic repulsion between the head-groups and entropic forces to orient and pack the lipid monolayer. Thus, an activation energy is associated with domain formation (Helm and Mohwald 1988). Upon expansion, surface tension gradients drive the domains to dissolve. However, the kinetics of domain dissolution is dominated by strong short-range van der Waals forces. Expansion of the monolayer, therefore, requires work to overcome these lateral van der Waals forces between the hydrophobic tails of the lipid (Israelachvili 1991) and expose the hydrophobic tails and gas interface to water.

Figure 2.8 shows a Langmuir isotherm of DBPC and PEG40 stearate mixture of the same composition as the lipid-encapsulated microbubbles. Compression of the monolayer resulted in a gradually increasing surface pressure, followed by a sharp rise in elasticity and monolayer collapse. The monolayer reached a maximum compression elasticity of 171 mN/m during compression. Hysteresis was observed upon expansion. The collapse plateau was not tracked back, indicating that collapse was irreversible (Nino et al. 2008). The compression elasticity
increased to 412 mN/m. The difference in elasticity suggested that the monolayer became more rigid upon compression. Hysteresis observed in compression-expansion cycles of surface pressure isotherms of lipid monolayers (Nino et al. 2008) may be due to the difference in activation energy between domain formation and dissolution.

Figure 2.8: Langmuir isotherm of a DBPC:PEG40-stearate monolayer mixture. The monolayer was compressed and then expanded at a rate of 380 mm$^2$/min. Compression and expansion are denoted by arrows on the hysteresis curve. Dotted lines show a linear fit to determine elasticity values. Cartoons show suggested monolayer behavior, as discussed in the text.

A parallel may be drawn between lipid shell break-up during microbubble expansion and hysteresis observed on Langmuir isotherms. The microbubble expansion rate is determined by the influx of gas into the microbubble, and ranged from 110 to 21,000 mm$^2$/min compared to the
380 mm²/min of the Langmuir-Blodgett trough. The microbubble may therefore have experienced “non-equilibrium” expansion. That is, the rate of expansion was greater than the rate of lipid domain dissolution. The domains may have “torn” apart, possibly along the inter-domain borders, to relieve the overpressure inside the microbubble. Domain tearing has been observed in other monolayer systems (Reda et al. 1996). The tensile force required to tear apart lipid domains may be the origin of the high break-up tension.

Lipid monolayer “tearing” may impact the behavior a lipid-coated microbubble under ultrasound pulsing. For example, higher defect densities within the monolayer shell may reduce the pressure buildup necessary for shell rupture, thus making the microbubble more compliant. One may then design the shell microstructure to amplify or dampen the acoustic response. However, this remains to be tested experimentally.

Dissolution of the lipid-encapsulated microbubble after the growth phase appeared to have three regions. In Region 1, immediately after monolayer expansion, the microbubble shrank rapidly back to its initial resting diameter (Fig. 2.6). Laplace pressure forced rapid dissolution of the gas core and compression of the monolayer back to a condensed phase. The surface tension decreased from the break-up tension and plateaus around 34 mN/m at the end of microbubble expansion. As the efflux of gas further drove surface compression, the monolayer reached maximum packing and began to collapse.

The onset of Region 2 occurred when the microbubble returned back to its approximate initial size. Region 2 was characterized by a nearly constant average microbubble boundary velocity. Non-spherical shapes and rapid jerks in diameter were also observed during dissolution (Fig. 2.3). Fluctuations in the effective surface tension suggested that monolayer collapse was
discontinuous, as previously observed in Langmuir monolayers of lipid mixtures (Gopal et al. 2006). Discrete collapse mechanisms such as vesicle formation and lipid monolayer buckling and folding may have caused the observed deviations in sphericity and fluctuations in surface tension (Gopal et al. 2006; Lee 2008; Lu et al. 2002). Though the effective surface tension fluctuated, the mean steadily decreased from around 10 mN/m to about 1 mN/m. The decrease in surface tension suggested that the shell was becoming increasingly rigid as the microbubble became smaller. The microbubble elliptical shape was characterized by an aspect ratio, which peaked around the transition between the Regions 2 and 3 (Fig. 2.6).

In Region 3, the diameter of the microbubble quickly dropped from 16 μm to 8 μm in diameter, and then stabilized. As the microbubble continued to dissolve very slowly, it became more spherical at diameters below ~6 μm. The microbubble may have been compressed to a point where the molecules or domains in the shell became ‘jammed’ (Yan et al. 2007), thus hindering further collapse. Interestingly, microbubble stabilization indicated that the monolayer becomes increasingly rigid at smaller diameters, suggesting that smaller microbubbles are more stable.

The surface tension from Figure 2.7 was re-casted as an excess pressure (Laplace pressure) and the area is represented by the fractional change in area, $\beta = \left( \frac{R}{R_0} \right)^2 - 1$, shown in Figure 2.9. He we observe the hysteretic behavior observed on the Langmuir isotherm. An excess pressure of approximately 45 kPa was required for monolayer break-up. The excess pressure was observed to decrease as the monolayer expands, indicating that the monolayer is relaxing and spreading once ruptured, demonstrating a “self healing” property.
2.5. Concluding Remarks

SF$_6$ microbubbles of 25-100 µm initial diameter were observed as they were suddenly immersed in single- and multi-gas environments using a modified perfusion chamber, and results were compared to a model derived to simulate gas exchange. Microbubbles coated with the soluble surfactant, SDS, and kept in a single-gas environment dissolved under constant surface tension as predicted by classic Epstein-Plesset theory. Microbubbles suddenly exposed to an air-saturated environment initially grew due to the influx of air and then dissolved under surface tension. The interior gas was rapidly exchanged. Interestingly, the coating surfactant had a strong effect on the reaction of the microbubble. While SDS-coated microbubbles grew and dissolved as predicted by the model, lipid-coated microbubbles deviated significantly. Surprisingly, the growth regime was less pronounced, and three dissolution regimes were found,
including (1) rapid dissolution back to the original diameter, (2) steady dissolution with a nearly constant wall velocity and (3) stabilization near ~10 µm diameter. The results indicate that the surface tension was dynamic, and the behavior can be explained by monolayer break-up, collapse and jamming. The role of the monolayer in the microbubble response has important implications on microbubble stability and size distribution, as well as the fate of the microbubble during intravenous injection and ultrasound insonification.
Chapter 3: Effect of Breathing Gas on Ultrasound Contrast Agents

3.1. Introduction

As discussed in Chapter 1.2, microbubbles are small gaseous particles stabilized by a lipid shell, and are currently implemented as ultrasound contrast agents (UCA), increasing the resolution of sonography images to the microvasculature level and providing a stimulus-response vehicle for drug delivery. These microbubble UCAs range between 1 – 10 µm in diameter and remain in the blood vessels, flowing with red blood cells. Their gaseous core is highly compressible, and as a result, their acoustic echo is much more than the surrounding tissue. Because of the compressibility difference, they strongly reflect acoustic signals. In fact, the echo is so strong that a single microbubble can be detected (Sboros 2008; Schneider 2008). The UCAs oscillate non-linearly, and their scattering patterns can be distinguished from the surrounding linear reflectors (e.g., tissue). Microbubble UCAs are currently being developed for diagnostic measurements (Trojan et al. 2010), characterization of microvascular blood flow (Moguillansky et al. 2011), detection for masses in the liver (Harvey et al. 2000), and molecular imaging of inflammation and angiogenesis (Kaufmann et al. 2007; Lindner 2009; Martin et al. 2010).

For all of the above applications, microbubbles are required to either be freely flowing through the bloodstream or directly targeted the region of application, through targeting specific molecular biomarkers, for example. Regardless of where the microbubbles need to be, UCAs must be administered via bolus injection or continuous IV drip during the time frame in which the ultrasound pulse is being applied. It is therefore critical to understand what factors contribute to the persistence of microbubbles in circulation. Factors such as the environmental conditions as
well as microbubble composition directly affect the lifetime in the bloodstream. Researchers have shown that the gas core composition and the make-up of the lipid encapsulation control the lifetime of the microbubble and therefore can be engineered for longer circulation times. The lipid encapsulation and surface microstructure retards gas diffusion, increasing the dissolution time (Borden and Longo 2002; Martin et al. 2010; Pu et al. 2005). The dissolution time can be further increased by using heavier hydrophobic gases, which have a tendency to be insoluble in aqueous mediums. Kabalnov et al. (Kabalnov et al. 1998) and, more recently, Reiss et al. (Riess and Krafft 2005) have shown that the persistence of microbubbles in circulation is increased using these large insoluble gases. While the composition of the gas core and lipid shell plays a critical role, environmental variables such as blood gas content also greatly affect the lifetime of a microbubble.

It has been established that gas saturation of the surrounding environment will affect the diffusion of the gas core, driving microbubbles to dissolve (Borden and Longo 2002; Duncan and Needham 2004; Epstein and Plesset 1950). For injected UCAs, dissolved gas content may provide to be an opportunity to improve their lifetime. Early studies showed that microbubbles injected into dog models inhaling different anesthesia gas carriers would result in different circulation times (Wible et al. 1998; Wible et al. 1996). These studies focused on the effect of breathing either medical air or pure oxygen on the circulation times of two “first-generation” air-core protein-shelled microbubbles: Albunex (Mallinckrodt Inc., USA) and Optison (GE Healthcare Inc., USA). For both microbubble types, the breathing of pure oxygen reduced the imaging contrast supplied by the UCAs in the dog’s bloodstream, indicating a loss in stability.
3.1.1. *Ultrasound Contrast Agent Lifetimes in Rats*

More recent studies extend this observation to “second-generation” lipid-coated microbubbles with perfluorocarbon gas cores (Mullin *et al.* 2011). An *in vivo* ultrasonography study was performed on five female Sprague Dawley rats (Harlan; Indianapolis, IN) to investigate the dependence between the lipid-coated microbubble UCA lifetime and the anesthesia gas carrier. Mullin *et al.* (Mullin *et al.* 2011) used the clinical imaging system Acuson Sequoia 512 (Siemens – Mountain View, CA) to obtain B-mode (at 14 MHz) and CPS-mode (at 7 MHz and mechanical index of 0.18) images of the rat’s kidneys. The kidneys were chosen to be the region of interest because of the large network of blood vessels, allowing a large degree of microbubble UCA perfusion. Before imaging, each rat was anesthetized through a breathing mask with aerosolized isofluorane – oxygen or air mixture (5% isofluorane). Once sedated, the concentration of anesthetizing agent was reduced from 5% to 2% for the duration of the experiment. The sedated rats were then injected in the tail vein with 25 µl of a suspension of DSPC:DSPE-PEG2K (9:1 molar ratio) perfluorbutane-core microbubbles, followed immediately by a 200 µl injection of sterile saline.

Video data of the kidney was collected across 20 minutes, starting 10 – 20 seconds before a bolus injection of microbubble UCAs was administered into the tail vein of the rat. To measure the residence time in the kidney of each lipid-coated UCA injection, a frame-by-frame mean pixel intensity was calculated over a region of interest in the kidney, which is displayed in Figure 3.1.
Figure 3.1: Two examples of a frame-by-frame grey scale intensity over the region of interest after a bolus injection of a microbubble suspension. Microbubble UCAs reside in the kidney longer in the rat inhaling air (grey squares) compared to the same rat breathing pure oxygen (black diamonds). Taken from Mullin et al. (Mullin et al. 2011).

They observed that the mean pixel intensity would sharply increase, hitting a peak as the bolus injection of microbubbles began to flow through the region of interest. As the microbubbles left the region of interest, the echo signal decayed and returned back to the baseline value. The microbubble lifetime was then calculated by measuring half-life (time for 50% of the signal to drop) and the quarter-life (time for 75% of the signal to decay). The study determined the half-life and quarter-life of the residence times of perfluorobutane microbubble UCAs in the kidney of rats breathing either medical air or pure oxygen. The study showed a clear increase in microbubble lifetime for rats breathing medical air (Fig. 3.2).
Figure 3.2: A comparison between five Sprague Dawley rats breathing the respective anesthesia gas carrier. (A) The time for 50% decay of the mean pixel intensity. The average microbubble UCA circulation half-life was significantly longer (p<0.05) for all of the rats inhaling medical air. (B) The time for 75% decay of the mean pixel intensity. The quarter-lifetimes were significantly (p<0.05) longer in 4 of the 5 rats breathing medical air. Taken from Mullin et al. (Mullin et al. 2011).

Circulation half-lives of microbubble UCAs in rats breathing medical air was 1.8 times longer in all of the rats when compared to pure oxygen as the breathing gas. Side-by-side comparisons of the rats inhaling medical air or pure oxygen showed a significant difference (p<0.05) in four of the five rats, with a 2.2 fold increase in quarter-lifetimes. The only difference between medical air and pure oxygen is the presence of nitrogen in medical air. The nitrogen gas was hypothesized to diffuse into the perfluorocarbon microbubble, increasing its diameter, and
prolonging the lifetime. To get a better understanding of the role nitrogen plays in extending the circulation time, theoretical estimations of the diameter-time behavior were calculated for a perfluorocarbon microbubble suddenly submerged in blood.

3.2. Theory

To investigate whether gas diffusion could account for differences in MCA lifetime, a mathematical model previously developed in Chapter 2 was employed which predicts the size of a microbubble suddenly immersed in a multi-gas environment, such as a PFB microbubble injected into blood. This model assumes that each gas acts independently to equilibrate at the gas/liquid interface and diffuse along its own chemical potential (partial pressure) gradient. All gases contribute to the total pressure and volume of the MCA gas core. Gas diffusion occurs through a stagnant aqueous layer equal in thickness to the microbubble radius (i.e., it is a purely diffusing sphere). Because the animal was breathing, the impact of the microbubble injection (~2 µl gas) on the dissolved gas contents of the blood pool was considered negligible. A set of coupled, nonlinear differential equations results from (i) a species balance over the microbubble, (ii) the diffusion equation, (iii) the Laplace pressure equation and (iv) the ideal gas law. Applying a finite difference method, the set of equations is discretized to the following form:

\[
\sum_{i=1}^{N} n_i^{\tau+1} = -4\pi R D_i K_{H,i} \left[ \frac{2\sigma}{R} - P_{\infty,i} f_i + P_H - \frac{3BT}{4\pi R^3} \sum_{j=1}^{N} n_j \right] + \sum_{i=1}^{N} n_i \tag{3.1}
\]

\[
0 = \frac{8\pi\sigma}{3BT} (R^\tau + 1)^2 + \frac{4\pi P_H}{3BT} (R^\tau + 1)^3 - \sum_{i=1}^{N} n_i^{\tau+1} \tag{3.2}
\]
where $N$ is the total number of gas species, $\tau$ is the numerical time step, $R$ is the microbubble radius, $\sigma$ is the microbubble surface tension; $P_H$ is the hydrostatic pressure, $B$ is the universal gas constant, $T$ is temperature; $n_i$ is the moles, $D_i$ is the diffusion coefficient, $K_{H,i}$ is Henry’s constant, $P_{sat,i}$ is the partial pressure at saturation, and $f_i$ is the ratio of the bulk dissolved gas content to that at saturation, each of component $i$. Solving equations 3.1 and 3.2 numerically allows prediction of the growth and dissolution of a microbubble subject to the simultaneous influx and efflux of different gas species as the system tends toward thermodynamic equilibrium, which occurs when the partial pressures in the gas core and surrounding medium are equal for every species. A variable time step was used to ensure that the moles inside the microbubble did not become negative. To determine the number of moles in the bubble, a forward wind difference method was applied, as seen in equation 3.1, which was solved step-wise. Equation 3.2 is non-linear and was solved using a Newton-Raphson method. In the simulation, 100 iterations were used. Finally, it should be noted that the model is limited in that it neglects the effects of convection in the surrounding medium, variations in blood gas concentrations and pressure as the microbubble passes through the venous-arterial circuit, and effects of the encapsulating shell, such as gas permeation resistance and viscous and elastic terms accounting for lipid monolayer expansion, break-up, compression, buckling and collapse.

### 3.3. Results and Discussion

To gain physical insight, we modeled microbubble dissolution times in blood by considering pure diffusion (no convection) of the different gas species into and out of the
Equations 3.1 and 3.2 were solved numerically using the Newton-Raphson method and MATLAB software for a 2-, 5- and 10-µm diameter microbubble initially filled with pure PFB and suddenly immersed in blood. Blood gas (O₂, N₂, H₂O and CO₂) partial pressure values were taken from Table 3.1 to simulate oxygen versus air as the carrier gas. The surface tension was set to 0 mN/m (Duncan and Needham 2004), and the hydrostatic pressure was set to 760 mmHg.

### Table 3.1: Blood Gas Values

<table>
<thead>
<tr>
<th></th>
<th>pO₂ (mmHg)</th>
<th>pCO₂ (mmHg)</th>
<th>pN₂ (mmHg)</th>
<th>pH₂O (mmHg)</th>
<th>Σpᵢ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Range</td>
<td>80-100</td>
<td>32-45</td>
<td>555-585</td>
<td>47</td>
<td>714-777</td>
</tr>
<tr>
<td>Breathing air (n=4)</td>
<td>70 ± 6</td>
<td>63 ± 9</td>
<td>560</td>
<td>47</td>
<td>740</td>
</tr>
<tr>
<td>Breathing oxygen (n=3)</td>
<td>546 ± 45</td>
<td>61 ± 15</td>
<td>0</td>
<td>47</td>
<td>654</td>
</tr>
</tbody>
</table>

Table 3.1: Mean partial pressures of dissolved O₂ and CO₂ gasses in arterial blood were measured from animals anesthetized with the two different carrier gases (Mullin et al. 2011). The values for N₂ and H₂O were taken from literature (Nunn 1987; West 1990). Σpᵢ is the sum of partial pressures. The pO₂ increased above the normal range while breathing pure oxygen, but the pCO₂ remained constant. Ventilation/perfusion mismatch increased for rats breathing pure oxygen, as expected (Nunn 1987; West 1990). These values were used to simulate the dissolution times of a single MCA within the different mixed gas environments.
Figure 3.3: Simulated response of a PFB microbubble suddenly immersed in arterial blood. The dissolved gas parameters used in these simulations, summarized in Table 3.1, were based on measured values. Microbubble diameter was normalized by the initial value shown.

