Ultrasound Microbubble Contrast Agents: Fundamentals and Application to Gene and Drug Delivery

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Abstract
This review offers a critical analysis of the state of the art of medical microbubbles and their application in therapeutic delivery and monitoring. When driven by an ultrasonic pulse, these small gas bubbles oscillate with a wall velocity on the order of tens to hundreds of meters per second and can be deflected to a vessel wall or fragmented into particles on the order of nanometers. While single-session molecular imaging of multiple targets is difficult with affinity-based strategies employed in some other imaging modalities, microbubble fragmentation facilitates such studies. Similarly, a focused ultrasound beam can be used to disrupt delivery vehicles and blood vessel walls, offering the opportunity to locally deliver a drug or gene. Clinical translation of these vehicles will require that current challenges be overcome, where these challenges include rapid clearance and low payload. The technology, early successes with drug and gene delivery, and potential clinical applications are reviewed.
INTRODUCTION

Small gas bubbles, used to enhance ultrasound contrast, were first reported by Gramiak & Shah (1). Air bubbles introduced without a stabilizing shell were very short lived, and therefore methods to stabilize the gas-liquid interface were developed. Simultaneously, low diffusivity gases were introduced to further increase the microbubble circulation time. Clinical ultrasound systems now incorporate contrast-specific imaging modes designed to take advantage of unique nonlinear properties of these small gas nuclei. Today, these stabilized gas bubbles are used to enhance the reflectivity of perfused tissues in applications spanning cardiology and radiology. Although molecularly targeted agents have not yet been applied clinically, successful preclinical studies have demonstrated application in angiogenesis and vascular inflammation, and are described in this review.

In the absence of an exogenous contrast agent, the safety of ultrasound energy has been widely studied, resulting in guidelines designed to control thermal and mechanical biological effects (2). Given that microbubble contrast agents can be expanded, moved, or fragmented by ultrasound, insonation of these agents requires additional safety consideration. Alterations in cell membrane and vascular permeability resulting
from in vitro and in vivo insonation of contrast agents are now well established (3–5). These studies present cautionary limitations for imaging studies and new opportunities for drug delivery. Creation of a clinically optimized microbubble-based drug or gene delivery vehicle is challenging because there exists a vast parameter space of potential shell materials, targeting ligands, cargos, and ultrasound parameters. Herein, engineering analyses of both the microbubble shell and gas core are reviewed. Aspects of the physicochemical properties and ultrasound response of microbubbles are presented in the first two sections. Later sections focus on their imaging and potential therapeutic applications. Finally, we conclude with a section on current limitations and challenges of ultrasound-guided gene and drug delivery with microbubbles.

**PHYSICOCHEMISTRY OF MICROBUBBLES**

**Microbubble Stability**

A newly formed microbubble without encapsulation will dissolve spontaneously and nearly instantaneously as a consequence of surface tension at the gas-liquid interface. Surface tension ($\sigma$) is a fundamental property of a fluid interface between two immiscible phases, and when the interface is curved, results in a higher pressure on the concave side. The pressure drop across a bubble interface ($\Delta P$) is given by the Laplace equation (6):

$$\Delta P = P_b - P_a = \frac{2\sigma}{r},$$

where $P_b$ is the pressure inside the bubble, $P_a$ is the hydrostatic pressure outside the bubble and $r$ is the bubble radius. The high curvature of a microbubble renders a significant pressure drop, on the order of 1 bar for a 2-μm-diameter bubble, which drives gas into the surrounding medium. An encapsulating shell—a barrier between the aqueous and gas phases—is necessary to sustain the gas cavity. The shell contributes two stabilizing components: a resistance to gas leaving the core and a reduction in surface tension, as modeled in a modified Epstein-Plesset (EP) equation (7, 8),

$$-\frac{dr}{dt} = \frac{L}{r/D_w + R_{shell}} \left( \frac{1 + 2\sigma_{shell}/P_r r - f}{1 + 3\sigma_{shell}/4P_r r} \right),$$

where $L$ is Ostwald’s coefficient, $D_w$ is the gas diffusivity in water, $R_{shell}$ is the resistance of the shell to gas permeation, $\sigma_{shell}$ is the surface tension of the shell, and $f$ is the ratio of the gas concentration in the bulk medium versus that at saturation. This model assumes a perfectly spherical geometry throughout dissolution and neglects changes in the shell properties as it deforms to accommodate the shrinking gas core. However, it provides useful simulations to illustrate the shell’s stabilizing effects.

The lifetime of a free microbubble is simulated by neglecting the shell resistance and applying the surface tension for a clean gas-liquid interface. As shown in Figure 1a, a free air microbubble completely dissolves in less than a second in a gas-saturated liquid ($f = 1$). One approach to increase stability has been to use insoluble gases, such as perfluorocarbons, which have water permeation resistances ($L^{-1}D_w^{-1}$)
Figure 1
Calculated microbubble dissolution kinetics based on the modified-EP equation. (a) Radius-time curves of a free microbubble composed of air or perfluorobutane (PFB). Model parameters were \( \sigma_{\text{shell}} = 72 \text{ mN m}^{-1}, R_{\text{shell}} = 0, P_x = 101.3 \text{ kPa, and } f = 1 \) (i.e., saturation). Diffusion parameters for air were \( L = 0.02 \) and \( D_w = 2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \); those for PFB were \( L = 0.0002, D_w = 0.7 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \). (b) Radius-time curves of lipid-coated microbubbles in degassed water \( (f = 0) \). Model parameters were the same as above, except \( R_{\text{shell}} = 10^4 \text{ s m}^{-1} \) for air and \( 10^7 \text{ s m}^{-1} \) for PFB and \( \sigma_{\text{shell}} = 0 \text{ mN m}^{-1} \). All curves were generated in MATLAB using a standard fourth-order Runge-Kutta algorithm.

that are several orders of magnitude higher than air (9, 10). For example, the water permeation resistance of perfluorobutane (PFB, \( n\text{-C}_4\text{F}_{10} \)) is \( \sim 300 \) times greater than that of air. Figure 1a shows, however, that the lifetime of a free PFB microbubble is less than a minute, which is far too short for use as a medical device.

