Therapeutic and Diagnostic Applications of Ultrasound Contrast Media
for Breast, Ovarian and Skin Cancers

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Dedication and Acknowledgements

I dedicate this thesis to my parents, Vandy and Chhunly Oum, who have encouraged me to go beyond my limits and achieve my goals.

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List of Abbreviations

**Ab**: Antibody

**Anti-CA-125-Ab**: Antibody specific for CA-125 antigen

**BMPH**: (N-[ß-Maleimidopropionic acid] hydrazide, trifluoroacetic acid salt), chemical crosslinker

**CA**: Contrast agent(s)

**CA-125**: antigen receptor for anti-CA-125 antibody

**DI water**: Deionized water

**DR4 and DR5**: Death receptors for TRAIL, also known as TRAIL R1 and TRAIL R2, respectively.

**DcR1 and DcR2**: Decoy receptors for TRAIL.

**DSPE-PEG-biotin** (or lipid-PEG-biotin): 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Biotinyl(Polyethylene Glycol)2000

**EDC**: (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride), chemical crosslinker

**EPR effect**: Enhanced permeability and retention effect

**FLIP**: FLICE-inhibitory protein

**GRGDS**: Glycine-Arginine-Glycine-Aspartate-Serine peptide

**GRDGS**: Glycine-Arginine-Aspartate-Glycine-Serine (scrambled RGD peptide)

**Mb**: Microbubble

**NHS**: N-hydroxysulfosuccinimide

**OC**: Ovarian Cancer

**PEG**: Poly (ethylene glycol)

**PEMA**: Poly (ethylene maleic acid) surfactant

**PEMA:PVA**: Ratio of blends of the two surfactants, Poly (ethylene maleic acid) and Poly (vinyl alcohol)
PFC: Perfluorocarbon gas

PLA: Poly (lactic acid)

PLA-BAM- (PEG): Microbubbles made with PLA and ammonium carbamate as a porogen, -PEG indicates addition of lipid-PEG-biotin into the shell.

PLA-BON- (PEG): Microbubbles made with PLA and ammonium carbonate as a porogen, -PEG indicates addition of lipid-PEG-biotin into the shell.

PLGA: Poly (lactic-co-glycolic acid)

PVA: Poly (vinyl alcohol) surfactant

RES: reticuloendothelial system

RGD: Arginine-Glycine-Aspartate peptide

TNF: Tumor Necrosis Factor

TRAIL: Tumor necrosis factor related apoptosis inducing ligand

TRAIL-Mb: Tumor necrosis factor related apoptosis inducing ligand bound to microbubbles

TRAIL-Mb-noXlink: Tumor necrosis factor related apoptosis inducing ligand conjugated to microbubbles without chemical crosslinkers (adsorbed TRAIL)

Velcade: Proteasome inhibitor drug made by Millenium Pharmaceuticals, Cambridge MA.

W/O/W: Water-oil-in water double emulsion
Using contrast enhanced ultrasound, molecular markers of disease can be noninvasively imaged. This method is dependent on the administration of a microbubble contrast agent (CA) made site-targeted by the surface attachment of engineered peptides or antibodies that bind to the disease related receptor molecules. The microbubbles selectively retained by the tissues can then be detected using ultrasound. The overall objective of this research is to engineer the surface poly (DL-lactic acid) (PLA) and poly (DL-lactic-co- glycolic acid) (PLGA) based microbubble CAs to facilitate efficient targeting of molecular markers in vitro that can potentially enable the early detection and treatment of breast, skin and ovarian cancers.

To enhance the diagnostic application of CAs, this research demonstrated that: (1) echogenic microbubbles can be created with a carboxyl containing surfactant (PEMA) which allowed more targeting ligand to be conjugated to its surface than that of microbubbles made with a hydroxyl containing surfactant (PVA) (p<0.05), although it did not improve cellular targeting ability (p>0.05); (2) a method of physically incorporating a lipid-ligand conjugate (DSPE-PEG-biotin) can be achieved for attaching a variety of targeting ligands; (3) rough-surfaces PLGA 50:50 based microbubbles have a significantly greater cellular attachment than smooth surface microbubbles once ligated with GRGDS (p<0.05); (4) an ultrasound CA targeting CA-125 receptors using anti-CA-
125 antibodies bound to the surface of the microbubbles created significant CA attachment (p<0.05) to OVCAR-3 cells.

To explore the potential for therapeutic applications a novel ultrasound CA that acts via signal transduction was examined. A TRAIL-modified CA induced a signaling cascade resulting in apoptosis in breast, ovarian and skin cancer cells. Furthermore, TRAIL-modified CA exhibited significantly greater apoptotic effects when combined with a proteasome inhibitor Velcade in melanoma cells (p<0.05).

The various aspects of this research were conducted with the intent of combining them with drug delivery in future applications. This would bring together the advantages of site-targeting, drug delivery, and signal transduction with the benefits of ultrasound imaging to effect a significant improvement in cancer detection and treatment.
1. INTRODUCTION

Using contrast enhanced ultrasound, molecular markers of disease can be noninvasively imaged. This method is dependent on the administration of microbubble contrast agents (CA) targeted by the surface attachment of engineered ligands or antibodies that bind to the disease related receptor molecules[1]. The microbubbles selectively retained by the tissues can then be detected using ultrasound [2, 3].

Since CAs have the potential to be tailored with a variety of targeting vectors, this technology has far ranging potential for the detection, monitoring and treatment of numerous diseases. Specifically, detection of $\alpha_v$-integrin expression in tumor neovessels may be particularly advantageous, since these integrins play a functional role in angiogenesis and have been identified as a marker of metastatic potential in tumors [4]. Modified CAs have the potential to elicit bioeffects such as apoptosis. Targeted CAs could also be used for early detection of other malignancies such as ovarian cancer. In addition, the CA can be loaded with a bioactive compound such as an anti-cancer drug.

To date, investigations of molecularly targeted ultrasound contrast agents have primarily focused on the development of lipid-coated perfluorocarbon microbubble system [5-8]. However, there is a lack of research on the targeting of polymeric, in particular poly-$\alpha$ hydroxy acids such as poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) - shelled, microbubble CAs. Polymeric microbubbles bear the advantage of being more stable and have a more controllable degradation than their lipid-coated counterparts. The rigid shell can also interact with ultrasound and the environment in a different way, and can carry a more substantial drug payload.
Combination of the two modalities (targeting and drug loading) in the form of a targeted drug carrier would have the advantage of being able to deliver a sustained dose of drug directly at the target site, with the added possibility of enhanced delivery triggered by ultrasound.

We have developed a double emulsion method for the fabrication of hollow, echogenic PLGA microbubbles[9]. PLGA is a well-known FDA approved biodegradable polymer for in vivo applications [10]. This polymer breaks down by hydrolytic degradation of its backbone, and has exposed carboxyl groups available for chemical modification with targeting vectors. Its nature allows the adjustable loading of drugs into its surface, shell or core. Overall, surface engineered PLGA CAs represent a highly adaptable cancer targeting system applicable to a variety of cancers by the addition of moieties that target specific entities on cell membranes with the added benefit of real-time ultrasound monitoring capabilities.

1.2 Thesis Objectives

The overall goal of this research is the in vitro development of PLA and PLGA based microbubble CAs that facilitate targeting to molecular markers enabling the early detection and potentially treatment of malignancies. Toward this goal we utilize a strategy composed of the following three Specific Aims:

Specific Aim 1 To characterize, optimize and develop methods of making biodegradable polymer contrast agents that have the capacity for carrying targeting
vectors and to assess cell attachment capabilities of the CA to breast cancer cells in vitro.

1. We have previously developed hollow echogenic microcapsules with demonstrated ability to carry the targeting ligand gly-arg-gly-asp-ser (GRGDS) with varying density. From these results we have chosen poly (lactic acid) PLA as a suitable carrier polymer, however the most uniform capsules to date are prepared from end-capped polymer, which presents fewer reactive groups on the surface available for chemical modification with ligand. We hypothesize that we can further functionalize the surface of the PLA by enhancing the number of carboxyl groups available for chemical conjugation. A surfactant, which coats the microbubbles with active carboxyl groups, polyethylene maleic acid (PEMA), will be synthesized and characterized. PEMA will be used to replace our original surfactant poly (vinyl alcohol) (PVA) and resultant CAs will be evaluated in terms of morphology, echogenicity, stability, and ligand carrying capacity (using flow cytometry analysis). Static cell attachment studies will be performed using MDA-MB-231 breast cancer cells and GRGDS modified CAs made with PEMA. Microbubble/cell adherence will be assessed at various time points with unmodified CAs as controls using light microscopy.

2. Another approach is the physical incorporation of lipid-ligand conjugates: this approach has been primarily explored in the formation of lipid-based contrast agents, and more recently with solid polymer microspheres but has not yet been extended to polymer-based CAs [11]. This technique allows the insertion of a lipid-poly (ethylene glycol) (PEG) - functional group into the shell of the microbubbles. We
hypothesize that by the addition of a lipid-PEG-biotin into our first emulsion we will be able to create microbubbles with incorporated lipid-PEG-biotin that can be further functionalized with targeting ligands. To achieve this, microbubbles will be synthesized, characterized acoustically and morphologically, and surface density of functional groups will be qualitatively assessed by fluorescence intensity.

3. The effect of microbubble morphology on cellular attachment is also a consideration in targeting. We hypothesize that by changing parameters in the double emulsion fabrication method we can produce bubbles that are of a rough texture and may have an enhanced binding to cancer cells once ligated. PLGA bubbles with rough surfaces may hold a different amount of peptide and may have an altered presentation of the peptide to cellular integrin receptors compared to smooth surfaced bubbles made of the same polymer. To achieve this we will vary aqueous/organic phase ratios, porogen amounts, surfactant molecular weights, homogenization and evaporation speeds during fabrication. The resulting smooth and rough microbubbles will then be evaluated for morphology, size and acoustic properties prior to being conjugated with GRGDS or control peptides using a carbodiimide reaction. The static attachment to MDA-MB-231 breast cancer cells will be assessed.

Specific Aim 2 Produce a novel bioactive ultrasound CA that elicits signal transduction, using TRAIL/apoptosis as a model.

The hypothesis is that the majority of cell surface receptors act as transducers of signals that are external to the cell to trigger an intracellular cascade, and that this
mechanism can be utilized to trigger a desired effect when a targeted contrast agent interacts with a cell surface receptor. Specifically, by conjugating the ligand tumor necrosis factor-related apoptosis inducing ligand (TRAIL) to the microbubble surface, cellular apoptosis can be achieved once the TRAIL-microbubbles bind to death receptors on the cell surface. TRAIL stimulates the tumor necrosis factor receptors, death receptor 4 and 5 (DR4 and DR5) which cause apoptosis in an extrinsic manner by triggering a capsase cascade within the cell. To achieve this, another method of conjugation will be investigated. A maleimide conjugation scheme utilizing a N-[β-Maleimidopropionic acid] hydrazide (BMPH) crosslinker/spacer will be developed and used to conjugate TRAIL to the surface of the microbubbles. These TRAIL-microbubbles will then be incubated on TRAIL-sensitive breast cancer, ovarian cancer and melanoma cell models. Controls include incubation with unmodified bubbles, TRAIL only and microbubbles with adsorbed TRAIL. Experiments examining the synergistic effect of proteasome inhibitors and TRAIL-microbubbles on cellular apoptosis will also be performed. Qualitative evaluation of apoptosis for each CA will be performed using a fluorescent apoptosis assay and fluorescent microscopy or flow cytometry. This novel concept has not been reported for any ultrasound CA currently in development.

Specific Aim 3 Produce a targeted contrast agent with the potential for early detection of ovarian cancer cells

- Ovarian cancer (OC) accounts for approximately 3% of all cancers in women and is the 5th leading cause of cancer-related death among women in the United States.
Ovarian cancer has the highest mortality of all cancers of the female reproductive system. This reflects a lack of both early symptoms and proven ovarian cancer screening tests. Thus, ovarian cancer is often diagnosed at an advanced stage, after the cancer has spread beyond the ovaries. Development of a contrast agent to target OC has not been reported to date. Contrast enhanced ultrasound is an ideal non-invasive early detection method for OC. *We hypothesize* that by coupling anti-CA-125 antibodies (Ab) specific to the naturally upregulated CA-125 receptor present in ovarian cancer to our microbubbles, we can develop an ovarian cancer detection model using an ultrasound contrast agent platform. The OC targeted agent is being developed for intraperitoneal injection for detection of surface lesions or for targeting by extravasation through leaky tumor vasculature. In order to find the optimal method for antibody immobilization, several methods will be examined. The first two methods include antibody fragmentations to create free thiol groups for conjugation to the microbubble surface at the antibody’s hinge region. Alternatively, a conjugation strategy using full anti-CA-125 antibodies conjugated to the microbubble surface by carbodiimide chemistry will be evaluated. Live OVCAR-3 ovarian cancer cell attachment assays will be performed to visually assess microbubble adhesion/cell at various time points. CA-125 receptor negative cells, A2058 melanoma cells, will be used as a control.
2. BACKGROUND AND LITERATURE REVIEW

This chapter presents background information along with a review of the pertinent literature on (1) ultrasound imaging, (2) ultrasound contrast agents with a focus on targeting and PLGA microbubbles (3) polymeric surface modification for attachment of bioactive compounds, and (4) cancer targeting and treatment.

2.1 Ultrasound Imaging

Ultrasound is a medical imaging technique that uses sound waves and their echoes to create images. The major benefits of this technique are that it is safe, non-invasive, portable, gives information in real-time, can be used for many different types of procedures, and is relatively inexpensive compared to x-ray and magnetic resonance imaging (MRI).

In diagnostic ultrasound, a transmitted sound wave (2MHz - 17 MHz) travels into the body and at the boundary between tissues (e.g. between fluid and soft tissue, soft tissue and bone) some of the sound waves get reflected back to the probe due to an impedance mismatch. Some of the signal is scattered, some is absorbed as heat and some travels on further until being reflected by another boundary. Impedance is the product of density and the speed of sound through a material. As the difference between the impedance for two media increases, the more signal will be reflected. This reflected signal, also known as an echo, returns to the transducer and is used to produce ultrasound images. To produce the images, a transducer receives the reflected ultrasound signal from the tissue and converts these mechanical waves into electrical signals. Transducers are usually made from piezoelectric or ferroelectric materials that change their thickness and
shape as a voltage is applied across them, and conversely create electrical potential when they are deformed. After being converted by the transducer, the ultrasound information is then processed by a receiver that alters the signal to remove noise and to adjust signals from deeper reflections for attenuation [12] The signals are then converted for display.

2.2 Ultrasound Contrast Agents

As with any imaging modality, there are limitations to the types of tissues that can be imaged with ultrasound. To increase diagnostic yield, contrast agents have been introduced to ultrasonography. Contrast agents usually consist of gas-filled microbubbles. The microbubbles act as signal enhancers, and operate by the basic mechanism present in all cases of diagnostic ultrasound, and in this case that the backscattered signal intensity is proportional to the change in acoustic impedance between the blood and the gas inside the bubbles [13]. In other words, a contrast agent provides an impedance mismatch where one did not exist or was not large enough to generate a visible signal. At the blood/gas interface the difference in acoustic impedance is very high; there is a perfect reflection of the incident sound wave, though not all the signal will return to the transducer due to scattering in other directions. Contrast agents should increase backscattered signal as much as possible without increasing the attenuation of the tissue.

A bubble at resonance has three orders of magnitude greater backscatter cross section, a measure of echogenicity, compared to a non resonating bubble [14]. It is desirable to maximize the resonant properties in the design of a contrast agent which takes into account size. The microbubbles must be small enough to cross the capillary bed (<7.5 microns) and the resonant frequency depends inversely on bubble diameter. It
is a fortuitous coincidence that the resonance frequency of free microbubbles 1–7 microns in diameter lies within the traditional 2–15 MHz medical ultrasound range. The amplitude of the contrast agent oscillations (and hence the scattering effect) is thus maximized [15].

The ability to produce sustained signals from contrast agents depends on the stability of the gas core, and the existence of mechanisms to prevent coalescence. Longevity is enhanced in bubbles by modification of the gas content and provision of a stabilizing shell. According to Epstein-Plesset models, gas loss from a free bubble is dependent on its size, the surface tension, and gas characteristics such as solubility and diffusion capacity [16]. Thus, the stability of contrast agents can be improved by using gases with low diffusion coefficients and low solubility in water/blood. Commonly used gases include air, nitrogen, sulfur hexafluoride and perflurocarbons, although air and nitrogen tend to be too soluble and low molecular weight [17]. The encapsulation of bubbles with shells made of proteins, lipids or polymers are also used to enhance stability, by reducing outward diffusion, reducing surface tension, and controlling microbubble size and coalescence [17].

Currently, there are three agents approved in the United States for clinical use in cardiology; Optison (GE Healthcare, Princeton, NJ), a perfluoropropane-filled agent with a phospholipid shell, Imagent (Alliance Pharmaceutical, San Diego, CA), a perfluroxane lipid shelled agent, and Definity (Lantheus Medical Imaging, North Billerica, MA), a perfluorotrien lipid shelled agent. Several other microbubble contrast agents have been approved in Europe and Canada [18], including Sonovue (Bracco, Princeton, NJ), a
sulfahexafluoride filled phospholipid shelled agent and Levovist (Schering, Kenilworth NJ), an air filled fatty acid shelled agent[19].

2.3 Polymeric Ultrasound Contrast Agents

2.3.1 PLGA

Many material choices have in the past been investigated for the formulation of microbubbles, including, polysaccharides (such as starch, alginate, and agarose), proteins (such as gelatin, and albumin), fats and fatty acids (such as palmitic and stearic acid), lipids and polymers [20].

Polymers have quickly become a widely applied material in the field of biomedical engineering and materials science. Poly (lactic – co – glycolic acid) (PLGA) polymers can be easily modified and processed, and their mechanical properties can be controlled. PLGA has been FDA approved for use in sutures and drug delivery devices [10]. The most important features of PLGA are that it is biodegradable and biocompatible. It degrades into lactic acid and glycolic acid. Lactic acid enters the tricarboxylic acid cycle and is metabolized and eliminated from the body as carbon dioxide and water. Glycolic acid is either excreted unchanged in the kidney or it enters the tricarboxylic acid cycle and is eliminated as carbon dioxide and water [10]. The \textit{in vivo} degradation of PLGA is affected by several properties, among them glass transition temperature ($T_g$), molecular weight, and crystallinity [21]. The porosity of a polymer construct (including capsules) will affect the degradation rate. As porosity increases, so does the surface area in contact with fluid and thus, the greater amount of area that can undergo degradation. Consequently, the greater the porosity, the faster the rate of
degradation. The structure of PLGA and the homo polymer PLA are depicted in figure 2.1.

![Chemical Structures of Poly(lactic acid) and Poly(lactic-co-glycolic acid)](image)

**Figure 2.1:** Chemical Structures of (A) Poly (lactic acid) and (B) Poly (lactic-co-glycolic acid)

### 2.3.2 Emulsions

One method to produce microcapsules is emulsification [9]. An emulsion is a mixture of 2 immiscible liquids. The solvent evaporation technique has been used to produce microbubbles of PLA and PLGA. These polymers have been studied extensively due to their biocompatibility. A polymeric solution is dispersed and emulsified in an aqueous continuous phase to form droplets and the organic solvent must evaporate at the air/water interface. During solvent evaporation, the microcapsules start to harden and can be collected [22]. This method has been used to prepare PLA and PLGA microbubbles with many different drugs such as anticancer drugs, narcotic agents, local anesthetics and steroids [22].

In a conventional O/W encapsulation there is an emulsification of an organic polymeric solution in an aqueous continuous phase. The emulsion is produced by agitating the two immiscible liquids and dispersing them via high shear with the use of
propeller blades, homogenization, microfluidization, or sonication [23, 24]. The greater the shear produced, the smaller the particles. Also, there is an influence of type and ratio of surfactant, rate of solvent evaporation, solvent type, agitation rate, and polymer molecular weight on the physiochemical characteristics. Sansdrape et al. found that microparticles became smaller when stirring rate was increased and that the mean diameter of the microsphere decreased as the volume of solvent increased [25].

In multiple emulsion systems such as a water-oil-water (W/O/W), the preparation conditions have a significant impact on the morphology and porosity of the microbubbles [22]. Investigating the effects of buffers or salts added to the internal and/or external aqueous solutions on the properties of somatostatin acetate-containing PLA microspheres, Hermann et al. [26] found that adding buffers or salts to the internal phase resulted in porous microspheres and that adding them to the external phase resulted in a dense homogenous polymer matrix. To investigate the effects of volume of the inner water phase on the internal and external structure of the polymer microspheres 5 batches of bovine serum albumin in PLGA microspheres were prepared using 1 to 22.7% inner water volume [27] and it was found that an initial inner aqueous phase volume fraction of 5.6% produced hollow microspheres with a dense nonporous polymer shell. However, with an initial volume fraction of 22.7% the hollow microspheres had porous surface structures.

A development in our laboratory has been the creation of polymeric microcapsules which were prepared by a double emulsion (W/O/W) solvent evaporation process [20]. In short, polymer and camphor were dissolved in methylene chloride and then a 4% ammonium carbonate was added to generate the (W/O) emulsion by probe sonication.
The (W/O/W) emulsion was created by pouring the (W/O) emulsion into a 5% poly vinyl alcohol (PVA) solution, which was then homogenized.

2.3.3 History of the Current Polymeric Ultrasound Contrast Agent

The focus of this thesis is the targeting of a novel polymeric ultrasound contrast agent originally developed by El-Sherif et al. [20]. The original contrast agent was made with 50:50 PLGA. The microcapsules prepared in this manner are about 1.2 µm in size and during \textit{in vivo} studies, the animals were not distressed after receiving up to 20 doses of the agent (in the range of 2 ml/kg of rabbit weight from a stock solution of 0.04g/ml). The capsules are also found to be echogenic \textit{in vitro}. The dose response was related to the frequency at which the capsules were insonated, producing an enhancement at 25°C of approximately 15, 25, 25 and 21 dB when insonated with 2.25, 5, 7.5 and 10MHz of ultrasound energy, respectively, for a dose of only 8μg of microcapsules/ml of buffer. \textit{In vivo} power Doppler imaging with the polymer contrast agent showed visual enhancement in comparison to the image taken without the agent.

