Published in final edited form as:
Bubble Sci Eng Technol. 2009 November ; 1(1-2): 3–17. doi:10.1179/175889709X446507.

Microbubble Compositions, Properties and Biomedical Applications

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Abstract

Over the last decade, there has been significant progress towards the development of microbubbles as theranostics for a wide variety of biomedical applications. The unique ability of microbubbles to respond to ultrasound makes them useful agents for contrast ultrasound imaging, molecular imaging, and targeted drug and gene delivery. The general composition of a microbubble is a gas core stabilized by a shell comprised of proteins, lipids or polymers. Each type of microbubble has its own unique advantages and can be tailored for specialized functions. In this review, different microbubbles compositions and physiochemical properties are discussed in the context of current progress towards developing novel constructs for biomedical applications, with specific emphasis on molecular imaging and targeted drug/gene delivery.

Keywords

Microbubbles; Microballoons; Gas-filled Microparticles; Hollow Microspheres; Colloidal Bubbles; Spherical Foams; Ultrasound Molecular Imaging; Targeted Drug and Gene Delivery

I. Introduction

Colloidal bubbles (microbubbles) are emerging as important contrast agents for imaging and carriers for targeted drug delivery.[1–7] A schematic structure of the biomedical microbubble is presented in Figure 1. The diameter of a microbubble is approximately equal to the size of a red blood cell (less than ~10 μm diameter), which allows it to display similar rheology in the microvessels and capillaries throughout the body.[8] The gas core comprises most of the particle volume and provides the mechanism for ultrasound backscatter and drug delivery. Gas bubbles of this size in aqueous media are inherently unstable owing to surface tension effects,[9] and therefore require a stabilizing shell. The shell may be composed of surfactants, lipids, proteins, polymers, or a combination of these materials. Because the interior gas is a poor solvent for drug molecules, loading strategies must be employed within or onto the shell. This is accomplished in a variety a ways, as illustrated in this review.

The key to success for microbubbles as drug delivery vehicles is their extreme activity when exposed to ultrasonic waves. The gas core expands during the rarefaction phase of the pressure

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wave and contracts during the compression phase. Depending on the ultrasound parameters, various phenomena may occur that facilitate ultrasound backscatter and/or the release and local delivery of drugs from the microbubble shell. These phenomena are summarized below and range from subtle effects such as acoustic radiation force, to highly energetic events such as inertial cavitation. Combinations of these phenomena allow for imaging, targeting, controlled release and vascular permeability enhancement.

Microbubbles are *theranostic* agents. That is, they provide simultaneous and co-localized contrast for imaging (diagnostics) and drug carrying and delivering capacity for targeted therapy. In this case, the imaging modality and therapeutic trigger is ultrasound. While ultrasound may be focused to mm-scale dimensions (roughly the size of a grain of rice), the bubbles further focus the mechanical energy to microscale events distributed throughout the insonified vasculature. Improvements in both microbubble design and ultrasound devices and methodology have made this technology clinically relevant. Herein, we present some important concepts in the rational design of microbubbles and review recent results on their performance under various imaging and drug delivery scenarios.

II. Microbubble Compositions and Physicochemical Properties

Protein Shells

Albumin shelled microbubbles were a pioneering formulation used in contrast ultrasound imaging. They paved the way for several subsequent formulations that could pass the lung capillaries and provide contrast in the left ventricle of the heart. The first albumin microbubble formulation to be approved by the US Food and Drug Administration (FDA) was Albunex (GE Healthcare). An Albunex suspension consists of roughly $7x10^8$ microbubbles/mL with a size range from 1 to 15 μm diameter.[10] Albunex is stable upon refrigeration for at least two years. Albumin-coated microbubbles are formed by sonication of a heated solution of 5% (w/v) human serum albumin in the presence of air. During sonication, microbubbles of air are formed which become encapsulated within a 15-nm thick shell of aggregated albumin. Heating is necessary to denature the albumin prior to sonication and facilitate encapsulation. Biochemical analysis suggested that the shell is a monomolecular layer of native and denatured albumin in multiple orientations.[11] The albumin shell is held together through disulfide bonds between cystein residues formed during cavitation.[12] Covalent cross-linking may explain the relative rigidity of albumin shells observed during ultrasonic insonification.[13] Following Albunex, an albumin formulation was developed encapsulating a perfluorocarbon gas core, named Optison™ (GE Healthcare). The low solubility of the perfluorocarbon gas gave these microbubbles much longer circulation persistence *in vivo*.[14] Optison™ is currently approved by the US FDA for contrast echocardiography.

Several proteins other than albumin have been used to coat microbubbles. This is not surprising given the amphipathic nature of many proteins, which makes them highly surface active. Disulfide bridging occurs between thiol groups found in cystein amino acid residues, which are present on most proteins. Cavalieri and coworkers used lysozyme to form microbubbles (see Figure 2A), which were found to be stable and retain their enzymatic activity for several months.[15] In their report, Cavalieri et al. were able to confirm the importance of disulfide bridging in forming a stable protein shell. Korpanty et al. [16] used a mixture of albumin and avidin to form microbubbles. Incorporation of the avidin into the protein shell in this study allowed biotin-mediated coupling of antibodies for targeting vascular endothelium.

Surfactant Shells

Microbubbles stabilized by mixtures of the synthetic surfactants SPAN-40 and TWEEN-40 were formulated by Wheatley et al.[17, 18] The SPAN/TWEEN solution was sonicated in the

presence of air to form stable microbubbles. Using a Langmuir trough, they were able to establish the correct ratio of SPAN to TWEEN (roughly 1:1) to use for maximum film stability. Interestingly, they showed that surfactant derived from sonicated microbubbles was more stable (i.e., was capable of reaching higher collapse pressures on the Langmuir trough) than that used in the precursor solution, indicating that the sonication process modified the surfactant to form a more stable film.[18]

Dressaire et al. [19] recently reported on stable microbubbles formed from sucrose stearate (mono- and di-ester) formed by a blending process at 70 °C in 75 wt% glucose syrup. These microbubbles were stable in suspension for over a year and showed remarkable polygonal domains on their surface. A thermodynamic analysis suggested that the unique domain morphology arose owing to the interplay between surface tension, domain boundary line tension and spontaneous curvature of the surfactant monolayer. While these particular microbubbles were not stable upon dilution, and therefore have limited biomedical utility, the study illustrates the importance of surface heterogeneity and domain bending with regard to microbubble stability.

Lipid Shells

Lipid-coated microbubbles are one of the most interesting and useful formulations used for biomedical imaging and drug delivery. The lipid shell is inspired by nature, as stable microbubbles found ubiquitously in the oceans and fresh waters of Earth are known to be stabilized by acyl lipids and glycoproteins.[20] The lipid shell of a microbubble is also bio-inspired, as it mimics the remarkable stability and compliance of lung surfactant.[21] Indeed, lung surfactant was discovered based on the observation of stable microbubbles formed from lung lavage.[22] Moreover, the ability of lung derived fluid to form stable microbubbles is being pursued as a clinical means of assessing lung surfactant viability in neonates.[23]

There are several commercially available lipid-coated microbubble formulations approved for clinical use in the US and abroad, including Definity (Lantheus Medical Imaging) and Sonovue® (Bracco Diagnostics). Lipid shells have several advantages. Phospholipids spontaneously self-assemble into a highly oriented monolayer at the air-water interface, such that their hydrophobic acyl chains face the gas and their hydrophilic headgroups face the water. Thus, the lipid monolayer will form spontaneously around a newly entrained gas bubble, just as for surfactants and proteins. Saturated diacyl phospholipids are capable of laterally compressing within the monolayer plane to reach a very low surface tension when below their main phase transition temperature, which is the temperature at which the membrane transforms from a crystalline or ‘gel-like’ state to a liquid-crystalline or ‘fluid-like’ state. This is important, because surface tension at the curved interface induces a Laplace overpressure which, in turn, forces the gas core to dissolve.[9] Thus, the low surface tension reached by the lipid monolayer stabilizes the microbubble.[24] In addition, lipid monolayers are highly cohesive owing to attractive hydrophobic and van der Waals interactions between the tightly packed acyl chains. The high cohesiveness gives the lipid shell a solid-like character.[25] These effects can be advantageous because microbubble stability is not dependent upon superoxide formation during sonication to facilitate disulfide bridging, as is the case with proteins. Hence, lipids are amenable to a variety of fabrication techniques aside from sonication, as recently described by Stride and Edirisinghe.[26] Additionally, the lipid molecules are held together by ‘weak’ physical forces, without chain entanglement, which makes the shell compliant to area expansion and compression during ultrasound insonification. Thus, lipid-coated microbubbles have exhibited favorable ultrasound characteristics, such as resonance with minimal damping and the ability to reseal around the gas core following fragmentation.[13, 27–33] Finally, lipid-coated microbubbles may be easily functionalized for drug delivery, molecular imaging or other purposes by incorporating different lipid headgroup species or post-production bio-

conjugation.[34, 35] Thus, the lipid-coated microbubble itself is a versatile platform technology. An example of a lipid microbubble is shown in Figure 2B, which depicts heterogeneity and phase separation of phosphatidyl choline and lipopolymers that are typically used to stabilize lipid microbubbles.[36] More specific details on the physicochemical properties of lipid-coated microbubbles can be found in a companion article by Longo et al.[37]

