Microbubbles as Ultrasound Contrast Agents for Molecular Imaging: Preparation and Application

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OBJECTIVE. The purpose of this review is to describe trends in microbubble application in molecular imaging.

CONCLUSION. Microbubbles are used for contrast ultrasound imaging as blood-pool agents in cardiology and radiology. Their promise as targeted agents for molecular imaging is now being recognized. Microbubbles can be functionalized with ligand molecules that bind to molecular markers of disease. Potential clinical applications of molecular imaging with microbubble-based ultrasound contrast agents are in the monitoring of the biomarker status of vascular endothelium, visualizing tumor vasculature, and imaging inflammation and ischemia-reperfusion injury zones and thrombi.

Microbubbles used for CEUS are gas-filled microbubbles that are injected intravenously (IV) and circulate non-recirculatingly until they are trapped in the capillaries. Their size is in the range of 1–10 μm. Microbubbles are used for CEUS as blood-pool contrast agents. They are visible with ultrasound as they scatter ultrasound energy. Microbubbles undergo oscillations in response to ultrasound and can be trapped in the capillaries by their size and viscosity, resulting in a signal enhancement (positive contrast effect).

Molecular imaging promises to expand the range of functional imaging, enable early disease detection and therapy monitoring, and provide spatial information for imaging-guided biopsy and imaging-guided therapy. Achieving the overarching goal of personalized medicine will depend on the success of molecular imaging techniques. Currently, SPECT and PET are in clinical use for molecular imaging, but they require bulky instrumentation and radio-isotopes. As an inexpensive, portable, real-time, safe, and popular modality, ultrasound imaging is being recognized. Microbubbles are being developed for clinical molecular imaging. Microbubbles are purely intravascular contrast particles, with a limiting size of several micrometers, allowing for a limited recirculation in the bloodstream. Targeted imaging with microbubbles requires decorating the bubble surface with suitable ligands—molecules with specific affinity for the biomarkers of disease (Fig. 1). Bubbles are injected IV, circulate, bind to the target receptors on vascular endothelium, and accumulate at the disease sites (Fig. 2). Echo from targeted bubbles allows visualization of the biomarker pattern. The purpose of this review is to describe trends in microbubble application for molecular imaging.

Microbubble Dynamics and Contrast Detection With Ultrasound

Microbubbles used for CEUS are gas-filled particles with an average diameter of several micrometers. Microbubbles larger than RBCs would be trapped in the capillaries, and submicron-size microbubbles scatter ultrasound poorly and have insufficient stability. Ultrasound is a pressure wave. Pulses generated by the transducer array cause rapid variation of pressure (up to several megapascals). Therefore, microbubbles in blood undergo volume oscillations at clinical diagnostic ultrasound imaging...
frequencies (1–15 MHz). Microbubbles vibrate, resonate, and reemit sound, and resulting backscatter ultrasound signal is detected. Reemitted ultrasound contains signal at harmonic frequencies [7]. Sensitive microbubble detection relies on the ability of the imaging system to differentiate bubble backscatter signal from tissue background. Modern ultrasound imaging techniques can depict individual bubbles with particle mass less than 1 pg [8], hence the required mass of ultrasound contrast material is low, comparable with the dose of some nuclear medicine agents. For clinical enhancement of the blood pool, a microbubble contrast aqueous dispersion volume of 0.1–5 mL (concentration, ≈10^9 bubbles/mL) is routinely injected or infused IV.