Figure 3.3 shows the simulation results for the two carrier gases. Theory predicted that a 2-μm diameter microbubble survives approximately 3.2 times longer when medical air is used instead of oxygen (Fig. 3.3). As the diameter is increased to 10 μm, the microbubble lifetimes are predicted to be 126 sec for oxygen-breathing and 409 sec for air-breathing, which is in good general agreement with the experimental in vivo imaging data. We should note that the simplified model is limited when making direct comparisons to in vivo contrast persistence data. Inspection of the microbubble contents over time provided an explanation for the extended lifetime (Fig. 3.4).
Figure 3.4: Simulated response of a PFB microbubble (MCA) suddenly immersed in arterial blood. The dissolved gas parameters used in these simulations, summarized in Table 1, were based on measured values (Mullin et al. 2011). Molar contents of each gas are plotted versus time for (A) medical air and (B) oxygen as the carrier gas.

Initially, the blood gases rapidly diffused into the microbubble just as PFB, which has a much lower solubility and diffusivity in water, slowly dissolved away. The inrush of blood gases resulted in rapid microbubble growth. Microbubble growth could not be measured acoustically here due to the complexity of the *in vivo* backscattered signal. However, microbubble growth has been predicted and measured as an increase in ultrasound attenuation in a more idealized system by Sarkar et al. (Chatterjee *et al.* 2005; Sarkar *et al.* 2009). The presence of nitrogen extended the growth phase by not only adding to the volume, but also diluting the other gas species, resulting in greater accumulation of the other blood gases and reduction in the rate of PFB efflux. After a short time, the microbubble was mainly composed of blood gases, and PFB contributed only slightly to the total volume. Eventually, all gases began to efflux from the microbubble as PFB dissolution continued and the partial pressures of $O_2$, $N_2$ and $CO_2$ in the gas core exceeded the partial pressures in the surrounding blood. The dissolution rate was
accelerated for the oxygen-carrier case owing to the greater degree of ventilation/perfusion imbalance. The sum of the partial pressures for oxygen-breathing (653 mmHg) was much lower than for air-breathing (740 mmHg). Since the ambient pressure was approximated to be 760 mmHg, the oxygen-breathing case represented a greater deviation from equilibrium and therefore a greater driving force for microbubble dissolution.

3.4. Concluding Remarks

Experiments performed by Mullin et al. (Mullin et al. 2011) first observed the relationship between the anesthesia gas carrier and the circulation lifetime in the kidneys of rats. They demonstrated that when breathing medical air, there was a significant increase in microbubble UCA persistence in the kidney at both the half-life (100% of the rats) and the quarter-life (80% of the rats). The increase in persistence was attributed to the presence of nitrogen. Indeed the classical Fickian diffusion model coupled to a molar balance for multiple gas species simulated that the nitrogen gas has a large influx. Microbubbles in the presence of arterial blood saturated with medical air showed a larger increase in diameter, increasing its dissolution time. The large ventilation-perfusion mismatch observed in the arterial blood of rats breathing pure oxygen provided a large driving force for dissolution, causing the microbubble to rapidly dissolve. These numerical results suggest the strong role gas diffusion plays in determining the lifetime of lipid-coated microbubble UCAs.
Chapter 4: Experimental Observations of Lipid-Coated Microbubbles in Multi-Gas Media

4.1. Introduction

In Chapter 1 we reviewed lipid-encapsulated microbubble used in medical (de Jong et al. 2009; Ferrara et al. 2007; Hernot and Klibanov 2008; Lentacker et al. 2009; Lindner 2004; Qin et al. 2009; Sirsi and Borden 2009; Stride and Edirisinghe 2008; Unger et al. 2004), food (Chang and Hartel 2002; Dressaire et al. 2008; Shen et al. 2008) and biotech (Bredwell and Worden 1998; Worden and Bredwell 1998) industries and are found in natural bodies of water (D'arrigo 1986; Johnson and Cooke 1981; Lozano et al. 2007). It was discussed that they appear in the oceans as plumes of gaseous particles, each one coated and stabilized by a monolayer of acyl-lipids and glyco-proteins (Fox and Herzfeld 1954). Natural microbubbles impact solid sedimentation rates, transport of salts and organics across the atmosphere-ocean interface and the reflection of sunlight (D'arrigo 1986; Leighton 1994; Seitz 2011). Synthetic microbubbles have shown promise in medicine as a platform for contrast-enhanced ultrasound imaging (de Jong et al. 2009; Lindner 2004; Qin et al. 2009), ultrasound-mediated drug and gene delivery (Ferrara et al. 2007; Hernot and Klibanov 2008; Lentacker et al. 2009; Sirsi and Borden 2009; Stride and Edirisinghe 2008; Unger et al. 2004) and intravenous oxygen delivery (Burkard and Van Liew 1994; Swanson et al. 2010; Unger et al. 2004). In order to understand the dynamics and stability of natural and synthetic microbubbles, it is important to gain knowledge of the effects of the lipid monolayer shell on the transfer of gas molecules between the microbubble core and the surrounding fluid.
In contrast-enhanced ultrasound, for example, microbubbles provide acoustic signals to delineate anatomical features, measure blood flow and indicate expression of vascular biomarkers. The contrast (to tissue) is provided by nonlinear echoes, which are strongest near the microbubble resonance frequency. Microbubble resonance depends on the resting diameter and the mechanics of the encapsulation, see Chapter 1.1.1. (de Jong et al. 1992). Since the microbubble resonance frequency may overlap with the imaging frequencies used commonly by clinical ultrasound scanners, the overall acoustic response of microbubble contrast agents in tissue depends strongly on the size distribution and encapsulation properties. Therefore, prediction and modeling of the \textit{in vivo} fate of intravenously injected microbubbles is critical for establishing contrast-enhanced ultrasound as a quantitative imaging modality.

Lipid-coated microbubbles used for medical applications typically comprise of a low-solubility gas, such as sulfur hexafluoride (SF$_6$) or perfluoropropane. Such microbubbles are remarkably stable (up to several months) in the storage vial, where the surrounding medium is saturated with the same gas that fills the microbubble core (Borden and Longo 2002; Klibanov 2002). However, once injected into blood, these same microbubbles are only stable for a few minutes. Microbubble dissolution is driven by under-saturation of the encapsulated gas in blood, and gas exchange between the microbubble and surrounding blood affects the rate of microbubble dissolution.

This effect was demonstrated recently by Mullin et al. (Mullin \textit{et al.} 2011), who reported on experiments to elucidate the contribution of anesthesia carrier gas on \textit{in vivo} microbubble persistence. They observed nearly a doubling in circulation half-life for rats breathing air compared to pure oxygen. The increased circulation time was attributed to nitrogen dissolved in
the blood, which diffused into the microbubble and diluted the other gas species, as well as the lower degree of alveolar ventilation-perfusion mismatch compared to pure oxygen.

The dynamics of bubble gas exchange has previously been studied (Burkard and Van Liew 1994; Cable and Frade 1987; Kabalnov et al. 1998; Kentish et al. 2006; Kwan and Borden 2010; Sarkar et al. 2009; Yung et al. 1989). Early theoretical models considered a constant surface tension at the gas/liquid interface and predicted an initial growth phase as the ambient gases diffused into the core, followed by Laplace pressure-driven dissolution as demonstrated in Chapter 2. We recently showed that this model works well for microbubbles coated with soluble surfactants, such as sodium dodecyl sulfate (SDS), but not for insoluble surfactants, such as lipids (Kwan and Borden 2010). Experimental observations showed that lipid microbubbles resisted both growth and compression beyond the equilibrium (initial) diameter. However, the microbubbles in these prior studies were relatively large (> 20 µm diameter) and not as relevant to biomedical and other applications.

Previous experiments have demonstrated the significant impact of the lipid encapsulation on the microbubble shell dynamics. Kim et al. (Kim et al. 2003) demonstrated that the lipid monolayer is a visco-elastic film with a yield stress that must be overcome to shear the monolayer. Borden and Longo (Borden and Longo 2002) demonstrated that the lipid shell results in bubble deformation and slows dissolution. They further observed that lipid-coated microbubbles dissolve in a step-wise profile: bending, folding and snapping back to a spherical shape in cycles as the surface area was compressed. Duncan and Needham (Duncan and Needham 2004) developed a micropipette technique to observe single microbubbles undergoing dissolution. They showed that soluble surfactant (SDS) coated microbubbles dissolve in accordance with the Epstein-Plesset theory (Epstein and Plesset 1950). Lipid-coated
microbubbles, on the other hand, were stable for long periods of time, which was attributed to “zero tension in the interface” that prevented Laplace pressure-driven dissolution. de Jong et al. (de Jong et al. 2007) showed that the lipid encapsulation may influence microbubble vibration in the ultrasound field, leading to interesting phenomena such as “compression-only” behavior. Wrenn et al. (Wrenn et al. 2009) showed that the lipid encapsulation affects the threshold for the onset of acoustic cavitation. Taken together, these results suggest that the lipid shell mechanics play a dominant role in the dynamics and stability of microbubbles.

Researchers have attempted to model microbubble dynamics by including explicit terms for the lipid shell. Fox and Herzfeld (Fox and Herzfeld 1954), and later Yount (Yount 1997), described the stabilization of a microbubble by a mechanical resistance to microbubble collapse. Borden and Longo described a resistance of the shell to gas transfer (Borden and Longo 2002). More recently, Katiyar et al. (Katiyar et al. 2009) incorporated a dilatational surface elasticity term, which resists changes in surface area. Ultrasound studies have been used to estimate the mechanical properties for vibrating microbubbles based on the acoustic response and high-speed videomicroscopy images (de Jong and Hoff 1993; de Jong et al. 1992; Doinikov and Dayton 2007; Doinikov et al. 2009; Marmottant et al. 2005; Sarkar et al. 2005). However, the relatively simple models employed in these prior studies did not capture the complex effects of the lipid shell on microbubble dynamics, for example, in Chapter 2 videomicroscopy was used to observe microbubbles subjected to multi-component gas environments (Kwan and Borden 2010).

The purpose of our study was to experimentally observe microbubbles of different sizes and shell compositions using the gas-exchange apparatus as a means of perturbing the microbubble in a way that will reveal the more subtle effects of the lipid shell. We investigated the influence of both shell composition and microbubble size, with values similar to
commercially available microbubbles used as ultrasound contrast agents. Table 4.1 shows physical property data reported in the literature for the lipid shells used in this study. The results of our study provide a new empirical data set for improving models of microbubble dynamics and revealing “design principles” for engineering microbubble suspensions for biomedical, food and biotech applications.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Emulsifier</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; [°C]</th>
<th>Shell Permeation Resistance [s/cm]</th>
<th>Yield Shear [mN/m]</th>
<th>Shear Viscosity [mN s/m]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC</td>
<td>PEG 40S</td>
<td>23</td>
<td>1 ± 1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>DPPC</td>
<td>PEG 40S</td>
<td>41</td>
<td>3 ± 1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>DSPC</td>
<td>PEG 40S</td>
<td>55</td>
<td>23 ± 3</td>
<td>0.9 ± 0.1</td>
<td>4 ± 0</td>
</tr>
<tr>
<td></td>
<td>DSPE PEG2K</td>
<td>386 ± 44</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>DBPC</td>
<td>PEG 40S</td>
<td>75</td>
<td>237 ± 20</td>
<td>4.1 ± 0.2</td>
<td>18 ± 7</td>
</tr>
</tbody>
</table>

Table 4.1: Properties of Lipid Encapsulation

Table 4.1: a – (Marsh) (Marsh 1990), b – (Longo et al.) (Borden and Longo 2002; Lozano and Longo 2009), c – (Kim et al.) (Kim et al. 2003)

4.2. Materials and Methods

4.2.1. Materials

1,2-dimyristoyl-<i>sn</i>-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-<i>sn</i>-glycerol-3-phosphocholine (DPPC), 1,2-distearoyl-<i>sn</i>-glycerol-3-phosphocholine (DSPC), 1,2-dibehenoyl-<i>sn</i>-glycerol-3-phosphocholine (DBPC), and 1,2-distearoyl-<i>sn</i>-glycerol-3-phosphoethanolamine-N-
[methoxy(polyethylene glycol)-2000] (DSPE-PEG) were purchased from Avanti Polar Lipids (Alabaster, AL) in powder form. Polyethylene glycol 40 stearate (PEG40S) and phosphate buffer saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO). Sulfur hexafluoride (SF₆) (99.8% pure) was purchased from Airgas (Radnor, PA). All reagents were used as received without further purification.

4.2.2. Preparation of Bulk Lipid Solution

Lipids and emulsifier (PEG40S or DSPE-PEG) were combined in powder form to a 9:1 molar ratio and suspended in 400 or 1000 mL filtered PBS to a final concentration of 2 mg/mL. The mixture was heated to approximately 10 °C above the phospholipid main phase transition temperature and bath sonicated to disperse the lipid, as evident by the dispersion becoming translucent and clear. The lipid solution was then split into two 250-ml serum vials and placed in a temperature-controlled water bath kept at 25 °C for the duration of the experiments. Air-saturated lipid solution was made by simply leaving one vial open to the atmosphere. SF₆-saturated lipid solution was generated by sealing and venting the second vial, and bubbling SF₆ gas for at least an hour prior to and then throughout the experiment.

4.2.3. Generation of Microbubbles

A 2.0 ml volume of SF₆-saturated lipid solution was transferred to a 3-ml serum vial, capped and sealed. The vial headspace was exchanged with SF₆ gas, as previously described (Kwan and Borden 2010). A vial shaker (Vialmix, Bristol-Myers Squibb) was used to generate the microbubbles. The microbubble suspension was diluted with SF₆-saturated lipid solution
before injection into the hollow microfiber in order to isolate single microbubbles for observation.

4.2.4. Observation of Single Microbubbles

A perfusion chamber containing a cellulose hollow microfiber and solution exchange apparatus was assembled as described previously (Kwan and Borden 2010). The perfusion chamber was modified to control temperature, maintained at 25 °C for these experiments, by attaching two flanking PID-controlled peltier devices (TE-Tech, Traverse City, MI) to the perfusion chamber casing. An Olympus IX-71 inverted microscope was fitted with a 50x or 100x water-immersion objectives, and a digital high-resolution CCD camera (Orca-HR, Hamamatsu Photonics, Japan) was used to capture images. The camera was controlled with Simple PCI software (Hamamatsu, Sewickley, PA). The perfusion chamber technique was used to isolate single microbubbles using a threaded 500-μL gas-tight syringe (Hamilton, Reno, NV) to control the placement of the microbubble in the hollow fiber, and then a syringe pump was used to rapidly replace the SF₆-saturated lipid solution surrounding the microfiber with air-saturated lipid solution, as previously described (Kwan and Borden 2010). The images were analyzed with the ImageJ (NIH) to determine the area, maximum and minimum Feret diameters, best-fit ellipse and aspect ratio.
4.3. Results

4.3.1 Overall Diameter-Time Behavior

Figure 4.1 shows diameter-time curves and single videomicroscopy snapshots for two representative SF₆-filled microbubbles growing and dissolving after sudden exchange of the surrounding medium from an SF₆-saturated lipid solution to an air-saturated lipid solution. Figure 4.1A shows a DMPC:PEG40S-coated microbubble with initial diameter \( D_0 \) of approximately 7 μm.

Figure 4.1: Growth and dissolution curves for typical microbubbles following gas exchange of the surrounding aqueous medium from SF₆ to air saturation. Diameter-time curves are shown above, and image snapshots at the labeled time points are shown below.
for: A) DMPC:PEG40S encapsulation, and B) DSPC:PEG40S encapsulation. Parameters used to define microbubble dynamics are marked on the plot: $D_0$ is the initial diameter, $D_{max}$ is the maximum diameter, $D_{S1}$ is the primary stabilization diameter, $t_\theta$ is the lag time before subsequent dissolution, $t_D$ is the dissolution time, and $D_{S2}$ is the secondary stabilization diameter (“stable diameter”).

The DMPC coating in this case was more fluid as evidenced by a relatively low main phase transition temperature ($T_m = 23 \, ^\circ{C}$). Upon introduction of the air-saturated fluid, the microbubble rapidly grew with the influx of nitrogen and oxygen to approximately 12.5 $\mu$m diameter. The microbubble then rapidly shrank back to a primary stabilization diameter ($D_{S1}$) that was slightly smaller than the initial diameter. The microbubble remained at this diameter, for approximately 65 sec before it began dissolving again. We gave this lag time between primary stabilization and the initiation of subsequent dissolution the symbol $t_\theta$ (Fig. 4.1A). Following $t_\theta$, the microbubble began dissolving at an accelerating pace. The time for complete dissolution (~45 sec) was marked as $t_D$ (Fig. 4.1A).

Figure 4.1B shows a DSPC:PEG40S-coated microbubble of approximately the same size and undergoing the exact same solution exchange process as for the microbubble described above. The response, however, is quite different for this lipid encapsulation, which is more solid as evidenced by a higher main phase transition temperature ($T_m = 55 \, ^\circ{C}$). The growth owing to the initial influx of nitrogen and oxygen was stunted, with the maximum diameter ($D_{max}$) being only a fraction of a micrometer larger than the initial diameter, $D_0$. Following growth, the microbubble rapidly shrank to the primary stabilization diameter, $D_{S1}$, which was slightly larger
than $D_0$. The lag time ($t_\theta \sim 120$ sec) and subsequent dissolution time ($t_d \sim 250$ sec) were much longer than for the DMCP-coated microbubble. Interestingly, for the DSPC-coated microbubble, dissolution was arrested at a secondary stabilization diameter ($D_{s2}$), which was approximately 1.5 \( \mu \text{m} \) diameter. The microbubble was stable at $D_{s2}$ indefinitely (over the experimental timeframe).

For the same encapsulation, DSPC:PEG40S, Figure 4.2A shows the effect of microbubble diameter on the growth and dissolution behavior. The largest microbubble (15-\( \mu \text{m} \) diameter) exhibited the most significant growth phase. Surprisingly, microbubbles of \( \sim 10 \) \( \mu \text{m} \) diameter or smaller exhibited significantly less growth than their larger counterparts. These results show that the initial size of the microbubble has a strong influence on the dynamics during the early gas-exchange phase.

**Figure 4.2: Effects of microbubble size and encapsulation type on diameter-time curves for typical microbubbles following gas exchange. A) Curves are shown for different sized microbubbles encapsulated with DSPC:PEG40S. B) Curves are shown for \( \sim 7 \) \( \mu \text{m} \) diameter**
microbubbles with different encapsulations, where the diameter was normalized by the initial diameter, $D_0$.

Representative diameter-time curves for the different lipid encapsulations are shown in Figure 4.2B. Each of the microbubbles had an initial diameter of ~7.0 μm, and the diameters were normalized to $D_0$ for the sake of comparison. The time scale is given in logarithmic units to show the different growth and dissolution regimes. Only microbubbles coated with the shortest acyl-chain lipid (DMPC) showed significant growth. DPPC- and DSPC-coated microbubbles exhibited stunted growth, with the longer acyl-chain lipid (DSPC) taking a slightly longer time to grow. In contrast, no growth was seen for the longest acyl-chain lipid (DBPC).