Polymer Shells

The term “polymer microbubble” typically refers to a special class of microbubbles that are stabilized by a thick shell comprising cross-linked or entangled polymeric species. The bulk nature of the polymer shell makes it more resistant to area compression and expansion than its lipid and albumin counterparts, which reduces the echogenicity and drug delivery activity. For example, polymer microbubbles have been observed to fracture during insonification, thereby releasing their gas core via extrusion through the shell defect.[32] The resulting gas bubble was unstable and rapidly dissolved according to the classical Epstein and Plesset equation.[9] The shell, on the other hand, remained intact and often propelled away from the gas core; this ballistic effect may be useful for drug delivery.

In 1990, Wheatley et al.[38] reported on a new polymer shelled microbubble, in which the shell was formed by the ionotropic gelation of alginate. The microbubbles were formed by concentric jets of air and alginate solution that were sprayed into a reservoir. The alginate adsorbed to the gas/liquid interface and was hardened upon plunging into the calcium solution. Sonication of the solution prior to spraying increased the microbubble yield. Microbubble size was primarily determined by the flow rate of air around the syringe needle. Microbubble diameters ranged between 30 and 40 μm and were therefore too large for intravenous administration.

In 1997, Bjerknes et al.[39] described a method for making microbubbles encapsulated by a proprietary double-ester polymer with ethylidene units using an emulsification, solvent evaporation method. The polymer microbubbles had a broad size distribution ranging from 1–20 μm diameter. Optical microscopy and cryogenic transmission electron microscopy (cryo-TEM) showed that the microbubbles had elongated, crumpled shapes. The polymer shell was typically 150–200 nm thick. Acoustic tests showed a dose-dependent increase in acoustic attenuation.

In 1999, Nayaran and Wheatley reported on microbubbles formed by the biodegradable copolymer poly(D,L-lactide-co-glycolide) (PLGA). The microspheres were made hollow by using a volatile solid core, which could be sublimed away. Microbubble size was controlled through manipulating the solution viscosity, polydispersity and shearing rate. The size distribution ranged from 2 to 20 μm diameter. The zeta potential of the microbubbles became less negative after incubation in serum, perhaps indicating opsonization of the surface. Interestingly, significant ultrasound contrast was observed in the right kidney of rabbits, thus showing that a significant population was capable of passing through the lung capillaries, which typically restrict particles greater than 10 μm in size.

In 2005, Cui et al.[40] described the fabrication of PLGA microbubbles through a double-emulsion, solvent evaporation method. Size analysis with a Coulter counter showed a monomodal distribution between 1–2 μm diameter. Scanning electron microscopy (SEM) showed spherical particles with smooth surfaces devoid of visible pores or cavities. Confocal scanning microscopy showed different internal morphologies, ranging from a single hollow core to more of a honeycomb structure, depending on the emulsification conditions. PLGA microbubbles filled with either air or perfluoropropane (PFB) were capable of opacifying the left ventricle (LV). Air and PFB microbubbles provided LV opacification in rabbits for 1–2 min and 7–8 min, respectively.

Also in 2005, Cavalieri et al.[41] described a method to create microbubbles coated with poly (vinyl alcohol) (PVA). PVA microbubbles were created by chemical cross-linking at the air/water interface during high-speed stirring (8000 RPM) of an acidic solution of telechelic PVA. The mean diameter was approximately 6 ± 1 μm. The shell thickness could be decreased from 0.9 to 0.7 μm by decreasing the operating temperature from room conditions to 4 °C. PVA microbubbles had a shelf life of several months and are capable of carrying hydrophobic drugs, charged polymers (e.g., DNA) and targeting ligands.

In 2006, Böhmer et al.[42] described a fascinating new method of creating polymer microbubbles using ink-jet printing. Remarkably monodisperse microbubbles encapsulated with the copolymer polyperfluorooctyloxycaronyl-poly(lactic acid) (PLA-PFO) in the 4–5 μm diameter range were created by injecting an organic phase containing the polymer into an aqueous solution. An additional non-solvent for the polymer was used to create the core/shell morphology, and the core was removed to create the hollow chamber (see Figure 2C).

Polyelectrolyte Multilayer Shells

A new class of polymer-surfactant shell hybrids was recently introduced that involves polyelectrolyte multilayer (PEM) shells on preformed microbubbles. The preformed microbubbles are coated with a charged surfactant or protein layer, which serves as a substrate for PEM deposition. The layer-by-layer assembly technique is used to sequentially adsorb oppositely charged polyions to the microbubble shell. Shchukin et al.[43] were the first to report PEM deposition onto microbubbles. They used the polymers poly(allylamine hydrochloride) (PAH) and poly(styrene sulfonate) (PSS) for the polyion pair. This system gave a relatively uniform PEM coating that provided the microbubbles with remarkable stability. Borden et al.[44] developed a PEM microbubble with phospholipid containing the cationic headgroup trimethylammonium propane (TAP) as the underlying shell and DNA and poly(L-lysine) (PLL) as the polyion pair. Interestingly, the PEMs formed as islands owing to phase separation of the phospholipid species in the shell. The formation of such islands may be useful for surface compartmentalization for multi-functional microbubbles that require both ligand-receptor mediated adhesion and drug release through ultrasound-triggered fragmentation. Lentacker et al.[45] described a multilayer microbubble in which albumin microbubbles were coated with DNA and PAH, where the latter layer served to bind and protect the DNA from enzymatic degradation.

III. Ultrasound Imaging

Useful Properties for Biomedical Applications

Microbubbles display numerous useful effects when they are insonified by ultrasound.[46, 47] A range of behaviors is available which depend not only on the ultrasound parameters, but also on the microbubble size and physicochemical properties. Subtle effects such as acoustic backscatter are used for diagnostic imaging. More violent effects such as inertial cavitation can be used for targeted drug delivery. An assortment of other useful effects lies between these extremes. Figure 3 shows a schematic of some useful ultrasound-mediated effects of microbubbles for biomedical applications.

At low acoustic pressures, an insonified microbubble produces a backscattered echo (Figure 3A). The echo can be used to detect and locate the microbubble. The microbubble can therefore be used as a contrast agent in ultrasound imaging. The echogenicity, or relative strength of the backscattered signal, is strongest near the microbubble resonance frequency. Bubbles of a few micrometers in diameter resonate at frequencies in the 1–10 MHz range which, fortuitously, is the range of typical ultrasound clinical imaging scanners. Thus, microbubbles are highly echogenic to conventional ultrasound. Additionally, microbubbles scatter ultrasound

nonlinearly. Imaging pulse sequences with modulated phase, frequency and amplitude can be used to separate the microbubble and tissue signals with high fidelity.[2] A single microbubble may be detected within an ultrasound imaging voxel.

The stably oscillating microbubble generates shear-field streamlines of fluid flow. These streamlines are asymmetric when a microbubble is adjacent to a surface (Figure 3B). Microbubble streaming not only increases local mixing through convective processes, but also may induce shear forces on cellular surfaces that enhance the intercellular and extravascular transport of nearby macromolecules.[7] This effect may be useful for drug delivery applications requiring subtle changes to the local microenvironment, such as in opening of the blood-brain barrier.[48]

At higher acoustic pressures, the microbubble may become unstable during oscillation and fragment into daughter bubbles (Figure 3C). Microbubble fragmentation generally occurs at a threshold peak negative pressure, and it arises due to an instability in the microbubble surface during the compression and expansion phase of oscillation.[31] Fragmentation is a useful means of eliminating the contrast agent signal within the transducer focus. Microbubble fragmentation is being employed to measure reperfusion in tumor and cardiac tissue and in ultrasound molecular imaging protocols.