This contrast agent was further modified by J. Lathia [28]. It was determined that the monomer ratio of lactic acid (LA) to glycolic acid (GA) impacts the performance of the contrast agent. \textit{In vitro}, polymer blends of PLGA 75:25, 85:15, and PLA showed sufficient acoustic enhancement of up to 20dB and acoustic stability for 15 minutes at both 25°C and 37°C in comparison with PLGA 50:50 [29]. \textit{In vivo}, all contrast agent blends showed sufficient acoustic enhancement of up to 20dB but the duration of contrast enhancement was dependent on the ratio of LA to GA since LA is more hydrophobic.
than GA. The decision to proceed making the contrast agent using PLA in this thesis stemmed from these finding that PLA lasted the longest under insonation at 37°C.

It was shown by K. Oum that the peptide-modified microbubbles could bind specifically to cells with integrin targets and that cellular binding was surface peptide density dependent with an optimal ratio of 0.5 Gly-Arg-Gly-Asp-Ser (GRGDS) peptide groups: total PLA carboxyl groups. GRGDS peptide modified microcapsules bound significantly (p<0.05) more to human breast cancer cells (MDA-MB-231) than unmodified PLA microcapsules in vitro [30].

The goals of this thesis are to enhance the targeting/treatment abilities of the above described polymeric ultrasound contrast agent by (1) functionalization of its surface both chemically and morphologically for cancer targeting, (2) bioconjugation of signal-inducing ligands and (3) targeting to novel receptors in ovarian cancer.

2.4 Targeting Microbubbles for Molecular Imaging

Many efforts are now being devoted to designing microbubbles that seek the molecular signature of diseased tissues, making them detectable by ultrasound [3, 5, 6]. Site-targeted microbubbles are expected to provide higher sensitivity and specificity than standard blood pool ultrasound contrast agents, allowing earlier and safe assessment of pathology. Because microbubbles normally do not escape the circulation, binding to targets is essentially restricted to pathologies that express specific ligands within the vascular lumen, typically endothelial cells, leukocytes and thrombi. Targeting of activated endothelial cells may be particularly useful for signaling thrombi, areas of inflammation, atherosclerotic plaques and angiogenesis in solid tumors [31].
There are two strategies generally employed for targeting of microbubbles to pathology; passive and active targeting.

### 2.4.1 Passive Targeting

Passive, or non-specific targeting of contrast agent relies on size and shell characteristics to preferentially produce accumulation in regions of interest. Examples of passive targeting include illumination of the lymphatic system [32, 33], accumulation in the reticuloendothelial system [34-36] within phagocytotic cells at inflammation sites [2], and within tumors.

Wisner et al. studied contrast agent enhancement in the lymphatic system, showing that subcutaneous injection of contrast media caused enhancement of 85% of sentinel lymph nodes in dogs [33]. Goldberg et al [32], examining whether lymphatic channels and sentinel lymph nodes with and without metastases can be detected after contrast agent injection in swine found it was not possible without contrast agent to determine lymph channels and sentinel nodes. After contrast agent injection, the lymph nodes became more echogenic as a result of passive contrast agent microbubble uptake. They found that lymphatic ultrasound with contrast agent administration can depict metastases within the sentinel lymph nodes.

Passive Targeting is also illustrated by the prolonged visualization of liver and spleen injury, including tumor metastases. As certain microbubbles are slowed down in the circulation of the liver and spleen or are taken up by phagocytic cells that are part of the reticuloendothelial system (RES), there is highlighting of normal tissue of the organs,
but not tumors [35] RES accumulation also allowed evaluation of hemorrhage within the liver and spleen in dogs [36].

Non-invasive assessment of inflammation is an important goal. Both albumin- and lipid-coated PFC microbubbles have been found to be retained in inflamed tissue [2]. Phagocytosed microbubbles remained acoustically active well after the blood pool concentrations had become negligible, providing a means of imaging inflammation and monitoring treatment [37]. Phagocytosed microbubbles are less easily destroyed by ultrasound than free microbubbles [37].

2.4.2 Targeting via EPR effect

It is a possibility that populations of the microbubbles may be able to extravasate into tissue from the large pores of leaky tumor vasculature. When aggregates of tumors achieve a diameter of 1-2mm they require neovasculature to supply nutrients. These vessels have an irregular, incomplete structure that includes pores. Yuan et al. measured the size of tumor vessel pores in LS174T human colon adenocarcinoma implanted in the dorsal skin chambers in immunodeficient mice [38]. They showed that tumor vasculature pores could be as large as 0.4 microns in diameter. Researchers, using techniques like electron microscopy, have identified structural abnormalities in the endothelium of tumor vasculature [39, 40]. These abnormalities included cellular openings with a mean diameter of 1.7 microns (range, 0.3-4.7 microns) as well as transcellular holes with a mean diameter of 0.6 microns in mouse mammary carcinoma [41, 42].

If the microbubbles can escape the tumor vasculature, microbubble retention could be enhanced through the enhanced permeability and retention (EPR) effect. There
are many abnormal characteristics of solid tumors that influence EPR effect including active angiogenesis and high vessel density, unregulated production of vascular mediators that facilitate extravasation, defective vascular architectures, and impaired lymphatic clearance of macromolecules from interstitial tissue [42]. Evans blue dye revealed the presence of the EPR effect in rodent tumors after injection with Evans blue bound albumin (67kDa), producing a blue color in only tumor tissues [43]. The blue color was not seen in normal tissues, except those which surround the tumor or inflammation site.

2.4.3 Active Targeting

Active targeting refers to the attachment of bioactive adhesion ligands to cause the accumulation of contrast agents at a particular site. Ligands belong to various classes of molecules including monoclonal antibodies, polysaccharides and peptides that recognize disease antigens. Currently there is much research focused on active targeting of inflammation [2], thrombus [44, 45] and angiogenesis [6, 7, 28].

Active targeting to the inflammation sites can be achieved using PFC microbubbles having antibodies to endothelial cell adhesion molecules that are expressed on the surface of endothelial cells activated during the inflammatory response [31]. Such molecules include intercellular adhesion molecule-1 (ICAM-1), P-selectin and certain integrins. F-butane microbubbles with anti-ICAM-1 monoclonal antibody covalently bound to a lipid component of the shell were reported to selectively attach to activated cultured endothelial cells in vitro, with minimal binding to non-activated cells [46]. In another approach, conjugation, via a biotin/avidin system, of antibodies against P-selectin
to \(F\)-butane/lipid microbubbles increased the retention of the microbubbles in inflamed tissue, allowing early detection of ischemia–reperfusion injury of the kidney in mice [47].

Detection of thrombus is another goal of active targeting. In vitro targeting and contrast enhancement of vascular thrombosis has been achieved with a lipid-coated PFC microbubble (MRX-408, ImaRx Pharmaceutical Corp., Tucson, AZ) having a peptide with an RGD sequence covalently attached via a PEG spacer to a lipid membrane component, dipalmitoylglycerol succinate [48]. The peptide binds selectively to the GPIIb/IIIa fibrinogen receptor that is expressed on the surface of activated platelets that attach to thrombi. \textit{In vivo} detection of intravascular and intracardiac thrombus was demonstrated in animal model using such microbubble [49].

A significant portion of this thesis is dedicated to targeting angiogenesis. Neovascularization in angiogenesis and arteriogenesis is important in wound healing, menses, tumor growth, invasion, and metastasis [17]. There is currently a lot of interest in influencing neovascularization for therapeutic reasons (especially in the case of anti-angiogenesis drugs) thereby creating a need for imaging of angiogenic markers in vivo for diagnostic purposes and measuring therapeutic effects [7, 50]. Targeted contrast enhanced ultrasound offers the possibility as well as providing information on microvascular perfusion and blood volume [17].

There are several studies targeting the integrin \(\alpha\nu\beta_3\), which is known to be required for angiogenesis [30, 51]. Targeting of \(\alpha\nu\beta_3\) has been accomplished with either monoclonal antibodies or peptides containing the RGD motif. Targeted ultrasound imaging with these microbubbles has been shown to detect neovascularization in matrigel plugs [7]. In a rat model of chronic ischemia targeting \(\alpha\nu\beta_3\) allowed the assessment of
baseline and growth factor induced angiogenesis [52]. Targeting of $\alpha \nu \beta 3$ has also been shown to detect tumor induced angiogenesis [6].

### 2.5 Surface modification of microbubbles

There are two general strategies for the attachment of the targeting ligand to the microbubble shell. In the first technique, the ligand is coupled to one of the shell-forming molecules (e.g. lipid) used as an anchor to retain the ligand prior to bubble fabrication. This approach works well for small organic molecule ligands, such as peptides, peptide mimetics, carbohydrates, hormones, and vitamins (such as biotin). Preparation of ligand-lipid conjugates has been used for ligand attachment to microbubbles either directly or via a PEG spacer arm [49, 53]. The main advantage of this approach is the ability to perform the synthesis and product purification in the fully controlled conditions of an organic chemistry setup, ensuring high product yield and purity. This method is common for lipid shelled contrast agents, but not used for polymeric shelled contrast agents, where harsh chemicals could destroy the ligand, or the ligand could become buried in the agent wall during synthesis.

When the bubble shell calls for covalent or noncovalent attachment of ligands to preformed microbubbles, as in polymeric contrast agents, a second general approach to ligand conjugation is used. This approach is most appropriate for the materials that are unstable in the conditions of continuous sonication, highspeed shear mixing, and resulting high temperatures, that are often associated with the preparation of microbubbles. Large protein molecules, such as antibodies or antibody fragments, are easily denatured in harsh conditions. To avoid denaturation of protein ligands, reactive
moieties that are stable enough to withstand bubble preparation conditions can be incorporated in the shell first (if reactive groups are not abundant naturally), functionalizing the surface, and then used for the attachment of the protein ligand to preformed bubbles.

2.5.1 Ligand Attachment to Microbubbles

The major methods of immobilizing a bioactive compound to a polymeric surface are adsorption via electrostatic interactions, ligand-receptor pairing (such as biotin-avidin), and covalent attachment as depicted in figure 2.2.

![Figure 2.2: Mechanisms of immobilizing bioactive compounds to polymer surfaces [54].](image)

Non-covalent adsorption is sometimes desirable, for example in certain drug delivery applications. The biotin avidin bond is the strongest non-covalent bond [55]. It is used by many researchers because of the number of biotinylated reagents available. There are drawbacks to using biotin avidin chemistry because endogenous biotin (vitamin H) competes in vivo for binding sites and streptavidin is an immunogenic foreign protein.
Also its cationic nature results in rapid complexing with anionic sites in the renal glomerular basement membrane [54]. Covalent attachment has advantages over both these methods by providing the most stable bond between ligand and surface. A covalent attachment has been shown to extend the half-life of a biomolecule, prevent its metabolism (desirable for agents anti-cancer agents that may be toxic once metabolized), or allow for continued bioactivity when used within medical devices [56, 57]. Covalent linking is the primary method of ligand attachment used in this thesis.

2.5.2 Functionalization of polymer surfaces

Several surface modification techniques have been developed to tailor surfaces with specific functional groups that would then be available linkage to a ligand. These include wet chemical modification [58], silane monolayer modification [59], UV irradiation [60], and ionized gas treatment (plasma) [61], coating [62] and physical incorporation [11]. Coating and physical incorporation were chosen for further study in this thesis. Plasma and UV irradiation are also options for introduction of carboxylic acid to PLGA surfaces, however these methods provide modification of only the top nanometers of a polymer surface, which may not be optimal for functional group enhancement of round microbubble surfaces.

2.5.3 Coating with carboxyl-carrying surfactants

Protocols for making PLGA microbubbles by emulsion techniques commonly use surfactants to stabilize the emulsion. Most commonly, poly(vinyl alcohol) (PVA) is used
as the stabilizer. When the emulsion is formed, the hydrophobic backbone of PVA partitions into the organic phase, while the hydrophilic hydroxyl side chains partition into the aqueous phase. Upon removal of the organic solvent, the surfactant is retained and the hydrophilic side chains remain exposed on the surface of the newly formed microspheres. This surface layer is suggested to be quite durable, remaining present even after repeated washing of the microbubbles, suggesting that the surfactant molecules are incorporated directly into the microbubble surface during fabrication [62]. Other functional groups aside from hydroxyls, however, would be more suitable for many surface modification chemistries.

Keegan et al. demonstrated an alternate stabilizer, poly(ethylene-alt-maleic acid) (PEMA) to produce microbubbles that allow the binding of ligands to the microbubble surface by carbodiimide chemistry [63]. The carboxylic acid side chains of PEMA, present at the microbubble surface, can be linked to primary amine groups of the desired ligand, forming stable amide bonds. They found that the capacity for ligand binding of PLGA/PEMA microspheres is substantially improved over those made of PLGA with acid end groups and PVA as stabilizer [63].

Keegan et al. reasoned that two features lead to the high density of functional carboxyl groups at the microbubbles surface. First, the carboxyl side chains are repeated along the entire length of the stabilizer molecule, providing a high density of functional groups as compared to PLGA molecules that are functionalized only at the ends of the polymer chain, and secondly the stabilizer molecules partition at the interface of the organic and aqueous phases of the emulsion that is formed during microbubble production. The surfaces of the microbubbles form at this interface during the solvent
evaporation step, meaning that the functional groups are localized at the microbubble surface and not buried within the core (they evaluated solid PLGA microspheres) [62].

In a subsequent study the researchers demonstrated that PLGA/PEMA microspheres could be conjugated to a variety of protein ligands. They found that these targeted microspheres could be retained in agarose columns and showed adhesion to cell surface receptors in Caco-2 cells [63].

2.5.4 Physical incorporation of Lipid-PEG-Biotin conjugates

The primary strategy of targeting contrast agents to specific sites involves modifying the surface of the contrast agent with ligands that home-in to specific sites of the vasculature. These ligands can either be attached covalently or by use of the biotin-avidin interaction as described previously. Another approach has been to use physical incorporation of lipid-ligand conjugates: this approach has been primarily explored in the formation of lipid-based contrast agents, and more recently with solid polymer microspheres [11], but has not yet been extended to polymer-based contrast agents. The physical incorporation method may overcome the difficulties of other methods of functionalization that lead to limited density of functional groups and decreased targeting effects due to desorption of the ligand during particle erosion.

In the work of Fahmy et al., avidin–palmitate conjugates were incorporated on the surface of solid PLGA particles [11]. The conjugate naturally positioned itself at the material surface: the palmitic acid preferentially partitioned into the hydrophobic PLGA matrix, whereas the hydrophilic avidin head group was display in the hydrophilic external
environment, facilitating the attachment of biotinylated ligands to the surface of their solid microspheres.

In this thesis a similar strategy is employed, at the suggestion of Dr. Joyce Wong of Boston University, with the incorporation of 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Biotinyl(Polyethylene Glycol)2000 (DSPE-PEG-biotin) into the shell. Using this method, biotin-avidin chemistry can be employed to conjugate bioactive ligands. Additionally, there are benefits to having PEG on microbubbles. Using a hydrophilic spacer molecule such as PEG may shield a compound from denaturation and improve bioactivity [64].

2.5.5 Morphological surface modification

Bubble morphology may be another factor in addition to chemical modification of bubbles enabling retention. A recent study by Rychak et al. featured “wrinkled” P-selectin targeted microbubbles created by application of a positive hydrostatic pressure of 750 mm Hg to a dispersion of spherical microbubbles, resulting in the formation of outward-projected structures on the surface [8]. They hypothesized enhanced microbubble capture could be achieved with the wrinkled bubbles because microvilli bearing clustered adhesion molecules [65] are responsible for efficient capture of leukocytes to inflamed endothelium in vivo[66]. The outward-projected structures on the surface of the wrinkled microbubbles resemble neutrophil microvilli. They report that microbubble bound by a single antibody molecule located at the tip of a 0.5 μm wrinkle experiences a 55% lower bond force than a spherical particle in which the ligand is flush with the microbubble surface resulting in the stabilization of a captured particle [8].
They found that wrinkled microbubbles form sustained adhesion events with higher efficiency than spherical microbubbles in vitro. The adhesion of P-selectin targeted microbubbles was examined in a flow chamber, and the wrinkled microbubbles exhibited significantly (p < 0.05) greater sustained adhesion than spherical microbubbles [8]. In addition, they found that wrinkled microbubbles exhibited enhanced adhesion in the inflamed mouse cremaster [8], in wild type mice (10 venules) approximately 14.5 ± 1.5 wrinkled microbubbles were retained versus 6 ± 1 retained for spherical microbubbles. A similar concept using rough surfaced PLGA microbubbles having outward projected structures is developed and evaluated in this thesis.

2.5.6 Immobilization of bioactive compounds

If a surface is functionalized or has functional groups naturally, it can be covalently decorated with targeting ligands. Common functional groups in bioconjugation chemistry include thiols, aldehydes, carboxylic acids, hydroxyls, and primary amines. The development of numerous cross linking agents, such as 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and (N-[ß-Maleimidopropionic acid] hydrazide) (BMPH), has expanded the array of usable conjugation chemistries. These chemistries are outlined in detail in a definitive text, Bioconjugate Techniques, by Hermanson [67]. Like the bioactive ligands themselves, crosslinkers have different optimum pH values, temperatures and solubilities and should be selected based on the conditions needed for the polymer/ligand conjugation. Crosslinkers can link the ligand directly to the surface (zero-length crosslinkers such as EDC) or they can introduce a spacer arm of several angstroms (such as PEG and BMPH) [67].
2.5.7 Surface Characterization

The type of analytical tools used for characterizing surface modified polymers depends on the anticipated nature of the modification and the specificity required.

Current non-spectral methods of characterization include dye assays using colorometric dyes (with a standard curve) that change color in the presence of a specific chemical group, protein assays such as the Bradford or Bicinchonic acid (BCA) assay which generate or change color when complexed with protein, assays for biological activity such as antigen binding, flow cytometry or cellular attachment analysis, and zeta potential testing which detects changes in charge on the surface of the polymer [54]. The research in this thesis utilizes protein assays, various biological assays and zeta potential testing for characterization of our modified polymers.

Current spectroscopic and microscopic analyses include X-ray photoelectron microscopy (XPS) which determines the atomic composition of a surfaces top several nanometers, time-of-flight secondary ion mass spectrometry (ToF-SIMS) to determine the type and quantity of ionizable chemical groups on a surfaces top nanometer, Fourier transform infrared spectroscopy (FTIR) which determines the chemical functionalities present in a sample using infrared radiation, and atomic force microscopy (AFM) and scanning electron microscopy (SEM) for surface morphology measurements. In this research FTIR and AFM characterization methods were applied, however SEM was found to be most useful for microscopic analysis of the microbubbles [54].
2.5.8 Challenges and Considerations of ligand conjugation

It is known that the activity of a bioactive ligand may change after immobilization onto a surface, most likely due to the differences between the bulk solution and surface microenvironmental conditions or alteration of the active site by distortion due to the chemical linkages. For example, enzymes linked to polycationic surfaces had their pH shifted to a more acidic value [68], and those on polyanionic surface shifted to a more alkaline value [69]. Also depending on the conjugation chemistry chosen, linking a bioactive ligand to a solid surface may introduce steric constraints. It is important to consider that ligand behaviors observed in measured bulk solution may not be representative of those occurring bound to the polymer surface.

When looking at the bioactivity of ligand:polymer conjugates its important to have the appropriate controls. Since surface functionalization is usually a multistep process, the surface charge and hydophilicity can change. Its not only important to compare the bioactivity of the ligand:polymer conjugate with the unmodified polymer, but also to compare the bioactivity of the surface modified polymer to which the ligand has not yet been attached; that is, adding a control of microbubbles that have gone through the functionalization or conjugation process without ligand addition. By including this process it can be identified whether a change in bioactivity in the microbubbles is due to the ligand or simply a change in microbubble surface chemistry. Also, in some applications it may be critical that the bond between ligand and polymer be covalent [54]. This can be confirmed by comparing samples made with and without the chemical crosslinker, ruling out simple adsorption.
It is important to optimize rather than just maximize the density of surface immobilized ligands. An example by Wheatley et al. [70] demonstrated that there is an optimal density of RGD ligands immobilized on the surface of polymer microbubbles for cellular attachment.

Lastly, a benefit of working with surface based chemistries is the relative ease of purification with processes such as wash steps to adequately rid the surface of unwanted physically adsorbed or electrostatically attached ligands. Wash steps must be included in conjugation procedures; samples made without wash steps may produce clouded results.

2.6 Cancer

Cancer is a disease characterized by the formation of abnormal tissue (neoplasm), and generally is a change in the way cells proliferate and differentiate. Tumors require the development of new blood vessels, known as angiogenesis, to provide the necessary nutrients for their growth.

Surgery, radiation therapy and chemotherapy are the current treatment options for cancer [71]. Local treatments, radiation therapy and surgery are used when the tumor has not metastasized. Once it is metastasized, chemotherapy, a systemic treatment, is also required. Anti-cancer agents affect different parts of the cell cycle and have side effects. Systemic treatment can affect all tissues, both normal and malignant [71].

Ultrasound contrast agents have been used to image and diagnose cancer, recently Forsberg et al. compared various modes of ultrasound with and without contrast agent to mammography for the diagnosis of breast cancer in 14 women. They suggested subharmonic imaging (which utilizes contrast agent) may increase the ability to diagnose
breast cancer [72]. The same group has also found that tumor neovascularity measured with contrast enhanced ultrasound (using Optison (GE Healthcare, Princeton, NJ) and Sonazoid (GE Healthcare, Princeton, NJ)) is significantly correlated with histological staining of vascular endothelial growth factor (VEGF), endothelial cells (CD31), and cyclooxygenase-2 (COX-2) in mice with melanoma tumors [50].

2.6.1 Angiogenesis

Angiogenesis is the growth of new blood vessels and plays a key role in fetal development, wound repair, menses, inflammation, rheumatoid arthritis, and cancer [4]. There has been much interest in developing therapeutic agents that inhibit angiogenesis. The detection, diagnosis, and monitoring of angiogenesis is also an application of ultrasound [50].

Angiogenesis is characterized by the invasion, migration, and proliferation of smooth muscle and endothelial cells [4]. Angiogenesis is essential for the growth of tumors past a few millimeters; if a tumor does not have vasculature to feed its growth, it will not develop into a larger tumor. If the tumor does begin to have vasculature develop around it, the growth and metastatic potential of the tumor increases [73].

The development of vasculature is initiated by the tumor, which releases factors that increase blood vessel growth and development. Next, the surrounding parental vessels vasodilate and the basement membrane of the vessels begins to degrade via proteolytic enzymes. Endothelial cells begin to migrate into the extracellular space and proliferate to form a leading edge. The endothelial cells form tubules with lumen and begin to synthesize basement membrane. Finally, the tubules anastamose and recruit
smooth muscle cells and pericytes to complete the vessel structure near or surrounding the tumor [73]. Due to rapid formation of new blood vessels into tumors, the new vascular is irregular and leaky. It is thought that the porous nature of the new vessels might aid metastasis, and also lead to the collection of fibrin in the extracellular matrix to promote angiogenesis [73].