As also can be seen from the plots, an increase in phospholipid acyl chain length resulted in greater microbubble stability. DMPC microbubbles dissolved completely, whereas the other encapsulations provided dissolution arrest (secondary stabilization) at the diameters shown on the plot. The shape of the dissolution curves was consistently smooth DMPC and DPPC. DSPC and DBPC exhibited a step-wise dissolution, as observed previously for air microbubbles dissolving in degassed media (Borden and Longo 2002).

The results above clearly indicate that microbubble dynamics depend not only on the initial size, but also the lipid composition of the encapsulation. The different regimes of growth and dissolution were parameterized as shown in Figure 4.1 and tabulated below in Table 4.2. In the following sections, we discuss each parameter in turn with respect to effects of size and encapsulation.
Table 4.2: Cumulative Results for Microbubble Growth and Dissolution Parameters

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Emulsifier</th>
<th>$N_{\text{total}}$</th>
<th>Initial Diameter [µm]</th>
<th>Relative Growth</th>
<th>%$_{DS1}$</th>
<th>$D_{S1}$ [µm]</th>
<th>$t_{\theta}$ [s]</th>
<th>$t_{d}$ [s]</th>
<th>%$_{DS2}$</th>
<th>$D_{S2}$ [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC</td>
<td>PEG 40S</td>
<td>24</td>
<td>6.9 ± 0.6</td>
<td>1.18 ± 0.07</td>
<td>96</td>
<td>6.6 ± 0.7</td>
<td>24 ± 23</td>
<td>28 ± 10</td>
<td>13</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>DPPC</td>
<td>PEG 40S</td>
<td>21</td>
<td>6.6 ± 0.5</td>
<td>1.06 ± 0.02</td>
<td>67</td>
<td>6.5 ± 0.5</td>
<td>35 ± 21</td>
<td>46 ± 28</td>
<td>67</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>DSPC</td>
<td>PEG 40S</td>
<td>20</td>
<td>7.1 ± 0.6</td>
<td>1.06 ± 0.02</td>
<td>80</td>
<td>7.3 ± 0.7</td>
<td>79 ± 93</td>
<td>220 ± 105</td>
<td>50</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>DSPE PEG2K</td>
<td>33</td>
<td>7.1 ± 0.4</td>
<td>1.03 ± 0.02</td>
<td>76</td>
<td>6.6 ± 0.8</td>
<td>73 ± 53</td>
<td>350 ± 180</td>
<td>55</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>DBPC</td>
<td>PEG 40S</td>
<td>23</td>
<td>6.9 ± 0.7</td>
<td>1.03 ± 0.01</td>
<td>83</td>
<td>7.0 ± 0.5</td>
<td>120 ± 69</td>
<td>800 ± 430</td>
<td>83</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>

Table 4.1: $N_{\text{total}} =$ total microbubbles analyzed; $D_0 =$ Initial diameter; $D_{\text{max}}/D_0 =$ Relative growth; %$_{DS1}$ = Percentage of microbubbles exhibiting primary stabilization, $D_{S1} =$ Primary stabilization diameter; $t_{\theta} =$ lag time; $t_{D} =$ dissolution time; %$_{DS2}$ = Percentage of microbubbles exhibiting secondary stabilization, $D_{S2} =$ secondary stabilization diameter or “stable diameter”.
4.3.2. Microbubble Growth

Figure 4.3 shows the effects of microbubble size and encapsulation on the degree of growth owing to the initial influx of oxygen and nitrogen. The maximum diameter reached during the experiment ($D_{\text{max}}$) was normalized by the initial diameter ($D_0$) for the sake of comparison. Figure 4.3A shows the effect of the initial diameter on the relative expansion for the DSPC:PEG40S encapsulation. Interestingly, there was a threshold near ~10 μm diameter, below which the growth was significantly stunted. Above this diameter, some of the microbubbles exhibited very large growth. Above 10 μm diameter, the tendency for large growth above did not appear to correlate with microbubble size.

Figure 4.3: Relative expansion ($D_{\text{max}}/D_0$) of microbubbles during the initial influx of air. A) Effect of microbubble size for DSPC:PEG40S encapsulation. Error bars represent error generated in image analysis owing to poor optical contrast of the microbubble edge or deviations from sphericity. The shaded region shows the diameter (10 μm) above which some microbubbles experienced large relative expansion. Below this threshold, all microbubbles experienced stunted growth. B) Effect of encapsulation reduced temperature,
as defined in the text, for 6-8 μm diameter microbubbles. Error bars represent standard deviation. The solid line is a mono-exponential fit to the data \( R^2 = 0.98 \) with \( y \)-intercept 1.0, pre-exponential term 0.15 and exponential term -2.11. The dotted vertical line shows the point where the main phase transition temperature is equal to the working temperature, 25 °C.

Figure 4.3B shows the effect of the lipid encapsulation on relative growth for 6-8 μm diameter microbubbles. The lipid encapsulation was parameterized by using the reduced temperature \( (T_r) \), defined as follows (Kim et al. 2003).

\[
T_r = \frac{T_m - T}{T_m}
\]

(4.1)

A higher value of \( T_m \) indicates a stiffer, more cohesive encapsulation. As can be seen from the plot, the relative expansion of the microbubbles decreased with increasing reduced temperature. The replacement of PEG40S with DSPE-PEG as the emulsifier in DSPC encapsulations reduced the relative growth to values similar to DBPC:PEG40S microbubbles (Table 4.2).

4.3.3. Primary Stabilization

Following growth, all of the microbubbles began to dissolve, and most of the microbubbles rapidly shrank to a primary stabilization diameter \( (D_{51}) \). Interestingly, the most
fluid encapsulation (DMPC:PEG40S) provided the greatest probability (96%) of primary stabilization (Table 4.2). Figure 4.4 shows a correlation between $D_{51}$ and the initial diameter, $D_0$, for DSPC:PEG40S-coated microbubbles.

![Figure 4.4: Primary stabilization diameter plotted versus initial diameter plotted for DSPC:PEG40S encapsulation. Solid line is a linear least-squares fit ($R^2 = 0.99$).](image)

For the diameters tested here (2-20 μm), there was a strong 1:1 correlation between $D_{51}$ and $D_0$ ($R^2 = 0.99$). Table 4.2 shows the same trend for the other encapsulations. Following growth, the microbubbles rapidly shrank back to their initial size.

4.3.4. Lag Time Between Primary Stabilization and Dissolution

Following the initial growth and dissolution stage, most of the microbubbles remained at a constant diameter for a significant period of time before they spontaneously began dissolving
again. We investigated the effects of initial microbubble size and encapsulation type on this lag time, $t_\theta$. Figure 4.5A shows that, for DSPC:PEG40S encapsulation, there was no clear trend between $t_\theta$ and $D_0$. A histogram of the $t_\theta$ values (inset) shows a Gaussian distribution with a value of $83 \pm 120$ sec (mean ± standard deviation). The median value was 150 sec. One out of the 25 microbubbles of this encapsulation experiencing primary stabilization persisted for over 1,000 sec before the initiation of the secondary dissolution stage.

Figure 4.5B shows the values for $t_\theta$ as a function of encapsulation reduced temperature for 6-8 μm diameter microbubbles.

![Figure 4.5: Lag time to dissolution. A) Scatter plot showing effect of microbubble size for DSPC:PEG40S encapsulation. Inset shows histogram with fit Gaussian distribution: $R^2 = 0.82$, mean ± standard deviation = 83±120 sec. The lag times for all other encapsulations also were normally distributed (data not shown). B) Effect of encapsulation reduced temperature for 6-8 μm diameter microbubbles. The solid line is a fit mono-exponential ($R^2 = 0.94$), with pre-exponential term equal to 11 sec and exponential term equal to 4.6.](image)
As expected, the lag time, which is a marker of stability, increased monotonically with increasing shell cohesiveness. Exchanging the emulsifier PEG40S with DSPE-PEG had a minimal effect on the total lag time. As evidenced by the large error bars (standard deviation from the mean), and also Figure 4.5A, there was significant variability in the duration of primary stabilization. The variability in lag time appeared to increase with increasing reduced temperature.

4.3.5. Dissolution Time

Upon the initiation of dissolution, each microbubble dissolved completely or arrested at a secondary stabilization diameter, where it remained at a nearly constant diameter over the experimental timeframe (tens of minutes). Figure 4.6A shows the dissolution time versus initial diameter (equivalently, the primary stabilization diameter) for DSPC:PEG40S encapsulations.

Figure 4.6: Dissolution time. A) Scatter plot showing effect of microbubble size for DSPC:PEG40S encapsulation. Inset shows histogram with fit Gaussian distribution: $R^2 =$
0.82, mean ± standard deviation = 270±140 sec. B) Effect of encapsulation reduced temperature for 6-8 μm diameter microbubbles. The solid line is a fit mono-exponential, $R^2 = 1.0$ with pre-exponential term equal to 0.69 sec, and exponential term equal to 10.6.

A very weak direct relationship was observed between $t_D$ and $D_0$ ($R^2 = 0.42$). This was surprising because it was taken as axiomatic that larger microbubbles would take longer to dissolve owing to their larger gas content, as clearly predicted by Epstein-Plesset theory (Epstein and Plesset 1950). At least for the size range investigated here (2-20 μm), it appeared that the encapsulation, and not the gas core volume, was the main determinate of the time for microbubble dissolution. The Figure inset shows a histogram of $t_D$ fit to a Gaussian distribution. The average time for dissolution was 270 ± 140 sec, which was longer than the average lag time mentioned above.

Figure 4.6B shows the effect of encapsulation on $t_D$ for 6-8 μm diameter microbubbles. As expected based on previous results (Borden and Longo 2002; Kim et al. 2003), the longer acyl chain lipids led to greater microbubble stability. The value of $t_D$ increased monotonically with the reduced temperature. The variability of the dissolution time also increased with increasing lipid acyl chain length. For DSPC, simply exchanging the emulsifier PEG40S with DSPE-PEG lead to an increase in dissolution time, indicating greater stability.
4.3.6. Stable Diameter

Some microbubbles completely stopped dissolving at a secondary stabilization diameter ($D_{S2}$). We termed this the “stable diameter” because these microbubbles appeared to remain unchanged indefinitely at this new diameter, at least under the conditions studied here. The percentage of microbubbles that had a stable diameter increased with lipid acyl chain length, but not with a change in the emulsifier (Table 4.2). Figure 4.7 shows a plot of the stable diameter versus the initial diameter for the DSPC:PEG40S encapsulation.

![Figure 4.7: Secondary stable diameter plotted versus the initial diameter for DSPC:PEG40S encapsulation. Solid line is a linear least-squared fit ($R^2 = 0.42$). Inset shows histogram with fit Gaussian distribution: $R^2 = 0.91$, mean ± standard deviation = 1.4±0.5 μm.](image)
A very weak direct relationship was observed between $D_{52}$ and $D_0$ ($R^2 = 0.42$). Most of the microbubbles stabilized in the 1-2 μm diameter range. Microbubbles below 1.0 μm diameter were difficult to characterize owing to the diffraction limit and onset of significant Brownian motion, which caused the microbubble to move in and out of focus. The inset shows a histogram fit with a Gaussian distribution, where the mean stable diameter was 1.4 ± 0.5 μm. The stable diameter did not change significantly with reduced temperature (Table 4.2).

4.4. Discussion

Our results show that the encapsulation plays a dominant role in the dynamics and stability of lipid-coated microbubbles. In the experiments detailed above, SF₆-filled microbubbles were suddenly transferred from an SF₆-saturated solution to an air-saturated solution. The initial influx of oxygen and nitrogen from the surrounding medium applied an isotropic, outward pressure on the microbubble shell. Larger microbubbles (> 10 μm diameter) were able to grow relatively unconstrained. However, smaller microbubbles with more solid encapsulations ($T_r > 1.0$) were inhibited in growth by the presence of the lipid monolayer encapsulation. The stiffest encapsulation tested here, DBPC:PEG40S, resulted in no growth at all. One may conclude then that the lipid monolayer not only resists dissolution, but also growth. This result is fortuitous for ultrasound contrast agents, in which growth following intravenous injection would be unwanted for obvious reasons. However, this result may have more far-reaching implications for a variety of other circumstances involving microbubbles. For example, it suggests that small, natural microbubbles rising under buoyancy may not grow and accelerate
their rise velocity with the decrease in hydrostatic pressure as they move up the water column, perhaps thereby increasing their persistence in the oceans. Likewise, the lipid monolayer lining the alveolus may similarly resist rupture during expansion in inhalation, possibly providing a mechanical stabilization that goes beyond simple reduction in surface tension. More research is warranted to test for these effects.

Following equilibration of air between the gas core and surroundings, the efflux of SF₆ led to subsequent microbubble dissolution (Kwan and Borden 2010). All microbubbles exhibited a rapid dissolution stage and then primary stabilization near the initial diameter. This result suggested that the ruptured lipid monolayer remains attached to the interface during expansion and then “self-heals” upon compression back to the initial diameter. Such behavior has been observed previously for lipid-coated microbubbles undergoing large-amplitude oscillation in an ultrasound field, where the microbubble rapidly stabilizes following to the acoustic pulse (Chomas et al. 2000). This appears to be a property unique to lipid monolayers, as albumin-coated microbubbles were not observed to self-heal and stabilize following insonification (Chomas et al. 2000).

Interestingly, the microbubbles were not completely stable upon shrinking back to their initial size. Following a lag time, which was highly variable but somewhat dependent upon the stiffness of the encapsulation, another dissolution stage was spontaneously initiated. The duration of this dissolution stage was also highly variable, surprisingly showing a very weak dependence on the initial microbubble size. The large variance in both \( t_\theta \) and \( t_D \) may be explained by differences in shell microstructures, leading to bubble-to-bubble differences in mechanical properties. The dissolution time, however, was strongly correlated to the stiffness of
the encapsulation. As noted previously, *ceteris paribus* longer acyl chain lipids provide more stable microbubbles (Borden and Longo 2002).

Finally, it was observed that many of the microbubbles with solid encapsulations \((T_r > 1.0)\) experienced a secondary stabilization at a very small diameter (typically, 1-2 µm). In most cases, these microbubbles appeared to persist indefinitely (at least for several tens of minutes) at this “stable diameter”. The phenomenon is reminiscent of the stable diameter observed previously for lipid-coated microbubbles undergoing “acoustic dissolution” by ultrasound pulsing (Borden *et al.* 2005). Remarkably, the stable diameter appeared to be independent of the initial diameter and the encapsulation. Incidentally, the 1-2 µm size is the most populated size range for the commercially available lipid microbubble Definity\textsuperscript{TM} (Lantheus Biomedical Imaging), which is primarily coated with DPPC, upon dilution in air-saturated media (Goertz *et al.* 2007).

### 4.5. Concluding Remarks

Lipid-coated microbubbles ranging in initial size from 2-20 µm diameter and encapsulated with a homologous series of lipids with increasing acyl chain lengths were observed as they were suddenly transferred from an SF\(_6\)-saturated environment, in which they were equilibrated, to an air-saturated environment. Analysis of the resulting diameter-time curves provided insight into the role of the lipid encapsulation on microbubble growth and dissolution. The encapsulation inhibited both growth and dissolution beyond the initial, equilibrium size. Following growth and dissolution back to the original size, the microbubbles dissolved with a highly variable lag time and dissolution time that did not strongly correlate with
microbubble size, but rather the encapsulation stiffness. Finally, the microbubbles tended to stabilize at a very small diameter, typically 1-2 µm, at which they appeared to persist indefinitely. These experimental results defy predictions from previous theoretical models of microbubble stability, which are based primarily on the kinetics of gas diffusion and simple models of the lipid shell mechanics. Our results suggest that more detailed models of the mechanics describing this remarkable nano-film are warranted. Such future modeling of microbubble stability and dynamics may find useful the empirical observations established here. This is the subject of Chapter 5.
Chapter 5: Theoretical Modeling of Growth and Dissolution: Analysis of Experiments

5.1. Introduction

Phospholipid monolayers are of great scientific interest and play critical roles in biological activities such as breathing (Alonso et al. 2004; Rooney 1985). These amphiphilic organic molecules contain hydrophobic waxy acyl-chain tails and a zwitterionic hydrophilic headgroup, and readily self-assemble into a single molecule thick membrane at the gas-liquid interface. It has long been known that these surfaces undergo phase transformations, forming gas-like states at lower pressures and waxy solid-like states at higher pressures (Edwards 1961; Gaines 1966; Kaganer et al. 1999; Petrov et al. 2001; Sharma and Radhakrishnan 2001). More recently, Borden and Longo (Borden and Longo 2004) demonstrated that these phospholipid surfaces can form complex liquid-crystalline phase states which are highly dependent on the chemical composition of the monolayer. They also observed that compressed monomolecular thick membranes exhibit a drop in compressibility from the elastic resistance to compression. In these states, the adsorbed molecules begin to imbue the two-dimensional surface with two-dimensional macroscopic mechanical properties such as elasticity and viscosity (Edwards 1961; Gaines 1966). These mechanical properties arise from the intrinsic properties of the monolayer (e.g., physiochemical properties of the lipid, thermal fluctuations, and electrostatic interactions) and greatly affect the behavior of systems like microbubbles, which are dominated by their surface area (Edwards 1961; Hoffmann 2005; Israelachvili 1991).
5.1.1. Role of Monolayer Mechanics

The presence of a monomolecular coating of organic molecules has long been established to mechanically stabilize the interface of a curved surface (Fox and Herzfeld 1954; Langmuir 1917). This stability due to an organic coating was first proposed by Fox and Herzfeld to explain the presence of small gas bubbles in the ocean (Fox and Herzfeld 1954). As mentioned in Chapter 1, they postulated that the presence of organic material in the ocean, such as glycol-proteins, encapsulate the gas bubble, acting as an elastic stabilizer and barrier for gas diffusion. Though they did not discuss in detail the gas permeability properties of the organic “skin”, they reasoned that the elasticity alone accounted for the stabilization of a microbubble. Fox and Herzfeld (Fox and Herzfeld 1954) showed that stability occurred under the condition:

\[
F_{\text{collapse}} > \sigma (1 - h) + \frac{R}{2} (P_H - P_{\text{sat}}) \left( 1 + \frac{R}{E'} \right)^{-1} \tag{5.1}
\]

\[
E' = \frac{3}{2} \gamma \frac{\delta E}{1 - \nu} \tag{5.2}
\]

where \( F_{\text{collapse}} \) is the tangential tension required to compress a microbubble, i.e., the collapse strength of the organic stabilizer, \( \sigma \) is the interfacial tension of the organic interface, \( h \) is the fraction of excess pressure due to surface tension, \( R \) is the outer diameter of the microbubble, \( P_H \) is the hydrostatic pressure, \( P_{\text{sat}} \) is the dissolved gas saturation pressure, \( \gamma \) is the adiabatic gas compressibility, \( \delta \) is the thickness of the stabilizing shell, \( E \) is the Young’s modulus of the shell, and \( \nu \) is the Poisson’s constant for the organic shell. From equations 5.1 and 5.2, Fox and Herzfeld (Fox and Herzfeld 1954) concluded that if the collapse strength was larger than the
surface tension of the gas-liquid interface (73 mN/m) the condition for microbubble stability is satisfied.