At acoustic pressures just below the fragmentation threshold, a microbubble will undergo dissolution (Figure 3D).[28, 30] A small volume of the gas core escapes with each cycle. Acoustic dissolution offers a more subtle means of eliminating the contrast signal than fragmentation and may also be useful for drug delivery.

A more violent activity may occur at high acoustic pressures and lower frequencies (Figure 3E). Inertial cavitation occurs after a prolonged expansion phase, where during the subsequent compression phase a strong inrush of water toward the microbubble center results in a violent implosion. The implosion emits a shockwave that may be detected by the ultrasound transducer as a broadband signal. The shockwave may also facilitate intercellular and extravascular transport of macromolecules. Asymmetric cavitation near a rigid boundary can result in an involuted jet (water hammer) that impinges on the surface and may further enhance drug delivery. It is presently unclear whether streaming, shockwave formation or jetting is the dominant mechanism of ultrasound-mediated drug delivery.[2]

Finally, at low acoustic pressures and at resonance frequency, each cycle of ultrasound results in a net force on the microbubble which displaces it away from the transducer (i.e., in the direction of the propagating acoustic wave) (Figure 3F).[27] Radiation force may be used to move the microbubble from the vessel lumen to the endothelium, thus facilitating ligand-receptor mediated adhesion and targeted drug delivery.[49–55] Long pulse trains may allow displacement velocities of greater than several mm/sec. A secondary radiation force may also come into play, where microbubbles oscillating in phase may attract each other, resulting in microbubble clumps that may significantly enhance the echo strength and drug delivery capability.[27, 56]

Contrast Agents for Ultrasound Imaging

As mentioned above, the oscillation of insonified microbubbles provides a strong backscattered echo that can be used to detect and locate microbubbles in the transducer scan plane. This contrast effect of microbubbles was serendipitously discovered by Gramiak and Shaw in the late 1960's.[57] However, it was not until the 1980's that microbubbles with sufficiently small size and circulation persistence were engineered to pass through the pulmonary capillary bed and opacify the left ventricle.[58] In the 1990's, a few of these microbubble contrast agents were approved by the FDA for echocardiography. Commercialization of advanced ultrasound scanner technology and contrast agent detection methods (e.g., Siemens' Cadence Pulse

Sequencing® mode) has made microbubble contrast agents even more effective. Currently, microbubbles are used clinically in the US for echocardiography[59] and in Europe, Canada and Asia for radiological applications as well, such as imaging focal lesions in the liver.[60]

Microbubbles make excellent contrast agents for ultrasound imaging. The strong echogenicity of microbubbles is a manifestation of their compressibility.[56, 61] Solid and liquid particles, which are relatively incompressible, produce much less backscattered signal to transmitted ultrasound and are therefore not as effective as microbubbles for imaging. Thus, microbubbles stand alone as the main contrast agent for one of the most widespread, inexpensive, portable and safe imaging modalities.

Ultrasound Molecular Imaging

The concept of ultrasound molecular imaging is to selectively adhere ligand-bearing contrast agent particles to endothelia expressing a target receptor and, by virtue of contrast agent accumulation and echo enhancement, to map and quantify the extent to which vasculature expresses that target receptor.[34, 50] Several pathologies have been targeted, including angiogenesis, inflammation and atherosclerosis, as recently reviewed elsewhere.[62, 2, 5]

Typically, an ultrasound molecular imaging scan goes as follows: (1) targeted microbubbles are injected intravenously and allowed to circulate and adhere to their target, (2) after waiting for some dwell time, the target tissue is scanned and the video intensity (i.e., contrast signal) in the region of interest is determined, (3) a ‘destruction’ pulse is then applied to fragment and dissolve all microbubbles within the field of view and (4) free microbubbles are allowed to flow back into the field and again the video intensity in the region of interest is determined. The signal from adherent (targeted) microbubbles is delineated from that of freely circulating microbubbles and tissue movement by comparing the video intensities before and after the destruction pulse.

Three attributes of microbubbles make ultrasound particularly attractive for molecular imaging.[35] First, their large size restricts them to the vascular space, and therefore one is assured that only target endothelium is being imaged. Second, their short circulation persistence (on the order of 10 minutes) results in a relatively rapid imaging session. Third, the ability to silence the microbubbles using a fragmentation pulse allows one to discern adherent from free contrast, as described above. These properties are unique and advantageous to ultrasound molecular imaging with targeted microbubbles.

Two additional attributes of microbubbles are being used to extend the utility of ultrasound molecular imaging. The first is radiation force, which allows the user to direct the contrast agent to the endothelium to enhance ligand-receptor mediated adhesion and thus more thoroughly interrogate endothelium for the target receptor.[52, 63] The second is the oscillation of the microbubble surface area during insonation, which allows new microbubble surface architectures to be used. In one example, a bimodal brush is used in which the ligand is tethered to a short chain and is thereby buried by the overbrush under normal conditions, but the ligand is exposed as the surface dilates when the microbubble is being insonified (Figure 4).[53, 55] These effects are unique to microbubbles and promise to enhance the sensitivity and accuracy of results from ultrasound molecular imaging scans.

IV. Gene and Drug Delivery

With the exception of contrast enhancement and molecular imaging, the utilization of microbubbles as targeted delivery vehicles is one of the most intensely researched applications of ultrasound contrast agents. Cavitation of bubbles in an ultrasound field can increase the permeability of an endothelial vasculature, allowing small molecules to enter into tissue from

the blood stream, a technique known as sonoporation[64]. The exact mechanisms that induce sonoporation are still in question, but are generally attributed to jetting that occurs during inertial cavitation [65] or streaming that occurs from stable cavitation [66] (see Figure 3). There are numerous comprehensive reviews on cavitiation and ultrasound biophysics that can be found in literature.[64 46, 67]

More recent studies demonstrate that microbubble oscillation near a cell membrane surface can cause hyperpoloraization of the membrane,[68, 69] which may promote endocytosis of external macromolecules. This mechanism of macromolecular entry into the cell is often overlooked in this field, but is particularly relevant for gene therapy applications in which plasmid DNA or small oligonuclotides are being delivered. A recent study by Lentacker et al. evaluated the uptake of lipoplexes in cultured cells after they had been released from microbubbles during insonifcation.[70] In this study, an endocytotic inhibitor (methyl-β-cyclodextrin) was shown to prevent endocytotic uptake of free liposomes incubated with cultured cells. When the liposomes were conjugated to the microbubble surface and released by ultrasound in the culture medium, however, methyl-β-cyclodextrin had no significant influence on the transfection levels indicating that endocytosis is not the primary mechanism of cell entry.

Meijering et al.[7] published a more recent study showing the opposite effect, specifically that low intensity ultrasound does promote endocytotic uptake of macromolecules. Fluorescently labeled dextrans of low and high molecular weights (4 kDa and 400 kDa) were used to transfect cultured cells in the presence of microbubbles and ultrasound. Utilizing clatherin- and caveolin-inhibitors and ATP depletion resulted in reduced uptake of low molecular weight dextrans and no uptake of high molecular weight dextrans, implying that cellular uptake via endocytosis did play a role in internalization of macromolecules. Cytosolic escape of dextran after it was preloaded into cells, then insonified with microbubbles, demonstrated transient pore formation also occurred that permitted passive leakage of small molecules. One of the conclusions drawn from this study was that the contribution of transient pore formation as the primary mechanism of entry is reduced as the molecular weight of the cargo is increased. This conclusion seemingly contradicts the findings by Lentacker et al. [70] However, it is important to mention that the parameters for the ultrasound exposure in this study were less intense than those used by Lentacker et al.[70] More violent cavitation may alter the mechanisms of macromolecule uptake.

The mechanisms of drug uptake following release from the microbubble carrier have important implications in designing rationale vectors for ultrasound triggered drug release. Certain drugs may easily diffuse through vasculature while larger macromolecules require more violent means of cell permeation to deliver their cargo. Finding a threshold where low intensity ultrasound could be used to promote endocytotic activity may be a favorable approach in situations where violent microbubble cavitation is not desired. Clearly, further work needs to be done in this area.

The unique ability of microbubbles to respond to ultrasound energy and to potentially cause a physiological response makes them ideally suited for targeted delivery applications. Over the last several years, there have been numerous literature reviews that provide a comprehensive overview on the topic of targeted drug/gene delivery using microbubbles.[2, 71–82] While many studies have shown successful site specific accumulation of a drug, many of the microbubbles used in these studies are commercially available formulations that were developed for contrast image enhancement in echocardiography applications.