For a suitable shelf-life (days to months), the microbubble shell must be solid to eliminate surface tension and impart a significant permeation resistance. Current ultrasound contrast agents typically comprise solid shells of various compositions, as discussed below. Removing the Laplace-overpressure term eliminates the impetus for dissolution in saturated media, as is easily demonstrated by Equation 1.2. This allows long-term storage of microbubbles, which are stable indefinitely in a sealed vial.

The effect of shell permeation resistance is enhanced for higher molecular weight gases. Permeation through surfactant monolayer films occurs by a thermally activated transition state (11–13), rather than partition-diffusion. The microbubble shell resistance for a high-molecular-weight gas, such as PFB, can be estimated from the oxygen resistance according to the relation (11, 14)

\[
\frac{R_{\text{PFB}}}{R_{\text{oxygen}}} = \exp \left( \frac{\pi \Pi}{4kT} (a_{\text{PFB}}^2 - a_{\text{oxygen}}^2) \right),
\]

where \( a \) is the collision diameter of the permeating species, \( \Pi \) is the surface pressure \( (\Pi = \sigma - \sigma_{\text{shell}}) \), \( k \) is Boltzmann’s constant, and \( T \) is the absolute temperature. The
values of \( a \) for oxygen and \( n-C_4F_{10} \) are 0.36 and 0.81 nm, respectively (15). Using these values, the ratio of resistances for PFB to air is estimated to be \( \sim 1400 \). This is an important point because it often is taken for granted that the shell resistance is negligible (9, 10, 16). Indeed, for high-molecular-weight gases, shell resistance is an important stabilizing term. For the sake of illustrating this effect, a comparison is made using the modified-EP equation under degassed conditions \( (f = 0) \). The oxygen permeability of solid lipid monolayers was measured to be approximately \( 10^2 \) s/cm (12, 17), and this value is used with Equation 1.3 to estimate the value for PFB at \( 10^5 \) s/cm. Figure 1b shows that the estimated microbubble lifetime is significantly enhanced, by five orders of magnitude, by the shell’s diffusion impedance to PFB. The low solubility and slow dissolution kinetics of lipid-coated, perfluorocarbon-filled microbubbles allows for the high degree of dilution necessary to modify the microbubbles for targeting and drug delivery.

Properties of the Lipid Shell

Lipids are a large class of compounds that consist of one or more hydrocarbon or fluorocarbon chains covalently attached to a hydrophilic headgroup, usually by a glycerol backbone. Lipid shells are less stable than other shell types (e.g., polymers), but they are easier to form and yield a more echogenic microbubble.

Lipids spontaneously adsorb from soluble aggregates (i.e., micelles and vesicles) to the gas-liquid interface and self assemble into a monolayer coating (18, 19). At the nanoscale, the molecules are oriented such that the hydrophobic tails face the gas phase and interact via hydrophobic and dispersion forces, which can be modulated by increasing or decreasing chain length. Lipids that are below the main phase transition temperature form highly condensed shells. Increasing chain length was found to diminish surface tension (16) and increase surface viscosity (20), gas permeation resistance (8, 12, 17), and buckling stability (8, 21, 22) of the shell, resulting in more robust microbubbles.

Recent findings have changed the prevailing paradigm concerning the structure of the lipid shell; it is now realized to be a complex, multiphase structure (Figure 2). Pioneering work by Kim et al. showed that the shell consists of planar microdomains (grains) separated by defects (grain boundaries), which influence the mechanical properties (20, 23). Pu et al. later showed that defect density also affects the gas permeability (17).

Research by Borden et al. also showed that the grain boundary regions are a separate, more labile phase that is enriched in certain monolayer components, such as lipopolymers, whereas the microdomains are composed mainly of lecithin (24). Both phases are necessary to stabilize the microbubbles (21, 25). Because the monolayer is compressed beyond the equilibrium spreading pressure, meta-stability of the shell is defined by the collapse rate (i.e., the speed of the 2-D-to-3-D transition). The two-phase system shifts the collapse transition from vesiculation to the more stable mode of buckling and folding (26). The folds, or wrinkles, can be stably attached and continuous to the monolayer shell (8, 21), and external shear forces can act to respread the shell over the elongated bubble surface (23, 27).
**Figure 2**
Phase separation in lipid-coated microbubbles. Shown are individual microbubbles imaged with epifluorescence or confocal microscopy that show preferential partitioning to different regions of the shell. Different species were labeled with dye, including (a) DiIC$_{18}$ membrane probe, (b) DiOC$_{18}$ membrane probe, (c) FITC-neutravidin bound to DSPE-PEG2000-biotin, (d) avidin-coated fluorescent nanobeads bound to DSPE-PEG2000-biotin, (e) NBD-cholesterol, and (f) YoYo-1-labeled DNA adsorbed to a cationic lipid.

Lipids can be chosen from a virtually unlimited library to present a shielding brush layer, express ligands, and bind to drugs, genes and other compounds. The lipopolymer and lecithin species can be mixed with ligand-conjugated or charged lipids, for example, and the surface microstructure and brush architecture can be engineered to yield novel properties. Additionally, lipid shells are highly compliant to severe area dilation and compression during ultrasound-induced oscillation. The lipid shell readily expands, ruptures, reseals, compresses, buckles, and respreads with each acoustic cycle (22, 28–32).