2.6.2 Integrins

Integrins are transmembrane receptor proteins, and are the main way cells bind and respond to the extracellular matrix. Integrins also influence many aspects of cell behavior such as morphology, cell adhesion, cell migration, and cellular proliferation and differentiation [74]. Many cell-signaling cascades, such as that for continued proliferation or death of a cell, begin with a ligand binding to integrin receptors. Thus, cell surface integrins represent a good target to prevent signaling cascades. As a tumor grows and develops it requires vasculature and it is theorized that chemotherapeutic agents bound to the growth factor receptors could result in localized cytotoxic effects [12]. Therefore, if these receptors can be targeted and blocked, angiogenesis may be effected and thus, the growth of the developing tumor can be reduced or even stopped.

The $\alpha_v\beta_3$ integrin is implicated in many pathological processes, such as osteoporosis, misregulated angiogenesis (e.g., rheumatoid arthritis or retinopathy) as well as tumor growth, and tumor metastasis [75]. This receptor is highly expressed on many tumor cells such as neuroblastomas, carcinomas of the lung, the breast, the prostate and the bladder [75]. It has been shown that the $\alpha_v\beta_3$ integrin is important during tumor-induced angiogenesis [4]. The phenomenon consists of the involved endothelial cells
expressing $\alpha_\nu\beta_3$ to adhere on the extracellular matrix during migration toward the tumor. It has been shown that the inhibition of the $\alpha_\nu\beta_3$ integrin with monoclonal antibodies or peptidic antagonists results in a drastic reduction of neovascularization around the tumor, leading to the starvation of the tumor [76].

The integrin $\alpha_\nu\beta_3$ binds to a wide variety of extracellular matrix proteins [51]. A common receptor recognition motif of these matrix proteins is the peptide sequence Arg-Gly-Asp (RGD).

### 2.6.3 RGD

The RGD sequence is the most often employed peptide sequence for stimulated cell adhesion on synthetic surfaces as well as surface targeting [77]. The tripeptide motif RGD was identified 18 years ago by Pierschbacher and Rouslahti as a minimal essential cell adhesion peptide sequence in fibronectin [78]. Since then, cell adhesive RGD sites were identified in many other ECM proteins, including vitronectin, fibrinogen, von Willebrand factor, collagen, laminin, osteopontin, tenasin and bone sialoprotein [78]. The RGD sequence is a non-specific binding sequence, however it can be made specific by the addition of extra amino acids. This binding specificity is dictated by many things including the cyclization of the peptide sequences and the conformation of disulfide bonds. About half of the 24 integrins have been shown to bind to ECM molecules in a RGD dependent manner [78]. In cancer biology, the main receptors of interest for angiogenesis targeting are cell surface integrins $\alpha_\nu\beta_3$ and $\alpha_\nu\beta_5$. 
A lock and key relationship has been identified between integrins overactive in angiogenesis, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, and the RGD peptide. Consequently, there have been attempts made to target angiogenesis using the RGD peptide sequence [77].

2.7 Apoptosis

Apoptosis, also known as programmed cell death, has critical roles in the development, homeostasis maintenance and host defense in multicellular organisms. It is characterized by DNA fragmentation, chromatin condensation, membrane blebbing and cell shrinkage [79]. Apoptosis occurs through extrinsic and intrinsic apoptotic pathways. The final step involved in each pathway is a caspase cascade, which cleaves regulatory and structural molecules, leading to cell death.

The extrinsic pathway of apoptosis is also known as the cytoplasmic or death receptor (DR) pathway. Extracellular agents such as tumor necrosis factor-related apoptosis inducing ligand (TRAIL), Fas ligand (FasL) and Tumor Necrosis Factor (TNF) induce the extrinsic pathway through the activation of specific DRs distributed on the plasma membrane. Specific DRs become activated by their specific ligands and as a result they form dimer/trimer complexes. Within seconds the activated DRs recruit adaptor molecules, which further transmit the death signals to DISC, where the 2 adaptor proteins FADD and TNF receptor-associated death domain have been shown to recruit caspase-8 or 10 to activate the extrinsic apoptotic pathway [79]. The intrinsic mechanism of apoptosis acts by perturbation of the mitochondrial membrane, and relies on the release of key apoptogenic factors such as cytochrome c and apoptosis-inducing factor [80]. These intrinsic and extrinsic pathways are depicted in figure 2.3 taken from [80].
Figure 2.3: Apoptosis: the ‘extrinsic’ and ‘intrinsic’ pathways to caspase activation. Two major apoptotic pathways are illustrated: one activated via death receptor activation (‘extrinsic’) and the other by stress-inducing stimuli (‘intrinsic’). Triggering of cell surface death receptors of the tumour necrosis factor (TNF) receptor superfamily, including CD95 and TNF-related apoptosis-inducing ligand (TRAIL)-R1/-R2, results in rapid activation of the initiator caspase 8 after its recruitment to a trimerized receptor-ligand complex (DISC) through the adaptor molecule Fas-associated death domain protein (FADD). In the intrinsic pathway, stress-induced apoptosis results in perturbation of mitochondria and the ensuing release of proteins, such as cytochrome c, from the inter-mitochondrial membrane space. The release of cytochrome c, from mitochondria is regulated in part by Bcl2 family members, with anti-apoptotic (Bcl2/ Bcl-XL/Mcl1) and proapoptotic (Bax, Bak and tBid) members inhibiting or promoting the release, respectively. Once released, cytochrome c binds to apoptotic protease-activating factor 1 (Apaf1), which results in formation of the Apaf1–caspase 9 apoptosome complex and activation of the initiator caspase 9. The activated initiator caspases 8 and 9 then activate the effector caspases 3, 6 and 7, which are responsible for the cleavage of important cellular substrates resulting in the biochemical and morphological changes associated with the apoptotic phenotype [80].

There have been several studies investigating the effect of ultrasound and ultrasound contrast agents on cellular apoptosis [81, 82].

Feril et al. [81] determined the effects of ultrasound contrast agents on ultrasound induced apoptosis and cell lysis in human lymphoma U937 cells. Cells in suspension
were exposed to 1 MHz continuous waves ultrasound for 1 min at various intensities with or without commercial contrast agents. Apoptosis was detected by flow cytometry. They determined Optison (GE Healthcare, Princeton, NJ) was effective in augmenting the ultrasound induced cell death, indicating that cavitation plays a role in the augmented effects. A similar study by Honda et al. [82] evaluating the effect of free radial formation during cavitation, found that ultrasound induced apoptosis of U937 cells, which is mitochondria-caspase dependent, was linked to cavitation. When the cells were insonated in the presence of Levovist; synergistic enhancement of secondary necrosis was observed at concentrations of more than 20 mg/mL [82]. Their results suggest that contrast agents; acting as cavitation nuclei, can enhance ultrasound-induced apoptosis/secondary necrosis due to an increase in cell membrane damage.

These findings support the concept that ultrasound contrast agents have the potential to be adjuncts in cancer therapy with, in particular those therapies which rely on apoptosis induction such as the TRAIL therapy.

2.7.1 TRAIL and TRAIL receptors

TRAIL is a ligand, which can induce apoptosis in tumor cells after binding to death inducing receptors TRAIL-R1 and R2, also known as DR4 and DR5. Activation of DR4 and DR5 lead to the induction of death inducing signals from the plasma membrane to the cytoplasm. It has been shown that TRAIL induces cell death in various cancer cells [83] such as breast cancer [84] and glioma [85]. TRAIL has a sequence homology similar to that of TNF and FasL [86]. The advantage of TRAIL over TNF and FasL is the fact that it is non toxic to normal cells [86]. The disadvantage of TRAIL is that it has no
specificity to tumor tissue alone. Binding to normal tissues, which also have TRAIL receptors results in a lower availability of TRAIL to tumor cells [87].

Ashkenazi et al. outlines why TRAIL is non-toxic to normal tissues, though TRAIL receptors are present [88]. Protection of normal tissues is based on a set of decoy receptors (DcRs), which compete with DR4 and DR5 for binding to TRAIL. DcR1 is a cell surface protein resembling DR4 and DR5, but lacks a cytoplasmic tail. DcR2 is another receptor that resembles DR4 and DR5, but as a truncated cytoplasmic death domain. Both DcR1 and DcR2 function as decoys that prevent TRAIL from binding to its death receptors. DR4 and DR5 are expressed in normal tissue and in many types of tumor cells, whereas DcR1 and DcR2 are expressed frequently in normal cells but infrequently in tumor cells [88]. This differential expression of death and decoy receptors might enable TRAIL to induce apoptosis in tumors while sparing normal cells.

The unique capacity of TRAIL has been exploited by pharmaceutical companies and various versions of TRAIL are currently being tested in clinical studies. Soluble TRAIL has entered phase I clinical trials (Genentech in collaboration with Amgen/Immunex). Also being tested are anti-DR4 and anti-DR5 monoclonal antibodies. Mapatumumab (Human Genome Sciences), an anti-human-DR4 antibody is currently enrolled in three phase II clinical trials, and at least five DR5-specific agonistic antibodies, including Lexatumumab are in late preclinical or in early clinical studies [79].

2.7.2 TRAIL and Velcade Induced Apoptosis

The ubiquitin-proteasome system is main degradation pathway for proteins involved in the regulation of cell survival, proliferation, apoptosis, and other critical cellular
functions [89]. It has recently been shown that the proteasome inhibitor Velcade, also known as PS-341 or Bortezomib, (Millenium Pharmaceuticals, Cambridge, MA) can interfere with tumor cell regulation, blocking proliferation and causing cell death [90-92].

Velcade induces apoptosis via an intrinsic cell death pathway, which relies on the release of proapoptotic proteins from mitochondria such as a second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low pI (SMAC/Diablo) and cytochrome c [89]. TRAIL induces apoptosis via an extrinsic pathway through interaction with death receptors/ direct activation of caspases [89]. The intrinsic and extrinsic apoptosis signaling pathways communicate with each other. Caspase-8 has been shown to cleave the proapoptotic Bcl-2 family member Bid. The cleavage of Bid by caspase-8 and the translocation of truncated Bid to the mitochondria to promote cytochrome c release provides a plausible mechanistic link between the extrinsic and intrinsic pathways [93]. This apparently amplifies the apoptotic signal following death receptor activation [94]. Conversely, activators of the intrinsic pathway can sensitize cells to extrinsic death ligands.

Recently, there have been many studies confirming a synergistic apoptotic effect of treating cancer cells with a combination of Velcade and TRAIL. It has been demonstrated in human pancreatic [91], human bladder [92], human prostate [92], renal [90], colon [90] and breast [90] cancer cell lines. A recent study by Conticello et al. [95] showed that the cytotoxic effect of Velcade in thyroid carcinoma cells was potentiated through combination treatment with TRAIL, predictably allowing the clinical usage of lower doses of both compounds. They also showed that because both intrinsic and
extrinsic cell death pathways are activated with TRAIL/Velcade combination treatment, mechanisms of death resistance could be overcome [95].

2.8 Ovarian Cancer

Ovarian cancer (OC) is the second most common and most lethal gynecologic malignancy in the USA. Epithelial OC comprises the majority of malignant ovarian tumors [96]. Over 70% of women with OC are diagnosed with advanced stage disease, with a 5 year survival rate 30%. This 5 year survival rate is 90% when diagnosed early (disease confined to the ovaries), but only 25% of cases are found in this early stage. Incidence rates remain high and mortality rates are virtually unchanged over the last 30 years [96]. Clinicians screen for OC by measuring CA-125 levels in the blood (elevated in most women with OC) in combination with transvaginal ultrasound.

2.8.1 CA-125 in as a Target Ovarian Cancer

CA-125 is a biomarker for ovarian cancer. The discovery of OC-125, and antibody that recognizes CA-125 was discovered by Bast and his colleagues in 1981 [97]. CA-125 is expressed as a membrane-bound protein at the surface of cells or is released in soluble form into bodily fluids [96].

Since OC occurs in the peritoneal cavity, the regional administration of therapy is possible. Therapies for epithelial OC are being developed and are in clinical trials. Oregovomab (Unither Pharmaceuticals, MA) is an immunotherapeutic agent for patients with ovarian adenocarcinomas expressing CA-125. Oregovomab operates with a unique
mechanism, the antibody forms a complex with serum soluble CA-125 and this complex binds to antigen presenting cells such as macrophages and dendritic cells. The complex triggers induction of CA-125 specific immune responses. Oregovomab was shown to increase the survival time of epithelial OC patients [98].

Targeted therapies with anti-CA-125 antibodies conjugated to anti-cancer drugs are currently under study in animal models. A recent publication compares the toxicity and efficacy of two such antibodies and reported efficacy in vitro and in vivo [99]. Efforts have been undertaken to develop anti-CA-125 antibodies specific for the cell-associated form of the antigen, which is of particular interest for targeted therapy [100].

CA-125 is a biomarker with a broad range of utility; risk, early detection, diagnosis, prognosis, monitoring and therapy. It also represents an attractive therapeutic target and therefore is studied as a target in this thesis.
3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Polymers

Poly (D,L-lactic-co-glycolic acid), Medisorb 50:50 3.5A lot # 0098-441, Medisorb 50:50 5A lot # 01-112-66, Medisorb 50:50 6A lot # 01-141-108, and Medisorb 100:0 6E lot # W2297-587) was purchased from Lakeshore Biomaterials, Birmingham, AL. The polymers designated E were endcapped with lauryl ester and those designated A were uncapped. The molecular weights and inherent viscosities of the polymers were as reported in Table 3.1.

Table 3.1: Polymer formulation molecular weight and inherent viscosity

<table>
<thead>
<tr>
<th>Polymer formulation (lactic acid: glycolic acid) + designation</th>
<th>Molecular weight (kDa)</th>
<th>Inherent Viscosity (dL/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:50_3.5A</td>
<td>51</td>
<td>0.4</td>
</tr>
<tr>
<td>50:50_5A</td>
<td>76</td>
<td>0.51</td>
</tr>
<tr>
<td>50:50_6A</td>
<td>91</td>
<td>0.6</td>
</tr>
<tr>
<td>100:0_6E</td>
<td>83</td>
<td>0.55</td>
</tr>
</tbody>
</table>

3.1.2 Targeting Ligands and Chemical Crosslinkers

The GRGDS (Gly-Arg-Gly-Asp-Ser) peptide sequence (Mw 490.5Da), human recombinant Tumor Necrosis Factor-related Apoptosis Inducing Ligand (TRAIL) (cat.# T9701), EDC (1-Ethyl-3-13-Dimethylamino-Propyl) carbodiimide, and NHS (N-
hydroxysuccinimide) was purchased from Sigma-Aldrich Chemical Co., St. Louis, MO. Murine anti-CA-125 monoclonal antibody was purchased from QED Bioscience, San Diego, CA (cat. #3330). 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Biotinyl(Polyethylene Glycol)2000 (DSPE-PEG-biotin) was purchased from Avanti Polar Lipids, Alabaster, AL (cat. # 880129). (N-[ß-Maleimidopropionic acid] hydrazide) (BMPH) was purchased from Pierce, Rockford, IL. Scrambled peptide used as a control, GRDGS (Gly-Arg-Asp-Gly-Ser) was from Biosynthesis Inc., Lewisville, TX.

3.1.3 Antibody Fragmentation and Detection Materials

Easy-Titer antibody detection kit was purchased from Pierce, Rockford, IL. Antibody fragmentation kits were purchased from Fisher Scientific, Springfield, NC. Alexa Fluor® 488 carboxylic acid, succinimidyl ester was purchased from Invitrogen, Carlsbad, California. Human Ovarian Cancer Antigen CA-125 was purchased from Meridian Life Science, Saco, ME (lot#1D09208).

3.1.4 Cell Lines and Cell Culture Materials

Human breast adenocarcinoma cells (MDA-MB-231) and human ovarian cancer cells (NIH:OVCAR-3) and human melanoma cells (A2058) were purchased from ATCC, Manassas, VA. Bovine insulin and penicillin/streptomycin antibiotic were purchased from Sigma Chemical Co., St. Louis, MO. RPMI-1640 medium (containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate) and Eagle's Minimum Essential Medium (EMEM) (containing
Earles Balanced Salt Solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate) were purchased from ATCC Manassas, VA. 0.25% Trypsin EDTA and fetal bovine serum (FBS) were purchased from Fisher Scientific, Springfield, NJ.

3.1.5 Fluorescent Probes and Cellular Assay Materials

Vybrant Apoptosis Assay Kit #2 containing alexafluor 488 -annexin V and propidium iodide, Hoechst blue 33342 nucleic acid stain, a cationic green dye 3,3’-diethyleneoxacarbocyanine iodide DiO(C$_2$)$_3$, and 5-aminoacetamido fluorescein were purchased from Invitrogen, Carlsbad, CA. Guava Nexin Reagent Kit for apoptosis detection was purchased from Guava Technologies, Hayward, CA. Nile Red was purchased from Sigma, St. Louis, MO.

3.1.6 Other Chemicals

Poly (vinyl alcohol) (PVA), 88% mole hydrolyzed, with a $M_w$ of 25kDa and Poly (ethylene maleic anhydride) (PEMA), with a $M_w$ of 100kDa was purchased from Polysciences, Inc., Warrington, PA. PVA, 88% mole hydrolyzed, with a $M_w$ of 133kDa, (2-[N-morpholino]ethanesulfonic acid) sodium salt (MES), thioglycolic acid, laboratory grade (1R)-(+) -camphor and laboratory grade ammonium carbamate was purchased from Sigma-Aldrich Chemical Co., St. Louis, MO. Pharmaceutical grade ammonium carbonate was purchased from J.T. Baker, Phillipsburg, NJ. Sodium chloride, methylene chloride, acetone, hexane, methanol, and isopropyl alcohol were all reagent grade and
purchased from Fisher Scientific, Springfield, NJ. Velcade (PS-341) was graciously supplied by Millenium Pharmaceuticals Cambridge, MA.

3.2 Contrast Agent Preparation and Characterization

3.2.1 Microcapsule Fabrication

Polymer microbubbles were prepared by a modified double emulsion process [70]. A detailed description of the procedure is given in Appendix A. Microbubbles were prepared by adapting the double emulsion (W/O)/W solvent evaporation process using ammonium carbamate as a sublimable core. PLGA or PLA (0.5 g) was dissolved in 10 ml of methylene chloride. To generate the first (W/O) emulsion, 1.0 ml of 4% ammonium carbonate solution was added to the polymer solution and probe sonicated at 115 Watts for 30 seconds. The (W/O) emulsion was then poured into a 5% PVA (at 4°C) solution and homogenized for 5 minutes at 9,500 rpm. The double emulsion (W/O)/W was then poured into a 2% isopropyl alcohol solution and stirred for 1 hour with a magnetic stirrer on a magnetic stir plate at a speed fast enough to create a vortex that spanned the entire beaker diameter. The microcapsules were collected by centrifugation for 5 minutes at 5000 times g force, washed three times with hexane, then once with deionized water and lyophilized, using a Virtis Benchtop freeze dryer, to remove the ammonium carbamate core.
3.2.2 Variations to fabrication procedure

3.2.2.1 Microbubbles with PEMA surfactant

Poly(ethylene-alt-maleic acid) was synthesized via an acid/base ring-opening of poly (ethylene maleic anhydride) (PEMA). Poly (ethylene maleic anhydride) was slowly added to a 1M NaOH solution, stirred overnight, corrected to pH 6.5 with HCl and filtered. 1, 3, 5 and 7% w/v solutions of PEMA were created. Blends of PEMA:PVA were created by blending the two surfactants at PEMA:PVA at weight ratios of 0:100, 25:75, 50:50, 75:25 and 100:0, using 5% w/v PEMA and PVA. During the second emulsion of the fabrication process the 50ml of PVA surfactant was replaced with 50ml of a PEMA solution or 50ml of a blend of PEMA:PVA. The fabrication process then proceeded as described in Appendix A.

3.2.2.2 Physical incorporation of DSPE-PEG-Biotin

For microbubbles with physically incorporated 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Biotinyl(Polyethylene Glycol)2000] (DSPE-PEG-Biotin), DSPE-PEG-Biotin was added the mixture of PLA and organic solvent, prior to the first emulsion, in a 1:100 ratio of lipid:polymer. The DSPE-PEG-Biotin was dispensed into a 50ml beaker with a glass syringe, the solution was dried under argon gas and desiccated under vacuum for 3 hours. With the DSPE-PEG-Biotin dried to the glass beaker, 10ml of methylene chloride solvent was added into the beaker. Next polymer was added and the fabrication process proceeded as described in Appendix A.
3.2.2.3 Nile Red Preparations

To physically incorporate the fluorescent probe Nile Red into the shells of the microbubbles, Nile Red was added at a 1:100 w/w ratio to the polymer in the organic phase prior to the first emulsion. This procedure was adapted from [101]. The fabrication process proceeded as described in Appendix A.

3.2.2.4 Shape studies

For microbubble studies examining the effect of surface morphology, parameters were varied in the fabrication process in order to create a range of microbubbles with varied morphology but made from the same class of polymer. The parameters altered included type of polymer (free acid or end capped), water to oil ratio in first emulsion (1:10 and 1:20), surfactant molecular weight, and amounts of porogens. Other parameters included stirplate speed (800rpm to 400rpm) used during solvent evaporation.

Fabrication began with dissolving 0.5g of 50:50 3.5A, 50:50 5A or 50:50 6A PLGA 50:50 polymer in either 10ml or 20ml of methylene chloride solvent. To this solution either 25mg or 50mg of camphor was added. Next, 1ml of a 20mg/ml or 40mg/ml solution of ammonium carbonate was added. The solution was sonicated for 30seconds in an ice bath in pulse mode 3seconds on, 1 second off. This first emulsion was then poured into a 5% w/v surfactant solution made with either 133kDa M_w PVA or 25kDa M_w PVA and homogenized at 10,500 rpm for 5 minutes. 100ml of 2% IPA solution was then added and the solution stirred on a stirplate at a speed of 400rpm for
1.5 hours. Microbubbles were then collected via centrifugation, washed with hexane 3x, frozen and lyophilized for 48 hours. Details of the microbubble fabrication procedure are described in Appendix A.