Over the last decade, there has been an effort to develop so-called “next generation” microbubbles, specifically designed to bind, carry, and deliver drugs to target specific sites in the body with greater efficiency (Table 2.). This section of the review focuses on the evolution

of ultrasound contrast agents, and how different modifications to the microbubble structure are being utilized for novel approaches pertaining to the development of drug/gene carrying microbubbles

Rationale for Using Microbubbles for Gene and Drug Delivery

The inability to deliver nucleic acids to target cells via systemic delivery is the biggest rate-limiting barrier in gene therapy applications. The use of ultrasound with microbubbles, however, is beginning to largely overcome this limitation, enabling target specific nucleic acid delivery following systemic delivery. Targeted gene delivery by co-injection of plasmid DNA with microbubbles can be effective for producing detectable levels of gene expression, but it often requires large amounts of DNA in order to produce quantifiable results.[91] This is due to the fact that poly(nucleic acids) are prone to nuclease mediated degradation and rapid clearance by the reticuloendothelial system (RES) when introduced into the blood stream.[92] Therefore, carriers are required to facilitate delivery. Several methods have been developed to utilize microbubbles as carriers for nucleic acids in order to increase their circulation time in the bloodstream, protect them from degradation, and improve specificity of targeted delivery. Several specialized formulations of microbubbles designed for this purpose are discussed in this review.

Targeted drug delivery can be accomplished by the incorporation of small molecules into the microbubble shell. The drug can be released upon destruction of the microbubble through ultrasound-mediated cavitation. Ultrasound focusing allows exquisite tissue selectivity. The same rationale for conjugating nucleic acids to microbubbles for gene delivery also applies to molecules for drug delivery applications. Unlike nucleic acids for gene delivery applications, drugs are rarely electrostatically bound to the microbubble surface. Rather, they are incorporated within or just beneath the microbubble shell. Alternatively, they can be loaded into a carrier which can then be linked to the microbubble surface. The articles presented in this review focus on how microbubbles of various shell types are utilized in different drug delivery applications.

Protein Microbubbles for Gene and Drug delivery

As described above, protein microbubbles are formed by a relatively simple method of disulfide crosslinking of the proteins during sonication. The relative simplicity of the formulation procedure lends this class of microbubbles to be an attractive tool for both drug and gene delivery applications. The 15-nm thick protein shell can accommodate the loading of nucleic acids or other macromolecules without significantly disrupting the acoustic properties of the microbubble. These macromolecules may be fully or partially incorporated within the shell during covalent crosslinking of proteins during the formulation stage. Alternatively, the charged protein surface is amenable to adsorption of nucleic acids without significantly altering the acoustic response. Table 2 illustrates some of the microbubble formulations based on protein shell microbubbles.

The simplest method of attachment is by incubating the desired compound with a microbubble solution to coat the surface. In 2000, Shohet et al. used this technique to attach adenoviral vectors encoding the *Escherichia coli* ß-galactosidase gene.[93] Albumin microbubbles were formed by sonicating a solution of containing 1% human serum albumin and 5% fructose with perflouropropane gas. The microbubbles were then mixed with adenoviral vectors for 2 hours. Buoyant microbubbles rose to the top of this solution and were collected while unattached adenovirus in the subphase was discarded. The adenovirus-loaded microbubbles were injected systemically into rats, and the cardiac region was exposed to ultrasound. The results of the study showed that ß-galactose expression was seen only in the myocardium following ultrasound-mediated destruction of microbubbles. Control experiments without ultrasound,

microbubbles, or both showed no significant ß-galactose expression compared to untreated tissue. This study was one of the earliest proof-of-concept studies that demonstrated the feasibility of organ specific targeting to deliver viral vectors.

A similar approach was taken by Kipshidze et al. in 2005.[94] In this study, albumin microbubbles were used for site-specific delivery of Rapamycin in order to treat intimal hyperplasia (thickening of the intima of blood vessels in response to vessel injury) and reduce restenosis (narrowing of the inner blood vessel diameter in response to hyperplasia). Rather than relying on ultrasound mediated destruction as a drug delivery mechanism, the authors of this study utilized a unique phenomenon in which albumin microbubbles have a tendency to adhere to sites of vascular injury. The results of the study showed significant concentrations of Rapamycin found in regions of vascular injury as well as reduced neointimal formation.

The mechanisms of drug/gene attachment were not discussed in these studies but may be attributed to adsorption of the compounds to the microbubble shell due to electrostatic and hydrophobic interactions. While coating of protein microbubbles may be a simple approach, the efficiency of drug loading remains a concern, especially when using drugs that are expensive or difficult to produce. Additionally, coating of the microbubbles for non-viral gene delivery is inefficient due to the negative charges on both the protein shell and the nucleic acid backbone.

One strategy for gene/drug delivery has been to incorporate macromolecules within the crosslinked protein matrix during microbubble formulation, which effectively entraps them until they are released by fragmentation of the protein shell. In 2002, Tuepe et al.[84] demonstrated the feasibility of entrapping plasmid DNA (encoding for LacZ) into the protein shell of a microbubble during formulation. The plasmid-loaded microbubbles were then perfused through a dissected porcine coronary artery while ultrasound was applied continuously. The results of this study showed that after perfusion of the microbubbles, LacZ protein was expressed only in the region of ultrasound application. The majority of the expression was seen in the endothelial cells, although some sporadic expression was seen in subintimal smooth muscle cells. β-galactosidase activity was significantly improved only when microbubbles were loaded with LacZ expressing plasmids and ultrasound was applied (nearly 5-fold greater expression compared to plasmids alone or plasmid-loaded microbubbles without ultrasound application). While this study used a relatively simple method of incorporating plasmid DNA into microbubbles, it was effective. This study did not contain controls in which the microbubbles were co-injected with free plasmid DNA, thus limiting the conclusions that can be made about the role of plasmid DNA incorporation into the microbubble shell.

In 2002, Frenkel et al.[95] published a study using a similar procedure to load plasmid DNA onto albumin-coated microbubbles in order to increase the amount of DNA delivered during microbubble destruction. This method of plasmid DNA loading showed relatively low loading efficiency; only 17% of the DNA was recovered from the microbubbles. However, high amounts of DNA were used for the loading studies (up to 10 mg per ml) resulting in bubbles containing up to 1.4 pg/μm^2 of DNA per bubble. This method of formulation shows a surprisingly high level of pDNA incorporation within the shell, but the result remains to be verified. Fluorescence microscopy was performed on labeled DNA to show uniform incorporation on the microbubble surface. Furthermore, gel electrophoresis was used on recovered DNA to demonstrate that the high-energy sonication method used to form the disulfide bonds between denatured albumin proteins did not fragment the plasmids during microbubble formulation. *In vitro* studies showed that ultrasound- assisted delivery of the microbubbles improved the level of expression compared to unloaded microbubbles co-transfected with free plasmid DNA. However, as noted in the results section of the manuscript, the dose range of the loaded microbubbles is estimated to be more than the amount of free

DNA that was co-injected, thus limiting the conclusions of the study. The transfection efficiency of the microbubble-treated cells was less (nearly half as much luciferase expression) compared with the same cells transfected with Lipofectamine™ (a commercially available lipid transfection agent). One reason for the low levels of microbubble-mediated transfection compared to lipofection could be incomplete plasmid DNA release from the fragmented microbubble shell upon microbubble destruction.

The approach utilized by Teupe et al.[84] and Frenkel et al.[95] is one of the simplest methods of incorporating plasmids into the microbubble shell and showed early promise as an effective gene carrier. The mechanism by which the DNA is physically trapped in the microbubble shell remains unexplained and may have important implications on acoustic properties of the bubble and the ability to release plasmid under flow conditions when used *in vivo*.

In 2006, Lentacker et al. [45] utilized a similar formulation of albumin microbubbles with a more sophisticated approach to bind plasmid DNA to the microbubble surface using a cationic polymer and the layer-by-layer assembly technique (see PEM assembly section). Positively charged PAH was used to coat the surface of negatively charged amphoteric albumin microbubbles, followed by the addition of a single layer of plasmid DNA. Polymer-coated albumin microbubbles showed a slightly lower yield of microbubbles, but the size distributions were about the same (90% of the bubbles between 1 μm and 5 μm). The loading capacity of the microbubbles in this study was measured to be 0.004 pg/μm^2 of pDNA per bubble, lower than methods incorporating the pDNA into the protein shell. The shelf life of microbubbles was evaluated by measuring the concentration of microbubbles over time. Interestingly, this study showed that the coating of albumin microbubbles with PAH improved the half-life of the bubbles from approximately 1.5 hours to 7 hours. For both coated and uncoated microbubbles, almost all the microbubbles were destroyed by ultrasound indicating that PAH-coated microbubble are still viable as cavitation nuclei. Furthermore, PAH/DNA-coated microbubbles showed minimal DNA degradation compared to free DNA in the presence of nuclease digestion enzymes, a result that is consistent with other literature reports utilizing cationic polymers to bind and protect DNA.[96–98]

PEM microbubbles may have important applications in gene delivery by improving the overall loading capacity of nucleic acids onto the microbubble surface, thus presumably increasing the payload that is delivered following ultrasonic destruction. Research has demonstrated that PEM assembly on microbubbles may have a potential advantage of increasing the stability and payload capacity. Both effects are expected to improve transfection efficiency *in vivo*.