**Other Shell Materials**
In the 1980s, ultrasound contrast agents were coated with an adsorbed layer of saccharide (33) or protein (34). The albumin-coated microbubbles Albunex$^{\text{TM}}$ and Optison$^{\text{TM}}$ (GE Healthcare) were the first commercially available, FDA-approved contrast agents. More recently, protein-shelled microbubbles have been functionalized to carry targeting ligands (35) and genetic payloads (36, 37). Albumin shells tend to be rigid (28), however, and introduce the typical immunogenicity issues associated with animal-derived materials.
Several methods have been described to encapsulate gas in a polymer shell. Alginic-acid-shelled microbubbles have been created by dispersion and ionic gelation (38). Several investigators have used organic solvents to dissolve and disperse the polymer, followed by resuspension of lyophilized products to form hollow polymer capsules (39–41). Partial filming of surfactant-coated microbubbles with nanoparticles has been described by Schmidt & Roessling (42). Polymerization at the air-liquid interface during agitation of an acidic medium was described by Paradossi et al. (43). Each of these methods has produced microbubbles with enhanced stability. However, chain entanglement and covalent bonds inherent in the polymer shells dampen the oscillation of the gas core (32, 44) until the shell is fractured by sufficient expansion.

**Targeting**

Physicochemical interactions can be used to increase the specificity of targeting. A simple example is the use of surface charge. Lindner et al. found that cationic microbubbles persist in the microcirculation of tissue undergoing ischemia/reperfusion and inflammation owing to interactions with the innate immune system (45). Electrostatic interactions generally are not sufficiently specific, however, given the complexity of biological milieu. Ligand-receptor interactions, on the other hand, yield high specificity in biological media. In this case, the microbubble surface is decorated with ligands that bind specifically to receptors on cells lining the lumen of vessels.

As discussed above, a lipopolymer is necessary to form stable microbubbles. The presence of the polymer brush necessitates a spacer between the ligand and monolayer shell for the ligand to interrogate its receptor on an apposing surface (46). Typically, the ligand is tethered with a spacer that is equal in length or longer than the chains of the surrounding brush. This gives maximum exposure of the ligand to the biological milieu.

Surface architectures designed to maximize ligand exposure to the target tissue also run the risk of boosting presentation of an immunogenic compound that leads to early particle clearance or, worse, a hypersensitivity response. For example, unpublished data in our laboratory clearly show that biotin-conjugated lipopolymer present on microbubbles activates the complement system in humans and mice (M. A. Borden and K. W. Ferrara, unpublished data). More research is necessary to test whether tethered antibody or peptide ligands also elicit an immune response. To account for the immunogenic effect, Borden et al. (47) showed that the ligand can be concealed by a polymeric overbrush to enhance circulation half-life, and then it can be locally revealed for binding to the target by ultrasound radiation force. A discussion of ultrasound radiation force is provided in a subsequent section.

There are two basic methods of attaching ligands to the microbubble surface: either through a direct covalent bond or through biotin-avidin linking. Biotin-avidin linkage is a straightforward technique in which a biotinylated ligand is coupled to a biotinylated microbubble via an avidin bridge. Although biotin-avidin linkage is useful for proof-of-concept and preclinical targeting studies, the immunogenicity precludes it from translation to humans. Covalent attachment is more desirable and
Microbubble shell

Receptor surface

Microbubble shell

Receptor surface

Figure 3

Ligand architectures. Cartoons show different schemes for the presentation of various ligand types: (a) small hydrophilic ligands can be covalently attached to the distal end of the carrier lipopolymer. The diffusion of the ligand is dictated by the polymer chain dynamics; (b) large, protein ligands can be attached by biotin (red) and avidin (yellow) linkage. The large size (60 kDa) and multiple binding pockets in the avidin create a sort of scaffold that is supported by the polymeric brush (2–5 kDa).

can be performed prior or subsequent to creation of the microbubble shell. Strategies for coupling onto preformed microbubbles include binding an amino group of the ligand to a carboxyl group on the microbubble shell by carbodiimide and N-hydroxysulfosuccinimide or, alternatively, binding a thiol group on the ligand to a maleimide on the microbubble shell. More details on the coupling chemistry can be found in a recent review by A.L. Klibanov (48). For lipid-coated agents, the advantage of using a preformed ligand-lipopolymer is that fewer steps are required in the clinical setting between microbubble production and administration into the patient. With postproduction linkage, however, a more efficient use of ligand is achieved through a series of modifications to preformed microbubbles.

Choice of the appropriate coupling chemistry and sequence of modifications depends on the type of ligand. An important consideration is ligand size and its effect on bioavailability (Figure 3). Small, hydrophilic molecules, such as metabolites and peptides, can be conjugated directly to the polymer spacer without significantly affecting the polymer dynamics (49). In contrast, large protein ligands, such as antibodies, are susceptible to denaturation owing to shear stresses and organic solvents involved with microbubble dispersion. For this reason, antibodies (∼120 kDa) typically are conjugated to the preformed microbubble surface by biotin-avidin linking. The resulting complex resembles more of a rigid scaffold than a free polymer chain (50), with the ligand well separated from the polymer brush (∼5 kDa) by the bulky avidin molecule (∼60 kDa).
Types of Ligands

Several classes of ligands have been conjugated onto microbubbles, including antibodies, peptides, and vitamins. Monoclonal antibodies, particularly those in the immunoglobulin-γ (IgG) family, have been used extensively for targeting cell surface receptors. Monoclonal antibodies are versatile and have a binding affinity in the nanomolar to picomolar range. However, they tend to be immunogenic when derived from murine origins. Antibody production for targeted imaging and drug delivery also tends to be expensive and time consuming with binding activity varying from batch to batch. Other limitations of antibodies as targeting agents include a limited shelf-life and temperature sensitivity (51).

Peptides are smaller molecules that offer chemical stability and low immunogenicity. The recent development of combinatorial peptide library methods has rapidly advanced the use of peptides as targeting ligands. A class of ligands that have not yet been used for targeting microbubbles is aptamers. Aptamers are RNA- or DNA-based ligands that bind targets with exceptional affinity and specificity. These ligands are created using a process of systematic evolution of ligands by exponential enrichment (SELEX) (51, 52). Because this process is based on chemical synthesis, some of the limitations encountered with antibody ligands are avoided. A downside is the natural activity of RNAses/DNAses.