3.3 Conjugation methods

3.3.1 Peptide Conjugation via Carbodiimide Linkage

Freeze-dried microbubbles (50mg) were suspended in 2ml of buffer (0.1M MES, pH 5.2) in a centrifuge tube. To this 0.8mg of EDC (2mM) and 2.2mg of NHS (5mM) was added. The solutions were shaken on an end-over-end shaker for 30 mins for EDC activation of microbubble surfaces. The solutions were then centrifuged and washed 3 times to remove excess EDC. GRGDS peptide was then added at a 0.5:1 molar ratio of peptide to total polymer carboxyl groups. Samples were shaken end-over-end for 3 hours, centrifuged and washed 3 times with buffer (PBS, pH 7.0), frozen and lyophilized for 48 hours. Detailed descriptions of the procedures are given in Appendix B, part A. The general reaction is as follows, figure 3.1:
3.3.2 Fluorescent Marker Conjugation

5-aminoacetamedio fluorescein was conjugated to the bubbles via the carbodiimide chemistry described previously. After the 30min EDC surface activation step, 5-aminoacetamido was then added at a 1:0.5 molar ratio of peptide to total polymer carboxyl groups. Samples were shaken end-over-end for 3 hours, centrifuged and washed 3 times, frozen and lyophilized for 48 hours. Amount of conjugated marker was quantified via flow cytometry.

3.3.3 TRAIL conjugation via maleimide chemistry

To conjugate TRAIL to the microbubbles using maleimide chemistry (figure 3.2), 60mg of freeze-dried microbubbles were suspended in 2-(N-morpholino)ethanesulfonic acid (MES buffer) (4ml, 50mM, pH 5.2) and shaken to create a suspension. Separately,
1ml of deionized water was added to 14.86mg of N-[β-Maleimidopropionic acid] hydrazide (BMPH) for a 25mM BMPH solution. 1ml of deionized water was added to 19.17mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) for a 50 mM EDC solution. 1 ml of deionized water was added to 12mg of N-hydroxysulfosuccinimide (NHS). To microbubble suspension was added 1ml of the BMPH, EDC and NHS solutions. The resulting suspension was shaken on end-over-end for 30 minutes. Microbubbles were collected via centrifugation, washed to remove unreacted EDC, and pellet resuspended in phosphate buffer solution (PBS, 4ml). To this solution was added Tumor necrosis (TNF)-related apoptosis-inducing ligand (1.2μg). The suspension was shaken end-over-end for 1.5 hours. Resulting TRAIL conjugated microspheres were collected via centrifugation, washed 3 times to remove unbound TRAIL, flash frozen, and lyophilized for 48 hrs. For a control microbubble group with only physically adsorbed TRAIL the same process was used, but the chemical crosslinkers EDC and BMPH were omitted. This conjugation strategy is outlined in detail in Appendix B, part B.

Figure 3.2: Maleimide coupling reaction. R=TRAIL.
3.3.4 Antibody Conjugation via Maleimide or Carbodiimide Chemistry

To link antibodies to the microbubbles via a maleimide reaction, free thiols in the hinge region of the antibody were created by reduction or reduction/digestion. Reduction/digestion procedures were followed according to manufacturer’s guidelines (Antibody fragmentation kit, Pierce, Rockford, IL) and are roughly as described in figure 6.1.

Fragmented antibodies were conjugated to the surface of the microbubbles via a maleimide reaction. 60mg of bubbles were added to 4ml (5.2 pH) MES buffer. 14.86mg BMPH, 19.17mg of EDC and 12ml NHS were added to 1ml of deionized water. Equal amounts of each solution (1ml of BMPH, EDC and NHS) were added to the bubble/MES buffer solution and end-over-end mixed for 30 mins for activation of groups. Solutions were centrifuged, resuspended and washed. After the final wash bubbles were resuspended in 4ml of PBS buffer and fragmented antibody solutions added. After end-over-end shaking for 3hrs solutions were centrifuged and bubbles washed 3x, flash frozen and lyophilized.

To link full anti-CA-125 antibodies to microbubbles a carbodiimide reaction was utilized. Freeze-dried microbubbles (50mg) were suspended in 2ml of buffer (0.1M MES, 0.3M NaCl, pH 6.0) in a centrifuge tube. To this 0.8mg of EDC (2mM) and 2.2mg of NHS (5mM) was added. The solutions were shaken on an end-over-end shaker for 30 mins for EDC activation of microbubble surfaces. The solutions were then centrifuged and washed 3 times to remove excess EDC. Anti-CA-125 antibody (100μg) was added.
Samples were shaken end-over-end for 3 hours, centrifuged and washed 3 times with buffer (PBS, pH 7.0), frozen and lyophilized for 48 hours.

3.4 Contrast Agent Characterization

3.4.1 Dose and Time Response

A detailed description of the procedure is given in Appendix C. Briefly, the freeze-dried microbubbles were weighed into a custom-made plexi-glass vessel equipped with an acoustic window. 100 ml of 37°C phosphate buffered saline (PBS) solution was added. The suspension was then placed in the acoustic set-up as shown in Fig. 3.3. The 5MHz single element ultrasound transducer (model V-309, center frequency 5.45 MHz, spectrum range 2.95-7.95 MHz, 0.75” diameter) used for acoustic enhancement was obtained from Panametrics (Waltham, MA). This frequency was chosen because it was shown to give the maximum enhancement for microbubbles previously developed in our laboratory [103]. The pulser/receiver (model 5072 PR, Panametrics, Inc, Waltham, MA) was used to pulse the transducer was set with the following parameters: pulse repetition frequency=100Hz, energy level=1 (~13 joules), damping level=3, and gain=40dB. These settings resulted in a peak pressure of 816 kPa at the focus (45 mm) of the 5MHz, 0.50” element diameter transducer [20]. The acoustic enhancement was analyzed using LabVIEW programs. Cumulative dose response curves were constructed for doses in the range of 0.015 mg/ml – 0.03 mg/ml at 37°C. Dose responses were performed cumulatively because dose response tests generally took less than 5 minutes to perform, and the CA was stable (as determined by time response) over this time period. After establishing the dose response curves, time response curves were performed for the
lowest steady state dose. Time response curves record data points once a minute for 15 minutes at 37°C.

Figure 3.3: *In vitro* acoustic set up. The 5MHz transducer was inserted in a deionized water bath and focused through an acoustic window of a 100ml custom made vessel located at a distance of 45 mm. A pulser/receiver was used to pulse the transducers at a pulse repetition frequency of 100 Hz. The received signals were amplified to 40 dB and fed to the digital oscilloscope, which is connected to the computer with LabView applications.

3.4.2 Environmental Scanning Electron Microscopy

The environmental scanning electron microscope used to capture images was a Phillips XL-30. Briefly, the samples were secured using carbon tape and gold-palladium sputter coated for 100 seconds prior to imaging. The samples were placed in the microscope in a round sample holder and put under vacuum. The imaging distance was approximately 10mm on the z-axis. After focusing, photos were taken at multiple magnifications and saved as high resolution TIF files.
3.4.3 Particle Sizing

Particle size was measured on a Malvern Nano ZS Particle Sizer (Worcestershire, UK) in order to characterize the mean size and distribution of the agent. Roughly one mg of dry sample was suspended in 1 ml of deionized water. Each sample was measured three times and the number percent results averaged.

Particles size and count were also measured using a Coulter Counter. Particles were diluted and suspended in electrolyte solution. The particles were drawn through an aperture in the glass vessel on the machine. Measurements of size and particle count were repeated three times and results averaged. Data is described in Appendix D.

3.4.4 Antibody Detection

Quantification of antibody conjugation was assessed indirectly using an Easy-Titer IgG assay (Pierce Biotechnology, Rockford, IL). The assay gives concentration of protein by absorbency. The assay procedure uses monodispersed polystyrene beads coated with anti IgG antibodies and absorb light at 340 and 405 nm. When beads are mixed with solution containing IgG they aggregate causing decreased adsorption of light. The decrease in absorption is proportional to IgG concentration. Standard curves were created by serially diluting anti-CA-125 antibody into 96 well plates, applying the Easy-Titer beads, shaking and measuring at 340/405nm. Samples were measured by collecting supernatant from the 3 washes during antibody conjugation, concentrating the solution in Amicon ultra filtraton tubes with 30k mW cutoff (Millipore, Billerica, MA), diluting
serially into 96 well plates, adding well-mixed Easy-Titer beads, shaking and measuring absorbance at 340/405 nm. Calculation of bound antibody was made by indirectly by calculating amount of IgG present in supernatant after conjugation.

Fluorescence detection was used to assess activity of anti-CA-125 antibody bound to microbubbles via incubation with fluorescently labeled CA-125 antigen. CA-125 antigen (Biodesign, Saco, ME), was fluorescently labeled with AlexaFluor 488-NHS (Invitrogen, Carlsbad, CA). The labeled CA-125 antigen was incubated with anti-CA-125 conjugated microbubbles for 1.5 hrs, washed 3 times and bubbles resuspended. The bubble solutions were diluted into 96 well plates and fluorescence intensity was measured. Fluorescence values were compared to those of a standard curve made with anti-CA-125 antibody incubated with labeled CA-125 antigen.

3.5 Cellular Analysis

3.5.1 Fluorescent Microscopy

Fluorescent microscopy was performed on samples using an Olympus IX80 microscope with 4x, 10x, 20x and 40x objective lenses and green (FITC), red (TRITC), blue (DAPI) filters. The microscope was interfaced to a PC. Images were acquired via Spot Advanced acquisition software (Diagnostic Instruments, Sterling Heights, MI).

3.5.2 Flow Cytometry

Flow cytometry was performed to assess model fluorescent ligand binding using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Microbubbles
were diluted in deionized water prior to placement in the machine. After flow cytometry measurement, data was analyzed using FloJo software (Tree Star Inc., Ashland, OR) focusing on changes in fluorescence intensity data.

Measurement of apoptosis in cell populations was achieving using a Guava EasyCyte flow cytometer (Guava Technologies, Hayward, CA). After treatments cells were detached from plate surface and incubated with fluorescent tags that indicate stages of apoptosis when measured with flow cytometry. Cells were diluted to 500 cells/μl prior to measurement. Data was analyzed using Guava analysis software.

3.6 Cell culture and cellular targeting studies

3.6.1 Cell Culture

There were 4 cell lines that were cultured in this project. All cell populations were incubated at 37°C in 95% humidified air and 5% CO₂ and passaged regularly at about 70-80% confluency. The media used for each cell model are as follows:

- MDA-MB-231 (human breast): RPMI 1640 90%, 9% Fetal Bovine Serum (FBS), 1% Penicillin Streptomycin (PS)
- MCF-7 (human breast): Dulbecco’s Modified Eagle’s Medium (DMEM) 90%, 9% FBS, 1% PS
- OVCAR-3 (human ovarian): RPMI 1640 90%, 20% FBS
- A2058 (human melanoma): Eagle's Minimum Essential Medium (EMEM) 90%, 9% FBS, 1% PS
3.6.2 Static Attachment Studies

For static attachment studies, cells were seeded on 48 well culture plates and permitted to grow to confluence. On the day of experimentation, special growth medium was prepared that contained either the microbubbles contrast agent or modified contrast agent in a concentration of 0.5 mg/ml. The cells were first washed with medium and then incubated with the 1ml of contrast agent containing medium for the specified time points. Controls included scrambled peptide conjugated microbubbles and unmodified microbubbles. For antibody targeting studies, cells were incubated with 0.5mg/ml of antibody-modified microbubbles or unmodified microbubbles. All studies were done in triplicate. At the specified time point, the medium was removed and the cells were washed with 1ml of growth media solution three times. Quantification of microbubble per cell attachment was assessed visually by light/fluorescent microscopy.

3.6.3 Apoptosis Studies

TRAIL-conjugated microbubbles, TRAIL-adsorbed microbubbles, unmodified microbubbles, or TRAIL by itself were incubated (0.5mg/ml or 10ng/well TRAIL) with cells and apoptosis was assessed at various timepoints 3, 8, 24, and 48 hours. An apoptosis assay was used that detects the externalization of phosphatidylserine in apoptotic cells using green-fluorescent Alexa Fluor 488 annexin and red-fluorescent propidium iodide nucleic acid stain. Propidium iodide stains necrotic cells with red fluorescence. Hoescht blue stain was added to stain the live cells. After treatment, live cells are positive for Hoescht stain (blue) negative for both annexin V and PI, early apoptotic cells are positive for annexin V (green) only and late apoptotic and necrotic
cells are positive for both dyes (green and red). Percent apoptosis was qualitatively assessed visually with fluorescent microscopy.

For studies evaluating the effect of Velcade on TRAIL-microbubble induced apoptosis. A2058 and OVCAR3 cells were grown to 70% confluence in 48 well plates (n=3). The cells were treated with either TRAIL, Velcade, TRAIL+Velcade, unmodified microbubbles, unmodified microbubbles+Velcade, Mb-TRAIL, Mb-TRAIL+Velcade, or left untreated (control). The concentration of agents were 100ng/ml TRAIL, 1μM Velcade, and 0.5mg/ml of unmodified or TRAIL modified microbubbles. After 24 incubation at 37C, 5% CO2, media was aspirated, cells trypsinized, media was replaced, 200ul was removed from each sample and treated with Guava apoptosis detection reagents consisting of Annexin V and 7-AAD. Apoptosis was quantified with flow cytometry using Guava EasyCyte flow cytometer. Data was analyzed using Cytosoft 4.1 software.

3.7 Statistical Testing

Statistical analyses were performed using Prism software (San Diego, CA, Graphpad Software). ANOVA analysis was used to determine significance. A one-way ANOVA was used to test for differences among two or more independent groups. For comparison between experimental groups, Tukey’s multiple comparison tests were performed and p-values were obtained. P-values greater than 0.05 were considered significant.
4. RESULTS AND DISCUSSION: FUNCTIONALIZATION OF CONTRAST AGENT SURFACES

A thorough description of the methods, related critical factors, results and analysis for each protocol and experiment are described within these chapters.

The results and discussion section of this thesis is divided into three chapters by specific aim.

- Chapter 4: Functionalization of contrast agent surfaces
- Chapter 5: A signal-inducing contrast agent
- Chapter 6: Ultrasound contrast agent for ovarian cancer detection

4.1 Functionalization of Contrast Agent Surfaces

The first specific aim of this thesis is to develop, optimize and characterize methods of making biodegradable polymer contrast agents that have the capacity for carrying targeting vectors and to assess cell attachment capabilities of the CA to breast cancer cells.

Degradable microbubbles of poly(lactic-co-glycolic acid) (PLGA) have been evaluated for use in a wide variety of specialized drug delivery applications. Investigators are approaching the problem of maintaining site specificity for microbubbles delivered by intravenous routes by modifying the surface properties of the microbubbles to increase their retention in tissues [2, 3, 6, 49, 70, 104]. Of particular interest is modification of the microbubble surface with ligands that bind to cell surface receptors. Targeting
microbubbles to specific cell types has great potential for increasing the efficiency of
drug delivery to the desired location, especially for microbubbles delivered intravenously.

A number of groups have devised techniques for immobilizing ligands to the
surface of PLGA microbubbles, including biotin-streptavidin complexes and
carbodiimide coupling of primary amines on the ligand to the carboxylic acid end groups
on microbubbles made of PLGA [30]. Carboxylated polystyrene microbubbles have been
used as model particles for targeting studies taking advantage of a high density of
carboxylic acid groups at the microbubble surface for ligand conjugation by a well-
established carbodiimide chemistry protocol [62].

Using carbodiimide chemistry to conjugate ligands to the surface of PLGA
microbubbles, however, is not as effective as linkage to the surface of the polystyrene
microbubbles. With carboxylic acid groups only at the end of polymer chains, PLGA
microbubbles have a limited number of available conjugation sites compared to a model
like carboxylated polystyrene microbubbles. In addition, common emulsion methods for
making PLGA microbubbles use surfactants such as poly(vinyl alcohol) (PVA) to
stabilize the emulsion. PVA has been shown to remain at the surface of the microbubbles,
even after repeated washing [105], refer to figure 3.1, creating a potential barrier for
efficient ligand conjugation to the acid end groups of PLGA.

The goal of specific aim #1 is to create and examine the ligand conjugation properties
of three different microbubble surfaces:

1. A surface with an increased number of surface carboxylic acid groups by
   replacing the traditional stabilizer PVA (which has hydroxyl side chains) with a
stabilizer bearing carboxylic acid side chains, namely poly(ethylene-alt-maleic acid) (PEMA). **Section 4.2**

2. A surface with incorporated lipid-PEG-biotin therefore laden with an outer layer of biotin available for functionalization by straightforward biotin-avidin coupling chemistry. **Section 4.3**

3. A surface with a varied, rough geometry, thereby having a greater modifiable surface area per microbubble as well as surface projections that may present ligands to receptors differently than a smooth-surfaced microbubble would. **Section 4.4**

### 4.2 Functional group enhancement via surfactant with carboxylic acid end chain

Protocols for making PLGA microspheres by emulsion techniques commonly use surfactants to stabilize the emulsion. Most commonly, poly(vinyl alcohol) (PVA) is used as the stabilizer. Other functional groups aside from hydroxyls, however, would be more suitable for many surface modification chemistries (see figure 4.1). Poly(ethylene-alt-maleic acid) (PEMA) is a surfactant with carboxyl end chains. The carboxyl side chains of PEMA, present at the microbubble surface, can be linked to primary amine groups of the desired ligand, forming stable amide bonds. In work by Keegan et al, PEMA has been used successfully to stabilize solid PLGA microspheres [62].

This group hypothesized that solid PLGA microspheres made with PEMA surfactant would have a greater amount of carboxyl groups on its surface and than those made with PVA surfactant, and as a result that they could bind more targeting ligands to the microspheres. We hypothesized that we could use PEMA as a surfactant to stabilize
hollow, echogenic contrast agent microbubbles. Though we were able to create micron sized bubbles with complete replacement of PVA by PEMA, these microbubbles were not acoustically active. Therefore, we chose to blend the two surfactants in order to achieve echogenic microbubbles with PEMA surfactant present on the microbubble surface. Further conjugation with a model ligand demonstrated that ligand conjugation increased with increasing amounts of PEMA, however cellular attachment did not increase with increasing amounts of PEMA. The results of these studies are presented in this section.

![Figure 4.1: Suggested arrangement of acid end groups present on the surface of microbubbles made with A) PVA and B) PEMA stabilizers. Use of PVA results in hydroxyl surface groups, use of PEMA results in carboxylic acid groups.](image)

4.2.1 PEMA Synthesis

Poly(ethylene-alt-maleic acid) was synthesized via ring-opening of poly (ethylene maleic anhydride). Two methods of ring opening were tested to create a 1% w/v solution. The first utilized a heating procedure similar to that used to make PVA. Poly (ethylene maleic anhydride) was slowly added to deionized water heated to 90 deg. C on a stirplate for 6 hours. This resulted in large aggregates of PEMA which did not go into solution. The heating procedure was modified by refluxing the solution in bottom flask a fitted
with a water-cooled condensation column and heating by means of a heating mantel for 72 hours. This resulted in smaller aggregates, however PEMA still did not go into solution.

The second attempt was an acid/base ring opening method. Poly (ethylene maleic anhydride) was slowly added to a 1M NaOH solution stirring in a round bottom flask for 24 hours (no heat). This method was successful; the resulting PEMA went into solution. After the procedure, pH was corrected to 6.5 and the solution was desalted via dialysis.

Using the acid/base ring opening method, 1, 3, 5 and 7% w/v solutions of PEMA were produced.

### 4.2.2 Complete surfactant replacement in microbubble fabrication

Following our microbubble fabrication procedure [9], the 50 ml of PVA surfactant in the second emulsion step was replaced with 50ml of either a 1, 3 or 5% w/v solution of PEMA surfactant. The resulting microbubbles were acoustically and morphologically evaluated.

It should be noted that the molecular weights of the two surfactants are not equal. PVA has a molecular weight of ~25,000. PEMA has a molecular weight of ~100,000. Therefore, theoretically 4 times more PEMA should be used to achieve the same surfactant molarity. However, creating a PEMA solution greater than 7% w/v using the ring-opening method of poly(ethylene maleic anhydride) (see materials and methods section 3.2.2.1) was not feasible. Therefore the following experiments were performed with are only 1-5% PEMA solutions.
It was observed that the 1% w/v PEMA surfactant did not fully stabilize the second emulsion. There were not enough PEMA groups at 1% w/v concentration for uniform stabilization, therefore large spheres of polymer were observed after the second emulsion. These spheres of polymer were confirmed by scanning electron microscopy (SEM) (Philips XL-30) (figure 4.2, A). The 3% and 5% solutions formed full emulsions and the resultant bubbles met size requirements (<8 microns), see figure 4.2.

![Figure 4.2](image)

Figure 4.2: Scanning electron micrographs of microbubbles made with (A) 1% PEMA, (B) 3% PEMA, and (C) 5% PEMA surfactant. 3000x, sizebar=10microns

The microbubbles were tested acoustically using the setup described in materials & methods section 3.4.1. The microbubbles made with 1% and 3% w/v PEMA solutions were weakly echogenic, having a maximum enhancement of 2 (±0.8) dB and 8 (±1.4) dB respectively. The microbubbles made with the 5% w/v PEMA solution had a maximum enhancement of 14 (±1.7) dB, significantly greater than microbubbles made with 1% and 3% PEMA (p<0.05). The average enhancement in our traditional microbubbles made with a 5% w/v PVA solution was 21 (±1.2) dB [30].

Therefore, complete replacement of PVA by PEMA surfactant in the second emulsion of our microbubble fabrication procedure yielded microbubbles that were not strongly echogenic. Due to the tradeoff between extra surface carboxyl groups (PEMA)
and the echogenicity of microbubbles (PVA), it was decided to study the effects of blending the two surfactants at PEMA:PVA.

4.2.3 Fabrication of microbubbles with blended surfactants

Blends of PEMA:PVA surfactant were created as described in materials and methods section 3.2.2.1. Surfactant solutions of volume ratios 0:100, 25:75, 50:50, 75:25 and 100:0 PEMA:PVA were made with 5% w/v PEMA and 5%w/v PVA. The 5%w/v PEMA solution was chosen for further study due to the superior acoustic and morphological characteristics over microbubbles made with 1% and 3% w/v PEMA.

The viscosity of the solutions of the blends linearly decreased with increasing PEMA:PVA ratios (Brookhaven viscometer, see figure 4.3). Microbubble samples were fabricated with 50ml of each blended surfactant in second emulsion process.