Lipid Microbubbles for Gene and Drug Delivery

Lipid microbubbles are more frequently used in drug and gene delivery applications than protein microbubbles. Fragmentation of the lipid monolayer following microbubble destruction is critical in targeted delivery applications where the drug being carried needs to be easily released when an ultrasound trigger is applied. Lipid microbubbles are more acoustically responsive and may serve as more desirable vehicles for ultrasound-triggered drug release. While the relatively thin shell on the microbubble does not allow a high payload of drugs to be incorporated, several research groups have presented novel methods of modifying the basic microbubble design to overcome this limitation.

Depending on the physiochemical properties, certain compounds may incorporate into the monolayer of lipid-stabilized microbubbles. In 2005, a study by Bekeredjian et al.[88] demonstrated that model drugs could be incorporated into a lipid shell of a microbubbles for ultrasound mediated delivery of proteins to the heart. Microbubbles were formulated in the presence of luciferase enzyme for incorporation into the shell (confirmed by monoclonal antibody staining). To determine their *in vivo* efficacy, luciferase loaded bubbles were infused

into Sprague-Dawley rats while ultrasound was applied to the thorax. The anterior and posterior portions of the heart were evaluated for luciferase activity and showed significant enhancement of protein delivery in both portions compared to control organs. In this study, the primary mechanism of protein delivery to the heart appears to be through ultrasonic destruction of microbubbles. This study by Bekeredijian et al.[88] demonstrates the clinical relevance of drug attachment to microbubbles for ultrasound drug delivery using luciferase as a model protein. While this strategy may be useful for a wide array of proteins, low loading efficiency may be a concern.

One of the earliest strategies to incorporate drugs into lipid-stabilized microbubbles was the development of acoustically active lipospheres (AALs). AALs are similar to lipid microbubbles, but they contain a thick oil layer separating the lipid shell from the gas core. Hydrophobic molecules can be loaded within the oil layer to create drug loaded AALs capable of releasing their contents upon microbubble disruption. The development of AALs for drug delivery applications was first described by Unger et al. in 1998. [87,99] In this study, paclitaxel (the hydrophobic anti-cancer drug) (paclitaxel) was loaded into AALs. Measurements on paclitaxel loading showed high efficiency of incorporation into AALS (measured at nearly 100% loading efficiency when loaded with 15mg of paclitaxel) with mean microbubble size of 2.9 μm in diameter. AALs were acoustically active, but they were not as stable as microbubbles formulated without the entrapped layer of oil. This initial study on AALs was successful in demonstrating a proof of concept for a novel method of loading hydrophobic drugs within acoustically active bubbles for targeted drug release application.

More recent work on novel paclitaxel containing AALs was reported by Tartis et al.[89] The authors formulated novel paclitaxel-loaded AAL with targeting potential to tumor vasculature using integrin binding RGD peptides. AALs in this study had a mean diameter of 1.4 μm and a loading capacity far less than reported earlier by Unger et al.[87], with an estimated 33% Paclitaxel loading efficiency (instead of the near 100% reported by Unger et al.[87]). This can be explained by the more rigorous washing steps performed in this study to remove submicron vesicles and excess lipid. A unique aspect of this study was the utilization of ultrasound radiation force and fragmentation ultrasound pulse sequences to facilitate drug deposition. In this novel ultrasound scheme, low intensity ultrasound pulses were applied that forced microbubbles against the vessel wall, followed by high intensity pulses used to fragment the shell and deposit the drug (see Figure 5). Drug release and targeted deposition of a model drug (fluorescent lipid) from RGD-bearing AAL's was demonstrated *in vitro* and *in vivo.* In both cases, the radiation-fragmentation pulse sequences were necessary to promote vascular adhesion and drug deposition in the area of ultrasound application. This study by Tartis et al. presents both a novel drug carrier and a novel method of ultrasound application that has the potential to significantly improve the efficiency of targeted drug delivery in a wide variety of applications.

While incorporation of drugs into the lipid layer of microbubbles, or in a thick oil layer in AALs, was shown to be an effective approach to enhancing drug deposition at the site of microbubble destruction,[82, 88, 100] the loading capacity remains a consideration. Large amounts of drug incorporation within or beneath the lipid shell may alter the echogenic properties of the microbubbles and production yield and cause instability. Recent studies are focusing on utilizing carrier systems attached to the surface of the microbubble in order to improve the loading capacity without altering the functionality of the microbubble.[86, 90]

In 2003, Christiansen et al. [85] performed a study designed to evaluate the effectiveness of plasmid bearing cationic microbubbles *in vivo*. Cationic lipids were introduced into the microbubble during formulation, which enabled them to electrostatically bind the negatively charged phosphate backbone of plasmid DNA. The size of the microbubbles was

approximately 3 μm in diameter, and the microbubbles were able to carry 0.001 pg of pDNA/μm^2. Cationic microbubbles were loaded with plasmid DNA (reporter gene encoding for firefly luciferase) and systemically delivered to rats. This study demonstrated successful expression of luciferase in hind-limb skeletal muscle and the heart, reportedly only within the ultrasound beam, following intra-arterial or intra-venous infusion of plasmid-loaded microbubbles. Furthermore, the localization of the plasmid DNA was evaluated, and the results showed deposition primarily onto the endothelial lumen only in the area where ultrasound was applied. This study is one of the first to utilize charge coupling of a lipid microbubble shell with plasmid DNA to improve the specificity of delivery and transfection efficiency.

A more recent study published by Haag et al.[101] in 2006 used the same cationic microbubble formulation to deliver tumor growth suppressing antisense oligodeoxynucleotides (ODNs) to prostate tumor cells both *in vitro* and *in vivo*. The ODN's in this study were designed to downregulate the androgen receptor (AR), a key component necessary for tumor growth and proliferation. Ultrasound was applied to Human LNCap prostate cancer cells through an opticell culture chamber in the presence of ODN bearing microbubbles. Fluorescence analysis using fluorescein-labeled ODN demonstrated that cationic microbubbles in the presence of ultrasound significantly improved ODN uptake into cells. Western blot analysis of androgen receptor (AR) expression showed downregulation of AR protein levels by nearly 37% when treated with ODN-bearing microbubbles in the presence of ultrasound. In this study, the authors were able to demonstrate uptake of digoxigenin-labelled ODN in the tumor regions of tumor-bearing nude mice (with most of the ODN located in the surrounding blood vessels and stromal tissue) when ultrasound was applied to ODN-loaded microbubbles delivered systemically. However, they were not able to show AR protein level knockdown *in vivo*. Interestingly, no statistical differences were seen between ODN-bearing microbubbles and free ODN in the presence of ultrasound for either the *in vitro* or *in vivo* studies, leading the authors to conclude that the ultrasound application was the driving force behind ODN delivery. Whether this result is specific to small ODN's compared to larger plasmid DNA or other macromolecules is not clear.

The application of cationic lipid microbubbles is not limited to plasmid DNA and oligonucloetides. In 2007, Taylor et al. demonstrated the retroviral vectors could be loaded onto the surface of cationic microbubbles and specifically delivered to focal regions where ultrasound is applied.[102] Envelope-deficient retrovirus carrying the nuclear localized β-galactosidase marker gene was electrostatically bound to cationic microbubbles and used to transfect cultured cells adhering to an Opticell™ acoustically transparent membrane. Trasnduction of cells within the ultrasound beam was enhanced by more than 100-fold when using the virus-loaded microbubbles and the optimal acoustic pressure. Importantly, since the retroviral vectors lacked the envelope proteins, they were unable to transduce cells without the aid of microbubbles and ultrasound exposure. The results of this study demonstrated a novel means of targeting retroviral transduction which is potentially safer and more specific compared to traditional viral vectors.