Payload

In addition to targeted imaging, ultrasound with microbubbles can be used to deliver a therapeutic payload. The simultaneous release and enhancement of vascular permeability, as discussed in more detail below, are attributes that are unique to ultrasound microbubble technology. A key component to engineering a microbubble formulation for intervention is loading the therapeutic agent onto the shell. The gas core is essentially a void that does not sequester organic compounds, and the lipid shell is too thin (~3 nm) to hold a sufficient cargo. One approach to enhance loading was to introduce oil into the lipid shell that dissolves hydrophilic or lipophilic drugs (53). This technique of forming acoustically active lipospheres (AALs) has shown success in delivering chemotherapeutics in vitro (53).

Charged therapeutics, such as DNA or RNA, can be coupled electrostatically onto the shell when cationic lipids or denatured proteins are present. This technique has been used extensively for gene transfection experiments. The loading capacity observed experimentally for lipid-coated microbubbles is on the order of 0.01 pg/um² (34, 55). We are currently testing an approach to improve loading capacity through the use of multiple strata to enhance the total surface area for electrostatic coupling, and preliminary data show a greater than tenfold increase in DNA loading capacity for five paired layers (M.A. Borden & K.W. Ferrara, unpublished data).

Loading on the microbubble surface also can be achieved using ligand-receptor interactions. For example, Lum et al. recently reported a study in which nanoparticles were bound to the shell by biotin-avidin linkage (56). The solid polystyrene nanoparticles served as a model system, which could be replaced by biodegradable
nanoparticles loaded with drugs or genes. Alternatively, soft nanoparticles, such as liposomes, have been loaded successfully onto microbubbles (48, 57). These results suggest a modular approach for loading, whereby the therapeutic compound is first loaded into a nanoparticle compartment, which is then loaded onto a microbubble carrier. Such an approach offers a versatile platform that can be tailored to the hydrophobicity, size, and release requirements of the specific therapeutic agent.

**MICROBUBBLE PHYSICS DURING INSONIFICATION**

**Microbubble Oscillation**

During an acoustic pulse, the highly compressible microbubble expands and contracts with the applied pressure rarefaction and compression. Capturing the expansion and breakup of the agent requires a shutter duration on the order of tens to hundreds of nanoseconds (Figure 4a). Observation of the microbubble collapse requires a shutter duration on the order of picoseconds where a peak wall velocity of hundreds of meters per second is observed (29). In the absence of a nearby boundary, spherical oscillations are observed, and the effect of ultrasound on contrast agents is approximately characterized by measuring the radial oscillations (radius-time curve) of the insonified agent using a radius-time “streak” image of a single line through the center of a microbubble (Figure 4b) (29, 30). For lipid-shelled microbubbles driven at a pressure up to several hundred kilopascals, the observed oscillation can be accurately predicted by Rayleigh-Plesset analysis (Figure 4c) (29). Although the applied ultrasound pulse is typically sinusoidal, radius-time images of microbubbles driven at a pressure greater than tens of kilopascals demonstrate that the oscillation contains a rich range of frequency components. Furthermore, the mapping of the radial oscillation into an ultrasound echo is dependent on the radial oscillation and its first and second time derivatives, accentuating higher frequency components. Thus, the received echoes from oscillating microbubbles contain submultiples and multiples of the transmission frequency spectrum.

Owing to their size-dependent resonance behavior, microbubbles with a diameter on the order of a few microns differ greatly in their response to ultrasound frequencies, which are on the order of megahertz. Parameters such as pulse phase, center frequency, and acoustic pressure alter the relative expansion and potential for fragmentation and can be optimized based on high-speed photography (Figure 5). Inversion of the transmitted pulse does not result in simple phase inversion of the radial oscillation or echo (Figure 5a). Decreasing the transmission center frequency increases the maximum diameter achieved during expansion and the likelihood of fragmentation (Figure 5b). Depending on the microbubble diameter and ultrasound frequency and pressure, the period corresponding to one entire microbubble oscillation may extend beyond the period of the transmitted pulse, resulting in echoes with components at submultiples (subharmonics) of the transmitted frequencies (Figure 5c).

Lipid-shelled agents have been studied most frequently and their oscillation can be predicted (29) and compared with that of albumin-shelled agents (58). Polymer
Oscillation of a microbubble in response to an ultrasound field. (a) Sequence of two-dimensional images, in which microbubble oscillates in response to a 2.25-MHz center frequency pulse, acquired with a camera shutter duration of 10 nanoseconds. (b) Concept of a radius-time image. Microbubble oscillates in response to the driving pressure and a distance-time image is acquired of a single line through the center of the microbubble demonstrating the increased and decreased diameter. (c) Radius-time image of a microbubble oscillating in response to a 2.25 MHz pulse with a peak negative pressure of 360 kPa. Transmitted pulse is shown in yellow, and predicted radius-time curve is shown in red. (Figure 4b reprinted with permission from Reference 29).

Shells undergo complex changes with insonation and models for their behavior are under development (59). Models and simulations of contrast agent oscillation have recently been extended to microbubbles constrained within small blood vessels or near boundaries (60–64). Within vessels with a diameter of about ten microns, microbubble oscillation amplitude is decreased, and fragmentation is not observed by a single ultrasound pulse.

Destruction of microbubble contrast agents has been observed during ultrasonic excitation. The mechanisms of destruction include fragmentation of the bubble into smaller bubbles and/or dissolution of the encapsulated gas. Relative expansion of a
Figure 5

Streak images of microbubble oscillation in response to varied pulse parameters. (a) Effect of the transmission phase of a single-cycle pulse. As compared with transmission of a rarefaction-first (180°) pulse, when the compressional half-cycle is transmitted first (0° pulse), expansion is longer, contraction occurs less rapidly, and the microbubble remains intact. (1) and (3) are streak images, (2) and (4) two-dimensional images acquired after insonation. (b) Effect of the transmission center frequency for matched peak negative pressure. As compared to a center frequency of 3.5 MHz, with a transmission center frequency of 1.5 MHz, expansion is larger and the microbubble fragments. (1) and (2) are streak images, (3–6) two-dimensional images (3) and (5) acquired at peak expansion, (4) and (6) acquired after insonation. (c) Effect of the transmission pressure with a transmission center frequency of 2.25 MHz. Streak images demonstrating harmonic and subharmonic oscillation of microbubble-driven with lower and higher transmission pressure, respectively. (Figure 5 reprinted with permission from References 30, 154).