Fox and Herzfeld (Fox and Herzfeld 1954) approached the organic interface as a continuous elastic sheet. Scriven (Scriven 1960), however, viewed the presence of a material interface as a Newtonian fluid with rheological properties. He proposed that the Newtonian interface conserved momentum during deformation. As a result, a two dimensional form of Cauchy’s law of motion was derived, shown below, predicting the stresses on the interface:

\[ \sigma \vec{A} = \vec{F} + T \vec{m} \]  

(5.3)

where \( \sigma \) is the interfacial tension, \( \vec{A} \) is the area position vector, \( \vec{F} \) represents the body force vector, \( T \) is the stress tensor in curvilinear coordinates, and \( \vec{m} \) is a unit surface vector normal to a line element. From the equation 5.3, Scriven derived a set of general equations to determine the rheological behavior of a material interface. For example, Scriven (Scriven 1960) determined from equation 5.3 that at a given radius, the normal pressures surrounding the interface are equal to:

\[ P_- - P_+ = \frac{2\sigma}{R} + \frac{4\kappa}{R^2} \frac{dR}{dt} \]  

(5.4)
where \( P \) represents a pressure normal to the interface, the subscripts “+” and “−” indicate positions above and below the interface, respectively, \( \kappa \) is the dilatational viscosity of the interface, and \( t \) represents time. The second term on the right hand side of equation 5.4 reflects the additional resistance to deformation due to the viscous nature of the Newtonian interface. This has been applied in several models to account for the anomalous behavior microbubbles exhibit under acoustic fields (Doinikov and Dayton 2007; Doinikov et al. 2009; Marmottant et al. 2005; Paul et al. 2010).

The presence of an organic shell not only stabilizes a microbubble, but also influences acoustic properties such as echogenicity. A metric for echogenicity is the contrast agent’s scattering cross-section, as shown in equation 1.1. From equation 1.1, it can be shown that the microbubbles resonant frequency, \( f_R \), strongly influences the scattering cross-section. de Jong et al. (de Jong et al. 1992) theorized that the microbubbles encapsulating shell will affect the microbubbles resonant frequency by imposing an additional restorative force, shown below:

\[
\begin{align*}
\omega_r^2 &= \omega_{r,g}^2 + \frac{S_{\text{shell}}}{m} \\
 f_R &= \frac{\omega_r}{2\pi}
\end{align*}
\] (5.5) (5.6)

where \( \omega_r \) is the angular resonant frequency of the microbubble, \( \omega_{r,g} \) is the angular resonant frequency of an unencapsulated gas bubble, \( S_{\text{shell}} \) is the shell stiffness and \( m \) is the effective mass of the system, and \( f_R \) is the resonant frequency of the microbubble. de Jong et al. (de Jong et al. 1992) showed that the contributions of the shell stiffness, \( S_{\text{shell}} \), can be calculated:
Using equations 5.5-5.7 and equation 1.1, de Jong (de Jong et al. 1992) demonstrated that the maximum scattering cross-sectional area decreases with increasing shell stiffness. From acoustic measurements of Albunex microbubbles they determined the shell parameter to be approximately $10^N/m$.

In addition to behaving like an elastic sheet during deformation, the lipid encapsulation on the microbubble also possesses viscous properties. Kim et al. (Kim et al. 2003) demonstrated that on a microbubble these viscous properties are quite prominent. They used a micropipette aspiration technique to deform the surface of a microbubble. From their experimental observations, shown in figure 1.11, they demonstrated that the phospholipid monolayer behaved as a Bingham fluid, not as a Newtonian fluid. As a result, the stresses on the interface were expressed as:

$$
\tau = \begin{cases} 
\tau_s & \text{no flow} \\
\tau_s & \text{deformation} \\
\tau_s & \text{flow}
\end{cases}
(5.6)
$$

where $\tau$ is the shear stress and $\tau_s$ is the yield shear. In the flow regime, the shear stress, $\tau$, was equal to:
\[ \tau = \tau_s + 2\eta_s V_s \]  \hspace{1cm} (5.7)

where \( \eta_s \) is the shear viscosity and \( V_s \) is the shear rate. By inducing shear and observing the flow of lipid into the micropipette, Kim et al. (Kim et al. 2003) determined both the onset of shear, \( \tau_s \), and the shear viscosity of the lipid monolayer from equation 5.7. They reported that the surface yield shear and viscous properties of the phospholipid monolayer coating on a microbubble greatly resist changes in shape and were dependent on both the acyl chain length of the lipid (\( \tau_s = 1 \frac{mN}{m} \) and \( \eta_s = 4 \frac{mNs}{m} \) for DSPC up to \( \tau_s = 4 \frac{mN}{m} \) and \( \eta_s = 16 \frac{mNs}{m} \) for DBPC) and microstructure of the encapsulation.

Duncan and Needham (Duncan and Needham 2004) demonstrated, and confirmed with the Epstein and Plesset microbubble dissolution model (Epstein and Plesset 1950), that a phospholipid monolayer has a profound impact on a microbubbles stability. Using this microbubble dissolution model, derived in Chapter 2 and shown below, they predicted that microbubble stability could only be obtained when the interfacial tension is reduced to zero in a gas saturated solution.

\[ \frac{dR}{dt} = -DK_HBT \frac{1 - f + \frac{2M_w\sigma}{\rho BTR}}{1 + \frac{4\rho w_0\sigma}{3\rho BTR}} \left( \frac{1}{R} \right) \]  \hspace{1cm} (5.8)
where $D$ is the diffusion of gas in the bulk medium, $K_H$ is the Henry’s constant, $B$ is the ideal gas constant, $T$ is the temperature, $f$ is the saturation fraction of dissolved gas, $\rho$ is the density of gas, and $M_w$ is the molecular weight of the gas. Fortunately, such low tension values could be obtained with phospholipid monolayers. This prediction, therefore, was confirmed when placing microbubbles encapsulated with insoluble monomolecular films composed of phospholipids in gas saturated solution. They observed that these lipid encapsulated microbubbles stabilize indefinitely in saturated solution.

Not only does the elastic shell reduce the scattering cross-section, mentioned earlier, but at low acoustic pressures, microbubbles exhibit a “compression-only” oscillatory behavior. To account for this behavior, Marmottant et al. (Marmottant et al. 2005) developed an elastic tension model of the encapsulating shell. Their model, shown below, predicts that the shell undergoes three distinct states, a buckled, elastic, and ruptured state.

\[
\sigma = \begin{cases} 
0 & A \leq A_{buckled} \\
\chi \left( \frac{A}{A_{buckled}} \right) - 1 & A_{buckled} < A < A_{ruptured} \\
\sigma_{g-l} & A \geq A_{ruptured}
\end{cases} \tag{5.9}
\]

where $A$ is the surface area of the encapsulation, $A_{buckled}$ is the surface area at which the monolayer begins to collapse and buckle, $\chi$ is the linear elasticity of the monolayer, $A_{ruptured}$ is the surface area at which the monolayer ruptures, and $\sigma_{g-l}$ is the surface tension of an air-water interface. In the buckled, or resting, state the interface is maximally compressed, resulting in a surface tension of zero. Upon initial expansion, the monolayer enters an elastic state, where the
encapsulation behaves similar to a continuous elastic sheet, akin to the models developed by Fox and Herzfeld, de Jong, and others (de Jong et al. 1992; Fox and Herzfeld 1954; Katiyar et al. 2009; Paul et al. 2010). After sufficient areal expansion, the interface tears and enters the ruptured state. In this state, the surface tension becomes that of the gas-liquid interface, \( \sigma_{g-l} \). Generally, this value is equal to \( 73 \, \frac{mN}{m} \) for an air-water interface. Using the model in equation 5.9, Marmottant et al. (Marmottant et al. 2005) reported that for a phospholipid encapsulation, the elasticity, \( \chi \), and the break-up tension, \( \sigma_{\text{rupture}} \), range from \( 0.5 - 1 \, \frac{N}{m} \) and \( 0.1 - 1 \, \frac{N}{m} \) respectively.

The restorative force of the material or organic interface greatly influences the acoustic properties of microbubbles. Katiyar et al. (Katiyar et al. 2009) proposed that mechanical stability from the elasticity of the encapsulating shell, therefore, also plays a dominant role as a stabilizing force. They incorporated an elasticity term for the microbubble shell into a two-component Epstein-Plesset diffusion equation. This term is similar in form to the model proposed by Marmottant et al. (Marmottant et al. 2005) in equation 5.9, but does not include a rupture tension. This two region model is shown below:

\[
\sigma = \begin{cases} 
0 & \sigma \leq 0 \\ 
\sigma_0 + E \left( \frac{R}{R_0} \right)^2 - 1 & \sigma > 0 
\end{cases} 
\]  

(5.10)

where \( \sigma_0 \) and \( R_0 \) are the surface tension and radius, respectively, of a stress free conformation, and \( E \) is the elasticity of the encapsulation. As a result, they demonstrated that when the
microbubble dissolved beyond the initial radius, the elasticity term \( E \left( \frac{R}{R_0} \right)^2 - 1 \) becomes negative. Stability, therefore, occurred when this term became equal and opposite of the stress free surface tension term, \( \sigma_0 \).

Though neglected when predicting the behavior of a dissolving microbubble (Duncan and Needham 2004; Epstein and Plesset 1950; Kabalnov et al. 1998; Katiyar et al. 2009), other rheological models described by Scriven (Scriven 1960) have been applied in equations predicting the diameter-time behavior of microbubbles perturbed by an acoustic wave (Doinikov and Dayton 2007; Doinikov et al. 2009; Paul et al. 2010). For example, Doinikov et al. modeled the “compression-only” behavior observed by others in phospholipid-coated microbubbles in the presence of an acoustic field (Doinikov et al. 2009; Marmottant et al. 2005). They separated the force contributions of the shell, \( S \), into viscous, \( S_{vis} \), and elastic, \( S_{el} \), components (de Jong et al. 1994):

\[
S = S_{vis} + S_{el} = 4\kappa_s \frac{R}{R} + 4\chi \frac{R}{R_e} \left( \frac{1}{R_e} - \frac{1}{R} \right)
\]

(5.11)

where \( \kappa_s \) is the dilatational viscosity, \( \dot{R} \) is the radial velocity, \( \chi \) is the elasticity of the monolayer, and \( R_e \) is the radius of the unstressed microbubble. Doinikov et al. (Doinikov et al. 2009) also suggested that the dilatational viscosity, \( \kappa_s \), is functionally dependent on the rate, \( \frac{\dot{R}}{R} \), and can be calculated by:
\[
\kappa_s \left( \frac{\dot{R}}{R} \right) = \frac{\kappa_0}{1 + \alpha \frac{\dot{R}}{R}} + \kappa_1 \frac{\dot{R}}{R} \tag{5.12}
\]

where \( \kappa_0 \) describes a shear-thinning behavior, \( \alpha \) is a time constant, and \( \kappa_1 \) describes the “compression-only” behavior. By applying fits to diameter-time curves of oscillating microbubbles undergoing “compression-only” behavior, Doinikov et al. (Doinikov et al. 2009) determined the coefficients, \( \kappa_0 \) and \( \kappa_1 \) in equation 5.12 to be approximately \( 1 \times 10^{-7} \frac{kg}{s} \) and \( 2 \times 10^{-15} \frac{kg}{s} \) for a 2 \( \mu \)m in diameter microbubble, assuming \( \alpha = 4 \mu s \).

Paul et al. (Paul et al. 2010) expanded the surface tension term and applied an exponential elasticity term to model the behavior of a strain softening encapsulation. Similar to the expansion shown in equation 5.10, Paul et al. (Paul et al. 2010) also assumed that the elasticity was dependent on the fractional change in area of the interface. The elasticity, \( E \), therefore, can be calculated by the following equation:

\[
E = E_0 e^{-\alpha \beta}
\tag{5.13}
\]

where \( E_0 \) is the exponential elasticity term, \( \alpha \) is an empirical constant, and \( \beta = \left( \frac{R}{R_0} \right)^2 - 1 \) represents the fractional area change of the interface. Combining equations 5.10 and 5.13, Paul et al. (Paul et al. 2010) formulated a strain-softening elasticity model. Using this strain-softening model, Paul et al. fit diameter-time curves of oscillating Sonozoid microbubbles at various
acoustic pressures. As a result, they determined the values of $E_0$ and $\alpha$ to be $0.55 \frac{N}{m}$ and 1.5, respectively.

In these studies, the interface’s elastic and rheological properties were addressed in both diffusion and acoustically driven systems. Though all of the different mechanical models presented utilize different methodologies in predicting the mechanical behavior of the material interface, all of the models presented share a common trait. All of the models assume that the surface is a continuous material interface and is time independent. Monolayer relaxations studies, however, have shown that the interface is indeed time dependent, and will relax when a stress is applied (Bois et al. 1988; Kato et al. 1992; Kato et al. 1989; Sakai and Umemura 2008). Though monolayers do relax when stressed, the relaxation times are on the order of hundreds to thousands of seconds. The assumptions made in the studies mentioned above may still be valid for oscillating microbubbles; the time scales of oscillations are on the order of microseconds. The time scales for diffusion driven microbubble deformation, as shown in Chapter 4, are over tens to hundreds of seconds. It is therefore important to account for the time-dependent kinetics of the lipid encapsulation during microbubble deformation. In addition to understanding the role of monolayer relaxation, the importance of gas diffusion across the microbubble encapsulation must also be addressed.

5.1.2. Gas Diffusion Across a Monolayer

It is clear from the studies mentioned above that the presence of an organic unimolecular film at the gas-liquid interface imposes a physical barrier. This monomolecular coating has also been shown to resist molecular transport from the bulk fluid phase to the bulk gas phase
Langmuir and Schaefer (1943). Zhang and Unwin (Zhang and Unwin 2003) observed that for phospholipid monolayers, the resistance to gas permeation increases exponentially upon compression, with reported values up to $10^2 - 10^3$ s/cm at high surface pressures. Blank and Roughton (Blank and Roughton 1960) reported that the gas permeation was exponentially dependent on the length of the acyl backbone of acyl-chain acids and alcohols. Though it is generally accepted that resistance is due to a partition-diffusion mechanism, Blank (Blank 1962; Blank 1964; Blank 1968; Blank and Musselwhite 1968; Blank and Roughton 1960) debated that the permeation through condensed insoluble monolayers occurs in discrete single-step jumps through “holes” in the monolayer. These gaps are provided by thermal fluctuations in the monolayer and are proportional to the cohesive energy provided by the acyl-chain backbone (Israelachvili 1991). Others have provided further evidence that gas permeation resistance of condensed monolayers of insoluble surfactant is mechanistically determined by an activation energy required for hole-formation (Barnes and Hunter 1990; Krustev et al. 1997; Lamer and Healy 1965).

As mentioned in Chapter 1, Fox and Hertzeld (Fox and Herzfeld 1954) described the organic shell stabilizing natural microbubbles, which persist in oceans, as a resistance preventing the contraction of the surface. Duncan and Needham (Duncan and Needham 2004) demonstrated, however, that though theoretically the monolayer should create a permeation barrier, even lipid-coated microbubbles with a near-zero surface tension will still obey the classical Epstein-Plesset theory if the gas-core is highly diffusive (e.g., air). While these findings may apply for “first generation” microbubbles like Albunex, “second” and “third generation” microbubble formulations such as SonoVue use perfluorcarbons for their gas core, which are very insoluble.
Furthermore, Borden and Longo (Borden and Longo 2002) showed that when increasing acyl-chain length on the main phospholipid component, the dissolution time also increased, which was not predicted by Epstein and Plesset (Epstein and Plesset 1950). To account for the deviation, they modified the classical model to incorporate a gas permeation resistance term for the organic shell. Though their results emulated the positive relationship between acyl-chain length and shell permeation resistance, they could not verify which mechanism, partition-diffusion or energy barrier, was appropriate. Borden and Longo (Borden and Longo 2004) reported an exponential decay in oxygen permeability with increasing acyl-chains on microbubbles encapsulated with a homologous series of diacyl saturated phospholipids, providing evidence in support for the energy barrier theory. With all of the evidence pointing to the importance of shell permeability, recent models for microbubble dissolution have been developed to incorporate the effects the organic film has on the gas diffusion from the core of the microbubble (Katiyar et al. 2009; Sarkar et al. 2009).