The strategy of binding plasmid DNA to the surface of lipid microbubbles has been further been explored by Borden et al.[44] by utilizing the technique of layer-by-layer polyelectrolyte assembly. In this study, cationic lipid-coated microbubbles, similar to the ones described by Christiansen et al.[85] and Haag et al.[101], were used to electrostatically bind plasmid DNA, giving the microbubbles an overall negative surface charge. Cationic polylysine could then be adsorbed to the surface, which yielded a net positive charge on the microbubbles, allowing further addition of plasmid DNA. By adding alternating coats of polylysine and DNA, multiple layers of polyelectrolytes could be formed, markedly improving the loading capacity of plasmid DNA onto the surface of the microbubbles. The size distribution of the microbubbles during polyelectrolyte assembly was less than 10 μm with a mean peak size at 1.8 μm.

Interestingly, the layer-by-layer polyelectrolyte assembly did not affect the size distribution of the bubbles while the loading capacity of the DNA was increased up to 0.12 pg/μm^2 (the highest loading capacity currently reported for lipid-based microbubbles). An interesting finding in this study was that that the addition of polyelectrolyte multilayers increased the stability of the microbubbles by mildly dampening the oscillation of microbubbles during insonification. The strategy of layer-by-layer polyelectrolyte assembly showed a substantial increase in DNA loading capacity without significantly affecting the structural properties and acoustic properties of the microbubble. Early *in vitro* evidence shows that the multilayer may affect bioavailability of the DNA (unpublished data), which is critical for transfection. This novel design holds promise towards generating more efficient carriers for targeted gene delivery, but bioavailability remains an important issue.

An alternative means of improving the loading capacity is to amplify the availability of binding sites on the surface of the microbubble. In 2007, Kheirolomoom et al.[90] described a system of covalently attaching liposomes to lipid microbubbles using a biotin-avidin linker system. In this system, microbubbles had a mean diameter of 1.8 μm while liposomes were measured to have a mean diameter of 100–200 nm. Liposomes were loaded with fluorescent cholesterol as a model hydrophobic drug for visualization and characterization purposes. A critical aspect of this study was the high binding efficiency of liposomes to the microbubble surface, showing upto 10,000 liposomes (100 nm mean size) were attached per microbubble with the optimized formulation parameters. Based on these reported numbers, the potential for higher levels of drug loading can easily be seen, even for hydrophobic drugs or proteins that may otherwise be incorporated into a microbubble surface. The surface area alone for 10000 liposomes would be on the order of 314.0 μm^2 whereas the surface area of a single microbubble with a 1.7 μm diameter, as used in this study, is only be 9.1 μm^2. The obvious advantage of being able to incorporate hydrophilic drugs into the liposome only further highlights the advantages of this delivery system. Insonification of the liposome-linked microbubbles *in vitro* showed targeted deposition of a model drug (fluorescent cholesterol incorporated in the liposome bilayer) onto cultured cell monolayers, which did not occur when the cholesterol was incorporated in the microbubble surface (rather than in the liposome), or with control experiments without ultrasound. Liposome-linked microbubbles have a clear advantage in terms of drug carrying capacity and targeted drug deposition. The use of this system is currently in its infancy and will require more testing to determine circulation persistence and *in vivo* drug deposition capabilities.

In 2008, Vandenbroucke et al.[86] published a study on a similar microbubble delivery system in which pegylated lipoplexes containing siRNA were conjugated to the surface of a microbubble using an avidin-biotin linker system. Biotin-labeled liposomes were formulated and loaded with luciferase-knockdown siRNA duplexes (forming so-called siPlexes), which were then conjugated to the surface of biotin-labeled microbubbles via an avidin linker. Lipoplex bound microbubbles were less than 10 μm in size with a mean diameter of approximately 2 μm. Following microbubble destruction, siRNA was still associated with the liposomes without a significant change in the siPlex size or zeta potential, indicating that ultrasound exposure did not disrupt the liposome complexes. Furthermore, the cellular distribution and siRNA knockdown efficiency of this novel microbubble system showed that siPlex-loaded microbubbles significantly increased the level of luciferase knockdown in the presence of ultrasound compared to controls. While this study showed a novel system of conjugating nucleic acids to microbubbles, the clinical relevancy remains to be tested. Cationic liposomes, and other cationic carriers, are notorious for having high efficiency of transfection *in vitro*, yet fail to produce quantifiable levels of transfection *in vivo*. The inability of cationic carriers to efficiently deliver nucleic acids *in vivo* is often associated with their inability to circulate for long periods of time through the blood stream owing to almost immediate clearance by the RES.[103] The conjugation of cationic carries to long-circulating microbubbles

may overcome this limitation and provide a novel and efficient means of delivering DNA to cells.

Polymer Microbubbles for Drug Delivery

Thick shelled gas-filled polymer microspheres have repeatedly been shown to be viable contrast agents for *in vivo* imaging and molecular targeting. However, the utilization of the agents as drug and gene delivery carriers is rarely found in published literature. One may infer that the thick crosslinked polymer shell may be more resistant to fragmentation and thus less effective for depositing genes/drugs, making lipid or protein microbubbles more attractive carriers. A recent publication by Mahier-Humbert et al.[104], however, does showed evidence that hard-shell polymer microbubbles are promising candidates for ultrasound-mediated gene delivery. Higher levels of transfection by sonoporation were achieved when comparing microbubbles with thick polymer shells (made using triglyceride or polystyrene) to lipid-based microbubbles, although higher acoustic pressures were required for the polymer shells. The authors suggest that the nature of fragmentation and jetting may be more violent for polymer microbubbles when higher acoustic pressure are applied, which may make them more efficient at propelling DNA into cells. Another advantage of polymer microbubble is that they are more favorable for incorporating a wide variety of hydrophobic or hydrophilic macromolecules within the thick crosslinked polymer matrix.

In 2001, Seemann et al.[83] published one of the first studies on polymer-based microbubbles designed for gene delivery. A double emulsion (w/o/w) technique was used to form PLGA microbubbles containing plasmid DNA complexed to a cationic polymer (Polylysine, protamin sulfate, or polyethylenimine). The approach used in this study is unique compared to lipid- and albumin-based microbubbles in that the DNA/polymer complexes can be encapsulated within the PLGA microbubble rather than bound to the surface or incorporated within the shell. DNA/polymer loaded microbubbles had a mean diameter ranging between 3.3 μm and 7.4 μm in size while maintaining strong acoustic properties. The maximum loading capacity was measured at 106 ng of plasmid DNA per milligram of lyophilized microparticle (microbubble concentration per milligram is not given) with a loading efficiency of 30%. Gel electrophoresis studies showed that cationic polymers bound to the plasmid DNA helped to keep the DNA in a supercoiled state. The release kinetics for encapsulated pDNA are reportedly slow, with approximately 10% of the encapsulated pDNA being released after 10 minutes of ultrasound application. To our knowledge, PLGA-based microbubbles containing pDNA on the surface have yet to be reported.

The PLGA microbubbles used in this study were evaluated for gene delivery applications in a subsequent study by Hauf et al.[105] Here, a plasmid encoding for ß-galactosidase was mixed with cationic polylysine and encapsulated within the PLGA microbubble in order to determine the efficacy of gene delivery to rodent tumor models. Rats used in preliminary studies were infused with the PLL/pDNA loaded PLGA microbubble suspension and treated with ultrasound in the tumor region. The ultrasound treated rats showed strong ß-galactosidase signal within the tumor, but not in the surrounding healthy tissue. The reason for this is unclear, however, it is plausible that the tumor cells more actively transcribed the plasmid DNA thus generating a signal only in the tumor regions. Microbubbles were also loaded with a plasmid DNA encoding for the p16 tumor suppressor gene and infused into tumor-bearing mice. Interestingly, late-stage tumor growth was significantly slowed during ultrasound-triggered release compared to control studies where ultrasound was not applied or pDNA was not used. The results of this study show that ultrasound mediated release of pDNA encapsulated within polymer microbubbles does have potential as a viable gene delivery system.

V. Summary and Conclusion

Over the last decade, there has been phenomenal progress in designing innovative microbubble formulations for imaging and drug delivery. The development of lipid, protein, and polymer based microbubbles has shown potential for a wide variety of imaging and therapeutic applications. Engineered microbubbles are ideally suited as theranostic agents to enhance the imaging and therapeutic capabilities of ultrasound.