Microbubble detection techniques, such as harmonic imaging, phase modulation, amplitude modulation, and a combination of phase and amplitude modulation improve microbubble detection sensitivity by suppressing background tissue signal. In harmonic imaging modes, the receiving transducers are tuned to a frequency higher or lower than the transmitted frequency (often, signal at the second harmonic frequency is monitored). Unfortunately for this approach, biologic tissues generate second harmonic backscatter signal in the absence of bubbles (e.g., a significant background signal in the heart muscle). A search of better bubble detection schemes led to the development of truly useful methods. In phase inversion, ultrasound pulse is sent and followed by a symmetric phase-inverted copy. The sum of received signals is zero for linear (tissue) reflections. Bubbles behave nonlinearly: They respond differently to compression-first and rarefaction-first ultrasound pulses. Therefore, nonlinear reflections from bubbles generate nonzero signals. Similarly, in amplitude modulation, two pulses with different intensities are sent [9]. Combination of their echoes results in elimination of the linear components, but non-zero signal is obtained for nonlinear (bubble) echoes. A combined phase and amplitude modulation approach with multiple pulses is implemented in ultrasound scanners to obtain excellent detection sensitivity and resolution [10, 11], very useful for molecular imaging with microbubble contrast.

**Microbubble Preparation**

**Shell Material and Gas**

The primary role of the shell is to maintain microbubble integrity after preparation and in vivo administration. This is achieved by reduction of surface tension and the Laplace pressure that causes rapid gas diffusion out of microbubbles [12]. The microbubble shell can be lipid, protein, or polymer. One of the first commercially approved bubble preparations, Albunex (Molecular Biosystems), with an air-bubble core, was made by sonication of albumin solution (so its shell consists of denatured albumin) [13] and could be stored for years refrigerated as an aqueous dispersion. Lipid stabilized bubbles, such as Levovist (Bayer Schering Pharma) [14], were first prepared by dissolution in water of a finely ground dry galactose matrix intermixed with palmitic acid. This preparation strategy provides an excellent storage profile for dry precursor in the vial, but the bubbles have to be used immediately after water is added. Lipid microbubbles have another advantage—lipid mixtures (including targeting ligand-lipid conjugates) can be easily formulated [15]. The shell of these bubbles (e.g., Definity [Lantheus Medical Imaging], SonoVue [Bracco], Imagent [Alliance...
Microbubble Contrast Preparation

The most commonly used method for manufacturing microbubbles involves high-shear mixing, such as sonication of the aqueous medium containing shell-forming materials. The solution (aqueous micellar dispersion, in case of lipids) is sonicated with a probe-type ultrasound generator while gas is sparged through the solution. Gas is dispersed in the aqueous phase by ultrasound-induced shear. Microbubble gas particles are stabilized with the shell components immediately deposited at the gas-liquid interface. Instead of sonication, other types of high-speed mixing can be used, as in dental amalgam processing. An enclosed vial with a sterile aqueous micellar lipid dispersion and a gas headspace is placed in the amalgamator. After a short (less than 1 minute) mixing at approximately 4000 rpm, bubbles are ready for injection. Definity microbubbles are manufactured this way [18].

Alternatively, microbubble formulations are prepared as dry precursors, similar to Levovist: Shell components (e.g., lipids, surfactants) are co-dissolved with a matrix agent (carbohydrate or PEG), placed in vials, and freeze-dried [19]. Vials are then filled with desired gas and sealed. Vials with bubble precursor matrix cake are very stable in storage. Immediately before injection, they are reconstituted with sterile water. As water moves into the lyophilizate cake and dissolves the matrix, it leaves behind isolated gas pockets that become microbubbles.

For preparation of polymer shell microbubbles, the polymer, such as poly(lactic-co-glycolic acid) is dissolved in a water-immiscible solvent and emulsified in the aqueous phase. The solvent is then removed by lyophilization to produce hollow microparticles [20, 21].

Attachment of Targeting Ligands

Molecular imaging applications require targeting to the receptors of interest after IV administration of contrast agents. Therefore, bubbles need to be functionalized with appropriate ligands that have relevant affinity and specificity to allow binding and retention of bubbles on the target in the physiologic flow conditions. Antibodies, antibody fragments, peptides, and carbohydrates have been conjugated to microbubble surface. For clinical use, antibodies have to be humanized to avoid immune response; for preclinical testing it is not needed. Small molecules such as biotin and peptides can be attached to the shell-forming material (protein or lipid) before bubble generation. Large proteins are denatured by ultrasound and are anchored to preformulated bubbles.