A microbubble is defined as the maximum radius, \( R_{\text{max}} \), divided by the resting radius, \( R_0 \), and has been correlated with the cavitation threshold and fragmentation (29, 30).

Radiation Force

The effects of radiation force were first reported in 1906 by C.A. Bjerknes and his son V.K.F. Bjerknes when they observed the attraction and repulsion of air bubbles in a sound field (65). Over the next 80 years, several research groups published theoretical analyses of radiation force on spheres, noting both a primary radiation force that translates the microbubble along the beam axis and a secondary radiation force resulting in microbubble attraction or repulsion (66–69). In these cases, the air bubbles studied were on the order of tens of microns in radius, and the acoustic frequency was on the order of tens of kilo-Hertz. More recent studies indicated that a strong radiation force effect is generated on micron-sized ultrasound contrast agents by clinical ultrasound instruments (53, 56, 60, 70–72). These agents are deflected a distance on the order of a fraction of a micron with each acoustic cycle when the driving pulse is near their resonance frequency. Although transmission of a pulse with a high-pressure amplitude would increase the deflection produced by radiation force, microbubbles are fragmented by a pressure on the order of hundreds of kiloPascals. Therefore,
a very long pulse, with a low pressure and frequency near the bubble’s resonance frequency is used. Owing to the polydisperse nature of commercial microbubble contrast agents, high-time-frequency bandwidth pulses have also been considered to maximize the volume of deflected microbubbles.

**MICROBUBBLE-SPECIFIC IMAGING TECHNIQUES**

With the addition of microbubble contrast agents, ultrasound becomes sensitive to capillary-sized vessels and very low flow rates, while maintaining the ability to detect morphological information from traditional B-mode imaging. Because they are highly compressible and result in strong scattering of ultrasound, microbubbles appear bright on an ultrasound image (30). When insonified, the expansion and contraction of these agents results in the generation of nonlinear signals (30, 73).

Power Doppler imaging involves the transmission and reception of a train of ultrasound pulses, where the motion of scatterers between pulses is used to detect blood flow. Combining power Doppler with ultrasound contrast agents results in improved detection of small vessels (Figure 6). A strong correlation was found between histologic microvascular density (MVD) and the number of intratumoral vessels identified with 2- and 3-D power Doppler sonography of human breast masses (74). Another study used the ratio of enhanced pixels to total pixels in the tumor to follow antiangiogenic treatment of xenografted tumors in mice. The signal-pixel rate was significantly decreased in the treated versus control group and correlated with MVD (75).

Various other approaches have been described to accentuate the nonlinear contrast agent echoes and repress the echoes generated from surrounding tissue. Harmonic imaging is a broad category of techniques that share the common feature of sending an incident beam at one frequency and listening for returned echoes at a harmonic (an integer multiple) of the incident beam (Figure 7) (73, 76). Although a useful technique, harmonic imaging has limitations. Most importantly, owing to inherent limitations.

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**Figure 6**

(a) B-mode ultrasound image of the left kidney of a 9-week-old mouse (bar = 0.5 cm). (b) The same kidney as in (a) is shown following intravenous administration of ultrasound contrast and application of power Doppler. Notice that the kidney vasculature is visualized to the level of the interlobular vessels (arrows). A = aorta.
In harmonic imaging, an ultrasound pulse of a specific frequency (fundamental signal) is emitted by the transducer. The transducer receives the emitted frequency as well as the harmonic. The returned signal is recorded as a combination of the fundamental and harmonic frequencies.

properties of the technique, there is typically a compromise that must be made between image contrast and spatial resolution (77). Additionally, owing to nonlinear sound propagation, tissue can also produce nonlinear echoes, thereby reducing contrast resolution (76).

To overcome these limitations, phase inversion imaging was developed (Figure 8) (77). Phase inversion involves multiple ultrasound pulses that are emitted sequentially, where the second pulse is inverted compared to the first. Linear reflectors will send

In a phase inversion scheme, multiple pulses are transmitted with differing phase. If the pulses are reflected by a linear reflector such as tissue, echoes appear similar to the transmitted pulses (top). The ultrasound system sums the returned echoes, which negate one another. If the reflector of the phase-inverted pulses is nonlinear, such as with a microbubble (bottom), the inverted pulses will be altered upon their return. Therefore, their sum will not negate, and a signal is detected.
the transmitted pulses back unchanged such that the sum of the paired waves will cancel and give no signal. However, nonlinear pulse pair responses will result in echo changes such that they no longer cancel when summed. Contrast-enhanced phase inversion imaging has been used to subjectively assess neovascularity (78). In another study, contrast-enhanced phase inversion harmonic imaging of xenografted tumors was shown to correlate with a semiquantitative scale of immunohistochemical staining with COX-2, a predictor of tumor angiogenesis (79).

Other multipulse contrast imaging schemes have been introduced, including contrast-specific sequence (e.g., CPS® from Siemens), which uses a combination of changes in pulse phase and amplitude to selectively minimize tissue echoes and enhance ultrasound contrast agent echoes. With selective scaling of each received echo and addition of all echoes, tissue signals are rejected and nonlinear microbubble echoes are retained (80, 81). An image registration feature is available on commercial ultrasound systems, minimizing motion and improving the accuracy of region-of-interest (ROI) measures.