Current Coating Strategies

There are three main categories of material that comprise the shell of a microbubble, namely (1) proteins, (2) lipids, and (3) polymers. Protein-based microbubbles form relatively rigid shells by disulfide bridging of proteins that surround and stabilize the gas core of the microbubble. These microbubble formulations are commonly made using albumin proteins. Recent research, however, has shown that other bioactive proteins such as lysozyme and avidin may be utilized as well. Incorporation of other bioactive proteins, or even therapeutic proteins, into the microbubble shell can potentially improve the functionality of microbubbles and significantly enhance their importance in biomedical applications. Clearly, this area of research should be explored further. Lipid-based microbubbles are formed by self-assembly of phospholipids into a monolayer at the gas-water interface of the microbubble, which are held together by weak hydrophobic and van der Waals interactions. The thin monolayer of the lipid-based microbubble makes it highly responsive to ultrasound which can be advantages for both molecular imaging (exposing buried ligands) and drug/gene delivery. Conversly, polymer-based microbubbles consist of thick bulky shells formed by crosslinking and entanglement of polymer chains. Polymer-based microbubbles can be fabricated using a wide variety of biocompatible materials which allows more versatility in their design. Polymer-based microbubbles are very stable and exhibit a severely dampened response in an acoustic field, however, they are less commonly used for drug and gene delivery applications.

Methods of Microbubble Drug/Gene Loading and Delivery

Loading drugs onto microbubbles can be advantageous for gene and drug delivery because (1) high velocity jetting during inertial cavitation can expel shell-loaded materials into target tissue, (2) microbubbles can facilitate circulation of a drugs/genes by protecting them from the biological milieu, and (3) ultrasound-mediated fragmentation of bubbles can control the release of drugs/genes thereby improving their target specificity. Designing microbubbles that can carry cargo with high efficiency and high target-specificity is particularly important for minimizing drug dosage, potentially reducing adverse side-effects, and lowering the overall cost of treatment.

Albumin-based microbubbles can potentially incorporate large amounts of plasmid DNA within the thick protein shell, although this remains to be verified. While this may improve the loading capacity of the microbubbles, the release of the shell-incorporated pDNA (or other drugs) needs to be studied further. Complete breakdown of the microbubble shell in a short time during insonification would be required to release all of the incorporated cargo, which may not occur with protein-based microbubbles. The same issue is apparent with thick-shell polymer-based microbubbles, which can easily encapsulate and protect DNA, but can be slow to degrade. Promoting adhesion of the polymer based microbubbles to the endothelial lumen where they can release their cargo over longer periods of time may significantly improve their functionality as drug/gene delivery vehicles. Further studies in this area of research are warranted.

Lipid-based microbubbles, which can rapidly break apart, may be a more favorable in terms of designing rationale vectors for drug/gene delivery. However, their drug carrying capacity

is relatively low. This limitation can be largely overcome by utilizing layer-by-layer assembly or chemically linking drug/gene carriers (i.e., liposomes) to the surface of microbubbles. Chemically linking the carriers to the surface of the microbubble can dramatically improve the surface area for DNA loading and improve transfection properties of DNA after ultrasound mediated release. This novel method of formulation is a recent and promising avenue of research but still requires *in vivo* testing to demonstrate efficacy.

Barriers in Drug/Gene Delivery

Most of the focus on developing more effective microbubble formulations has been placed on improving drug carrying capacity and targeted release. There are still other potential barriers to drug/gene delivery efficiency that need to be addressed. The majority of drug/gene deposition occurs primarily in the endothelial lumen and subluminal intima of the vasculature. This is a major rate-limiting barrier to drug uptake for targets that lie beyond the endothelial layer, especially in gene delivery applications. Small molecules such as anticancer drugs, oligonucleotides, and siRNA, may more easily escape the vicinity of the vasculature to penetrate the interstitium, however this area of research needs further exploration. Currently, most *in vivo* research focuses on targeting tumors or other organs with fenestrated endothelial lining. In these scenerios, the vasculature barrier is largely removed. In order to expand on the usefulness of microbubbles in biomedical applications, more focus needs to be placed on designing more efficient drug carriers to promote intracellular uptake and drug transport. Some groups are beginning to investigate this area,[61, 37] however further research in these topics are warranted.

Conclusion

The potential utility of microbubbles in biomedical applications is continually growing as novel formulations and methods emerge. Microbubbles provide a unique range of responses to ultrasound, which makes them useful for contrast ultrasound imaging, identifying molecular expression and targeting drugs to specific tissue sites. Advances in our understanding of the underlying physicochemical properties has led to a recent burst in the development of novel constructs, including the use of bimodal brushes, polyelectrolyte multilayers, surface compartmentalization, and nanoparticle-microbubble hybrids. Further development of microbubble formulations will enhance the practical use of ultrasound as an imaging modality and generate further clinical interest in microbubbles for therapeutic applications.

Acknowledgments

This work was funded by NIH R01 EB009066 and R21 CA139173 to MAB.

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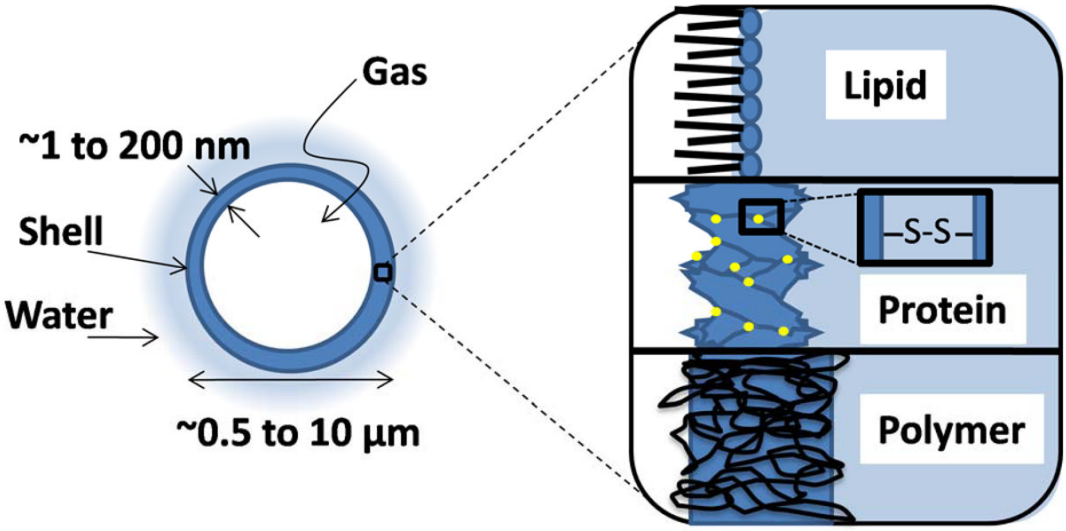

Figure 1.

Cartoon showing structure of a typical microbubble with different shell compositions. Microbubbles used for biomedical purposes are typically between 0.5 and 10 µm diameter (the upper limit for passage through the lung capillaries). The gas core is a single chamber and comprises a large majority of the total particle volume. The shell acts as a barrier between the encapsulated gas and the surrounding aqueous medium. Different shell materials may be used, including lipid (~3 nm thick), protein (15–20 nm thick) and polymer (100–200 nm thick). The lipid molecules are held together through physical force fields, such as hydrophobic and van der Waals interactions. The protein is cross-linked by covalent disulfide bonds. The polymer chains are covalently cross-linked and/or entangled to form a bulk-like material.

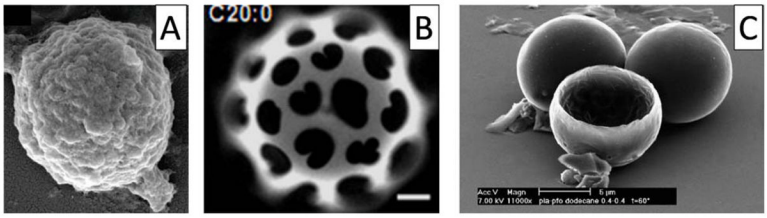

Figure 2.

Microbubble shell morphologies. (A) A lysozyme protein microbubble imaged with SEM taken from Calaveri et al.[15] The microbubble diameter is roughly 1 μm. (B) A diC20:0 phospholipid microbubble imaged with fluorescence microscopy taken from Borden et al. Scale bar denotes 20 μm. (C) A PLA-PFO polymer microbubble imaged with SEM taken from Böhmer et al.[42] All images have been reproduced with permission.