![Figure 4.3: Viscosity of PEMA: PVA blended surfactants. Viscosity decreases with increasing percentage of PEMA in surfactant. 1 CPS = 1 mPa·s](image)
Morphology was assessed using SEM as seen in figure 4.4. The microbubbles were spherical and ranged in size from 0.5-8 microns. Microbubble size was confirmed by light scattering. Size increased with increasing ratio of PEMA:PVA used in microbubble fabrication as seen in figure 4.5, though not significantly (p>0.05) between ratios 0:100, 25:75, 50:50 and 75:25. The size of microbubbles made with 100% PEMA were significantly larger than those made with the other ratios (p<0.05). Acoustic testing revealed that microbubbles made with blended surfactants yielded maximum enhancements of: 0:100=21(± 1.5), 25:75=20(± 1.7), 50:50=17(± 1.3), 75:25=8(± 0.9) and 100:0 =2(±0.5) dB (see figure 4.6). Microbubbles made with 75:25 and 100:0 PEMA:PVA ratios had significantly less maximum acoustic enhancement than bubbles made with 0:100 and 25:75 PEMA:PVA ratios (p<0.01), for both pre- and post-modification with GRGDS.

**Figure 4.4:** Scanning Electron Micrographs of poly(lactic acid) (PLA end-capped, M₊ 84.5kDa) microbubbles made using blends of PEMA:PVA as a stabilizer in the second emulsion of the W/O/W emulsion. A) 0% PEMA: 100% PVA B) 25% PEMA: 75% PVA C) 50% PEMA: 50% PVA D) 75% PEMA: 25% PVA E) 100% PEMA: 0% PVA size bar= 10 microns
Figure 4.5: Size distribution (A) measured by light scattering (number percent) of microbubbles made with blends of PEMA:PVA surfactant (B) Chart of microbubble diameters (Z-average) by surfactant ratio. ± standard error of the mean, n=3.

<table>
<thead>
<tr>
<th>PEMA: PVA surfactant ratio used in microbubble fabrication</th>
<th>Size (Z-average, in microns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:100 PEMA:PVA</td>
<td>2.65 +/- 0.4</td>
</tr>
<tr>
<td>25:75 PEMA:PVA</td>
<td>2.99 +/- 0.4</td>
</tr>
<tr>
<td>50:50 PEMA:PVA</td>
<td>3.12 +/- 0.6</td>
</tr>
<tr>
<td>75:25 PEMA:PVA</td>
<td>3.43 +/- 0.5</td>
</tr>
<tr>
<td>100:0 PEMA:PVA</td>
<td>5.85 +/- 0.8</td>
</tr>
</tbody>
</table>

Figure 4.6: Maximum enhancement of microbubbles made with PEMA:PVA blended surfactants. Maximum enhancement decreases with increasing amount of PEMA surfactant used in microbubble fabrication. Closed circles: unmodified microbubbles, closed squares microbubbles conjugated with GRGDS peptide. ± standard error of the mean, n=3.
Therefore, we were able to successfully create echogenic microbubbles with blends of surfactants that have the potential to display enhanced functional groups on their surface. We hypothesized that having an increased amount of PEMA in the microbubble fabrication would yield more surface carboxyl groups and therefore more ligand conjugation to the microbubbles. To confirm this we conjugated a model fluorescent ligand and assessed conjugation via flow cytometry.

4.2.4 Ligand Conjugation Assessment

The degree of surface modification was assessed using a model fluorescent ligand, 5-aminoacetamideo fluorescein, which was conjugated to the microbubbles made with blended surfactants using the carbodiimide linking chemistry as described in materials and methods section 3.3.2. The control group consisted of microbubbles contacted with the model ligand, but without the EDC chemical crosslinker present, to account for nonspecific adsorption.

Fluorescence of the PEMA:PVA microbubble samples was measured at 492nm using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Relative ligand conjugation was assessed by plotting the geometric mean intensities of the fluorescence data. (figure 4.7). The rightward shift in the green population in figure 4.6 indicates that bubbles made with PEMA (figure 4.7B) held more ligand than bubbles made with PVA (figure 4.7A). There was significantly more conjugated ligand (p<0.05) on microbubbles made with 50:50, 75:25, and 100:0 blended PEMA:PVA surfactants over
that of microbubbles without PEMA (0:100) and microbubbles made with a 25:100 volume ratio of PEMA:PVA (see figure 4.8).

**Figure 4.7**: Representative flow cytometry plots of fluorescence shift demonstrating that PEMA (B) surfactant enhanced microbubbles held more fluorescent ligand than PVA (A) stabilized microbubbles.
This experiment confirms that our ligand can be successfully linked to our microbubbles via carbodiimide chemistry. It also demonstrates that we have an significantly (p<0.05) increased number of functional groups on the surface with the addition of PEMA (above 50% volume ratio PEMA:PVA), since we were able to conjugate an increased amount of the fluorescent ligand. We hypothesized that having an increased amount of available targeting ligand on our bubbles will allow greater targeted microbubble attachment to cellular receptors. We tested this hypothesis with a cellular adhesion assay.

4.2.5 Static Adhesion Study with PEMA enhanced GRGDS microbubbles

The 0:100, 25:75, 50:50, 75:25 and 100:0 of PEMA:PVA microbubbles were conjugated with GRGDS using a carbodiimide reaction as described above. MDA-MB-231 cells were grown in 48well plates in DMEM media supplemented with 10% FBS. Microbubble solutions were made with 0.5mg/ml microbubbles in media with the additional unmodified microbubble group as a control. At 70% confluency cells were incubated with the CA modified media (n=3) for 4 timepoints 0, 5, 10 and 15 minutes. At the specified time points media was aspirated and cells washed 2x before imaging using light microscopy. Microbubble per cell attachment was assessed through blinded, visual inspection of images (figure 4.9).
**Figure 4.9:** Cellular Attachment Testing of PEMA surfactant enhanced ligated microbubbles. MDA-MB-231 cells incubated with 0.5 mg/ml of microbubbles made with blends of surfactant (a) 0PEMA:100PVA, (b) 25PEMA:75PVA, (c) 50PEMA:50PVA, (d) 75PEMA:25PVA, (e) 100PEMA:0PVA and conjugated with GRGDS peptide, or (f) unmodified microbubbles. Pictures taken at 10 mins incubation, 40x, size bar=50um.

**Figure 4.10:** Cellular attachment (microbubbles/cell) of GRGDS ligated microbubbles made with PEMA:PVA blends of surfactant. ± standard error of the mean.

It was determined that contrary to our hypothesis that increased amounts of GRGDS resulting from an increase in of carboxyl groups by PEMA would lead to increased attachment, attachment decreased with increasing amounts of PEMA (figure
4.10). At 10 and 20 minutes, microbubbles made without PEMA (100% PVA) had significantly more microbubble/cell attachment than microbubbles made with 50:50, 75:25, and 100:0 PEMA:PVA blends (p<0.001 for all groups except 50:50 at 10min which was p<0.01). A possible explanation for this phenomenon is that there is an optimal density for targeting ligand on the microbubble surface for cellular adhesion. This is suggested by several researchers [54, 77]. In a previous study by our group [70] it was determined that 0.5 GRGDS groups: total PLA carboxyl groups was optimal for our application. In this experiment it was this same ratio that gave the best cellular attachment. Perhaps having more carboxyl groups resulted in an oversaturation of targeting ligand on the surface of the bubbles. Therefore this may have led to reduced bioactivity of GRGDS due to overcrowding on the microbubble, reducing the chance for the ligand to adhere to its target. Other possible explanations include the fact that PEMA results in a greater net negative surface charge on the microbubbles, which inhibits attraction of the microbubbles to the cells, or that PEMA is deleterious to the health of the cells in some fashion that PVA is not.

4.2.6 Conclusion: Functional group enhancement via surfactant with carboxylic acid end chains

We were able to produce echogenic ultrasound contrast agents with an enhanced ability to carry surface ligands, with agents made using blends of two surfactants, PVA (hydroxyl end groups) and PEMA (carboxyl end groups). The acoustic enhancement provided by these bubbles decreased with increasing percentage of PEMA surfactant. As we hypothesized, agents prepared with greater amounts of PEMA were able to carry
more conjugated ligand as determined by flow cytometry, suggesting a greater number of carboxyl groups available on the microcapsule surface. However, unexpectedly there was a reduction in cellular attachment with increasing amounts of PEMA. A possible explanation is that the optimal density of GRGDS was exceeded by increasing the amount of available modifiable groups on the microbubbles.

### 4.3 Novel Targeted Lipid-Polymer Microbubbles

The primary strategy of targeting contrast agents to specific sites involves modifying the surface of the contrast agent with ligands. The techniques of surface modification with ligands typically used are either the covalent attachment of ligands, or physical incorporation of ligands into the shell of the contrast agent. The physical incorporation of lipid-ligand conjugates has been primarily explored in the formation of lipid-based contrast agents. This aim explores the feasibility of combining the physical incorporation of lipid-ligand conjugates into polymer ultrasound contrast agents. We hypothesize that by adding DSPE(lipid)-PEG-biotin conjugate into the first emulsion, with the subsequent association of the conjugate’s lipid portion within the hydrophobic polymer shell, we will create bubbles with surface PEG spacer-biotin groups adaptable to a variety of biotinylated targeting molecules.

#### 4.3.1 Development of DSPE-PEG-Biotin incorporated microbubbles

Microbubbles were formed in our double emulsion process [70] using either ammonium carbonate (BON) or ammonium carbamate (BAM) as a porogen. In order to fabricate the biotinylated microcapsules, 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Biotinyl(Polyethylene Glycol)2000] (DSPE-PEG(2000)Biotin)
was added to the first mixture of PLA and organic solvent in a 1:100 ratio of lipid:polymer. Briefly, 1mL of 5M solution (5mg lipid / 1mL CHCl₃) was aspirated to the bottom of a 50ml max beaker with a glass syringe, dried under a stream of argon gas, and dessicated under vacuum for 3 hours. The 50 ml beaker containing the dried DSPE-PEG-Biotin was then used to dissolve the polymer in organic solvent prior to the first emulsion. The resulting microbubbles have the DSPE lipid in the shell, a PEG spacer and biotin on the surface for modification with streptavidin and further conjugation with a biotinylated ligand as seen in figure 4.11

![Figure 4.11](image)

**Figure 4.11**: Schematic of microbubble with incorporated DSPE-PEG-biotin plus streptavidin and biotinylated targeting ligand.

### 4.3.2 Characterization of DSPE-PEG-Biotin incorporated microbubbles

Biotin presence was confirmed by first incubating the capsules with fluorescently labeled streptavidin followed by visualization of fluorescence with a fluorescent microscope and confocal microscope. To examine microbubble morphology, microcapsules were mounted on metal stubs with double-sided tape and coated with platinum for imaging with an SEM. Additional size information was obtained from light
scattering experiments. *In vitro* acoustic dose and time response curves were collected using our custom built set-up.

The four varieties of polymer microbubbles fabricated were PLA formed with ammonium carbonate as a porogen (PLA-BON), PLA formed with ammonium carbamate as a porogen (PLA-BAM), and each of those two formed with the addition of the lipid-ligand conjugate (PLA-BON-PEG and PLA-BAM-PEG). Confocal and fluorescent microscopy confirmed the presence of fluorescently labeled streptavidin bound to the biotinylated surfaces of PLA-BON-PEG and PLA-BAM-PEG, and not to the control microcapsules which lack the DSPE-PEG-Biotin component (figure 4.12) The PLA-BAM and PLA-BAM-PEG microbubbles were relatively monodisperse, hollow, with smooth surfaces (figure 4.13, A & B). The PLA-BON and PLA-BON-PEG were similarly monodisperse and hollow, but with a rougher surface morphology (figure 4.13, C &D). Light scattering size distributions supported the ESEM observations of microbubble diameters ranging from 300nm-2.5 microns. The acoustic signal enhancement for the spheres after maximal dose was 18 (±0.6) dB for PLA-BON, 22 (±1.7) dB for PLA-BON-PEG, 19 (±0.9) dB for PLA-BAM, and 24 (±0.6) dB for PLA-BAM-PEG (± standard error of the mean, n=3) (figure 4.14, A). There was no significant difference in maximum enhancement with the addition of the lipid-ligand conjugate (p>0.05). The percent loss of the original signal over 15 minutes was 18% for PLA-BON, 20% for PLA-BAM, 50% for PLA-BON-PEG, and 60% for PLA-BAM-PEG (figure 4.14, B). There was 40% more signal loss from microbubbles made with lipid-ligand conjugate after 15 minutes of insonation, significantly greater than that of microbubbles made without the conjugate (p<0.01).
The time response data follow what would be expected based on the dose response data. Addition of lipid into the microbubble shell results in a higher acoustic enhancement probably due to the resultant larger, thinner shelled bubbles created by the addition of a lipid into the first emulsion. Lipid is a viscosity enhancer, and it has been shown that increased viscosity in the polymer phase of an emulsion leads to greater microsphere size [106]. These bubbles are also more likely to burst which explains why the bubbles with lipid within in the shell lost about 30-40% more signal after 15 minutes of insonation than those without lipid.

**Figure 4.12:** Fluorescence detection of ligand. Confocal micrographs of microbubbles (A-D). The left column is brightfield (A&C), while the right column is fluorescence (B&D). The top column is the control of microbubbles without DSPE-PEG-biotin in the shell (A&B) and the bottom column is the experimental group of microbubbles with DSPE-PEG-biotin in the shell (C&D) Both control and experimental microbubbles were incubated with streptavidin prior to imaging. E) Histogram of the detection of Oregon green-labeled Streptavidin bound to microbubbles. The shift in fluorescence with the biotin group shows the labeled streptavidin binds to the microbubbles made with DSPE-PEG-biotin, but not control bubbles without DSPE-PEG-biotin.
**Figure 4.13**: Scanning Electron Micrographs of (A) PLA-BAM microbubbles, (B) PLA-BAM-PEG microbubbles (C) PLA-BON microbubbles, and (D) PLA-BON-PEG microbubbles, depicting microbubbles 300nm-2.5microns in size. 6000x, size bar=5 microns.

**Figure 4.14**: Acoustic Testing. Cumulative dose response curve (A) and acoustic stability (B) of microbubbles with DSPE-PEG-biotin incorporation (PLA-BAM-PEG, PLA-BON-PEG) and without (PLA-BAM, PLA-BON). ± standard error of the mean, n=3.
4.3.3 Conclusion: DSPE-PEG-Biotin incorporated microbubbles

Proof of concept studies demonstrate that DSPE-PEG-Biotin conjugates can be successfully incorporated into PLA microbubbles without serious effect on echogenicity or morphology. In addition, it is noted that the incorporation of the lipid-ligand caused a 40% decrease in acoustic stability after 15 minutes of insonation. The acoustic response of these microcapsules can possibly be tuned by changing amounts of incorporated lipid. As demonstrated by fluorescent ligand, this model system can potentially be extended to physiologically relevant ligands by either using lipids conjugated to the physiologic ligand or linking biotinylated ligands to the current system. This novel approach for functionalization of microbubble contrast agents represents a flexible disease targeting system; it can be extended for use with any targeting ligand that can be biotinlyated.

4.4 Targeted microbubbles with smooth and rough textured surfaces

The last component of specific aim 1 was to examine the hypothesis that shape of the polymeric contrast agent will have an effect on the adhesion of microbubbles to cells due to a change in the presentation the ligand to its target. Researchers have found that adhesive properties of lipid-based contrast agents with microvilli-like projected structures on their surface enhanced the cellular binding properties of the microbubbles [5]. In our laboratory, it has been shown that microbubbles made with polymer PLGA 50:50 can produce echogenic particles with a rough architecture of projected structures which maintain high echogenicity. In order to compare only the effect of the morphology on cellular targeting it was necessary to first produce echogenic microbubbles with both smooth and rough surfaces, but made of the same polymer.
4.4.1 Development of smooth and rough textured microbubbles

In order to produce echogenic microbubbles with either smooth or rough surfaces using the same polymer a systematic microbubble fabrication study was performed. Samples were fabricated by systematically varying both chemical and physical steps in our double emulsion process. These included:

- Inherent viscosity of polymer (PLGA 50:50 from 0.3-0.70 dL/g I.V.)
- Water to oil ratio (1:10 or 1:20) in first emulsion
- Surfactant molecular weight (25k or 133k M_w PVA) in second emulsion
- Porogen types and concentrations (camphor, ammonium carbonate)
- Homogenization and solvent evaporation parameters

Based on previous work in our laboratory, we were already aware of which parameters in fabrication resulted in rough textured microbubbles. In fact, it was applying the same fabrication procedure we use to create our spherical PLA based microbubbles to PLGA 50:50 (3.5A) polymer. However, to that date we did not have a method to create smooth spherical microbubbles using the PLGA 50:50 polymer.

**Inherent Viscosity.** We began by evaluating inherent viscosity of the polymer, which equates with the molecular weight. PLGA 50:50 was available from the manufacturer (Lakeshore Biomaterials, Birmingham, AL) in 2A, 3.5A, 4A, 5A and 6A forms. The number in the designation refers to the range of inherent viscosity and letter refers to end group (e.g. 6A is 0.60-0.70 dL/g I.V. with an acid end group). The PLA we use to create smooth microbubbles has a much higher I.V. (0.55-0.65 dL/g) than PLGA...
50:50 3.5A (0.35-0.45 dL/g). Therefore, we decided to evaluate only those polymers with I.V.'s equal or greater than 0.35 dL/g. 4A was not evaluated due to its very similar I.V to 3.5A. We fabricated microbubbles using 3.5A, 5A and 6A PLGA 50:50 polymers. See table 3.1 for a list of polymers and characteristics.

**Water:Oil Ratio.** It has been shown that the oil-phase volume plays a critical role in determining morphology. Yang et al. varied oil phase volume (9,12 and 15 ml) in their double emulsion process to produce microspheres [107]. They found that a microspheres with a low oil volume yielded microspheres with numerous pores. They reasoned that since a low methylene chloride (oil) content results in a more viscous solution, it was more difficult to break up the internal water into smaller droplets in the first emulsion. The larger water droplets left empty spaces after freeze-drying creating the pores. Also since water has a high surface energy, it was likely that the internal droplets coalesced with one another when the oil volume was low, yielding a porous internal matrix [107]. Since we are seeking to reduce the porosity, we chose to evaluate doubling our oil volume in the water:oil ratio from 1:10 water:oil to 1:20 water:oil.

**Surfactant concentration.** In order to form spherical particles it is essential to have enough stabilization groups in the surfactant. In the past our lab has evaluated a 6,000, 25,000 and 133,000 M_w PVA for creating microbubbles [14]. We hypothesized that increasing the size of the PVA would help stabilize the microbubbles and retain the smooth surface in the second emulsion. It has been shown that due to the high molecular weight of PVA, its presence in the external water phase of the double emulsion may increase the emulsion viscosity which increases difficulty of breaking the emulsion into smaller droplets and may lead to increased microbubble size [107]. Offsetting this is the
stabilization effect of the PVA in the external water phase, stabilizing the emulsion
droplets against coalescence, resulting in smaller stabilized droplets [107]. To increase
the stabilization effect and create spherical microbubbles, we’ve chosen to test a higher,
133,000 M_w surfactant along with our traditional 25,000 M_w PVA. To account for the
predicted increase in particle size due to an increase in emulsion viscosity from higher
M_w PVA, a higher homogenization speed, 10,500 rpm, was used to break up the more
viscous emulsion.

Porogen Types and Concentrations. Past research in our laboratory has
demonstrated that acoustic properties can be tweaked by varying the sublimable core and
shell porosity of the microbubble [20]. Originally, our process uses a 40 mg/ml
concentration of ammonium carbonate and the 50mg of camphor in the shell. It is known
that without ammonium carbonate and camphor in the double emulsion process, smooth
but solid microspheres are formed [11, 62]. Solid microspheres are not echogenic. We
hypothesized that by incrementally increasing the amount of ammonium carbonate and
camphor from 0 (smooth solid/not echogenic) up to the original concentration
(rough/echogenic) in increments of 25mg for camphor and 20mg/ml ammonium
carbonate (1M), we can create hollow smooth surfaced echogenic microbubbles.

Homogenization/Solvent Evaporation. Lastly, we evaluated physical parameters
for creation of smooth microbubbles. We chose to increase at homogenization speed
because a faster rate of homogenization can help the second emulsion form and stabilize
micron sized bubbles. Furthermore, increasing the viscosity of the polymer and of the
surfactant creates a more viscous emulsion that must be homogenized at a higher speed in
order to break up the larger droplets. We increased the rate from 9500 rpm to 10500 rpm,
however we could not further increase due to equipment limitations. Because the bubbles are not yet hardened during the 1.5 hour solvent evaporation step, we decreased the stirplate speed from 800 to 400 rpm to make the conditions less harsh, but still stirring at a rate fast enough for proper evaporation of solvent. We hypothesized that the microbubbles may be smoother with reduced stirspeed because they aren’t colliding as much with other bubbles.

Using the above mentioned parameters we created the samples described in Table 4.1. Acoustic testing and SEM was used to screen each sample.

4.4.2 Characterization smooth and rough surfaced microbubbles

Characterization included size analysis with dynamic light scattering, acoustic testing in our custom setup, and morphology analysis using SEM.