As noted above, ultrasound contrast agents can be destroyed when subjected to ultrasound of appropriate frequency and sufficient pressure (30, 82, 83). A destructive pulse of ultrasound can be used to fragment the contrast agent within the region being imaged (Figure 9). Lower pressure, higher frequency, nondestructive pulses can be used to visualize the replenishment of intravascular contrast agent into the imaging region. The echo amplitude can be measured as the contrast agent refills the region, and the rate of return can be estimated. A destruction-replenishment method was described in 1998 by Wei et al. for the assessment of blood flow velocity in the myocardium (84). Continued research has assessed microbubble replenishment after acute myocardial infarction (84–87) and in abdominal organs such as kidney and liver (88, 89). An adaptation to the Wei model of replenishment kinetics has recently been described for single bolus intermittent contrast-enhanced power Doppler imaging of tumor perfusion in small animals (90). Nonlinear imaging techniques, such as CPS, can be combined with destruction-replenishment sequencing for the assessment of regional blood flow (Figure 10).

**IMAGING THE RESPONSE TO THERAPY**

Combining a targeted imaging modality with lesion-directed therapy results in the ability to determine several biologically relevant facts regarding the likelihood of a positive treatment response. Of particular interest are questions regarding whether the target is present, if the agent is reaching the target, and whether the intended target is truly what is being treated. There are a variety of interesting biological processes amenable to the application of targeted ultrasound imaging for monitoring the efficacy of drug delivery.

Our group has described a contrast-enhanced ultrasound technique combined destruction-replenishment ultrasound with subharmonic phase inversion imaging to improve spatial resolution and to differentiate contrast echoes from tissue echoes (91). During the nondestructive imaging pulses, sound was transmitted from the transducer at a specified frequency, whereas the receive function detected
Figure 9
Ultrasound contrast agent is freely circulating in the vasculature. In a destruction-replenishment scheme, a strong ultrasound pulse destroys the agent in the imaging plane. Low-pressure pulses are then used to observe the contrast agent returning to the imaging plane.

Subharmonic frequencies of the original frequency. Subharmonic oscillations are uniquely generated by ultrasound contrast agents and not surrounding tissue (92), resulting in substantial subharmonic echoes from contrast agents within vessels and little to no signal from surrounding tissue. Quantitative parametric maps of flow velocity and overall integrated intensity were generated (93) and measures of perfusion compared favorably with gold-standard techniques (94). This technique was used to monitor the response of experimental tumors treated with antiangiogenic agents and identified different levels of response to therapy (95).

CHANGES IN CELL MEMBRANE AND VASCULAR PERMEABILITY

Electron microscopy has demonstrated that small holes produced within a cell membrane are associated with the collapse of a microbubble and production of a jet (96).
Depending on the ultrasound parameters, pores produced within a cell membrane may be short-lived, resulting either in cell death or successful introduction of an exogenous material into the cytoplasm (5, 97). In addition to changing cell membrane permeability, the application of ultrasound to small vessels containing microbubbles can change blood vessel wall permeability, resulting in the extravasation of particles into the interstitial space (3, 4). This change in capillary permeability is dependent on bubble size, shell composition, and the ratio of capillary diameter to bubble diameter. Varying ultrasound parameters, such as the acoustic pressure and pulse interval, and physical parameters, such as injection site and microvascular pressure, can maximize local drug delivery of microspheres. A pressure of 0.75 MPa at an ultrasound center frequency of 1 MHz is sufficient to produce capillary rupture in the exteriorized rat muscle microcirculation (98). The ultrasound pulse interval affects both the number of points at which extravasation is observed and the volume of material delivered, with both maximized at a pulse interval of 5 s (98). It is believed that maximizing the volume of material delivered requires the replenishment of microbubbles into the region between pulses. It also was shown that as capillary blood pressure increases, microsphere transport across the capillary wall increases.

**APPLICATIONS: POTENTIAL IMAGING AND THERAPEUTIC TARGETS**

Ultrasound contrast agents are uniquely suited for local drug delivery because they can be forced to oscillate within a region with instantaneous dimensions as small as hundreds of microns or as large as centimeters. One approach is to systemically inject the pharmaceutical simultaneously with an ultrasound contrast agent and to then apply ultrasound to the target region. In smaller blood vessels, the oscillations of
Ultrasound contrast agents are freely circulating in small vessels along with drug particles (blue). Once a sufficiently strong ultrasound pulse is applied to the area, the contrast agent expands rupturing the endothelial lining. Drug is then able to extravasate. Drug-laden ultrasound contrast agents are freely circulating throughout the vasculature. A pulse of ultrasound is applied and ruptures the contrast agent, thereby liberating the drug payload. Because ultrasound is only applied in the region of interest, drug is preferentially delivered locally. Drug-laden ultrasound contrast agents baring surface ligands targeted to specific endothelial receptors are freely circulating. The ligand preferentially binds the ultrasound contrast agent in the target region, increasing local agent accumulation. An ultrasound pulse is then applied liberating the drug payload.

As defined earlier, radiation force has been described as a method to push the microbubbles toward the vascular wall prior to destroying them. The close proximity of the bubble will alter the vessel walls, allowing for extravasation of the pharmaceutical agent (Figure 11a) (3, 99). The feasibility of extravascular drug delivery has been evaluated by co-injection of microbubbles with particles and dyes (4, 99). Co-injection of microbubbles with gadolinium followed by MRI has shown that gadolinium extravasates (100, 101). Alternatively, drug may be incorporated into the microbubble and increased local delivery achieved by rupturing the microbubbles selectively in the feeder vessels of the lesion (Figure 11b) (55, 102). However, these methods do not eliminate wash-out and systemic distribution of released drug by flowing blood. Successful demonstrations of reduced neointimal formation, endothelial transfection, and clot dissolution by microbubbles have been reported (103–107). Although the volume of the microbubble payload delivered thus far has been small, delivery of drugs or genes across the blood brain barrier (BBB) is a promising application of microbubble-based delivery, since few alternative methods can change BBB permeability to such a wide range of cargos (100, 108, 109).

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the microbubble to the vascular wall will effectively paint the drug onto the endothelium upon rupture of the delivery vehicle. This method has resulted in deposition of a tenfold increase in fluorescently labeled oil to cells in vitro in comparison to ultrasound alone (53).