It is clear from the above studies that the monolayer encapsulation plays a profound role in gas diffusion across the interface. These studies measured gas permeation as a function of surface pressure, surface microstructure, and lipid acyl-chain length. Though the results from these studies have been implemented in determining a microbubbles dissolution behavior, the dynamic aspects of the gas permeation resistance of the lipid shell have been neglected. Taking the information from these studies, we plan on implementing a gas permeation resistance model that accounts for the dynamic behavior of the phospholipid encapsulation.
5.2. Generalized Microbubble Dissolution Model

The expressions for microbubble behavior under multi-gas environments have been developed in Chapter 2. Kabalnov et al. and others (Cable and Evans 1967; Kabalnov et al. 1998; Katiyar et al. 2009; Paul et al. 2010) have developed analytical models describing the behavior of microbubbles in a two-gas system. These models predicted that microbubbles grow in the presence of a dissolved foreign gas that diffuses faster than the gaseous core. In clinical settings, however, microbubbles are exposed to blood which is a complex fluid with several different dissolved gases. As a result, a numerical model that accounts for multiple dissolved gases was developed in Chapter 2 that incorporates \( N \) number of gas species. Following the procedure in Chapter 2, we first focused on the molar gas exchange across the interface.

\[
\frac{dn_i}{dt} = -4\pi R^2 J_i
\]

(5.14)

where \( n \) is the moles of gas in the microbubble core, \( i \) represents the gas species, \( t \) is the time coordinate, \( R \) is the radius of the microbubble, and \( J \) is the molar flux across the interface. The molar flux, \( J \), can be determined by:

\[
J_i = -D_i \left( \frac{\partial c_i}{\partial r} \right)_{r=R} = k_i \left( c_{ib} - c_{i,s}(R) \right)
\]

(5.15)
where $D$ is the diffusion of gas in the aqueous medium, $C$ is the molar concentration of gas (where “b” and “s” are points inside the gas core and on the interface respectively), $r$ is the radial dimension, and $k$ is the mass transfer coefficient of gas across the phospholipid monolayer. In order to determine the concentration profile $C_i$, and consequently, the molar flux, we implemented Fick’s second law for a spherical particle in a quiescent medium.

\[
\frac{\partial C_i}{\partial t} = D_i \left[ \frac{1}{r^2} \frac{\partial}{\partial r} \left( r^2 \frac{\partial C_i}{\partial r} \right) \right] \tag{5.16}
\]

Assuming that the concentration profile develops instantaneously, equation 5.16 can be reduced to:

\[
0 = D_i \left[ \frac{1}{r^2} \frac{\partial}{\partial r} \left( r^2 \frac{\partial C_i}{\partial r} \right) \right] \tag{5.16}
\]

with the boundary conditions:

\[
@r = R; \quad -D_i \left( \frac{\partial C_i}{\partial r} \right)_{r=R} = k_i \left( C_{i,b} - C_{i,s}(R) \right) \tag{5.17}
\]

and,
where $C_\infty$ is the concentration of dissolved gas in the bulk medium at infinite distance for gas species $i$. Solving equation 5.16 and applying the boundary conditions (equation 5.17 and 5.18) we obtain:

$$\left. C_i \right|_{r = \infty} = C_\infty + \frac{1}{\frac{1}{k_i} \frac{R}{D_i}} \int_0^R \left( \frac{C_{b,i} - C_\infty,i}{1 + \frac{R}{D_i}} \right) \frac{1}{r} dr$$

From equation 5.19, the molar flux, $J_i$, can be determined:

$$J_i = -D_i \left( \frac{\partial C_i}{\partial r} \right)_R = \frac{C_{b,i} - C_\infty,i}{1 + \frac{R}{k_i} \frac{1}{D_i}}$$

and as a result, the molar gas exchange across the interface becomes:

$$- \frac{dn_i}{dt} = 4\pi R^2 \left( \frac{C_{b,i} - C_\infty,i}{1 + \frac{R}{k_i} \frac{1}{D_i}} \right)$$
The interfacial concentration of species $i$ is given by:

$$C_{b,i} = K_{H,i} \left( P_E + P_H - \frac{3BT}{4\pi R^3} \sum_{j=1}^{N} n_j \right) \text{ where } j \neq i$$

(5.22)

where $K_H$ is the molar Henry’s constant, $P_H$ is the hydrostatic pressure, $P_E$ is the pressure exerted by the interface onto the gas core, $B$ is the ideal gas constant, and $T$ is the temperature of the system. Here, $j$ represents a gas species that does not correspond to gas species $i$. The saturation fraction, $f$, relates the bulk partial pressure with the saturation pressure ($P_{\infty}$).

$$C_{\infty,i} = K_{H,i} P_{\infty,i} f_i$$

(5.23)

Substituting equations 5.22 and 5.23 to the molar balance 5.21 gives:

$$\frac{d n_i}{dt} = -\frac{4\pi R K_{H,i}}{\Omega_{s,i} + \Omega_{w,i}} \left( P_H + P_E - \frac{3BT}{4\pi R^3} \sum_{j=1}^{N} n_j - f_i P_{\infty,i} \right) \text{ where } i \neq j$$

(5.24)

where $\Omega_s = \frac{1}{k}$ is the resistance to gas permeation through the lipid shell and $\Omega_w = \frac{R}{D}$ is the resistance of gas diffusion through the surrounding aqueous medium. Assuming $\Omega_s = 0$ and
\[ P_E = \frac{2\sigma}{R}, \] where \( \sigma \) is the interfacial surface tension, equation 5.24 becomes identical to the equations presented in Chapter 2.

In order to obtain a general understanding of the excess pressure, \( P_E \), we focus on the momentum transport at the interface. The interface can be defined as a discontinuity separating two bulk fluids and the properties of each fluid and the discontinuity can be represented as:

\[ \mathbb{V} = \overline{\mathbb{V}} + \mathbb{A} \quad (5.25) \]

where \( \mathbb{V} \) is a volumetric property and \( \mathbb{A} \) is an areal property. Here the accent \( \overline{\cdot} \) indicates every point excluding the interface. Starting with the bulk volumetric property, \( \mathbb{V} \), the momentum transport can be determined using Newton’s second law.

\[ \mathbf{F}_B = \frac{d}{dt} \int_{V} \rho \vec{v} dV \quad (5.26) \]

where \( \mathbf{F}_B \) represents the body force tensor, \( \rho \) is the density of the volume, \( V \) is the control volume of the system, and \( \vec{v} \) is the velocity vector. Applying Leibniz rule to equation 5.26:

\[ \mathbf{F}_B = \frac{d}{dt} \int_{V} \rho \vec{v} dV = \int_{V} \frac{d\rho}{dt} \vec{v} dV + \int_{S} (\vec{n} \cdot \vec{v}) \rho \vec{v} dS \quad (5.27) \]
where $\vec{n}$ is the unit normal direction, $\vec{v}_s$ is the surface velocity vector, and $S$ represents the surface area. Applying the divergence theorem on equation 5.27:

$$F_B = \int_V \frac{d\rho \vec{v}}{dt} dV + \int_V \nabla \cdot (\rho \vec{v} \vec{v}) dV$$  \hspace{1cm} (5.28)

The left hand side of equation 5.28 can be broken into two terms, volumetric acting forces, $F_V$, or surface acting forces, $F_S$.

$$F_B = F_V + F_S$$ \hspace{1cm} (5.29)

$$F_B = \int_V \rho \vec{g} dV + \int_V \nabla \cdot \vec{\gamma} dV$$ \hspace{1cm} (5.30)

where $\vec{g}$ is the gravitational vector and $\vec{\gamma}$ is the stress vector. Combining equation 5.27 with equation 5.30, we obtain:

$$\int_V \rho \vec{g} - \nabla P + \nabla \cdot \vec{\tau} dV = \int_V \rho \frac{d\vec{v}}{dt} + \rho \vec{v} \cdot \nabla \vec{v} dV$$ \hspace{1cm} (5.31)
The stress tensor, $\gamma$, can be determined by:

$$
\gamma = -\nabla P + \nabla \cdot \tau
$$

(5.32)

And as a result, equation 5.31 becomes:

$$
0 = \int_V \left( \frac{d\rho_v}{dt} + \nabla \cdot (\rho_d \vec{v}) + \nabla P - \vec{F} - \nabla \cdot \tau \right) dV
$$

(5.33)

where $\vec{F} = \rho \vec{g}$ represents the force vector due to gravity. From the definition of an areal property (equation 5.25) and the arbitrary chosen control volume, we used equation 5.33 to determine the interfacial momentum transport:

$$
\int_A \left( \frac{\partial \rho_s \vec{v}_s}{\partial t} + \nabla (\rho_s \vec{v}_s \vec{v}_s) - \vec{F}_s \right) dA + \Phi_s = 0
$$

(5.34)

where $\rho_s$ is the surface density, $\vec{F}_s$ is the surface force vector, analogous to the volumetric force vector, $\vec{F}$, $A$ is the control area, and $\Phi_s$ is the diffusive flux tensor of the surface momentum analogous to the three-dimensional stress tensor, $\gamma$. The diffusive flux of the surface momentum, $\Phi_s$, can be determined by:
\[ \Phi_s = \int_A \vec{n} \cdot \|\vec{P}\| dA + \int_L \Sigma \vec{m} dL + \int_L \tau_s dL \quad (5.35) \]

where \( \vec{P} \) is the pressure normal to the interface, where the double bracket, \( \| \| \), represents a jump condition \( (\vec{n} \cdot \|\vec{P}\| = (P_s - P_c)\vec{n}) \), \( \Sigma \) is the tension of the interface, \( \vec{m} \) is the unit normal vector of control contour \( L \), and \( \tau_s \) is the deviatoric stress tensor of the interface. Applying the surface divergence theorem and rearranging equation 5.35, we obtain:

\[ \Phi_s = \int_A (\vec{n} \cdot \|\vec{P}\| + \nabla_s \Sigma + 2\Sigma H \vec{n} + \nabla_s \cdot \tau_s) dA \quad (5.36) \]

where \( H \) is the curvature of the surface (for a sphere, \( H = \frac{1}{R} \)). Substituting equation 5.36 into equation 5.34:

\[ \int_A \left( \frac{\partial \rho_s \vec{v}_s}{\partial t} + \nabla (\rho_s \vec{v}_s \cdot \vec{v}_s) - \vec{F}_s + (\vec{n} \cdot \|\vec{P}\| + \nabla_s \Sigma + 2\Sigma H \vec{n} + \nabla_s \cdot \tau_s) \right) dA = 0 \quad (5.37) \]

Again, because the control area is arbitrary and therefore infinitely small, equation 5.37 becomes:
It can be shown that equation 5.38 can reduce to equation 5.4 under the proper assumptions. To obtain the classical Young-Laplace equation we assume that the momentum profile develops instantaneously, surface body forces are negligible, and the interfacial tension, $\Sigma$, is isotropic and homogenous, and surface deformation is negligible. With these assumptions, equation 5.38 can be converted into:

\[
\frac{\partial \rho_s \bar{v}_s}{\partial t} + \nabla (\rho_s \bar{v}_s \bar{v}_s) - \bar{F}_s + \bar{n} \cdot \|\bar{P}\| + \nabla \Sigma + 2 \Sigma \mathcal{H} \bar{n} + \nabla \cdot \tau_s = 0
\]  

(5.38)

For a spherical gaseous particle, equation 5.39 becomes:

\[
(P_+ - P_-)\bar{n} + 2\Sigma \mathcal{H} \bar{n} = 0
\]  

(5.39)

Here, the term on the right hand side of equation 5.40 represents the excess pressure, $P_E$, seen in equation 5.24.
5.3. Materials and Methods

5.3.1. Langmuir Isotherms

Filtered phosphate buffer saline (PBS) (Sigma-Aldrich, St. Louis, MO) solution was used as the sub-phase on a Langmuir trough (KSV, Espoo, Finland). Stock solution of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) (Avanti Polar Lipids, Alabaster, AL) and polyethylene glycol 40 (PEG40) stearate (Sigma-Aldrich, St. Louis, MO) were dissolved into chloroform at a molar ratio of 9:1 and a concentration of 2 mg/ml. The stock solution was then deposited onto the PBS sub-phase. After the chloroform evaporated (~30 min), the deposited monolayer was compressed and expanded at a rate of 1020 mm$^2$/min. The monolayer was compressed from 100 cm$^2$ to 240 cm$^2$ and then expanded back to 27.6 cm$^2$, corresponding to a fractional area change, $\beta$, of 0.14. After expansion, the monolayer’s surface pressure was measured over time.

5.4. Simulations

To examine if the organic shell’s visco-elastic properties and gas permeability could account for the unique behavior observed for lipid-coated SF$_6$ microbubbles suddenly submerged in air-saturated solution, the mathematical models previously developed above, in Chapter 2 and used in Chapter 3 were modified step-wise. The model was broken into two categories: (1) a priori determination of the microbubble radius using the fitted surface tension or (2) solving for the excess pressure by fitting measured radius-time curves. The modified Epstein-Plesset model for multi-gas species assumes that each gas acts separately and equilibrates at the gas-liquid interface. Each gas diffuses along its own chemical potential gradient, and independently contributes to the total pressure and volume of the microbubble gas core. Again, convection around the microbubble is assumed negligible and therefore gas diffusion occurs through a
quiescent aqueous solution. Diffusion through the medium is assumed to be faster than the changes in the microbubble boundary, thus allowing for fully developed gradient approximations to remain valid. For all of the simulations the molar composition of the microbubbles was determined by a first-order forward finite difference discretized molar balance that incorporates Fickian diffusion.

Using methods used in Chapter 2, the diameter-time behavior was predicted \textit{a priori}. A minimization algorithm was applied to calculate the next time step diameter from the recently determined molar composition. To minimize propagation of error, a dynamic time step (equation 2.11) was applied. The molar flux equation and diameter estimation equation were iterated to produce the diameter-time curves.

To solve for the excess pressure from the measured diameter, data from optical microscopy images were imported into Matlab 2010b. To avoid errors from large time steps, a linear interpolation was used between each diameter-time point. To account for outlier results, the experimental data was smoothed before evaluated. Again, the future molar composition was initially determined, and with the measured diameter the excess pressure could be calculated. The excess pressure fit simulations also used the dynamic time step shown in equation 2.11.

\section*{5.5. Results and Discussion}

\subsection*{5.5.1. Assumption of Zero Surface Tension}

We initially hypothesized that the diameter-time behavior of a sulfur hexafluoride (SF$_6$) microbubble coated with a lipid monolayer shell and suddenly immersed in an air-saturated environment could be analyzed using our multi-gas Epstein-Plesset model with a constant
surface tension. First, we assumed that the surface tension was zero owing to the solid-like properties of the lipid monolayer encapsulation discussed in Chapter 1. From equation 5.24 and assuming \( \Omega_s = 0 \), the accumulation of each species \( i \) in the microbubble gas core is given as (Kwan and Borden 2010):

\[
\frac{dn_i}{dt} = \frac{-4\pi R^2 K_{Hi}}{\Omega_{w,i}} \left( P_H + P_E - \frac{3BT}{4\pi R^3} \sum_{j=1}^{N} n_j - f_i P_{x,i} \right)
\]  

(5.41)

The excess pressure, \( P_E \), is assumed here to be equal to the Laplace pressure, as shown below:

\[
P_E = \frac{2\sigma}{R}
\]  

(5.42)

Initially, it was assumed that the surface tension term is zero or, equivalently, that the gas pressure inside the bubble is always equal to the ambient hydrostatic pressure. Matlab R2010b was used with equations 5.41 and 5.42 to simulate the diameter-time behavior of an \( \text{SF}_6 \) microbubble suddenly immersed in an air-saturated environment (Appendix A.1.). Figure 5.1A shows the diameter-time behavior for theoretical and experimental microbubbles initially filled with pure \( \text{SF}_6 \) and surrounded by an \( \text{SF}_6 \)-saturated medium, where the medium is suddenly exchanged to be saturated in air and depleted of \( \text{SF}_6 \) at \( t = 0 \) s. Figure 5.1B shows the gas composition inside the theoretical microbubble as a function of time.
Figure 5.1: A) Theoretical and experimental diameter-time curves for an SF$_6$ microbubble suddenly immersed in an air-saturated medium. Three typical experimental curves are shown (empty points). The theoretical curve (black) was calculated from equation 5.41 assuming that the resistance to gas permeation and the surface tension both had values of zero. B) Gas composition in the theoretical microbubble as a function of time. Numbers indicate the serial number of the microbubble.

The model predicted an immediate growth in diameter as the rapidly diffusing oxygen and nitrogen molecules entered the microbubble, shown in Figure 5.1B. The predicted growth of 72% in diameter occurred in less than 10 seconds. Growth stopped once the partial pressure difference of air between the gas-core and surrounding environment had equilibrated (~10 s). These modeling results are important because they show that the efflux of SF$_6$ along its concentration gradient did not drive ultimate microbubble dissolution. The gas core was simply replaced with air. Equilibrium was allowed to occur because the surface tension was set to zero and, as a result, there was no “restorative force” to drive the microbubble to shrink back to its original size.
The theoretical model did not accurately predict the experimental results for the three representative DSPC:PEG40S microbubbles, shown in Figure 5.1A. As described in Chapter 4, DSPC:PEG40S microbubbles showed four distinct regions: 1) growth and dissolution, 2) primary stabilization, 3) dissolution and, 4) secondary stabilization. Here, we focus only on the initial growth and subsequent dissolution because the model was not designed to account for the complex mechanics and dynamics that occur during monolayer collapse post primary stabilization. As expected, the model predicted the initial expansion, but overestimated the magnitude and failed to predict the subsequent dissolution observed in the experiment. The discrepancy between the theoretical and experimental results indicated that the lipid monolayer shell developed a non-zero surface tension, supplying the restoring force for the initial resistance to growth and for the subsequent dissolution to occur.

5.5.2. Non-Zero, Constant Surface Tension

We next hypothesized that the lipid monolayer reduces the surface tension to a non-zero value, which remains constant throughout time. This assumption is valid for a “soluble” surfactant monolayer, such as the SDS microbubble discussed in Chapter 2. It is possible that a monolayer that maintains a constant surface density of lipid as the surface expands and contracts would also have a constant surface tension. One could imagine, for example, that lipid material attached to the monolayer in the form of surface folds can be pulled into and pushed out of the monolayer plane to maintain a constant lipid surface density.

If the constant surface density is valid, then the surface tension of the microbubble would remain a constant. It was assumed therefore that the lipid monolayer maintained the equilibrium surface density, which is defined as the surface density at which the flux of lipid molecules out
of the monolayer plane is equal to the flux of lipid molecules into the monolayer plane. It has been reported that a DSPC monolayer has an equilibrium surface tension of 19 mN/m (Lee et al. 2001), and this value was chosen for the model. Appendix A.1. shows the code for predicting the diameter time behavior under constant surface tension. Figure 5.2A shows a comparison between the model prediction and the experimental results. Figure 5.2B shows the gas composition inside the theoretical microbubble.

![Figure 5.2A](image1)

**Figure 5.2:** A) Theoretical and experimental diameter-time curves for an SF₆ microbubble suddenly immersed in an air-saturated medium. Three typical experimental curves are shown (empty points) for DSPC:PEG40S encapsulation. The theoretical curve (black) was calculated from equation 5.41 assuming that the surface tension had a value of 19 mN/m. The predicted excess pressure (dashed line) was calculated from equation 5.42. B) Gas composition in the theoretical microbubble as a function of time. Numbers indicate the serial number of the microbubble.

Growth was predicted to be rapid and overshot the maximum diameter observed experimentally by approximately 35%. Thus, the non-zero surface tension better estimated the magnitude of
growth. However, the model predicted that complete dissolution of the microbubble occurred immediately after the initial period of expansion. The non-zero surface tension provided a restoring force which forced gas out of the microbubble even in saturated conditions, as shown in Figure 5.2B. Dissolution time (~5 s) was calculated to be two orders of magnitude faster than the duration of the experiment (~400 s). As a result, the constant surface tension model failed to accurately portray the observed microbubble behavior.

The model was at least successful in predicting a finite lifetime for the microbubble and was therefore more accurate than the previous model of zero surface tension. We next asked the question of whether the delay in growth and dissolution for the experimental microbubbles may be due to the kinetics of gas transfer, rather than the driving force. Perhaps the divergence of our simulation from experimental observation may have been attributed to a gas permeation resistance of the lipid encapsulation.