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Solvent (ml)</th>
<th>PVA Mw</th>
<th>Camphor</th>
<th>Porogen</th>
<th>Stir/Homo. Rpm</th>
<th>Max dB</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.5A</td>
<td>10</td>
<td>133k</td>
<td>25mg</td>
<td>20mg/ml</td>
<td>400 / 10500</td>
<td>7</td>
<td>very rough</td>
</tr>
<tr>
<td>2</td>
<td>3.5A</td>
<td>10</td>
<td>25k</td>
<td>25mg</td>
<td>20mg/ml</td>
<td>400 / 10500</td>
<td>6</td>
<td>very rough</td>
</tr>
<tr>
<td>3</td>
<td>3.5A</td>
<td>10</td>
<td>133k</td>
<td>50mg</td>
<td>40mg/ml</td>
<td>400 / 10500</td>
<td>12</td>
<td>*</td>
</tr>
<tr>
<td>4</td>
<td>3.5A</td>
<td>10</td>
<td>25k</td>
<td>50mg</td>
<td>40mg/ml</td>
<td>400 / 10500</td>
<td>20±1.1</td>
<td>Rough</td>
</tr>
<tr>
<td>5</td>
<td>3.5A</td>
<td>20</td>
<td>133k</td>
<td>25mg</td>
<td>20mg/ml</td>
<td>400 / 10500</td>
<td>8</td>
<td>round rough</td>
</tr>
<tr>
<td>6</td>
<td>3.5A</td>
<td>20</td>
<td>25k</td>
<td>25mg</td>
<td>20mg/ml</td>
<td>400 / 10500</td>
<td>11</td>
<td>*</td>
</tr>
<tr>
<td>7</td>
<td>3.5A</td>
<td>20</td>
<td>133k</td>
<td>50mg</td>
<td>40mg/ml</td>
<td>400 / 10500</td>
<td>12</td>
<td>round rough</td>
</tr>
<tr>
<td>8</td>
<td>3.5A</td>
<td>20</td>
<td>25k</td>
<td>50mg</td>
<td>40mg/ml</td>
<td>400 / 10500</td>
<td>17</td>
<td>Rough</td>
</tr>
<tr>
<td>9</td>
<td>5A</td>
<td>20</td>
<td>133k</td>
<td>25mg</td>
<td>20mg/ml</td>
<td>400 / 10500</td>
<td>16</td>
<td>semi-smooth</td>
</tr>
<tr>
<td>10</td>
<td>5A</td>
<td>20</td>
<td>25k</td>
<td>25mg</td>
<td>20mg/ml</td>
<td>400 / 10500</td>
<td>18</td>
<td>less rough</td>
</tr>
<tr>
<td>11</td>
<td>5A</td>
<td>20</td>
<td>133k</td>
<td>50mg</td>
<td>40mg/ml</td>
<td>400 / 10500</td>
<td>15</td>
<td>semi-smooth</td>
</tr>
<tr>
<td>12</td>
<td>5A</td>
<td>20</td>
<td>25k</td>
<td>50mg</td>
<td>40mg/ml</td>
<td>400 / 10500</td>
<td>19</td>
<td>Rough</td>
</tr>
<tr>
<td>19</td>
<td>6A</td>
<td>20</td>
<td>133k</td>
<td>25mg</td>
<td>20mg/ml</td>
<td>400 / 10500</td>
<td>18±0.8</td>
<td>smooth</td>
</tr>
<tr>
<td>20</td>
<td>6A</td>
<td>20</td>
<td>133k</td>
<td>50mg</td>
<td>40mg/ml</td>
<td>400 / 10500</td>
<td>19</td>
<td>semi-smooth</td>
</tr>
</tbody>
</table>

* samples not imaged
We began our study by varying each of our parameters solvent amount, PVA $M_w$, camphor and porogen for the 3.5A polymer. As expected using the original microbubble fabrication procedure applied to the 3.5A polymer created echogenic (20±1.1 dB) rough microbubbles. We found that the most spherical appearing particles, though still rough surfaced, were created with the 1:20 ratio of water:oil (solvent). We chose to use 20ml of solvent for the 5A polymer, varying only the surfactants and porogens. After this experiment we noted that those microbubbles made with the 133k $M_w$ surfactant had semi-smooth surfaces. Testing our last and highest I.V. polymer, 6A, we chose to fix both solvent and surfactant varying only porogen amounts. It was found that the 6A sample with higher amounts of porogen was slightly more echogenic than that with less porogen, however its surface was not as smooth, so the 6A sample with half the amount of porogens was chosen for further study.

SEM demonstrated smooth and rough morphologies from PLGA 50:50 microbubbles (figure 4.16, A & D). The size distribution of the 20 samples described in table 4.1 is depicted figure 4.15A and shows a general size range of 0.3-6 microns for all the various samples. The size distribution of the microbubbles made with the chosen parameters for smooth and rough morphology is seen in figure 4.15B. Mean diameter for these smooth microbubbles was 2.86 ± 0.2 microns, and for the rough microbubbles the mean diameter was 4.1 ± 0.3 microns (z-average, ± standard error of the mean, n=3). The optimal fabrication parameters selected to create smooth and rough surfaced PLGA 50:50 microbubbles are summarized in table 4.2. The optimal parameters for making smooth and rough surfaced bubbles from the PLGA 50:50 polymer were as hypothesized.
**Figure 4.15**: Size distribution by number as determined by light scattering for (A) all variants (1-20) of PLGA 50:50 microbubbles, and (B) chosen smooth and rough surfaced PLGA 50:50 microbubbles.

**Table 4.2**: The fabrication parameters selected for fabrication of smooth, column (A), and rough, column (B), surfaced PLGA 50:50 microbubbles.

<table>
<thead>
<tr>
<th></th>
<th>(A) Smooth</th>
<th>(B) Rough</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer I.V.</td>
<td>0.60-0.70 dL/g</td>
<td>0.30-0.40 dL/g</td>
</tr>
<tr>
<td>Water : Oil</td>
<td>1:20</td>
<td>1:10</td>
</tr>
<tr>
<td>Ammonium Carbonate</td>
<td>20 mg/ml</td>
<td>40 mg/ml</td>
</tr>
<tr>
<td>Camphor</td>
<td>25mg</td>
<td>50mg</td>
</tr>
<tr>
<td>PVA Mw (Da)</td>
<td>133,000</td>
<td>25,000</td>
</tr>
<tr>
<td>Homogenization Speed</td>
<td>10,500 RPM</td>
<td>9,500 RPM</td>
</tr>
<tr>
<td>Stirplate Speed during Solvent Evaporation</td>
<td>400 RPM</td>
<td>800 RPM</td>
</tr>
</tbody>
</table>
4.4.3 Cellular adhesion of targeted smooth and rough surfaced microbubbles

These above described agents were chosen for ligand conjugation with either the peptide GRGDS or a scrambled peptide GRDGS using a covalent carbodiimide linkage. These GRGDS modified microbubbles should have an affinity to the $\alpha_v\beta_3$ receptors present on cancer cells. MDA-MB-231 human breast cancer cells were grown in 48 well plates until 70% confluence. Solutions of cellular media made with 0.5 mg/ml of the modified (RGD/scrambled) or unmodified microbubbles were incubated on the cells for 0, 10, 20 or 60 min (37degC, 5% CO₂). After washing cells were examined under light microscopy (figure 4.16, B,C,E F) and binding quantified with blinded visual inspection (figure 4.17).

For all time points past 0 min the GRGDS ligated rough textured microbubbles had significant cellular attachment over the controls of unmodified microbubbles and microbubbles conjugated with a scrambled peptide ($p < 0.05$). Furthermore, at 20 and 60 minutes the GRGDS ligated rough textured microbubbles had significantly greater cellular attachment than the GRGDS ligated smooth surfaced bubbles ($p < 0.05$). (one-way ANOVA, Tukey’s multiple comparison test). It should be noted that the unmodified rough textured microbubbles exhibited greater cellular attachment than the unmodified smooth microbubbles and scrambled peptide-modified smooth microbubbles, however this difference in attachment was not statistically significant ($p > 0.05$).
Figure 4.16: SEM of smooth and rough microbubbles with corresponding light micrographs demonstrating cellular attachment of the modified and unmodified agents to MDA-MB-231 cells. A) SEM smooth bubbles, size bar =10um, B) Light micrograph of unmodified smooth CA incubated on cells C) Light micrograph of RGD modified smooth CA incubated on cells D) SEM of rough bubbles, size bar=10um, E) Light micrograph of unmodified rough CA incubated on cells C) Light micrograph of RGD modified rough CA incubated on cells (all cellular photos taken after 20 min incubation).

Figure 4.17: Microbubble attachment/cell of smooth and rough surfaced microbubbles (Mb), unligated (Mb-unmod), modified with a scrambled peptide (Mb-ScramPep), or modified with GRGDS peptide (Mb-RGD). Filled symbol indicates rough texture, open symbol indicates smooth textured microbubbles. Incubated with 0.5mg/ml CA, evaluated at 0,10,20 and 60 min. n=3 ± standard error of the mean.
4.4.4 Conclusion: Smooth and rough surfaces microbubbles for targeting

It has been shown that through the systematic analysis of chemical and physical parameters in the fabrication process it is possible to create echogenic PLGA 50:50 contrast agents with both smooth and rough surfaces. Furthermore we demonstrated that conjugation with GRGDS allowed attachment of these agents to αvβ3 expressing breast cancer cells, with microbubble/cell attachment of rough-CA significant (p<0.05) as compared to smooth CA at both 20 and 60 minutes incubation. Similar studies performed by Rychak et al [8] offer an explanation of this observed phenomenon.

Rychak et al. claims that the outward projected structures on their microbubbles reduced the force on the adhesive bond of antibody to target by increasing the lever arm between the center of the microbubble and the rearmost anchoring bond. They calculated that a microbubble bound by a single antibody molecule located at the tip of a 0.5 micron wrinkle experiences a ~55% lower bond force than a spherical microbubble in which the ligand is flush with the surface. This reduced load due to lever arm resulted in increased bubble stabilization; they found that their wrinkled bubbles remained adherent long enough for additional stabilizing bonds to form. However, they could not conclude whether it was the geometry alone or the deformable nature of the wrinkled bubbles that was ultimately responsible for the sustained adhesion. To compare our findings to Rychak’s, the projected structures as seen in figure 4.18 are also in the 0.5 micron range.
We found that at 20 and 60 minutes of incubation we had significant (p<0.05) cellular attachment of our GRGDS ligated rough microbubbles compared to GRGDS ligated-smooth microbubbles. Rychak’s model predicted that the projections in the microbubbles increased sustained adherence since it allowed the particle to stay attached long enough for stabilized bonds to form. We believe that our increased adhesion may be due to the same phenomenon, though it should be noted that their model was based on liposomes and that the deformability of a polymer-shelled agent is less than a lipid-shelled agent.

Alternatively, the observation that unmodified rough textured microbubbles exhibited greater cellular attachment than unmodified smooth microbubbles suggests there could be greater entanglement with the rough textured microbubbles within the glycocalyx layer at the cellular surface. Glycocalyx is a carbohydrate-rich zone on the cell exterior, mainly consisting of glycoproteins and proteoglycans. The glycocalyx on
the surface of cells is partly responsible for the adhesive properties of the cell and carries a net negative charge [108]. A model by Agarwal et al. suggests that glycocalyx presence on the endothelial cell surface has an effect on the binding of nanocarriers [109]. Leung et al. states that any increase in contact area and establishment of physical entanglement at the interface strengthens the force of cellular adhesion [108]. Hence, factors that favor enhanced physical entanglement, such as our rough textured microbubbles, could potentially increase cellular adhesion strength.

4.5 Conclusion: Surface functionalized microbubbles

Specific aim 1 evaluated three new methods of enhancing the surface of our microbubbles for cellular targeting purposes. The first method enhanced the ability of our agent to carry targeting ligands by the addition of PEMA, a carboxyl containing surfactant. This surfactant was successfully used to form solid PLGA microspheres [62], but has never been applied to polymer based contrast agents. Furthermore, this research presents the first example blending PVA and PEMA surfactants to create acoustically active contrast agents and is the first to examine the effects of the surfactant blends on CA size, morphology, acoustic properties and RGD mediated cellular adhesion to $\alpha_v\beta_3$ receptors in breast cancer cells. For this application, increased surface ligands did not lead to an increase in cellular attachment. This may be attributed to an oversaturation of surface-bound ligands leading to overcrowding, hence hindering the ligand from attaching to its target and reducing cellular adhesion. Other possible explanations include the fact that PEMA results in a greater net negative surface charge on the microbubbles,
which inhibits attraction of the microbubbles to the cells, or that PEMA is deleterious to the health of the cells in some fashion that PVA is not.

The second method was the evaluation of the incorporation of a lipid conjugated with PEG and a functional group into the shell of our CA microbubbles. Though this concept has been demonstrated in solid microspheres [11], this thesis presents the first report of using lipid-PEG-biotin to functionalize a contrast agent platform. As the lipid preferentially associated with the polymer shell, it created a PEG layer with surface modifiable groups (biotin). Incorporation of the lipid did not significantly affect the CA’s maximum acoustic enhancement (p>0.05), however it significantly reduce its stability over 15 minutes of insonation by 30-40% (p<0.05). This incorporated lipid-conjugate concept could be extended to create other functional groups on the microbubble surface and hence the CA could be tailored for a variety or targeting ligands.

The last method of surface functionalization explored the cellular attachment behavior of microbubbles with varied morphological features. A systematic study of varying microbubble fabrication parameters produced echogenic microbubbles made of PLGA 50:50 polymer with either a rough surfaced or smooth surfaced morphology. Cellular attachment studies revealed that rough textured microbubbles that were ligated with GRGDS exhibited significant (p<0.05) cellular adhesion over smooth ligated microbubbles and controls at 20 and 60 mins. It was noted that even without ligation, rough microbubbles tended to adhere more the cells. Reasons for this behavior may be the deformability of the rough microbubbles allowing the extended features holding the ligand to reach its integrin target or alternatively that the morphology of the rough bubble creates entanglement within the glycocalyx layer surrounding the cells.
Specific Aim 1 demonstrated that our polymeric ultrasound contrast agent platform is highly adaptable. It can be tuned to various applications by changing the functional groups available on its surface through surfactants or incorporation of lipid conjugates as well as modifying its morphology to improve cellular adhesion.
5. RESULTS AND DISCUSSION: A SIGNAL-INDUCING ULTRASOUND CONTRAST AGENT

5.1 A signal-inducing ultrasound contrast agent

   The goal of specific Aim #2 was to develop methods to produce a novel bioactive ultrasound contrast agent that elicits signal transduction, using TNF related apoptosis-inducing ligand (TRAIL also known as Apo-2L) and apoptosis as a model. This was done by first conjugating TRAIL to microbubbles using maleimide chemistry and then assessing the apoptotic inducing ability of TRAIL-microbubbles on TRAIL-sensitive cell populations. We hypothesized apoptosis of cancer cells via signal transduction can be achieved by incubation with TRAIL-conjugated microbubbles.

   TRAIL/ Apoptosis was chosen as a model for several reasons. It is well documented that TRAIL induces cell death selectivity in tumor cells [86, 110, 111]. There are death receptors (DR4 and DR5) for TRAIL on normal cells, however protection of normal tissues happens by way of decoy receptors (DcRs), which compete with DR4 and DR5 for binding to TRAIL (see figure 5.1,B). DcR1 and DcR2 are cell surface protein resembling DR4 and DR5, but lack cytoplasmic components. DR4 and DR5 are expressed in normal tissue and in many types of tumor cells, whereas DcR1 and DcR2 are expressed frequently in normal cells but infrequently in tumor cells [88]. This differential expression of death and decoy receptors enables TRAIL to induce apoptosis in tumors while sparing normal cells. The first reason TRAIL was chosen for our application was this cell selectivity, a desirable attribute, since our agent is administered
systemically. The TRAIL-microbubbles will theoretically induce apoptosis in only tumor cells.

Furthermore, TRAIL-microbubbles may enter and be retained within tumor tissue. While some believe that micron-sized contrast agents remain only within the vasculature, it is a possibility that populations of the microbubbles may be able to extravasate into tissue from the large pores of leaky tumor vasculature. When aggregates of tumor cells achieve a diameter of 1-2 mm they require neovasculature to supply nutrients. These vessels have an irregular, incomplete structure that includes pores. Yuan et al. found the size of tumor vessel pores in LS174T human colon adenocarcinoma implanted in the dorsal skin chambers in immunodeficient mice could be as large as 0.4 microns in diameter [33 background]. Using SEM, researchers have identified structural abnormalities in the endothelium of tumor vasculature including cellular openings with a mean diameter of 1.7 microns (range, 0.3-4.7 microns) [39-42]. If the microbubbles can escape the tumor vasculature, TRAIL would be able to reach its receptors and exert its effects. Coincidentally retention of the microbubbles in tumors is enhanced through the retention mechanisms at play in the enhanced permeability and retention (EPR) effect [42].

Lastly, we considered the benefit of delivering apoptosis inducing TRAIL on an ultrasound contrast agent platform. There have been several studies investigating the effect of ultrasound and ultrasound contrast agents on cellular apoptosis [81, 82]. Feril et al. [81] investigated the effects of ultrasound contrast agents on ultrasound induced apoptosis in human lymphoma cells, determining that Optison was effective in augmenting ultrasound induced cell death due to the cavitation of the agent. A similar
study by Honda et al. [82] evaluating the effect of free radial formation during cavitation, found that ultrasound induced apoptosis of cancer cells was linked to cavitation. When their cells were insonated in the presence of Levovist; synergistic enhancement of cell death was observed at concentrations of more than 20 mg/mL [82]. This results suggest that contrast agents, acting as cavitation nuclei, can enhance ultrasound-induced apoptosis due to an increase in cell membrane damage. Therefore, there is added apoptotic benefit to delivering TRAIL via an ultrasound contrast agent platform over TRAIL therapy alone.

Currently, there is no published research regarding the use of pro-apoptotic agents, such as TRAIL, as a ligand on an ultrasound contrast agent. Furthermore, we present a method of enhancing TRAIL-Mb apoptosis by the synergistic effect of concurrent treatment by a proteasome inhibitor, Velcade (Millenium Pharmaceuticals, Cambridge, MA). There is currently no published research on the Velcade-enhanced TRAIL apoptosis using TRAIL on a polymeric carrier.

Figure 5.1: Schematic of apoptosis inducing polymeric contrast agent. A) TRAIL is conjugated to the PLA microbubble with a BMPH linking arm. Binding of TRAIL-CA to receptors on cancer cells induces apoptosis. B) TRAIL receptors and decoy receptors present on cellular surface, DR4 and DR5 binding trigger caspase signaling cascade and apoptosis.
5.1.1 Conjugation of TRAIL to microbubbles

As a proof of concept, a method of coupling TRAIL to PLA microbubbles was established using a maleimide reaction with a BMPH heterobifunctional spacer arm (8.1 angstroms in length). Control samples were also made with TRAIL but without the BMPH chemical crosslinking agent in the conjugation process, therefore any TRAIL present is not chemically linked to the bubble, only adsorbed.

Briefly, to conjugate TRAIL to the microbubbles using maleimide chemistry, 60mg of freeze-dried microbubbles were suspended in 4ml MES buffer. 1ml of deionized water was added to 14.86mg of BMPH (25mM) and 1ml of deionized water was also added to 19.17mg EDC (50 mM). Equal amounts of the BMPH solution (0.5ml) and the EDC solution (1ml) were added to microbubble suspensions and shaken on end-over-end for 30 minutes to activate surfaces. Microbubbles were collected via centrifugation, washed 3 times to remove unreacted EDC, and resuspended in PBS. TRAIL was added (1.2μg) and suspension shaken end-over-end for 1.5 hours. Resulting TRAIL conjugated microspheres were collected via centrifugation, washed 3 times to remove unbound TRAIL, flash frozen, and lyophilized for 48 hrs. For a control microbubble group with only physically adsorbed TRAIL, the same process was repeated with the omission of chemical linkers EDC and BMPH.
5.1.2 Cellular studies with TRAIL-microbubbles and Velcade

To establish a proof of concept we used a breast cancer cell line, MDA-MB-231, which has receptors for TRAIL and with which we had significant experience. Once confirming that it was possible to induce apoptosis by TRAIL-Mb in breast cancer cells by fluorescent microscopy, we chose to expand the focus of this thesis to other cancers, namely ovarian cancer and melanoma.

In our second experiment we grew these two cancer cell lines, OVCAR-3 and A2058, and evaluated the amount of live, dead and apoptotic cells once exposed to TRAIL-Mb at various time points using fluorescent microscopy.

Based on these findings we decided to explore options for enhancing the apoptosis capability of our TRAIL-Mb. Proteasome inhibitors have emerged as a way to enhance TRAIL based treatments [89, 90, 95]. The mechanism by which proteasome inhibitors enhance TRAIL apoptosis is explained in detail in figure 2.3. Briefly, TRAIL triggers an external apoptosis mechanism by creating a caspase cascade once bound to its DR4/DR5 receptor. However, not all tumors respond to TRAIL. This lack of response may be attributed either to unfavorable ratios of death and decoy receptors or because of intracellular resistance mechanisms [112]. With respect to intracellular resistance mechanisms, the FLIP protein has been identified as a blocker of apoptosis induced by TNF family death receptors. FLIP binds to and neutralizes pro-caspase and pro-apoptotic proteins normally recruited to the cytosolic domains of apoptosis-inducing TRAIL receptors upon ligand stimulation, thus interrupting early steps in TRAIL signaling. Furthermore, overexpression of FLIP protein has been documented in cancers.

Proteasome inhibitors like Velcade trigger angiogenesis intrinsically by spurring the
release of pro-apoptotic proteins from mitochondria. Therefore, the internal signaling mechanism of Velcade helps overcome TRAIL intracellular TRAIL resistance and synergistically causes apoptosis in cancer cells. Millenium Pharmaceuticals graciously supplied us with their proteasome inhibitor, Velcade, which is currently undergoing clinical trials.

Looking for a robust way to quantitatively measure the apoptotic effects of TRAIL-Mb and TRAIL-Mb + Velcade on Ovarian and Melanoma cancer cells, we decided to move from fluorescent microscopy to flow cytometry. Our third experiment is the flow cytometry based apoptosis measurement of ovarian and melanoma cancer cells treated with TRAIL-Mb with and without Velcade.

Prior to starting studies with TRAIL-Mb and Velcade we performed several proof of concept experiments of TRAIL-Mb induced apoptosis which are discussed in the sections following.

5.2 TRAIL-induced apoptosis in breast cancer cell model

MDA-MB-231 breast cancer cells were grown in 48 well plates. TRAIL conjugated microbubbles were incubated (0.5mg/ml) with MDA-MB-231 breast cancer cells and apoptosis was assessed at 3, 24 and 48 hours. Several TRAIL induced apoptosis studies assessed apoptosis at 24 hours [85, 89, 113]. Therefore this time point was tested along with an earlier and later time point to observe shorter and longer term effects. We used an apoptosis assay that detects the externalization of phosphatidylserine in apoptotic cells using green-fluorescent Alexa Fluor 488 annexin and red-fluorescent propidium iodide nucleic acid stain. Propidium iodide stains necrotic cells with red fluorescence.
After treatment, live cells are negative for both annexin V and PI, early apoptotic cells are positive for annexin V (green) only and late apoptotic and necrotic cells are positive for both dyes (green and red). The results seen in figure 5.2 demonstrate that there is little apoptosis in the controls of unmodified bubbles and bubbles made without crosslinking agents (figure 5.2, A&B respectively). The control group of TRAIL applied directly to the cells (no microbubbles present) and the experimental group of microbubbles conjugated with TRAIL (figure 5.2, C&D respectively) showed similar amounts of apoptotic activity (p= 0.58) as quantified by counting the fluorescently labeled apoptotic cells in each population (assay performed at 3 hours, t-test, n=3).

Figure 5.2: Fluorescence Imaging of MDA-MB-231 human breast cancer cells incubated with A) unmodified microbubbles B) adsorbed TRAIL on microbubbles (conjugated without chemical linker) C) TRAIL ligand alone (no microbubbles) D) Microbubbles conjugated with TRAIL. Timepoint for all images= 3 hrs. Green indicates apoptotic cell, red indicates dead cell, and green & red together indicates late apoptotic or dead cell.