More specific drug delivery may be achieved by attaching a ligand that is directed at a designated surface marker to the outside of the drug-laden microbubble. For example, endothelial surface markers are particularly attractive targets as certain markers are overexpressed in areas of angiogenesis and targeted microbubbles have been shown to adhere to these markers (110). Ultrasound can be applied locally to target bound microbubbles resulting in delivery of drug selectively in the areas where the surface marker is expressed (Figure 11) (111).

**Thrombolytic Therapy**

The first successful targeted ultrasound contrast agents were developed in the late 1990s using avidin-biotin adhesion (112, 113). For in vivo imaging, a three-step process was developed (113). First, a biotinylated monoclonal antibody was administered that bound to fibrin within the clot. Avidin was administered, which bound the biotin on the monoclonal antibody. Finally, biotinylated ultrasound contrast agents were given, which bound the exposed end of the avidin molecule. This method of ultrasound contrast targeting resulted in a fourfold increase in acoustic signal from clots (113).

More recently, Unger et al. have developed an ultrasound contrast agent, MRX408, targeted to activated platelets. This agent uses an alternative binding method with an arginine glycine aspartic acid (RGD) molecule attached directly to the surface of the contrast agent (114). RGD binds to glycoprotein IIB/IIIA receptors present on activated platelets. MRX408 has proven to increase the visibility of thrombi and better characterize the extent of the thrombus both in vitro and in vivo (115, 116).

Ultrasound has been shown to enhance thrombolysis with and without the addition of microbubbles and typically in conjunction with intravenous administration of thrombolytic agents (117–119). Effective thrombolysis with minimized treatment-associated hemorrhage has been demonstrated with an ultrasound frequency of 1-2 MHz. Targeted or free microbubbles can be administered intravenously or directly into the clot. The mechanism behind ultrasound-guided thrombolytic therapy involves the mechanical properties of the microbubbles themselves. When insonified at low frequency and high power, contrast agents expand and contract and can potentially break apart the clot. In addition, thrombolytic agents such as t-PA can be incorporated into the bubble and deposited into the thrombus when the bubbles are ruptured. The combination of cavitation and thrombolytic agent delivery has proven more successful for dissolving clots than ultrasound with contrast agents alone.

**Anti-Cancer Therapy**

For years, lipid-soluble anticancer agents have been incorporated into delivery vehicles so as to avoid systemic toxicity. As described above, it is now possible to
incorporate hydrophobic agents into the lipid outer layer of the imaging microbubbles (53) or to attach hydrophilic molecules to the bubble shell (120, 121). Alternatively, it is possible to immerse a hydrophobic drug into an oil layer of acoustically active liposomes (AALs) (111). Toxicity studies indicate a tenfold reduction in toxicity when AAL-encapsulated paclitaxel was systemically administered to mice and compared to unencapsulated paclitaxel.

Angiogenesis is an essential process for the growth and spread of cancer (122). Endothelial cells and receptors associated with angiogenesis are readily accessible to intravascular agents such as ultrasound microbubbles. The placement of ligands onto the surface of ultrasound contrast agents has already proven successful for imaging purposes. Integrins, and particularly $\alpha_v\beta_3$, play an important part in angiogenesis with roles in cell adhesion, cell migration, and signal transduction (123). Lindner’s group (124) has conjugated monoclonal antibodies and RGD peptides with a high affinity for $\alpha_v$-integrins to the surface of microbubbles using an avidin-biotin system. In mouse models, ultrasound detected a greater signal from these bubbles in angiogenic areas where $\alpha_v$-integrins were upregulated.

Endoglin (CD105), the receptor for transforming growth factor, is a proliferation-associated, hypoxia-inducible protein that is highly expressed on angiogenic endothelial cells (125). Immunoscintigraphy using $^{99m}$Tc-labeled monoclonal antibodies targeting endoglin has shown substantial uptake in tumors. More recently, a novel method for conjugating monoclonal antibodies specific for endoglin to microbubbles has been described (35). Avidin was incorporated into the shell of microbubbles by sonication and then bound to the monoclonal antibody by biotin. Ligand directed accumulations of microbubbles targeting endoglin were demonstrated in vitro (35). Given the ability to attach peptides and monoclonal antibodies to microbubbles, one can envision targeted ultrasound agents for the imaging of tyrosine kinase receptors for vascular endothelial growth factor (VEGF) (126), fibroblast growth factor (FGF), and tissue inhibitors of metalloproteases (TIMPs) (127).

**Atherosclerotic Therapy**

One of the earliest indicators of atherosclerosis is the activation and attachment of monocytes to endothelial cells (128). This is mediated by the upregulation of leukocyte adhesion molecules (LAMs) such as intercellular adhesion molecule-1 (ICAM-1). In 1997, ultrasound contrast agents with albumin shells used for routine myocardial contrast echocardiography were seen to have a slow transit time through the myocardium under certain pathologic conditions (129). These microbubbles preferentially adhered to endothelial cells expressing LAMs in vitro. Subsequently, ultrasound contrast agents bearing monoclonal antibodies targeted to ICAM-1 were shown to have good binding efficiency in vitro and in vivo (130). Active targeting of inflammation using microbubbles was described by Villanueva et al. (131, 132) and others (27, 133–135), where endothelial cells activated during the inflammatory response were targeted using a microbubble. Takalkar et al. (135) used a parallel-plate flow chamber to determine the adhesiveness of microbubbles targeted with anti-ICAM-1 to endothelial cells artificially activated with interleukin-1$\beta$. A 40-fold increase in
microbubble adhesion occurred with targeted microbubbles compared with a non-targeted control. Microbubbles adhered at shear rates up to 100 s⁻¹, which is characteristic of larger venules.

Other leukocyte adhesion molecules are up-regulated in inflammatory and ischemia-reperfusion injuries. Of particular interest is P-selectin, which has been targeted with ultrasound contrast agents in a mouse model of inflammation (133). Rychak et al. recently demonstrated targeted adhesion of deformable microbubbles to P-selectin (27).