5.5.3. Constant Gas Permeation Resistance

To help answer this question, we assumed that gas escaping the microbubble must first permeate through the monolayer at a non-negligible rate before diffusing through the aqueous medium. As a result, equation 5.24 was used to determine the molar rate of gas transfer in the microbubble. Again, the excess pressure was equated to the Laplace pressure in equation 5.42. This would be true, for example, if the surface density remained constant due to lipid adsorption from folds. In this scenario, the shell resistance term also would be constant, an assumption which has been explored by Paul et al. (Paul et al. 2010). With the constant lipid surface density, the gas permeation resistance depends only on the rate at which thermally active gaps form on
the monolayer. The holes allow for the passing of single gas molecules. The relationship between gas permeation resistance and the lipid acyl chain length and collision diameter of the gas has been derived from an energy barrier theory, as shown below (Blank and Mussellwhite 1968).

\[
\Omega_{s,i} = \Omega_0 e^{\frac{\pi r_i^2}{R_B \Pi_{eq}}}
\]  

(5.43)

where \( \Omega_0 \) is an empirical constant for each lipid encapsulation species, \( k_B \) is Boltzmann’s constant, \( \Pi_{eq} \) is the surface pressure at the equilibrium lipid surface density, and \( r \) is the collision radius for each gas species \( i \). Table 5.1 lists the empirical constant \( \Omega_0 \) for each lipid species, as determined by Borden and Longo (Borden and Longo 2004).

**Table 5.1: Empirical Coefficients**

<table>
<thead>
<tr>
<th>Lipid Species</th>
<th>Gas Permeation Resistance [s/cm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC</td>
<td>40.9</td>
</tr>
<tr>
<td>DPPC</td>
<td>46.2</td>
</tr>
<tr>
<td>DSPC</td>
<td>133</td>
</tr>
<tr>
<td>DBPC</td>
<td>227</td>
</tr>
</tbody>
</table>

The collision diameters are in units of meters, and were determined using an empirical model previously described by Tee et al. (Tee et al. 1966).

\[
r_i = 0.118 \times 10^{-9} \left( \frac{p_{ci}}{T_{ci}} \right)^{-1/3}
\]

(5.44)
where $P_{c,i}$ is the critical pressure of gas species $i$ and $T_{c,i}$ is the critical temperature of gas species $i$. Table 5.2 lists the calculated collision diameters and GPRs for each gas.

<table>
<thead>
<tr>
<th>Gas Species</th>
<th>Gas Permeation Resistance [s/cm]</th>
<th>Collision Diameter [Å]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td>691</td>
<td>0.34</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>863</td>
<td>0.37</td>
</tr>
<tr>
<td>Water Vapor</td>
<td>656</td>
<td>0.34</td>
</tr>
<tr>
<td>Sulfur Hexafluoride</td>
<td>3358</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Table 5.2: Gas Permeation Resistances

Using equation 5.24 and the values in Table 5.2, the theoretical diameter-time behavior could be calculated. The Matlab code for the simulation is given in Appendix A.1. Figure 5.5A shows a comparison between the model prediction and the experimental results. Figure 5.5B shows the gas composition inside the theoretical microbubble.
Figure 5.3: A) Theoretical and experimental diameter-time curves for an SF₆ microbubble suddenly immersed in an air-saturated medium. Three typical experimental curves are shown (empty points) for DSPC:PEG40S encapsulation. The theoretical curve (black) was calculated from equation 5.24 using the resistances to gas permeation given in Table 5.2 and a surface tension value of 19 mN/m. The predicted excess pressure (dashed line) was calculated from equation 5.42. B) Gas composition in the theoretical microbubble as a function of time. Numbers indicate the serial number of the microbubble.

The model predicted a similar growth-dissolution trend (inset of Figure 5.3) as seen in Figure 5.2. Addition of the GPR term improved the accuracy of the model by increasing the time for growth and dissolution (~500 s) to a scale on the order of magnitude of the experiment. This increase in microbubble lifetime was due to the slower efflux of SF₆ (Fig. 5.3B). Consequently, there was a larger influx of air (Figure 5.3B) into the microbubble, resulting in a 71% increase in maximum diameter compared to the experiment. Thus, accounting for the slow kinetics of gas transport through the lipid encapsulation led to a better estimate of microbubble lifetime, but a poorer estimate of the total growth. Furthermore, the shape of the diameter-time behavior did not match the observed experimental results. Behaviors such as stunted growth and
the stabilized diameters were not seen in the model results. The discrepancy between the theoretical and experimental results suggests that the surface density of the lipid is not constant during the growth and expansion of the microbubble.

5.5.4. Dynamic Surface Tension

We next hypothesized that the surface density of lipid covering the gas-liquid interface changes as the microbubble grows and dissolves. This is consistent with the behavior of insoluble monolayers on flat Langmuir troughs. Since surface tension depends on the lipid surface density, it would follow that the surface tension changes with the surface area of the microbubble. The previous models maintained that the excess pressure is given by the simple Laplace pressure, equation 5.5. This expression for the excess pressure, however, ignores the deviatoric stress components, or forces that are dependent on the direction (tangential or orthogonal) of movement of the Newtonian surface, or a surface that is material dependent with stress linearly dependent with strain (Edwards 1961). Deviatoric stresses can be broken to two major components, shear stress (tangential) or dilatational stress (orthogonal). Normal forces that apply stress in plane to the interface, such as those that originate from growth or dissolution of the microbubble, are dilatational (expansive or compressive) stresses. The excess pressure can be estimated by accounting for viscous dissipation by the bulk medium and surface viscoelasticity (Edwards 1961; Paul et al. 2010; Scriven 1960):

\[ P_E = 4\mu_R \frac{R}{R} + 4\mu_s \frac{R}{R^2} + \frac{2\Sigma(R)}{R} \]  

(5.45)
where $\mu$ is the bulk fluid viscosity, $\dot{R}$ is the radial shell velocity, $\mu_s$ is the dilatational surface viscosity and $\Sigma(R)$ is the interfacial tension. It should be noted that because the boundary $r = R$ and the system is assumed to be spherical, the excess pressure, $P_E$, is only affected by normal forces, and shear stresses will not affect the changes in diameter.

In addition to a viscous term, the surface tension is assumed to be the elastic component and therefore we no longer assume it to be constant. As the monolayer stretches, the surface tension attempts to restore it back to the original shape. If the surface tension changes linearly with strain, the elasticity will be constant. Studies have shown, however, that lipid monolayers decrease in elastic force with increasing expansion (Doinikov et al. 2009). This behavior is a result of the intermolecular forces between the lipid molecules decreasing as the distance between lipid molecules increases, indicated by the increasing fractional change in area. Lipid monolayers, therefore, are “strain softening” interfaces. To account for a strain-softening lipid monolayer, an empirical relationship developed by Paul et al. (Paul et al. 2010), can be used to calculate the dynamic surface tension:

$$\Sigma(R) = \Sigma_0 + \beta E_0 e^{-\alpha \beta}$$  \hspace{1cm} (5.46)

where $\Sigma_0$ is the unstrained (equilibrium) tension, $\beta$ is the fractional change in area $\left(\frac{R}{R_0} - 1\right)$, $E_0$ is the linear elasticity (for small strains), $\alpha$ is an empirical exponential term, and $R_0$ is the initial diameter. The first term on the right hand side of equation 5.46 represents the equilibrium
tension of the lipid monolayer, while the second term represents the elastic strain softening portion. Table 5.3 shows the values used by Paul et al. (Paul et al. 2010) in their model.

<table>
<thead>
<tr>
<th>Table 5.3: Property Values for Model Constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Viscosity $\mu\ [\text{kg*m/s}]$</td>
</tr>
<tr>
<td>8.9\times10^{-4}</td>
</tr>
</tbody>
</table>

While the literature reports that the surface viscosity of the microbubble encapsulation was indeed non-negligible, they were determined from microbubbles perturbed by ultrasound where the dilatation rates are on the order of $1 \times 10^6 \ \mu\text{m}^2/\text{s}$ (Doinikov et al. 2009). Our experimental results, however, showed that the dilatation rates of a microbubble perturbed by gas diffusion alone are at least five orders of magnitude lower ($\sim 1 – 10 \ \mu\text{m}^2/\text{s}$). As a result, the viscous portions of equation 5.46 can be ignored, resulting in a strain-softening elastic model. Maintaining a constant GPR (Table 5.2), the diameter-time behavior of a strain-softening lipid-encapsulated SF$_6$ microbubble submerged in a multi-gas (air) medium was estimated using equations 5.2, 5.8 and 5.9. The MATLAB code is given in Appendix A.5. Figure 5.8A shows the results of the simulations.
Figure 5.4: A) Theoretical and experimental diameter-time curves for an SF\textsubscript{6} microbubble suddenly immersed in an air-saturated medium. Three typical experimental curves are shown (empty points) for DSPC:PEG40S encapsulation. The theoretical curve (black) was calculated from equation 5.2 assuming that the resistance to gas permeation was constant (Table 5.2). The dynamic surface tension was determined using equations 5.8 with the values listed in Table 5.3, neglecting the viscous terms. Predicted excess pressure (dashed) was determined using equations 5.8 and values in Table 5.3. B) Gas composition in the theoretical microbubble as a function of time. Numbers indicate the serial number of the microbubble.

The simulation predicted a stunted growth that closely mimics the behavior observed experimentally. During initial microbubble expansion, the excess pressure rapidly increased to approximately 50 kPa (Figure 5.4A). The microbubble could not rapidly grow from the influx of air, shown in Figure 5.4B owing to the elastic terms that resist expansion of the lipid monolayer. The resulting increase in excess pressure reduced the total influx of air and increased the efflux rate for SF\textsubscript{6}. This reduction in total gas accumulation is represented by the slower rate of expansion on the diameter-time curve (Fig. 5.4A, B). Although the maximum diameter reached
in the simulation overshot the experimentally observed maximum, it was minimal (<10%), and
the general shape of the growth-dissolution region was in relatively good agreement. Again, the
overshoot can be attributed to the slow diffusion of SF$_6$ out of the gas core, as seen in the earlier
model. The simulated microbubble stabilized as the excess pressure approached zero (not
shown) and occurred at a later time than was observed experimentally. This stabilization arose
from the exponential component of the areal fractional change term, $\beta$, becoming negative at
values less than the initial radius. Once the elastic term, $\beta E_0 e^{-\alpha\beta}$, in equation 5.46 became
equal and opposite of the equilibrium value, $\Sigma_0$, stability was reached, and the microbubble
dissolution halted.

5.5.5. Dynamic Gas Permeation Resistance

The analysis above shows that the assumption of a variable lipid surface density
improves the predictive capability of the microbubble dissolution model. Since the gas
permeation resistance through the monolayer also depends on lipid surface density, it follows
that the GPR should change with the surface area of the microbubble. The dependence of gas
permeation through a lipid monolayer on surface density was discussed in Chapter 1 and has
been explored experimentally by Zhang and Unwin (Zhang and Unwin 2003) and Borden and
Longo (Borden and Longo 2004). The relationship between the surface pressure and the GPR of
a phospholipid monolayer is as follows (Borden and Longo 2004):

$$\Omega_{s,i} = \Omega_0 e^{\frac{\pi r_i^2}{k_B T}}$$  (5.47)
where $\Pi = \sigma_{g-l} - \Sigma$ is the surface pressure on the interface, and can be determined by the dynamic interfacial tension in equation 5.46. Appendix A.1. shows the Matlab code used to determine the diameter-time behavior. A comparison of the theory to the observed behavior is shown in Figure 5.9A.

![Figure 5.5: A) Theoretical and experimental diameter-time curves for an SF$_6$ microbubble suddenly immersed in an air-saturated medium. Three typical experimental curves are shown (empty points) for DSPC:PEG40S encapsulation. The theoretical curve (black) was calculated from equation 5.2. The resistance to gas permeation was estimated with equations 5.7 – 5.10. The dynamic surface tension was determined using equation 5.9, again neglecting the viscous terms, with the values listed in Table 3. Predicted excess pressure was calculated using equation 5.7 and Table 2. B) Gas composition in the theoretical microbubble as a function of time. Numbers indicate the serial number of the microbubble.](image-url)
As the GPR decreased during growth, the gas diffused in and out of the microbubble more rapidly. As a result, the initial growth period was more rapid than for the case of constant GPR. Figures 5.5A-B showed that the initial growth period approximately 5 seconds. The more rapid exchange of gases also reduced the maximum diameter. One would expect a larger excess pressure to reduce the maximum diameter; however, the peak excess pressure shown in Figure 5.8A was roughly 10 kPa less than the constant GPR model predicted (with dynamic surface tension). The reduction in excess pressure can be attributed to the increased rate of SF$_6$ efflux mentioned earlier. As expansion occurred, the GPR decreased while the excess pressure increased. The net of these combined factors increased the efflux of SF$_6$ out of the microbubble. As a result, the magnitude of growth matched what was observed, but the duration of growth and dissolution did not. The growth and dissolution peak was observed to be broad, whereas the predicted behavior was narrower. Because no changes were made to the constitutive expression for the excess pressure, the microbubble was predicted to stabilize for the same reasons discussed earlier, namely the elastic term that resisted both growth and dissolution beyond the initial size.

5.5.6. Determination of Interfacial Mechanical Properties

In order to determine the cause of the deviations between the current theory and the experimental data, we looked at the excess pressure ($P_E$) as a fitting parameter to see its response to the microbubble diameter-time behavior. Figure 5.6 show the results for the calculated excess pressure and gas core contents (Appendix A.2.).
Figure 5.6: A) Representative fitted excess pressure curve (black), fitted diameter-time curve (grey), and experimental diameter-time curve (empty circle) for an SF$_6$ microbubble suddenly immersed in an air-saturated medium. The excess term in equation 5.2 was used to fit experimental data for a DSPC:PEG40S encapsulation assuming dynamic resistance to gas permeation. B) Gas composition in the fitted theoretical microbubble as a function of time. Numbers indicate the serial number of the microbubble.

The excess pressure, which was determined by fitting the dynamic GPR model to the experimental data, had the characteristic initial peak from the sudden influx of air being greater than the slow efflux of SF$_6$ (Fig. 5.6B). The peak marked a change in behavior of the interface from a linear positive slope, to a negative exponential decay in excess pressure. Interestingly, this exponential decay in excess pressure occurred, in part, while the microbubble was growing, possibly suggesting that interface was undergoing a relaxation process. The excess pressure approached zero as the microbubble stabilized before undergoing further dissolution post collapse. The excess pressure was also plotted alongside the strain rate, $\frac{\partial \rho}{\partial t}$, shown in figure 5.7, to demonstrate the speeds at which the interfaces are moving and its affect on the excess pressure.
Figure 5.7: Strain-rate (grey) and excess pressure (black) curves of representative SF$_6$ microbubbles encapsulated with DSPC:PEG40S and suddenly immersed in an air-saturated medium assuming a dynamic resistance to gas permeation. Horizontal line (dotted) indicates the zero strain-rate. Numbers indicate the serial number of the microbubble.

From figure 5.7, it is clear that once break-up occurs, there is a decrease in growth rate. This decay suggests a relaxation phenomena occurring post monolayer rupture. Following dissolution back to the initial diameter, the model lost accuracy in its fitting capabilities for reasons mentioned previously and as a result, was not shown.
Using the fitted excess pressure values and assuming that $P_E = \frac{2\Sigma}{R}$, a stress-strain curve of the microbubble’s interface was generated and shown in figure 5.8.

Figure 5.8: Stress-strain curves of representative SF₆ microbubbles encapsulated with DSPC:PEG40S and suddenly immersed in an air-saturated medium assuming a dynamic resistance to gas permeation. Horizontal line (dotted) indicates the surface tension of an air-water interface (0.073 $\frac{N}{m}$). Arrows indicate direction of time. Numbers indicate the serial number of the microbubble.
The stress-strain curves show an initial linear rise in surface tension. This increase occurred during the early stages of growth ($\beta < 0.02$) and was followed by a gradual decrease in surface tension. Similar to figure 5.6A, figure 5.8 clearly demonstrated that this decrease in surface tension occurred during both growth and dissolution. A decrease in surface tension during microbubble growth has not been documented before and as a result, Langmuir Isotherm relaxation studies were performed and used to derive a molecular mechanism for the observed stress-strain behavior.

5.5.7. Phospholipid Monolayer Relaxation

The results from the fit indicated that there was an increase in surface tension followed by a decrease during interfacial expansion, which upon first inspection, contradicted our initial expectations. Marmottant et al. (Marmottant et al. 2005) suggested that an increase in surface tension above that of a pure gas-liquid interface was possible, but exceeding this “rupture” tension would result in an instantaneous decrease in surface tension to the gas-liquid interface (73 $\frac{mN}{m}$) where it remains constant until compression below the $A_{rupture}$ (equation 5.9). The strain-softening model also predicts that the surface tension can increase an order of magnitude above the pure gas-liquid interface. At fractional area changes above the maximum surface tension ($\beta = 0.7$), the model predicts a slow decrease in surface tension to an equilibrium spreading tension (Paul et al. 2010). In our experimental observations, the fractional area change never exceeded more than 0.2 and as a result, neither of these models would account for the trends found in figure 5.8.
We propose the lipid encapsulation undergoes three distinct regions: (1) elastic resistance to expansion until interfacial rupture, (2) domain dissolution due to interfacial surfactant concentration gradients, and (3) compression of the interface. Figure 5.9 shows a cartoon illustrating our hypothesis.

Figure 5.9: Cartoon illustrating (A) initial state of the lipid encapsulation, where the dark hexagons represent the lipid and the light circles represent the emulsifying agent, (B) interfacial strain due to microbubble growth (Region 1), (C) rupture at the interstitial regions between lipid domains, (D) domain dissolution during microbubble expansion (Region 2), (E) recompression of the interface (Region 3), and (F) reformation of the lipid domains. Arrows indicate direction of strain and lines between the circles represent intermolecular forces such as van der Waals and hydrophobic interactions (Israelachvili 1991).
To verify our hypothesis we employed a Langmuir trough study, measuring the surface tension of a DSPC:PEG40S interface, post expansion, over a period of 5,000 seconds, shown in figure 5.10.

![Langmuir Trough Study](image)

**Figure 5.10:** Average and standard deviation of nine DSPC:PEG40-stearate monolayer mixture Langmuir isotherms. The monolayer was compressed and then expanded to a fractional area change of 0.14 at a rate of 1020 mm$^2$/min. The area was remained constant and the surface tension was measure over the course of 5,000 seconds. The horizontal dotted line indicates the plateau ($25 \frac{mN}{m}$).