Therefore we have demonstrated the proof of concept that TRAIL conjugated to a polymeric ultrasound contrast agent can induce apoptosis in breast cancer cells and that conjugating the TRAIL ligand to a CA not diminish its abilities as a pro-apoptotic agent. For our next experiment we move from breast cancer to ovarian and melanoma cancer cells. We also add a live-cell stain to evaluate the relative amount of live cells in each treated population.
5.3 TRAIL induced apoptosis on melanoma and ovarian cancer cells by microscopy

Two cell lines were chosen for this experiment. OVCAR-3 ovarian cancer cells are epithelial type derived from adenocarcinoma. They are resistant to clinically relevant concentrations of adriamycin, melphalan and cisplatin and often used as a model system in which to study drug resistance in ovarian cancer [114]. OVCAR-3 are known to have DR4 receptors as reported by Siervo et al. [113]. A2058 melanoma skin cancer cells are derived from metastatic lymph node site. They are noted to be a highly invasive cell line [114], and have also been shown to have DR4 receptors present on their surface [115].

To qualitatively assess apoptosis induced by Mb-TRAIL by fluorescent microscopy, OVCAR-3 and A2058 cells were first seeded in 48 well plates (n=3) and at 80% confluency were aspirated and treated with modified media supplemented with 0.5 mg/ml Mb. Sample groups included untreated cells, microbubbles only, TRAIL only, TRAIL- microbubbles made without crosslinkers, and TRAIL modified microbubbles. At timepoints 3, 12, and 24 hrs modified media were aspirated off the cells and cells were washed. The study timepoints were shortened to 24 hrs from the previous breast cancer cell study because of the non-sterile microbubbles. Following protocol from Invitrogen (Vybrant apoptosis assay kit #2 V13241), cells were stained with Annexin V (green), PI (red), and live cell stain Hoescht (blue) dye.

Fluorescent Microscopy results for A2058 melanoma are depicted in figure 5.3 and from OVCAR-3 are seen in figure 5.4. Green color indicates apoptotic cell, pink color indicates dead cell, green/pink indicates late apoptotic or dead cell and blue indicates live cell. Photographs are all at 24 hrs incubation.
Figure 5.3: Fluorescence Imaging of OVCAR-3 ovarian cells A) untreated  B) treated with Mb-only C) adsorbed TRAIL on microbubbles (conjugated without chemical linker)  D) TRAIL ligand alone (no microbubbles) E) Microbubbles conjugated with TRAIL. Timepoint for all images= 24 hrs. Green indicates apoptotic cell, pink indicates dead cell, green & red together indicates late apoptotic or dead cell, blue indicates live cell. 10x, sizebar=200um
Figure 5.4: Fluorescence Imaging of A2058 Melanoma cells A) untreated  B) treated with Mb-only C) adsorbed TRAIL on microbubbles (conjugated without chemical linker)  D) TRAIL ligand alone (no microbubbles) E) Microbubbles conjugated with TRAIL. Timepoint for all images= 24 hrs. Green indicates apoptotic cell, pink indicates dead cell, green & red together indicates late apoptotic or dead cell, blue indicates live cell. 10x, sizebar=200um
These results were very encouraging. Both OVCAR-3 and A2058 experienced greater amounts of apoptosis once exposed to TRAIL-Mb than Mb alone and Mb with adsorbed TRAIL, as measured by visual inspection. Also, it appears that they also experience similar or greater amounts of apoptosis than treatment with TRAIL alone (at an equal concentration to that present on the Mb in each sample), though this statement will have to be confirmed with quantitative evidence (flow cytometry).

It should be noted that even after 24 hours of incubation with TRAIL and TRAIL-modified Mb there is a large population of alive (blue) OVCAR-3 and A2058 cells resistant to apoptosis. Ovarian cancer cells are known to be resistant to common anti-cancer agents, however they have had some limited success with TRAIL based therapy [116]. Researchers have examined sensitizing OVCAR-3 to TRAIL induced apoptosis using proteasome inhibitors [116]. Melanoma cells are more susceptible to TRAIL-induced apoptosis, however apoptosis may also be enhanced in this population using a proteasome inhibitor like Velcade.

5.4 TRAIL induced apoptosis on melanoma and ovarian cancer cells by flow cytometry

To quantitatively assess the amount of apoptosis induced by TRAIL-Mb and TRAIL-Mb + Velcade of OVCAR-3 cells and A2058 cells, flow cytometry studies were performed.

To prepare OVCAR-3 and A2058 cells were first seeded in 12 well plates (n=3) and at 80% confluency were aspirated and treated with TRAIL, Velcade, microbubble modified media, or combinations of these treatments. Sample groups included: Untreated
cells, cells treated with Velcade only (1μM), cells treated with TRAIL only (100ng/ml),
cells treated with Velcade + TRAIL, cells treated with microbubbles (0.5mg/ml) only,
cells treated with microbubbles + Velcade, cells treated with TRAIL-modified
microbubbles (0.5mg/ml), and lastly cells treated with TRAIL-modified microbubbles +
Velcade.

After 24 hours of incubation, cells were aspirated (saving the old media that may
contain dead/apoptotic cells) and trypsinized. 100ul of well mixed cells was added to
100ul of Guava Nexin Reagent consisting of Annexin V and 7-AAD stain, and incubated
for 20 mins at room temperature in the dark.

Measurement of apoptosis in cell populations was achieving using a Guava EasyCyte
flow cytometer. Cells were diluted to 500 cells/μl prior to measurement. Data was
analyzed using Guava analysis software.

The analysis from Guava software plotting Annexin V vs. 7-AAD staining for
OVCAR-3 cells is displayed in figure 5.5, and A2058 in figure 5.7. In these diagrams
viable cells appear in the bottom left hand quadrant, apoptotic cells in the bottom right
hand quadrant and dead/necrotic cells in the top right quadrant. Quadrants were
determined by first calibrating the instrument with a positive and negative control. The
 corresponding numerical data appears in graphical form in figure 5.6 for OVCAR-3 and
in figure 5.8 for A2058.
Figure 5.5: Flow cytometry dot plots of Annexin V staining vs. 7-AAD of OVCAR-3 ovarian cancer cells after treatment at 24 hours with (A) Untreated (B) TRAIL only (C) Velcade Only (D) TRAIL + Velcade (E) unmodified microbubbles (F) unmodified microbubbles + Velcade (G) TRAIL modified microbubbles (H) TRAIL modified microbubbles + Velcade. Top right quadrant refers to dead cells, bottom left with viable cells, and bottom right with apoptotic cells.

Figure 5.6: Cell death of OVCAR-3 ovarian cancer cells after 24 hour treatment with TRAIL, Velcade, Unmodified microbubbles, TRAIL modified microbubbles or combinations of treatments as indicated. ± standard error of the mean, n=3.
**Figure 5.7:** Flow cytometry dot plots of Annexin V staining vs. 7-AAD of A2058 melanoma cells after treatment at 24 hours with (A) Untreated (B) TRAIL only (C) Velcade Only (D) TRAIL + Velcade (E) unmodified microbubbles (F) unmodified microbubbles + Velcade (G) TRAIL modified microbubbles (H) TRAIL modified microbubbles + Velcade. Top right quadrant refers to dead cells, bottom left with viable cells, and bottom right with apoptotic cells.

**Figure 5.8:** Cell death of A2058 melanoma cells after 24 hour treatment with TRAIL, Velcade, unmodified microbubbles, TRAIL modified microbubbles or combinations of treatments as indicated. ± standard error of the mean, n=3.
Flow cytometry of treated OVCAR-3 cells revealed that TRAIL-Mb have significant (p<0.05) apoptotic effect as compared to control of unmodified-Mb. This finding is in agreement with florescent microscopy observations. However, there was not a significant (p>0.05) synergistic apoptotic effect from TRAIL and Velcade treatment by themselves or Mb-TRAIL + Velcade, though there was a slight additive effect.

OVCAR-3 has shown limited sensitivity to TRAIL. Lane et al. states that intrinsic (natural) resistance to TRAIL-induced apoptosis occurs in 50% of ovarian carcinoma cell lines [116] and that the molecular mechanisms underlying this resistant TRAIL phenotype in human ovarian carcinoma cells is still unclear. Unlike the results we found, Lane et al. found that the addition of proteasome inhibitors to a TRAIL-resistant line of OVCAR-3 cells they created resulted in 40–50% more cell death at 24 hrs [116]. However, we did find cell viability results similar to that of Kim et al [112] after treatment with TRAIL. They found that after 24 hours of incubation with 100ng/ml of TRAIL, OVCAR-3 had a cell viability of 30%, which is in agreement with our result that 70% of OVCAR-3 cells died after 24 hrs of TRAIL (100ng/ml) incubation.

The A2058 cells treated with Mb-TRAIL + Velcade had significantly (p<0.01) more cell death than Mb-TRAIL. This phenomenon was also observed in the control groups of Velcade and TRAIL only on A2058 cells. The cells treated with TRAIL (100ng/ml) and Velcade (1μM) underwent apoptosis significant (p<0.001) from TRAIL treatment or Velcade treatment alone. Therefore, flow cytometry of treated A2058 cells revealed that there was a significant TRAIL + Velcade apoptotic effect. Lastly, it was confirmed as observed in fluorescent microscopy that cells treated with Mb-TRAIL underwent apoptosis significantly different (p<0.01) from unmodified Mb.
Though there is not literature on the TRAIL sensitization of A2058 cells by proteasome inhibitors, there is a study of TRAIL sensitization of A2058 cells by E1A oncogene [117]. Since E1A does not function within the ubiquitin-proteasome pathway, their results are not comparable to our results with Velcade. However, in this study by Routes et al. they found that ~50% of A2058 melanoma cells underwent cell death after treatment with 100ng/ml TRAIL (16hrs) [117]. This is very similar to our finding that 50% of A2058 cells died after treatment with 100ng/ml TRAIL (24 hrs).

5.5 Conclusion: A signal-inducing ultrasound contrast agent

In this research we have successfully developed a signal-inducing ultrasound contrast agent using TRAIL/apoptosis as a model. There is benefit to having TRAIL targeted to receptors via a microbubble platform. For the microbubble, TRAIL is a relatively biologically safe targeting ligand for systemic administration because it exerts its effects selectively in cancer cells. For TRAIL, an ultrasound contrast agent platform could allow the ligand to reach its targets outside the leaky tumor vasculature and be retained via EPR effect. Also, the apoptosis of the targeted cancer cells could be enhanced by cavitation of the microbubble under insonation causing membrane damage and cell death. Lastly, for cancers that are TRAIL resistant having a platform with dual TRAIL and Velcade delivery would be ideal. That is, Velcade could be loaded into the shell of our microbubbles and TRAIL bound to its surface, allowing the exploitation the synergistic apoptotic effect of TRAIL and Velcade if it exists for the particular cell line.
6. RESULTS AND DISCUSSION: AN ULTRASOUND CONTRAST AGENT FOR OVARIAN CANCER DETECTION

6.1 Ultrasound contrast agent for ovarian cancer detection

The goal of the third specific aim in this thesis is the development of an ultrasound contrast agent for detection of ovarian cancer.

Ovarian cancer (OC) is the second most common and most lethal gynecologic malignancy in the USA [96]. Over 70% of women with OC are diagnosed with advanced stage disease, with a 5 year survival rate 30%. This 5 year survival rate is 90% when diagnosed early (disease confined to the ovaries), but only 25% of cases are found in this early stage. Partly due to a lack of symptoms and early screening procedures, OC incidence rates remain high and mortality rates are virtually unchanged over the last 30 years [96]. Clinicians currently screen for OC by measuring CA-125 levels in the blood (elevated in most women with OC) in combination with transvaginal ultrasound. However, there is a need for more robust early OC detection methods.

We decided to pursue this goal using CA-125 as our model target receptor. CA-125 is a biomarker for ovarian cancer. CA-125 is expressed as a membrane-bound receptor at the surface of cells or is released in soluble form into bodily fluids [96]. CA-125 is a biomarker with a broad range of utility; risk, early detection, diagnosis, prognosis and monitoring. It also represents an attractive therapeutic target and therefore is studied as a target in this thesis. In order to target this receptor we chose to use as a ligand the anti-CA-125 antibody, OC125.
There is no reported research on the development of a targeted ultrasound contrast agent for ovarian cancer. There are however, several CA-125 therapies under development that support the possibility of creating a CA-125 antigen targeted contrast agent using an anti-CA-125 antibody. Researchers have demonstrated target-specific polymeric micelles that can detect specific target proteins such as ovarian cancer-specific antigen CA-125 in vitro from serum [118]. Targeted therapies with anti-CA-125 antibodies conjugated to anti-cancer drugs are currently under study in animal models. A recent publication compares the toxicity and efficacy of two such antibodies and reported efficacy in vitro and in vivo [99]. Lastly, efforts have been undertaken to develop anti-CA-125 antibodies specific for the cell-associated form of the antigen, which is of particular interest in the future for a targeted applications such as ours [100].

### 6.1.2 Project Summary and Challenges

This project’s primary challenge was finding a proper method for antibody conjugation that would allow the antibody to be efficiently coupled to the microbubble in a manner that would not affect its bioactivity. The project has had several setbacks. This section will describe the actions/experiments taken to correct them.

To summarize what will be described in this chapter, it was decided that digestion and/or digestion and reduction of the antibody would allow it to be conjugated to the bubble at its hinge region only using a maleimide coupling reaction. Anti-CA-125 antibody was from Biocare, Inc and was reduced or reduced/digested and maleimide conjugated to bubbles. Immunohistochemical (IHC) studies were performed by incubating bubbles on slices of CA-125 positive tumor tissue and treating with secondary antibody
chromophore to see if the CA-125 targeted bubbles bound specifically to CA-125 positive areas of slices. Unfortunately the IHC process was too harsh for the bubbles, requiring solvents that dissolved them. However, it was discovered that in sample tissues treated with the reduced Ab-microbubbles CA-125 areas were lightly stained, indicating post conjugation activity of reduced but not reduced digested antibody, so reduced antibody only was chosen for future studies.

The static cell attachment study of digested antibody microbubbles revealed no significant cellular adhesion. Re-evaluating our processes we discovered that the protein BSA was used by the suppliers as a preservative in the CA-125 antibody and, therefore BSA may interfere with the conjugation of CA-125 to our bubbles. Co-conjugation could easily obscure the antibody active sites, preventing close contact between the ligand and the cell surface receptor. A BSA-free anti-CA-125 antibody supplied by QED Biosciences was identified, This antibody was reduced, conjugated to the microbubbles, and the resulting activity was studied via a cellular attachment study. Neither method demonstrated significant results. Since the reduced Ab was bound to the surface at the hinge region, we concluded that perhaps the remaining Fc fragment of the reduced Ab is causing steric hindrance, blocking the Ab’s active site and reducing bioactivity. We decided to move on and attempt conjugating the full anti-CA-125 antibody using a carbodiimide reaction. Conjugating full antibodies by carbodiimide chemistry for targeting purposes has been used successfully by other researchers, but it has the drawback of random conjugation. Ottoboni et al. used a carbodiimide conjugation to create solid microspheres linked with full antibodies directed towards ICAM-1 and VCAM-1 [119]. By assay, we found that we were able to achieve excellent conjugation
efficiency of the full antibody to the microbubbles using this strategy. Finally, we performed static attachment studies with full anti-CA-125 antibody conjugated microbubbles and found significant binding to CA-125+ and not CA125- cells.

6.2 Experimental methods and results

6.2.1 Antibody preparation and maleimide conjugation

Commercially available anti-CA-125 antibody was obtained from Biocare, Inc. and two fragments of the antibody were made for further testing, a reduced antibody (Fab retaining the Fc portion) and a reduced and digested antibody (Fab). Procedures were followed according to manufacturer’s guidelines (Antibody fragmentation kit, Pierce, Rockford, IL) and are roughly as described in figure 6.1.

![Figure 6.1: Schematic of antibody digestion and reduction.](image-url)
Antibodies were fragmented in order to expose the free thiol groups in the hinge region for conjugation. Fragmented antibodies were conjugated to the surface of the microbubbles via a maleimide reaction. Briefly, 60mg of bubbles was added to 4ml MES buffer (5.2pH). 14.86mg BMPH and 19.17mg of EDC were added to 1 ml of deionized water (for 25umol BMPH, 50umol EDC solutions), equal solutions (0.5ml each) of BMPH and EDC were added to the bubble/MES buffer solution and end-over-end mixed for 30 mins for activation of groups. Solutions were centrifuged, resuspended and washed 3x. After final wash bubbles were resuspended in 4ml of PBS buffer and antibody solutions added. After end-over-end shaking for 3hrs solutions were centrifuged and bubbles washed 3x, frozen and lyophilized.

6.2.2 Immunohistochemical analysis

To test the hypothesis that microbubbles conjugated with CA-125 antibody fragments will adhere specifically to areas of CA-125 expression in ovarian cancer tissue IHC was performed. Tumors were excised from patients (Dr. Brooks, DUCOM), sliced, fixed, and assayed to determine CA-125 expression. The slices were made serially to ensure they would have corresponding areas of CA-125 expression. CA-125 was stained by applying anti-CA-125 antibody and applying a secondary antibody/chromophore (DAB). Tissue slices were exposed to digested and reduced anti-CA-125-Mb, reduced anti-CA-125-Mb, and unmodified Mb (control) prior to IHC chemical staining. Areas of the Mb attachment will be correlated with known CA-125 positive areas through visual inspection. A schematic of the envisioned staining procedure is seen in figure 6.2.
Figure 6.2: Schematic of immunohistochemical studies. Full anti-CA-125 antibodies and microbubbles with fragmented antibodies will bind to CA-125 positive cells. A secondary Ab with chromophore will attach to the anti-CA-125 antibodies and create a brown stain. Unmodified microbubbles will not cause staining.

The resulting micrographs were not as expected. Microbubbles were not present, and it was assumed they were dissolved in the solvent based washing steps of the IHC procedure. Staining of the CA-125 receptors was successful in tissue, seen as the brown colored areas in figure (6.3, A). Though microbubbles were not seen in the microbubble incubated tissue slices (data not shown), CA-125 staining was visible in tissue slices incubated with reduced-antibody microbubbles as seen in figure (6.3, B). This staining was not seen in reduced-digested samples (figure 6.3, C), therefore we decided to discontinue reduced and digested antibody and continue studies with reduced antibody. Based on the results of this experiment, IHC analysis of microbubble cellular adhesion was discontinued. Because of the harsh solvents and numerous wash steps involved in IHC, we chose to move onto static attachment studies to evaluate microbubble cellular adhesion.
Figure 6.3: Immunohistochemical staining of excised CA-125 receptor positive tumor tissue with secondary antibody- DAB chromophore. (A) anti-CA-125 antibody (B) reduced anti-CA-125 antibody conjugated microbubbles (C) reduced and digested anti-CA-125 antibody conjugated microbubbles.

6.2.3 Static attachment studies of reduced-anti-CA-125 antibody microbubbles

We discovered through a first unsuccessful static attachment study that we have BSA preservative in our bulk solvent of CA-125 antibody that may interfere with conjugation. We changed antibody manufacturers to QED Biosciences, Inc. The new CA-125 antibody was reduced and maleimide conjugated to the microbubbles. Another static attachment study was performed using OVCAR-3 (CA-125+) and A2058 (CA-125-) cells.

Cells were plated in 48 well plates (n=3). Reduced-CA-125 antibody conjugated microbubbles and unmodified microbubbles (0.5 mg/ml) were incubated on the each cell lines for 0, 15, 30, and 60 minutes. After each time point cells were washed and imaged with light microscopy. Attachment was assessed through visual inspection.

The results showed that there was no significant attachment of any microbubble population to CA-125+ or CA-125- cells. We decided to test the activity of the reduced-antibody once conjugated to the bubbles by determining its antigen binding ability.
6.2.4 Conjugation of Full anti-CA-125 antibody, with incorporated Nile Red

Because of our unexpected results using fragmented antibodies we decided to try conjugating the full, unfragmented anti-CA-125 antibody to our microbubbles by carbodiimide chemistry. This method has been used successfully by other researchers to conjugate antibodies to particles, but it has the drawback of random conjugation to amine groups present on the antibody.

Prior to conjugation it was also decided to incorporate nile red, a marker not fluorescent in aqueous phases, into microbubbles for imaging purposes. Nile red was added to organic phase of the first emulsion 1:100 w/w ratio with PLA.

Conjugation of the full antibody was completed by suspending nile-red loaded freeze-dried microbubbles (50mg) in 2ml of MES buffer (pH 6.0) in a centrifuge tube. To this 0.8mg of EDC (2mM) and 2.2mg of NHS (5mM) was added. The solutions were shaken on an end-over-end shaker for 30 mins for EDC activation of microbubble surfaces. The solutions were then centrifuged and washed 3 times to remove excess EDC. 100ug of full anti-CA-125 antibody was added to the sample. Samples were shaken end-over-end for 3 hours, centrifuged and washed 3 times with PBS buffer (pH 7.0), frozen and lyophilized for 48 hours.

6.2.5 Characterization of full anti-CA-125 antibody on microbubbles

By antibody assay, we found that we were able to achieve excellent conjugation efficiency of the full antibody to the microbubbles. Supernatant from the 3 wash steps in the conjugation procedure was concentrated in amicon tubes with a molecular weight
cutoff filter and then diluted into 96 well plate. Pierce Easy-Titer antibody assay kit beads were applied, incubated on a shaker and absorbance measurement by made by plate reader. The Easy-Titer IgG assay kit (Pierce) measures concentration of protein by absorbency. The assay uses monodispersed polystyrene beads coated with anti-IgG Ab that absorb light at 340 and 405 nm. When beads are mixed with a sample containing IgG, they aggregate, causing decreased absorption of light. Low IgG concentrations yield high absorbance values and high IgG concentrations yield low absorbance values. The decrease in absorption is proportional to IgG concentration.

We developed a standard curve with full CA-125 antibody, figure 6.4. The calculated absorbance was 0.88±0.05 (n=6). We calculated, based on pierce protocol, that approximately 70μg of the original 100μg of full CA-125 antibody was conjugated to the bubbles; a 70% conjugation efficiency.