There are several conjugation techniques (covalent and noncovalent) to couple ligands to the bubbles. First, a suitable reactive moiety (biotin, carboxylate group, thiol, or maleimide) is attached to a shell-forming material. Avidin–biotin interaction is among the strongest noncovalent bonds and is widely used in biomedical research and analytic practice, as in immunoassays. Commercial biotinylated antibodies are abundant. They can be linked to premanufactured biotinylated bubbles via a streptavidin linker. Because foreign proteins like avidins can cause an immune response, this coupling scheme is useful only in preclinical research.
Another attractive noncovalent strategy is to use the interaction of a metallochelating lipid complex (such as nickel-nitrilotriacetyl-lipid) with a multiplicity of histidine residues (His-tag) of a protein. This method offers an easy way to attach to the bubble surface a genetically engineered protein with a His-tag, a commonly applied insert in recombinant protein preparation [22].

Covalent coupling does not require foreign proteins or His-tags, so the chances of immune response to the ligand are low. Two methods have been proposed for protein coupling. In the early method, a carboxyl group on a bubble is activated with carbodiimide in the presence of N-hydroxysulfosuccinimide, forming active ester [23]. This N-hydroxysulfosuccinimide ester reacts with the protein amino group, forming an amide bond. Alternatively, a maleimide on the shell is coupled with a thiol group on the ligand, forming thioether [24]. The advantage of the latter approach is oriented coupling—if a ligand protein has a single thiol (e.g., a cysteine residue placed far from the binding segment), then a single point attachment to the bubble shell will retain affinity of the ligand to its target. Proteins possess multiple lysine residues, so coupling via amide bond is random and may interfere with ligand-receptor interaction. Another disadvantage of the N-hydroxysulfosuccinimide technique is low coupling yield at the reactant concentrations typical for microbubble preparations [24]. Loss of expensive humanized antibodies or other recombinant protein ligands is undesirable. Overall, selective oriented coupling of thiol protein with a maleimide-carrying microbubble looks more attractive.

Although ligands can be attached to the bubble shell directly, it may be more helpful to connect the ligand to lipid via an extended flexible spacer arm, such as a lengthy (n ≈ 45, 75, or 140) PEG. In the extended state, this polymer spacer brings the ligand so far as 30 nm from the surface of the bubble [25], acting as a “fishing line” [26]. This spacer approach allows more efficient ligand binding to the target and improves microbubble adhesion [27] and retention [28] on the receptor-coated surfaces. Targeted microbubble agent BR55, which has reached clinical trial stage, has a combination peptide ligand attached to the bubble shell via a PEG spacer arm [29].

Behavior of Microbubbles in Biologic Systems

Micron-size bubbles circulate in the bloodstream for minutes. The bubble clearance mechanism is somewhat different from clearance of other types of microparticles and nanoparticles: Gas is cleared through gas exchange via expiration by the lungs. For example, within 6 minutes after IV administration of perflutren (Optison, GE Healthcare) to patients, 99.9% of C$_2$F$_6$ is exhaled [30]. Residual microbubble shell material and some intact bubbles are cleared by phagocytosis (the rate of this process depends on the bubble shell material and PEG coat [17]). The major sites for microbubble accumulation are the liver (Kupffer cells) and spleen (macrophages) [31, 32].

Ultrasound contrast agents are generally considered safe; serious adverse events are rare [33]. In 2007, the U.S. Food and Drug Administration introduced a “black box” warning for microbubble use owing to risk of serious cardiopulmonary reactions in some patients. The warning was later relaxed because it was recognized that the rate of adverse reactions of microbubbles is comparable to or lower than that of other types contrast agents [34], and use of microbubbles does not affect mortality in an undesired way [35].

Microbubble Targeting: In Vitro and In Vivo Considerations

Targeted microbubbles are the choice of CUSM material for molecular and cellular imaging. Although much of the field is still at the preclinical stage, clinically translatable microbubbles have been devised for tumor vasculature targeting [36] and have successfully completed a small-scale exploratory clinical trial. For cellular imaging, Sonazoid microbubbles (approved in Japan) have found clinical use for cancer node detection in hepatology (a rough estimate of 300,000 studies by 2012). In this section we examine the logic of targeted bubble development by reviewing the history of in vitro and preclinical in vivo studies using microbubbles. Particular emphasis is paid to angiogenesis, inflammation, and thrombus imaging.