In this figure, the interface was expanded from a fully compressed monolayer ($\sigma = 73 \frac{mN}{m}$) to a fractional area change of 0.14 ($\sigma = 45 \frac{mN}{m}$). Once expanded, the monolayer surface tension exponentially decreased to $25 \frac{mN}{m}$, indicating a relaxation of the monolayer. Though this expansion-relaxation has not been observed in Langmuir trough studies before, compression relaxation of surfactant coated monolayers have been documented previously (Bois et al. 1988).
Boise et al. (Bois et al. 1988) attributed the relaxation to molecular re-ordering and nucleation and growth of lipid domains. Because of the time scales at which relaxation occurs, the kinetic effects of domain growth or dissolution are not observed in traditional Langmuir isotherms. By observing microbubble growth, however, we expect to resolve the effects of domain dissolution.

To explain the multifaceted behavior that the phospholipid interface exhibits during microbubble growth and dissolution, we put forward a three tier model. In the first section of the stress-strain curve, the lipid monolayer was assumed to be fully compressed (Fig. 5.9A) until suddenly expanded due to the sudden influx of air. The interface was presumed to be stretched elastically and was modeled using the following equation (Israelachvili 1991; Marmottant et al. 2005):

\[ \Sigma(\beta) = k_E \beta \]  \hspace{1cm} (5.48)

where \( k_E \) is the linear elasticity of the interface (Fig. 5.9B). In the second region, the interface was expanded past a critical point, causing monolayer rupture (Fig. 5.9C). Because the interface has separated, a gas-liquid interface forms and causes interfacial mass transfer of lipid due to a concentration gradient between domains and gas-liquid interface (Fig. 5.9D). We used the relaxation model proposed by Bois et al. (Bois et al. 1988) to account for the behavior of the interface due to domain dissolution, as shown in the equation below:

\[ \Sigma(t) = \Sigma_{EQ} + (\Sigma_{\text{break}} - \Sigma_{EQ})e^{-t/t_d} \]  \hspace{1cm} (5.49)
where $\Sigma_{\text{break}}$ is the break-up tension of the interface, $\Sigma_{\text{EQ}}$ is the effective equilibrium tension of the monolayer due to domain dissolution, and $\tau_E$ is the effective relaxation rate constant that accounts for the diffusion of the domains. In the third region, the net gas flux is outward and the microbubble begins to shrink, compressing the lipid encapsulation (Fig. 5.9E-F). Again, we used the linear elasticity model to determine the surface tension (Israelachvili 1991):

$$\Sigma(\beta) = \Sigma(\beta_{\text{max}}) + k_C(\beta - \beta_{\text{max}})$$

(5.50)

where $\Sigma(\beta_{\text{max}})$ is the tension at maximum expansion ($\beta_{\text{max}}$) and $k_C$ is the compressive elasticity of the monolayer. Combining these three models, the surface tension can be defined by the equation below:

$$\Sigma(\beta, t) = \begin{cases} 
  k_E \beta & \beta \leq \beta_{\text{break}} \\
  \Sigma_{\text{EQ}} + (\Sigma_{\text{break}} - \Sigma_{\text{EQ}}) e^{-\tau_E t} & \beta_{\text{break}} < \beta \leq \beta_{\text{max}} \\
  \Sigma(\beta_{\text{max}}) + k_C(\beta - \beta_{\text{max}}) & \beta > \beta_{\text{max}} 
\end{cases}$$

(5.51)

This three tier model (equation 5.51) was used to fit the following parameters, $k_E$, $\tau_E$, and $k_C$, to the three regions in the stress-strain curve shown in figure 5.7. The equilibrium tension, $\Sigma_{\text{EQ}}$, was assumed to be constant ($25 \frac{mN}{m}$) in agreement to the relaxation studies we performed. The fit was applied to each lipid encapsulation formulation described in Chapter 4. Figure 5.11
shows the results of the three tier model fit (equation 5.51) for representative DSPC:PEG40S stress-strain curves and Table 5.4 lists the tabulated results for all of the lipid encapsulations used in Chapter 4.

Figure 5.11: Stress-strain curves (empty points) of representative SF$_6$ microbubbles encapsulated with DSPC:PEG40S and suddenly immersed in an air-saturated medium assuming a dynamic resistance to gas permeation with three tier fit overlaid (dashed line). Horizontal dotted line indicates the surface tension of an air-water interface (0.073 $\frac{N}{m}$). Numbers indicate the serial number of the microbubble.
<table>
<thead>
<tr>
<th>Lipid</th>
<th>Emulsifier</th>
<th>$N_{\text{total}}$</th>
<th>$k_E$ [N/m]</th>
<th>$\beta_{\text{break}}$</th>
<th>$\Sigma_{\text{break}}$ [mN/m]</th>
<th>$\tau_E$ [1/s]</th>
<th>$k_C$ [N/m]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC</td>
<td>PEG 40S</td>
<td>15</td>
<td>10 ± 10</td>
<td>0.02 ± 0.01</td>
<td>76 ± 5</td>
<td>0.8 ± 0.07</td>
<td>0.09 ± 0.09</td>
</tr>
<tr>
<td>DPPC</td>
<td>PEG 40S</td>
<td>22</td>
<td>6.0 ± 3.6</td>
<td>0.02 ± 0.01</td>
<td>74 ± 5</td>
<td>0.6 ± 0.05</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>DSPC</td>
<td>PEG 40S</td>
<td>21</td>
<td>3.6 ± 2.6</td>
<td>0.04 ± 0.03</td>
<td>73 ± 5</td>
<td>0.2 ± 0.04</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>DSPE PEG2K</td>
<td>33</td>
<td>6.4 ± 4.8</td>
<td>0.02 ± 0.01</td>
<td>75 ± 3</td>
<td>0.2 ± 0.02</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td>DBPC</td>
<td>PEG 40S</td>
<td>17</td>
<td>3.4 ± 1.7</td>
<td>0.02 ± 0.01</td>
<td>70 ± 6</td>
<td>0.1 ± 0.02</td>
<td>0.7 ± 0.3</td>
</tr>
</tbody>
</table>

Table 5.4: Cumulative Results for Three Tier Fit of Stress-Strain Curves

$N_{\text{total}}$ = total microbubbles analyzed; $k_E$ = linear expansion elasticity; $\beta_{\text{break}}$ = fractional area change at interfacial rupture; $\Sigma_{\text{break}}$ = break-up tension; $\tau_E$ = effective rate constant; $k_C$ = linear compression elasticity. Averages were determined omitting outlier values.
In region 1, the results of the three tier fit (equation 5.51) showed no significant differences between the different encapsulation formulations for the linear elastic expansion region, $k_E$, and the rupture point, $\beta_{\text{break}}$, or the break-up tension, $\Sigma_{\text{break}}$. A non-significant difference in linear elasticity, rupture point, and break-up tension may reflect the notion that rupture occurred at the interstitial regions between domains. The interstitial regions are primarily PEG40S and would suggest that the linear elasticity recorded in Table 5.4 correspond to the elasticity of the PEG40S. These non-significant differences also suggested that increases in acyl-chain length may not be the primary cause for the maximum growth differences observed experimentally. Instead, the gas permeation through the monolayer would account for the reduced growth observed for increasing acyl-chain lengths of the primary lipid encapsulation.

In region 2, there was a significant change in the rate constant, $\tau$, as the acyl-chain length was increased. The significant decrease in the rate constant, seen in table 5.4, was indicative of reduced lipid domain diffusion rates as the encapsulating lipid becomes more insoluble. As a result, the rate constant was attributed to two different processes. First, before a lipid molecule diffuses across the interface, it must first detach from the lipid domain, breaking van der Waals and hydrophobic interactions between the hydrophobic tails of the phospholipid. Second, after the lipid molecule breaks away from the domain, the molecule diffuses across the interface. Similar to gases, one expects that the diffusion of the lipid would inversely scale with its molecular weight. The larger lipid, therefore, requires more time (or a slower relaxation rate) to reduce the overall tension experienced on the interface.

The microbubbles exhibited extremely slow expansion rates ($\sim 7 \frac{\mu m^2}{s}$). At such slow rates, the strain may be considered negligible compared to the dissolution rates of the lipid
domains and, therefore, comparable to the Langmuir trough relaxation studies. The relaxation results from the Langmuir trough of a DSPC:PEG40S monolayer show a relaxation rate of about $0.04 \frac{1}{s}$. The relaxation rate of the DSPC domains on the microbubble were determined to be approximately $0.2 \frac{1}{s}$, and order of magnitude faster. This discrepancy may be attributed to two key differences between the trough and the microbubble. First, the Langmuir trough is at least five orders of magnitude larger in area than the microbubble. The microbubble, therefore, can be considered as a micro-trough and as a result, is more sensitive to short-time kinetics. Second, the Langmuir trough expands an-isotropically whereas the microbubble expands isotropically. This difference may explain why there is a large rate constant difference between the relaxation results from the trough. If a plane is expanded an-isotropically and uni-directionally, such as the Langmuir trough, a line contour is formed between the two sides orthogonal to the direction of expansion. The lipids, therefore, only diffuse in the direction normal to the line that is formed. The microbubble, however, expands isotropically. The contour formed during expansion is not a line that bisects a plane, but a circle that surrounds the lipid rafts. This results in a larger perimeter of gas-liquid interface surrounding the lipid domains per unit of expansion, and therefore, a faster rate of diffusion of lipid. The microbubble encapsulation length scales are also on the same order as these diffusion length scales.

The fitted values for region 3 showed a significant increase in the linear elastic compression region, $k_C$, as the acyl-chain length was increased. This increase in elasticity indicated that stiffer shells resisted compression, which was expected.
5.6. Conclusions

Here we presented several different attempts to theoretically describe the phenomena observed when lipid-coated SF₆ microbubbles are suddenly exposed to air-saturated aqueous solution. First, we demonstrated that surface tension was required to act as the restoring force which drives the microbubble to dissolve. Surface tension alone, however, was shown to dissolve the microbubble orders of magnitude faster than what was observed. A constant resistance to gas permeation was therefore applied to the diffusion model and indeed increased the dissolution time. A strain softening elasticity model and the effects of dilatational viscosity were appended to the excess pressure equation, and greatly improved the accuracy of the model. The presence of elasticity provided the resistive force which stunted the predicted growth to values near the ones observed experimentally. The notion of a dynamic surface tension due to the expansion of the monolayer violates the assumption that the surface density remains constant. As a result, a variable gas permeation model replaced the constant permeation term. Interestingly, using the elasticity terms from Paul et al. (Paul et al. 2010) for the dynamic surface tension, the predictions from this model lowered the accuracy. The drop in accuracy indicates that the two extremes, constant surface density and a density that is both homogenous and variable are not valid and that the true behavior lies somewhere in between. Stress-strain curves of the expansion-dissolution region displayed a peak in excess pressure, followed by decay in excess pressure as the microbubble expanded. Unfortunately, neither the predictive model nor the fit could account for the unique phenomena such as a primary stabilization at the initial diameter and a secondary stabilization in the 1 – 2 µm range. As a result, focus was placed on the growth and initial dissolution to stabilization regions of the microbubble. A three tier model
was implemented to fit the stress-strain curves generated in the excess pressure fits which implied that linear elasticity, rupture point, and break-up tension did not correlate to acyl-chain lengths but instead may relate to the break-up of the interstitial regions lining the lipid domains. The relaxation rate constant decreased with increasing acyl-chain length of the lipid encapsulation formulation. This decrease was attributed to the time required for detachment and subsequent transport away from the lipid domain. The exponential decay in tension on the microbubble monolayer was verified Langmuir trough expansion relaxation studies. The difference in rate constants was determined to be a result of differences in the nature of the system. The compression elasticity and showed a significant increase when the acyl-chain length was increased, indicating that more cohesive shells resisted compression.
Chapter 6: Conclusions and Future Work

6.1. Accomplishment of Specific Aims

6.1.1. Microbubble Dissolution in a Multi-Gas Environment

An experimental technique to directly observe microbubbles under different gas saturated mediums was successfully developed along with a multi-gas microbubble diffusion model. The apparatus consisted of a hollow microfiber threaded through a perfusion chamber. A threaded gas-tight syringe was used to spatially control the microbubbles. The perfusion chamber set-up was situated on top of an inverted microscope. The dissolution of soluble surfactant coated (SDS) and insoluble surfactant (DBPC) SF₆-microbubbles (ranging from 25 – 100 µm initial diameters) was observed, and the diameter-time behavior measured. The diameter-time results were compared to a model derived to simulate gas exchange. Microbubbles coated with SDS and kept in an SF₆-gas saturated environment dissolved under the increasing presence of Laplace pressure as predicted by classic Epstein-Plesset theory. Soluble surfactant coated microbubbles suddenly submerged in an air-saturated environment were shown to initially grow, due to the influx of nitrogen and oxygen, and then dissolve under surface tension. This observation was also predicted by the diffusion model. Lipid-coated microbubbles deviated significantly from the SDS-coated microbubbles. The expansion regime was less prominent, and three dissolution regimes were found, including (1) rapid dissolution back to the original diameter, (2) steady dissolution with a nearly constant wall velocity and (3) stabilization near ~10 µm diameter. To explain the observations, it was suggested that the surface tension is not constant, and that the monolayer tears upon expansion and jams when compressed.
6.1.2. Experimental Observations of Lipid-coated Microbubbles in Multi-gas Mediums

The goal of our this study was to clarify the features observed previously using microbubbles of 2-20 µm diameter, and a homologous series of diacyl-chain lipid encapsulations (DMPC, DPPC, DSPC, and DBPC). The lipid-encapsulated SF₆-microbubbles grew when submerged in an air-saturated medium, with one exception. DBPC microbubbles did not grow beyond the resolution capabilities of optical microscopy. For DSPC, growth was severely repressed below an apparent threshold size (~10 µm diameter). The amount of growth was shown to decrease with increasing reduced temperature of the lipid encapsulation. Following growth, all microbubbles dissolved, most of them back to their original size before stabilizing, indicating the “self-healing” capability of the lipid monolayer shell. The microbubbles experienced a lag before the spontaneous onset of a second dissolution stage. The lag and dissolution times were highly variable and more correlated to the reduced temperature of the lipid-encapsulating molecule, rather than the initial diameter. Astonishingly, most of the microbubbles with a reduced temperature greater than one stabilized again at a diameter of 1-2 µm. This “stable diameter” appeared to be universal and not correlated with either the initial microbubble size or the rigidity of the encapsulation indicating it was geometric in origin.

6.1.3. Theoretical Modeling and Analysis of Experimental Results

Different theoretical models were implemented to describe the phenomena observed when lipid-coated SF₆ microbubbles are submerged in air-saturated aqueous solution. It was demonstrated that surface tension was required to act as the restoring force, contradicting the notion that the lipid-encapsulation maintained a constant surface tension of zero. Surface tension alone predicted that a microbubble would dissolve orders of magnitude faster than what was
experimentally observed. These results indicated that constant surface tension is inadequate at describing the observed diameter-time curves. Next, a constant resistance to gas permeation was applied to reduce the rate of gas transfer. The dissolution time increased. However, the model could not exhibit unique phenomena, such as a primary stabilization at the initial diameter and a secondary stabilization in the 1 – 2 µm range. A strain-softening elasticity model was incorporated into a constitutive mechanical stress equation, which improved the accuracy of the model. The presence of elasticity provided the resistive force necessary to stunt the growth from gas influx. Though these results were promising, the notion of a dynamic surface tension violates the assumption that the surface density remains constant. As a result, a variable gas permeation model was employed. Unfortunately, this addition reduced the accuracy in the predictive model and indicated that a different set of constants are necessary. It was proposed that the drop in accuracy may indicate that the two extremes, constant surface density and a density that is both homogeneous and variable, are not valid and that the true behavior lies somewhere in between. Fitted excess pressure hysteresis curves displayed a peak in excess pressure during the initial influx of gas, followed by decay in excess pressure as the microbubble expanded. Elasticity and relaxation parameters were determined from the fitted model using a three-tier model. The three-tier model suggested that the linear elasticity and rupture of the interface was controlled by both the PEG40S interstitial regions and the gas permeation resistance of the lipid domains. The rate constant corresponded to the domain dissolution which caused the interface to relax and decreased as the lipid acyl-chain length increased. The effective compression elasticity was dependent on the encapsulation formulation and demonstrated that stiffer shells resisted compression more.
6.2. Impact on the Field

The modeling of gas diffusion and the influx of nitrogen provided and explanation for the observations of increased microbubble persistence times for animals breathing air compared to oxygen. The initial single- and multi-gas experiments on soluble surfactant microbubbles validated the proposed diffusion model, setting up a basis from which the shell mechanics could be qualitatively and quantitatively described. Though these discoveries were important and set up the framework for the subsequent studies, the observations of lipid-encapsulated microbubbles have the greatest impact. Prior to this study, it was assumed that lipid-encapsulated microbubbles grow when injected into a patient. The idea that a microbubble can grow when subjected to blood poses a problem. If the hypothetical growth was sufficiently large to cause an embolism, this would be a major concern for doctors and patients (even when studies clearly indicate that this does not happen). Our observations clearly show that lipid-encapsulated microbubbles in the medically relevant sizes grow, but not to any appreciable sizes which would cause concern. The resistance to growth may add to the factors which cause the long persistence in oceans. For example, it suggests that small, natural microbubbles may not accelerate as they rise to the surface from buoyancy, due to decreasing hydrostatic pressures. With the lipid shell resisting both growth and dissolution, microbubbles (sub 5 µm) may persist indefinitely because the Brownian motion on that size range are similar in magnitude to the buoyant forces. Similarly, the lipid monolayer lining the alveoli in the lungs may resist rupture during inhalation. The lipid coating may provide additional mechanical stability beyond just reducing the surface tension, which is the classical model.
The inception of a yield stress was first observed by de Jong et al. (de Jong et al. 2007) and later explained by Marmottant et al. (Marmottant et al. 2005). The results from the theoretical fitting models, discussed in Chapter 5, demonstrated that during expansion there was a peak in excess pressure, providing further evidence that the lipid monolayer is not a simple Newtonian interface. Our model also was able to back out a surface tension at very low strain rates, which may be similar to rates experienced on the surface of the alveolus. The results showed that during expansion, there is a sudden increase followed by a non-linear decrease in interfacial tension. This follows that the surface relaxes during expansion due to lipid domain dissolution. This has implications on what may be occurring to lung surfactant (which comprises mostly phospholipids) during breathing.