![Figure 6.4: Standard curve of absorbance vs. CA-125 antibody concentration for antibody assay, Easy-Titer kit (Pierce, Rockford, IL).](image)
6.2.6 Static Cellular Attachment Study of full anti-CA-125 conjugated microbubbles

The last experiment of this chapter is the evaluation of cellular attachment to OVCAR-3 (CA-125+) and A2058 (CA-125-) cells by full anti-CA-125 antibody conjugated microbubbles. We have confirmed that these bubbles have antibody conjugated to their surfaces. We proceeded by seeding the two cell lines in 48 well plates. Full-CA-125 antibody conjugated microbubbles and unmodified microbubbles (0.5 mg/ml) were incubated on the each cell lines for 0, 15, 30, and 60 minutes. After each time point cells were washed and imaged by both light and fluorescent microscopy. Attachment was assessed through visual inspection; counting the number of nile red fluorescent microbubbles attached to the cells. An example image of the nile-red loaded targeted anti-CA-125 antibody microbubbles binding to ovarian cancer cells (15 minute time point) is seen in figure 6.5.
Figure 6.5: Light micrograph with fluorescent micrograph overlay of OVCAR-3 cells at 15 mins exposure to anti-CA-125 antibody conjugated microbubbles.

Quantification of cellular attachment demonstrated that using the full-CA-125 antibody as a ligand on our microbubbles enabled our microbubbles to bind significantly to CA-125 positive ovarian cancer cells. Significant cellular attachment of antibody conjugated microbubbles over controls was observed at 30 min (p<0.05) and 60 min (p<0.01) as seen in figure 6.6.
Figure 6.6: Microbubble/cellular attachment of anti-CA-125 antibody conjugated microbubbles or unmodified microbubbles on CA-125+ (OVCAR-3) or CA-125- (A2058) cells at 0, 15, 30 and 60 mins incubation. ± standard error of the mean.

6.3 Conclusion: targeted ultrasound contrast agent for early detection of ovarian cancer

This research presents a novel method that can potentially be used for early detection of ovarian cancer. Currently, there is no published research on a targeted ultrasound contrast agent for ovarian cancer. This technology could perhaps be used for intraperitoneal injection for surface lesion detection since epithelial ovarian cancer comprises the majority of malignant ovarian tumors [96]. Targeting could also be possible if the contrast agent were able to escape out of the leaky tumor vasculature, a possibility that may be enhanced by ultrasound treatment. Lastly, this targeting technique could perhaps be combined with another therapy for ovarian cancers such as loading of Velcade/TRAIL for targeted delivery to chemo-resistant ovarian cancer tumors.
7. CONCLUSIONS AND FUTURE RECOMMENDATIONS

7.1 Conclusions and Contributions to Science

The overall objective of this thesis was to develop \textit{in vitro} improved PLGA based microbubble CAs that facilitate efficient targeting to molecular markers enabling the early detection and potentially treatment of malignancies.

This research examined the engineering of particle surfaces. It demonstrates that adding modifiable carboxyl groups to surfaces via a surfactant allows more targeting ligand to be conjugated, but does not improve cellular targeting. It presents a physical method to add modifiable PEG-biotin surface groups successfully. Also developed were methods to produce rough surfaced microbubbles which had significant cellular binding over smooth surfaced microbubbles once modified.

Introduced was a novel ultrasound contrast agent that acts via signal transduction. The TRAIL-modified agent induced a signaling cascade resulting in apoptosis in breast, ovarian and skin cancer cells. Furthermore, TRAIL-modified agents exhibited synergistic apoptotic effects when combined with the proteasome inhibitor Velcade.

Lastly, this thesis presents an ultrasound contrast agent targeted to CA-125 receptors in ovarian cancer which has the potential to enable early detection of the disease.

The various aspects of this research are being conducted in hopes of combining them in the future with drug delivery. This would bring together the advantages of site-targeting, drug delivery, and signal transduction along with the valuable benefits of ultrasound.
Overall in the field of ultrasound contrast agents this research is unique because of its hollow polymeric contrast agent platform, new adaptable surface functionalization techniques, cellular signal-inducing capabilities and its novel targets and ligands for breast, ovarian and skin cancer detection and treatment.

7.2 Future Recommendations

Future recommendations for research include combining the cellular targeting methods developed in this thesis with ultrasound-triggered drug delivery. Drugs could be loaded into the microbubble prior to conjugation with a targeting ligand. Once ligated these receptor-targeted drug loaded contrast agents could be exposed to cells and drug delivery assessed. An early proof of concept is presented in appendix E.. Once these methods are established, the research could continue into small animal investigations to establish \textit{in vivo} safety and efficacy of these targeted drug delivery agents while under insonation.

Specific Aim 2 presents the never before reported use of TRAIL as surface bound ligand on an ultrasound contrast agent, creating an apoptotic signaling agent. Hence, this is also the first report of proteasome inhibitors (Velcade) being used in combination with a TRAIL-ligated ultrasound contrast agents for exploitation of the synergistic apoptotic effect of proteasome inhibitors + TRAIL. Having the TRAIL on an ultrasound contrast agent may also potentiate the apoptotic effect, as there are reports that contrast agents by themselves can enhance apoptosis. Though delivery of Velcade on a polymer platform has not yet been reported, perhaps in the future a TRAIL-targeted contrast agent loaded
with Velcade could be created. If effective, this therapy could be used for a variety of malignancies resistant to traditional chemotherapy treatments.

The targeting methods developed in this thesis would also be beneficial for future adaptation to nano-scale agents. Moving to a nano-scale would allow easier extravasation out of tumor vasculature allowing the CAs to reach the tumor tissue. Being able to reach the tumor tissue opens the doors to targets that are not present within the vasculature. Binding to these targets by nanobubbles could be achieved by surface modification with ligands. This thesis demonstrates the adaptability of our polymeric ultrasound contrast agent for modification with targeting ligands (e.g. GRGDS peptide and anti-CA-125 antibodies) and signaling ligands (e.g. TRAIL).

Lastly, sterilization methods should also be explored. Most sterilization methods, especially those involving heat, lead to polymer degradation and subsequent loss of acoustic enhancement from our microbubbles. Recently, plasma sterilization has emerged as a possible way to sterilize our microbubbles. This method has the potential benefit of creating functional groups (carboxyl) on the microbubble surface. The effectiveness of the plasma as a sterilization method should be investigated, along with the changes in functional groups on the surface, which may also alter the cellular attachment properties of the contrast agent once ligated.
REFERENCES


Appendix A: Protocol for Preparing Microcapsules

Preparing fresh microcapsules is essential for all studies and takes about three hours if done in triplicate.

1. Weigh out the following chemicals: polymer (0.5g, record lot number as well), and porogens ammonium carbamiate (0.78g) or ammonium carbonate (0.4g) and camphor (0.05g-if used)
   a. Note: Polymer should always be brought to room temp. before opening so that condensation does not form on the beads and cause degradation. For convenience you should take ~5g out of the stock solution and place it in a sealed glass vial in the refrigerator.

2. Combine the polymer and 10ml of methylene chloride in a beaker (40ml max volume) with a stir bar and stir on a magnetic stir plate, cover with a layer of wax paper topped by a layer of parafilm, until camphor and polymer are dissolved (~15min).
   a. Note: The ratio of polymer to solvent should is important. If the cover opens during stirring, some methylene chloride may evaporate. You should be careful, but if needed you can add more methylene chloride to bring the level to 10ml after stirring. Also, remember to wear gloves when using methylene chloride.

3. Combine porogen with 10ml of deionized water in a beaker (40ml max volume) with a stir bar and stir on a magnetic stir plate.

4. Measure out 50ml of 5% poly vinyl alcohol (PVA) and put it in a beaker (600ml max volume) and place it in the refrigerator.
5. Measure out 100ml of 2% isopropyl alcohol (IPA) and keep it in the graduated cylinder.

6. When the camphor and polymer are dissolved, remove stir bar and put in 1ml of the porogen solution and sonicate with Misonix probe sonicator at 110W for 30 seconds. Sonicate in an ice bath 3 seconds on 1 second off.

7. After sonication, pour the solution into the cold PVA and homogenize with Brinkmann PT 3100 homogenizer with attached Polytron PT-DA 3020/2 generator for 5 minutes at 9,500 rpm.

8. After homogenization, pour the 100ml of IPA in the solution, add a larger stir bar, and stir on a magnetic stir plate for 1 hour. Make sure the vortex spans the diameter of the beaker so the solvent can fully evaporate.
   
   a. After homogenization remove the blade and pour the IPA over it into the beaker to prevent the loss of yield.

9. After an hour, combine the solution in at least 4 centrifuge tubes (50ml max volume) and then centrifuge for 5 minutes at 5,000 rpm (the equivalent of approximately 5,000 g).

10. Decant the liquid and combine the microcapsules into 1 tube and recentrifuge for 5 minutes at 5,000 rpm.

11. Decant the liquid and wash the microcapsules three times with hexane. Lay the centrifuge tube under the hood and let excess solvent evaporate for about 30 min.

12. When the microcapsules appear to be thick and pasty, add water and recentrifuge for 5 minutes at 5,000 rpm.
13. Decant the water, mix (fluff) the microcapsules, put a kimwipe on top of the tube with a rubber band, and flash freeze using nitrogen (swirling the tube in nitrogen to create a cone shaped pellet).

14. Put the frozen microcapsules in a freeze dryer vessel and on the freeze dryer for at least 48 hours.
Appendix B: Chemical Conjugation Methods

Conjugating ligands to polymer microbubbles occurs after the microbubbles have already been made.

Part A: Carbodiimide Conjugation

1. Weigh out 50mg of freeze-dried microbubbles into a centrifuge tube
2. Weigh out 0.8mg of EDC and 2.2mg of NHS
3. Add 2ml of buffer to the centrifuge tube with microbubbles (0.1M MES pH 5.2)
4. To this solution add the 0.8mg of EDC (2mM) and 2.2mg of NHS (5mM)
5. Shake this solution on an end over end shaker for 30 mins for EDC activation of the microbubble surfaces.
6. Centrifuge the solution and wash to remove excess EDC
7. Add ligand: Add GRGDS peptide or 5-aminoacetamedio fluorescein at a 1:0.5 molar ratio of peptide to total polymer carboxyl groups. For antibody conjugation add 100ug of anti-CA-125 antibody
8. Shake the samples on an end-over-end shaker for 1.5 hours. If the sample is fluorescent, wrap the centrifuge tubes in aluminum foil to protect from light.
9. Centrifuge to collect and washed 3 times with buffer (PBS, pH 7.0) to remove unbound ligand.
10. Freeze the samples with liquid nitrogen, swirling the centrifuge tube to create a cone shaped pellet
11. Uncap the centrifuge tube, cover with a kimwipe and lyophilize the sample for 48 hours.
Part B: Maleimide Conjugation

1. Weigh out two 60 mg of freeze-dried microbubbles into a centrifuge tube

2. Add to the microbubble sample 4ml of MES buffer (pH 5.2)

3. Weigh out BMPH (14.86 mg, 25mM) and EDC (19.17mg, 50mM) and NHS (12mg, NHS:EDC ratio of 0.6) into eppendorf tubes

4. Add1ml DI water to each eppendorf tube (BMPH, EDC and NHS)

5. Add 1ml of each solution (BMPH, EDC and NHS) into the centrifuge tube containing the suspension of 60mg microbubbles in 4ml MES. For control samples made without crosslinkers, omit these agents.

6. Shake on end-over-end shaker for 30 mins.

7. Centrifuge and wash to remove unreacted BMPH (washed with PBS).

8. Resuspend pellet in 2mL of PBS

9. Add ligand: 1.2μg of TRAIL or fragmented antibody

10. Shake sample end over end for 1.5 hours

11. Add thioglycolic acid (5mM, 50μl) to vial to stop reaction

12. Shake sample end over end for 10 mins.

13. Centrifuge, wash 3x, flash freeze by swirling in tube in liquid nitrogen, and lyophilize for 48 hours.

Note: Thioglycolic acid (92.12 M_w). We want to use 5mM in the conjugation, but 0.347μl of acid is too small to measure out, so a stock solution is made and used by:
1. Make 0.1M stock solution by adding 5ul of acid to 720 ul of DI water.

2. Add 50μl to each centrifuge tube to stop maleimide conjugation process
Appendix C: Protocol for Conducting Dose and Time Response

The purpose of the dose response is to find the optimum dose where the ultrasound enhancement is the maximum. The purpose of a time response is to measure acoustic stability of the contrast agent. The time response should be done following a dose response at a dose less than the maximum dose on the linear portion of the dose response curve.

For Dose Response:

1. Weigh out 3mg of contrast agent and suspend it in 200µl of Phosphate Buffered Saline (PBS pH 7.4). This is your stock solution.
2. Fill the sample vessel with 100 ml of PBS and a small stir bar, turn on the oscilloscope and pulser, and focus the sample vessel.
3. For each dose, starting with 0.003mg/ml, inject 20µl of stock solution into the sample vessel and begin the LabView computer program.
4. Run the program.
5. For the next dose, 0.006mg/ml, inject another 20µl of stock solution into the sample vessel. Do not remove the original contents if this is a cumulative dose response.
6. Continue dose response up to 0.03mg/ml or until shadowing is evident.

Computer Operation for Dose Response:

1. Create a folder with appropriate subfolders.
2. Open up the Main Ultrasound Program from LabVIEW and then open up the Main Ultrasound Program.vi.

3. Using the open file program, choose a directory path in which the data should be saved by opening the folder path, selecting which folder to save the data in, and hitting select current directory.

4. To begin taking the dose response, push the Run button.

5. When completed, to view the results, first under the Analyze Data tab, push Export Calculated Text and chose a folder to save the data to.

6. To view results, open up the dose data file using Excel and the enhancement in will be displayed for each dose in mV and dB.

For Time Response:

1. Choose dose on the dose response curve that is not the maximum (it should be on the linear part of the curve) and prepare a solution of that concentration, using procedure described above in the dose response section.

2. Fill the sample vessel with 100 ml of PBS and a small stir bar, turn on the oscilloscope and pulser, and focus the sample vessel.

3. Inject dose into the sample vessel and begin the LabView computer program (Main Ultrasound Program.vi).

4. Run the program for 15 minutes.

Computer Operation for Time Response:

1. Create a folder with appropriate subfolders.
2. Open up the Main Ultrasound Program from LabVIEW and then open up the Main Ultrasound Program.vi.

3. Using the open file program, choose a directory path in which the data should be saved by opening the folder path, selecting which folder to save the data in, and hitting select current directory.

4. To active Time Response, select button next to “Capture waveform on set time interval” and set the interval and number of time points to acquire.

5. Hit run button to begin Time Response.

6. To view the results when completed, go to the Analyze Data tab, select Export Calculated Text and chose a folder to save the data to.

7. To view results, open up the time data file using Excel and for each minute the enhancement will be given in mV and dB.
Appendix D: Coulter Counter Data

Microbubble size and microbubble count per mg were measured with a Coulter Multisizer II. Microbubbles were diluted to 0.002mg in electrolyte and were drawn through a 30μm aperture. Particles displace their own volume, increasing the impedance of the aperture. Coulter Principle states that the pulse is directly proportional to the volume of the particle that produced it. The overall size range measurable with the Coulter Counter is 0.4μm to 1200μm. Each result given by the Coulter Counter is displayed graphically as a percentage of channel content, which can be selected to display measurements by volume(weight), number(population) or surface area.

Basic Coulter Multisizer II Operating Instructions:

1. Fill the buffer reservoir with Isoton II and empty the waste reservoir.
2. Turn on the power to the electrical unit and the sample stand. Allow the instrument to warm up 10 minutes.
3. Press SETUP and select manual. Verify that your settings for your count are correct. The manometer select should be set to 500μl.
4. Check the diameter of the orifice that is located on the aperture tube. The 30-50 micron tube is commonly used for microbubbles.
5. Remove the blue solution and install a small plastic beaker containing Isoton.
6. Turn the knobs from CLOSE to FILL and from COUNT to RESET. This allows the blue solution to be flushed from the aperture tube. Turn from RESET to COUNT and from FILL to CLOSE for the bottom knob. (Note: turn clockwise)
7. Press FULL. Remove the beaker containing Isoton and install sample after several inversions. (Note: Sample in PBS)

8. Press reset in order to clear the screen and turn the knob from COUNT to RESET. The light in the sample stand should come on and the Mercury level should drop. Turn the knob from RESET to COUNT. The instrument will stop counting when 500ul of sample pass through the orifice of the aperture tube.

9. When finished counting, install the plastic beaker containing the blue solution and turn the knob from COUNT to RESET. Turn the knob from RESET to COUNT. Repeat 4X.

Results of these measurements on PLA based microbubbles are presented in the figures D.1 and D.2.
Figure D.1: Coulter analysis. Size distribution of PLA microbubbles by (A) number (population)- mean diameter 1.45 μm, and (B) volume (weight)- mean diameter 4.53 μm.
Figure D.2: Coulter analysis results. For 0.002mg of PLA microbubbles there were approximately 70,000 microbubble counts. Therefore there are approximately 35,000,000 microbubbles/mg of the PLA contrast agent.
Appendix E: Proof of Concept Drug Delivery Experiments

This appendix describes the delivery of dye from surfactant blended microbubbles into 2D and 3D MDA-MB-231/MCF-7 cells.

Microbubbles made with PEMA blends were ligated with GRGDS. The freeze-dried bubbles were then labeled with a model for a membrane soluble drug; the cationic green dye DiOC2(3), (3,3’-diethyleneoxacarbocyanine iodide Mw 460.31 Da). A cationic dye was chosen due to negatively charged surface of the microbubbles.

The protocol for the adsorbing the dye to the microbubbles is as follows. 100mg of dye was dissolved in 2.3ml of DMSO (2M), a 2ul stock was added into 18.9 ml MES for a 30mM solution. 10mM of the solution was incubated with 25mg of bubbles, and shook end over end for 30min, washed vigorously 3 times with MES and freeze-dried.

To confirm fluorescence, bubbles were imaged under fluorescent microscope. As expected, the bubbles with the dye fluoresced, while the unlabeled bubbles did not (figure E.1, A&B).

To determine if the dye on the bubbles would be taken up by cells, MDA-MB-231 cells were grown in culture plates to 70% confluence and incubated with ligated dye-labeled bubbles, unligated dye-labeled bubbles, and bubbles without dye. As seen in figure (E.1, C&D) the dye from the bubbles is delivered to the membranes of the cells, while there was no fluorescence from the control cells incubated with unlabeled bubbles (not shown). This was true for all bubbles with various surfactant blends. There was no significant difference noted between experiment groups in regards to amount of dye delivered to cells. The dye was uptaken by the cells in a similar manner with both ligated and unligated populations of bubbles, therefore it was concluded ligation was not
essential for uptake. The model cationic dye was released from the bubbles regardless of ligation.

Tumors made of both MDA-MB-231 and MCF-7 cells were grown in vitro in a 3D bioreactor with the assistance of A. Vamvakidou. Tumors were incubated with microbubbles that were conjugated with targeting vector GRGDS and labeled with the model dye. Pictures were taken using a fluorescent microscope at 0, 10 and 20 mins. As seen in figure (E.1, E) GRGDS ligated microbubbles were present on the tumor and also released dye that was taken up into the tumor cells, figure (E.1, F). It should be noted however that specific binding due to GRGDS ligation could not be confirmed; the control of unligated bubbles were also present on the tumors. There was no noted difference due to type of surfactant used in fabrication. All tumors incubated with bubbles labeled with dye became fluorescent.

This is a proof of concept that a dye adsorbed to the bubbles ligated with GRGDS can be uptaken by cells.
Figure E.1: Fluorescent Micrographs. A) Bubbles labeled with cationic dye, light microscopy: inset, under fluorescent microscopy B) Unlabeled bubbles, light microscopy: inset, under fluorescent microscopy demonstrating fluorescence in dye labeled bubbles only. C) Light microscopy of MDA-MB-231 cells incubated with 0.5mg/ml dye labeled microbubbles, 10 minutes. D) Fluorescent microscopy of MDA-MB-231 cells incubated with 0.5mg/ml dye labeled microbubbles, 10 minutes demonstrating uptake of dye from bubbles into membranes of cells. E) Light micrograph of MDA-MB-231/MCF tumors incubated with 0.5mg/ml dye labeled microbubbles, 20 minutes F) Fluorescent micrograph of MDA-MB-231/MCF tumors incubated with 0.5mg/ml dye labeled microbubbles, 20 minutes demonstrating presence of bubbles and uptake of dye into tumors in vitro. Size bars = 50 microns.
Appendix F: Dose Response of PLA contrast agent insonated at 2.25, 5, 7.5 and 10 MHz

Dose response tests were performed using PLA microbubbles prior to (figure F.1) and after modification with GRGDS peptide (figure F.2) at the frequencies 2.25, 5, 7.5 and 10 MHz. These results confirm that 5MHz is an appropriate insonation frequency for the \textit{in vitro} characterization of the contrast agents both before and after ligand conjugation.

\textbf{Figure F.1}: Dose response curves of unmodified PLA contrast agent insonated at 2.25, 5, 7.5 and 10 MHz. \(n=3\), ± standard error of the mean
**Figure F.2:** Dose response curves of GRGDS ligated PLA contrast agent insonated at 2.25, 5, 7.5 and 10 MHz. n=3, ± standard error of the mean
VITA

Kelleny Oum has received her Bachelors of Science and Masters of Science degree in Biomedical Engineering from Drexel University in 2004. As an undergraduate she held co-op positions at Merck Research Labs and Dupont Hospital for Children. She pursued her doctoral degree immediately thereafter also at the School of Biomedical Engineering at Drexel University. As a first year graduate student she was awarded the prestigious National Science Foundation Graduate Research Fellowship as well as an American Heart Association Pre-Doctoral Fellowship.

While at Drexel Kelleny enjoyed presenting at various scientific conferences and garnered several awards for research including Best Research at Drexel Research Day, Best Poster at the International Society for Pharmaceutical Engineering’s Graduate Research Competition, and Best Poster at the BEACON BioNanotechnology Symposium.

She was involved in leadership, co-founding the Drexel Biomedical Graduate Student Association and was recognized by several organizations for leadership including being named Woman of the Year and Outstanding Alumnae Woman in Leadership by Drexel’s Panhellenic Council, and was given the Rita Schaffer National Undergraduate Leadership Award by the Biomedical Engineering Society.

Lastly, Kelleny aspired to be involved in all aspects of biotechnology, filing a patent and writing business plans focusing on the commercial perspective of technology. During her studies she helped create two ideas for companies, Focus Therapeutics and Puragent, for which she authored business plans and represented at competitions, allowing her and her team to be awarded Grand Prize at the Drexel University Business Plan Competition as well as top positions in the Wharton Venture Finals Competition, MIT $100K Entrepreneurship Competition, the Purdue National Life Sciences Business Plan Competition, and Grand Prize in the Loring Entrepreneurial Business Plan Competition.