In vitro assays are the first step in testing microbubble targeting and understanding the molecular biomechanics of binding. The receptor molecule is adsorbed on a plastic or glass surface, and microbubbles are allowed to bind to the target under static (dish) or dynamic (flow chamber) conditions. Microbubble adhesion to the surface can be quantified with optical microscopy or ultrasound imaging. A radial or parallel-plate flow chamber is often used. Full characterization of microbubble binding efficiency under controlled flow rates and various receptor-ligand concentrations can be obtained [37]. Adhesive behavior patterns, such as transient versus firm adhesion in flow can be assessed [38]. Detachment of bound microbubbles from the targeted surface, an indirect measure of avidity, can be quantified [37]. The performance of advanced strategies such as targeting with combinations of ligands can be tested [39, 40]. Visualization and quantification of microbubble binding to endothelial cell monolayers can be performed [23].

In vivo bubble adhesion to the vasculature can be mediated by nonspecific and specific ligand-receptor interactions. Retention of bubbles in the inflamed microvasculature is possible owing to nonspecific attachment to activated leukocytes. For albumin bubbles this interaction is mediated by leukocyte integrins, and for lipid-shelled bubbles it is mediated by serum complement [41]. Microbubbles remain acoustically active even after phagocytosis by neutrophils [42]. Complement activation aids microbubble adherence in the microvasculature. Microbubbles with a net negative surface charge were found to have higher retention in capillaries than neutral bubbles; this interaction was complement-mediated [43]. Incorporating phosphatidylserine in the lipid shell of the microbubble also resulted in higher binding in ischemia-reperfusion injury models of kidney and heart [44, 45]. The mechanism of the latter effects is most likely via direct bubble uptake by phagocytic cells. Sonazoid, a clinical ultrasound contrast agent approved in Japan for liver radiology, relies on uptake of phosphatidylserine bubbles by Kupffer cells in normal liver parenchyma, with lower contrast accumulation in cancer nodes [46].

The potential of molecular ultrasound imaging can only be realized with targeting of a variety of molecular markers. Targeting to inflammatory markers is relevant for atherosclerosis, ischemia-reperfusion injury, and inflammatory bowel disease. Uptregulation of vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule 1 (ICAM-1) is detected on endothelium of atherosclerotic plaques and early-stage lesions. ICAM-1 and VCAM-1 targeting with microbubbles has been found in cultured endothelial cells under flow [23, 47] and in murine models [47]. Feasibility of early-stage inflammatory event detection before obstructive plaque development has been found in animal models [48]. Selectors are also upregulated in inflammation. In ischemia-reperfusion injury models
of the heart and kidney, targeted P-selectin CEUS using antibodies has been reported [49, 50]. Targeting can also aid in detecting and assessing inflammatory bowel diseases such as Crohn disease and ulcerative colitis. In experimental animal studies, successful targeting using microbubbles has been achieved against mucosal addressin cellular adhesion molecule 1 [51] and P-selectin [52]. VCAM-1 (which is also known to be upregulated on the tumor endothelium) was recently targeted via a new class of small (≈15 KDa) recombinant antibody fragment of camelid origin, nanobody, attached to the bubbles; anti–VCAM-1 nanobody bubbles selectively accumulated in the tumor vasculature [53].