Another important observation is the secondary re-stabilization to 1 – 2 µm in diameter after the spontaneous dissolution. This result appears to be ubiquitous across all lipid microbubbles and independent from the initial diameter, though the frequency of their presence is dependent on the lipid encapsulation. It was proposed that this stability was due to the jamming of the lipid as it is compressed, but because the size range of these stable microbubbles is so ubiquitous, there may be another mechanism that causes stability. This stable size may give an explanation as to why it is the most prominent size class when microbubbles are generated through mechanical agitation. This stable size range is a recurring theme across different microbubble studies. Borden et al. (Borden et al. 2005) observed that DSPC:DSPE-PEG2K encapsulated microbubbles will stop dissolving under acoustic pulses, stabilizing at 1 – 2 µm in diameter. Rossi et al. (Rossi et al. 2008) reported that contrary to belief, DMPC encapsulated microbubbles 1 – 2 µm in diameter persisted longer in solution than 5 µm microbubbles. These results have profound impact on microbubble applications that involve ultrasound. Both
experimental and theoretical evidence suggests that lipid-coated microbubbles are less acoustically active in the 1 – 2 µm size range (de Jong and Hoff 1993; de Jong et al. 1992; Medwin 1977; Sirsi et al. 2010). Because this size range absorbs, not scatters, acoustic signals, understanding the mechanics of what causes this ubiquitous stable size is necessary for the development of better acoustically active microbubbles.

6.3. Future Work

There are several immediate extensions of this work that can be pursued. More control over the microbubble, possibly generating single bubbles instead of populations, may allow one to expand the dissolution studies. One could investigate the impact that different variables such as microbubble aging, gas saturation fractions, emulsifying compositions and shell microstructure have on the different regions of dissolution. Such studies would further link shell properties to the microbubble stability and dynamics in solution. Such studies may also elucidate some of the unique mechanics observed when monolayers are highly compressed.

The current studies demonstrate the behavior of single microbubbles, but it is left unclear if these same phenomena will occur in populations of microbubbles. Dissolution studies on microbubble suspensions may provide useful information on the dynamics of in vivo microbubbles. It has been proposed that binding molecules, such as biotin, will adhere to linker molecules like avidin on the surface of the phospholipid encapsulation, inducing folds that greatly modify the surface structure (Chen and Borden 2010). The study of monolayer expansion during growth would be appropriate here.
Another path to take is to focus on improving the constitutive model. To account for a varying surface density of lipid, one could replace the empirical models of monolayer isotherms with lipid adsorption kinetics that account for the varying surface density. It may also be possible to utilize more powerful fluid dynamic model simulators like finite element analysis to aid in adding transient effects and convection around the microbubble. Additional techniques, such as the Langmuir trough, pendant drop and microscopy, can be employed to quantify monolayer elasticity, surface viscosity and permeability, which is necessary to fully characterize the microbubble monolayer encapsulation.

Finally, one intriguing direction may be to focus on what factors contribute to the secondary stable diameter. A systematic study on microbubble dissolution, while adjusting relative concentrations of emulsifier, surface structure, and production technique may elucidate why the 1 – 2 µm size range is so dominant. Other measuring techniques such as confocal microscopy and freeze fracture transmission electron microscopy may help in visualizing what is occurring on the surface at these size ranges. Pursuing this may also provide understanding as to why microbubbles appear to form multimodal size distributions with peaks at 1 – 2, 4 – 5, and 6 – 8 µm. Is it coincidence that these sizes roughly occur at intervals of this stable diameter?
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Appendix


function MCGD_RPredict_GEN_Model

clear all
clc
close

tic
R = [Enter Value]; % Initial Radius [m]
R0 = R;
ft = [Enter Value];% End Time [s]
i = [Enter Value];
j = [Enter Value];
tspeed = [Enter Value];
model = [[Enter Value] [Enter Value] [Enter Value]];
L = model(1); %[DMPC DPPC DSPC DSPCP2K DBPC]
Lipid = {'DMPC' 'DPPC' 'DSPC' 'DSPCP2K' 'DBPC'};
LFull = {'DMPCPEG40S' 'DPPCPEG40S' ...
'DSPCPEG40S' 'DSPCDSPEPEG2K' 'DBPCPEG40S'};

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

%mentalData--ExperimentalData--ExperimentalData--ExperimentalData--ExperimentalData--Experime
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

initdiam = num2str(round(2*R*10^6));
strmodel = 'GEN';

foi = [datestr(now,'HHMMSS'),'-',char(Lipid(L)),...
'','-','MCGD_RPredict_GEN_Model_111011',... 
'-',strmodel,'-',datestr(now,'yymmdd'),'-',initdiam,'-um','.txt'];

foj = [datestr(now,'HHMMSS'),'-',char(Lipid(L)),...
'','-','MCGD_RPredict_GEN_Model_111011',... 
'-',strmodel,'-',...
'Parameters-',datestr(now,'yymmdd'),'-',initdiam,'-um','.txt'];

ProcessPath = ['C:\Users\FJPKWANJ\Desktop\Research\Research Work\'...
,'Papers\2011 Lipid Microbubble Dynamics Theoretical - Kwan\',...
'Model Results\',char(LFull(L)),'\Predicted\'];

pathname = strcat(ProcessPath,datestr(now,'yymmdd'));

if exist(pathname,'dir') == 0
    mkdir(pathname)
end

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%alConstants--GlobalConstants--GlobalConstants--GlobalConstants--GlobalCons
%==========================================================================
Pinf = [20619 77569 3137 0]; %Partial Pressures at inf. [Pa]
D = [2.42 2 2.1 1.2]*10^-9; %Diffusivity [m^2/s]
kc_g = [3.948 1.713 315.2 3.46]*10^-4; %Henry Constant [(g/m^3)/Pa]
MW = [32 28 18 146]; %Molecular weight [g/mol]
T = 25 + 273.15; %Temperature [K]
Ph = 1.01325*10^5; %Atmos. Pressure [Pa]
kc = kc_g./MW; %Converts Henry's constant to [(mol/m^3)/Pa]
Tc = [155 126 647 318]; %Tc - Critical Temperature [K] (Vector: 1 x N)
Pc = [50.4 33.9 220.6 37.7]; %Pc - Critical Pressure [atm] (Vector: 1 x N)

%Order [DMPC DPPC DSPCPEG40S DSPCDSPE-PEG2K DBPC]
if model(3) == 1
  Ohm0 = [Enter Value]*100;
elseif model(3) == 2
  Ohm0Lipids = [40.9 46.2 133 133 227]*100;
  Ohm0 = Ohm0Lipids(L);
end

%Physical Constants
sigc = [Enter Value];
chi = [Enter Value];
E1 = [Enter Value];
aE = [Enter Value];
E2 = [Enter Value];
aD = [Enter Value];
betab = [Enter Value];

%==========================================================================

%enFile--OpenFile--OpenFile--OpenFile--OpenFile--OpenFile--OpenFile--OpenFile--Parameters

fo1 = fullfile(pathname,foj);
fidw = fopen(fo1,'w');
fiw = fopen(fo1,'w');
fprintf(fidw,'model-Lipid\t');
fprintf(fidw,'model-\tST\t');
fprintf(fidw,'model-GPR\t');
fprintf(fidw,'sigc [N/m]\t');
fprintf(fidw,'chi [N/m]\t');
fprintf(fidw,'betab [#]\t');
fprintf(fidw,'E1 [N/m]\t');
fprintf(fidw,'aE [#]\t');
fprintf(fidw,'betam [#]\t');
fprintf(fidw,'E2 [N/m]\t');
fprintf(fidw,'aD [#]\t');
fprintf(fidw,'Ohm0 [s/m]\t');
fprintf(fidw,'Ohm Oxygen [s/m]\t');
fprintf(fidw,'Ohm Nitrogen [s/m]\t');
fprintf(fidw,'Ohm Water Vapor [s/m]\t');
fprintf(fidw,'Ohm Sulfur Hexafluoride [s/m]\n');
%--------------------------------------------------------------------------
% Data

fo = fullfile(pathname,foi);
fidw = fopen(fo,'w');
fida = fopen(fo,'a');
fprintf(fidw,'Time [sec]\t');
fprintf(fida,'Radius [um]\t');
fprintf(fida,'Excess Pressure [kPa]\t');
fprintf(fida,'Beta [#]\t');
fprintf(fida,'Surface Tension [N/m]\t');
fprintf(fida,'Nitrogen [pmole]\t');
fprintf(fida,'Water Vapor [pmole]\t');
fprintf(fida,'Sulfur Hexafluoride [pmole]\n');

%==========================================================================
% Calculations- Calculations- Calculations- Calculations- Calculations- Calculations-Calculation

%--------------------------------------------------------------------------
% Calculations- Calculations- Calculations- Calculations- Calculations- Calculations-Calculation

%--------------------------------------------------------------------------
% Initial Condition--------------------------------------------------------------------------

t = 0;
R1 = R;
k = 1;

[Pe,sig] = PePredictGEN (R,R0,sigc,chi,E1,aE,model(2));

f = [1 1 1 0]; % Saturation Fraction [fraction]
n = initCond(R,Ph,T,Pe);
figure('WindowStyle','docked')

%==========================================================================
% Solution--------------------------------------------------------------------------

while t <= ft && R >= 10^-10

beta = (R/R0)^2 - 1;

if k >= i || (t <= tspeed && k >= j)
    rdata = [t 2*R*10^6 Pe*10^-3 beta sig n'*10^12];
    dlmwrite(fo,rdata,’-append’,’delimiter’,’\t’,’precision’, 6);
    subplot(2,2,1:2), hold on, plot(t,2*R*10^6)
    title(’Microbubble Diameter [um]’)
    subplot(2,2,3), hold on, plot(beta,sig*1000)
    title(’Surface Tension [mN/m]’)
    subplot(2,2,4), hold on, plot(t,n(1)*10^12,t,n(2)*10^12,...
        t,n(3)*10^12,t,n(4)*10^12)
    title(’Moles of Gas [pmol]’)
    drawnow
    k = 0;
end

%--------------------------------------------------------------------------
% Solve future n & R Values

n = FluxEq(R,D,kc,T,Tc,Pc,Ph,f,Pinf,Ohm0,n,sig,model(3));

[R] = NRSolverGEN(R0,Ph,sigc,chi,R1,T,n,aE,E1,model(2));

%--------------------------------------------------------------------------
% Solve Excess Pressure


[Pe, sig] = PePredictGEN(R, R0, sigc, chi, E1, aE, model(2))

%------------------------Increase Time Step
    R1 = R;
    k = k + dTime(R, D);
    t = t + dTime(R, D);
    if R < R0*0.1, break, end

end

% Final Data Point
rdata = [t 2*R*10^6 Pe*10^-3 beta sig n'*10^12];
dlmwrite(fo, rdata, '-append', 'delimiter', '	', 'precision', 6);
subplot(2,2,1:2), hold on, plot(t, 2*R*10^6); title('Microbubble Diameter [um]')
subplot(2,2,3), hold on, plot(beta, sig*1000);
title('Surface Tension [mN/m]')
subplot(2,2,4), hold on, plot(t, n(1)*10^12, t, n(2)*10^12, ...
    t, n(3)*10^12, t, n(4)*10^12);
title('Moles of Gas [pmol]')
drawnow

% Parameters
Ohmi = WShell1111111(sigc, Ohm0, T, Tc, Pc, model(3));

Parameters = [model(1) model(2) model(3)...
    sigc chi betab Msigb E1 aE betam E2 aD...
    Ohm0 Ohmi(1) Ohmi(2) Ohmi(3) Ohmi(4)];
dlmwrite(fo1, Parameters, '-append', 'delimiter', '	', 'precision', 6);

syst = toc;
A.2. Fitted Excess Pressure: Matlab Code

function [foi,syst,D0,D0STDV,Dmax,DmaxSTDV] = MCGD_PeFit_GEN_110908...
(RawData_dir,RawData_Filename,...
Fit_Data_dir,t0,span,L,Raw_Data_dir,Check_Data_dir,tf,delay)

close all
%
%--------------------------
Global Constants
%--------------------------

[t_temp,R_temp,STDV_temp] = gatherDataSet110901...
(RawData_dir,RawData_Filename); %Gather data [s,m] (Vector: N x 1)

functionname = ['MCGD_PeFit_GEN_110908-','Coupled-','...'
'Delay-',num2str(delay)];

[foi] = DataStorage110315(RawData_Filename,functionname);
[~,t0_index] = min(abs(t_temp - t0));
D0 = 2*R_temp(t0_index)*10^6;
D0STDV = 2*STDV_temp(t0_index)*10^6;
Dmax = 2*max(R_temp(t0_index:end))*10^6;
DmaxSTDV = 2*max(STDV_temp(t0_index:end))*10^6;
R_smooth = smooth(t_temp, R_temp, span, 'rloess');
t0_index = t0_index - delay;

t_exp = t_temp(t0_index:end) - t_temp(t0_index);
t_temp = t_temp(t0_index:end) - t_temp(t0_index);
R_exp = R_smooth(t0_index:end);
R_temp = R_temp(t0_index:end);
STDV_temp = STDV_temp(t0_index:end);

%==========================================================================
%alConstants--GlobalConstants--GlobalConstants--GlobalConstants--GlobalCons
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

Pinf = [20619 77569 3137 0]; %Partial Pressures at inf. [Pa]
D = [2.42 2 2.1 1.2]*10^-9; %Diffusivity [m^2/s]
kc_g = [3.948 1.713 315.2 3.46]*10^-4; %Henry Constant [(g/m^3)/Pa]
MW = [32 28 18 146]; %Molecular weight [g/mol]
T = 25 + 273.15; %Temperature [K]
Ph = 1.01325*10^5; %Atmos. Pressure [Pa]
kc = kc_g./MW; %Converts Henry's constant to [(mol/m^3)/Pa]
Tc = [155 126 647 318];
Pc = [50.4 33.9 220.6 37.7];

%Order [DMPC DPPC DSPCPEG40S DSPCDSPE-PEG2K DBPC]
Ohm0Lipids = [40.9 46.2 133 133 227]*100;
Ohm0 = Ohm0Lipids(L);

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%openfile%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
fo = fullfile(Fit_Data_dir,foi);
fopen(fo,'w');
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%calulations%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%-----------------------------Initial Condition-------------------------------
R0 = R_exp(1); %Initial Radius [m] (Scalar)
R1 = R0;
sig = 0;
f = [1 1 1 0]; %Saturation Fraction [fraction]
n = initCond(R0,Ph,T,(2*sig)/R0);
DATA = zeros(1000,3);
k = 1;
t = 0;
z = 1;
i = [ENTER VALUE];
j = [ENTER VALUE];
tspeed = [ENTER VALUE];
figure('WindowStyle','docked')
%-----------------------------Solution--------------------------------------
while t < t_exp(end)
    R = interp1(t_exp,R_exp,t,'linear');
    R2 = interp1(t_exp,R_exp,t+dTime(R,D),'linear');
    dr_dt = Rdot(R1,R2,R,D);
    beta = (R/R0)^2 - 1;
    if R < R0*0.93, break,
    elseif t > tf, break, end

%-----------------------------Solve future n Values-----------------------------------
    n = FluxEq(R,D,kc,T,Tc,Pc,Ph,f,Pinf,Ohm0,n,sig);

%-----------------------------Solve Excess Pressure-----------------------------------
    Pe = Pbub(T,R2,n) - Ph;
    sig = (1/2)*Pe*R2;

%-----------------------------Store Data-----------------------------------
    if k >= i || (t <= tspeed && k >= j)
rdata = [t R*10^6 dr_dt sig n'*10^12];

dlmwrite(fo,rdata,'-append','delimiter','\t','precision', 6);

subplot(2,2,1:2), hold on, plot(beta,sig*1000)
title('Surface Tension [mN/m]')

subplot(2,2,3), hold on, plot(t,2*R*10^6)
title('Microbubble Diameter [um]')

subplot(2,2,4), hold on, plot(t,n(1)*10^12,t,n(2)*10^12,...
    t,n(3)*10^12,t,n(4)*10^12)
title('Moles of Gas [pmol]')

drawnow
k = 0;
DATA(z,:) = [t R sig];
z = z+1;

end

%---------------------------------------------------Increase Time Step---------------------------------------------------
k = k + dTime(R,D);
t = t + dTime(R,D);
R1 = R;
end

DATA = DATA(1:(z-1),:);
A.3. Important Functions: Matlab Code

```matlab
function [n_temp] = FluxEq(R,D,kc,T,Tc,Pc,Ph,f,Pinf,Ohm0,n,sig)

    n_temp = n - (((SArea(R)*dTime(R,D))./...
    (OhmW(R,D)+WShell(sig,Ohm0,T,Tc,Pc)))...
    .*((kc'.*((Ph+(2*sig)/R)-Pbj(T,R,n)))-(kc.*f.*Pinf)'));

    m_temp = n_temp*6.02*10^23;
    check = find(m_temp < 1); %Make moles zero if below 1 molecule
    if check > 0
        n_temp(check,end) = 0;
    end

function [GPRShell] = WShell(sig,Ohm0,T,Tc,Pc)

    sig0 = 0.073;%Surface Pressur [N/m] (Scalar)
    a = ColDiam(Tc,Pc); %Collision Diameter [m] (Vector: 1 x N - Function)
    a = a'./2; %Collision Radius [m] (Vector: 1 x N - Function)
    k  = 1.38*10^-23; %Boltzmann Constant [(m^2*kg)/(s^2*K)] (Scalar)
    GPRShell = Ohm0*exp((pi.*a.^2/(k*T))*(sig0-

function [R] = NRSolverGEN(R0,Ph,sigc,chi,R1,T,n,aE,E1,Rmax,model)

    B = 8.314; %Gas Constant [(m^3*Pa)/(mol*K)]
    betamax = (Rmax/R0)^2 - 1;
    N = sum(n);
    if model == 1
        f = @(x)Ph*x.^3 + 2*sigc.*x^2 - (3*B*N*T)/(4*pi);
    elseif model == 2
        f = @(x)Ph*x.^3+ 2*sigc.*x^2 + ...
        2.*x.^2.*(x.^2/R0^2 - 1).*E1.*exp(-aE.*(x.^2/R0^2 - 1)) - ...
        (3*B*N*T)/(4*pi);
    end
    R = fzero(f,R1);
    if R < 0
        R = 0;
    end
```
function [a] = ColDiam(Tc,Pc)
a = (0.23647*(Pc./Tc).^(1/3))*10^-9;

function [Pe,sig] = PePredictGEN(R,R0,sigc,chi,E1,aE,model)

beta = (R/R0)^2 - 1;
betamax = (Rmax/R0)^2 - 1;

if model == 1
    sig = sigc;
elseif model == 2
    sig = sigc + beta*E1*exp(-aE*beta);
end

Pe = (2*sig)/R;