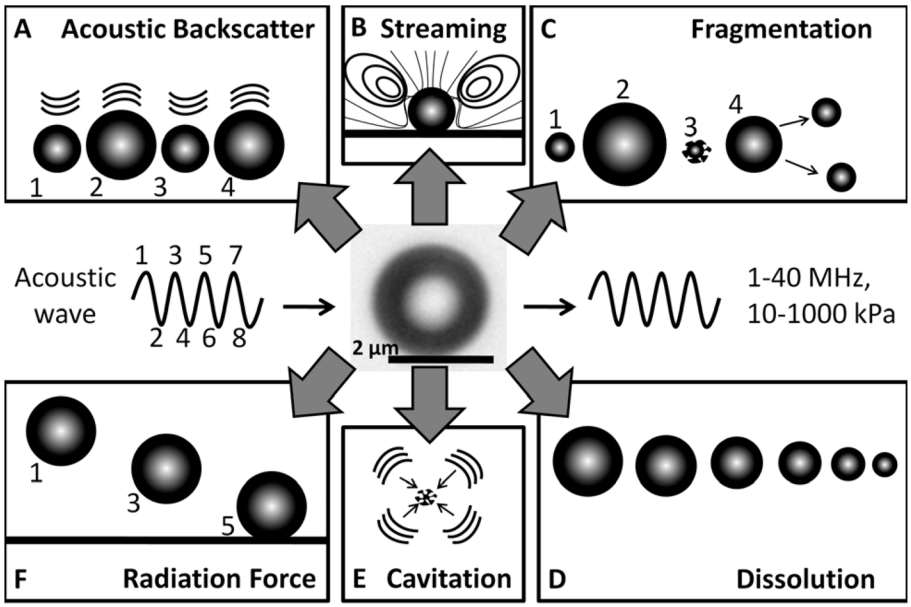

Figure 3.

Useful ultrasound-mediated effects of microbubbles. Microbubbles insonified at MHz frequencies produce a variety of effects which may be beneficial for ultrasound imaging or drug delivery. (A) Oscillation of the gas core at moderate pressures produces a detectable backscatter. (B) Streaming of the fluid around the oscillating microbubble creates shear forces that may facilitate drug release and uptake by nearby cells. (D) Insonation at high pressures results in microbubble fragmentation. (D) Insonation at moderate pressures below the fragmentation threshold results in dissolution of the gas core. (E) Insonation at lower frequencies and higher pressures results in inertial cavitation, which can produce shock waves and involuted jets (water hammer). (F) Insonation at low pressures near microbubble resonance results in radiation force, which displaces the microbubble away from the transducer.

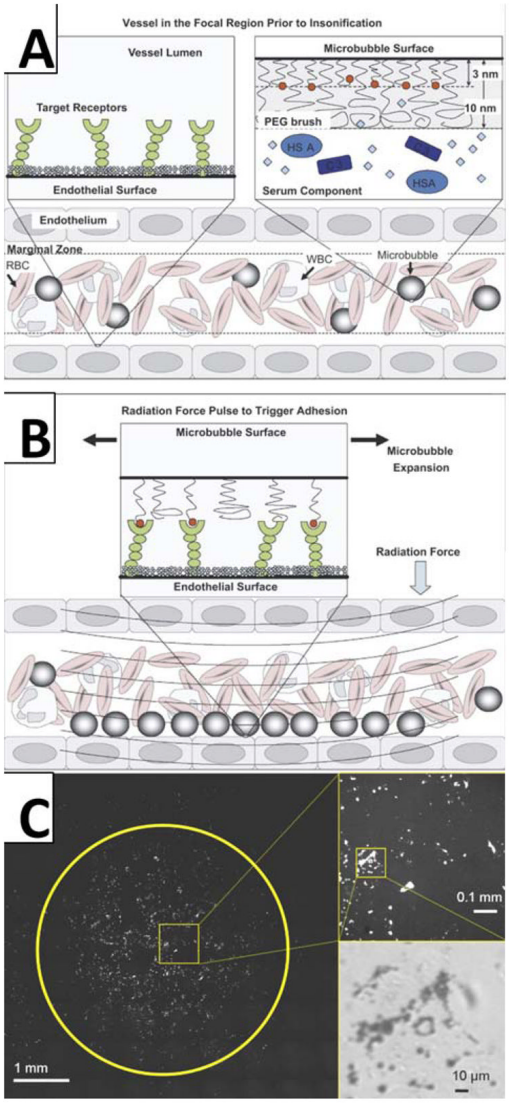

Figure 4.

Schematic showing technique to enhance targeting selectivity using a microbubble with ultrasound-gated adhesiveness. (A) shows the microbubble traveling to the target site, where the ligand is buried by an overbrush layer of methylated PEG. This protects the microbubble from adhering to non-target tissue and protects the ligand from binding to serum proteins, such as C3b, which would alter the ligand binding specificity and lead to premature clearance by the immune system. (B) shows microbubble activity during insonification, where ultrasound radiation force drives the microbubbles up against the target endothelium and oscillation of the gas core transiently reveals the ligand for binding to promote firm adhesion. (C) optical microscopy images showing selective adhesion of RGD-microbubbles with the buried-ligand architecture to plated HUVEC cells expressing $\alpha_V\beta_3$ integrin, where adhesion is only observed within the transducer focus. Images taken from Borden et al. [55] All images have been reproduced with permission.

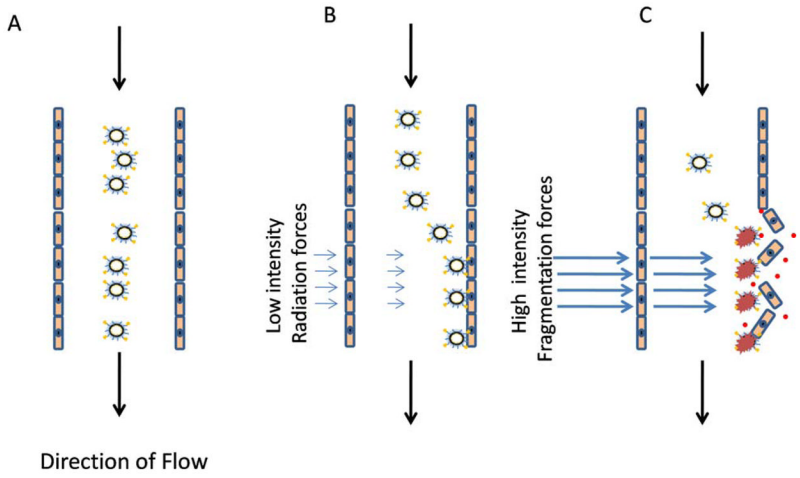

Figure 5.
Schematic representation of radiation-fragmentation pulse. A) Microbubbles passing through the vasculature are B) given low intensity ultrasound pulses to effectively force the bubbles against the endothelial layer. C) High intensity pulses are then applied to fragment the microbubble in order to release the drug cargo in proximity to the endothelium.

Table 1

Shell compositions, properties and ultrasound effects.

Shell Type	Thickness	Compliance	Stability	Drug Payload	Ultrasound Effects
Protein	15–20 nm	Medium	Medium	Medium	High echogenicity; Shell does not reseal after rupture
Lipid Surfactant	3 nm	High	Low to Medium	Low to Medium	High echogenicity; Shell reseals after rupture
Polymer	100–200 nm	Low	High	High	Low echogenicity; Shell does not reseal after rupture
PEM hybrid	10–200 nm	High	High	High	Unknown

Table 2

Summary of Research on Novel Microbubbles for Drug and Gene Delivery

Gene Delivery			
	Group	**Year**	**Research Summary**
A	Seemann et al. [83]	2002	Development of PEI-DNA loaded PLGA Microbubbles
B	Teupe et al. [84]	2002	Utilization of DNA loaded Albumin-based Microbubbles
C	Christiansan et al. [85]	2003	Development of DNA-bearing Cationic Lipid Microbubbles
D	Lentacker et al.[45]	2006	Development of Polymer Coated Albumin Microbubbles for DNA Binding
E	Borden et al. [44]	2007	Layer-by-Layer Assembly of DNA/Polylysine multilayers onto Lipid Microbubbles
F	Vandenbroucke et al. [86]	2008	Development of siRNA/Lipoplex Loaded Microbubbles
Drug Delivery			
	Group	**Year**	**Research Summary**
G	Unger et al.[87]	1998	Development of Acoustically Active Lipospheres for Paclitaxel Delivery
H	Bekeredjian et al. [88]	2005	Development of Cardiac Protein loaded Lipid Microbubbles for Targeted Delivery to the Heart
I	Tartis et al.[89]	2006	Development of Targeted Acoustically Active Lipospheres for Paclitaxel Delivery
J	Kheirolomoom et al. [90]	2007	Development of Liposome Loaded Microbubbles for Hydrophobic Drug Delivery