Angiogenesis is a hallmark of tumor growth. Vascular endothelial growth factor receptor 2 (VEGFR-2) and αvβ3 integrins are prominently expressed angiogenesis markers. Targeted CEUS provides an opportunity to visualize tumors and evaluate response to therapy. The feasibility of targeting angiogenic vasculature was first found in a matrigel model. Neovessels in subcutaneous matrigel plugs exhibited higher retention of αv, integrin–targeted microbubbles compared with control nontargeted microbubbles. In tumor models, αvβ3 integrin has been targeted with microbubbles carrying echistatin [54], cyclic arginine-glycine-aspartic acid (RGD) peptide [55], and knottin peptides [56]. Microbubble targeting of the angiogenic marker VEGFR-2 has been performed with anti–VEGFR-2 antibody [57] and a single-chain VEGF construct [58]. With the new microbubble formulation (BR55, Bracco) functionalized with a dual-peptide ligand for VEGFR-2, ultrasound contrast-aided monitoring of tumor growth looks promising [36, 59].

One potentially useful application of targeted bubbles is ultrasound molecular imaging of ischemic memory (hours after transient ischemia events, selectins on vascular endothelium are upregulated). Targeted ultrasound contrast imaging with anti–P-selectin antibody or other selectin ligands [49, 69] such as sialyl Lewisx or P-selectin glycoprotein ligand 1 may assist emergency department decision making.

In the bloodstream, microbubbles depend on the flow of blood for transport to the target receptor site. Microbubble concentration is higher in the bulk flow compared with the immediate vessel wall proximity. Therefore, most of the bubbles, especially in laminar flow through larger vessels, have no chance of contact with the target vessel wall and pass by with the bulk flow. Ligands can only bind to receptors within direct molecular interaction range, so most of the ligand-coated bubbles (which are micrometers or even millimeters away from the wall) never touch the target. There is a potential solution to this problem: Microbubbles in an acoustic field are subject to radiation forces. Microbubbles are translated away from the acoustic source by primary radiation force [70], so bubbles can be concentrated at the vessel wall with targeting enhancement. The ability of radiation force to displace bubbles toward the vessel wall was tested in a cremaster model with nontargeted bubbles [71]. Radiation force–enhanced microbubble targeting to avidin and αvβ3 integrin was first found in vitro [72]. Targeting under high shear flow was also possible with radiation force [73]. In vivo targeting of antibody microbubbles to P-selectin in the cremaster venules and femoral vein and artery was found to be higher under the action of radiation force [74]. This effect was especially pronounced in the femoral artery, where a nearly 20-fold increase of targeting bubbles is ultrasound molecular imaging.
in microbubble retention was observed. Similar targeting improvement effects were found for tumor targeting via VEGFR-2 endothelium marker for BR55 CEUS in rats (Frinkin P, et al., presented at the 2012 European Symposium on Ultrasound Contrast Agents).

Another reason to apply radiation force may be not only to improve targeting but also to reduce the contrast dose. In the recently reported BR55 prostate cancer clinical trial, the best results were achieved with the highest injected dose of bubbles (0.05 mL/kg). At a low contrast dose (0.01 mL/kg), tumor was not well detected (Wijkstra H, et al., 2012 European Symposium on Ultrasound Contrast Agents). Reducing the required targeted microbubble dose will reduce the cost of contrast material and minimize potential side effects.

Radiation force also helps target bubbles with “buried” ligand architecture, where bubbles are hidden within a thick PEG brush, and ligand is not exposed unless sonication is performed. The advantage is lower nonspecific adhesion of the bubbles in the tissues outside the region of interest [75].

**Future of Ultrasound Molecular Imaging**

The future of ultrasound molecular imaging is limited by the ultrasound contrast imaging modality itself. Ultrasound does not depict lungs and bone owing to poor ultrasound propagation. The low cost of ultrasound is an issue (i.e., acquiring modalities offer high reimbursement rates to hospitals, and ultrasound equipment does not bring high profit margins). The latter may explain the limited attention of equipment manufacturers to contrast media. The excellent bubble detection technique of pulse inversion amplitude modulation is a decade old yet still not available for most probes and imaging systems. This insufficient technologic support may change—especially in view of the exciting potential of the use of molecularly targeted microbubble contrast media for early noninvasive diagnostics, image guidance of biopsy, and therapy to structures with a small FOV setting, such as prostate and breast, or ischemic memory imaging of the heart and kidney.

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