Gene Therapy

One potential therapeutic application of targeted ultrasound contrast agents is for the delivery of gene-based therapies. Nonspecific regional delivery of adenoviral and plasmid reporter genes has been accomplished using ultrasound-directed methods. More specifically, adenoviral or plasmid vectors have been incorporated into albumin-based ultrasound contrast agents and delivered to the myocardium using ultrasound to destroy the microbubbles in the target region (136, 137). Microbubbles carrying plasmids encoding VEGF have been used to induce angiogenesis in the myocardium of rats following ultrasound application (138). However, traditional microspheres are negatively charged and have a low efficiency for cell transfection of negatively charged RNA and DNA molecules. Tiukinhoy et al. developed a positively charged liposome with ultrasonographically detectable echogenic properties (139). Using an intravascular ultrasound system, they were able to deliver and detect luciferase gene expression in HUVEC cells following ICAM-1-targeted, ultrasound-directed gene transfection.

Incubation of DNA and microbubbles can result in fusion of the DNA to the shell (140), thus facilitating co-injection. Early studies demonstrated gene delivery to the myocardium following intravenous injection of albumin microbubbles, incorporating plasmid DNA on the shell, together with ultrasound (137). Subsequent studies have developed techniques to incorporate DNA within lipid microbubble shells with similar local transfection after intravenous injection and insonation (56, 120, 136, 138, 141–144). Although successful transfection has been reported using venous injection, a study comparing intravenous with intra-arterial injection of plasmid-containing microbubbles concluded that intra-arterial infusion was 200-fold more efficient in achieving local tissue transfection (54).

Delivery of therapeutic levels of drug or therapeutic gene delivery has yet to be demonstrated with intravenously injected of clinically-relevant concentrations of microbubbles. Cardiac gene transfection in the rat utilized 1 ml of intravenously injected ultrasound contrast agents with a concentration on the order of 1 × 10⁹ microbubbles/ml (136). Effective delivery of a therapeutic gene to the rat pancreas was achieved with injection of 1 ml of microbubbles containing the gene within the shell, injected at a concentration of 5 × 10⁹ microbubbles/ml (55). These studies employed a dose much larger than that recommended for human imaging. The development of microbubble agents capable of producing successful transfection with a small dose of intravenously injected microbubbles will be important for future translational
studies. It is not clear, however, whether high concentration is required due to the low payload capacity of the microbubbles or the need for a high concentration of gas bubbles to be present when ultrasound is applied. Alternatively, interventional techniques in which a high concentration of microbubbles is injected within a muscle or artery to achieve local drug or gene delivery may be considered. Intramuscular injection of microbubbles and plasmids produced consistent local transfection in small preclinical studies (145). With intramuscular injection of plasmid and microbubbles, the injection of microbubbles alone enhanced transfection without ultrasound (146). Coinjection of plasmid DNA and microbubbles into the renal artery, when combined with transient vascular compression and ultrasound, has been shown to produce local gene expression in the kidney (147). Coinjection of plasmid DNA and microbubbles into the cerebrospinal fluid, coupled with ultrasound, produced DNA transfer into the rat central nervous system (148). Tsunoda et al. (149) showed an order of magnitude increase in reporter gene transfection to the heart after local injection of microbubbles and plasmid DNA to the left ventricle compared to injection via the tail vein.

CHALLENGES AND LIMITATIONS

Several important challenges face the field of targeted imaging and drug delivery. The most difficult yet essential questions to answer include the following. Is the target present in this individual lesion? Is the ligand specific for the target, or could it bind some other site? Is the target abundant enough to bind detectable amounts of the agent? Will the drug payload remain locally or wash out into the systemic circulation?

Additional issues are more specific to ultrasound contrast agents as drug delivery vehicles. First, ultrasound contrast microbubbles are relatively large compared with traditional pharmaceuticals. Microbubbles are typically 1–10 μm in diameter. Tumor vessels are particularly permeable and often have large endothelial gaps (150); however, contrast microbubbles are typically too large to exit the vasculature. This poses a particular problem when trying to target receptors that may be present in the tumor tissue rather than on the vascular endothelium. In a recent article by Wheatley et al., a nanoparticle ultrasound contrast agent (450 nm diameter) was described with good acoustic properties (151). This agent resulted in good renal opacification in experimental rabbits.

Although the circulation time of ultrasound contrast agents has increased in the past several years, this is also a concern for ultrasound drug delivery. For example, the elimination half-life of Sonovue is 6 min (152). Uptake of Albunex occurs in the liver, lung, and spleen of rats and pigs, with 70% cleared from the bloodstream in 3 min (153). If the agent is taken from circulation by the reticuloendothelial system, the circulation time may not be long enough to allow higher amounts of drug to be delivered to the target region. Contrast agents are typically administered into a peripheral vein so only a small amount of agent will pass through a tumor in a given circulatory cycle. Multiple circulations are necessary to allow destruction of enough agent to increase local concentration significantly. Polymer-shelled agents may provide a greatly increased circulation time (32).
Although ultrasound microbubbles are relatively large agents, the amount of drug that can be attached to the bubble surface or incorporated into the internal lipid layer is a concern. In a study by Tartis et al., approximately 4 mg/ml paclitaxel was incorporated into the internal oil layer of AALs (111). Currently, the recommended systemic paclitaxel dose approaches 175 mg/m² (Taxol, FDA product insert, 1998), although it is administered at a low concentration to reduce toxicity. Since typical strategies for paclitaxel administration deliver less than 5% of the injected dose to a solid tumor, local microbubble delivery may allow regional delivery of a greater volume than other strategies. Adding liposomes or other particles to the microbubble shell also can substantially increase the efficiency of the vehicle (57).

Finally, the bioeffects associated with ultrasound contrast agents are not fully understood and are strongly dependent on concentration and imaging parameters. These effects will need to be considered as clinical translation proceeds.

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